Multiple cis-Elements Mediate the Transcriptional Activation of Human fra-1 by 12-O-Tetradecanoylphorbol-13-acetate in Bronchial Epithelial Cells*

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Recent studies indicate a potential role for Fra-1, a heterodimeric partner of activator protein 1 (AP1), in toxicant-induced epithelial injury, repair, and cellular transformation. Here, we have investigated the transcriptional regulation of fra-1 by 12-O-tetradecanoylphorbol-13-acetate (TPA) in human bronchial epithelial (HBE) cells, which are the direct targets of inhaled toxins/carcinogens. In contrast to a transient induction by H₂O₂, TPA persistently activated fra-1 transcription, principally at the transcriptional level. A deletion analysis of the fra-1 promoter revealed that several cis-elements located between −105/+32 and −283/−105 bp mediate minimal and basal promoter activities, respectively. A region between −379 and −283 bp, which harbors a putative TPA response element, a GC box, and an Ets-like binding site, was required for high level TPA-inducible expression. Mutations in any of these cis-elements markedly reduced both basal and TPA-inducible expression. Thus, cooperative interactions between factors binding to multiple cis-elements of the −379/−283 promoter region appear to regulate TPA-induced fra-1 transcription in HBE cells. Consistent with this finding, electrophoretic mobility shift assays indicated the formation of multiple complexes consisting of the AP1-, Sp-, and ETS-specific family of transcription factors with the −379/−283 fragment. Members of the AP1 family distinctly regulated the fra-1 promoter. In particular, coexpression of c-Jun, Jun-D, and Fra-2 up-regulated fra-1 transcription. Chromatin immunoprecipitation assays revealed an enhanced recruitment of c-Jun, Jun-D, and Fra-2 to the endogenous fra-1 promoter upon TPA stimulation. These results underscore the regulatory role of c-Jun, Jun-D, and Fra-2 in TPA-inducible fra-1 expression in HBE cells in vivo.

The Jun (c-Jun, Jun-B, and Jun-D) and Fos (c-Fos, Fos-B, Fra-1, and Fra-2) families of transcription factors form heterodimeric complexes referred to as activator protein 1 (AP1). AP1 has been shown to act as a “master switch” for diverse stimuli and to regulate the expression of genes involved in various biological processes (1–4). Upon activation, specific combinations of AP1 proteins bind to TPA response elements (TREs) and induce the transcription of various genes in a context-dependent manner (1–4). AP1 proteins can also form heterodimers with other members of the leucine zipper superfamily of transcription factors, such as activation transcription factors (ATFs/CREBs), Nrf/Mafs, and CCAAT/Enhancer-binding proteins (5). These interactions not only result in differential DNA binding of AP1 proteins to various promoters but also increase the repertoire of genes controlled by AP1 proteins in a given cell type (5).

A critical part of such regulation is the maintenance of optimal intracellular levels of specific AP1 proteins. Thus, the regulation of AP1 protein levels itself contributes to the strength of gene expression and, as a result, influences the outcome of various biological responses (5). Since TREs are often embedded in antioxidant response elements, which are commonly found in the regulatory regions of several antioxidant enzymes (6), AP1 proteins have been suggested to play both pro- and antiregulatory roles in pulmonary insult, injury, and repair (7).

Gene ablation studies have demonstrated that c-jun, jun-B, and fra-1 are critical for normal embryonic development, whereas jun-D, c-fos, fos-B, and fra-2 are required for proper postnatal growth (4, 8). In contrast, studies of transgenic mice have indicated that some AP1 proteins are associated with specific and distinct phenotypes, including cellular transformation (4). Although several contrasting roles for AP1 proteins have been suggested in the literature (4), Passague et al. (8) have shown by use of transgenic techniques that reconstitution of jun-B into the c-jun locus can overcome the liver and cardiac defects observed in c-jun−/− mice. However, expression of jun-B in c-jun−/− mice or, conversely, c-jun in jun-B−/− mice cannot restore normal function. These observations suggest that temporal and spatial expression of AP1 family members rather than their protein structure per se plays a critical role in controlling various biological processes (8, 9). Indeed, a comparison of the regulatory (promoter) regions of the AP1 proteins did not reveal a significant conservation of the cis-acting motifs, suggesting that their expression is differentially controlled.
Emerging evidence indicates that fra-1 plays a central role in pulmonary defense, injury repair, and transformation (7). For example, tobacco smoke (10), silica (11), and asbestos (12–14) persistently activate fra-1 (but not fra-2) expression in lung cells both in vitro and in vivo (7). Moreover, fra-1 is highly overexpressed in asbestos-induced lung tumors in a rat model (14) and has been reported to play a critical role in the transformation of mesothelial cells (15). A differential requirement for Fra-1, but not Fra-2, in TPA- and EGF-induced transformation of epithelial cells has also been documented in other studies (16). We have recently shown that Fra-1 and Fra-2 have diametrically opposite effects on the gene expression associated with airway squamous metaplasia (17, 18). Consistent with this finding, a high level of fra-1 expression is found in stomach (19) and esophageal (20) squamous cell carcinomas, breast tumor cells (21), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (a tobacco carcinogen)-induced lung tumors (10). Also consistent with these observations, formation of lung tumors has been noted in transgenic mice overexpressing fra-1 (22). Taken together, these observations highlight a major role for Fra-1 in toxicant-induced gene expression and cellular transformation.

Although an enhanced expression of fra-1 correlates with a malignant phenotype (22), the mechanisms regulating fra-1 in human bronchial epithelial (HBE) cells in response to various toxicants, carcinogens, and mitogens have not been defined. We hypothesized that the sustained expression of fra-1 in response to toxicants/mitogens is distinctly regulated by unique and/or common factors. In the present study, we have investigated the role of Fra-1 in toxicant-induced gene expression and cellular transformation.

**EXPERIMENTAL PROCEDURES**

**Gene Expression Analyses—**A549, a human alveolar type II-like epithelial cell line, was grown in RPMI 1640 supplemented with 5% fetal bovine serum and antibiotics. For Northern blot analyses, cells were stimulated with appropriate agents, and ChIP was performed using a commercial kit (Upstate Biotechnology, Inc., Lake Placid, NY). Total RNA (15 μg/lane) was separated on a 1.2% agarose gel, blotted onto a nylon membrane, and hybridized with 32P-labeled cDNAs of fra-1 and 18 S RNA as previously described (17, 18). A semiquantitative reverse transcription–PCR was performed to detect specific mRNAs as described previously (17, 18). In brief, total RNA (750 ng) was reverse-transcribed into cDNA, and PCR was performed using human fra-1 (forward, 5′-GGCGATGTTTGGCAGACTTCG-3′; reverse, 5′-TCCAAAGGGAGGTTTG-3′) and β-actin gene-specific primer pairs (forward, 5′-GGAGAAAATCTCGGACACAAAC-3′; reverse, 5′-TACCCCTCCTGATATGGGCC-3′). The number of cycles was empirically optimized to amplify the products in a linear range (17, 18).

For Western blot analyses, total protein was extracted using a kinase lysis buffer (20 mM Tris, pH 7.5, with 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 5 mM β-glycerophosphate, and 1 μg/ml leupeptin). A comparable quality of protein from each sample was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride nylon membrane (Bio-Rad). The membrane was then probed with specific polyclonal antibodies and developed using ECL-Plus reagent (Amersham Biosciences).

**Chromatin Immunoprecipitation (ChIP) Assays—**Cells (~106) were stimulated with appropriate agents, and ChIP was performed using a commercially available kit (Upstate Biotechnology, Inc., Lake Placid, NY). Chromatin was cross-linked by adding formaldehyde to the tissue culture medium to a final concentration of 1% and incubating the mixture for 10 min at 37 °C. Cells were washed twice with ice-cold phosphate-buffered saline containing protease inhibitor mixture (Sigma) and suspended in 0.2 ml of lysis buffer (50 mM Tris-HCl, pH 8.1, with 1% SDS, 5 mM EDTA, and protease inhibitor mixture). Samples were sonicated on ice to an average length of 500–1000 bp and centrifuged at 12,000 rpm. Solubilized chromatin was diluted 10-fold with dilution buffer (16.7 mM Tris-HCl, pH 8.1, with 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and 167 mM NaCl) and used for ChIP assays. A fraction of the soluble chromatin (1%) was saved for total chromatin input. Chromatin was precleared with salmon sperm DNA/protein A-agarose for 30 min and then incubated with specific antibodies for 18 h at 4 °C. The immunoprecipitated products were washed, and DNA was eluted. DNA from the immunoprecipitated products was extracted after proteinase K digestion and used for PCR (40 cycles) with fra-1 promoter-specific primers: –379 F (5′-GGCGAGCTTGGGAAACCCAGG-3′) and –220 R (5′-GGCTATAGCCCTCTGAGTACGACCTCAT-3′). These primers generate a 160-bp DNA product.

**Plasmin and Mutagenesis—**The 5′-flanking region of human fra-1 (~861 to +32 bp) was amplified with Pfu DNA polymerase using the genomic DNA isolated from HBE-1, a nontumorigenic human bronchial epithelial cell line, as a template. The resulting product was cloned into the MluI site of the firefly luciferase (LacZ) reporter gene. Specific flank and Neh sites of the pGL3 basic vector (Promega). Various mutants were generated using PCR and verified by DNA sequencing. pCMV expression vectors bearing the wild-type and dominant negative (mutations in the transactivation domain) c-jun were generously provided by Michael Birrer (NCI, National Institutes of Health, Bethesda, MD); jun-B and jun-D cloned in the SR-α vector were obtained from Michael Karin (University of California at San Diego). pCMV-c-fos and pCMV-fos-B, pCMV-fra-1, and pCMV-fra-2 were obtained from R. Dohner (University of Massachusetts Medical School, Worcester, MA), Eugene Tulchinsky (Institute of Cancer Biology), and Donna Cohen (Australian National University), respectively.

**Electrophoretic Mobility Shift Assays (EMSAs)—**To generate stable transfectants, A549 cells were transfected with 10 ng of reporter construct along with 5 ng of pC1-Neo vector. Stable clones were isolated after selection with G418 (400 μg/ml). Transient transfection was performed as described in our earlier publications (17, 18). Cells were transfected with 100 ng of reporter construct, 1 ng of Renilla luciferase (pRL-TK; Promega), and 25–200 ng of empty or expression plasmids. After 18–24 h, cells were serum-starved for 24 h and treated with either vehicle (Me2SO) or TPA (100 ng/ml) for 5 h. Cell extracts were assayed for firefly and Renilla luciferase activities using a dual luciferase kit (Promega). Firefly luciferase activity was normalized to that of Renilla luciferase (17, 18). All experiments were repeated at least 3–4 times in triplicate. Data are presented as the mean luciferase activity ± S.E. The statistical significance of the differences between groups was determined using a Student’s t test.

**RESULTS**

**Toxicant-induced Expression of fra-1 in HBE Cells—**To understand the regulation of human fra-1 expression, A549 cells were exposed to TPA (100 ng/ml), EGF (50 ng/ml), H2O2 (200 μM), and lipopolysaccharide (10 μg/ml). RNA was isolated, and Northern blot analysis was performed using a 32P-labeled human fra-1 cDNA probe. As shown in Fig. 1A, TPA induced a strongly enhanced level of fra-1 mRNA expression (lanes 8–14) as early as 30 min after initial exposure, peaked at 3 h, and remained elevated thereafter. The two alternatively spliced
mRNA transcripts of fra-1 (3.3 and 1.7 kb) (19) were similarly induced. Although fra-1 mRNA was transiently induced by H2O2 (90 min), it was undetectable at 6 h after stimulation. In contrast to TPA and H2O2, lipopolysaccharide did not have an appreciable effect on fra-1 expression when evaluated for 180 min, with the exception of a modest induction of the smaller RNA species (1.7 kb) after 6 h (lanes 1–7). As shown in Fig. 1B, EGF caused an increase in fra-1 message levels as early as 30 min after initial treatment. Message levels peaked at 120 min and remained above the basal level for 14 h. The induction of fra-1 mRNA expression by TPA or EGF also correlated with a corresponding increases in its protein levels (Fig. 1C) and DNA binding (data not shown). A similar pattern of fra-1 induction by TPA or EGF was observed in two other HBE cell lines, HBE1 and H441 (data not shown). The differential patterns of fra-1 induction by H2O2 (an oxidant), TPA, and EGF suggest a stimulus-specific regulation of fra-1 expression in HBE cells. Studies using actinomycin D and cycloheximide indicated that TPA, H2O2, and EGF-inducible expression of fra-1 was mainly regulated at the transcriptional level and did not require de novo protein synthesis (data not shown). These observations are consistent with the reported regulation of other AP1 genes in different cell types (1, 25, 26).

Mapping of the fra-1 Promoter Required for TPA-inducible Transcription—To further elucidate the mechanisms controlling the inducible expression of fra-1 by TPA, we generated luciferase reporter constructs bearing various portions of the 5’-flanking region of human fra-1. Fig. 2A shows these constructs and the positions of the various putative cis-elements. These reporters were transfected into A549 cells, and luciferase activities were monitored. When compared with 68-Luc (which displayed a promoter activity no greater than pGL3 basic vector), the 105-Luc reporter had a slightly higher (>30%) luciferase expression (Fig. 2B). This observation indicates that the DNA sequences between −105 and +32 probably contain the minimal fra-1 promoter region. Compared with 105-Luc, 466-Luc, and 861-Luc, the 283-Luc construct had an ~4-fold higher basal activity, suggesting that the region between −283 and −105 bp contains sites responsible for the basal expression of fra-1. As shown in Fig. 2B, cells transfected with the 68-Luc and 105-Luc constructs showed no appreciable change in luciferase activity following TPA treatment. The 283-Luc construct had displayed a 2–4-fold higher luciferase activity in response to TPA. However, an increase of nearly 20–30-fold in TPA-inducible promoter activity was noticed with the 466-Luc, 570-Luc, and 605-Luc constructs. Although TPA markedly stimulated 659-Luc and 861-Luc (~10–15-fold increase), the induction was not as dramatic as that observed for 466-Luc, 570-Luc, and 605-Luc. Taken together, these results suggest that the regions between −105 and +32, −283 and −105, and −466 and −283 contain necessary cis-elements for directing minimal, basal, and TPA-inducible expression of fra-1, respectively. To better define the coordinates of the TPA inducible cis-elements, additional deletion mutants were generated and used in transfection analysis. As shown in Fig. 2C, cells transfected with 379-Luc showed a high level of luciferase activity in response to TPA, as compared with other constructs. Although 328-Luc showed a 10-fold greater stimulation than did 283-Luc and was nearly comparable with that of 466-Luc, the magnitude of the induction was half of that observed for 379-Luc. This result indicates that the −379/−283 region contains elements necessary for directing high level TPA-inducible fra-1 transcription. Similar results were obtained in other HBE lines, H441 and HBE1 (data not shown). Therefore, we focused the rest of our studies on this region.

Multiple cis-Elements within the −379/−283 Region of the fra-1 Promoter—The −379/−283 region harbors several putative cis-acting elements, including an Ets-like binding site (EBS), a GC box (also known as an Sp1 binding site), and a TRE (see Fig. 3A). To assess the contribution of these motifs to TPA-inducible fra-1 promoter activity, we introduced mutations into these sites. Mutations in any of the individual cis-elements markedly reduced (~50–80%) the basal level expression of the reporter (Fig. 3B). In particular, mutations in either of these sites profoundly reduced the TPA-inducible fra-1 promoter activity. Because of its profound inhibitory effect on transcription, we infer that the TRE at −318 is essential for transcriptional regulation of fra-1 in both the unstimulated and stimulated state.

Analysis of Transcription Factors Binding to the −379/−283 Fragment in HBE Cells—Since mutations in the −379/−283 region ablated promoter activity, we used EMSAs to determine whether this region bound to specific transacting factors. When incubated with the A549 nuclear extracts, the −379/−283 bp

**Fig. 1. Differential induction of fra-1 in response to various stimuli in HBE cells.** A, A549 cells were serum-starved for 24 h prior to stimulation with vehicle (ctr), TPA (100 ng/ml, H2O2 (200 μM), or lipopolysaccharide (LPS) (10 μg/ml). Northern blot analysis was carried out as described under “Experimental Procedures.” The picture below is the ethidium bromide-stained gel showing the 18 S rRNA. B, total RNA from cells treated with EGF (50 ng/ml) was used for Northern blot analysis. C, an equal amount of total cellular protein was separated on 10% SDS-PAGE, and Western blot analysis was performed using anti-Fra-1 antibodies. Each experiment was repeated at least two times to obtain consistent results.
fragment formed three major protein complexes, designated I, II, and III. The formation of these complexes was strongly enhanced by TPA treatment (Fig. 4A, compare lanes 1 and 2). The specificity of formation of these complexes was determined using competition assays with unlabeled oligonucleotides bearing consensus TRE, EBS, and GC box sequences (see Fig. 5A). Each of these oligonucleotides selectively inhibited the formation of specific protein-DNA complexes with the −379/−283 fragment. Interestingly, the unlabeled TRE probe not only completely blocked the formation of complex I but also significantly diminished the binding intensity of complexes II and III (lanes 3 and 4). A similar effect on complex I was observed with the EBS and GC box oligonucleotides (lanes 5–8), which strongly reduced the formation of complexes II and III. In contrast to the individual oligonucleotides, the unlabeled −379/−283 fragment (self, lane 9) blocked the formation of all three complexes. These observations suggest the formation of multi-protein complexes with the −379/−283 region.

We next performed EMSAs using a −379/−283 fragment bearing mutations in each of the functional element(s). As shown in Fig. 4B, the −318 TRE mutation markedly reduced the overall intensity of complexes I, II, and III when compared with the wild-type fragment. In particular, mutations within the EBS and TRE (double mutant; DM) or EBS/GC box/TRE (triple mutant; TM) profoundly diminished the intensity of all three complexes. The weak complexes observed with double or
A multiple cis-acting elements control the TPA response of the human fra-1 promoter. A, putative cis-acting elements located between −379 and −283 bp are indicated: EBS (−377/−367), a GC box (−343/−334), and TRE (−318/−312). Lowercase letters indicate the mutated bases within the EBS, TRE, and GC boxes. B, mutant constructs were transfected into cells, and luciferase activity was determined as in Fig. 2B. Both the basal and TPA-inducible promoter activities of various constructs are shown. Each data point represents mean luciferase activity ± S.E. of triplicate samples. At least two independent experiments were performed in this case.

The −379/−283 fra-1 promoter forms several complexes with nuclear proteins. A, an EMSA was performed with the 32P-end-labeled −379/−283 fra-1 fragment and 3 μg of nuclear extract isolated from A549 cells. Lane 0, no nuclear extract (F); lane 1, Me2SO (−); lanes 2–9, TPA (+). For competition experiments, nuclear extracts were incubated for 10–15 min with a 20- or 100-fold excess of the indicated unlabeled double-stranded oligonucleotides or the −379/−283 promoter (self) before the addition of labeled probe. In lanes 3–9, various consensus oligonucleotides were used as competitors: cTRE (lanes 3 and 4), consensus EBS (cEBS; lanes 5 and 6), cGC box (lanes 7 and 8), and self (lane 9) were employed. The consensus sequences (upper strand) of the TRE, EBS, and GC box are shown in Fig. 5A. B, specificity of transcription factor complexes formed with the −379/−283 fragment. Nuclear extracts were incubated with the indicated labeled probes. Minus and plus signs indicate no treatment and TPA treatment, respectively. F, free probe. The open triangle and arrow indicate the nonspecific (NS) complex and unbound probe, respectively. A representative EMSA autoradiogram from two independent experiments is shown. WT, wild type; DM, double mutant; TM, triple mutant.

The triple mutant may be attributed to other flanking sequences present in the promoter. Nevertheless, these data suggest that complexes consist of multiple proteins such as the AP1, Sp, and ETS families of transcription factors, which bind to the −379/−283 region and regulate fra-1 expression in HBE cells.

We then made use of specific antibodies to characterize the nature of the protein complexes binding to the −318 TRE, −343 GC box, and −377 EBS of the fra-1 promoter and then compared these binding patterns with those of the consensus TRE, GC box, and EBS sequences (Fig. 5A). Since the multiprotein complexes formed with −379/−283 fragment often yielded a smeared pattern, we used the oligonucleotides bearing −318 TRE, −343 GC box, and −377 EBS for supershift assays. As seen in Fig. 5B, TPA strongly enhanced protein complex formation with −318 TRE, which could be competed out by unlabeled probe and consensus TRE (cTRE; lane 5). In contrast, the GATA oligonucleotide had no such effect (Fig. 5B, lane 6). Mutation of TRE (Fig. 5B, right panel) effectively
Fig. 5. The nature of transcription factors complexes formed with the functional elements of the fra-1 promoter. A, DNA sequences of the oligonucleotide probes used for EMSA analysis. The sequence of only the upper strand is shown in each case. cEBS, consensus EBS. EMSA was performed with two different batches of nuclear extracts, and similar results were obtained in both cases. Different binding buffers (see
reduced the formation of complexes, indicating the binding of protein to −318 TRE. We next compared the patterns of AP1 protein binding with −318 TRE (Fig. 5D) and cTRE (Fig. 5C). We chose a 90-min exposure to TPA because inducible expression of fra-1 mRNA was maximal at this time point (Fig. 1). In unstimulated cells, Jun-D, c-Fos, and Fra-2 predominantly bound to cTRE (Fig. 5C, left panel). Jun-B binding was weak but noticeable (lane 2). TPA strongly induced the binding of c-Jun, Jun-B, and Fra-1 as well as Jun-D, c-Fos, and Fra-2 to the cTRE (Fig. 5C, right panel). Specific antibodies recognizing c-Jun, Jun-B, or Fra-2 decreased the binding of the AP1 complex with −318 TRE. This discrepancy may be related to the influence of the sequences within or flanking the respective TREs.

We next characterized the nature of the protein complexes bound to the −343 GC box of the fra-1 promoter (Fig. 5E, right panel). A consensus GC box (cGC box) was used as a control in these experiments (Fig. 5E, left panel). Nuclear extracts from unstimulated cells formed three distinct complexes (I, II, and III) with the cGC box. Incubation with antibodies recognizing Sp1, Sp3, or EGR1 specifically caused a “supershift” or blockade of complexes, I, II, and III, respectively (Fig. 5E, lanes 1–4). TPA did not noticeably alter the formation of these complexes (Fig. 5E, lanes 5–8). In contrast to the cGC box, the −343 GC box of the fra-1 promoter formed only weak complexes with the nuclear extracts from unstimulated cells. TPA clearly enhanced the protein binding to the −343 GC box. Supershift analyses with specific antibodies revealed the presence of Sp1, Sp3, and EGR1 proteins in these complexes (Fig. 5E, lanes 14–17). Sp1- and Sp3-specific antibodies blocked the formation of protein complexes I and II, respectively. Although the EGR1-specific antibody predominantly blocked complex III (Fig. 5E), the formation of Sp1 and Sp3 complexes was also weakly diminished. Protein binding to the consensus EBS (cEBS in Fig. 5E, left panel) and −377 EBS of the fra-1 promoter (Fig. 5E, right panel) revealed the formation of a single complex, whose formation was diminished by Ets-1/2-specific antibodies.

AP1 Family Members Distinctly Regulate fra-1 Transcription—The data presented above suggested a role for EBS, GC box, and TRE binding proteins in the regulation of TPA-induced fra-1 expression in A549 cells. Therefore, we analyzed the effects of specific co-expressed AP1 proteins on the fra-1 promoter (Fig. 6). Among the jun subfamily of proteins, c-Jun and Jun-D strongly (>10-fold) stimulated luciferase gene expression, whereas Jun-B had no effect (Fig. 6A). With respect to the fos family, overexpression of fra-2 strongly (~15-fold) induced the reporter, with fos-B (6-fold), c-fos (~3-fold), and fra-1 (~1-fold) showing a lesser effect. In contrast to the fra-1 promoter, overexpression of Fra-1 or Fra-2 strongly suppressed reporter gene expression driven by the cTRE, whereas Jun-B had an opposite effect (Fig. 6B). This result suggests that the promoter context of a TRE plays a critical role in its response to specific AP1 proteins. Taken together, these results indicate that AP1 proteins differentially regulate the fra-1 promoter.

Ectopic Expression of AP1 Family Members Up-regulates Endogenous fra-1 mRNA Expression—To further demonstrate the importance of AP1 proteins in endogenous fra-1 regulation, cells were transfected with expression plasmids for the individual Jun and Fos family members. Their effect on endogenous fra-1 mRNA levels was then analyzed by semiquantitative reverse transcription-PCR. As shown in Fig. 7, fra-1 mRNA expression was very low in cells transfected with empty vector. However, overexpression of c-Jun and Fra-2 strongly (>5-fold) increased the basal levels of mRNA expression, in contrast to other AP1 family members, which had a modest effect (~2-fold increases). These observations are consistent with the data obtained in our transient transfection assays, in which the co-expression of c-Jun and Fra-2 markedly induced the fra-1 promoter.

c-Jun, Jun-D, and Fra-2 Bind to the fra-1 Promoter in Vivo—The data presented above demonstrated that among the members of the AP1 family of proteins, c-Jun, Jun-D, and Fra-2 markedly up-regulate the expression of fra-1. Therefore, we next used a ChIP assay to determine whether these proteins are recruited to the endogenous fra-1 promoter in response to

*Experimental Procedures* were used for detecting ETS and Sp1 complexes. B, the −318 TREwt and −318 TREmt probes were incubated with nuclear extracts as in Fig. 4. F, free probe. Lanes 4–6, nuclear extracts incubated with a 50-fold molar excess of unlabeled double-stranded −318 TREwt oligonucleotide (SELF), cTRE or GATA (GAG) sequence, respectively; prior to their use in EMSA. C and D, supershift analysis of the complexes formed with the consensus TRE and −318 TRE using various antibodies. Ig, nonimmune IgG; cJ, c-Jun; JB, Jun-B; JD, Jun-D; cF, c-Fos; F1, Fra-1; F2, Fra-2; FB, Fos-B; −, no treatment; +, TPA treatment. F, free probe. The partial blocking of the NS band by the indicated antibodies in D is not consistently seen between EMSAs. E, supershift analysis of the protein; DNA complexes formed with cGC and −343 GC boxes using antibodies specific for Sp1, Sp3, or EGR1. F, analysis of Ets protein binding to the fra-1 promoter. Labeled probes used for EMSA are indicated above the panels. Other labels and symbols are defined in the legend to Fig. 4.
TPA. Soluble cross-linked chromatin was isolated after appropriate treatment and immunoprecipitated using antibodies specific for c-Jun, Fra-2, or Jun-D. Nonimmune IgG was used as a negative control. The immunoprecipitated DNA fragments were amplified using fra-1 promoter-specific primers with the aid of PCR. As expected, ChIP assays using nonimmune IgG showed no amplification of the fra-1 promoter. As shown in Fig. 8, Jun-D was present in the basal transcriptional complex formed with the fra-1 promoter in unstimulated cells. c-Jun and Fra-2 were present at very low or undetectable levels under these conditions. However, TPA stimulation enhanced the recruitment of c-Jun to the fra-1 promoter after 30 min of exposure, and Fra-2 recruitment was enhanced after 60 min of exposure. Although Jun-D was associated with the promoter in unstimulated cells, TPA treatment only marginally enhanced its binding to the promoter. c-Jun, Jun-D, and Fra-2 were no longer detectable at 6 h after TPA stimulation. These results underscore the regulatory role of c-Jun, Jun-D, and Fra-2 in TPA-inducible fra-1 expression in HBE cells in vivo. To further determine whether c-Jun, Jun-D, and Fra-2 induce fra-1 transcription through −318 TRE, expression vectors of these transcription factors were co-transfected into the cells along with the wild-type or −318 TREmt construct of the −379/+32 bp fra-1 promoter. As shown in Fig. 8D, mutations within −318 TRE dramatically reduced c-Jun-, Jun-D-, and Fra-2-enhanced promoter activity when compared with the wild-type promoter. This result indicates a requirement for −318 TRE in AP1 protein-enhanced fra-1 transcription.

c-Jun-enhanced fra-1 Promoter Activity Requires EBS, GC Box, and TRE—To further emphasize the role of c-Jun in regulating fra-1 transcription, we used a transactivation-deficient c-Jun mutant (TAM) that inhibits AP1-dependent gene expression (27). In unstimulated cells, TAM enhanced (−2-fold) the basal promoter activity (compare bar 1 with bar 2; Fig. 9A), probably by opposing basal c-Jun activity. TPA-inducible gene expression of the reporter was strongly suppressed in the presence of TAM. In addition to −318 TRE, mutational analysis revealed that the GC box and EBS, located at −343 and −377, respectively, are also critical for basal and TPA-inducible fra-1 transcription (Fig. 3). To ascertain the role of the EBS and GC boxes in c-Jun-enhanced transcription, we transfected the wild-type and mutant 379-Luc promoter constructs into cells along with a c-Jun expression vector. As shown in Fig. 9B, mutations within the −318 TRE drastically reduced c-Jun-enhanced fra-1 transcription. Moreover, mutations within the −343 GC box and the −377 EBS had a similar effect. In particular, the EBS...
mutation dramatically inhibited c-Jun-induced transcription of the reporter. These observations suggest the requirement of the EBS, GC box, and TRE for maximal stimulation of the fra-1 promoter by c-Jun and TPA.

The −379 to +32 bp Region Confers TPA-induced fra-1 Transcription in the Context of Chromatin—To determine whether the −379 to +32 bp region confers TPA inducibility in vivo, the wild-type (379-Luc) and TRE mutant (379-TREmt) plasmids were stably transfected into A549 cells. Cells were serum-starved for 24 h prior to TPA treatment. As shown in Fig. 10, the stably transfected cell clones containing the wild-type fra-1-Luc displayed a level of induction (~20-fold) analogous to that observed for the transient transfections. However, the magnitude of the TPA inducibility was drastically reduced in cell clones expressing a luciferase gene driven by the −379/+32 bp fra-1 promoter (379-Luc). C, quantification of ChIP data. D, the −318 TRE contributes to the c-Jun-, Jun-D-, and Fra-2-enhanced fra-1 promoter activation. Cells were transfected with the wild-type 379-Luc (WT) or 379-Luc bearing a mutation within −318 TRE (TREmt) in the presence of empty or expression vectors for the indicated API1 proteins. Luciferase activity was determined as in Fig. 2. Values are mean ± S.E. of triplicate samples of two independent experiments.

DISCUSSION

We have shown here that at least three different elements, an EBS, a GC box, and a TRE located in the −379/−283 region, are necessary for the TPA-induced expression of the human fra-1 gene in HBE cells. The fact that a mutation in any of these elements significantly reduced the level of gene induction and that competition with any of the corresponding oligonucleotides...
interfered with the binding of transcription factors at other sites in this enhancer strongly suggest that cooperative interactions between transcription factors are obligatory for driving transcription from the promoter. As would be expected for the cooperative multiprotein complexes formed with this type of enhancer, such cooperativity was also associated with preferential recruitment of only specific members of a family of structurally similar but functionally distinct transcription factors. Indeed, the fra-1 enhancer was preferentially activated by c-Jun, Jun-D, and Fra-2 among the several members of the AP1 family. The dependence of fra-1 transcription on c-Jun is further supported by the complete lack of its induction by serum in c-jun−/− mouse embryonic fibroblasts (28, 29).

We have also demonstrated for the first time that Fra-2 can control fra-1 transcription. The observation that a loss of c-fos delays, but does not ablate, serum-stimulated fra-1 induction (28, 29) indicates that Fra-2 may compensate for c-Fos in regulating fra-1 expression. Intriguingly, in contrast to the fra-1 promoter, gene expression driven by the consensus TRE was strongly suppressed by Fra-2. This observation indicates that Fra-2 differentially regulates TRE-dependent gene expression, depending on the promoter context. Recently, Schweneger et al. (30) have shown that a Fra-2-based AP1 complex is required for inducing human interleukin-5 gene transcription in T-cells. Both transient transfection and overexpression studies indicated a role for Jun-D in the up-regulation of fra-1 transcription, although EMSA and ChIP assays showed that Jun-D binds to the fra-1 promoter even in the absence of any stimulus (Figs. 5 and 8). Therefore, we infer that an AP1 complex composed of Jun-D alone is insufficient to induce fra-1 transcription. However, TPA-induced posttranslational modifications of Jun-D, combined with the binding of other AP1 proteins such as c-Jun and Fra-2, probably contribute to the activation of fra-1 transcription. For instance, Jun-D is not phosphorylated by c-Jun N-terminal kinases because it lacks a functional docking site for these kinases (31), but dimerization with c-Jun allows the phosphorylation of Jun-D by c-Jun N-terminal kinase, thereby enhancing its transcriptional activity (31). Although overexpression of Jun-D activates the fra-1 promoter (Fig. 6), this response may not be physiologically relevant, given the observation that most cell types have abundant Jun-D levels (32).

We have noticed that 328-Luc, which contains the critical TRE but lacks the EBS and GC boxes, can still respond to TPA, albeit less robustly than 379-Luc. Mutation of −318 TRE not only abolished the formation of API complexes (Fig. 5B) but also dramatically inhibited (>50%) TPA-inducible fra-1 transcription (Figs. 4 and 10). These results suggest that −318 TRE is necessary but not sufficient for optimal transcription. In accord with our findings, Tsuchiya et al. (33) have previously shown an important role for −318 TRE (also known as Fos AP1-binding element) in serum- and Tax1- (a viral protein) induced fra-1 transcription. Thus, −318 TRE is necessary for inducing the human fra-1 promoter in response to disparate stimuli in several cell types. In contrast to human fra-1 (our studies), Bergers et al. (26) have reported that a −710-bp 5′-flanking region and an intronic sequence (located between positions +1112 and +1162) are important for regulating AP1-induced rat fra-1 transcription. The intronic 52-bp DNA fragment contains a perfect TRE and two variant TREs, whose positions and order are conserved between mouse and rat (26, 29). Comparison of human (GenBank™ accession number AP009470), mouse, and rat gene sequences have also identified a similarly conserved sequence in the intronic region (located between +957 and +1008) of human fra-1. However, transient transfection analyses (Figs. 2, 3, and 10) and ChIP assays (Fig. 8) have clearly demonstrated that the −379 to +32 bp region of fra-1 is sufficient for TPA-induced expression. Based on these observations and a previous report showing the involvement of the −379 to +32 bp region in Tax-1- and serum-inducible fra-1 promoter activity (33), we propose that intronic TREs are not required for TPA-inducible fra-1 expression in HBE cells. However, it cannot be ruled out that the intronic sequences may contribute to the overall fra-1 expression under some currently unidentified circumstances.

The loss of TPA-inducible expression following mutation of the −377 EBS and −343 GC box, despite the presence of a functional TRE, is consistent with the EMSA data (Fig. 4). These observations suggest that cooperative interactions between trans-acting factor(s) binding to the −377 EBS, −343 GC box, and −318 TRE are critical for optimal regulation of fra-1 expression. A similar role for GC/CAC boxes and EBBS in the regulation of other AP1 genes has been documented (34, 35). For example, the human c-fos also contains two CAC/GT boxes located between positions −97 and −76, and mutation of these sites severely cripples both basal and TPA- and EGF-inducible expression of c-fos (36). A similar involvement of EBBS in the regulation of AP1 genes has also been shown. The ETS family members p62TCF, Elk1, and SAP1a, and SAP1b, in conjunction with serum response factor, regulate c-fos and fos-B promoters in response to various stimuli in different cell types (35, 37). Ets-1 and Ets-2 strongly transactivate the jun-B promoter (38) but not those of c-jun and jun-D promoters, which lack EBBS (35). Although our results indicate that ETS and Sp family members play a role in fra-1 regulation, further investigation is needed to assess the individual contributions of these factors to fra-1 regulation. Such interactions may permit the integration of various signaling networks into the −379 to −283 bp promoter in empirically regulating fra-1 transcription in different cell types in response to diverse agents, such as oxidants, carcinogens, inflammatory cytokines, and mitogens.

Fra-1 expression is tightly correlated with tumor growth progression. It is also likely that cooperation between ETS proteins and an activated c-jun (a known oncogene) (39, 40) sustains cell growth via induction of Fra-1. In this context, it is interesting to note that ETS-1 is also a known oncprotein (41). It is tempting to speculate that Fra-1 is a convergence point for oncogenic transcription factors.
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