Activator Protein Accelerates Dihydropyrimidine Dehydrogenase Gene Transcription in Cancer Cells

Kei Ukon,1,2 Keiji Tanimoto,1 Tatsushi Shimokuni,1 Takuya Noguchi,1 Keiko Hiyama,1 Hiroaki Tsujimoto,3 Masakazu Fukushima,1 Tetsuya Toge,1 and Masahiko Nishiyama1

Departments of 1Translational Cancer Research and 2Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan; and 3Cancer Research Laboratory, Hanno Research Center, Taiho Pharmaceutical Co., Ltd., Saitama, Japan

Abstract
Dihydropyrimidine dehydrogenase is the most extensively investigated predictive marker for individual response to 5-fluorouracil. Clinical responses to the anticancer agent, along with various reports, have clearly shown that dihydropyrimidine dehydrogenase activity is closely correlated to its mRNA levels, but the regulatory mechanisms of its expression have remained unclear. We attempted to clarify the mechanisms and found that activator protein (AP-1) is probably one of the key factors in the transcriptional regulation of DPYD in cancer cells, and that phorbol 12-myristate 13-acetate (PMA) plus ionomycin treatment enhances transcription of DPYD via AP-1 activation. In this study, we characterized our previously subcloned 5’ region of human DPYD, an ~3.0-kb fragment (accession no. AB162145). Luciferase reporter assay showed that the clone showed strong promoter activities in 293T and HSC42 cells, and comparative analysis using 5’ deletion mutants suggested the existence of several positive and negative regulatory regions, including putative binding sites for AP-1, SP-1, and nuclear factor-κB. PMA/ionomycin treatment increased the mRNA level of DPYD in HSC42 cells, and electrophoretic gel mobility shift assay showed that the complex on the putative AP-1 binding site was drastically induced by PMA/ionomycin treatment. The complexes formed were competed out by preincubation with the cold-consensus AP-1 binding site, and the DNA binding complex formed on the site contained c-Jun and c-Fos, which are components of AP-1 transcription factor. We further identified the functional AP-1 binding site (nucleotide positions from -290 to -280), whose nucleotide mutations abolished PMA/ionomycin-induced DPYD promoter activation. (Cancer Res 2005; 65(3): 1055-62)

Introduction
Among a variety of predictive markers identified to date, dihydropyrimidine dehydrogenase (DPD) has been the most extensively investigated for individual response to 5-fluorouracil (5-FU), as well as thymidylate synthase (1–5). The initial rate-limiting enzyme in the pyrimidine catabolic pathway rapidly converts 5-FU (over 80% of given dose) to inactivated metabolites, and this activity varies considerably among both individuals and tumor cells (6). DPD deficiency is now well known as the primary cause of severe or life-threatening 5-FU toxicity, and DPD in cancer cells is widely recognized as an important determinant of sensitivity to 5-FU (7, 8). Despite the critical role of DPD activity as a biomarker of 5-FU response, its regulatory mechanisms are still poorly understood. A clearer understanding of these mechanisms could lead to the development of more powerful 5-FU regimens with less toxicity, and eventually to an individualized 5-FU chemotherapy if the prediction markers of the response can be identified.

The original extensive studies of molecular mechanisms showed that DPD deficiency is caused by several polymorphisms or mutations of DPD gene (DPYD), such as IVS14 + 1G > A mutation (7). However, recent studies have suggested that such DNA variations alone cannot adequately explain either polymorphic DPD activity in vivo or reduced DPD activity in the majority (>85%) of cases with 5-FU toxicity (7, 8). In recent DNA variation studies, interest has thus increasingly focused on the regulatory mechanisms of DPYD expression. Various reports have clearly shown that DPD activity is closely correlated to its mRNA levels, indicating that the DPD phenotype is controlled, at least in part, by some transcriptional or posttranscriptional mechanisms in both normal and cancer cells (9–11). The details, however, have resisted clarification. To identify the more prominent regulators of DPD activity, the regulatory mechanisms of DPYD expression are now being intensively investigated (10–13).

By using molecular cloning and functional characterization of 1.2 kb of the 5’ region of the human DPYD gene, Shestopal et al. (12) found two regulatory regions apparently essential for DPYD promoter activity, both located near the transcription start site. Collie-Duguid et al. (13) also reported the cloning of 1.85 kb of the 5’ region of the human DPYD gene and its subsequent transcriptional activity in cultured cancer cells, and they identified a single nucleotide polymorphism in a putative γ-IFN response element in a cancer patient with reduced DPD activity and severe 5-FU toxicity. The greater parts of the suggested sequences, however, differed from one another, so the regulatory mechanisms of its expression remained unclear, unlike the well characterized expression profiles of DPYD in cells.

The purpose of this study was to clarify the mechanism. Because the details of the sequence of the 5’ region are still controversial, we used our original clone, an ~3.0-kb fragment (nucleotide positions -2,918 to +83) fragment of 5’ region of DPYD from a human placenta genome library (11). We attempted to clarify the transcriptional regulatory mechanisms of DPYD by identifying several regulatory regions, including putative binding sites for transcriptional factors such as activator protein (AP-1), SP-1, and nuclear factor-κB in cancer cell lines: we discovered that phorbol 12-myristate 13-acetate (PMA) and ionomycin treatment increased the mRNA level of DPYD in HSC42 cells via one of the functional AP-1 binding sites. We think this is a large step toward understanding the molecular mechanisms of regulation of DPD...
activity and 5-FU response in cancer cells, and that it will ultimately contribute to the development of personalized medicine.

Materials and Methods

Chemicals. All chemicals were analytic grade and were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma (St. Louis, MO).

Cell Lines and RNA Preparation. A human gastric cancer cell, HSC42, and a transformed renal cell, 293T (the Japanese Cancer Research Resource Bank), were maintained in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) or DMEM (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (BioWhittaker, Verviers, Belgium) plus penicillin (50 IU/mL) and streptomycin (50 μg/mL) with passage every 3 days. For expression analyses of DPYD and c-Jun protein with PMA and ionomycin treatment, cells were incubated in FBS-free medium for 12 hours before treatment, and then PMA (Sigma-Aldrich Japan) and ionomycin (Sigma-Aldrich Japan) were simultaneously added to the medium (final concentrations, 1 or 10 ng/mL of PMA; 0.5 μmol/L of ionomycin). Cells were harvested after incubation in the presence of PMA

Figure 1. Molecular organization of the cloned 5' region of DPYD gene and its transcriptional activities in cell lines. A, the 5' region (nucleotides -2,918 to +83) of DPYD was subcloned into pGL3 basic plasmid vector. In these schematics, the transcription start site and the translation initiation site for the DPD protein are indicated as +1 and +99, respectively. B, the promoter activity of the 5' region of DPYD with luciferase reporter gene was measured by transient transfection in 293T or HSC42 cells. Luciferase activities of reporter were normalized with pRL-TK in each cell line. Columns, mean of three independent experiments; bars, SD. The P values were calculated using Student’s t test.

Figure 2. Comparative analysis of transcriptional activity using 5' deletion mutants of DPYD promoter. Left, a series of deletion mutants of DPYD promoter. In this schematic, the transcription start site is indicated as +1. Right, promoter activities of the deletion mutants of DPYD promoter were measured by transient transfection in 293T or HSC42 cells. Luciferase activities of reporter were normalized with pRL-TK in each cell line and are represented as a percentage of full-length promoter activity in each cell line. Columns, mean of three independent experiments; bars, SD. The P values were calculated using Student’s t test.
and ionomycin for different treatment periods (1, 3, 6, 12, or 24 hours). Total RNA was prepared from frozen cell pellets by using Qiagen RNeasy mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s instruction.

Reverse Transcription-PCR. Two-micrograms of total RNA extracted from each cell line was reverse-transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time reverse transcription-PCR was done using TaqMan system on ABI Prism 7700 sequence detector (Applied Biosystems) according to the manufacturer’s instructions. A one-hundredth aliquot of the cDNA was subjected to real-time reverse transcription-PCR using Assays-on-Demand Gene Expression (Applied Biosystems) for DPYD and predeveloped TaqMan Assay Reagents (Applied Biosystems) for ACTB as internal control. Reactions were carried out under the following conditions: 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 50 cycles at 95 °C for 15 seconds, and 60 °C for 1 minute. Subsequently, the Ct, the cycle number at which the amount of amplified target crossed a fixed threshold, was determined. Three independent measurements were averaged and relative gene expression levels were calculated as a ratio to ACTB expression of each cell line.

**Figure 3.** PMA and ionomycin treatment increases endogenous expression of DPYD in HSC42 cells. DPYD expressions in HSC42 cells were analyzed by real-time reverse transcription-PCR method after treatment with different concentrations (1 or 10 ng/mL) of PMA and 0.5 μmol/L of ionomycin (A), and different treatment times (0, 1, 6, 12, or 24 hours) (B). ACTB expression was used as an internal control and relative expressions are represented as a ratio to the expression in nontreated control cells. Columns, mean of three independent experiments; bars, SD. The P values were calculated using Student’s t test.

**Reporter Plasmid Construction.** The 3.0-kb DNA fragment (nucleotide positions –2918 to +83) including the 5’ region, as well as the noncoding exon 1 of DPYD gene, was subcloned from a human placenta genome library (accession no. AB162145; ref. 11). The HindIII and XhoI fragment was subcloned into MluI and XhoI sites of a luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI), which encodes firefly luciferase as a reporter. The construct was designated pGL3-DPYD Pro3.0. A series of 5’ deletion mutants of pGL3-DPYD Pro was constructed by PCR method using internal specific primer sets with pGL3-DPYD Pro3.0 as a template. These mutants contained the regions from nucleotide positions –2,494 to +83 (pGL3-DPYD Pro2.5), –2,170 to +83 (pGL3-DPYD Pro2.2), –1,519 to +83 (pGL3-DPYD Pro1.5), –1,154 to +83 (pGL3-DPYD Pro1.2), –674 to +83 (pGL3-DPYD Pro0.6), –453 to +83 (pGL3-DPYD Pro0.45), –338 to +83 (pGL3-DPYD Pro0.34) and –64 to +83 (pGL3-DPYD Pro0.06). Base-exchanged mutants of putative AP-1 binding site in –304/–279 region, mutations in the 5’ half of –290/–280 (MT1), the 3’ half of the site (MT2), and all of the site (MT3) in pGL3-DPYD Pro0.34, were generated by PCR-based site-directed mutagenesis as previously reported (14). All constructs were confirmed by sequence analysis using RVprimer3, GLprimer2, (Promega) and several internal primers with Big Dye Terminators and ABI Prism 310 Genetic Analyzer (Applied Biosystems).

**Luciferase Reporter Assay.** Transient transfection was done as follows: pGL3-DPYD Pro3.0 or mutants (0.5 μg/15 mm well) and Renilla luciferase vector (pRL-TK, 0.01 μg/15 mm well; Promega) as an internal control were mixed with 1.0 μL of FuGENE 6 Transfection Reagent (Roche Diagnostic Co., Indianapolis, IN). Cells were incubated for 36 hours prior to analysis of luciferase reporter activity. PMA and ionomycin treatment was started (final concentration, 10 ng/mL of PMA, 0.5 μmol/L of ionomycin) after 12 hours of incubation with transfection mixtures, then cells were harvested after incubation with reagents for 12 hours. The luciferase luminescence was measured by a single-sample luminometer, the Mini Lumat LB 9505 luminometer (Berthold Technologies GmbH and Co., KG, Bad Wildbad, Germany) using Dual Luciferase Assay Kit (Promega). DPYD promoter activity was calculated as a ratio to Renilla luciferase activity.

**Electrophoretic Gel Mobility Shift Assay.** Nuclear extracts were prepared from frozen cell pellets as previously described (15). Five double-stranded oligoprobes, an AP-1 consensus binding sequence and four similar sequences in DPYD promoter region, were synthesized as follows: AP-1, sense, 5’-GGGCGTTCGATGGAGCAGGCTGGAAGAAGGCTAGGCTAAGCGGAG-3’; antisense, 5’-GTTGCGGTGCTGAGTCCACCAGAAGCTCAGGCGG-3’. Results were visualized on a Bio-Rad videodoc system and quantified using Quantity One software. To determine the specific binding of the nuclear extracts to each probe, competition experiments were performed in which the nuclear extract was preincubated with increasing concentrations of unlabeled double-stranded oligonucleotides of the probe (100-fold excess) before incubation with 32P-labeled probe.

**Immunoblot Analysis.** To analyze c-Jun or phosphorylated c-Jun protein expression, whole cell extracts were prepared with or without PMA and ionomycin treatments as previously described (16). One hundred
micrograms of protein were blotted onto nitrocellulose filters following SDS-PAGE. Anti-c-Jun (Chemicon) or anti-phospho-c-Jun (Ser63; Upstate, NY, USA) was used as primary antibodies, diluted 1:500 in TBS containing 0.1% Tween 20 (TBS-T) and 1% nonfat milk. After several washes, a 1:1,000 dilution of anti-mouse or anti-rabbit IgG horseradish peroxidase conjugate (Amersham Life Science) in TBS-T buffer containing 1% nonfat milk was used as a secondary antibody and incubated with the sample for 1 hour at room temperature. After extensive washing with TBS-T buffer, immunocomplexes were visualized using enhanced chemiluminescence reagent, ECL Plus (NEN Life Science Product, Inc., Boston, MA).

Statistical Analysis. In all experiments, significance was calculated using Student’s \( t \) test.

Results

Subcloning of the 5’ Region of \( DPYD \) and its Promoter Activities in Cancer Cells. In this study, we characterized our previously subcloned 5’ region of human \( DPYD \), an \( \sim 3.0 \)-kb fragment from nucleotide positions \(-2,918 \) to \(+83 \) when transcription start site was designated as \(+1 \) (accession no. AB162145; Fig. 1A). We first did a transient transfection of luciferase reporter plasmid driving by the 5’ region of \( DPYD \) (pGL3-\( DPYD \) Pro3.0) in 293T or HSC42 cells to confirm its transcriptional activity (Fig. 1B). The analysis showed significant promoter activities in 293T or HSC42 cells. Observed \( DPYD \) promoter activities in 293T or HSC42 cells were 25.4 (\( P < 0.0001 \)) or 5.9 (\( P < 0.01 \)) times stronger, respectively, than those of empty vector controls, suggesting that the clone contains important regulatory cis-elements activated in these cell lines.

Comparative Analysis of Transcriptional Activities Using 5’ Deletion Mutants of \( DPYD \) Promoter. Next, we investigated the regulatory regions using luciferase assay in which a series of deletion mutants of the 5’ region were transiently transfected into 293T or HSC42 cells (Fig. 2). The luciferase reporter assay revealed that the regulatory mechanisms of \( DPYD \) transcription could vary among cell lines: \( DPYD \) promoter in the two cell lines had several putative positive- and negative-regulatory regions that differed from one another. Deletions from \(-1,519 \) to \(-1,155 \) and from \(-338 \) to \(-65 \) resulted in a loss of luciferase activity, whereas deletions from \(-1,154 \) to \(-675 \) enhanced the activity in HSC42 cells. Likewise, several regulatory regions were suggested in 293T cells: two negative (from \(-2,494 \) to \(-2,171 \) and from \(-1,519 \) to \(-1,155 \)) and three positive (from \(-2,918 \) to \(-2,495 \), from \(-1,154 \) to \(-675 \), and \(-338 \) to \(-65 \)) regulatory regions in 293T cells. Despite such differences, the region from \(-338 \) to \(-65 \) of \( DPYD \) alone was found to be common in the two cell lines: This deletion significantly attenuated promoter activities in both cells, and especially in HSC42, luciferase activity was inhibited below the control level (pGL3 basic), suggesting that the region probably contains essential cis-elements for the transcription of \( DPYD \).

AP-1 as a Putative Transcriptional Activator of \( DPYD \) and the Role of its Activator in Endogenous \( DPYD \) Expression. A sequence-homology search using a database from the National Institute of Advanced Industrial Science and Technology (http://www.cbrj.jp/research/db/TFSSEARCH.html) suggested that several transcriptional factors, such as AP-1, SP-1, and nuclear factor-kB, might regulate \( DPYD \) promoter, and that AP-1 was the most likely among them. Their consensus sequences were identified in the region from \(-338 \) to \(-65 \) (\( >70\% \) homology), and pGL3-\( DPYD \) Pro0.34 was shown to contain as many as four consensus sequences of AP-1 binding sites: nucleotide positions from \(-290 \) to \(-280 \), \(-14 \) to \(-8 \), \(+18 \) to \(+24 \), and \(+63 \) to \(+69 \). To determine whether AP-1 actually participates in the transcriptional activation of \( DPYD \), the effect of an activator of AP-1, PMA plus ionomycin, on \( DPYD \) mRNA level was investigated in HSC42 cells. As expected, treatment with PMA and ionomycin increased \( DPYD \) expression, and the increase was in a dose- and time-dependent manner until 12 hours (Fig. 3A and B). \( DPYD \) expression increased up to 2.6-fold over that of the control by treatment with 10 ng/mL PMA and 0.5 \( \mu \)g/mL ionomycin (Fig. 3A), and the expression level reached the maximum at 12 hours (Fig. 3B).

\( DPYD \) Transcription Activated by PMA/Ionomycin Treatment via Core Promoter Region. Treatment with PMA and ionomycin (PMA/ionomycin) has clearly been shown to increase \( DPYD \) expression, suggesting that activation of AP-1 may be a key factor in the regulation of \( DPYD \) expression. It is, however, still not clear whether transcriptional regulation is involved in the increase. To clarify this, we transiently transfected HSC42 cells with the full-length \( DPYD \) 5’ region of \( DPYD \) Pro3.0 and two deletion mutants (pGL3-\( DPYD \) Pro0.34 and pGL3-\( DPYD \) Pro0.06), and the luciferase activities were measured after treatment with or without PMA/ionomycin. Subsequent analysis showed that, of the transcriptional activities, pGL3-\( DPYD \) Pro3.0 and Pro0.34 increased up to 2-fold after treatment, whereas pGL3-\( DPYD \) Pro0.06 did not (Fig. 4). These results indicated that PMA/ionomycin increased \( DPYD \) expression through its core promoter activation which involved one of the consensus AP-1 binding sites.

AP-1, Activated with PMA/Ionomycin, Binds to the Putative Binding Site on \( DPYD \) Promoter. To show that putative AP-1 binding sites on \( DPYD \) core promoter are truly active and that AP-1 clearly binds to the sites, an electrophoretic gel mobility shift assay (EMSA) with \( ^{32} \text{P} \)-labeled probes was done. For the assay, nuclear extract of HSC42 cells was prepared after treatment with or without PMA/ionomycin. EMSA showed that the AP-1 probe-specific DNA binding complexes had shifted, and that the complex on the putative AP-1 binding site was...
drastically induced by PMA/ionomycin treatment (Fig. 5A). Furthermore, the complexes formed were competed out by preincubation with the cold-consensus AP-1 binding site. Immunoblot analysis also showed that c-Jun in HSC42 cells was activated by PMA/ionomycin treatment: we confirmed an increase of phosphorylated c-Jun under the same treatment condition (Fig. 5D). Interestingly, PMA/ionomycin treatment was also found to increase the DNA binding complex on probe $-304/-279$, and the shifted band, likely a single complex, was completely competed-out by preincubation of the cold probe itself or the consensus AP-1 binding site. Moreover, supershift assay using anti-c-Jun or anti-c-fos antibody revealed that this complex contained c-Jun and c-fos, which are components of AP-1 transcription factor (Fig. 5B). In contrast, on the other AP-1 consensus binding sites, none of the DNA binding complexes were induced by PMA/ionomycin treatment, although AP-1 consensus probes competed-out some of the shifted bands (Fig. 5C). These results show that AP-1 definitely participates in regulation of DPYD transcription, and that the AP-1 binding site in probe $-304/-279$ is vital in the activation mechanism of DPYD transcription induced by PMA/ionomycin treatment.

**Role of the AP-1 Binding Site in the Activation of DPYD Transcription Induced by PMA/Ionomycin Treatment.** To understand in greater detail the function of the AP-1 binding site on PMA/ionomycin-induced activation, we generated several mutant-type DPYD promoter reporters by using PCR-based site-directed mutagenesis. Nucleotides of the 5' half of $-290/-280$ site (MT1), the 3' half of the site (MT2), and all of the site (MT3)
in pGL3-DPYD Pro0.34 were replaced by adenine (Fig. 6A). Wild- or mutant-type of pGL3-DPYD Pro0.34 was transiently transfected into HSC42 cells and the luciferase activities were measured after treatment with or without PMA/ionomycin. When cells were not treated, the transcriptional activities of those reporters remained the same. In contrast, treatment with PMA/ionomycin resulted in remarkable differences in the transcriptional activities of reporters: nucleotide changes significantly attenuated or abolished promoter activation by PMA/ionomycin treatment. pGL3-DPYD Pro0.34 WT showed 1.77-fold ($P < 0.0001$), MT1 showed 1.57-fold ($P = 0.44$) compared with untreated control. These results suggest that the −290/−280 site, especially the 3′ half of this site, is critical in the PMA/ionomycin-induced DPYD transcription.

**Discussion**

It has been suggested that the mechanisms responsible for the observed variations in DPD activity may involve alterations in gene transcription, and it has been shown that several factors, including circadian rhythm (17, 18), gender differences (19), and treatment with 5-FU (20), tegafur (21), epidermal growth factor, and transforming growth factor-α (22, 23), could alter DPD expression, but the transcriptional mechanisms and their responsible factors are still undefined (24). In this study, we attempted to clarify the mechanisms, and showed for the first time that AP-1 is probably one of the key factors in the transcriptional regulation of DPYD in cancer cells, because its activation by PMA/ionomycin significantly increased DPYD expression in a gastric cancer cell line.

There have been several studies on the transcriptional mechanisms of DPD, but even the sequence of the 5′ region has remained controversial (12, 13). Shestopal et al. (12) subcloned 1.2 kb of the 5′ flanking region of the DPD gene from human placenta genomic library, and Collie-Duguid et al. (13) also subcloned a 1.85-kb of the 5′ flanking region of the human DPD by RAGE. Both of the 5′ regions showed their transcriptional activities, but the greater parts of the suggested sequences differed from one another. Although the reasons for the discrepancy are still unknown, the sequence study from the −3.0-kb fragment of the 5′ region showed that the subclone contained a region consistent with the sequence reported by Shestopal et al. (from the nucleotide positions −1,152 to +83), along with a novel upstream region. The clone lacked the typical TATA or CCAAT box, and contained 60 CpG sites located near the transcriptional start site (11). The 5′ region had strong transcriptional activity, suggesting that the region from −338 to +83, especially the region from −338 to −65, might be essential for the transcriptional activation. These findings are partially consistent with the results reported by Shestopal et al., in that they pointed to two key regulatory regions, and one of them, the region from −72 to −51, was shown to act on the transcriptional activation. However, the transcription factors for these cis-elements could not be identified. We also found another possible regulatory region, from −1,519 to −1,155, in the novel upstream region (from −2,918 to −1,153). Interestingly, this region is likely regulated differently among the two cell lines investigated here; the region probably acts as a positive element in HSC42 and a negative element in 293T cells. Because this element could be of key importance in explaining the differential transcriptional regulation of DPD in cancer cells, because its activation by PMA/ionomycin significantly increased DPYD expression in a gastric cancer cell line.

![Figure 6. The AP-1 binding site was responsible for the activation of DPYD transcription by PMA and ionomycin treatment. A, schematic representation of mutant-type DPYD promoter reporters: nucleotides of the 5′ half of −290/−280 site (MT1), 3′ half of the site (MT2), and all of the site (MT3) were replaced by adenine residues. B, promoter activities of wild- or mutant-type of pGL3-DPYD Pro0.34 were measured by transient transfection in HSC42 cells with or without PMA and ionomycin treatment. Luciferase activities of reporter were normalized with pRL-TK and are represented as a percentage of full-length promoter activity in nontreated cells. Columns, mean of three independent experiments; bars, SD. The P values were calculated using Student’s t test.](https://cancerres.aacrjournals.org/doi/fig/10.1158/0008-5472.CAN-04-0874-fig6)
expression of **DPYD** in various types of cells, its characterization is our next subject.

In the present study, by focusing on the region from –338 to +83, we attempted to clarify its detailed function. Comparative analysis showed that the region acted as a positive element in 293T and HSC42. The region contained numerous CpG sites, which included several putative SP-1 binding sites as well as four putative AP-1 binding sites, and Shestopal et al. (12) had previously reported that SP-1 and SP-3 were not involved in the complex formation on the probe of the **DPYD** promoter. These findings led us to hypothesize that AP-1 and the putative AP-1 binding sites might play an important role in **DPYD** transcription. In actuality, the DNA binding protein complex proved to be formed on some of the consensus sequences for AP-1 binding site, and these complexes were competed-out by cold-consensus probe for AP-1 in EMSA. Moreover, we found that PMA/ionomycin treatment significantly increased **DPYD** expression. Because PMA/ionomycin is a well-known activator of a protein kinase C pathway and various transcription factors positioning in the downstream, including AP-1 (25, 26), this new finding strongly suggested the participation of AP-1 in **DPYD** transcription via the AP-1 binding site. We further showed that the region from –290 to –280 in **DPYD** promoter played an important role in the mechanisms, despite the fact that the site was likely not active without PMA/ionomycin treatment. Although the nucleotide sequence of this site is not completely identical to the AP-1 consensus sequence, our super-shift assay showed that the DNA binding complex formed on the site contained c-Jun and c-Fos, which are components of the AP-1 transcription factor. These results not only support our hypothesis on the significant role of AP-1 but also suggest that AP-1 could be a good molecular target for cancer therapy combined with 5-FU, because inhibition of AP-1 by the dominant-negative form of c-Jun has been shown to inhibit the growth of breast cancer through c-Jun blocking activities on multiple signal transduction pathways (27, 28). Thus, overexpression of **DPYD** in cancer cells might be related to carcinogenesis, because the roles of c-Jun and c-Fos in carcinogenesis are well established along with that of PMA in tumor promotion (29).

Nevertheless, it is obvious that further studies are necessary to precisely define the detailed functions of the core promoter region of **DPYD**. Several other consensus sequences, such as SP-1 and nuclear factor-κB, were found in the region, and PMA/ionomycin alone did not activate AP-1. The DNA binding complexes detected in EMSA without treatment were insufficient to explain the specific role of AP-1 in **DPYD** transcription. All probable transcriptional factors need to be studied along with AP-1. Additionally, a comparative view of the mechanism of **DPYD** transcriptional regulation is still undetermined. Our analyses on the transcriptional activities of a series of deletion mutants suggested that multiple regulatory regions may exist in the **DPYD** promoter, and that such regions are likely cell-specific, which differs from the findings by Shestopal et al. (11). A computer-homology search indicated several possibly important regulatory regions, such as consensus sequences for the binding sites of Oct-1, C/EBPα, nuclear factor-κB, and HNF-3β; these might be essential for the up-regulation or down-regulation of **DPYD** gene. The functional roles of these cis-elements and trans-acting factors are now being intensively investigated in our laboratory.

**References**

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