The American Journal of Pathology, Vol. 170, No. 1, January 2007
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DOI: 10.2353/ajpath.2007.060722

Epithelial and Mesenchymal Cell Biology

Pre-B-Cell Leukemia Transcription Factor 1 Regulates Expression of *Valosin-Containing Protein*, a Gene Involved in Cancer Growth

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Valosin-containing protein (VCP) is involved in a wide variety of cellular functions. Our previous studies showed that the enhanced expression of VCP in cancer cells correlated with invasion and metastasis of cancers. Here, the regulatory mechanism for *VCP* transcription was investigated. Luciferase reporter constructs containing serially deleted 5′-flanking region of the *VCP* gene were transfected into MCF7 mammary carcinoma cell line, in which VCP was abundantly expressed. The deletion and mutation at the two binding motifs for pre-B-cell leukemia transcription factor 1 (PBX1) reduced the luciferase activity, indicating that these two PBX1 motifs mediated the transactivation of the *VCP* gene. Chromatin immunoprecipitation assay showed the binding of PBX-1 to the 5′-flanking region of the *VCP* gene. The knockdown of PBX1 by siRNA decreased the expression level of VCP. VCP is reported to maintain cell viability after the treatment of tumor necrosis factor-α. The viability of tumor necrosis factor-α-treated cells was significantly reduced in PBX1 knockdown MCF7. These findings indicate that PBX1 plays a crucial role in VCP expression and function and that the PBX-VCP pathway might be important for cell survival under cytokine stress. ***(Am J Pathol 2007, 170:152–159; DOI: 10.2353/ajpath.2007.060722)***

Valosin-containing protein (VCP, or p97), a member of the ATPases associated with various cellular activities (AAA) superfamily, is essential for a wide variety of cellular functions such as ubiquitin/proteasome-dependent protein degradation, membrane fusion, cell cycle regulation, and endoplasmic reticulum-associated degradation.[1–5] Notably, VCP is involved in activation of nuclear factor (NF)-κB, a transcription factor promoting antiapoptosis, cell proliferation, and invasion.[6] VCP is abundant in all kinds of cells, accounting for more than 1% of total cellular protein.[1]

Recently, we showed that VCP is associated with metastatic potential of murine osteosarcoma cell line by using mRNA subtraction technique: the cell lines transfected with *VCP* showed the constant activation of NF-κB, decreased apoptosis rates after stimulation with tumor necrosis factor (TNF)-α, and increased metastatic potential.[7] Subsequent studies on the clinical samples showed that the high level of VCP expression in cancer cells correlated with the increase in recurrence rate and poor prognosis of patients with cancer of the liver, stomach, prostate, colorectum, pancreas, esophagus, and lung.[8–14] A clear correlation in VCP expression between mRNA and protein levels was found, indicating that the up-regulation of *VCP* transcription resulted in the enhanced VCP expression in cancers, which is advantageous for cancer cells to survive and metastasize.[8,10,13] This implies that *VCP* transcription would be a novel target for cancer therapy.

Previous study on the basal transcription activity of mouse *VCP* gene revealed the importance of the 410-bp sequence of the 5′-flanking region, which contains consensus binding sites for several transcription factors.[15] However, the precise mechanism for *VCP* transcription has not been clarified yet. No study has been done on the human *VCP* promoter. In the present study, 5′-flanking DNA sequence of the *VCP* gene in the human genome was analyzed, revealing the pre-B-cell leukemia transcription factor 1 (PBX1) to be a key factor for transcription of *VCP*.

Accepted for publication October 10, 2006.

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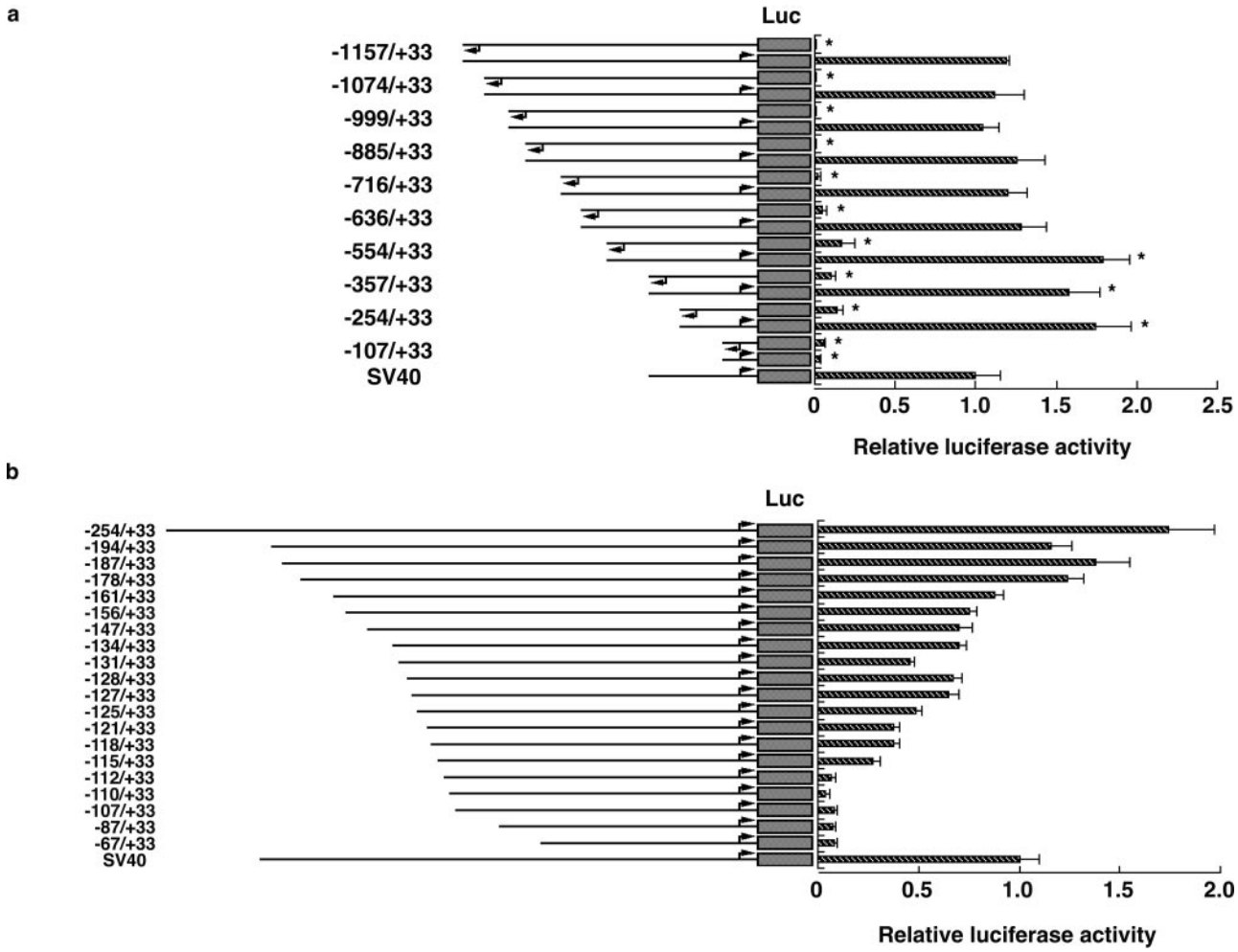

Figure 1. a: Reporter assay of serially deleted constructs containing different lengths of the 5′-flanking sequence of the *VCP* gene. The plasmids were transiently transfected into MCF7 cells, and the reporter activity was analyzed 12 hours after transfection. The values are shown as relative ratio to that of pGL3 control vector containing SV40 promoter. Bars represent mean ± SE of at least three independent experiments. *Significant difference ($P < 0.05$) compared with the −1157/+33 construct. Luc, luciferase. Significant decrease of reporter activity was observed in the plasmid containing −107/+33 construct compared with others. **b:** Reporter assay with serial 5′-deletions between −254 and −67. The deletion between −115 and −112 abolished the luciferase activity.

Materials and Methods

Cell Lines and Culture

Human cell lines MCF7 (breast cancer), PC3 (prostate cancer), and SW480 (colon cancer) were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma) in an air incubator with 5% CO_2 at 37°C.

Analysis of Promoter Activity

The human genomic sequence of the 5′-flanking region of the *VCP* gene including the transcription start site was obtained from GenBank (accession no. NM_007126). DNA extracted from the peripheral blood cells of a healthy volunteer with DNAzol (Invitrogen, Carlsbad, CA) was used as a template. A primer set of 5′-GCTGGCTCCTGATCCGCGAG-3′ and 5′-CTCTGTGGGGAGGGCTTTCT-3′ was used to amplify the 1.5-kb DNA fragment using polymerase chain reaction (PCR). Fragments with various lengths were cloned into pGL3-basic vector (Promega, Madison, WI) either by using Gateway cloning system (Invitrogen) or by ligation of *Xho*I/*Sac*I-digested PCR fragments and vector. Sizes of the subcloned fragments are shown in Figure 1.

Cells (1×10^5) were plated in each well of 24-well plates and after 24 hours were transiently transfected with 0.5 μg of pGL3 plasmid construct containing 5′-flanking region of *VCP* gene including the transcription start site using Lipofectamine 2000 (Invitrogen). phRL-TK vector (0.05 μg; Promega), containing a *Renilla reniformis* luciferase reporter gene, was co-transfected as internal control. The cells were incubated with DNA-Lipofectamine 2000 complex for 9 hours, washed gently with phosphate-buffered saline (PBS), and cultured in fresh serum-supplemented media for an additional 12 hours. Cell lysates were collected and used for measurement of the relative promoter activity of each fragment with the dual luciferase reporter assay system (Promega) according to the manufacturer's protocol. pGL3-control vector (0.5 μg) containing the

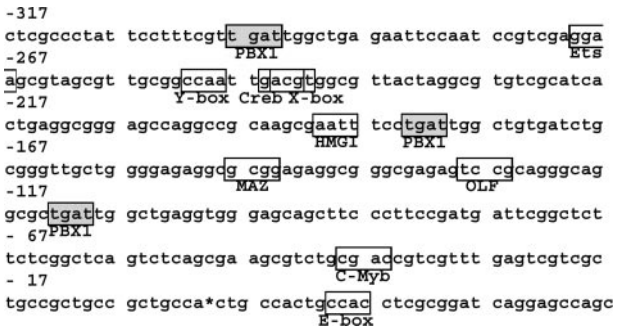

Figure 2. Nucleotide sequence of the 5′-flanking region of the human *VCP* gene. The 5′ 317-bp sequences are numbered with respect to the transcription start site (*). MatInspector software revealed several potential binding sites of transcription factors. Among them, three PBX-1 binding sequences were observed. Another PBX-1 binding sequence was observed at −694.

simian virus 40 (SV40) promoter region was used as a positive control.

Bioinformatics Resources

Transcription factor binding site analysis combined with a literature search was performed using MatInspector (Genomatix Software, Munich, Germany).

Site-Directed Mutagenesis

Experiments of site-directed mutagenesis were performed with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The primers containing mutated/deleted sequences of PBX1 binding sites were synthesized (Figure 2). The pGL3-basic plasmid containing 1.5-kb sequence of 5′-flanking region of the *VCP* gene was used as the parental clone. Plasmids containing the mutation/deletion at PBX1 binding sites were sequentially generated (Figure 3). All constructs were sequenced to confirm the accuracy of mutagenesis. The *VCP* promoter (mutation −113) is 5′-CGCAGGGCAGGCGC**TGAT**TGGCTGAGGTGG-3′ to 5′-CGCAGGGCAGGCGC**GACT**TGGCTGAGGTGG-3′, and *VCP* promoter (deletion −184) is 5′-GCGAATTTCC**TGAT**TGGCTGTGATCTGCGGG-3′ to 5′-GCGAATTTCC**TGAT**GCTGTGATCTGCGGG-3′ (bold letters represent the core sequences of the PBX1 binding region, and underlined letters represent mutated/deleted nucleotides.)

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay was performed using a kit from Upstate (Lake Placid, NY) according to the manufacturer's protocol. In brief, MCF7 cells were treated with formaldehyde for protein-DNA crosslinking. Soluble chromatin was incubated with either anti-PBX1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or preimmune IgG serum. Immunoprecipitation was performed overnight at 4°C with rotation. After washing and elution, DNA was purified with phenol/chloroform extraction and resuspended in 20 μl of water. Two μl of DNA solution was used as a template for 35 cycles of PCR amplification with primers for the 5′-flanking region of the *VCP* gene: 5′-CGGAGAGGGGGGCGAGAGTC-3′ and 5′-GCTGGCTCCTGATCCGCGAG-3′. Template DNA applied for immunoprecipitation with each antibody was used for PCR as positive controls. As a negative control, PCR without template was performed. PCR products were sequenced to confirm the accuracy of the assay.

Small Interfering RNAs

Oligonucleotides to produce plasmid-based small interfering RNA (siRNA) were cloned into pSilencer 4.1-CMV hygro vector (Ambion, Austin, TX) using *Bam*HI/*Hind*III restriction sites. siRNA target sequence to *PBX-1* was 5′-CGACAGAAATCCTGAATGA-3′. Target sequence used as a negative control was 5′-ATCCGCGCGATAGTAGTACGTA-3′. The plasmids producing siRNA were transfected into MCF7 cells with Lipofectamine 2000 according to the manufacturer's protocol, and clones resistant to hygromycin were selected.

Antibodies

The monoclonal antibody against VCP was purchased from Progen Biotechnik (Heidelberg, Germany), poly-

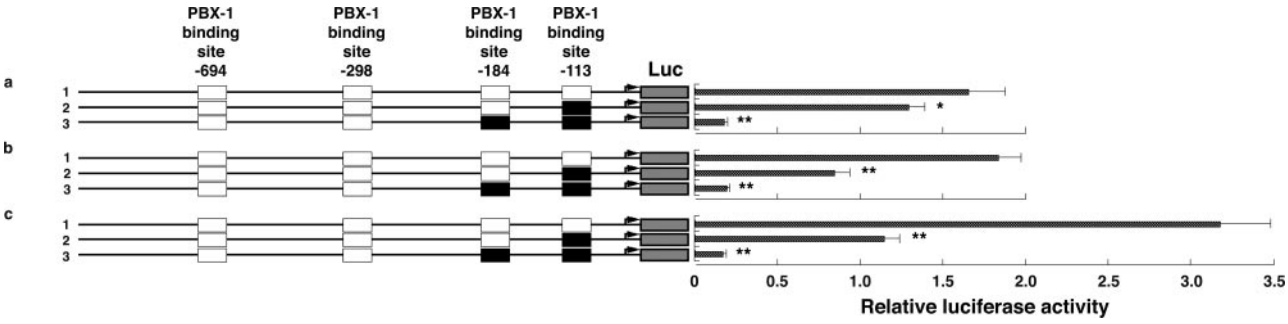

Figure 3. Mutation analysis of transcription factor binding sites of the *VCP* promoter. The pGL3 basic plasmid containing 1.5-kb sequence of 5′-flanking region of the *VCP* gene was used as the parental clone.[1] Plasmids containing mutation at −113[2] and both of −113 and −184[3] were sequentially generated and transfected to MCF7 (**a**), PC3 (**b**), and SW480 (**c**) cells. The luciferase activity showed a significant decrease with the transfection of the plasmid mutated at −113 and was completely lost with the transfection of the plasmid mutated at both −113 and −184. Bars represent mean ± SE of at least three independent experiments. *, **Significant difference ($P < 0.05$, $P < 0.001$, respectively) compared with the parental construct. Luc, luciferase.

clonal antibody against PBX1 and against β-actin from Santa Cruz Biotechnology and Sigma, respectively. Anti-mouse IgG antibody and anti-rabbit IgG antibody conjugated with horseradish peroxidase purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK), and Alexa Fluor 488 goat anti-mouse IgG antibody and Alexa Fluor 594 goat anti-rabbit IgG antibody from Invitrogen were used as secondary antibodies for Western immunoblotting and immunofluorescence, respectively.

Western Immunoblot Analysis

MCF7 cells were washed with ice-cold PBS and lysed with lysis buffer (2% sodium dodecyl sulfate and 125 mmol/L Tris-HCl, pH 6.8). Protein concentration was measured with a protein assay kit (Bio-Rad, Hercules, CA). An aliquot of 10 μg from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transblotted onto polyvinylidene difluoride transfer membranes (Millipore, Bedford, MA). After blockage of nonspecific binding with 5% nonfat milk in PBS-0.1% Tween 20 (PBST), the blotted membranes were reacted with primary antibodies and then visualized with electrochemiluminescence detection system (Amersham Pharmacia Biotech).

Immunofluorescence Confocal Microscopy

MCF7 cells were cultured in Lab Tek II chamber slides (Nunc Nalgene, Naperville, IL), fixed in methanol at −20°C for 10 minutes, washed in PBST, preincubated in PBS with 1% bovine serum albumin for 1 hour, and then incubated with primary antibodies at a concentration of ×200 in PBS. After washing with PBST three times, the slides were incubated with the secondary antibodies, washed, and examined with Zeiss LSM510 confocal microscope (Carl Zeiss, Jena, Germany). As a control, fluorescent immunohistochemistry with anti-VCP antibody and nonimmunized rabbit serum, and anti-PBX1 antibody and nonimmunized mouse serum, was performed.

Cell Viability Assay

MCF7 cells were plated on 96-well plates at 4×10^3 cells per well, treated with TNF-α (Sigma) for 30 minutes at a concentration of 5 ng/ml, and incubated in Dulbecco's modified Eagle's medium with 0.5% fetal bovine serum for 6 hours. Cell viability was measured with a cell Titer-Glo luminescent cell viability assay kit (Promega).

Statistical Analysis

Data were expressed as mean ± SE. The significance of differences of the mean values was determined by Student's *t*-test. *P* values of less than 0.05 were considered as statistically significant.

Results

Region Mediating VCP Transactivation

To identify regions mediating *VCP* transactivation, reporter plasmids containing the serially deleted 5′-flanking sequence of *VCP* gene were constructed. The constructed reporter plasmids were transfected into MCF7 cells that endogeneously expressed VCP at a high level. Plasmids containing the sequences starting from −1157, −1074, −999, −885, −716, −636, −554, −357, and −254 (+1 means transcription initiation site) showed the constant strong promoter activities, whereas plasmid starting from −107 showed the 96% decrease in promoter activity compared with that from −254 (Figure 1a). To examine further the regulatory mechanism of *VCP* expression, luciferase reporter plasmids with serial 5′-deletions between −254 and −67 were constructed. When the region between −254 and −115 was serially deleted, the promoter activity gradually decreased. In contrast, promoter activities almost disappeared below −112 (Figure 1b).

Regulation of VCP Promoter Activity by PBX1

MatInspector software was used to identify the sequences responsible for *VCP* transcription. The deletion between −115 and −112 abolished the luciferase activity (Figure 1b), indicating that the sequence between −115 and −112 mediated the transactivation of *VCP* gene. With MatInspector software, one transcription factor binding site was found in this sequence, the PBX1 binding motif located between −110 and −113 (Figure 2). Hereafter, we called this motif PBX-113. Besides PBX-113, three PBX1 binding motifs were found in the examined 5′-flanking region of *VCP* gene, the motifs starting from −184, −298, and −694. These were termed PBX-184, PBX-298, and PBX-694, respectively (Figure 2). Among them, the contribution of PBX-298 and PBX-694 to *VCP* transactivation seemed to be low because the luciferase activity remained high even when these two motifs were deleted (Figure 1a). In contrast, PBX-184 and PBX-113 seemed to be important for *VCP* transactivation because the construct deleting them showed significantly low luciferase activity (Figure 1b). However, the gradual decrease of luciferase activity between −178 and −115 suggests that PBX1 is necessary, but not sufficient, for normal regulation of *VCP* transcription (Figure 1b). To confirm the importance of PBX-184 and PBX-113 for *VCP* transcription, the reporter plasmids starting from −1494 and mutated at PBX-113 and/or PBX-184 were constructed. When the construct mutated at PBX-113 was transfected to MCF7, the luciferase activity decreased by one third. When the construct mutated at both PBX-113 and PBX-184 was transfected into MCF7, the luciferase activity was completely lost (Figure 3a). This was also observed in VCP-expressing cell lines PC3 and SW480 (Figure 3, b and c).

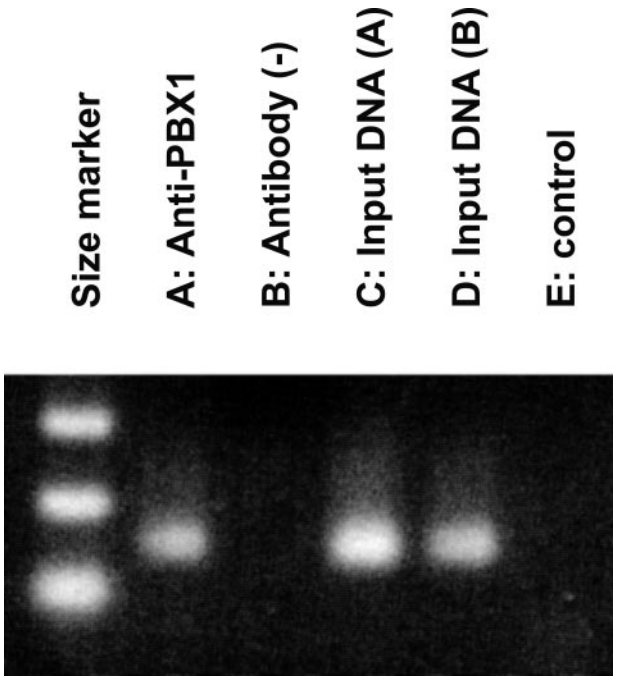

Figure 4. Analysis of PBX1 binding to the 5′-flanking region of the human VCP gene. Chromatin immunoprecipitation assay was performed using MCF7 cells. Precipitated DNA fragments with (**lane A**) or without (**lane B**) the addition of anti-PBX1 antibody together with the input DNA samples used for immunoprecipitation with anti-PBX1 antibody (**lane C**) or preimmune IgG serum (**lane D**) were PCR-amplified with primers specific for the VCP promoter region. Result of PCR without template was shown (**lane E**). PCR products were separated on 2.5% agarose gel and stained with ethidium bromide. Positive band in the lane of the sample precipitated with anti-PBX1 antibody (**lane A**) was seen at the same size with those of input DNA (**lanes C** and **D**).

Binding of PBX1 to the 5′-Flanking Sequence of VCP Gene

To confirm the binding of PBX1 to the 5′-flanking sequence of *VCP* gene in a cell line, chromatin immunoprecipitation assay was performed. After treatment with formaldehyde to crosslink protein and DNA, soluble chromatin extracted from MCF7 cells was immunoprecipitated with anti-PBX1 antibody. PBX1 binding sequence at the *VCP* promoter region was successfully amplified by PCR. The amplified fragment was confirmed to be the *VCP* promoter region by sequencing. No bands were detected when PCR was performed using DNA precipitated without anti-PBX1 antibody and without template. The result showed the binding of PBX1 to the 5′-flanking sequence of *VCP* gene (Figure 4).

siRNA Interference of PBX1

Plasmid expressing *PBX1* siRNA was stably transfected to MCF7 cells. As a control, plasmid expressing negative siRNA was transfected. Western immunoblotting of the cloned cells showed a marked decrease in PBX1 expression in *PBX1* siRNA clone compared with negative siRNA clone and original MCF7 cells (Figure 5). Expression level of VCP in *PBX1* siRNA clone was lower than that in the negative siRNA clone and MCF7 cells (Figure 5). Fluorescent immunohistochemical analysis showed that PBX1 and VCP expression in *PBX1* siRNA clone was weaker than that in negative siRNA clone and MCF7 cells (Figure 6). Co-localization of PBX1 and VCP was observed (Figure 6). Control analysis showed the fluorescence for the specific antibody, but not for the nonimmunized serum, affirming specificities of the antibodies and absence of bleed-through of the fluorophores (Figure 6). Association between *PBX1* expression and cell viability after TNF-α treatment was measured by the cell Titer-Glo luminescent cell viability assay kit (Promega). Proportion of viable cells in *PBX1* siRNA clone was lower than that in MCF7 cells and negative siRNA clone (Figure 7).

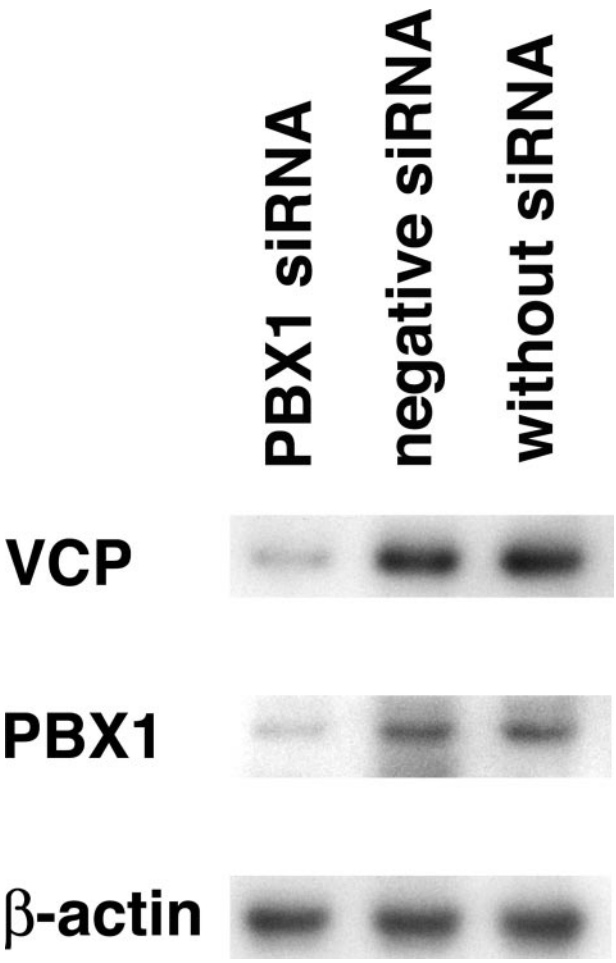

Figure 5. Western immunoblot analysis of *PBX1* siRNA-transfected MCF7 cells. Equal amounts of whole cell lysate protein were subject to immunoblotting with the indicated antibodies. Expression level of PBX1 and VCP in *PBX1* siRNA clone was lower than that in the negative siRNA clone and MCF7 cells.

Discussion

VCP is associated with diverse cellular activities that are essential for cells to survive, such as endoplasmic reticulum-associated degradation, cell homotypic membrane fusion, cell cycle control, and anti-apoptosis.[1–5] Together with its co-factor Ufd1-Npl4, VCP extracts ubiquitylate substrates from the membrane for subsequent delivery to

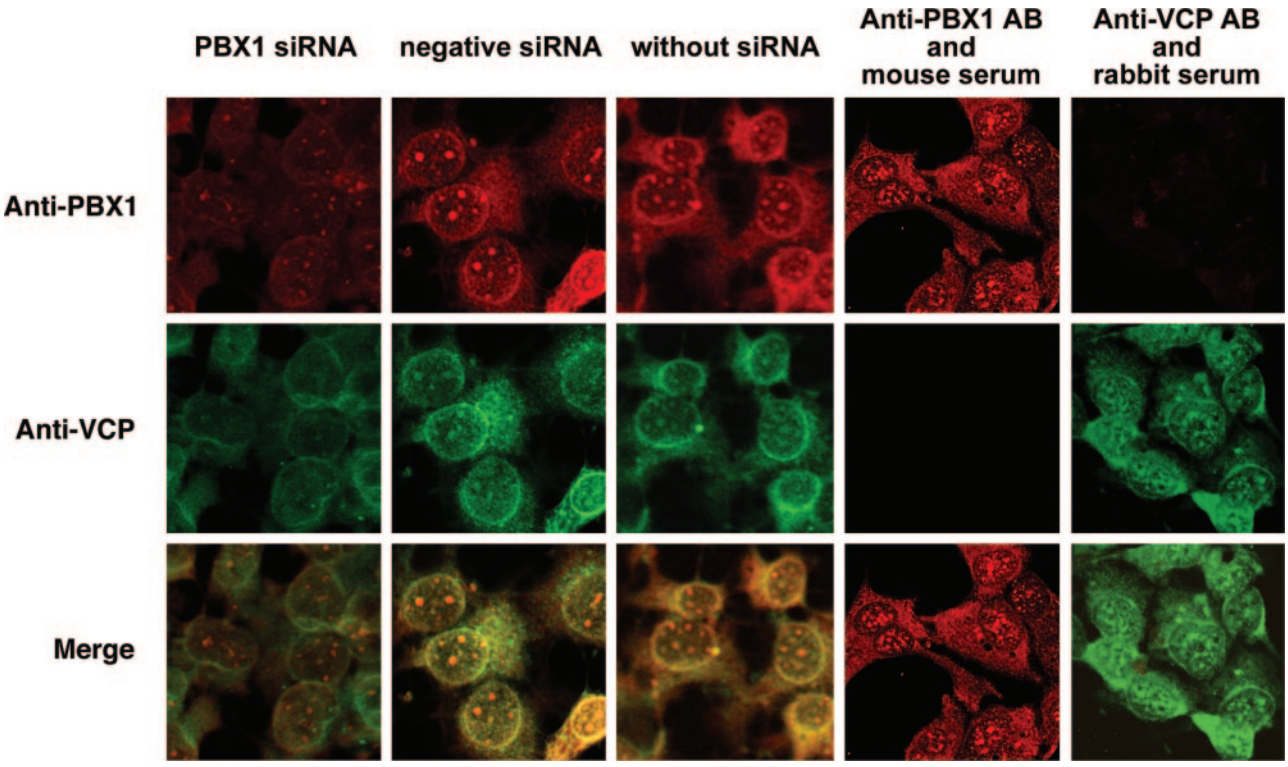

Figure 6. Fluorescent immunohistochemical analysis of *PBX1* siRNA-transfected MCF7 cells. Immunofluorescence confocal microscopy was conducted for PBX1 (red) and VCP (green). Merged images are shown. PBX1 and VCP expression in *PBX1* siRNA clone was weaker than those in negative siRNA clone and MCF7 cells. Control analyses with nonimmune rabbit or mouse serum as primary antibodies instead of anti-PBX1 and anti-VCP antibodies showed the fluorescence for the specific antibody, but not for the nonimmunized serum, indicating the specificity of primary antibodies and absence of bleed-through of the fluorophores. AB, antibody.

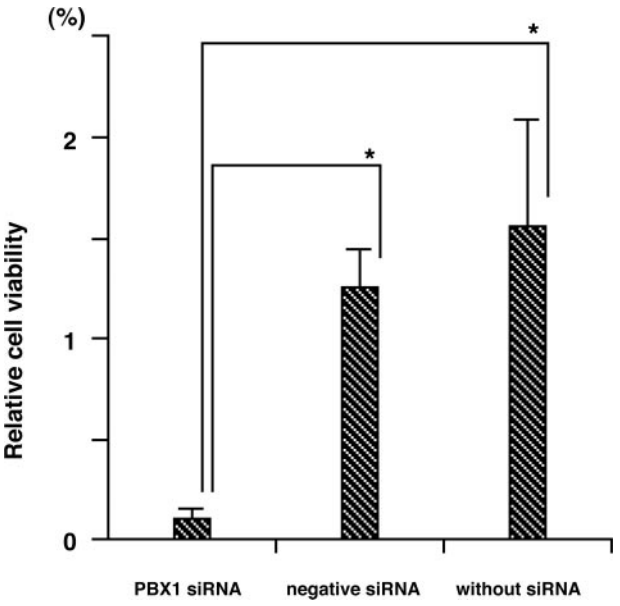

Figure 7. Cell viability assay after TNF-α treatment. Cells were treated with TNF-α for 30 minutes at a concentration of 5 ng/ml and incubated in Dulbecco's modified Eagle's medium with 0.5% fetal bovine serum for 6 hours. Cell viability was measured by the Cell Titer-Glo luminescent cell viability assay kit (Promega) and the relative ratio to cells without TNF-α treatment was calculated. Bars represent mean ± SE of at least three independent experiments. *Significant difference ($P < 0.01$). Proportion of viable cells in *PBX1* siRNA clone was significantly lower than that in MCF7 cells and negative siRNA clone.

the proteasome during endoplasmic reticulum-associated degradation.[4] VCP and its other co-factor p47 are involved in fusion of endoplasmic reticulum, Golgi, and nuclear membrane.[3] VCP interacts with DNA unwinding factor (DUF) in *Xenopus* egg extracts, and the VCP-DUF complex may produce a chromatin structure favorable for DNA replication.[16] VCP is involved in NF-κB activation, which works in an anti-apoptotic function.[6,7] Dysfunction of VCP results in vacuole and inclusion body formation, ultimately leading to cell death.[17] Mutated VCP causes inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia.[18]

VCP is a highly conserved and ancient protein, homologues of which could be found in most organisms ranging from archaebacteria to human.[1] Because of the abundance in cells and its important role in basic cellular function including cell proliferation, VCP is considered to be ubiquitously expressed,[19] although its expression level varies among cells and tissues.[15] Analysis of 5′-flanking region of *VCP* has revealed the multiple consensus binding sites for several growth factors; therefore, VCP expression is supposed to be regulated in a complex and manifold manner.[15] In fact, expression of VCP homologue is regulated temporally and tissue specifically in Drosophila, undetectable in larvae, and predominantly expressed in the brain and gonads in imago and adult fly.[20] As for mouse, Muller and colleagues[15] reported that expression levels of VCP protein and mRNA were

high, but their regulations depend on cell-type and differentiation.

The present analysis revealed PBX1-binding sequence in the 5′-flanking region of human *VCP* to be a key sequence for the transcription. Several consensus sequences for major transcription factors such as Ets-binding motif, X-box, and Y-box were present in the 5′ region of *VCP*, but the mutation analysis of these sequences did not alter the reporter activities (data not shown).

There are four PBX1 binding sites in the 5′-flanking region of *VCP*, ie, at −113, −184, −298, and −694. Among them, mutation analysis revealed that PBX-113 and PBX-184 mediated the *VCP* transcription. Actual binding of PBX1 was shown by chromatin immunoprecipitation assay. Transfection of siRNA for *PBX1* decreased the expression level of VCP, as well as PBX1. The decrease in PBX1 expression induced susceptibility of MCF7 cells to TNF-α treatment. These findings showed that PBX1 is essential for the transcription of *VCP* gene. The PBX1-VCP pathway might be important for cell survival under cytokine stress.

The *PBX1* gene was initially identified as the chromosome 1 participant of the t(1;19) chromosomal translocation in pre-B-cell acute lymphoblastic leukemia.[21,22] The translocation creates the *E2A-PBX1* chimeric gene, which is essential for development of myeloid and T-lymphoid leukemia in animal model and induces tumorigenic conversion of NIH3T3 fibroblasts as assayed by tumor formation in nude mice.[23–25] Subsequently, normal PBX1 was shown to act as a transcription factor in cooperation with Homeobox (Hox) proteins and regulates proliferation and differentiation of normal cells and cancer cells.[26]

PBX1 is known to act as co-factor for MyoD as well as Hox.[27] Binding of PBX1 to the MyoD-dependent promoter occurs before MyoD binding, then muscle differentiation starts.[27] From these findings, it is postulated that PBX penetrates into condensed chromatin and changes the local condition from silent to active stage ready for binding of gene-specific transcription factor.[27,28]

The fluorescent immunohistochemical analysis showed the co-localization of PBX1 and VCP in the cytoplasm. PBX1 has two nuclear localization signals, which are masked by intramolecular interaction with the N terminus.[29] PBX1 is actively transported from the nucleus to the cytoplasm via the Exportin1 (CRM1)-dependent pathway.[30] In addition, nonmuscle myosin II heavy chain B acts as a co-factor for cytoplasmic localization of PBX1.[30] Taken together, further investigation is necessary to identify the interaction between PBX1 and VCP in the cytoplasm and its biological importance.

VCP is involved in cancer cell proliferation and metastasis.[7] Its expression level in various kinds of cancer cells correlated with recurrence of cancers and prognosis of patients with cancers,[8–14] indicating that VCP plays a crucial role for cancer biology. Because PBX1 plays an important role in *VCP* transcription, the PBX1 expression pattern could be used as a new tool both for the stratification and treatment of patients with cancers. Modulation of PBX1 expression could be a target for treatment of cancers.

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