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Identification of cDNAs for Sox-4, an HMG-Box Protein, and a Novel Human Homolog of Yeast Splicing Factor SSF-1 Differentially Regulated during Apoptosis Induced by Prostaglandin A_2/Δ^{12}-PGJ_2 in Hep3B Cells

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We have examined specific genes whose expression is altered during apoptosis induced by prostaglandin (PG)A_2 and Δ^{12}-PGJ_2 in human hepatocellular carcinoma Hep3B cells. Using mRNA differential display, we have identified two genes: one is specifically up-regulated and encodes for human Sox-4 (Sry-HMG box gene) and the other is significantly down-regulated and is the human homolog of yeast Ssf-1, a novel splicing factor. Northern blot analysis confirmed their differential expressions. Interestingly, Sox-4 was highly expressed in subcutaneous tumors grown in nude mice as a xenograft from Hep3B cells. These results suggest that the expression of Sox-4 may be related to the apoptosis pathway leading to cell death as well as to tumorigenesis, and that Ssf-1 gene may serve as a negative regulator of PGA_2/Δ^{12}-PGJ_2-mediated Hep3B cell apoptosis.

Cyclopentenone prostaglandins (PGs) such as PGA_2 and Δ^{12}-PGJ_2, enzymatic dehydration products of PGE_2 and PGD_2 respectively, have antiproliferative activities on the growth of various tumor cells including hepatoma cells (1, 2) with characteristic morphological and biochemical findings of apoptosis (3, 4, 5).

Several cardinal findings of apoptosis induced by PGA_2/Δ^{12}-PGJ_2 in L1210 and SK-Hep-1 cells were inhibited by pretreatment with cycloheximide (3, 4). It has also been reported that PGA_2/Δ^{12}-PGJ_2 induced the production of several proteins such as heme oxygenase (6), gadd 153 (7), γ-glutamylcysteine (8), acute phase reactants (APRs) (9) and heat shock proteins (10) in tumor cells, while the steady-state level of c-myc was down-regulated during PGA_2-mediated apoptosis in HL-60 cells (11, 12). The overexpression of HSP70 showed an increasing resistance to apoptosis induced by PGA_2/Δ^{12}-PGJ_2 and other apoptotic stimuli in various tumor cells (5, 13, 14). APRs, such as α_1-acid glycoprotein and α_1-antitrypsin specifically inhibited the induction of apoptosis in hepatocytes by TNF/galactosamine *in vivo* (15). We have previously described that the transfection of c-myc antisense oligomer into Hep3B cells significantly delayed HSP70 expression and blocked the formation of DNA fragmentation induced by PGA_2/Δ^{12}-PGJ_2 (5). These results emphasized that the expression of up- and down-genes and their products induced by PGA_2/Δ^{12}-PGJ_2 in tumor cells may serve as a positive or negative mediator in the PGA_2/Δ^{12}-PGJ_2 induced apoptotic pathway.

In this study, we have identified two functionally distinct genes following PGA_2/Δ^{12}-PGJ_2 exposure to human hepatocarcinoma Hep3B cells. One, Sox-4, is predominantly an up-regulated member of the family of DNA-binding HMG box proteins, and the other is a significantly down-regulated gene which is the human homolog of yeast Ssf-1, a novel splicing factor. These results provide the possibility that these two newly identified genes by differential display may serve as modulators in PGA_2/Δ^{12}-PGJ_2 mediated apoptosis of tumor cells.

MATERIALS AND METHODS

Reagents and cell culture. PGA_2 was purchased from Sigma Co. (St. Louis, MO) and Δ^{12}-PGJ_2 was supplied by BioMol (Plymouth Meeting, PA). The human hepatocellular carcinoma cell line Hep3B was acquired from American Type Culture Collection (Rockville, MD). Hep3B cells were cultured in RPMI 1640 (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum and 50 μg/μl gentamicin at 37°C in a humidified atmosphere containing 5% CO_2 reaching about 80% confluence.

Hep3B cells xenograft. 2×10^6 Hep3B cells were inoculated subcutaneously into 6-8 week old athymic nude mice (BALB/c-nu, slc, Jan). For inoculation, cells were harvested by treatment with typsin-EDTA to allow cell detachment and suspended in phosphate-

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buffered saline (PBS) to give a suspension of 2×10^6 cells in a volume of 100 μl. After 4-6 weeks, tumor masses was approximately 0.8 × 81.2 cm, were observed.

Total cellular RNA isolation and Northern blot analysis. Total RNAs from Hep3B cells and hepatoma tissues derived from Hep3B cell xenografts were isolated with RNA zol B reagent (Biotech Laboratories, Houston, TX). RNAs were separated on a 1.5% agarose gel containing 2.2 M formaldehyde and then transferred to a nylon membrane (Boehringer Mannheim, Germany). Reamplified or cloned cDNA probes were purified by agarose gel electrophoresis using QIAEX kit from QIAGEN (Chatsworth, CA). cDNA probe was labeled with digoxigenin (DIC-11-UTP) using a specific primer by polymerase chain reaction (PCR). Hybridization was performed by the standard procedure (16). After hybridization, the filters were washed twice in 2× SSC and 0.1% SDS for 5 min at room temperature. The washed filters were incubated with alkaline phosphatase-conjugated anti-DIG (Digoxigenin) antibody followed by immunodetection with chemiluminesence substrates (Boehringer Mannheim, Germany).

Differential display (DD). DD PCR was performed by modifications of procedures previously described (17). Three-base anchored oligodeoxythymidylate primers, $T_{12}CA$ (5′-TTTTTTTTTTTTCA-3′), $T_{12}A$ (5′-TTTTTTTTTTTTA-3′) and $T_{12}CG$ (5′-TTTTTTTTTTTTCG-3′) were used to reverse transcribe total RNA from cell lines into first-strand cDNAs, and subsequently amplified by PCR with [α-^{32}P]dCTP (3000 μCi/mmol, Amersham, UK) using 3 different arbitrary primers, Ram1 (5′-CTCTGCAGCC-3′), Ram2 (5′-CTTGATTGCC-3′) and Ram3 (5′-GTCTGCAGGT-3′). PCR conditions used were the same as described previously (17). PCR products were analyzed on a 6% DNA sequencing gel. The recovery and reamplification of cDNA fragments from a dried DNA sequencing gel were described previously (17).

Cloning and DNA sequencing. The amplified cDNA fragments were cloned into pBluescript SK(+) (Stratagene, La Jolla, CA) and pCR2.1 vectors. Both strands of the DNA fragments were sequenced with a sequenase kit (USB Biochemicals Co., Cleveland, Ohio) using T3 and T7 primers.

Reverse transcription (RT)-PCR of the coding region of Sox-4. Complementary strands of RNA that were isolated from human Jurkat cells were generated by RT reactions containing random hexamer (dN_6), 0.2 mM dNTP, 50 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, MuLV reverse transcriptase (Boehringer Mannheim, Germany). The cDNA was amplified by long PCR using primer 5′-CAGCCGAGAGACAGCAAACT-3′ and 5′-AACTCTTCGTCTGTC-CTTTTCGTT-3′. The 1.8 kb fragment was used as a probe for Northern blot analysis.

RESULTS

We and others have previously shown that PGA_2 and Δ^{12}-PGJ_2 have antiproliferative activity on the growth of various tumor cells with characteristic morphological and biochemical features of apoptosis (1–5). Identification of PGA_2-induced alterations in gene expression can provide essential clues for understanding the molecular mechanism of apoptosis mediated by PGs. First, we analyzed genes which altered their expressions in Hep3B cells undergoing apoptosis mediated by PGs. A representative result of differential display (DD) for mRNA present in untreated and PGA_2-treated Hep3B cells is illustrated in Fig. 1. cDNAs amplified by nine different combinations of three anchor primers and three short arbitrary primers were separated simultaneously to compare the differential expression between both control and PGA_2-treated Hep3B cells. The analysis of the expression patterns generated by each set of primers showed that the intensity of most bands did not differ when they were treated by PGA_2. However, a limited number of bands showed comparable differences between control and PGA_2-treated HepB3 cells (indicated by the arrows). The bands (indicated by the arrows) were excised from the 6% denaturing sequencing gel to be further characterized (Fig. 1). We isolated various kinds of cDNA fragments preferentially expressed in either PGA_2-treated or control Hep3B cells.

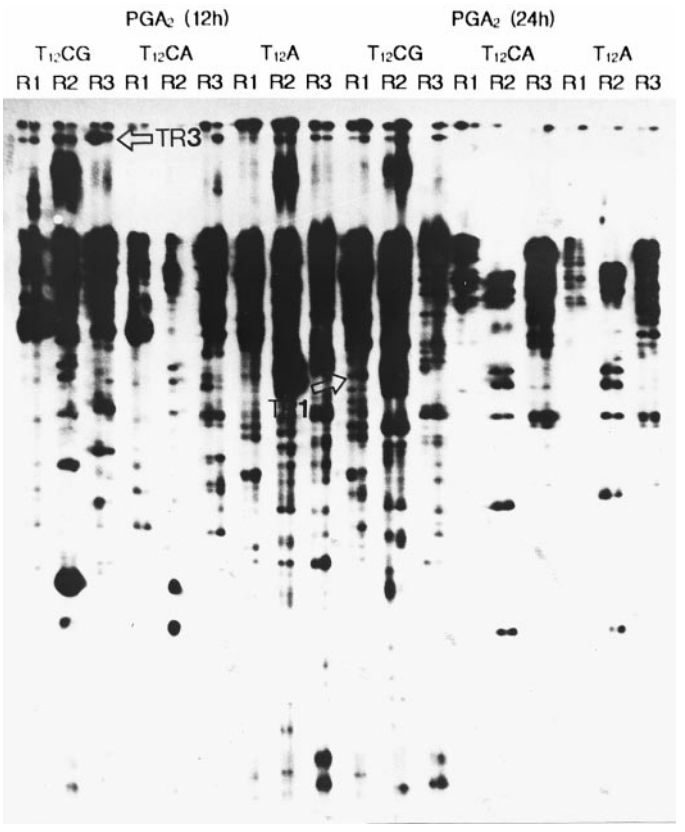
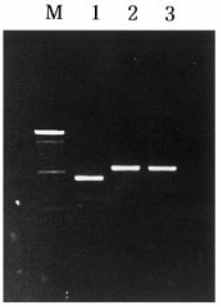


FIG. 1. Identification by differential display of altered gene expression in Hep3B after PGA_2 (20 μg/ml) treatment. A differential display was carried out using a 5′ arbitrary primer, Ram-1 (5′-CTCTGCAGCC-3′), Ram-2 (5′-CTTGATTGCC-3′), Ram-3 (5′-GTCTGCAGGT-3′), and anchored primer, $T_{12}CA$, $T_{12}GC$, $T_{12}A$, and resolved by electrophoresis. Differentially expressed candidate genes are marked with arrows (TR1, TR3). Abbreviations: R1, R2, and R3; arbitrary 10-mer.

The band, TR1, (indicated by the arrow) came out in PGA_2-treated Hep3B cells, but not in untreated Hep3B cells (Fig. 1). To confirm that the pattern of the identified fragment is displaying different expressions, the cDNA was reamplified and used as a probe for Northern blot analysis (Fig. 2A). The reamplified TR1 fragment was subcloned into a pCR 2.1 vector and sequenced (Fig. 2A,B). The 330-bp cDNA fragment, TR1,

A



B

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1          10         20         30         40         50
CTCTGCAGCC GGAGGAGGAG ATGTTGAGGG GAGGAGGCCA GCCAGTGTGA
51         60         70         80         90         100
CCGGCGCTAG GAAATGACCC GAGAACCCCG TTGGAAGCGC AGCAGCGGGA
101        110        120        130        140        150
GTAGGGGCGG GGGCGGAGGA GGACACGAAC TGGAAGGGGG TTCACGGTCA
151        160        170        180        190        200
AACTGAAATG GATTTGCACG TTGGGGAGCT GGCGGCGGCG GCTGCTGGGC
201        210        220        230        240        250
CTCCGCCTTC TTTTCTACGT GAAATCAGTG AGGTGAGACT TCCCAGACCC
251        260        270        280        290        300
CGGAGGCGTG GAGGAGAGGA GACTGTTTGA TGTGGTACAG GGGCAGTCAG
301        310        320        330
TGGAGGGCGA GTGGTTTCGA AAAAAAAAAA A
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C

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1          10         20         30         40         50
GTCTGCAGGT TCGTAAGAAG AACTCGCTGA AGGACTGCGT GGCCTGGCTG
51         60         70         80         90         100
GGCCCTCGGT CACACACTTT CTGATCCTGA GCAAAACAGA GACCAATGTC
101        110        120        130        140        150
TACTTTAAGC TGATGCGCCT CCCAGGACGG CCACCTTGAC CTTCAGGTCA
151        160        170        180        190        200
AGAAGTACTC GCTGGTGCGT GATGTGGTCT CTCACTGCGC CGGCACCGCA
201        210        220        230        240        250
TGACGAGCAG CAGTTTGCCC ACCCACCCCT CCTGGTACTC AACAGCTTTG
251        260        270        280        290        300
GCCCCCATGG TATGCATGTC AAGCTCATGG CCACCATGTT CCAGAACCTG
301        310        320        330        340        350
TTCCCCTCCA TCAACGTGCA CAAGGTGAAC CTGAACACCA TCAAGCGCTG
351        360        370        380        390        400
CCTCCTATCG ACTACAACCC CGACTCCCAG GAGCTGACTT CCGCCACTAT
401        410        420        430        440        450
AGCATCAAAG TTGTTCCTGT GGGGCGAGTC CTCGGGATGA AGAAGCTGCT
451        460        470        480        490        500
CCAGGAGAAG TTCCCCAACA TGAGCCGCCT GCAGGACATC AGCGAGCTGC
501        510        520        530
TGCCGCACGG GCGCGAAAA AAAAAAAA
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FIG. 2. Reamplifiction and nucleotide sequence of two differentially expressed cDNA fragments. Reamplified cDNA fragment was cloned into pCR 2.1 vector. (A) The M lane shows the 100 bp ladder as molecular weight marker (BRL). Lane 1, up-regulated gene (Sox-4); lanes 2, 3, down-regulated gene (Ssf1). (B) Nucleotide sequence of Sox-4 cDNA fragment. (C) Nucleotide sequence of Ssf1 cDNA fragment.

has 98% homology to the 3′ untranslated region of Sox-4, a member of the *Sry* (sex determining region Y)-type HMG box family of transcription factors (GenBank Accession Number X70683). Consistent with the DD results, an RNA protection assay (RPA) also showed that the mRNA levels of Sox-4 was increased in the PGA_2-treated Hep3B cells in a time dependent manner (data not shown). Subsequently, the full-length coding region of Sox-4 (1.3 kb) was amplified by PCR using the specific primer set which corresponds to the reported sequence for Sox-4 mRNA and cloned to be further characterized. In contrast, the TR3 amplified product was expressed at lower levels in PGA_2-treated Hep3B cells as compared to untreated control cells, indicating that the expression of the TR3 amplified product could be down-regulated by PGA_2. The TR3 fragment (530 bp) recovered from the dried denaturing polyacrylamide gel was reamplified using the corresponding primer sets (Fig. 2A), followed by subcloning into pCR2.1 vector and sequencing (Fig. 2C). Two overlapping EST cDNA clones [Clone identification numbers: 267831 (N34073) and 686731 (AA258103)] exhibited over 97% sequence identity to 530 bases of the TR3 amplified product and were shown to be similar to yeast SSF1 (second-step splicing factor 1; *Saccharomyces cerevisiae Ssf1p*) protein.

Next, to confirm whether the corresponding RNA expressions correlated with the expression patterns of the DD-PCR amplification products selected by mRNA differential display or whether they were modulated by PGA_2/Δ^{12}-PGJ_2, Northern blot analyses were performed with RNAs isolated from control and PGA_2/Δ^{12}-PGJ_2-treated Hep3B cells. The cDNAs shown in Fig. 2A were labeled with digoxigenin by PCR and used as probes for Northern blot analysis. Northern blot comparisons of messages for the TR1 and TR3 PCR products from PGA_2/Δ^{2}-PGJ_2-treated and control Hep3B cells revealed striking discrepancies in the levels of steady-state mRNAs that were consistent with differential display. The TR1 probe was shown to hybridize to a major transcript of about 5 kb which corresponds to the reported size for Sox-4 mRNA (Fig. 3A). The levels of Sox-4 transcript significantly increased after 12 hr treatment with PGA_2 (20 $\mu g/\mu l$) and were up-regulated in a time-dependent manner, while its transcript started to significantly increase after 3 hr treatment with Δ^{12}-PGJ_2 (5 $\mu g/\mu l$) (Fig. 3B). TR3, a transcript of about 2.3 kb, seems to be down-regulated by PGA_2 treatment until hour 6 (6 hr) and then the signal was no longer detected (Fig. 4A). On the other hand, Δ^{12}-PGJ_2 completely down-regulated the message by hour 3 (3 hr) treatment, but resumed expression of the message at hour 24 (24 hr) (Fig. 4B). Consequently, the up- and down-regulated gene products differentially amplified in DD-PCR were reconfirmed by Northern analyses.

Also, we established human Hep3B cell xenografts in nude mice. The expression of genes involved in the course of carcinogenesis was characterized. Because many regulatory proteins control cellular fate by reg-

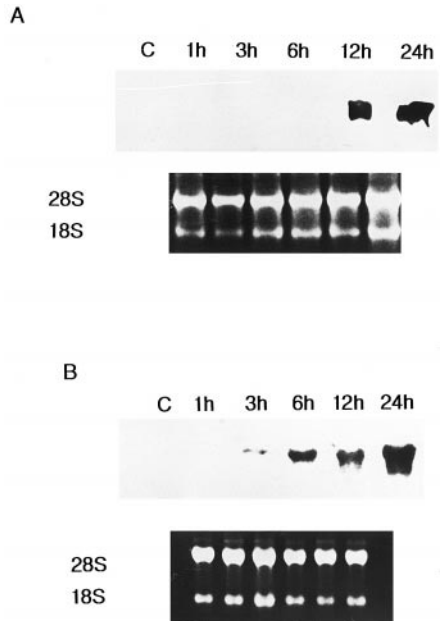

FIG. 3. Northern blot analysis of Sox-4 by PGA_2/Δ^{12}-PGJ_2. Hep3B cells were incubated for different time periods after treatment with PGA_2/Δ^{12}-PGJ_2 at PGA_2 (20 μg/ml) or Δ^{12}-PGJ_2 (5 μg/ml), respectively. 10 μg of total RNA was loaded on each lane of formaldehyde-agarose gel, ethidium bromide staining to demonstrate similar loading of undegraded RNA in each lane.

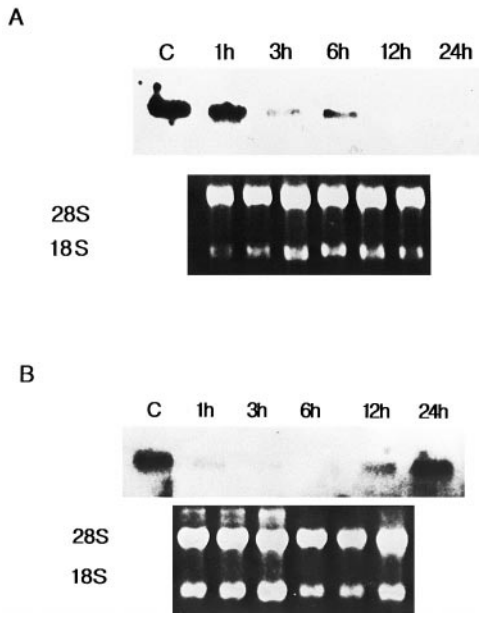

FIG. 4. Down-regulated gene as measured by Northern blotting on total RNA extracted from Hep3B cells. Hep3B cells were incubated for different time periods after treatment with PGA_2/Δ^{12}-PGJ_2 at PGA_2 (20 μg/ml) or Δ^{12}-PGJ_2 (5 μg/ml), respectively. 10 μg of total RNA was loaded on each lane of formaldehyde-agarose gel, ethidium bromide staining to demonstrate similar loading of undegraded RNA in each lane.

ulating gene expression during apoptosis and tumorgenesis, we examined whether Sox-4 is also related to tumorgenesis. Interestingly, we could detect overexpression of Sox-4 in xenograft tumors. Densitometric scanning revealed that these levels were at least 30-fold higher than those observed in normal hepatic tissue. We therefore suggest that Sox-4 expression may be closely associated with hepatic tumorigenesis (Fig. 5).

Finally, to investigate the possible role of Sox-4 in regulating the PG-mediated apoptosis of Hep3B cells, the steady-state mRNA levels of Sox-4 were measured by Northern blot analysis after treatment with PGA_2/Δ^{12}-PGJ_2 at various time points. The levels of Sox-4 were gradually increased in response to PGA_2/Δ^{12}-PGJ_2 after 6 hr and 12 hr, respectively (Fig. 3). In addition, a number of studies demonstrate that PGs regulate the levels of transcripts for several immediate early genes including c-myc and hsp70 in many tumor cells (5, 10, 11, 12, 18). To examine the relationship between endogenous Sox-4 and constitutive expression of hsp70 on PGs-regulated gene expression, cells transfected with either a control vector or hsp70 clone were treated with PGA_2/Δ^{12}-PGJ_2 and Northern blot analysis was performed (Fig. 6). The levels of endogenous Sox-4 transcripts were accumulated by PGA_2/Δ^{12}-PGJ_2 in vector-transfected control cells (Fig. 6A). However, hsp70 transfectants expressed high levels of Sox-4 mRNA by overexpression of HSP70 in PG-untreated control. The steady state levels of Sox-4 mRNA were remarkably reduced within 6 hr in PGs-treated cells, and then it gradually recovered to control levels. HSP70, which was constitutively expressed in Hep3B cells, has been previously demonstrated to correlate with resistance to apoptosis (5, 14). Taken together, these results suggest that there is a possible involvement between Sox-4 and HSP70 in regulation of apoptosis induced by PGA_2/Δ^{12}-PGJ_2.

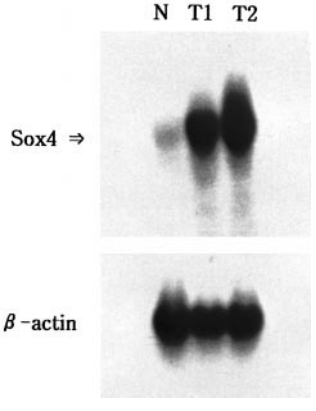

FIG. 5. Northern blot analysis of Sox-4 gene from malignant tissues. 10 μg total RNA per each lane was analyzed. Lane 1, normal liver tissue of nude mice; lanes 2 and 3 (T1, T2), malignant tissue of Hep3B cell xenograft.

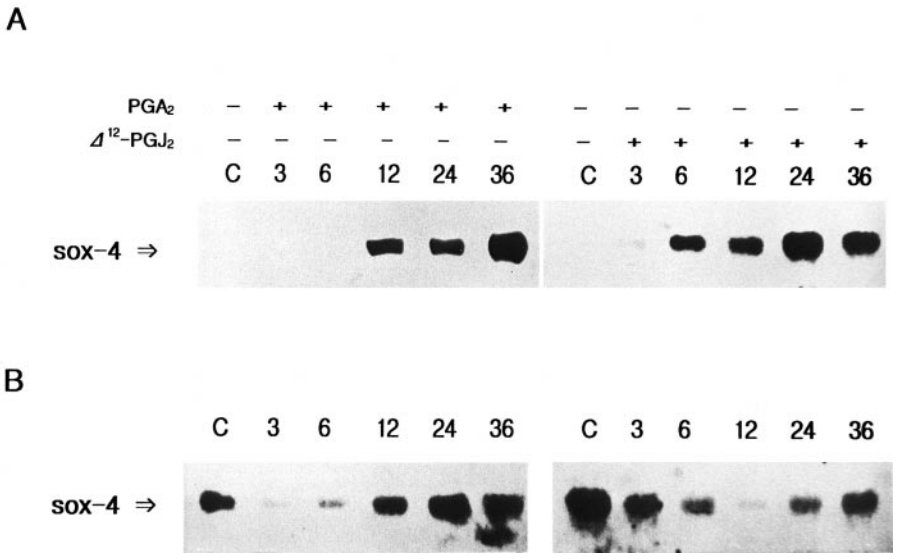

FIG. 6. Expression of Sox-4 in HSP70-transfected Hep3B cells. The transfected cells were treated with PGs at the times indicated. Hep3B cells transfected with vector alone (A) or HSP70 (B) were treated with either 20 μg/ml PGA_2 or 5 μg/ml Δ-PGJ_2, respectively.

DISCUSSION

In this paper, we described two newly identified up- and down-regulated genes, Sox-4 and a human homolog of yeast splicing factor, Ssf1, respectively, mediated by PGA_2/Δ^{12}-PGJ_2 in differential display. The Sox-4 gene was originally identified as a gene carrying an HMG box similar to that of sex-determing factor *Sry* (19–22, 25, 26). The precise biochemical function of most Sox genes is still not clear, but recent studies revealed that mutation of Sox-4 (Sox-$4^{-/-}$) in the embryos of mice leads to premature death at embryonic day 14 due to impaired development of the endocardial ridges (23). In 1998, Southard-Smith *et al.* reported that premature termination of Sox-10, a member of the Sox family, disrupted neural crest development due to apoptosis in Dom Hirschsprung mouse models (24). These results increase the possibility that Sox genes, including Sox-4, may be involved in tumor cell apoptosis induced by various apoptotic agents. A novel splicing factor, SSF1, whose bases were identical to our tested gene in over 97% of the positions over a span of 530 bases, is absolutely required for the splicing of actin pre-mRNA *in vitro* (27). Schwer *et al.* reported that SSF1 was required, in concert with PRP16, to promote progression through the second catalytic step of splicing (27). However, the mechanism of SSF1 has not been clarified. Further studies and the identification of the gene encoding hSSF1, human homolog of yeast SSF1, are necessary to determine their physiological function in mammalian cells.

In this study, we have also demonstrated that Sox-4 is highly expressed in subcutaneous tumors grown in nude mice as xenografts from Hep3B. The expression of Sox-4 in Hep3B xenograft tumors appears to correlate with its tumorigenic potential *in vivo.* In 1993, Miyamoto *et al.* demonstrated that *lck,* the *src* family tyrosine kinase, was transcriptionally activated by the aberrant activity of Sox-4 or related proteins. Therefore, these genes may have a potential role in colorectal tumorigenesis (28). Thus, analysis of the genetic variations of the Sox-4 gene will provide a basis for understanding cellular growth and might be important in deciphering the multistep pathway of tumorigenesis and the dysregulation of apoptosis.

We suggest that the expression of Sox-4 and the human homolog of Ssf1 can be altered by PGA_2/Δ^{12}-PGJ_2 treatment in Hep3B cells, which was closely associated with apoptosis induced by PGA_2/Δ^{12}-PGJ_2 in Hep3B cells. Thus, they may be excellent candidates for mediators of apoptosis and will provide more information to further understand PGA_2/Δ^{12}-PGJ_2-mediated apoptosis.

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