Analysis of the TPA Regulatory Element in the Genomic Poly(ADP-ribose) Synthetase Gene in Human Leukemia U937 Cells

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ABSTRACT: The human leukemia U937 cells differentiate into monocyte/macrophage-like cells when treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). We observed that during this process, both protein and mRNA levels for PARS markedly decreased in U937 cells. Through deletion analysis of the PARS regulatory gene, we found that the sequence within the first intron region was responsible for the TPA-dependent repression. Electrophoretic mobility shift assays (EMSAs) and Southwestern blot analysis indicate that this element bound specifically to a nuclear protein. TPA treatment abolished the binding of the protein in U937 cells but not in HeLa cells. DNase I footprinting data show that the cis regulatory element is located between residues 328 and 383. We further examined the function of this cis element (BS207) in a basal promoter regulatory reporter construct and found that this cis element (BS207) functions as an enhancer via the binding of an unknown trans-acting factor. TPA treatment diminished the binding activity of the factor in U937 cells, resulting in a decrease in the enhanced activity to the basal level. These results suggest that abolishment of the binding of a special nuclear protein to the first intron of the PARS gene is related to the TPA-responsive downregulation of PARS in U937 cells.

Poly(ADP-ribose) synthetase (PARS)[1] (NAD^+ ADP-ribosyltransferase, EC 2.4.2.30) is a chromatin-bound enzyme, which catalyzes DNA-dependent transfer of the ADP−ribose moiety of NAD to a variety of nuclear proteins, including itself. The covalent modification of the nuclear proteins was suggested to involve some nuclear functions such as DNA repair, DNA replication, and cell differentiation (*1*−*4*). In addition, recent findings suggest a possible involvement of PARS in apoptosis (*5*).

PARS enzyme activity decreases during differentiation of murine teratocarcinoma cells (*6*) and rooster spermatogenesis (*7*). We also observed a downregulation of PARS enzyme activity during NGF-promoted neurite outgrowth in rat pheochromacytoma PC12 cells (*8*), erythrocytic differentiation of K562 cells (*9*), and TPA-induced differentiation of human leukemia THP-1 cells (*10*). Recently, Bhatia et al. (*11*) also observed a downregulation of PARS gene expression in NB4 cells after a 36 h treatment with *all-trans*-retinoic acid. Although involvement of PARS in cell differentiation has not been well understood, studies on the regulation of PARS gene expression during differentiation may help facilitate a better understanding of the biological roles of PARS and, furthermore, may provide fundamental insights into the mechanism responsible for the regulation of gene expression during differentiation.

Since human leukemia U937 cells are induced to differentiate into monocyte/macrophage-like cells after exposure to TPA (*12*), we studied the mechanism of downregulation in PARS gene expression after treatment of U937 cells with TPA. Here we demonstrated that a regulatory cis element within the first intron region of the human PARS gene was identified to play an important role in TPA-responsive downregulation of PARS gene expression in U937 cells.

EXPERIMENTAL PROCEDURES

Plasmids. The 3 kb genomic DNA fragment corresponding to nucleotides −1952 to +1027 of the human PARS gene was ligated into a unique *Hin*dIII site of the promoterless chloramphenicol acetyltransferase (cat) gene reporter plasmid (pCAT-Basic, Promega) to generate Hz61 (number +1 of the PARS nucleotide sequence was defined as a cap site of the gene) (GenBank accession number M60436) (*13*). Hz61A was created by removing all PARS 3′ nucleotides from residue +136 of the 3 kb PARS genomic DNA and ligating them to the cat gene. Hz61S was constructed by deleting the *Sac*II fragment (nucleotides −9 to +421) from Hz61. The *Sac*II fragment (nucleotides −9 to +421) was filled up by T4 DNA polymerase and ligated into the *Sma*I site of the pUC19 plasmid (pUC19/SS430). The pUC19/SS430 was digested with *Sal*I and *Bss*HII, and a PARS DNA fragment (nucleotides −9 to +214) was removed. The resultant plasmid DNA was religated after filling up both restriction sites (pUC19/BS207). A reporter plasmid β-actin-Luc was constructed by replacing the SV40 promoter of

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[1] Abbreviations: PARS, poly(ADP-ribose) synthetase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EMSA, electrophoretic mobility shift assay; G418, 2-deoxystreptamine antibiotic.

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pGV-P2 (Toyo Ink Co. Ltd., Tokyo, Japan) with the human β-actin promoter (nucleotides −305 to +63) (*14*, *15*). The human β-actin gene was provided by T. Kawamoto (Department of Biochemistry, Hiroshima University School of Dentistry, Hiroshima, Japan). BS207/β-actin-Luc was constructed by inserting the BS207 (*Bss*HII and *Sac*II fragment of Hz61, nucleotides 215−421) into the *Sma*I site of plasmid β-actin-Luc. The orientation of inserted BS207 DNA was confirmed by digestion with restriction enzymes. Chicken β-actin-β-gal was a gift of S. Ishii (Laboratory of Molecular Genetics, Tsukuba Life Science Center, Riken) (*16*).

Western Blot Analysis. Western blot analysis was performed according to the method of A. Domingo and R. Marco (*17*). Nuclear proteins (5 μg) were subjected to a 10% SDS−PAGE and electrotransferred onto a PVDF membrane; the membrane was incubated with the antibody against bovine PARS (*8*) in GENT solution [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, and 0.05% Triton X-100] and horseradish peroxidase-labeled second antibody (Zymed Laboratories, Inc., San Francisco, CA). PARS on the membrane was visualized by staining by a chemiluminescence detection (ECL) system (Amersham).

Northern Blot Analysis. Total RNA was extracted from $2-3 \times 10^7$ cells by the guanidine thiocyanate/CsCl ultracentrifugation method and analyzed as described previously (*18*). Probe DNA was labeled with [α-^{32}P]dCTP by random primer procedures (Boehringer Mannheim).

Isolation of Permanent Transfectants and CAT Assay. Plasmids Hz61, Hz61A, and Hz61S, together with pSV$_2$-neo (a gift from H. Ohkubo, Institute for Medical Genetics, Kumamoto University Medical School), were cotransfected into U937 cells in saline G [137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 6.1 mM glucose, and 1.1 mM sodium phosphate (pH 7.2)] at 1.5 kV/cm with a capacity of 25 μF by electroporation with a Gene Pulser (Bio-Rad) according to the Gene Pulser operation manual, and permanent transfectants were selected with RPMI1640 medium containing 10% fetal calf serum and 400 μg/mL 2-deoxystreptamine antibiotic (G418). G418-resistant clones were cultured independently. The clones were cultured in the presence or absence of TPA (10 or 20 ng/mL) for 1 or 2 days. The cells were suspended in 100 mM Hepes (pH 7.75) and disrupted by sonication. Cell extracts containing equal amounts of protein (100 μg) were treated at 70 °C for 5 min and then incubated with 0.1 mCi of D-threo-[*dichloroacetyl*-1-^{14}C]chloramphenicol and 70 μg of acetylcoenzyme A at 37 °C for 2 h. The reaction products were separated by thin-layer chromatography and visualized by autoradiography. The radioactivity of the bands was measured using a Bio-image analyzer (BAS2000, Fujix Co. Ltd., Tokyo, Japan).

Preparation of Nuclear Extracts. Nuclear extracts were prepared according to the procedure of Dignam et al. (*19*). Briefly, the cells were resuspended in 4 volumes of buffer A [10 mM Hepes (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 1 mM pepstatin]. The cells were disrupted with 30 strokes in a pestle of a type B Dounce homogenizer. The homogenate was centrifuged at 10000*g* for 10 min to isolate crude nuclei. The crude nuclei were suspended in 4 volumes of buffer C [20 mM Hepes (pH 7.4), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, and 10 mM pepstatin]. The suspension was rotated slowly for 60 min at 4 °C and then centrifuged at 10000*g* for 20 min. The resulting clear supernatants were stored at −80 °C and used as a nuclear extract. Protein concentrations were determined by the Bradford method (*20*).

Electrophoretic Mobility Shift Assay. EMSA was performed as described previously (*21*) with slight modifications. Briefly, the double-stranded wild-type BS207 fragment was used as the probe. They were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Each 10 μL reaction mixture contained 5000 cpm (∼6 ng) of double-stranded end-labeled BS207 DNA fragment and 1.5 μg of nuclear protein and 1.5 μg of poly(dI-dC) in binding buffer [10 mM Tris-HCl (pH 7.5), 5 mM NaCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 5% glycerol, and 1 mM EDTA]. The mixture was incubated at 25 °C for 30 min and then electrophoresed in a 3.5% polyacrylamide gel in 0.5× TBE at 100 V for 1 h. The DNA−protein complexes were visualized by autoradiography. Competition experiments were performed by adding the unlabeled oligonucleotides before the addition of the labeled probe. For supershift assay, U937 nuclear extracts (1.5 μg) were incubated for 30 min with the ^{32}P-labeled BS207 probe in an EMSA cocktail to form a DNA−protein complex. Then anti-Sp1 IgG was added to the complex and the mixture incubated for a further 20 min on ice. For antibody blocking assay, the nuclear extracts (1.5 μg) were incubated with 1 or 2 μg of anti-Sp1 IgG for 30 min on ice, followed by the addition of ^{32}P-labeled BS207. The anti-Sp1 antibody used was a rabbit affinity-purified polyclonal antibody against 436−454 amino acid residues of human Sp1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

DNase I Footprinting. DNase I footprinting was performed essentially as described by Cereghini et al. (*22*). The BS207 DNA fragment was isolated and subcloned into the *Sma*I site of pUC19. Single-end-labeled fragments were prepared by using restriction sites within the polylinker region of this vector and were labeled with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase I. Nuclear proteins (100 μg) were incubated in 100 μL of reaction mixture containing 2 μg of poly(dI-dC), 10 mM Tris-HCl (pH 7.5), 5 mM NaCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 5% glycerol, 1 mM EDTA, and 100 000 cpm (∼4 fmol) of end-labeled probe at 25 °C for 30 min. After the incubation, 1.0 μg/mL DNase I was added, and digestion was carried out at 25 °C for 60 s. The reaction was stopped by the addition of 200 μL of DNase I stop solution containing 1% SDS, 200 mM NaCl, and 20 mM EDTA. The products were extracted with phenol/chloroform (1:1) and then separated by electrophoresis on 8% polyacrylamide/8 M urea gels. The gel was then dried and subjected to autoradiography. G and A reaction was performed by the Maxam and Gilbert method (*23*).

Oligonucleotide-Directed Mutants. The pUC19/BS207 plasmid DNA was subjected to PCR-based site-directed mutagenesis (*24*). Two primary PCRs were performed to produce two overlapping DNA fragments bearing the same mutation using four primers; the outermost two primers were M13 primers (M13 M4 upper primer and M13 RV lower primer, Takara Co. Ltd., Kyoto, Japan), and the inner two primers contained mutations indicated in Figure 5A (mu

upper primer, gggggcgtgcttgcaccgggagcg, and mu lower primer, cgctcccggtgcaagcacgccccc). The upstream outermost primer and the downstream inner primer were used as one pair and the upstream inner primer and the downstream outermost primer as another pair in the primary PCR amplifications. Excess primers were removed by gel electrophoresis. Small aliquots of purified, primary PCR products were then mixed, denatured, reannealed, and subjected to five cycles of PCR amplification without primers to allow the two fragments to recombine and extend. The resulting products then served as templates in the secondary PCR amplifications, using only the outermost two primers. The secondary PCR products were digested by *Eco*RI and *Pst*I, purified, and cloned into the identical site in pUC19. The sequence of the mutant was verified by DNA sequencing using an ABI PRISM Genetic Analyzer (model 310), and the ABI PRISM dye-terminator Cycle sequencing ready reaction kit (Applied Bio-systems). PR I and PR II competitor DNA fragments were produced by PCR of the pUC19/BS207 template DNA with either the M13 M4 upper primer and the mu lower primer or the mu upper primer and the M13 RV lower primer, respectively.

Southwestern Blot Analysis. Southwestern blot analysis was performed as described by Bramson et al. (*25*). Nuclear protein (24 μg) from the cells was subjected to SDS−PAGE and then electrotransferred onto a PVDF membrane. The membrane was soaked in Hepes/salt buffer [20 mM Hepes (pH 7.9), 5 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT] for 15 min, followed by 5% Blotto (Hepes/salt buffer with 5% skim milk) at 25 °C for 1 h. Subsequently, the membrane was soaked overnight in 0.5% Blotto with the ^{32}P-labeled BS207 DNA probe (6×10^5 cpm/20 mL). The membrane was washed three times (10 min each) with Hepes/salt buffer, air-dried, and subjected to autoradiography to visualize the radiolabeled DNA−protein interaction.

Transient Transfection. Reporter plasmid DNA (2.5 μg) was mixed with 1 μg of internal control plasmid chicken β-actin/β-gal (*15*) and cotransfected into U937 cells by electroporation with a Gene Pulser (Bio-Rad) under the same electroporation conditions used in permanent transfections. The cells were cultured in RPMI 1640 medium with 10% fetal calf serum and incubated for 48 h. To examine the effect of TPA on the expression of reporter plasmids, cells were treated with 10 ng/mL TPA. Luciferase activity was determined according to the manufacturer's protocols.

RESULTS

Change in the Level of PARS mRNA and PARS Protein during TPA-Induced Differentiation of U937 Cells. We studied monocytic differentiation by treating human leukemia U937 cells with TPA. When U937 cells were cultured in the presence of 10−50 ng/mL TPA for 1 day, the cells differentiated into monocyte/macrophage-like cells, which exhibited phagocytosis activity. We examined the effect of TPA treatment on PARS content during the differentiation process by Western blot analysis (Figure 1A). TPA treatment caused a marked decrease in the level of PARS protein in U937 cells. On the other hand, TPA treatment did not cause any morphological change in HeLa cells and a remarkable decrease in the PARS protein level was not detected.

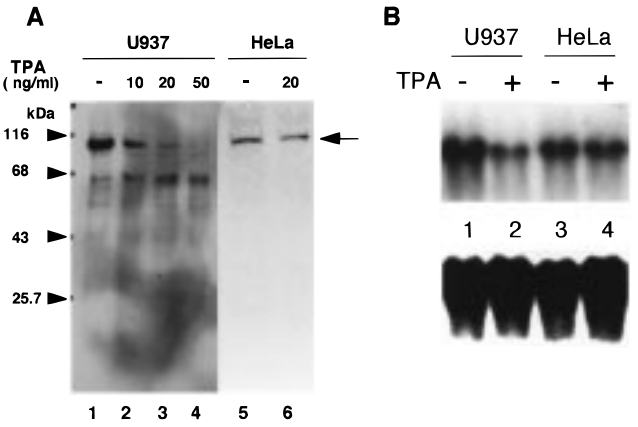

FIGURE 1: (A) Effect of TPA treatment on the PARS protein level in U937 cells and HeLa cells. U937 cells (lanes 1−4) and HeLa cells (lanes 5 and 6) were incubated with 10 (lane 2), 20 (lanes 3 and 6), and 50 ng/mL TPA (lane 4) or without TPA (lanes 1 and 5) for 1 day. Equal amounts of nuclear proteins (5 μg) were subjected to electrophoresis on a SDS−polyacrylamide gel and transferred onto a PVDF membrane. The amount of PARS protein was estimated by Western blot analysis as described in Experimental Procedures. An arrow indicates the band corresponding to the intact PARS. The positions of molecular mass standards (STD) are indicated. (B) Effect of TPA treatment on the PARS mRNA level in U937 cells and HeLa cells. Total RNA was extracted from U937 cells (lanes 1 and 2) and HeLa cells (lanes 3 and 4) treated with (lanes 2 and 4) or without (lanes 1 and 3) 10 ng/mL TPA for 1 day. An equal amount of RNA (20 μg) was loaded in each lane, and Northern blot analysis was performed as described in Experimental Procedures. RNA on the membrane was hybridized with ^{32}P-labeled PARS cDNA (upper panel) (*18*) and the 28S ribosomal RNA gene, provided by J. Kominami (Department of Biochemistry, School of Medicine, Niigata University) (lower panel).

To determine whether the decrease of the PARS protein level in U937 cells is due to TPA-induced suppression in PARS gene expression, we examined PARS mRNA by Northern blot analysis. The level of PARS mRNA was decreased markedly in U937 cells 1 day after TPA treatment, while the level of PARS mRNA did not decrease significantly in HeLa cells (Figure 1B). The result suggests that the decrease in the PARS protein level caused by TPA treatment in U937 cells is at least in part due to a transcriptional response to TPA. Thus, there must be some regulatory elements responsive to TPA in the PARS gene.

Location of the TPA-Responsive Element(s) within the Human PARS Gene. We first constructed a reporter gene which consisted of a promoter of the human PARS gene linked to a promoterless chloramphenicol acetyltransferase (CAT) gene (Hz61A) (Figure 2), as described in Experimental Procedures. This reporter construct was transfected into U937 cells, and the neoresistant transfectants were treated with or without TPA. The CAT activity was not suppressed by TPA treatment. Thus, we used another cat reporter construct (Hz61) (Figure 2) and examined the effect of TPA treatment on the CAT activity. This construct exhibited a downregulation of cat activity by TPA treatment. To identify the cis-acting elements responsible for the TPA-dependent repression of the PARS gene, a plasmid Hz61S was created by deleting the *Sac*II fragment (nucleotides −9 to +422) from Hz61 (Figure 2). We transfected these constructs into U937 cells, isolated some independent neoresistant clones, and used them to examine the effect of TPA treatment on these reporter constructs. The CAT

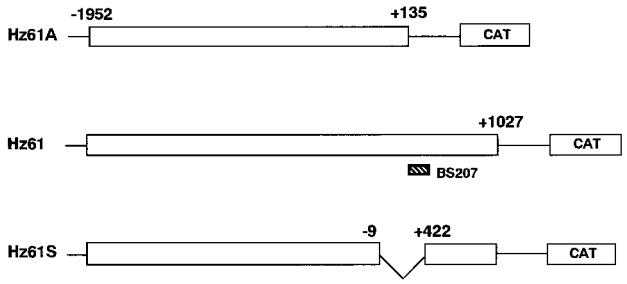

FIGURE 2: Schematic representation of reporter plasmids Hz61A, Hz61, and Hz61S used for transfection in examining the effect of TPA treatment on the expression of CAT reporter genes controlled by PARS regulatory genes. A hatched box shows the fragment BS207 (nucleotides 215−421) which was used as a probe in EMSA and Southwestern blot analysis.

activity in Hz61-transfected clones was decreased to 35% of the control by TPA treatment (Table 1). In contrast, the CAT activities of Hz61A and Hz61S clones were not decreased by TPA treatment (Table 1). This indicates that the cis element responsible for TPA-induced repression is located within the deleted region of Hz61S and Hz61A.

The TPA-dependent repression of the PARS gene expression may be caused by the binding of nuclear protein(s) to the cis element. Thus, we isolated a BS207 fragment (nucleotides 215−421), which represents the major part of the deleted region of Hz61S and Hz61A, and investigated whether some nuclear proteins could bind to this element in vitro. EMSA was performed by using nuclear extracts prepared from U937 cells and a ^{32}P-labeled BS207 fragment as a probe (Figure 3A). DNA−protein complexes were obtained with nuclear extracts from both U937 and HeLa cells (Figure 3B). Interestingly, TPA treatment abolished DNA−protein complex formation with nuclear extract of U937 cells, while it did not cause any significant change in

Table 1: Effect of TPA Treatment on CAT Activity in Stably Transfected Clones[a]

	% conversion of CAT activity		
treatment	Hz61	Hz61A	Hz61S
without TPA	3.70 ± 0.13	4.13 ± 0.12	2.30 ± 0.32
with TPA	1.31 ± 0.40	7.20 ± 1.71	3.83 ± 1.44
with or without TPA	0.35	1.74	1.67

[a] Percentage conversions of CAT activity in untreated and TPA-treated U937 cells transfected with the Hz61, Hz61A, or Hz61S reporter gene were depicted. Each reporter plasmid was independently transfected into U937 cells, and neoresistant clones (three Hz61 clones, one Hz61A clone, and two Hz61S clones) were selected. Each clone was cultured in the absence or presence of 20 ng/mL TPA for 2 days. The total protein (100 μg) was subjected to CAT assay. Experiments were repeated at least three times for each clone, and the radioactivity of the bands was measured using a Bio-image analyzer (BAS2000, Fujix Co. Ltd.). The mean ± SD was calculated from the results obtained from each reporter gene.

a DNA−protein complex formed with HeLa cell extracts (Figure 3B). When U937 cells were treated with 50 ng/mL TPA for 2 days, dead cells appeared. To demonstrate that the decrease in the binding activity by TPA treatment was not due to cell death or variations in the efficiency of nuclear isolation and extraction, we performed EMSA using the same nuclear extract that was used in the experiment whose results are depicted in Figure 3B and a consensus oligonucleotide sequence for AP-1. AP-1 binding activity was enhanced by TPA treatment in U937 cells (Figure 3C). This result agrees well with the previous observation (*12*) and suggests that the decrease in the binding activity caused by TPA treatment was not due to a difference in the efficiency of the nuclear protein extraction in this experiment.

To further define the specific binding sites of the protein(s), we performed DNase I footprinting experiments using nuclear extract from U937 cells and the BS207 DNA labeled

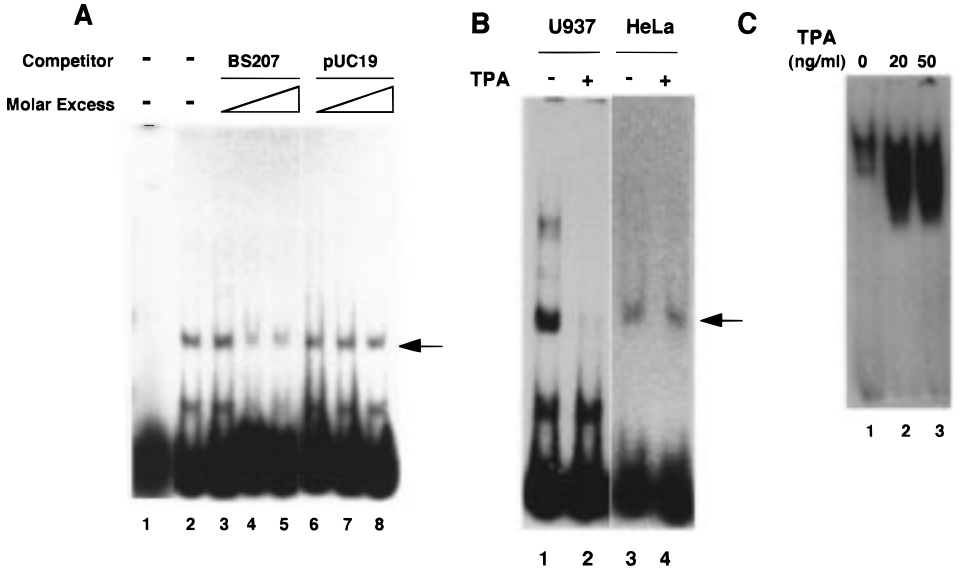

FIGURE 3: Transcription factors binding to the BS207 DNA fragment. (A) The specificity of the protein binding to the BS207 probe DNA was examined by competitive EMSA with unlabeled BS207 DNA (lanes 3−5) and pUC19 plasmid DNA digested with *Hae*III (lanes 6−8). An arrow indicates the DNA−protein complex. The amount of competitors is expressed as a 10-, 50-, and 100-fold molar excess of the labeled probe used. Lanes 1 and 2 represent the labeled probe DNA alone and EMSA of U937 nuclear extracts without competitor, respectively. (B) Effect of TPA treatment on the binding activity of the nuclear factor(s). U937 cells (lanes 1 and 2) and HeLa cells (lanes 3 and 4) were treated with (lanes 2 and 4) or without (lanes 1 and 3) 20 ng/mL TPA for 1 day, and then nuclear extracts were prepared from the cells and incubated with the labeled BS207 DNA. An arrow indicates the DNA−protein complex. (C) Induction of AP-1 binding activity by TPA in U937 cells. Nuclear extracts were prepared from cells treated with 20 (lane 2) or 50 ng/mL TPA (lane 3) for 1 day, and equal amounts of proteins (1.5 μg) were incubated with the end-labeled oligonucleotide for AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′).

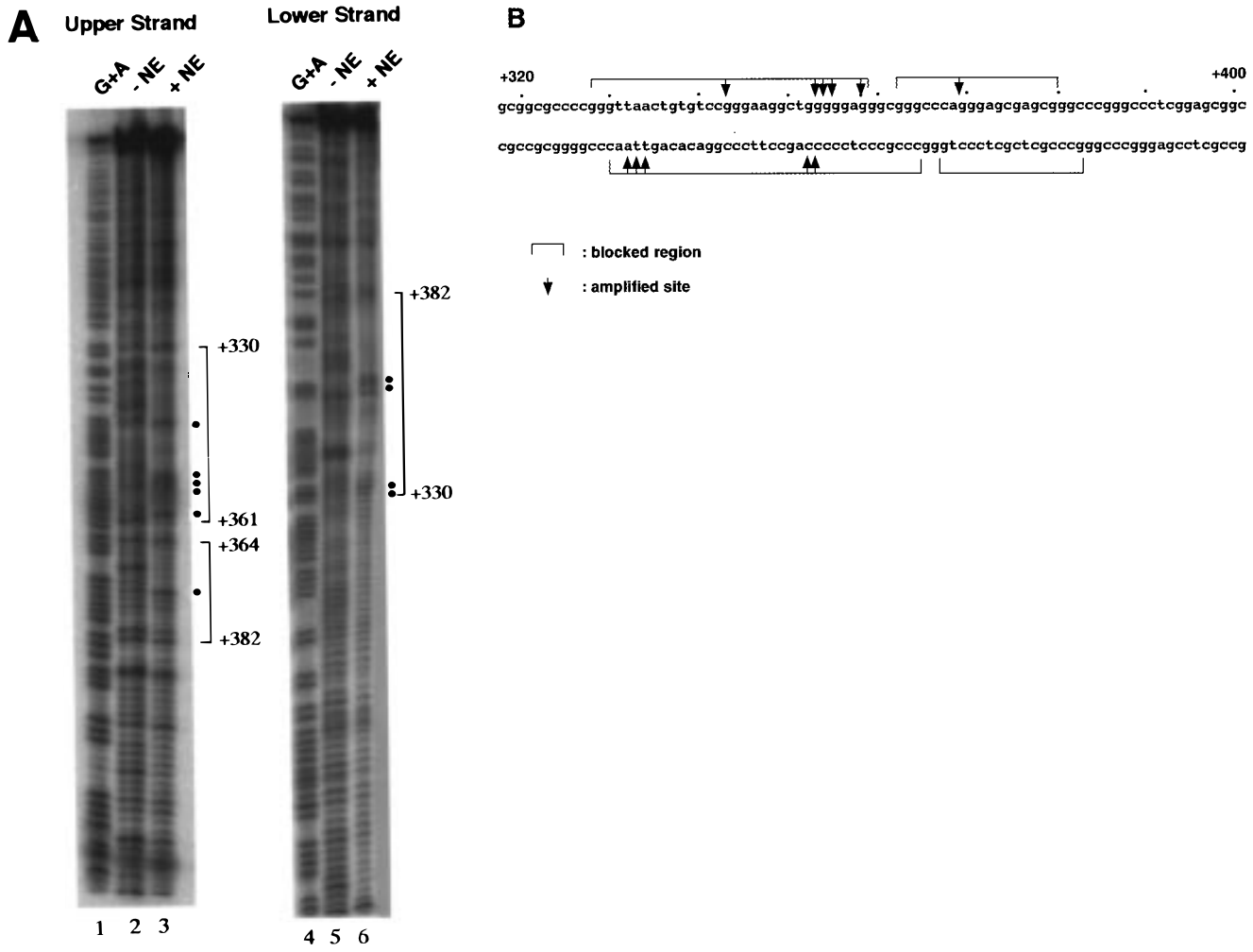

FIGURE 4: DNase I footprinting analysis of the human PARS first intron region. (A) The upper (left) or lower (right) strand was end-labeled with [α-^{32}P]ATP by using Klenow fragment as described in Experimental Procedures, incubated with (lanes 3 and 6) or without (lanes 2 and 5) the nuclear extract of U937 cells, subjected to limited DNase I digestion, and analyzed in an 8% sequencing gel along with a position marker that had been partially cleaved at A and G bases (lanes 1 and 4). The area of specific protection is marked by a bracket. Numbers represent nucleotide positions with respect to the transcription initiation site as position 1. G+A represents a Maxam−Gilbert sequencing ladder. Amplification sites of DNase I digestion are indicated by dots. (B) Localization of the protected site in nucleotide sequence from nucleotides 320 to 400 of the human PARS gene. The region protected from DNase I digestion is marked by a bracket above the sequence. Arrows indicate amplification sites of DNase I digestion.

either at the upper or at the lower strand (Figure 4A). Nuclear proteins of U937 cells protected two adjacent protected regions, designated PR I (nucleotides 328−359) and PR II (nucleotides 362−380), on each strand (Figure 4B). Sites amplified with DNase I digestion are also observed bordering and within the complex (Figure 4B, arrows).

Search for a Binding Protein That Binds to the BS207 DNA Responsible for TPA-Dependent Repression of the PARS Gene. DNase I footprinting analysis suggests that a protected region extending from nucleotides 328 to 383 is responsible for the DNA−protein interaction. A transcription factor binding site profile database search suggested that a potential Sp1-binding site (nucleotides 355−367) is located in this region. Thus, we analyzed protein binding to an oligonucleotide containing base pair substitution in the Sp1 site. We constructed a mutated DNA fragment muBS207 by site-directed mutagenesis (Figure 5A) and used it as a competitor for EMSA. The labeled BS207 probe DNA competed for DNA−protein complex formation with muBS207 DNA (Figure 5B, lanes 5−7) as well as the BS207 DNA (Figure 5B, lanes 2−4). The result suggests that the Sp1 binding site was not responsible for the binding of the TPA-responsive protein. Thus, we investigated further to identify which of the two protected regions are responsible for the binding. Two oligonucleotides containing either PR I or PR II were incubated in the gel mobility reaction mixture as competitors, using nuclear protein(s) from U937 cells and BS207 as a probe. The binding was almost completely abolished when a 100-fold molar excess of either PR I (BS207 upstream sequence down to nucleotide 359) or PR II (BS207 downstream sequence from nucleotide 355) oligonucleotide was added to the reaction mixture (Figure 5C). The result indicates that both of the protected regions are able to compete with BS207, suggesting that both of the protected regions are involved in the DNA−protein interaction. To further rule out the possibility that Sp1 is present in the complex, we used an anti-Sp1 antibody for EMSA. The binding reaction mixture containing U937 nuclear extracts and BS207 probe DNA was further incubated with purified anti-Sp1 antibody prior to electrophoresis. Incuba-

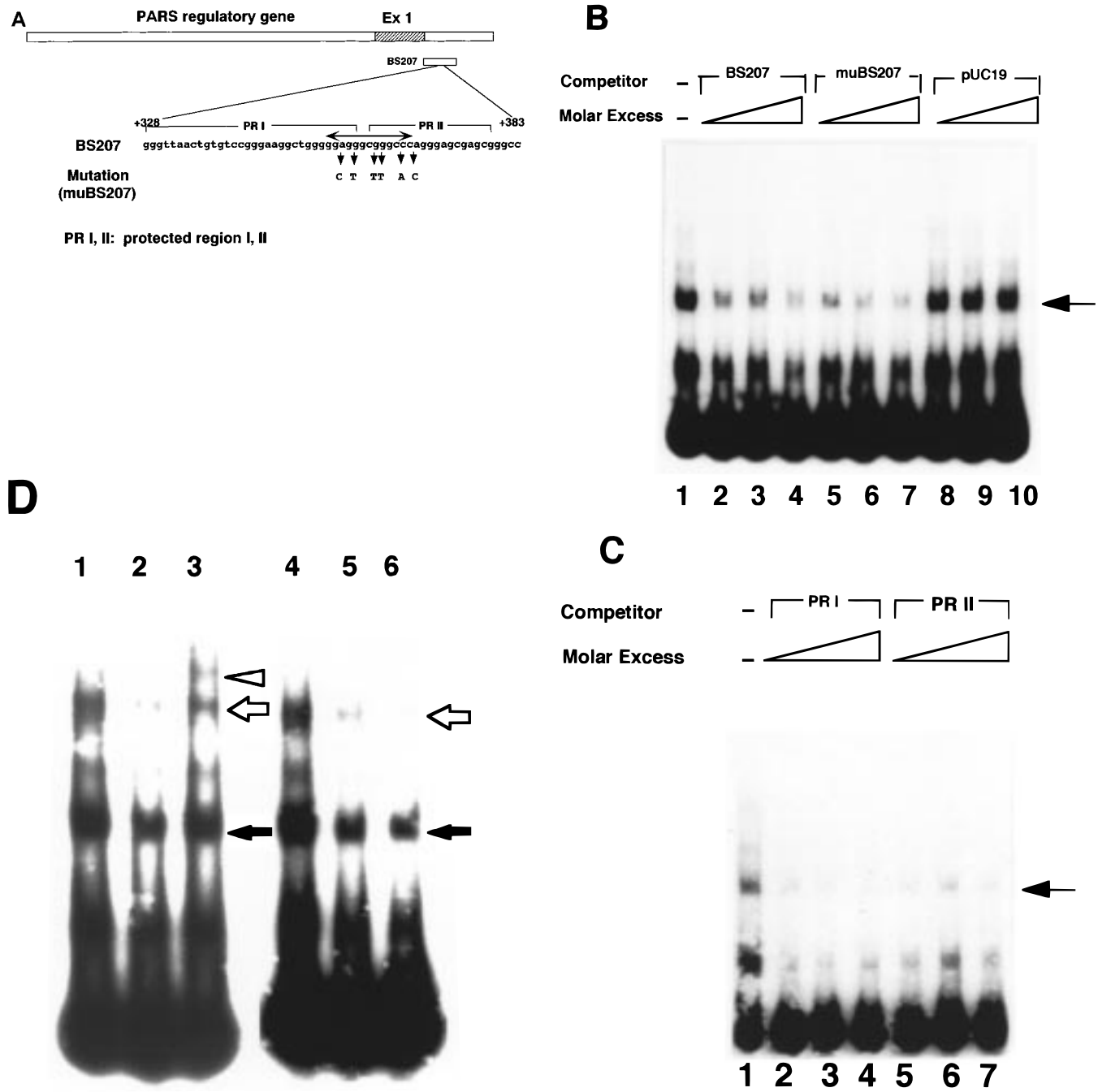

FIGURE 5: Oligonucleotide mutagenesis and competition EMSA for precisely mapping the cis element. (A) Schematic representation of the wild type and the mutant oligonucleotide of BS207. The region marked by an arrow is a potential Sp1-binding site which was deduced by searching a transcription factor binding site profile database. (B) Complexes formed between BS207 and nuclear extracts from U937 cells were analyzed by competition with various unlabeled competitors: lane 1, no competitor; lanes 2−10, a 50-, 100-, or 200-fold excess of various unlabeled competitors as indicated; BS207, wild-type BS207 DNA (lanes 2−4); muBS207, mutant oligonucleotide of BS207 (lanes 5−7); and pUC19, pUC19 plasmid DNA digested with *Hae*III (lanes 8−10). An arrow indicates the DNA−protein complex. (C) Competitive EMSA was performed as described for panel B except that the competitor DNA PR I or PR II containing either upstream protected block (PR I) or downstream protected block (PR II) as described for panel A was used. An arrow indicates the DNA−protein complex. (D) U937 nuclear extracts (1.5 μg) were incubated with the ^{32}P-labeled BS207 probe in EMSA cocktail. After 30 min, none (lane 1), 1 μg (lane 2), or 2 μg (lane 3) of anti-Sp1 IgG was added to the mixture and the mixture incubated for another 20 min on ice. U937 nuclear extracts (1.5 μg) were incubated with none (lane 4), 1 μg (lane 5), or 2 μg (lane 6) of anti-Sp1 IgG for 30 min on ice, followed by addition of ^{32}P-labeled BS207. The white arrow indicates Sp1 or Sp1-like protein which binds weakly to BS207. The black arrow indicates the TPA-responsive protein−DNA complex.

tion with anti-Sp1 antibody resulted in the formation of a supershifted band shown by a white arrowhead (Figure 5D, lanes 2 and 3). The nuclear extracts were incubated with anti-Sp1 antibody and then mixed with BS207. The upper band was abolished (Figure 5D, lanes 5 and 6), but the major complex shown by a black arrow, whose level is diminished by TPA treatment, was not affected by the antibody. Therefore, we conclude that the nuclear protein(s), which is responsible for TPA-dependent regulation of the human PARS gene in U937 cells, is not Sp1.

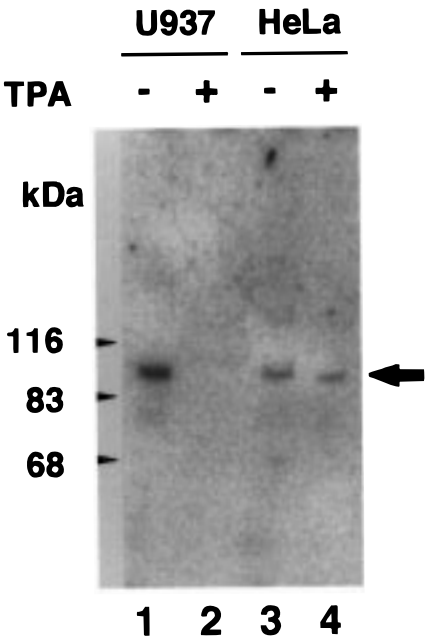

FIGURE 6: Southwestern blot analysis of nuclear protein(s) from U937 cells and HeLa cells with the BS207 DNA probe. U937 cells (lanes 1 and 2) and HeLa cells (lanes 3 and 4) were treated with (lanes 2 and 4) or without (lanes 1 and 3) 20 ng/mL TPA for 1 day. An equal amount of nuclear proteins (24 μg) prepared from both cells was loaded in each lane. Proteins on the membrane were incubated with ^{32}P-labeled BS207 DNA probe overnight. Protein interactions with the labeled DNA probe were visualized by autoradiography.

To characterize the protein(s) binding to BS207 DNA, Southwestern blot analysis was performed using end-labeled BS207 DNA as a probe. Southwestern blot analysis of nuclear extracts of U937 cells revealed a specific interaction of BS207 with a single protein band with an apparent molecular mass of 90−95 kDa (Figure 6). An identical band was also observed in nuclear extract of HeLa cells, but no band was detected in TPA-treated U937 cells. It is consistent with our EMSA observations. These results clearly indicate that the TPA-dependent repression of PARS expression coincides well with the abolishment of binding of a specific factor with a molecular mass of 90−95 kDa to the TPA regulatory element located in the first intron of the human PARS gene.

TPA-Inducible Downregulation of the BS207 Cis Element Linked to a Basal Promoter in U937 Cells. To examine the function of the BS207 cis element in a reporter construct with a basic promoter, we inserted the BS207 DNA fragment upstream of a human β-actin promoter (nucleotides −305 to +63) (*13*, *14*) and constructed a luciferase reporter gene. The human β-actin regulatory luciferase reporter gene with or without the BS207 cis element was transfected into U937 cells, and the cells were cultured for 48 h in the presence or absence of TPA. A chicken β-actin promoter-driven β-galactosidase (β-gal) was cotransfected, and luciferase activity was normalized by the β-gal activity. Insertion of the BS207 cis element increased the basal activity 3.7-fold in the absence of TPA, and this increase in activity was diminished by TPA treatment (Figure 7). This result indicates that the BS207 cis element functions as an enhancer with the aid of an unknown binding protein, and TPA-induced downregulation seems to be due to the loss of the binding activity of the unknown protein caused by TPA treatment.

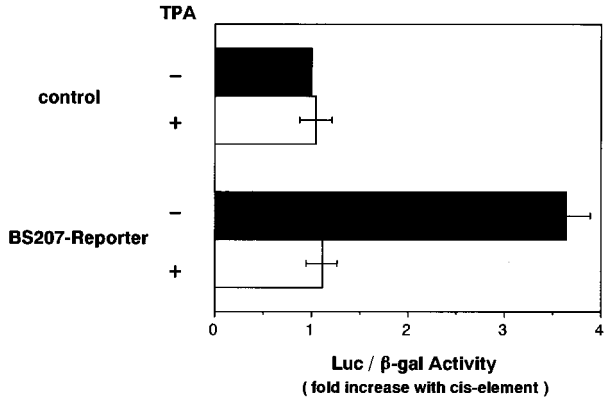

FIGURE 7: TPA responsiveness of BS207 DNA-linked human β-actin promoter. U937 cells were transfected with β-actin-Luc or BS207/β-actin-Luc and treated with TPA (10 ng/mL). Luciferase and β-galactosidase activities were determined 48 h after transfection, and luciferase activity was normalized to results for cotransfected chicken β-actin promoter-driven β-galactosidase (β-gal). Data are expressed as the fold increase over β-actin-Luc (mean ± SE, n = 3).

DISCUSSION

A downregulation of PARS gene expression was observed during differentiation of some cell lines by inducers (*6*, *8*, *10*, *11*). In this study, we identified a TPA-responsive cis-acting element, which represses the transcription of PARS. TPA-responsive genes include various cellular proto-oncogenes, collagenase (*26*), stromelysin (*27*), human metallothioneine IIA (*28*), and SV40 genes. The promoter region of TPA-responsive genes is well-characterized and shares a conserved 9 bp motif (TGAGTCAG) (*29*). The TPA-responsive elements are recognized by a common cellular protein, AP-1 (*29*). However, the cis element found in this study is a GC-rich sequence and is different from the TPA-responsive upregulation element (*30*). The GC-rich cis element, as shown by DNase I protection, contains a typical Sp1 consensus sequence (*31*); however, site-directed mutagenesis of the Sp1 consensus sequence and supershift assay with an antibody against Sp1 revealed that it was not responsible for the binding of the TPA-responsive downregulation factor (Figure 5B). A transcription factor motif search of the DNase I-protected DNA sequence indicates that a STAT family protein (*32*) or an Ikaros gene product, Ik-2 (*33*), is a possible candidate for interaction with the cis element. Involvement of these proteins in TPA-dependent downregulation of PARS gene expression is currently under investigation. The interaction between DNA binding proteins and cis-acting elements plays an important role in controlling gene expression. The same DNA sequence may function as either a positive or negative control element, depending on the DNA binding factors present in different cell types or differentiation stages. One possibility is competition with various activators for DNA binding sites. Because the factor and other activators interact with the same sequence or an overlapping sequence, the binding of the factor might induce directly transcriptional repression. In fact, the cis-acting element contains the consensus sequence of Sp1 and also the sequence homologous to AP-2 (CCGCCCGCG) (*34*). Some transcriptional repressor proteins in mammalian systems have been reported previously. Among them, a GC factor recognizes a GC-rich consensus motif (GCGGGC) in β-actin, EGFR, and CANP promoters (*35*). The cDNA of

this GC factor encodes a 91 kDa protein, which is a molecular mass similar to that of the TPA-responsive downregulation factor determined in this study by Southwestern blot analysis (Figure 6). A similar protein, YY1, (human GLI-kruppel-related protein), also represses transcription of the adeno-associated virus P5 promoter, and the transcriptional repression was relieved by the adenovirus E1A protein (*36*). But the YY1-binding element in the P5 promoter is not homologous to this TPA-inducible downregulation factor-binding element. Recently, TPA-inducible downregulation factors, thyroid transcription factor 1 (TTF-1), and hepatocyte nuclear factor 3 (HNF3) were reported (*37*), but the consensus motif was different from the BS207 cis element of PARS.

Although the TPA-responsive downregulation factor of PARS exists in HeLa cells and TPA treatment did not affect its binding activity, TPA treatment of U937 cells abolishes its binding activity. We speculate that the mechanism by which the binding property is lost is as follows. One is that the factor may be phosphorylated and therefore lose its binding property. H7, an inhibitor of protein kinase C, partially blocked the loss of binding (data not shown). Another possibility is that the factor may interact with another TPA-inducible protein to block the cis element binding domain. The Southwestern blot result reveals that SDS-denatured nuclear proteins in TPA-treated U937 cells did not associate with the DNA probe (Figure 6, lane 2); therefore, the latter possibility may be excluded. We further examined the function of the BS207 cis element in a basal promoter regulatory reporter construct and found that the BS207 cis element functioned as an enhancer via the binding of an unknown trans-acting factor. The TPA treatment diminished the binding activity of the factor in U937 cells but not in HeLa cells, resulting in a decrease in the enhanced activity to the basal level. The TPA-responsive downregulation of PARS takes place only in cells where differentiation is induced by TPA treatment. Thus, the loss of the activity of binding in the unknown trans-acting factor to the BS207 cis element via TPA treatment may provide us a clue for solving the mechanism of macrophage-specific cell differentiation.

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