MOLECULAR AND CELLULAR BIOLOGY, May 1997, p. 2401–2412 Vol. 17, No. 5
0270-7306/97/$04.00+0

Rapid Phosphorylation of Ets-2 Accompanies Mitogen-Activated Protein Kinase Activation and the Induction of Heparin-Binding Epidermal Growth Factor Gene Expression by Oncogenic Raf-1

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Received 1 November 1996/Returned for modification 19 December 1996/Accepted 28 January 1997

Heparin-binding epidermal growth factor (HB-EGF) gene transcription is rapidly activated in NIH 3T3 cells transformed by oncogenic Ras and Raf and mediates the autocrine activation of the c-Jun N-terminal kinases (JNKs) observed in these cells. A 1.7-kb fragment of the promoter of the murine HB-EGF gene linked to a luciferase reporter was strongly induced following activation of ΔRaf-1:ER, a conditionally active form of oncogenic human Raf-1. Promoter activation by ΔRaf-1:ER required a composite AP-1/Ets transcription factor binding site located between bp −974 and −988 upstream of the translation initiation site. In vivo genomic footprinting indicated that the basal level of occupancy of this composite AP-1/Ets element increased following ΔRaf-1:ER activation. Cotransfection of Ets-2 and p44 mitogen-activated protein (MAP) kinase expression vectors strongly potentiated HB-EGF promoter activation in response to ΔRaf-1:ER. Potentiated activation required both p44 MAP kinase catalytic activity and threonine 72 in the Pointed domain of Ets-2. Biochemical assays demonstrated the ability of the p42 and p44 MAP kinases to phosphorylate Ets-2 on threonine 72. Importantly, in intact cells, the kinetics of phosphorylation of Ets-2 on this residue closely mirror the activation of the p42 and p44 MAP kinases and the observed onset of HB-EGF gene transcription following ΔRaf-1:ER activation. These data firmly establish Ets-2 as a direct target of the Raf-MEK-MAP kinase signaling pathway and strongly implicate Ets-2 in the regulation of HB-EGF gene expression.

Inducible changes in the phosphorylation of transcription factors are pivotal to the alterations in gene expression which govern such fundamental cellular processes as proliferation and differentiation (17, 35–38). Deregulation of the signaling pathways that elicit these changes can lead to oncogenic transformation of cells, as the normal stringent control of gene expression is left unchecked (3). The Ras–Raf–MEK–mitogen-activated protein (MAP) kinase cascade is a prominent signaling pathway that regulates cellular proliferation and differentiation (14, 28, 31, 44, 45, 50, 65, 66). The functional loss of components of this pathway gives rise to a variety of developmental defects in organisms from simple eukaryotes to mammals (2, 15, 26, 41, 51, 55). Furthermore, constitutive activity of this pathway promotes oncogenic transformation of mammalian cells and is firmly implicated in human tumorigenesis (5, 12, 42, 64). For this reason, the transcription factors regulated by the Ras-Raf-MEK-MAP kinase pathway and the genes upon which they impinge are of considerable interest.

One of the best-studied mechanisms through which the Ras-Raf pathway modulates gene expression involves phosphorylation of Elk-1, a member of the Ets family of transcription factors which acts in conjunction with the serum response factor to transiently stimulate transcription of target genes containing serum response elements, such as the c-Fos gene (30, 43). ERK-mediated phosphorylation of Elk-1 in its carboxy-terminal transactivation domain is believed to be important in serum-induced c-*fos* transcriptional activation, although recent evidence has indicated a greater degree of complexity of c-Fos regulation than initially suspected (11, 22, 32, 48, 58). Ras-mediated signaling may also regulate the activity of genes containing binding sites for the Fos and Jun family of transcription factors (AP-1) through the activation of a second family of MAP kinase-like enzymes known as the c-Jun N-terminal kinases–stress-activated protein kinases (JNKs-SAPKs) (13, 29, 36, 39, 61, 67). Another major route through which Ras signaling regulates gene expression is via composite binding sites for both Ets and Fos-Jun transcription factors. First identified in the Ras-responsive element of the polyomavirus enhancer (69), AP-1/Ets sites have since been located in several cellular genes and mediate responses to Ras and other oncogenes, including Raf (6, 24, 25, 53, 57, 63).

Recent work has demonstrated that, in mammalian cells, Ras regulates the activities of the Ets-1 and Ets-2 transcription factors. Both Ets-1 and Ets-2 contain a Pointed domain (by analogy to the *Drosophila melanogaster* Ets domain transcription factor Pointed-P2) that contains a potential MAP kinase phosphorylation site (PLLTP). Mutation of the phospho-acceptor threonine (T72 in Ets-2; T38 in Ets-1) within this site in either protein abolishes responsiveness to activated Ras. Furthermore, this site is phosphorylated in cells transiently transfected with Ets-2 and activated Ras (73). The kinases responsible for Ets-1 and Ets-2 phosphorylation in response to

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oncogenic Ras in mammalian cells have not yet been identified.

We have previously employed a conditional form of the Raf-1 protein kinase, ΔRaf-1:ER, to study signaling downstream of Raf kinases (46, 47, 59, 60). The kinase activity of ΔRaf-1:ER can be selectively and conditionally stimulated in cells by addition of β-estradiol or its analogs to the cell culture medium (60). Using differential-display PCR, we have identified several genes whose expression is regulated following activation of ΔRaf-1:ER in NIH 3T3 cells. Among these is the gene encoding heparin-binding epidermal growth factor (HB-EGF). ΔRaf-1:ER activation results in a rapid increase in HB-EGF gene transcription, leading to sustained induction of HB-EGF mRNA expression and secretion of mature HB-EGF from cells which in turn is responsible for the delayed activation of the JNKs that is observed in cells transformed by ΔRaf-1:ER (46).

Aside from highlighting genes that may be involved in the transformation process, identification of ΔRaf-1:ER target genes provides an excellent opportunity to study the mechanisms by which the Raf-MEK-MAP kinase cascade signals to the nucleus. In this study, we have focused attention on the regulation of the HB-EGF promoter by ΔRaf-1:ER and have identified a route through which Raf-1 signaling regulates gene expression, namely, MAP kinase-dependent phosphorylation of Ets-2 and activation of an AP-1/Ets element in the HB-EGF promoter.

MATERIALS AND METHODS

Transient transfection of C2 cells. NIH 3T3 cells expressing the ΔRaf-1:ER fusion protein (46) were grown to confluence in 60-mm cell culture dishes. After 24 h at confluence, the cells were transfected with the indicated DNA constructs by the DEAE dextran method or, where indicated, by a modified calcium phosphate procedure as described previously (18, 27, 73). Amounts of DNA transfected by the DEAE dextran method were as follows: HB-EGF promoter reporters, 500 ng; pGCN-Ets-2/PEA3 expression plasmids, 750 ng; pCEP-p44 expression plasmids, 250 ng. The final DEAE dextran concentration was 20 μg/ml, and the final chloroquine concentration was 80 μM. The cells were incubated for 3 h and then subjected to a dimethyl sulfoxide shock by incubation for 1 min in a solution of phosphate-buffered saline (PBS) containing 10% (vol/vol) dimethyl sulfoxide. Following an overnight incubation, each plate of cells was replated into 4 wells of a 24-well tissue culture plate, and 2 wells were stimulated with 1 μM β-estradiol or the pure estrogen receptor antagonist ICI 164,384. Ethanol (0.1%, vol/vol) was used as a solvent control in all experiments. β-Estradiol and ICI 164,384 were used interchangeably and gave identical results. After 24 h in culture, the cells were lysed in 250 μl of reporter lysis buffer (Promega) per well, and luciferase activity was measured with a luciferase assay reagent (Promega) and a Berthold AutoLumat luminometer. Data are presented as the mean (± standard error of the mean) of the duplicate stimulations. Each experiment is representative of at least three similar experiments. The data are expressed as activity relative to the luciferase activity obtained from cells transfected with the HB-EGF–luciferase vector in which ΔRaf-1:ER was inactive or as indicated in the figure legends. Where indicated, data were corrected for transfection efficiency by cotransfection with 500 ng of the plasmid pPGK-lacZ. β-Galactosidase activity in cell lysates was measured with the Galactolight kit (Tropix).

HB-EGF promoter deletion and mutation. A series of deletions of the HB-EGF promoter (8) from −1884 to −477 (see Fig. 1A) were generated by PCR. Details of the oligonucleotides used to construct these plasmids are available upon request. PCR products were ligated to *Nhe*I/*Bgl*II-digested pGL2 (Promega). All of the numbering of the upstream region of the HB-EGF gene is with respect to the A of the initiator methionine of the HB-EGF protein as the +1 nucleotide (8). In addition, a *Hin*dIII fragment of the promoter was ligated into the *Hin*dIII site of pGL2, generating a deletion to position −911. Constructs were sequenced to verify their integrity. During sequencing, we noticed a 46-bp insertion between positions −1051 and −1052 and an additional C between −1045 and −1046 compared to the original published sequence (8). This 46-bp insertion was not a PCR-generated artifact, as resequencing of the original HB-EGF promoter fragment revealed the presence of the 46-bp insertion that was apparently overlooked at the time of the original sequencing. Thus, the full HB-EGF genomic fragment used here is 1,730 bp in length, spanning nucleotides −155 to −1884 of the murine HB-EGF gene. All construct numbering refers to this modified DNA sequence. The GenBank submission of this sequence has been amended accordingly.

Based on previous studies of similar sites in the collagenase (25), stromelysin (70), and keratin-18 (53) genes, mutations were introduced into a composite AP-1/Ets site of the HB-EGF promoter by PCR as indicated in Fig. 2A. Details of the oligonucleotides used to construct the plasmids are available upon request. The integrity of the introduced mutations was confirmed by DNA sequence analysis.

DMS genomic footprinting of the AP-1/Ets site in HB-EGF promoter. Genomic footprinting experiments were performed as described previously (20, 21). Briefly, C2 cells were either untreated or treated with 1 μM ICI 164,384 for different periods of time, at which point they were exposed to the membrane-permeable alkylating agent dimethyl sulfate (DMS), and then genomic DNA was isolated. As a control, EL4 cells, which express little HB-EGF mRNA, were similarly treated with DMS. As a further control for unprotected DNA, deproteinated genomic DNA was prepared from C2 cells and subjected to in vitro methylation. After in vivo or in vitro methylation, the DNA was treated with piperidine and subjected to ligation-mediated PCR in order to visualize the methyl-guanosine-specific DNA sequence ladder. HB-EGF gene-specific primers were used to detect interactions on one strand of the AP-1/Ets-containing region. Details of the oligonucleotides used and the conditions for the ligation-mediated PCR are available upon request. The ^{32}P-labeled ligation-mediated PCR products were separated on a 6% polyacrylamide sequencing gel. Quantitation of band intensity was performed with a Molecular Dynamics PhosphorImager. The intensity of each band was quantitated by normalization to a band that displayed an invariant signal intensity.

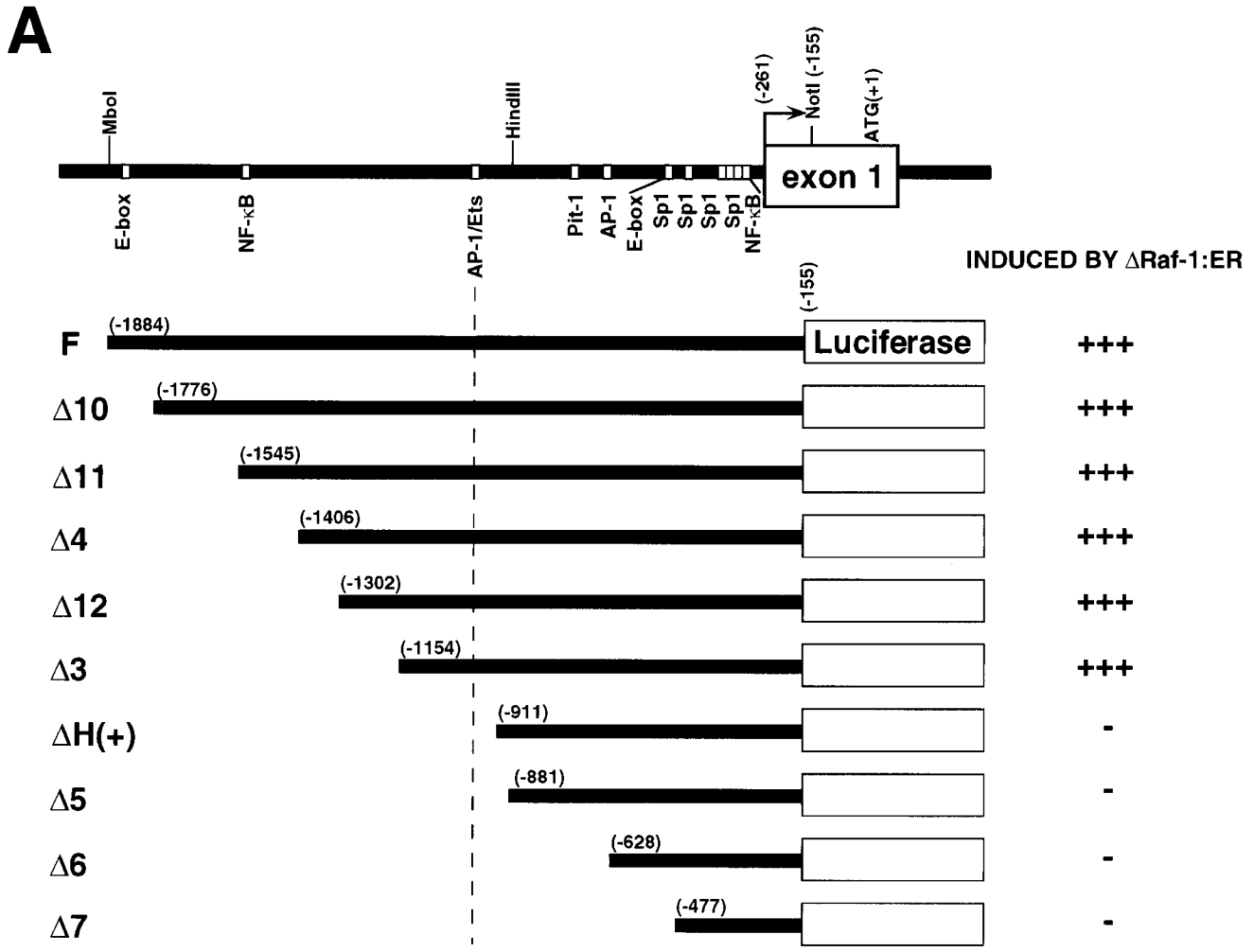
Preparation of cytosolic and nuclear extracts for Western analysis. Cell extracts were prepared by lysis of cells in Gold lysis buffer containing protease and phosphatase inhibitors as previously described (60). The pelleted Triton X-100-insoluble fraction was reextracted in boiling radioimmunoprecipitation assay buffer (Gold lysis buffer containing 0.1% [wt/vol] sodium dodecyl sulfate [SDS] and 0.5% [wt/vol] sodium deoxycholate) to yield a crude nuclear fraction. Following extraction in radioimmunoprecipitation assay buffer, insoluble material was removed by ultracentrifugation in a Beckman TL-100 ultracentrifuge at 30,000 rpm for 30 min.

Ets-2 Pointed domain expression and purification. *Nde*I-*Bam*HI PCR fragments encoding amino acids 59 to 177, encompassing the Pointed domain of Ets-2 and encoding either a threonine (Pd-T72) or an alanine (Pd-A72) in position 72, were isolated and subcloned into pET15b for expression as polyhistidine fusion proteins in *Escherichia coli* BL21 under the control of IPTG (isopropyl-β-D-thiogalactopyranoside). The Pointed domains were purified by standard techniques on a 20-ml Ni-nitrilotriacetic acid column with 10 to 100 mM imidazole to elute the proteins. Fractions containing the Pointed domains were pooled, dialyzed extensively, and snap frozen for long-term storage. The yields of Pd-T72 and Pd-A72 were 5.7 and 3.0 mg/liter, respectively, with purities of ~90 and ~93%, respectively, as estimated by Coomassie blue staining.

Ets-2 Pointed domain in-gel protein kinase assay. The in-gel protein kinase assay was carried out as has been described previously (23, 43, 60). Briefly, 100-μg aliquots of cell extracts were electrophoresed through a 12.5% polyacrylamide gel containing 250 to 500 μg of either purified Pd-T72 or Pd-A72 per ml as described above which had been copolymerized into the gel. After electrophoresis, the gel was subjected to a denaturation-renaturation procedure, and in-gel kinase reactions were performed exactly as described previously (60). The gel was dried and exposed to X-ray film for 24 to 48 h.

p42 MAP kinase immunoprecipitation and immune-complex kinase assays. Immune-complex MAP kinase assays using the Ets-2 Pointed domain as a substrate were conducted exactly as previously described, except that instead of using 20 μg of myelin basic protein as a substrate we used 1 μg of Pd-T72 or Pd-A72 as substrates (60). The products of the kinase reaction were subjected to polyacrylamide gel electrophoresis followed by Western blotting and were quantitated with a Molecular Dynamics PhosphorImager. Alternatively, the Western blots were probed with an antiserum that specifically recognizes Ets-2 only when phosphorylated on threonine residue 72 (anti-PT72), the isolation of which is described elsewhere (52a). The rabbit polyclonal antibody that recognizes the Ets-2 protein was generated against a TrpE fusion protein containing Ets amino acids 163 to 331. This region does not include the Pointed or Ets domains and has not been found to cross-react with other Ets family proteins (26a).

p42-p44 MAP kinase immunodepletion. Cell extracts were freshly prepared from cells expressing ΔRaf-1:ER that were either untreated or treated with ICI 164,384 for 1 h. These extracts were subjected to four successive rounds of immunodepletion in a total volume of 25 μl with either normal rabbit serum or a mixture of antisera raised against p42 and p44 MAP kinases, and immune complexes were collected. Each of the successive immunoprecipitates and the remaining soluble fractions was assayed for the presence of kinase activity specific for threonine residue 72 of Ets-2, using 1 μg of either Pd-T72 or Pd-A72 and [γ-^{32}P]ATP as substrates as described above. The products of the kinase reaction were subjected to polyacrylamide gel electrophoresis followed by Western blotting and were quantitated with a Molecular Dynamics PhosphorImager. Following quantitation, the Western blots were probed with antisera that recognize p42 and p44 MAP kinases to demonstrate the presence of these proteins in either the immune complexes or soluble fractions.



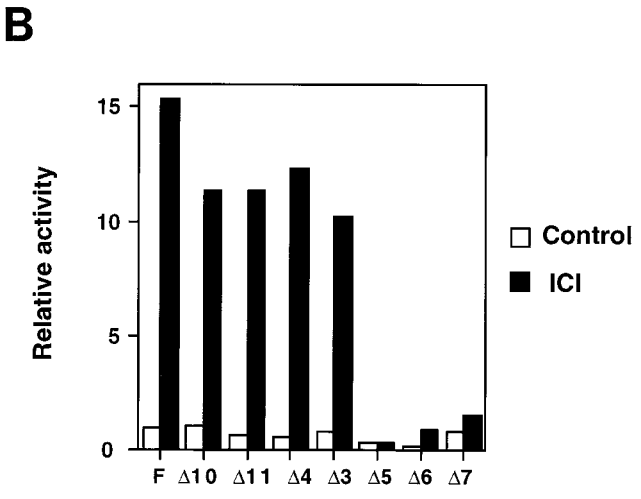


FIG. 1. Deletion analysis of the HB-EGF promoter. (A) Structures of the HB-EGF promoter region as defined previously (8) and the deletion mutants generated in this work. The positions of putative transcription factor binding sites, the transcription initiation site (−261), and the methionine that is used to initiate translation [ATG(+1)] are indicated. Deletions were prepared by PCR amplification from a luciferase reporter construct, pGL-HB-EGF, containing the full-length (F) HB-EGF promoter comprising sequences between the indicated 5′ *Mbo*I site and a *Not*I site at −155 in exon 1. For reference purposes, the relative position of the AP-1/Ets site is indicated by the dotted line. (B) Activities of the various HB-EGF promoter deletion mutants in transiently transfected C2 cells. Constructs were transiently transfected into C2 cells, and after 24 h the cells were stimulated with either 0.1% (vol/vol) ethanol (solvent control) or 1 μM ICI 164,384 to activate ΔRaf-1:ER for a further 24 h, at which time relative luciferase activity was determined as described in Materials and Methods. All of the data were normalized to the luciferase activity obtained from cells transfected with the full-length HB-EGF promoter fragment (F) in the absence of ΔRaf-1:ER activation, which was assigned an arbitrary value of 1.

RESULTS

Activation of the HB-EGF promoter by ΔRaf-1:ER requires a composite AP-1/Ets element. To investigate the mechanism by which ΔRaf-1:ER activation leads to elevated transcription of the HB-EGF gene, we transiently transfected cells expressing ΔRaf-1:ER (C2 cells) with a reporter construct containing a 1.73-kb region of the murine HB-EGF gene encompassing sequences from positions −1884 to −155 upstream of the translation initiation codon in exon 1 (Fig. 1A). This region of the HB-EGF gene, which contains putative transcription factor binding sites for NF-κB, AP-1, Ets, E-box binding factors, Pit-1, and SP-1, has previously been shown to operate as a functional promoter in myoblasts (8). In addition, we generated a series of deletion mutants to facilitate the mapping of Raf-responsive elements in the HB-EGF promoter (Fig. 1A). For comparison to the HB-EGF promoter, a reporter construct containing the promoter of the murine c-Fos gene was

also transfected into C2 cells. Activation of ΔRaf-1:ER led to a 16-fold activation of the full-length HB-EGF promoter in C2 cells (Fig. 1B). By comparison, activation of ΔRaf-1:ER stimulated the c-Fos promoter approximately 9-fold (data not shown). Both the c-Fos and HB-EGF promoters were responsive to tetradecanoyl phorbol acetate (TPA) (three- and ninefold, respectively) and were induced by ΔRaf-1:ER in the absence or in the presence of serum growth factors (data not shown).

We next utilized deletion mutants of the HB-EGF promoter to identify *cis*-acting sequences that confer Raf responsiveness on the gene. Successive deletion of the promoter from −1884 to −1154 had no discernible effect on promoter activation by ΔRaf-1:ER (Fig. 1B and data not shown). However, deletion of the promoter to position −881 resulted in an almost complete ablation of the response to ΔRaf-1:ER. Consistent with this observation, further deletions through to positions −628 and −477 were largely unresponsive to ΔRaf-1:ER activation (Fig. 1B). Interestingly, basal promoter activity was still detected even in those deletion constructs that were no longer responsive to ΔRaf-1:ER activation. Consistent with these observations, digestion of the promoter with *Hin*dIII, which cleaves at position −911, generated a promoter fragment that was also nonresponsive to ΔRaf-1:ER activation (data not shown). These results suggested that sequences between positions −911 and −1154 are required for full activation of the HB-EGF promoter by ΔRaf-1:ER. We also tested the responsiveness of the HB-EGF promoter to serum stimulation, since HB-EGF mRNA is serum responsive in NIH 3T3 cells (46). Serum stimulation of HB-EGF promoter activity also showed dependence on sequences located between positions −881 and −1154 (data not shown).

We next investigated whether specific transcription factor binding sites between positions −881 and −1154 were required for induction of the HB-EGF promoter by ΔRaf-1:ER. As indicated in Fig. 1A, DNA sequence analysis of the murine HB-EGF promoter had identified a composite AP-1/Ets site located between positions −988 and −974 (8). Composite AP-1/Ets elements have been implicated in the responsiveness of a number of viral and cellular genes to oncogenes, notably Ras and Raf (4, 6, 57, 72). We therefore introduced mutations into the Δ12 (−1302) deletion construct to ablate either the AP-1 site, the Ets site, or both sites (Fig. 2A). These mutant constructs were then tested for their responsiveness to ΔRaf-1:ER by transfection into C2 cells as described above (Fig. 2B). The Δ12 construct retained full responsiveness to activation by ΔRaf-1:ER similar to that observed with the 1.8-kb promoter fragment. Point mutations of either the AP-1 site, the Ets site, or both sites resulted in an almost complete loss of responsiveness to ΔRaf-1:ER activation. Consistent with the deletion analysis, basal promoter activity was largely unaffected by these mutations. The small residual responsiveness to ΔRaf-1:ER of the mutated HB-EGF promoter may be a consequence of the presence of upstream sites for transcription factors such as AP-1 and NF-κB. It is clear, however, that full transcriptional activation of the HB-EGF promoter requires the presence of the composite AP-1/Ets binding site described above.

Occupancy of the AP-1/Ets site in the HB-EGF promoter in vivo. In order to provide further evidence for the role of the AP-1/Ets site identified above in mediating the activation of HB-EGF expression, we conducted an in vivo genomic footprinting analysis of the HB-EGF promoter (20, 21). C2 cells, either untreated or treated with 1 μM ICI 164,384 for different periods of time, were treated with DMS, and genomic DNA was isolated as described in Materials and Methods. As a control for unprotected DNA, we isolated naked genomic DNA from C2 cells and subjected it to DMS treatment in vitro. As a further control, the mouse T-cell line EL4, which expresses little HB-EGF mRNA, was treated with DMS. Genomic footprinting analysis was conducted as described in Materials and Methods.

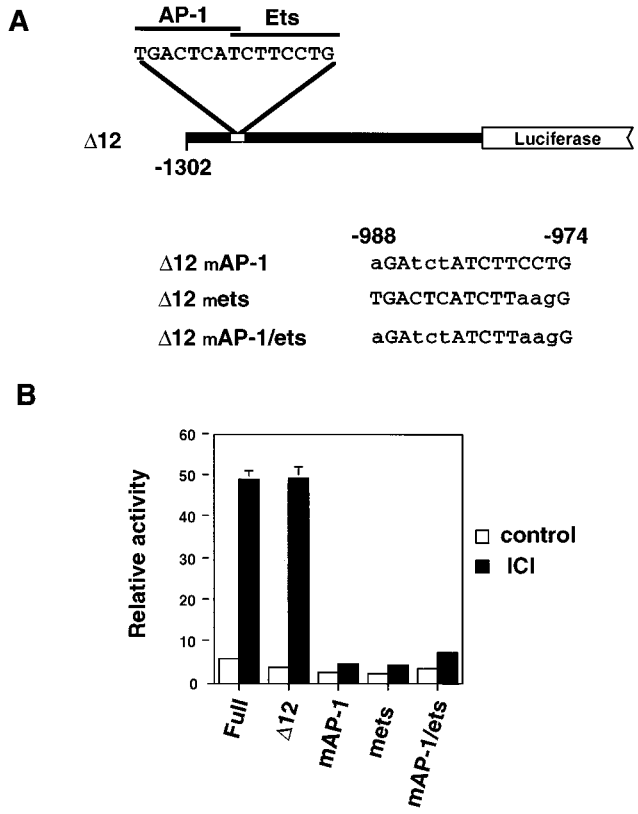


FIG. 2. Point mutation of an AP-1/Ets element within the HB-EGF promoter abrogates responsiveness to ΔRaf-1:ER activation. (A) The HB-EGF promoter contains a composite AP-1/Ets site at positions −988 to −974. The indicated mutations were introduced into either the AP-1 site (Δ12 mAP-1), the Ets site (Δ12 mets), or both sites (Δ12 mAP-1/ets) in the context of the Δ12 deletion (−1302), which retained full responsiveness to ΔRaf-1:ER. (B) Activities of the mutated constructs. Constructs were transiently transfected into C2 cells, and 1 day later the cells were stimulated with either 0.1% (vol/vol) ethanol (solvent control) or 1 μM ICI 164,384 to activate ΔRaf-1:ER for a further 24 h, at which time relative luciferase activity was determined. Full, full-length promoter; Δ12, −1302 deletion; mAP-1, mutated AP-1 site; mets, mutated Ets site; mAP-1/ets, mutated AP-1 and Ets sites. All of the data were normalized to the luciferase activity obtained from cells transfected with the full-length HB-EGF promoter fragment in the absence of ΔRaf-1:ER activation, which was assigned an arbitrary value of 5.

In vivo protein-DNA interactions render guanosine residues in transcription factor binding sites either hypo- or hypersensitive to DMS-mediated methylation. Subsequent piperidine cleavage and ligation-mediated PCR allow the generation of a DNA sequence ladder that is specific for the presence of methylated guanosine residues, thus allowing the comparison of the footprinting patterns of in vitro DMS-treated DNA (naked DNA) and DNA from cells treated with DMS in vivo as shown in Fig. 3. We detected differences in the DNA sequence ladders corresponding to the aforementioned AP-1/Ets site between naked DNA (lanes 1 and 8) and DNA isolated from untreated C2 cells (compare lane 2 with lanes 1 and 8). This analysis indicated that the two guanosines in the AP-1 site and the two guanosines in the adjacent Ets site as indicated in Fig. 3 were protected compared to naked genomic DNA isolated

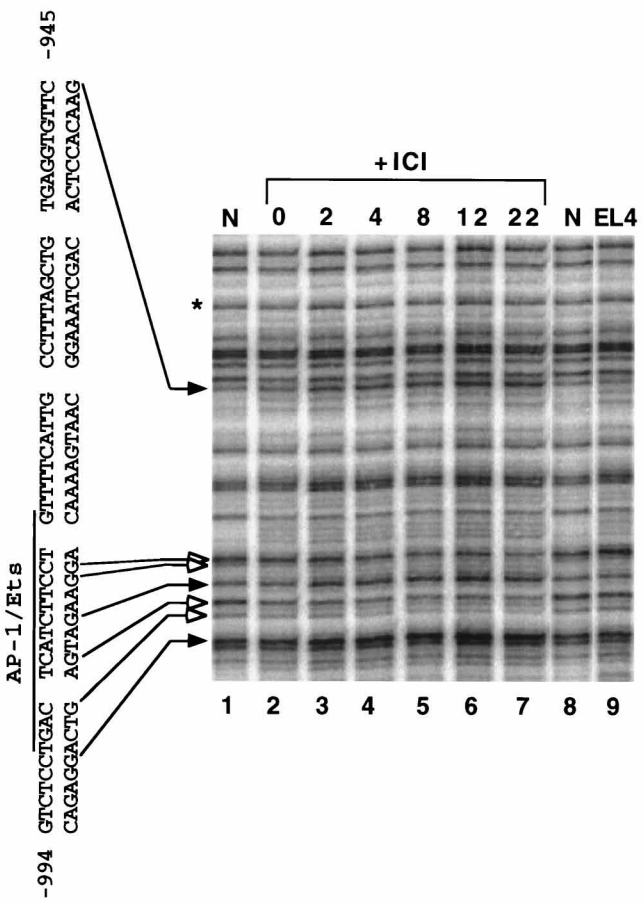


FIG. 3. Genomic footprint analysis of the AP-1/Ets element of the HB-EGF promoter. C2 cells (2×10^7 cells per time point) were either untreated (lane 2) or treated with 1 μM ICI for 2, 4, 8, 12, or 22 h, at which point they were exposed to 0.1% (vol/vol) DMS for 2 min at 37°C and washed twice with warm PBS, and then genomic DNA was isolated as described in Materials and Methods. Naked genomic DNA (lanes 1 and 8) was prepared in parallel from C2 cells and subjected to in vitro methylation with 0.1% (vol/vol) DMS for 2 min at room temperature and then ethanol precipitated. As an additional control, the mouse T-cell line EL4 was treated with 0.1% (vol/vol) DMS in vivo, and genomic DNA was isolated (lane 9). After piperidine cleavage and ligation-mediated PCR with HB-EGF gene-specific primers, the ^{32}P-labeled PCR products were separated on a 6% polyacrylamide sequencing gel. Quantitation of band intensity was performed with a Molecular Dynamics PhosphorImager. The intensity of each band was quantitated by normalization to a band that displayed an invariant signal intensity (indicated by an asterisk). Footprints are indicated by linking the G residue in the ladder to the corresponding sequence displayed at the side. Filled arrowheads indicate ΔRaf-1:ER-induced hypersensitivity, and open arrowheads indicate ΔRaf-1:ER-induced protection.

from C2 cells. These protections were not due to intrinsic variations between naked DNA and in vivo DMS-treated DNA, because DNA isolated from DMS-treated EL4 cells showed no sign of protection of the AP-1/Ets site compared to naked DNA from C2 cells (Fig. 3, lanes 8 and 9). After ΔRaf-1:ER activation, we detected changes in the pattern of protected guanosines in the AP-1/Ets element. All four of the guanosines described above displayed a greater extent of protection from methylation that increased with time following ΔRaf-1:ER activation. In addition, the guanosine residue located between the AP-1 and Ets sites as indicated in Fig. 3 became more sensitive to DMS methylation after ΔRaf-1:ER activation, as demonstrated by enhanced intensity of the indicated band. Two additional guanosine residues displayed increased sensitivity to DMS methylation. One is located immediately upstream of the AP-1 site and the other is located ~28 bp downstream of the Ets site (Fig. 3, lanes 3 to 7). All of the changes in intensity of genomic footprints were observed as early as 2 h after ΔRaf-1:ER activation and became more intense with time thereafter. Although the changes in the genomic footprint are undoubtedly subtle, qualitatively similar results were obtained in at least three independent experiments.

Ets-2 and MAP kinase potentiate HB-EGF promoter activation. Previous work has demonstrated synergistic activation of an AP-1/Ets site in the urokinase-type plasminogen activator (uPA) promoter by Ras in combination with either Ets-1 or Ets-2. Synergy requires the Ras-induced phosphorylation of a conserved threonine residue within the Pointed domain of the Ets proteins (63, 73). In order to explore a possible role for these proteins in the activation of the HB-EGF promoter by ΔRaf-1:ER, we investigated whether the addition of vectors expressing either normal p44 MAP kinase (p44) or catalytically inactive p44 MAP kinase (p44KR) along with normal (T72) or mutated (A72) Ets-2 to the transient transfection assays had an effect on the activation of the HB-EGF promoter by ΔRaf-1:ER (Fig. 4A). The expression of proteins encoded by these constructs in mammalian cells has previously been well documented (63, 73). Cotransfection of vectors encoding either p44 MAP kinase (p44) or catalytically inactive p44 MAP kinase (p44KR) had no effect on the 15-fold activation of the HB-EGF promoter (Δ12; −1302) ΔRaf-1:ER (Fig. 4A). Cotransfection of a vector encoding Ets-2, but not the A72 form of the protein, led to a 30-fold activation of the HB-EGF promoter. However, cotransfection of p44 MAP kinase and Ets-2 resulted in a 50-fold activation of the reporter construct. This potentiated activation was not observed upon transfection of a catalytically inactive form of p44 MAP kinase (p44KR) with Ets-2, indicating that p44 MAP kinase catalytic activity is required for the potentiation of reporter activity. Moreover, wild-type p44 MAP kinase failed to potentiate reporter activation when transfected with the alanine 72 form of Ets-2 (T72A). PEA3, which does not possess a Pointed domain (69), did not potentiate promoter activity either alone or in combination with p44 MAP kinase. Taken together, these data suggest a simple model in which the phosphorylation of Ets-2 on threonine 72 by MAP kinase is involved in the transcriptional activation of the HB-EGF promoter in response to ΔRaf-1:ER activation.

HB-EGF gene expression is induced by both the Raf and Ras oncogenes, so we tested whether promoter activation could be potentiated by oncogenic H-Ras(61L) (46). Transfection of the full-length HB-EGF promoter linked to luciferase gave a low level of reporter activity that was potentiated eight- to ninefold by cotransfection with a vector encoding oncogenic H-Ras(61L) (Fig. 4B). Cotransfection of Ets-2 with H-Ras(61L) led to a 28-fold activation of the HB-EGF promoter, which was not observed with the A72 form of Ets-2.

Although many Ras-responsive genes are regulated by AP-1/Ets elements, the promoters of at least two Ras-responsive genes, stromelysin and *junB*, are regulated by tandem inverted Ets transcription factor binding sites with no apparent role for AP-1 (10, 18, 70). We therefore tested whether ΔRaf-1:ER could activate expression of the synthetic Ras-responsive reporter construct E18-luciferase, which contains tandem Ets binding sites inserted adjacent to a minimal promoter (18). Activation of ΔRaf-1:ER gave rise to a sevenfold induction of the E18-luciferase reporter construct (Fig. 4C). Cotransfection with the Ets-2 expression vector gave rise to a slight increase in basal promoter activity, but activation of ΔRaf-1:ER gave rise to a 24-fold activation that was not observed with the A72 form

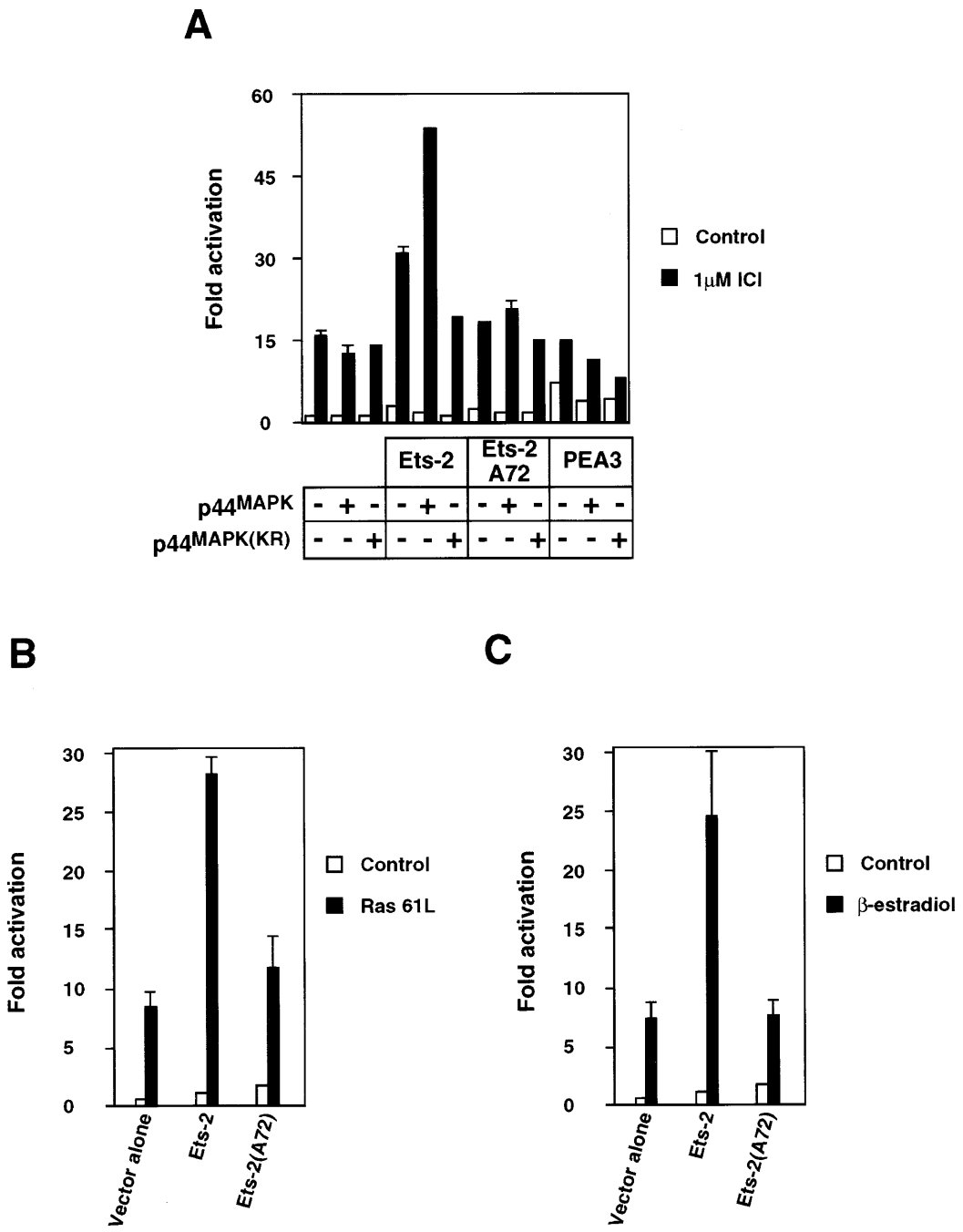


FIG. 4. Synergistic activation of the HB-EGF promoter by Ets-2 and p44 MAP kinase. (A) C2 cells were transiently transfected with the full-length HB-EGF promoter construct and the indicated combinations of expression plasmids encoding either catalytically active p44 MAP kinase ($p44^{MAPK}$), catalytically inactive p44 MAP kinase [$p44^{MAPK(KR)}$], wild-type mouse Ets-2 (Ets-2), wild-type mouse Ets-2 encoding alanine at position 72 (Ets-2 A72), or wild-type mouse PEA3 (PEA3). Twenty-four hours after transient transfection, the cells were stimulated with either 0.1% (vol/vol) ethanol (solvent control) or 1 μM ICI 164,384 to activate ΔRaf-1:ER for a further 24 h, at which time relative luciferase activity was determined. All of the data were normalized to the luciferase activity obtained from cells transfected with the full-length HB-EGF promoter construct in the absence of ΔRaf-1:ER activation, which was assigned an arbitrary value of 1. (B) C2 cells were transiently transfected by a modified calcium phosphate protocol with 250 ng of HB-EGF promoter-luciferase construct and 500 ng of either empty expression vector (vector alone) or an expression vector encoding H-Ras(61L). Cells were also cotransfected with either 12.5 ng of wild-type mouse Ets-2 (Ets-2) or wild-type mouse Ets-2 encoding alanine at position 72 [Ets-2(A72)] as described previously. Luciferase activity was measured 48 h after transfection. The data were normalized to the luciferase activity obtained from cells transfected with the full-length HB-EGF promoter construct in the absence or in the presence of the backbone vector used to express H-Ras(61L), which was assigned an arbitrary value of 1. (C) C2 cells were transiently transfected by a modified calcium phosphate protocol with 375 ng of the E18-luciferase promoter in which luciferase expression is promoted by a tandem Ets binding site (18, 27, 73) either alone or with 12.5 ng of expression vectors encoding wild-type mouse Ets-2 (Ets-2) or mouse Ets-2 encoding alanine at position 72 [Ets-2(A72)] as described above. Twenty-four hours after transient transfection, the cells were stimulated with either 0.1% (vol/vol) ethanol (solvent control) or 1 μM β-estradiol to activate ΔRaf-1:ER for a further 24 h, at which time luciferase activity was measured and fold activation relative to that with the reporter alone was determined. All of the data were normalized to the luciferase activity obtained from cells transfected with the E18-luciferase construct in the absence of ΔRaf-1:ER activation, which was assigned an arbitrary value of 1.

of the protein, consistent with the results obtained with the HB-EGF promoter in Fig. 4A.

Phosphorylation of threonine 72 of Ets-2 by p42 and p44 MAP kinases in vitro. Although potentiated activation of AP-1/Ets elements by Ras and Raf oncogenes appears to be mediated by phosphorylation of threonine 72 of Ets-2 (73), the kinase(s) responsible for this phosphorylation has not been identified. Given the potentiation of Raf-induced HB-EGF promoter activity by Ets-2 and MAP kinase, we next investigated whether the MAP kinases could phosphorylate threonine 72 of Ets-2 in vitro.

The Pointed domain of Ets-2 (Pd-T72) and a mutant Pointed domain in which threonine 72 was mutated to alanine (Pd-A72) were expressed in *E. coli* and used as substrates for in-gel kinase assays (23, 43, 60) to determine the nature of the major Ets-2 T72 kinases in C2 cells following ΔRaf-1:ER activation (Fig. 5A). Extracts were prepared from either quiescent C2 cells or C2 cells in which ΔRaf-1:ER was activated for 72 h (Fig. 5A, lane QI_{72}) or that had been stimulated with serum (lane QS) or TPA (lane QP) as described in Materials and Methods and as indicated. Two major Pd-T72 kinases of 42 and 44 kDa were activated in quiescent C2 cells following activation of ΔRaf-1:ER or stimulation with serum or TPA. Time course analysis indicated that both the 42- and 44-kDa kinases were detectably stimulated within 6 h after ΔRaf-1:ER activation and that their activity continued to increase for at least 48 h (Fig. 5A, lanes +ICI). Furthermore, the activity of these protein kinases was significantly potentiated when ΔRaf-1:ER was activated in the presence of sodium orthovanadate (Fig. 5A, lanes $+VO_4$ +ICI), a treatment that potentiates ΔRaf-1:ER-mediated MAP kinase activation (60). These data are highly consistent with the molecular masses and activation kinetics of the p42 and p44 MAP kinases in response to the various stimuli described above. Furthermore, no inducible Ets-2 kinases were observed when Pd-A72 was used as a substrate (Fig. 5B), although a single prominent band was detected that is likely due to the autophosphorylation of a protein from the cell extract, as it is observed in the absence of any substrate protein in the gel (data not shown).

The in-gel kinase assays suggested that the p42 and p44 MAP kinases are the major renaturable kinases responsible for phosphorylation of threonine 72 of Ets-2 following activation of ΔRaf-1:ER in NIH 3T3 cells. To further establish the ability of the p42 and p44 MAP kinases to phosphorylate threonine 72 of Ets-2, we prepared immunoprecipitates of p42 MAP kinase from extracts of C2 cells made at different times following ΔRaf-1:ER activation. Such immunoprecipitates were able to phosphorylate Pd-T72 but not Pd-A72 in vitro (Fig. 5C). The specific activity of p42 MAP kinase towards Pd-T72 increased approximately fivefold after 1 h of ΔRaf-1:ER activation and was maintained thereafter, consistent both with the kinetics of p42 MAP kinase activation that we have previously described and with the in-gel kinase assays described above (60).

To ensure that the site of p42 MAP kinase phosphorylation was indeed threonine 72, we performed an identical immunoprecipitation kinase assay in the presence of excess nonradioactive ATP. The reaction products were then Western blotted and probed with a phospho-T72-specific antiserum that recognizes the Pointed domain of Ets-2 only when phosphorylated on threonine 72 (Fig. 5D). A rapid and sustained increase in the ability of p42 MAP kinase to phosphorylate threonine 72 of Pd-T72 observed within 1 h after ΔRaf-1:ER activation was consistent with the phosphorylation of Pd-T72 observed in the experiments whose results are shown in Fig. 5A and C.

The in-gel and immunoprecipitation kinase assays indicated that the p42 and p44 MAP kinases are capable of phosphorylating Ets-2 on threonine 72, but there may be additional Raf-activated kinases that can do so. In order to address this issue, we conducted an immunodepletion experiment to determine if the p42 and p44 MAP kinases are the major Raf-activated Pd-T72 kinases in extracts of NIH 3T3 cells. Fresh cell extracts were prepared from C2 cells that were either untreated (Fig. 6A to D, lanes 1, 3, 5, and 7) or treated with ICI 164,384 to activate ΔRaf-1:ER for 1 h (Fig. 6A to D, lanes 2, 4, 6, and 8). These extracts were subjected to four successive rounds of immunoprecipitation with either a mixture of anti-p42 and anti-p44 MAP kinase antisera (anti-p42/p44 MAP kinase) (Fig. 6A and C) or normal rabbit serum (Fig. 6B and D). The kinase activity of the immunoprecipitates (Fig. 6A and B) and the remaining soluble proteins (Fig. 6C and D) was assessed with either Pd-T72 or Pd-A72 (data not shown) as substrates as described in Materials and Methods.

After 1 round of anti-p42/p44 MAP kinase immunoprecipitation, the majority of the Raf-activated Pd-T72 kinase activity was detected in the immunoprecipitates, and only a small amount of activity remained in the soluble fraction (Fig. 6A and C). This remaining activity was removed by subsequent rounds of immunoprecipitation (Fig. 6C, lanes 3 to 8). The residual phosphorylation observed in the soluble fraction after the third and fourth rounds of immunoprecipitation was not Raf dependent and was identical to the background level of phosphorylation observed in this experiment when Pd-A72 was used as a substrate (Fig. 6C, lanes 5 to 8 and data not shown). Probing these Western blots with anti-MAP kinase antisera revealed that greater than 95% of the p42 and p44 MAP kinases was removed from the cell extract after one round of immunodepletion and that the remainder was removed following the second round (data not shown), indicating that the extent of phosphorylation of Pd-T72 correlated well with the presence of p42 and p44 MAP kinases in the different samples.

Little or no Pd-T72 kinase activity was detected in immunoprecipitates of cell extracts made with normal rabbit serum (Fig. 6B). In this case, all of the Raf-activated Pd-T72 kinase activity remained in the soluble fraction of the cell extract and was largely unaffected by the immunodepletion procedure (Fig. 6D, lanes 2, 4, 6, and 8). Probing of these Western blots with the anti-p42/p44 MAP kinase antisera revealed that 95% of the p42 and p44 MAP kinases remained in the soluble material after four successive rounds of immunoprecipitation with normal rabbit serum (data not shown).

Rapid phosphorylation of threonine 72 of Ets-2 following ΔRaf-1:ER activation in intact cells. The stringent control of the MAP kinase pathway afforded by cells expressing ΔRaf-1:ER allowed a kinetic analysis of the phosphorylation of Ets-2 on threonine 72. Activation of ΔRaf-1:ER leads to activation of the MAP kinases within minutes and to the appearance of mature HB-EGF mRNA within 1 to 2 h (46, 60). We would therefore predict that, if phosphorylation of Ets-2 plays a role in induced HB-EGF gene expression, it should occur with kinetics that mirror the activation of MAP kinase and the induction of HB-EGF mRNA. We therefore prepared nuclear extracts of C2 cells as indicated in the legend to Fig. 7, in which ΔRaf-1:ER was either inactive (lane 0) or had been activated for 30 min or 1, 2, or 24 h. A Western blot of these lysates was prepared and probed with the antiserum raised against threonine 72-phosphorylated Ets-2 as described in Materials and Methods. Prior to ΔRaf-1:ER activation, there was a low basal level of threonine 72-phosphorylated Ets-2 that was significantly increased within 30 min and was maintained for at least 24 h following ΔRaf-1:ER activation (Fig. 7A). Although we also detected an increase in Ets-2 protein expression after 24 h of ΔRaf-1:ER activation (Fig. 7B), no change was detectable at

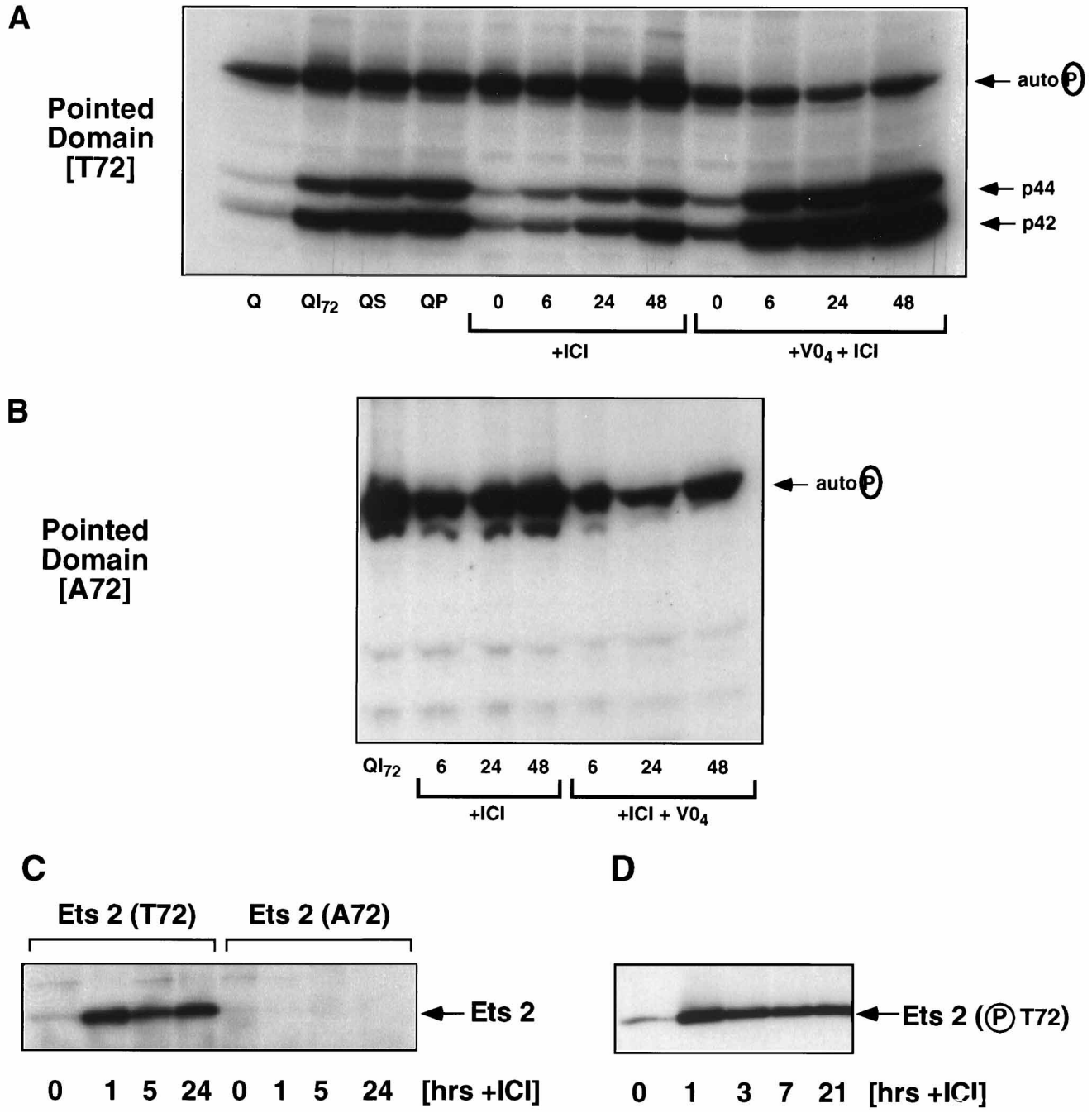


FIG. 5. Threonine residue 72 of Ets-2 is a target for p42-p44 MAP kinase-mediated phosphorylation in vitro. (A and B) In-gel assay for protein kinases able to phosphorylate threonine 72 in the Pointed domain of Ets-2 (Pd-T72). Two polyacrylamide gels that contained (per milliliter) 500 μg of the Pointed domain of Ets-2 encoding either a threonine (Pd-T72) (A) or an alanine (Pd-A72) (B) at amino acid 72 were prepared. Cell extracts were prepared from quiescent C2 cells that were either unstimulated (lane Q) or stimulated with either 1 μM ICI 164,384 for 72 h (lane QI72), 20% serum for 15 min (lane QS), or 50 nM TPA for 10 min (lane QP). Additional extracts were prepared from C2 cells grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum that were either untreated (lane 0) or treated with 1 μM ICI 164,384 to activate ΔRaf-1:ER for 6, 24, or 48 h in the absence (+ICI) or in the presence ($+VO_4$+ICI) of 50 μM sodium orthovanadate. After the lysates were electrophoresed, the gel was subjected to successive denaturation and renaturation steps to detect protein kinases able to phosphorylate Pd-T72 (A) but not Pd-A72 (B). The positions of renaturable Ets-2 kinases of 42 and 44 kDa (p42 and p44, respectively) as well as a band that is believed to be due to the autophosphorylation of a cellular protein (auto P) are indicated. (C and D) p42 MAP kinase phosphorylates Ets-2 on threonine-72. (C) Cell extracts were prepared from C2 cells that were either untreated (lane 0) or treated with 1 μM ICI 164,384 for the indicated times. Immunoprecipitates of p42 MAP kinase were prepared and incubated for 30 min at 30°C in a reaction mix containing [γ-^{32}P]ATP and either Pd-T72 or Pd-A72 as substrates as indicated. Reaction products were separated by SDS-polyacrylamide gel electrophoresis, and Ets-2 phosphorylation was visualized by autoradiography. (D) Immunoprecipitates of p42 MAP kinase were prepared from either untreated C2 cells or cells treated with 1 μM ICI 164,384 for the indicated times and incubated for 30 min at 30°C in a reaction mix containing 1 mM unlabeled ATP and Pd-T72 as a substrate. Reaction products were separated by SDS-polyacrylamide gel electrophoresis and Western blotted into an Immobilon polyvinylidene difluoride membrane, and Ets-2 phosphorylation was visualized by probing with an antiserum that recognizes the threonine 72-phosphorylated Pd-T72.

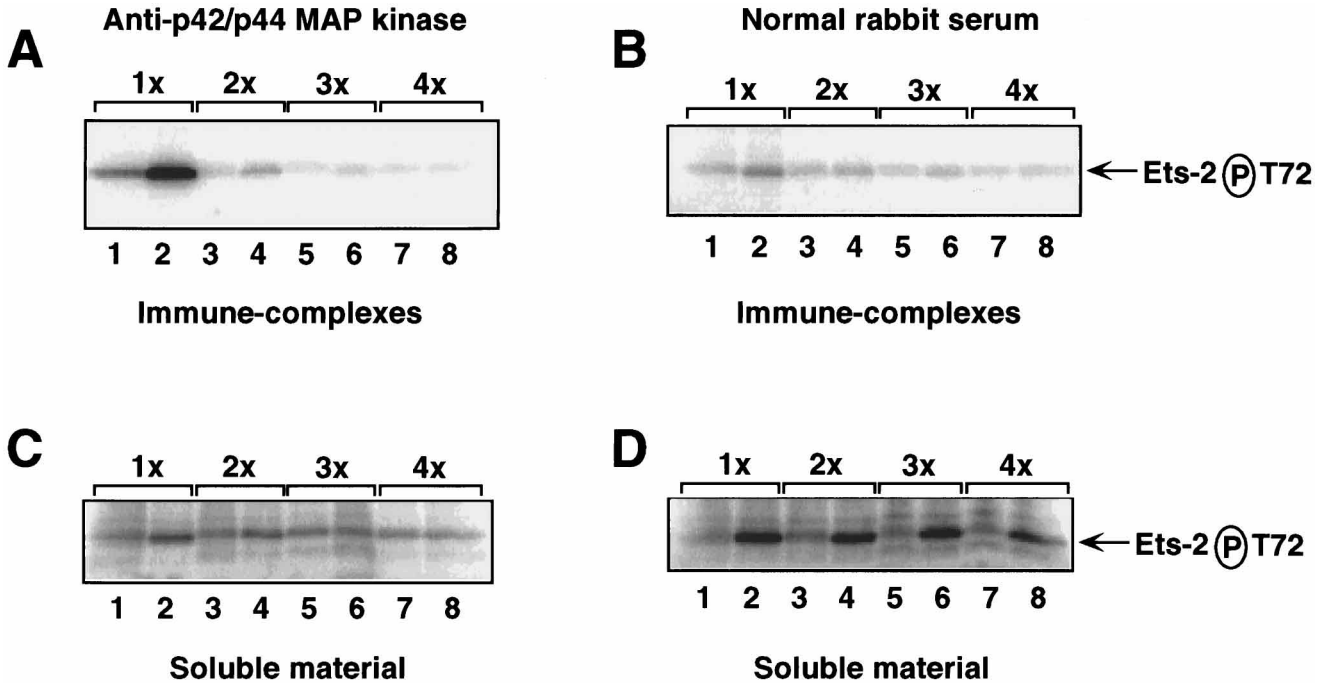


FIG. 6. Immunodepletion of Pd-T72 kinase activity from extracts of Raf-transformed cells. Extracts were prepared from NIH 3T3 cells expressing ΔRaf-1:ER that were either untreated (all lanes 1, 3, 5, and 7) or treated for 1 h with ICI 164,384 (all lanes 2, 4, 6 and 8). The cell extracts were subjected to four successive sequential rounds of immunoprecipitation (1× to 4× as indicated) with either a mixture of anti-p42 and anti-p44 MAP kinase antisera (A and C) or normal rabbit serum (B and D). Pd-T72 kinase activity was measured in the immune complexes (A and B) or in the remaining soluble fractions (C and D) as described in Materials and Methods.

early time points when the increase in Ets-2 phosphorylation was readily observed. The induction of HB-EGF mRNA by ΔRaf-1:ER occurs in the absence of de novo protein synthesis (46). Consistent with this observation, the appearance of threonine 72-phosphorylated Ets-2 was insensitive to treatment of cells with cycloheximide (data not shown). After 24 h of ΔRaf-1:ER activation, a second immunoreactive species of approximately 46 to 48 kDa, recognized by both the phospho-T72-specific and the Ets-2-specific antisera, was observed. Although the identity of this protein is unknown, we suspect that it may correspond either to an alternatively spliced form or to a proteolytic degradation product of Ets-2.

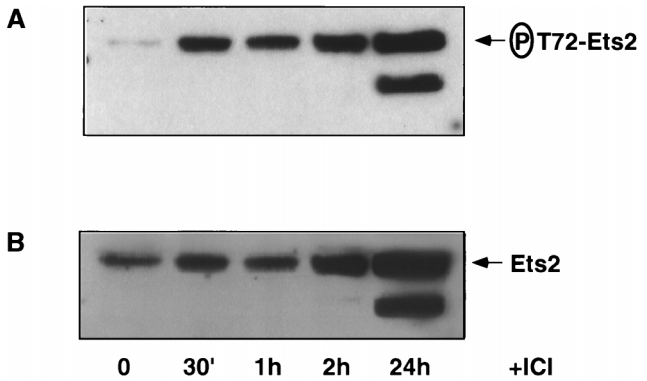


FIG. 7. Rapid phosphorylation of Ets-2 on threonine 72 following ΔRaf-1:ER activation. Quiescent C2 cells were either untreated (lane 0) or treated with 1 μM ICI 164,384 to activate ΔRaf-1:ER for 30 min or 1, 2, or 24 h as indicated. Crude nuclear extracts were prepared as described in Materials and Methods and analyzed by Western blotting and probing with either the anti-phospho-T72 Ets-2-specific antiserum (A) or an antiserum that recognizes the protein backbone of Ets-2 (B).

DISCUSSION

In order to understand the process of oncogenic transformation, we have identified genes that are regulated by the Ras-Raf signaling cascade and are trying to unravel the transcriptional machinery that underlies their regulation (66). We have previously shown that the gene encoding HB-EGF is an immediate-early transcriptional target of this signaling cascade. Given the importance ascribed to autocrine growth factors in tumorigenesis, it is important to understand the mechanisms of HB-EGF regulation in response to activation of the Ras-Raf pathway (1). In this study, we have extended our initial observations of the induction of HB-EGF mRNA to a more detailed investigation of the mechanism through which ΔRaf-1:ER activates transcription from the HB-EGF promoter.

Using transient transfection assays, we identified a composite AP-1/Ets binding site located 713 bp upstream of the transcription start site (positions −988 to −974) that was required for promoter activation by ΔRaf-1:ER. A large number of AP-1/Ets sites have been found to be important in oncogene- and receptor-mediated activation of promoters for genes encoding uPA (63), collagenase (25), transforming growth factor β (57), keratin-18 (53), the heavy chain of immunoglobulin H (24), and the polyomavirus enhancer (69). Since the positioning of AP-1/Ets elements varies considerably among responsive genes, they work as position-independent enhancers.

The AP-1/Ets element in the HB-EGF promoter most closely resembles the AP-1/Ets element found in the 5′ region of the uPA gene (63). Each is located upstream of the transcription start site, and both the AP-1 and Ets binding sites are required for responsiveness to Ras-Raf signaling. Like induction of the HB-EGF gene by ΔRaf-1:ER, induction of uPA mRNA by colony-stimulating factor 1 (CSF-1) is immediate early, and prolonged mRNA expression is observed following

CSF-1 stimulation. It has therefore been suggested that AP-1/Ets elements may mediate the sustained changes in gene expression observed in oncogene-transformed cells (30, 43).

In vivo genomic footprinting analysis revealed that the AP-1/Ets site of the HB-EGF promoter was occupied in vivo prior to ΔRaf-1:ER activation. The relatively weak footprint at the AP-1/Ets site in untreated C2 cells suggests that there is either a low-affinity protein-DNA interaction or a low stoichiometry interaction at this site prior to the activation of ΔRaf-1:ER. This observation is consistent with the fact that these cells have a low level of basal HB-EGF gene transcription prior to ΔRaf-1:ER activation. Following ΔRaf-1:ER activation, there was increased protection of both the AP-1 and the Ets sites in accord with the observed stimulation of transcription, suggesting that ΔRaf-1:ER induces the binding of transcription factors at this site. An alternative possibility is that the actual occupancy of the AP-1/Ets site is unchanged after ΔRaf-1:ER activation but that the recruitment of transcriptional coactivators or the regional structure of the chromatin around the AP-1/Ets site is altered, leading to the observed changes in DNA footprinting.

Despite the apparent importance of AP-1/Ets elements in the regulation of gene expression, the precise mechanisms through which these elements regulate transcription are not fully understood. Ets transcription factors are oncogenic in their own right and are implicated in the ability of Ras and erbB2/Neu to transform cells (19, 40, 68, 71, 72). Ets-2 acts synergistically with oncogenic Ras to activate reporter constructs containing AP-1/Ets elements. In these experiments, synergy was observed with Ets-1 and Ets-2 but not with other Ets domain-containing proteins such as Fli-1, Elf-1, or PEA3. Furthermore, in cells transiently transfected with activated Ras, Ets-2 becomes phosphorylated on threonine 72 located within the amino-terminal Pointed domain of the protein (73). In this report, we focus primarily on the role of Ets transcription factors in the activation of the HB-EGF promoter by ΔRaf-1:ER.

Activation of the HB-EGF promoter by ΔRaf-1:ER was potentiated by cotransfection with vectors encoding p44 MAP kinase and Ets-2. Potentiation was not observed with vectors encoding either catalytically inactive p44 MAP kinase or Ets-2(A72). These data suggested that phosphorylation of Ets-2 on threonine 72 may enhance its ability to stimulate transcription from the HB-EGF promoter. These data are in accord with the fact that the ability of oncogenic forms of the three different mammalian Raf kinases to activate HB-EGF gene expression in NIH 3T3 and Rat1a cells correlates with their ability to activate the MAP kinase pathway (46).

Although threonine 72 lies within a potential MAP kinase phosphorylation site, the cellular kinase(s) responsible for Ets-2 phosphorylation has not previously been identified. Using both in-gel and immunoprecipitation kinase assays as well as immunodepletion experiments, we identified the p42 and p44 MAP kinases as the major ΔRaf-1:ER-activated Ets-2(T72) kinases. Neither the JNKs-SAPKs, $p38^{HOG1}$ (52a), nor any of the D-type cyclins in complex with either cdk4 or cdk6 were able to phosphorylate threonine 72 of Ets-2 (41a) in vitro. These data strongly suggest that there is defined transcription factor substrate specificity within the family of proline-directed protein kinases. These results do not, however, rule out the possible existence of protein kinases that phosphorylate sites outside of the Pointed domain of Ets-2.

In addition to the biochemical data described above, we demonstrated that, following ΔRaf-1:ER activation in intact cells, the kinetics of phosphorylation of Ets-2 on threonine 72 closely mirrored both the activation kinetics of the p42 and p44 MAP kinases and the accumulation of HB-EGF mRNA (46, 60). Following ΔRaf-1:ER activation, we consistently detected the appearance of an altered form of Ets-2 of 46 to 48 kDa that appeared to be phosphorylated on threonine 72. A similar form of Ets-2 is also detected in response to CSF-1 stimulation of NIH 3T3 cells engineered to express the CSF-1 receptor (52a). It is not clear if this protein is a proteolytic degradation product of Ets-2 or the product of an alternatively spliced mRNA, but it is certainly possible that this protein plays a role in the regulation of transcription from Ets elements.

One of the most striking aspects of these observations is the conservation of the Raf-MEK-MAP kinase-Ets signaling pathway from simple eukaryotes to mammalian cells (28, 66, 67). In *Drosophila*, the activation of the Sevenless receptor leads, through the Ras-Raf-MEK-MAP kinase cascade, to the phosphorylation of both Pointed-P2 and the *Drosophila* homolog of c-Jun (D-Jun). Pointed-P2 is an Ets domain-containing protein that is phosphorylated on a threonine residue analogous to that described in mammalian Ets-1 and Ets-2. Pointed-P2 acts in conjunction with D-Jun to stimulate the transcription of genes such as Phyllopod that are involved in R7 photoreceptor cell specification (7, 16, 52, 66).

In *Drosophila*, there are, however, additional levels of complexity so far only hinted at in mammalian cells. In order for Pointed-P2 to activate transcription, the effects of an Ets domain-containing transcriptional repressor, Yan, have to be abrogated (56). MAP kinase-mediated phosphorylation of Yan appears to both destabilize the protein and cause its relocalization to the cytoplasm. Recently, a mammalian Ets domain-containing transcriptional repressor, Erf, has been isolated (62). Although rather dissimilar to Yan in overall sequence and organization, it has the capacity to abrogate Ets-1-induced gene expression and transformation and appears to be regulated by MAP kinase-mediated phosphorylation. We are presently analyzing C2 cells for the presence of Erf or related proteins to determine if they play a role in HB-EGF gene regulation. Furthermore, in addition to Elk-1 and Ets-2, an additional Ets transcription factor, ER81, has recently been shown to be responsive to MAP kinase activation; therefore, it is likely that multiple Ets family members may be responsive to the Ras-Raf-MEK-MAP kinase signaling module (33, 49).

Induction of the HB-EGF promoter through the AP-1/Ets element required the presence of binding sites for both Ets and AP-1. Although the role of Fos and Jun in this system remains unclear, it is well established that the genes encoding members of the Fos and Jun family are transcriptionally activated by Ras and Raf and that Ras-mediated transformation requires the expression of c-*jun* (4, 10, 34). Cycloheximide experiments have, however, indicated that de novo protein synthesis is not required for the early phase of HB-EGF mRNA induction (up to 8 h), but these experiments cannot address the sustained phase of HB-EGF expression (>8 h) due to the toxic effects of cycloheximide. It is, therefore, possible that the early phase of HB-EGF gene transcription is mediated by phosphorylated Ets-2 working either alone or in combination with the low level of constitutively expressed AP-1 in the cell. The sustained phase of induction may be potentiated both by the induction of AP-1 components and the autocrine activation of the JNKs that occur 16 to 24 h after ΔRaf-1:ER activation (46).

The ability of Ets proteins to activate transcription does not, however, uniformly rely on the presence of adjacent AP-1 transcription factor binding sites (68). The induction of the rat stromelysin and the murine *junB* genes by Ras is mediated by tandem Ets binding sites in the genes' promoters (10, 18, 70). Indeed, reporter constructs containing tandem Ets binding sites were efficiently activated by oncogenic Ras and ΔRaf-

1:ER and required the presence of threonine 72 to do so (Fig. 4B and C) (18).

The consequences of MAP kinase-mediated Ets-2 phosphorylation for transcriptional transactivation are not yet clear. Since the major transcription transactivation domains of Ets-2 map to either side of the Pointed domain (68), it is possible that the phosphorylation of threonine 72 leads to a conformational change that unmasks these activation domains. Indeed, Ets-2 transactivation is increased by Ras, since a fusion protein consisting of the amino terminus of Ets-2 fused to a heterologous DNA binding domain is responsive to Ras and requires the presence of threonine 72 for this response (19a). Alternatively, phosphorylated Ets-2 may recruit a transcriptional coactivator in a manner analogous to the binding of CBP to phosphorylated serine 133 of CREB (9, 54). These models are presently being investigated.

In summary, we have demonstrated a requirement for an AP-1/Ets element in the regulation of HB-EGF expression by oncogenic Raf. We have demonstrated rapid phosphorylation of threonine 72 of Ets-2 following ΔRaf-1:ER activation and implicated the p42 and p44 MAP kinases in this process. It remains to be demonstrated, however, that the MAP kinases phosphorylate Ets-2, leading to HB-EGF promoter activation in the intact cell. Nonetheless, we believe that these data provide compelling evidence that Ets-2 is a direct target of the Raf-MEK-MAP kinase cascade and that the regulation of Ets transcription factors likely plays an important role in the induction of gene expression that occurs in response to oncogene activation.

ACKNOWLEDGMENTS

We thank Esteban Masuda, Douglas Woods, Natasha Aziz, Elizabeth Bosch, Mike Samuels, and Emma Lees for helpful comments and advice; Dan Mahony and Emma Lees for the cyclin D-cdk4 and cdk6 assays; Mary Weiss and Satish Menon for expert assistance with protein purification; Melanie Cobb for the p44 MAP kinase expression plasmids; Lori Nelsen for expert technical assistance; and Laura Routhier and Leah Anderson for help in the preparation of the figures and the manuscript, respectively.

This work was supported by NCI grants CA53271 (M.C.O.) and CA63130 (C.A.H.). DNAX Research Institute is supported by Schering Plough Corporation.

REFERENCES

1. **Aaronson, S. A.** 1991. Growth factors and cancer. Science **254:**1146–1153.
2. **Ambrosio, L., A. P. Mahowald, and N. Perrimon.** 1989. Requirement of the Drosophila raf homologue for torso function. Nature **342:**288–291.
3. **Bishop, J. M.** 1995. Cancer: the rise of the genetic paradigm. Genes Dev. **9:**1309–1315.
4. **Bortner, D. M., S. J. Langer, and M. C. Ostrowski.** 1993. Non-nuclear oncogenes and the regulation of gene expression in transformed cells. Crit. Rev. Oncog. **4:**137–160.
5. **Bos, J. L.** 1989. Ras oncogenes in human cancer: a review. Cancer Res. **49:**4682–4689.
6. **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.
7. **Chang, H. C., N. M. Solomon, D. A. Wassarman, F. D. Karim, M. Therrien, G. M. Rubin, and T. Wolff.** 1995. phyllopod functions in the fate determination of a subset of photoreceptors in Drosophila. Cell **80:**463–472.
8. **Chen, X., G. Raab, U. Deutsch, J. Zhang, R. M. Ezzell, and M. Klagsbrun.** 1995. Induction of heparin-binding EGF-like growth factor expression during myogenesis. Activation of the gene by MyoD and localization of the transmembrane form of the protein on the myotube surface. J. Biol. Chem. **270:**18285–18294.
9. **Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy, and R. H. Goodman.** 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature **365:**855–859.
10. **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.
11. **Coso, O. A., M. Chiariello, J. C. Yu, H. Teramoto, P. Crespo, N. Xu, T. Miki, and J. S. Gutkind.** 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell **81:**1137–1146.
12. **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.
13. **Derijard, B., M. Hibi, I. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R. J. Davis.** 1994. JNK1: a protein kinase stimulated by UV light and Ha-ras that binds and phosphorylates the c-jun activation domain. Cell **76:**1025–1037.
14. **Dickson, B., and E. Hafen.** 1994. Genetics of signal transduction in invertebrates. Curr. Opin. Genet. Dev. **4:**64–70.
15. **Dickson, B., F. Sprenger, D. Morrison, and E. Hafen.** 1992. Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. Nature **360:**600–602.
16. **Dickson, B. J., M. Dominguez, D. S. A. Van, and E. Hafen.** 1995. Control of Drosophila photoreceptor cell fates by phyllopod, a novel nuclear protein acting downstream of the Raf kinase. Cell **80:**453–462.
17. **Egan, S. E., and R. A. Weinberg.** 1993. The pathway to signal achievement. Nature **365:**781–783.
18. **Galang, C. K., C. J. Der, and C. A. Hauser.** 1994. Oncogenic Ras can induce transcriptional activation through a variety of promoter elements, including tandem c-Ets-2 binding sites. Oncogene **9:**2913–2921.
19. **Galang, C. K., R. J. Garcia, P. A. Solski, J. K. Westwick, C. J. Der, N. N. Neznanov, R. G. Oshima, and C. A. Hauser.** 1996. Oncogenic Neu/ErbB-2 increases ets, AP-1, and NF-kappaB-dependent gene expression, and inhibiting ets activation blocks Neu-mediated cellular transformation. J. Biol. Chem. **271:**7992–7998.

19a. **Garcia-Ramirez, J., and C. Hauser.** Unpublished observations.

20. **Garrity, P. A., D. Chen, E. V. Rothenberg, and B. J. Wold.** 1994. Interleukin-2 transcription is regulated in vivo at the level of coordinated binding of both constitutive and regulated factors. Mol. Cell. Biol. **14:**2159–2169.
21. **Garrity, P. A., and B. J. Wold.** 1992. Effects of different DNA polymerases in ligation-mediated PCR: enhanced genomic sequencing and in vivo footprinting. Proc. Natl. Acad. Sci. USA **89:**1021–1025.
22. **Gille, H., T. Strahl, and P. E. Shaw.** 1995. Activation of ternary complex factor Elk-1 by stress-activated protein kinases. Curr. Biol. **5:**1191–1200.
23. **Gotoh, Y., E. Nishida, T. Yamashita, M. Hoshi, M. Kawakami, and H. Sakai.** 1990. Microtubule-associated-protein (MAP) kinase activated by nerve growth factor and epidermal growth factor in PC12 cells. Identity with the mitogen-activated MAP kinase of fibroblastic cells. Eur. J. Biochem. **193:** 661–669.
24. **Grant, P. A., C. B. Thompson, and S. Pettersson.** 1995. IgM receptor-mediated transactivation of the IgH 3′ enhancer couples a novel Elf-1-AP-1 protein complex to the developmental control of enhancer function. EMBO J. **14:**4501–4513.
25. **Gutman, A., and B. Wasylyk.** 1990. The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. EMBO J. **7:**2241–2246.
26. **Han, M., A. Golden, Y. Han, and P. W. Sternberg.** 1993. C. elegans lin-45 raf gene participates in let-60 ras-stimulated vulval differentiation. Nature **363:** 133–139.

26a. **Hauser, C.** Unpublished observations.

27. **Hauser, C., J. K. Westwick, and C. J. Der.** 1995. Ras-mediated transcription activation: analysis by transient cotransfection assay. Methods Enzymol. **255:** 412–425.
28. **Herskowitz, I.** 1995. MAP kinase pathways in yeast: for mating and more. Cell **80:**187–197.
29. **Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin.** 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. **7:**2135–2148.
30. **Hill, C. S., R. Marais, S. John, J. Wynne, S. Dalton, and R. Treisman.** 1993. Functional analysis of a growth factor-responsive transcription factor complex. Cell **73:**395–406.
31. **Hill, C. S., and R. Treisman.** 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell **80:**199–211.
32. **Hill, C. S., J. Wynne, and R. Treisman.** 1995. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell **81:**1159–1170.
33. **Janknecht, R.** 1996. Analysis of the ERK-stimulated transcription factor ER81. Mol. Cell. Biol. **16:**1550–1556.
34. **Johnson, R., B. Spiegelman, D. Hanahan, and R. Wisdom.** 1996. Cellular transformation and malignancy induced by ras require c-jun. Mol. Cell. Biol. **16:**4504–4511.
35. **Karin, M.** 1991. Signal transduction and gene control. Curr. Opin. Cell Biol. **3:**467–473.
36. **Karin, M.** 1994. Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. Curr. Opin. Cell Biol. **6:**415–424.
37. **Karin, M.** 1990. Transcriptional control and the integration of cell-autonomous and environmental cues during development. Curr. Opin. Cell Biol. **2:**996–1002.
38. **Karin, M., and T. Hunter.** 1995. Transcriptional control by protein phos-

phorylation: signal transmission from the cell surface to the nucleus. Curr. Biol. **5:**747–757.
39. **Kyriakis, J. M., P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett.** 1994. The stress activated protein kinase subfamily of c-jun kinases. Nature **369:**156–160.
40. **Langer, S. J., 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.
41. **MacNicol, A. M., A. J. Muslin, and L. T. Williams.** 1993. Raf-1 kinase is essential for early Xenopus development and mediates the induction of mesoderm by FGF. Cell **73:**571–583.
41a. **Mahony, D., and E. Lees.** Unpublished observations.
42. **Mansour, S. J., W. T. Matten, A. S. Hermann, J. M. Candia, S. Rong, K. Fukasawa, W. G. Vande, and N. G. Ahn.** 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science **265:**966–970.
43. **Marais, R., J. Wynne, and R. Treisman.** 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell **73:**381–394.
44. **Marshall, C. J.** 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Genet. Dev. **4:**82–89.
45. **Marshall, C. J.** 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80:**179–185.
46. **McCarthy, S. A., M. L. Samuels, C. A. Pritchard, J. A. Abraham, and M. McMahon.** 1995. Rapid induction of heparin binding epidermal growth factor/diphtheria toxin receptor by Ras and Raf oncogenes. Genes Dev. **9:**1953–1964.
47. **Minden, A., A. Lin, M. McMahon, C. Lange-Carter, B. Derijard, R. J. Davis, G. L. Johnson, and M. Karin.** 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science **266:**1719.
48. **Minden, A., A. Lin, F. X. Claret, A. Abo, and M. Karin.** 1995. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell **81:**1147–1157.
49. **Monte, D., L. Coutte, J. L. Baert, I. Angeli, D. Stehelin, and Y. de Launoit.** 1995. Molecular characterization of the ets-related human transcription factor ER81. Oncogene **11:**771–779.
50. **Nishida, E., and Y. Gotoh.** 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biochem. Sci. **18:**128–131.
51. **Nishida, Y., M. Hata, T. Ayaki, H. Ryo, M. Yamagata, K. Shimizu, and Y. Nishizuka.** 1988. Proliferation of both somatic and germ cells is affected in the Drosophila mutants of raf proto-oncogene. EMBO J. **7:**775–781.
52. **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.
52a. **Ostrowski, M.** Unpublished observations.
53. **Pankov, R., A. Umezawa, R. Maki, C. J. Der, C. A. Hauser, and R. G. Oshima.** 1994. Oncogene activation of human keratin 18 transcription via the Ras signal transduction pathway. Proc. Natl. Acad. Sci. USA **91:**873–877.
54. **Parker, D., K. Ferreri, T. Nakajima, V. J. La Morte, R. Evans, S. C. Koerber, C. Hoeger, and M. R. Montminy.** 1996. Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. Mol. Cell. Biol. **16:**694–703.
55. **Pritchard, C. A., L. Bolin, R. Slattery, R. L. Murray, and M. McMahon.** 1996. Post-natal lethality and neurological and gastrointestinal defects in mice with targeted disruption of the A-Raf protein kinase. Curr. Biol. **6:**614–617.
56. **Rebay, I., and G. M. Rubin.** 1995. Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras/MAPK pathway. Cell **81:**857–866.
57. **Reddy, M. A., S. J. Langer, M. S. Colman, and M. C. Ostrowski.** 1992. An enhancer element responsive to ras and fms signaling pathways is composed of two distinct nuclear factor binding sites. Mol. Endocrinol. **6:**1051–1060.
58. **Robertson, L. M., T. K. Kerppola, M. Vendrell, D. Luk, R. J. Smeyne, C. Bocchiaro, J. I. Morgan, and T. Curran.** 1995. Regulation of c-fos expression in transgenic mice requires multiple interdependent transcription control elements. Neuron **14:**241–252.
59. **Samuels, M. L., and M. McMahon.** 1994. Inhibition of platelet-derived growth factor- and epidermal growth factor-mediated mitogenesis and signaling in 3T3 cells expressing delta Raf-1:ER, an estradiol-regulated form of Raf-1. Mol. Cell. Biol. **14:**7855–7866.
60. **Samuels, M. L., M. J. Weber, J. M. Bishop, and M. McMahon.** 1993. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human Raf-1 protein kinase. Mol. Cell. Biol. **13:**6241–6252.
61. **Sanchez, I., R. T. Hughes, B. J. Mayer, K. Yee, J. R. Woodgett, J. Avruch, J. M. Kyriakis, and L. I. Zon.** 1994. Role of SAPK/ERK kinase-1 in the stress activated pathway regulating transcription factor c-jun. Nature **372:**794–798.
62. **Sgouras, D. N., M. A. Athanasiou, G. J. Beal, Jr., 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.
63. **Stacey, K. J., L. F. Fowles, M. S. Colman, M. C. Ostrowski, and D. A. Hume.** 1995. Regulation of urokinase-type plasminogen activator gene transcription by macrophage colony-stimulating factor. Mol. Cell. Biol. **15:**3430–3441.
64. **Stanton, V. P., Jr., D. W. Nichols, A. P. Laudano, and G. M. Cooper.** 1989. Definition of the human *raf* amino-terminal regulatory region by deletion mutagenesis. Mol. Cell. Biol. **9:**639–647.
65. **Sternberg, P. W., A. Golden, and M. Han.** 1993. Role of a raf proto-oncogene during Caenorhabditis elegans vulval development. Phil. Trans. R. Soc. Lond. **340:**259–265.
66. **Treisman, R.** 1996. Regulation of transcription by MAP kinase cascades. Curr. Opin. Cell Biol. **8:**205–215.
67. **Wassarman, D. A., M. Therrien, and G. M. Rubin.** 1995. The Ras signaling pathway in Drosophila. Curr. Opin. Genet. Dev. **5:**44–50.
68. **Wasylyk, B., S. L. Hahn, and A. Giovane.** 1993. The Ets family of transcription factors. Eur. J. Biochem. **211:**7–18.
69. **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.
70. **Wasylyk, C., A. Gutman, R. Nicholson, and B. Wasylyk.** 1991. The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. EMBO J. **10:**1127–1134.
71. **Wasylyk, C., S. M. Maira, P. Sobieszczuk, and B. Wasylyk.** 1994. Reversion of Ras transformed cells by Ets transdominant mutants. Oncogene **9:**3665–3673.
72. **Wasylyk, C., B. Wasylyk, G. Heidecker, M. Huleihel, and U. R. Rapp.** 1989. Expression of *raf* oncogenes activates the PEA1 transcription factor motif. Mol. Cell. Biol. **9:**2247–2250.
73. **Yang, B.-S., C. A. Hauser, G. Henkel, M. S. Colman, C. Van Beveren, K. J. Stacey, D. A. Hume, R. A. Maki, and M. C. Ostrowski.** 1996. Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. Mol. Cell. Biol. **16:**538–547.