Expression of Human Squamous Cell Differentiation Marker, SPR1, in Tracheobronchial Epithelium Depends on JUN and TRE Motifs*

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Tracheobronchial epithelial (TBE) cells that normally do not express the squamous cell differentiation marker gene, SPR1, can be induced to produce it by 12-O-tetradecanoylphorbol-13-acetate (TPA). The regulation of SPR1 gene expression by TPA occurs in part, at the transcriptional level in primary human and monkey TBE cells. Using a transient transfection assay, we observed that TPA stimulates the activity of the reporter gene, chloramphenicol acetyltransferase, by 2–4-fold in transfected TBE cells. However, this chloramphenicol acetyltransferase activity is cell-type-specific with significantly less activity in transformed epithelial cell lines and no activity in non-epithelial cell types. TPA-dependent stimulation can also be demonstrated by cotransfection with plasmid DNAs that overexpress the JUN family of proteins, especially c-JUN. Overexpression of c-JUN and TPA treatment synergistically stimulate the SPR1 promoter activity by more than 40-fold. Deletion analysis of the promoter region demonstrates that the DNA fragment of the first 98 base pairs of the 5′-flanking region contains the basal promoter activity, while the region between –162 and –96 contains the cis-enhancer elements for both the basal and TPA/c-JUN-stimulating promoter activities. This observation is supported by in vivo genomic footprinting studies that reveal persistent protections in the following motifs of this region: –141 TRE, –131 GT, –123 ETS-like, and –111 TRE-like motifs and in the enhanced protections in –141 TRE and –111 TRE-like motifs in cells after the TPA treatment. Site-directed mutagenesis in this region demonstrates the involvement of both –141 TRE and –111 TRE-like motifs in TPA/c-JUN-dependent stimulation as well as enhanced basal transcriptional activity. However, it is primarily the –111 TRE-like motif that is involved in the mediation of the enhanced basal promoter activity of the human SPR1 gene. These results are further supported by gel mobility shift assays that demonstrate the involvement of c-JUN and these TRE motifs in the formation of the DNA-protein complex.

The small proline-rich protein (SPR)1 family with a molecular mass ranging from 10 to 30 kDa was first reported by Kartasova and van de Putte in 1988 (1). They demonstrated that the synthesis of SPR proteins is rapidly induced in human keratinocyte cultures after UV irradiation or treatment with TPA. Both of these treatments enhance the cornification of keratinocytes in culture. Two distinct groups of SPR cDNA clones were subsequently isolated using the differential hybridization technique, and their sequences were determined (1, 2). Immunohistochemical studies, using a polyclonal antibody specific to the C-terminal peptides of the SPR1 protein, demonstrate the presence of a SPR1 antigen in the suprabasal cell layer of various human squamous tissues such as the epidermis and esophagus (3). A close association between the expression of SPR genes and squamous epithelial cell differentiation has been further demonstrated by Northern blot analysis (4) and in situ hybridization (5).

A unique feature of the structure of the SPR gene family is that the central segments of the encoded polypeptides are built up from tandemly repeated units of either eight (SPR1 and SPR3) or nine (SPR2) amino acids with the general consensus XKKPPEXX (6). The function of such a repeated peptide unit is currently unknown. Backendorf and Hohl (7) suggested that the SPR proteins are potential substrates involved in squamous cell cornification based on a comparison of both the N- and C-terminal amino acid sequences of SPR proteins with involucrin and loricrin, the cornified envelope proteins. Marvin et al. (8) have suggested that, based on Western blot analysis, the SPR proteins are part of the cornified envelope. We have recently demonstrated a similar finding. Unexpectedly, we also observed SPR1-like antigens in the nucleus (9). A similar observation was recently made by Hohl et al. (5) in epithelium. This finding suggests that the SPR1-like protein may also play a regulatory role in gene expression.

In contrast to squamous tissues, the presence of SPR proteins is very low in respiratory tract epithelia that normally express mucociliary functions; however, we have demonstrated a rapid increase of SPR1 gene product in isolated human and monkey airway epithelial cells upon plating on a culture dish (4). This increase can be reduced by supplementing the culture medium with vitamin A or its synthetic retinoid derivatives (4). Another study has demonstrated that vitamin A down-regulates the stability of SPR1 mRNA (10). We have also observed increased expression of SPR1 protein and mRNA in the patchy

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1 The abbreviations used are: SPR, small proline-rich protein; TPA, 12-O-tetradecanoylphorbol-13-acetate; TBE, tracheobronchial epithelium; TRE, TPA-responsive element; CRE, cAMP-responsive element; tk, thymidine kinase; CAT, chloramphenicol acetyltransferase; DMS, dimethyl sulfoxide; GT, GGTGG motif; ETS, E-26 transformation specific; HSV, herpes simplex virus; PCR, polymerase chain reaction.
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Squamous cell metaplasia of monkey tracheal tissues after maintaining them under a vitamin A-free organ culture condition (data not shown). TPA, a potent squamous cell differentiation inducer, also stimulates SPR1 gene expression at the transcriptional level (11). These results establish a close relationship between the up-regulation of SPR1 gene expression and squamous airway epithelial cell differentiation.

The expression of squamous cell function in respiratory tract epithelium is a phenomenon that is frequently associated with injury. Squamous cell metaplasia has been implicated in the development of bronchogenic cancer (12, 13). The nature of the induction of squamous cell differentiation in the conducting airway epithelium is still unresolved. Therefore, studies of SPR1 gene expression in non-squamous airway epithelial cells may be different from those carried out in epithelial cells that express only the differentiation of skin-like properties such as keratinization and cornification. Results obtained from studying squamous cell differentiation may provide essential understanding of the mechanism underlying the divergent pathways of cell differentiation in conducting airway epithelium (14).

We have isolated the human SPR1 genomic clone and have completed the DNA sequencing of the 5'-flanking region (11). The purpose of this communication is to use the transient transfection study, in vivo genomic footprinting, site-directed mutagenesis, and the gel mobility shift assay to elucidate the elements essential for both the basal, uninduced and TPA-inducible promoter activities. We observed that the expression of the human SPR1 gene in conducting airway epithelium is dependent on JUN and TREC motifs located between −141 and −111 of the 5'-flanking region. Furthermore, the expression is cell-type-specific, with a decrease in the promoter activity from primary epithelial cells to established cell lines, with no activity in the non-epithelial cell type.

MATERIALS AND METHODS

Cell Isolation and Culture Conditions—Primary TBE cells were isolated from rhesus monkey and human tracheobronchial tissues, which were obtained from the California Regional Primate Research Center and the Medical Center of the University of California at Davis, respectively. All procedures involved in the tissue procurement were approved by the University of California at Davis Animal Protocol Review Committee and the Human Subject Research Review Committee. Epithelial cell isolation and the culture conditions were carried out as described previously (15, 16). Experiments were carried out between 7 and 14 days of age, and the various cell lines, HepG2 (ATCC HB8065, a hepatocellular carcinoma), Caco-2 (ATCC HTB37, a colon adenocarcinoma), and A172 (ATCC CRL1629, a glioblastoma), were obtained from the American Type Culture Collection (ATCC), and they were maintained in serum-supplemented culture conditions according to the supplier’s data sheet.

Plasmids and Construction of CAT Reporter Constructs.—Plasmids pBL-CAT3 contains the herpes simplex virus (HSV) thymidine kinase (tk) promoter in front of the CAT structural gene. Plasmid pBL-CAT3 is a promoterless construct. The expression plasmids encoding JUN B and JUN D DNAs are pBR322-based vectors driven by a long terminal repeat promoter of the mouse sarcoma virus. The other expression plasmid encoding c-Jun DNA was derived from pSVL vector (Phar- macia Biotech, Inc.) driven by the SV40 early promoter. The control plasmid pUC119 encodes β-galactosidase cDNA, which is driven by the SV40 promoter.

Various 5'-flanking regions (between −2000 and −9 relative to the transcription start site) of the human SPR1 gene were amplified from human genomic clone (11) by polymerase chain reaction (PCR) with two restriction sites attached to the two specific primers: Sall to the 5'-primer and XbaI to the 3'-primer. Positive clones containing the appropriate inserts were determined by PCR screening and confirmed by restriction mapping and DNA sequencing. The generation of various chimeric constructs is described in Fig. 1. The 2000-CAT3, 622-CAT3, 557-CAT3, 162-CAT3, 113-CAT3, 98-CAT3, and 67-CAT3 constructs consist of DNA fragments between −2000 and −9, −622 and −9, −557 and −9, −162 and −9, −113 and +9, −98 and +9, and −67 and +9, respectively, of the SPR1 promoter in the pBLCAT3 vector under XbaI and Sall cloning sites. The chimeric constructs 622/81-tk-CAT2, 622/540-tk-CAT2, 193/81-tk-CAT2, and 162/96-tk-CAT2 contain various 5'- flanking sequences between −622 and −81, −540, −193 and −81, and −162 and −96 relative to the transcription start site, respectively, in the pBLCAT2 vector under XbaI and Sall cloning sites.

The PCR reactions were carried out in a total volume of 100 μl containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.2 mM MgCl₂, 0.01% gelatin, 0.001% each of dATP, dGTP, dCTP, and dTTP (Pharmacia). Initial denaturation was set at 95°C for 5 min followed by 30 cycles of 94°C denaturation (1 min), 55°C annealing (1 min), and 72°C extension (4 min), with a final 72°C extension for 7 min in an automated thermal cycler (Perkin-Elmer Corp.).

The 451-CAT3 and 135-CAT3 constructs contain regions between −451 to −9 and −135 to −9 of the SPR1 promoter, respectively, were constructed by ligation of SpeI-XbaI and HindIII-XbaI DNA fragments, digested from 622-CAT3 construct into pBLCAT3 vector.

DNA Transfection and CAT Assays—DNA transfection was performed using a liposome technique (Lipofectin™) according to the procedure suggested by the manufacturer (Life Technologies, Inc.). Each culture dish at 70−80% confluence was washed with serum-free medium and then transfected with 3 μg of pUC18 plasmid DNA. 1 μg of pCH110 plasmid DNA, and 1 μg of expression plasmid DNA when the cotransfection experiments were carried out. The pUC18 plasmid DNA was added to ensure that all the individual experiments contained the same total amount of DNA. TPA was added to transfected cultures at 10 ng/ml for 48 h before the harvesting. Cell extracts were prepared by freezing thawing in 0.25× Tris-HCl (pH 8.0). The protein concentration of cell extracts was determined by a modified Bradford technique (Bio-Rad). Equal amounts of protein were assessed for CAT activity by either a liquid scintillation method (11) or an enzyme-linked immunosorbent assay kit (Boehringer Mannheim) following the manufacturer’s suggested protocol. The β-galactosidase activity was normalized as the internal control that normalized transfection efficiency, was determined by a β-galactosidase enzyme assay system (Promega).

Nuclear Extract Preparation and Gel Mobility Shift Assay—Nuclear extracts were prepared according to the method of Dignam et al. (17) from cultured cells, except that during extraction 600 μM NaCl was used instead of 400 μM NaCl. The binding was performed in a 20-μl reaction volume containing 25 mM HEPES, pH 7.9, 10% (w/v) glycerol, 30 mM NaCl, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 5 mM MgCl₂, 0.5 mM EDTA, 0.5 μg of salmon sperm DNA, and 50–100 ng of poly(dI-dC). After incubation on ice for 10 min with 3–5 μg of nuclear extract, 0.1–0.5 ng of end-labeled probe (~20,000 cpm) was added and incubated at room temperature for 15–20 min. The DNA-protein complexes were resolved in native polyacrylamide gel (20% acrylamide to bisacrylamide) in 0.5× Tris borate-EDTA buffer. In supershift analysis, nuclear extracts were mixed with 1–2 μg of anti-c-Jun UN antibody (Santa Cruz Biotechnology, CA) and incubated on ice prior to adding the labeled DNA probe. For competition experiments, cold consensus AP1 oligonucleotide (Promega) was added to the reaction mixture prior to adding labeled probe.

In Vivo Dimethyl Sulfate (DMS) Footprinting—The method of in vivo DMS footprinting was described by Mueller and Wold (18). The primary TBE cells were cultured to 70–80% confluence followed by incubation with or without TPA (10 ng/ml, Sigma) for 12 h. For in vivo DMS treatments, cells were treated with 0.1% DMS (Aldrich) for 2 min at room temperature. The DNA-DMS treated cells were lysed in SDS buffer (0.5% SDS, 20 μM Tris-Cl, pH 8.0, 200 μM NaCl, and 20 mM EDTA) and then incubated with protease K (250 μg/ml) (Sigma) at 37°C for 12 h. Genomic DNA was then isolated by phenol/chloroform extraction. As an in vitro control, protein-free genomic DNA was prepared and treated with DMS for 20–30 s at room temperature. It was then cleaved by proteinase K (Aldrich) and labeled by the ligation-mediated PCR method of genomic DNA sequencing (19).

DNA fragments were analyzed on 6% denaturing urea-polyacrylamide gels. The bands on the autoradiogram were detected after 24–48 h of exposure. The reproducibility of the in vivo footprinting data was checked by analyzing genomic DNA samples prepared from three or more separate batches of DMS-treated cells.

Statistical Analysis—The StatView™ statistical program on the Macintosh computer was used to perform analysis of variance between different CAT reporter constructs. The Fisher PLSD (Protected Least Significant Difference) test was used to determine the significance of the differences in the CAT activities. The results were considered to be statistically significant when the p-value was less than 0.05.
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RESULTS

Basal Promoter Activity—Previous research based on the nuclear run-on assay and transient transfection study suggests that the treatment of primary airway epithelial cells with TPA stimulates the expression of the SPR1 gene at the transcriptional level. DNA sequence analysis of genomic clones revealed the following motifs: 'TATA' box at -28; three TRE-like sites at -49, -111, and -472; ETS at -55 and ETS-like at -123; GT motif (GGTGG) at -131; TRE site at -141; and CRE-like at -588 relative to the transcription start site in the 5'-flanking region of the human SPR1 gene. The -49 TRE-like site and -55 ETS site are merged together. To elucidate the functional roles of these putative cis-acting elements, a number of chimeric constructs containing various lengths of the 5'-flanking region and the CAT reporter gene were prepared. Significant differences were observed in 162-CAT3, but not in 135-CAT3, implies that the DNA fragment between -162 and -135 contains a sequence responsible for the enhanced expression. In contrast, cells transfected with 451-CAT3 construct, which contains the 162-CAT3 DNA fragment plus the flanking region between -451 and -162, did not exhibit enhanced basal promoter activity. This implies that the region between -451 and -162 may contain a sequence that down-regulates this enhanced activity.

To further elucidate the region involved in the basal promoter activity, a 67-CAT3 chimeric construct that includes -55 ETS, -49 TRE-like, and -28 TATA was studied. As presented in Fig. 3, the relative CAT activity in cells transfected with this construct is very low but significantly higher (2-fold) than the promoterless pBL-CAT3 transfected cells; however, this activity is less than 10% of the 98-CAT3 transfection. To clarify this residual activity, site-directed mutations in both -55 ETS and -49 TRE-like sites were prepared. Single or double mutations in these sites have no effect on this residual CAT activity, which suggests that neither site is involved in this residual CAT activity.

Cell Type-Specific Basal Promoter Activity—Both immunohistochemistry and mRNA analyses have demonstrated that the expression of the SPR1 gene is closely associated with squamous epithelial cell types. This specificity is preserved in the basal promoter analysis. As illustrated in Fig. 4, 162-CAT3 transfected primary human TBE cells exhibited the highest CAT activity as compared with the immortalized human normal TBE cell line, 5 clone, and other ATCC cell lines. The non-epithelial cell line A172 exhibited no CAT activity. In some cases of transfection with the 98-CAT3 construct, which contains no enhanced sequence, all TBE cells, regardless of their origin, exhibited significantly lower levels of basal promoter activity.
activity. Transfected non-TBE cells exhibited no activity.

TPA/c-JUN Inducible Promoter Activity—The relative CAT activity in 622-CAT3 transfected cells was further enhanced 4-fold by TPA (Fig. 2). This enhancement of promoter activity by TPA is maintained in various deletion-construct transfected TBE cells, until the TRE-like motif at –111 is deleted in such constructs as 98-CAT3 and 67-CAT3. These results imply that the CRE-like motif at –588 and the two TRE-like motifs at –472 and –49 are not involved in mediating the TPA response.

We observed that TPA transiently stimulates the expression of the JUN family gene products (data not shown) in TBE cells prior to the stimulation of SPR1 gene expression. We then examined whether overexpression of JUN family proteins can enhance the CAT activity in the absence of TPA treatment. Cotransfection with one of the JUN family genes, c-JUN, cotransfection was the most active. TPA treatment of these c-JUN cotransfected cells resulted in a 40-fold increase of CAT activity; however, TPA has no effect on the expression of JUN family proteins in cotransfected cells (data not shown). These results suggest that the SPR1 promoter is synergistically stimulated by TPA and c-JUN.

To further elucidate whether the regulatory sequences at the 5'-flanking region can enhance the promoter activity in heterologous constructs, several selective DNA fragments of SPR1 promoter were cloned to the pBL-CAT2 vector carrying the heterologous HSV-tk promoter (Fig. 1C). As shown in Fig. 6, in the absence of TPA and c-JUN cotransfection, the relative CAT activities in both 622/81-tk-CAT2 and 193/81-tk-CAT2 transfected cells were 13- and 11-fold higher than the pBL-CAT2 control. However, this enhanced activity cannot be seen in 622/540-tk-CAT2 transfected cells. This suggests that the enhanced activity located at the –193 and –81 5'-flanking region is capable of stimulating a heterologous promoter. The stimulations by TPA and c-JUN cotransfection are not as significant
as in the homologous promoter system, only a 20–40% stimulation. However, this stimulation was not seen in 622/540-tk-CAT2 transfected cells.

These results suggest that the DNA sequence located between –193 and –81 contains the cis-element that is also capable of activating the heterologous tk promoter in response to TPA/c-j UN treatment. To elucidate the site(s) responsible for this stimulation, we carried out site-directed mutations in the SPR1 promoter region. The relative CAT activity was further normalized to the 622-CAT3 construct alone in transfected cells.

Genomic Footprinting of SPR1 Promoter—To further understand the nature of the regulation of SPR1 gene expression in vivo, the interactions between the promoter DNA sequence and transcriptional proteins were studied by DMS footprinting. Using appropriate primers as described in Fig. 8A, the DNA-protein interaction sites of the SPR1 promoter region were mapped. Genomic footprinting data of the DNA fragment between –164 and –94 are displayed in Fig. 8B. The G residue at –140 of the coding strand of the TRE motif was partially protected, while the flanking G residue at –143 was hyperreactive in primary TBE cells. This protection at –140 G residue was slightly enhanced by TPA. In addition, a displayed protection pattern at –138 G residue was observed only in TPA-treated cells. Other protection residues in the coding strand included the G residues at –139, –127, –126, –121, –120, which are parts of the GT (–131 to –127) and ETS-like (–123 to –116) motifs. At the –111 TRE-like motif site, the G residue at –110 was partially protected, and this protection was slightly enhanced in cells after the TPA treatment. The G residues at –113, –112, and –99 in other flanking regions were partially protected, whereas the G residue at –94 was hyperreactive to piperidine cleavage in both TPA-treated or untreated cells. On the noncoding strand, only G residues at –106 displayed protections (Fig. 8B).

Other DMS footprints were detected around the CRE-like motif. On the coding strand, similar protections occurring at –138 G residue in TRE-treated cells were observed in both TPA-treated and untreated cells (Fig. 8B).

A summary of these footprinting studies is presented in Fig. 10. There are multiple protections in the region between the –141 TRE and the –111 TRE-like motifs. These protections, presumably due to the interactions between the trans-activation proteins and the DNA sequence, are further enhanced on both –141 TRE and –111 TRE-like motifs by TPA. In contrast,
the protections in the CRE-like motif at −588 are not affected.

Identification of Nuclear Factors That Bind to the −162/−96 Fragment of the SPR1 Promoter—The DNA-protein interactions were further studied by using the gel mobility shift assay. A −162/−96 probe containing the TRE sites (−141 TRE and −111 TRE-like) forms a retarded complex in S-cell nuclear extracts (Fig. 11 A, lane 2). The specificity of the DNA-protein complex was demonstrated by a self-competition with the unlabeled DNA probe of the region (Fig. 11 A, lane 3) and by the DNA fragment known to bind the AP1 transcriptional protein complex (Fig. 11 A, lanes 4–6). Furthermore, this binding is cell type-specific. As shown in Fig. 11, incubation of the nuclear extracts isolated from fibroblasts (A172) (Fig. 11 A, lane 7) with −162/−96 probe did not show any retarded complex. Site-directed mutagenesis in either of these two TRE sites has no effect on the DNA-protein complex formation (data not shown); however, double mutations in both TRE sites (−141 and −111) result in a loss of DNA-protein complex formation (Fig. 11B). These results suggest that these two TRE sites participate in DNA-protein complex formation.

To further characterize this DNA-protein complex, anti-c-Jun antibody was used to demonstrate a supershift in this gel mobility shift assay experiment. As shown in Fig. 11C, a preincubation of S-cell nuclear extracts with the anti-c-Jun antibody significantly reduced the retarded complex and caused a supershift in gel mobility shift assay. The control experiment indicated no complex formation between the DNA probe and the antibody used in this study (Fig. 11C, lane 3). Furthermore,

**Fig. 8.** In vivo DMS footprinting of the −193 to −81 5′-flanking region of the SPR1 promoter. A, identification of the locations and orientations of the primers used in the Sequenase and PCR amplification (indicated by arrows). B, both coding (left) and noncoding (right) strands of −193 to −81 regions were analyzed. The vertical open bars indicate the positions of different motifs. Protected and hyperreactive G residues are denoted by open and closed circles, respectively, and the numbers indicate the SPR1 promoter sequence. Sizes of the circles indicate the relative extents of DMS protection in vivo. Lane 1, control (C), protein-free genomic DNA; lane 2, genomic DNA from untreated primary TBE cells; lane 3, genomic DNA from TPA-treated primary TBE cells.

**Fig. 9.** In vivo DMS footprinting of the −622 to −540 5′-flanking region of the SPR1 promoter. A, identification of the locations and orientations of the primers used in the Sequenase and PCR amplification (indicated by arrows). B, both coding (left) and noncoding (right) strands of −622 to −540 regions were analyzed. Symbols and labels used are the same as those in Fig. 8.

**Fig. 10.** Summary of in vivo DMS footprinting analyses of the SPR1 promoter in primary TBE cells. A, data from Fig. 8; B, data from Fig. 9. Symbols and labels used are described in Fig. 8.
ments that work in concert to regulate the basal promoter activity in cells without the TPA treatment. First, we observed that the first 98-base pair DNA fragment at the 5′-flanking region of the human SPR1 gene contains sufficient information for the basal promoter activity. A deletion between −98 and −67 substantially reduces this activity. Neither the −55 ETS or the −49 TRE-like motif is involved in the residual activity. This is consistent with the presence of the "TATA" box at the −28 position in this DNA fragment.

Our second finding regarding the regulation of the basal promoter activity of the human SPR1 gene was that the enhanced element in the 5′-flanking region was identified. In the homologous promoter system, the fragment at −162 to −135 is recognized as the enhancer that elevates the basal promoter activity. However, in a heterologous promoter system such as the pBL-CAT2, which contains the HSV-tk promoter, the enhanced activity is located at −162/−96. Double mutations at the −141 TRE and −111 TRE-like motifs can eliminate this enhanced activity, suggesting the participation of both motifs in enhancing the promoter activity. The −111 TRE-like motif plays a more important role in this enhanced activity since a mutation in this site reduced the activity more than 50%. These studies are consistent with the in vivo footprinting data.

The third element in the regulation of SPR1 promoter activity is the suppressor sequence that is located at the 5′-flanking region between −451 and −162. The nature of this suppressive effect has not been characterized.

We also identified the regulatory elements that are involved in TPA-induced promoter activity. Several potential TREs are identified in the 5′-flanking region of the human SPR1 gene based on the DNA sequence information; however, not all of the potential motifs are involved. This is consistent with the report of Morrow et al. (20) that the TRE motif in the human glutathione S-transferase gene promoter is unresponsive to both TPA and the JUN/FOS protein activation. Based on the results from transient transfection studies and the DMS footprinting data, we hypothesize that the DNA fragment between −162 and −96 is involved in the mediation of TPA responsiveness. This region contains −141 TRE, −131 GT, −123 ETS-like, and −111 TRE-like motifs.

This mediation occurs not only on the homologous promoter but also, less actively, on heterologous promoters such as the HSV tk promoter. The reason for the decreased activity on the HSV tk promoter is not clear. It is possibly due to the fact that this region contains strong basal enhanced elements that can stimulate the tk promoter more than 30-fold, and further stimulation by TPA and c-JUN is restricted. Nevertheless, it is possible to demonstrate that double mutations in both −141 TRE and −111 TRE-like motifs will knock out the c-JUN-dependent stimulation. A single mutation in either site cannot eliminate this enhanced activity. These results suggest that either the −141 TRE or the −111 TRE-like site can be used for TPA/c-JUN-mediated activation. This notion is further supported by the gel shift and in vivo genomic footprinting analysis. Gel shift analysis using S-cell nuclear extracts reveals that the mutation of TRE motifs (−141 and −111) significantly blocks the protein-binding complex (Fig. 11) at the −162/−96 5′-flanking region. Competition experiments using the consensus AP1 oligonucleotide completely abolishes the binding of nuclear factors, indicating that the −162/−96 region is probably bound by AP1 proteins. Further, preincubation of nuclear extracts with an anti-c-JUN antibody is able to supershift the DNA-protein complex (Fig. 11C). These studies indicate that TRE motifs at the 5′-flanking region are occupied by transcriptional factors such as AP1 proteins and c-JUN.

In vivo genomic footprinting data also revealed a persistent protection of G residues in this DNA fragment; however, the protections on the G residues in both the −141 TRE and −111 TRE-like motifs were further enhanced in cells treated with TPA. Actually, a new protection on the −138 G residue of the −141 TRE motif was induced in cells after the TPA treatment (Fig. 10). The −141 TRE (TGAGTCA) has a perfect nucleotide sequence, which matches a known consensus sequence of a TRE. While the −111 TRE-like (TGAAATCA) has A substituted
for G in the fourth position, the motif should retain 25–75% of the binding activity of the JUN-FOS complex (20). We have also demonstrated that this −111 TRE-like motif plays a more important role than the −141 TRE motif in mediating both the basal and stimulated enhanced activities.

However, the mediation by the −111 TRE-like motif is not that straightforward. We observed less inducible CAT activity by TPA in cells transfected with the 113-CAT3 construct despite the fact that the −111 TRE-like motif is included in this construct. In contrast, TPA responsiveness was demonstrated in cells transfected with 135-CAT3, which includes the flanking region between −135 and −111 in addition to the −111 TRE-like motif. These data imply that the presence of a flanking sequence between −135 and −111 is critical for the −111 TRE-like motif to mediate the TPA responsiveness, especially in the homologous promoter system. The DNA fragment between −135 and −111 contains both GT and ETS-like motifs at −131 and −123, respectively. Interestingly, the in vivo DMS footprinting study demonstrated that the multiple protection persistently appeared on the G residues of these motifs, regardless of the TPA treatment. This result further supports the notion that the flanking region is important to the TRE-like motif in mediating TPA response. Further experiments are needed to elucidate the role of this flanking region in mediating the human SPR1 promoter activity.

We also demonstrated that c-jun is involved in the activation of promoter activity. Cotransfection with c-jun expression plasmid DNA can mimic the action of TPA and can also cooperate with the TPA treatment to synergistically stimulate the promoter activity. A number of TPA-inducible genes have been characterized (21–28), and the TRE-like motif, consensus sequence TGA(G/C)TCA, in their promoter/enhancer region has been recognized (29–31). This study is consistent with the notion that the TRE activity is mediated by the AP1 transcription factor, which is composed of both the JUN and the FOS family of proteins (32–34). The JUN family proteins (35–37), c-jun, JUN B, and JUN D, can either homodimerize or heterodimerize with the FOS family proteins, c-FOS, FOS B, FRA-1, and FRA-2 (38). These dimers bind to the TRE site, thereby regulating gene expression in response to TPA (39–41). Northern blot analysis demonstrates that TPA transiently enhances the c-jun message in primary TBE cells (data not shown). In contrast, the messages for JUN B and JUN D are very low in primary TBE cells (data not shown). Therefore, it is possible that one action of the TPA is to enhance the c-jun synthesis. This would explain why c-jun cotransfection also stimulates the promoter activity; however, this action is different but very complementary to the TPA treatment.

We observed a synergistic activation of the SPR1 promoter activity by both c-jun cotransfection and the TPA treatment. This suggests that complementary pathways are activated by these treatments in the enhancement of the SPR1 promoter activity. It is necessary to point out that TPA treatment did not enhance the expression of c-jun protein in c-jun cotransfected cells. Therefore, the main mechanism of the TPA effect does not depend on the c-jun production per se but rather on the activation of various kinases that further activate the c-jun. One potential mechanism to account for the increase in c-jun transcription factor activity would be the dephosphorylation of phosphoserine and phosphothreonine residues adjacent to the DNA binding domain of c-jun protein. These residues are a target for casein kinase II and, when phosphorylated, inhibit the ability of c-jun to bind to DNA (42). TPA usually activates the protein kinase C (PKC), which appears to be the regulator for c-jun phosphatase that dephosphorylates these residues, thereby stimulating c-jun binding and transcriptional activation.

Lastly, we demonstrated that the basal and the enhanced promoter activities of the SPR1 gene observed in TBE cells cannot be demonstrated in other non-TBE cells. The nature of this cell type-specific mechanism is not understood; however, a similar cell type-specific and tissue-specific SPR1 gene expression has been demonstrated by immunohistochemistry (1) and Northern blot hybridization (4). This study suggests that the cell type specificity occurs at the transcriptional level. Since transcriptional regulation involves the DNA-protein interactions, it is likely that these non-TBE cells either lack transcriptional factor(s) to recognize this SPR1 DNA sequence or contain inhibitory factor(s) that interfere with the transcription of the SPR1 gene. Gel mobility shift assays using nuclear extracts from SPR1 expressing (TBE cells) and nonexpressing (nonepithelial) cell lines show formation of cell type-specific retarded complex at the SPR1 promoter region. Further detailed study may help elucidate the nature of this cell type specificity.

In summary, our results suggest the involvement of −141 TRE and −111 TRE-like motifs and c-jun expression in conferring the maximal expression of the human SPR1 gene in TBE cells. Both −141 TRE and −111 TRE-like motifs are involved in the basal and TPA-stimulated enhanced activities. Other TRE sites, such as −472 TRE-like and −49 TRE-like, are not involved. The exact molecular basis of these motifs and the corresponding binding factors involved in the SPR1 transcription, however, remain to be determined.

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