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Enhancement of Gene Expression by Transcription Factor AP-1 Is Dependent on Orientation of AP-1 Element

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Transcription factor AP-1 induced by 12-O-tetradecanoylphorbol-13-acetate treatment of LLC-PK1 cells binds specifically to an AP-1 oligonucleotide. To study the effect of interaction of transcription factor AP-1 with its AP-1 element on gene expression, an AP-1 consensus sequence was cloned into a reporter vector. Expression of the reporter gene in transfected cells was greatly enhanced in the presence of the reverse-oriented AP-1 element upstream of a SV40 promoter. Mutation (G to C) of the central base in the AP-1 motif (TGAGTCA) abolished the enhancement for the reporter gene expression. These results indicate that the orientation of AP-1 element relative to the transcription machinery is important for its regulatory function. Moreover, sequences that flank the AP-1 motif are also required for its regulatory function. © 1997 Academic Press

AP-1 and AP-1-like elements have been identified in the 5′ flanking region of several genes (1, 2), including the gene encoding the heavy subunit of γ-glutamylcysteine synthetase, which catalyzes the rate-limiting step in cellular glutathione synthesis (3, 4). Cellular glutathione concentrations are regulated by feedback inhibition of γ-glutamylcysteine synthetase by glutathione (5, 6), but the presence of AP-1 and AP-1-like elements in the 5′ flanking region of the gene encoding γ-glutamylcysteine synthetase indicates that cellular glutathione concentrations may also be regulated at the gene level. c-Jun and c-Fos induced by various stimuli interact with AP-1 and AP-1-like elements, which results in up-regulation of those genes (7-9). The orientation and spacing of AP-1 and AP-1-like elements are highly conserved (10, 11). Hence, the orientation of AP-1 and AP-1-like elements relative to the transcription site may be critical for the function of AP-1 and AP-1-like element in gene regulation.

To study the effect of orientation on the interaction of transcription factor AP-1 with the AP-1 element on gene expression, a consensus AP-1 sequence was cloned into a firefly luciferase reporter vector. LLC-PK1 cells were cotransfected with the firefly luciferase reporter vector and a *Renilla* luciferase reporter vector, which served as an internal control. c-Jun and c-Fos induced by incubation of LLC-PK1 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) increased the expression of the firefly luciferase gene in transfected cells when a reverse-oriented AP-1 consensus sequence was inserted upstream of a SV40 promoter in the firefly luciferase reporter vector. When the central base in the AP-1 motif (TGAGTCA) was mutated from G to C, cells transfected with reporter vectors containing AP-1 element in either 5′ → 3′ or 3′ → 5′ orientations did not show increased expression of the reporter gene in response to TPA. These findings indicate that the up-regulation of genes by the transcription factor AP-1 is dependent on the orientation of the AP-1 element relative to the transcription site and that both the AP-1 motif and flanking sequences contribute to enhancement of gene expression.

MATERIALS AND METHODS

Electrophoretic mobility shift assay. LLC-PK1 cells (ATCC) were maintained in Delbecco's modified Eagle medium (DMEM) (GibcoBRL, Grand Island, NY), supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin. Nuclear extracts were prepared by differential centrifugation of LLC-PK1 cells homogenized with a Dounce homogenizer (type B pestle) (Kontes, Vineland, NJ). A sample of the nuclear extract (5 μg protein) was incubated with ^{32}P-labeled AP-1 oligonucleotide (5′ CGCTTGATGAGTCAGCCGGAA 3′), 2 μg poly(dI-dC), 20 mM Hepes (pH 7.5), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol (4). After incubation at room temperature for 25 min, the protein-DNA complex was resolved on 5% polyacrymide gels containing 0.5 x TBE (1 x TBE contains 89 mM Tris-HCl, pH 7.5, 89 mM boric acid, and 2.5 mM EDTA). The gels were dried and analyzed by autoradiography.

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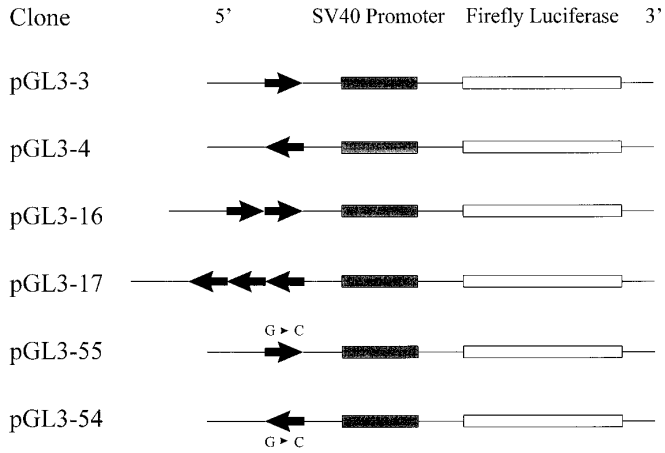

FIG. 1. Clones containing AP-1 elements in pGL3 promoter vectors. The filled arrow represents the orientation of AP-1 oligonucleotide. The nucleotide at the central position of the AP-1 motif (TGAGTCA) was mutated from G to C in pGL3–54 and pGL3–55.

Cloning of AP-1 oligonucleotide in pGL3 promoter vector. A double-stranded AP-1 consensus oligonucleotide (5′ CGCTTGATGAGTCAGCCGGAA 3′) and a mutated AP-1 oligonucleotide (G to C mutation at position 11) were phosphorylated with T4 polynucleotide kinase and cloned at the *Sma* I site of pGL3 promoter vector (Promega, Madison, WI). The AP-1 sequence and orientation in each clone were verified by didioxy DNA sequencing analyses. DNA clones containing one copy of the AP-1 element in the either 5′ → 3′ (pGL3-3) or 3′ → 5′ (pGL3-4) orientations, two copies of the AP-1 element in the 5′ → 3′ orientation (pGL3-16), or three copies of the AP-1 element in the 3′ → 5′ orientation (pGL3-17) were selected to study whether AP-1 orientation and copy number affected enhancement of the firefly luciferase gene expression (Fig. 1). pGL3-55 and pGL3-54 contained one copy of the mutated AP-1 element in the 5′ → 3′ and 3′ → 5′ orientations, respectively.

Luciferase activity in transfected LLC-PK1 cells. Cells (5×10^4) were transfected with 2 μg lipofectamine (GibcoBRL), 0.05 μg pRL-SV40 DNA (Promega) as an internal control, and 0.5 μg of pGL3 DNA in 300 μl of Opti-MEM I reduced-serum medium (GibcoBRL). The Opti-MEM medium was replaced with supplemented DMEM 6 h later. After 46 h of transfection, 100 nM TPA (GibcoBRL) was added to the culture medium, and the cells were incubated for 6 h. The cells were washed twice with phosphate-buffered saline (PBS) and harvested by addition of 100 μl of passive lysis reagent (Promega). Luciferase activities were measured with a dual luciferase assay system (Promega).

Statistical analyses. Data were analyzed by analysis of variance with the Tukey-Kramer Multiple Comparison Test by Instat 2.05a (GraphPad, San Diego, CA). A level of ≤ 0.05 was chosen for acceptance or rejection of the null hypothesis.

RESULTS AND DISCUSSION

Induction of transcription factor AP-1 by TPA in LLC-PK1 cells was determined by electrophoretic mobility shift assays. Incubation of LLC-PK1 cells with TPA induced the formation of transcription factor AP-1 compared with control cells (Fig. 2A, lane 3 vs. lane 2). Excess unlabeled AP-1 oligonucleotide prevented binding of transcription factor AP-1 to the AP-1 consensus oligonucleotide (Fig. 2B, lane 3 vs. lane 2), indicating that binding was selective for the AP-1 consensus oligonucleotide.

To study the effect of the transcription factor AP-1 to AP-1 element on gene expression, the AP-1 consensus sequence was cloned into a luciferase reporter vector, pGL3 promoter vector that contained the SV40 promoter but lacked enhancing elements. The DNA clones analyzed are shown in Fig. 1. Significantly higher relative luciferase activities were detected in cells transfected with the pGL3 control vector compared with cells transfected with the pGL3 promoter vector (Fig. 3). Relative luciferase activity did not differ among cells transfected with pGL3 promoter and pGL3-3, pGL3-4, pGL3-16, or pGL3-17 (Fig. 3). The small increase in relative luciferase activities in cells transfected with the pGL3 promoter vector containing the AP-1 element reflected the basal expression of transcription factor AP-1 in cells.

Relative luciferase activity was measured in trans-

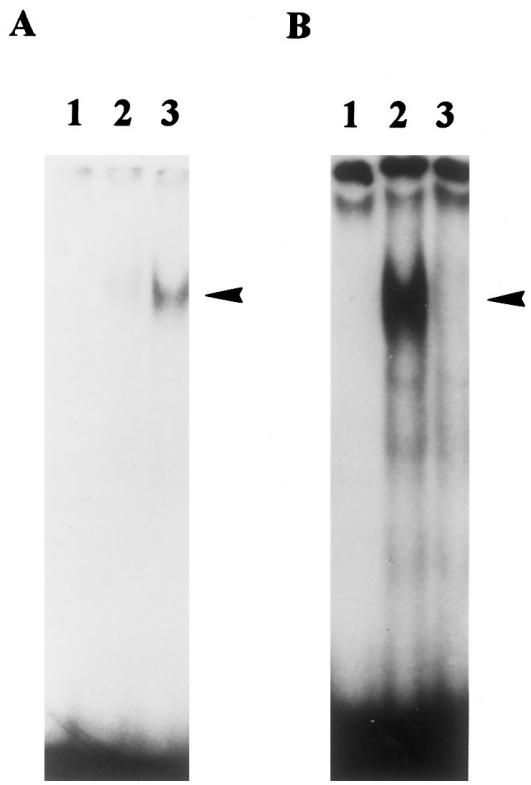

FIG. 2. Induction of transcription factor AP-1 by TPA in LLC-PK1 cells. Electrophoretic mobility shift assays were performed as described in Materials and Methods. A. Lane 1, no nuclear extract in the reaction mixture. Lane 2, nuclear extract from control cells. Lane 3, nuclear extract from cells treated with 100 nM TPA for 1 h. B. Lane 1, no nuclear extract in the reaction mixture. Lane 2, nuclear extract from cells incubated with 100 nM TPA for 1 h. Lane 3, nuclear extract plus 100-fold excess of unlabeled AP-1 oligonucleotide in the reaction mixture. The protein–DNA complex is indicated by an arrow.

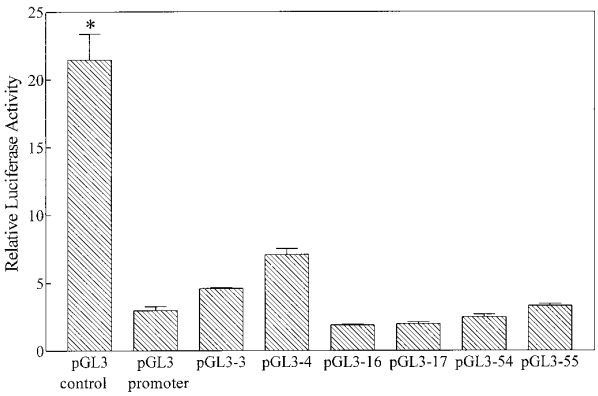

FIG. 3. Relative luciferase activity in transfected cells. Cells were transfected with pGL3 control vector, pGL3 promoter vector, or pGL3 promoter vectors containing AP-1 oligonucleotide in the orientations shown in Fig. 1. Cells were harvested 48 h after transfection, and luciferase activities were measured as described in Materials and Methods. Relative luciferase activity (mean ± SD, n 3) was calculated by normalizing firefly luciferase activity with *Renilla* luciferase activity. *pGL3-control vs pGL3-promoter, pGL3–3, pGL3–4, pGL3–16, pGL3–17, pGL3–54, or pGL3–55, $p < 0.05$.

fected cells after incubation with TPA (Fig. 4). Relative luciferase activity in cells transfected with pGL3-4 was significantly higher than in cells transfected with pGL3 promoter or pGL3-3. There was no difference in the relative luciferase activities among cells transfected with pGL3 promoter and pGL3-3, pGL3-16, or pGL3-17. In contrast to the luciferase activities observed in cells transfected with pGL3-3, TPA markedly increased the relative luciferase activities in cells transfected with pGL3-4, indicating that the interaction of induced transcription factor AP-1 was more favorable with the 3′ → 5′-oriented AP-1 element in pGL3-4 than with the 5′ → 3′-oriented AP-1 element in pGL3-3. Interestingly, cells transfected with pGL3 promoter DNA containing two or three copies of AP-1 element exhibited significantly lower luciferase activity than pGL3-4. This may indicate that the spacing between AP-1 elements in pGL3-16 and pGL3-17 is not optimal, because a certain relative orientation and spacing of the two AP-1 elements may be required to exert transcription enhancement (10, 12).

The AP-1 motif (TGAGTCA) is asymmetric. Hence, the central base may play a determining role in enhancing the gene transcription. Accordingly, the central base was mutated from G to C in the AP-1 motif. The mutated oligonucleotide was inserted upstream to the SV40 promoter (Fig. 1). As shown in Fig. 4, luciferase activities were not enhanced by TPA in cells transfected with clones pGL3-54 and pGL3-55. The change of the central base of the AP-1 motif from G in pGL3-4 to C in pGL3-54 abolished the up-regulation of reporter gene expression in response to TPA, indicating that the central base in the AP-1 motif is critical for its function. pGL3-4 and pGL3-55 contained the same AP-1 motif, whereas pGL-4 and pGL3-55 differed in the AP-1 motif-flanking sequence. Therefore, sequences that flank AP-1 motif are also important for functioning of the AP-1 consensus sequence.

Mutagenesis studies showed that the sequence of AP-1 element had a marked impact on the binding by c-Jun and c-Fos. The interaction of c-Jun and c-Fos with AP-1 was reduced by certain base substitutions within the AP-1 element (13). Crystallographic analysis of c-Jun and c-Fos with the AP-1 consensus sequence revealed that the basic regions of c-Jun and c-Fos interacted with the bases of the AP-1 site through hydrogen bonds and van der Waals forces. In addition, the heterodimer bound to the DNA in a about a 2:1 ratio of the two orientations (reverse orientation was dominant) (14). Moreover, other DNA binding proteins influenced the interaction of the c-Jun and c-Fos heterodimer with the AP-1 element. By application of the affinity-cleaving technique, it was demonstrated that the nuclear factor of activated T-cells (NFATp) directed the c-Jun and c-Fos heterodimer through protein-protein interactions to interact with one strand of a double-stranded AP-1 sequence (15). The results presented herein indicate that the configuration (sequence, orientation and spacing) of the AP-1 element is important for its regulatory function. c-Jun and c-Fos may be fixed in a certain position by other transcription factors so that the interaction of c-Jun and c-Fos with the 3′ → 5′-oriented AP-1 element in pGL3-4 was favorable. Currently, pGL3-4 is being used in our laboratory to facilitate the understanding of the role of the AP-1 element in regulation of γ-glutamylcysteine synthetase expression under oxidative stress.

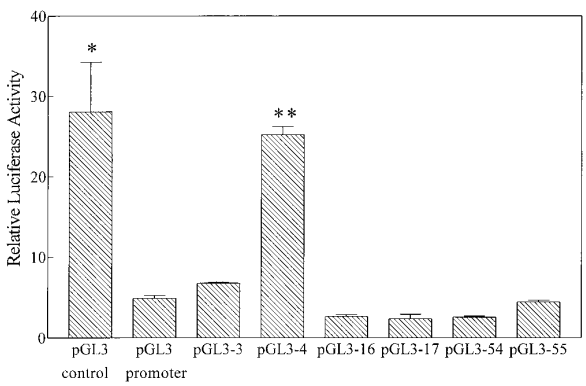

FIG. 4. Relative luciferase activity in transfected cells after incubation with TPA. Cells were transfected with pGL3 control vector, pGL3 promoter vector, or pGL3 promoter vectors containing AP-1 oligonucleotides in the orientations shown in Fig. 1 for 46 h. TPA was added to the culture medium, and the cells were incubated for 6 h. Cells were harvested, and luciferase activities were measured. Relative luciferase activity (mean ± SD, n 3) was calculated by normalizing firefly luciferase activity with *Renilla* luciferase activity. *pGL3-control vs pGL3–4, $p > 0.05$; **pGL3–4 vs pGL3-promoter, pGL3–3, pGL3–16, pGL3–17, pGL3–54, or pGL3–55, $p < 0.05$.

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