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Negative regulation of human U6 snRNA promoter by p38 kinase through Oct-1

Bor-Ruei Lin* and **Ven Natarajan***

SAIC-Frederick, National Cancer Institute at Frederick, 1050 Boyles Street, Frederick, MD 21702-1201, USA

Abstract

Recruitment of Oct-1 protein to the octamer sequence of U6 promoter is critical for optimal transcription by RNA polymerase III. Here we report that p38 kinase inhibitors, SB202190 and SB203580, stimulated U6 promoter activity and this stimulation can be observed only in the presence of octamer sequence. SB202190-treated cell nuclear extract had about 50% increase in Oct-1 binding activity suggesting that the increased U6 promoter activity by p38 kinase inhibitor is mediated through Oct-1. Mutation in octamer sequence significantly reduced the SB202190-stimulated U6 promoter transcription and the distance between octamer and proximal sequence element of U6 promoter is also critical for the p38 kinase inhibitor-stimulated activity. Exogenous Oct-1 expression showed a concentration-dependent activation of U6 promoter that was further stimulated by the p38 kinase inhibitors. When cells were treated with p38 kinase inducer, hydrogen peroxide or phorbol 12-myristate 13-acetate (PMA), U6 promoter activity was down regulated and this inhibition was reversed by p38 kinase inhibitors. Over-expression of p38 α kinase down-regulated U6 promoter activity and this inhibition was further enhanced by PMA and p38 kinase inhibitors reversed this inhibition. p38 kinase inhibitor-treated cells had 50% more U6 RNA than the control cells. Taken together, our results show a negative correlation between the p38 kinase levels and Oct-1 binding on U6 promoter, suggesting that U6 promoter is negatively regulated by p38 kinase.

Keywords

U6 promoter; Oct-1; p38 kinase; SB202190; MAPK

1. Introduction

The spliceosome contains U1, U2, U4, U5 and U6 snRNAs and they are highly conserved in eukaryotic cells (Wahl et al., 2009). Among these snRNAs, U6 snRNA in particular is transcribed by RNA Pol III machinery and has been studied extensively (Kunkel and Pederson, 1988; Willis, 1993). The U6 snRNA promoter contains a TATA box, proximal sequence element (PSE) and a distal sequence element (DSE) (Kunkel and Pederson, 1988). Optimal transcription from U6 promoter is achieved by the recruitment of transcription factor Oct-1 to DSE, followed by a cooperative binding of snRNA activating-protein

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*Corresponding authors. Tel: +1 301 846 5089; Fax: +1 301 846 6762; linbr@mail.nih.gov (B.-R. Lin).. *Tel: +1 301 846 1248; Fax: +1 301 846 6762; vnatarajan@mail.nih.gov (V. Natarajan)..

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complex (SNAPc) to PSE (Mittal et al., 1996), and a positioned nucleosome binding to the region between PSE and DSE of U6 promoter (Stunkel et al., 1997; Zhao et al., 2001).

Transcription factor Oct-1 is a widely expressed protein in mammalian cell nucleus and recognizes a conserved octamer sequence (5'-ATTTGCAT-3' or 5'-ATGCAAAT-3') in different housekeeping or tissue-specific gene promoters, such as histone H2B, U6 snRNA, immunoglobulin heavy chain, and interleukin 2 (Klemm et al., 1994; Zhao et al., 2001; Sturm et al., 1988; Phillips and Luisi, 2000; Ullman et al., 1991). Oct-1 belongs to the POU family which is named from the founder member of *Pit-1*, *Oct-1/2* and *Caenorhabditis elegans Unc-86*, and contains a core bipartite DNA binding domain consisting of a POU-specific and a POU-homeo subdomains joined by a linker (Dekker et al., 1993; Sturm et al., 1988; Phillips and Luisi, 2000). The orientation of the conserved octamer sequence does not affect Oct-1 POU domain binding and the recruitment of SNAPc to PSE by Oct-1 due to the highly flexible linker sequence (Mittal et al., 1996; Zhao et al., 2001).

Oct-1 is suggested as a cell stress sensor in response to DNA damage by different stimuli and regulates the genes involved in cellular oxidative and metabolic stress (Tantin et al., 2005; Zhao et al., 2000; Meighan-Mantha et al., 1999; Schild-Poulter et al., 2003). Cell stress signals can also activate mitogen-activated protein kinase (MAPK) signal transduction pathways, including extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs) and p38 kinases (Ono and Han, 2000; Kyriakis and Avruch, 2001; Johnson and Lapadat, 2002; Zarubin and Han, 2005; Ashwell, 2006). In particular, p38 kinase as a cellular stress sensor is activated by radiation, genotoxic agents, oxidative stress, or DNA double strand breaks (Lee et al., 2001; Thornton and Rincon, 2009; Pedraza-Alva et al., 2006; Wang et al., 1998). In this study, we report that U6 promoter activity is stimulated by p38 kinase inhibitors and Oct-1 was negatively regulated by p38 kinase by preventing Oct-1 from binding to DSE on U6 promoter.

2. Materials and Methods

2.1. Cell culture

Jurkat (clone E6-1) cell was obtained from American Type Culture Collection and maintained in complete RPMI-1640 medium supplemented with 10 mM HEPES, 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM L-glutamine (Quality Biological), and 10% heat-inactivated fetal bovine serum (Hyclone Laboratories) at 37 °C in 5% CO₂ atmosphere. 293FT cell was purchased from Invitrogen and maintained in DMEM (Cellgro) supplemented with 0.5 mg/ml G-418 (Sigma-Aldrich), 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum.

2.2. Preparation of U6 promoter constructs

U6 promoter region (GenBank ID: M14486) from -264 to +23 base pair (bp) was amplified by polymerase chain reaction (PCR) from human peripheral blood mononuclear cells using primers with restriction sites XhoI and HindIII at the forward and reverse primers respectively (Integrated DNA Technologies), and cloned into pGL4.10[luc2] vector (Promega) to obtain pU6-264 (Fig. 1A). A series of promoter deletion constructs were made by PCR and cloned into pGL4.10[luc2] vector (Fig. 1A). The octamer mutation from ATTTGCAT to ACGTGCAG was made using QuikChange site-directed mutagenesis kit (Stratagene) as per manufacture's instruction (Fig. 1E). Constructs containing octamer adjacent to -200, -150, -100 and -50 bp of U6 promoter were made by cloning octamer containing sequence obtained by annealing 5'- TCGAATATTTGCATAT -3' and as 5'- TCGAATATGCAAATAT -3' oligonucleotides and ligating to XhoI digested pU6-200,

pU6–150, pU6–100 and pU6–50 to generate Oct-200, Oct-150, Oct-100 and Oct-50 respectively (Fig. 1E). All U6 promoter constructs were verified by DNA sequencing.

2.3. Oct-1 and p38 α kinase expressing plasmids

Human *oct-1* gene was amplified by PCR from cDNA clone purchased from PlasmID Database and ligated into pCMV6-Entry vector (OriGene) with SgfI and MluI sites to obtain pCMV6-Oct-1-FLAG. pcDNA3-FLAG-p38 α expressing p38 α kinase was purchased from Addgene.

2.4 pCMV-luc plasmid

The pCMV-luc plasmid was a gift from Dr. Colburn (National Cancer Institute, Frederick, MD) and was constructed by ligating a luciferase gene from pGL3-basic (Promega) into pcDNA3.1(+) (Invitrogen) vector which contains a RNA polymerase II-driven 588 bp of CMV promoter (Yang et al., 2004).

2.5. Transfection and treatment

Jurkat cells were pelleted at 800 rpm for 10 min at room temperature and resuspended in pre-warmed RPMI-1640 medium without supplements. Electroporation was performed by Gene Pulser Xcell Electroporation System (Bio-Rad). Ten million cells in 250 μ L RPMI-1640 medium were mixed with plasmids, loaded in 4 mm cuvette and placed in ShockPod chamber. The parameters were set as 250V in voltage, 1000 μ F in capacitance (750 μ F used in Fig. 3, Fig. 4 and Fig. 5), and ∞ ohm in resistance. After electroporation, pre-warmed RPMI-1640 complete medium was added to the cuvette and cells were transferred to a 12-well tissue culture plate incubated for 24 h.

293FT cells were seeded prior to the day of transfection in 6-well plate with DMEM containing 10% heat-inactivated fetal bovine serum. Transfection was performed by mixing DNA and lipofectamine 2000 with a ratio of 1:2 (w:v) in DMEM. After 24 h, medium was replaced by pre-warmed DMEM containing 10% heat-inactivated fetal bovine serum. Cells were then treated with dimethylsulfoxide (DMSO) or 20 μ M SB202190 or SB203580 (EMD Biosciences) for 2 h prior to 25 μ M hydrogen peroxide (Sigma-Aldrich) or 50 ng/ml PMA (Sigma-Aldrich) for 16 h to 18 h. For α -amanitin (Sigma) treatment, the medium was replaced 5 h after transfection by pre-warmed DMEM containing 10% heat-inactivated fetal bovine serum with different concentrations of α -amanitin and cells were harvested after 2 days.

2.6. Luciferase assay

Transfected Jurkat cells were harvested, washed with phosphate-buffered saline (PBS, pH 7.4) and lysed in 1x lysis buffer (Promega) as per manufacture's instruction. Luciferase activity was measured by mixing 25 μ L cell lysate with 50 μ L firefly luciferase substrate (Promega) at room temperature. Normalized luciferase activity was calculated by dividing the luciferase activity by total protein concentration ($n = 2$). The relative luciferase activity was calculated by taking values of untreated cells transfected with pU6-264 as 1. The data are representative to minimum of 3 individual experiments as mean with SEM ($n \geq 3$).

2.7. Cell nuclear extract preparation

Jurkat cells were pelleted at 800 rpm and washed twice in PBS (pH 7.4). 293FT cells were harvested by adding 0.05% trypsin-EDTA, washed in PBS (pH 7.4) and pelleted at 800 rpm. Nuclear extracts were prepared by NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to manufacture's instruction. Cell nuclear extracts were used immediately or stored at -80°C until needed.

2.8. Western Blot

Nuclear extracts were electrophoresed in a 10% NuPAGE Bis-Tris Gel with NuPAGE MES-SDS running buffer in XCell SureLock Mini-Cell and then transferred to a polyvinylidene difluoride membrane with Xcell II Blot Module (Invitrogen). Mouse anti-DDK(FLAG) monoclonal antibody (OriGene), mouse anti-Oct-1 monoclonal antibody (Santa Cruz), rabbit p38 kinase antibody (Cell Signaling), rabbit anti-phospho-p38 kinase antibody (Cell Signaling), rabbit anti-histone H4 (Upstate) or rabbit anti- β -actin (abcam) were used as primary antibodies. IRDye680 and IRDye800 fluorophore-labeled antibodies were then applied as secondary antibodies and membrane was scanned by Odyssey Infrared Imaging System under channel-700 or channel-800 (LI-COR Biosciences).

2.9. Electrophoretic mobility shift assay (EMSA)

Sense and anti-sense nucleotides of octamer sequence flanked by 10 nucleotides from U6 promoter (5'-GATTCCTTCATATTTGCATATACGATAC-3'; 5'-GTATCGTATATGCAAATATGAAGGAATC-3') were labeled at 5'-ends of both strands with IRDye-700 infrared (IR) dye (Integrated DNA Technologies). Five microgram of nuclear extracts were incubated with annealed labeled-octamer DNA (25 nM) for 40 minutes at room temperature in dark with reagents supplied from Odyssey Infrared EMSA kit (LI-COR Biosciences). For supershift, Oct-1 antibody (Santa Cruz Biotechnology) or normal mouse IgG (Santa Cruz Biotechnology) was added to DNA-nuclear extract mixture after 20 min and incubation continued for another 20 min. Specificity of the binding was assessed by the addition of unlabeled wild type or mutant octamer (5 μ M) DNA. Mixtures were then run in a nondenatured 6% TBE gel (Invitrogen) for 1 h at room temperature in dark. Binding signals were detected by Odyssey infrared imaging system (LI-COR Biosciences). Amount of probe bound by protein was quantitated using Odyssey application software. Relative increase in Oct-1 binding shown in Fig. 2B and 2C is calculated by the equation: [binding in inhibitor-treated extract — binding in untreated extract] / binding in untreated extract. A Paired, one-tailed method in *t* test was used for statistical comparison when $p < 0.05$ was considered significant.

2.10 Real-time PCR quantification of U6 RNA in Jurkat cells

Jurkat cells (1.5×10^6) were treated with SB202190 (20 μ M) or DMSO (untreated) for 18 h. Cells were harvest and total RNA was isolated by RNazol-RT (Molecular Research Center, Inc.) as per manufacture's instruction, followed by RNase-free DNase I treatment (Ambion). cDNA was synthesized by using Superscript II reverse transcriptase system (Invitrogen) and random primers. Relative levels of U6 cDNA were estimated by real-time PCR in a 7500 Fast Real-Time PCR System (Applied Biosystems) using U6 specific primers (forward: 5'-CTCGCTTCGGCAGCACA -3'; reverse: 5'- AACGCTTCACGAATTTGCGT -3') and RT² SYBR Green/ROX FAST Mastermix from Qiagen. Levels of human β -actin or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific RNAs were also quantitated in these samples by real-time PCR using reagents from Applied Biosystems. The results were analyzed by 7500 software (v. 2.0.5, Applied Biosystems) and fold change values for U6 RNA was calculated using $\Delta\Delta C_t$ (comparative cycle threshold) method.

3. Results

3.1. Stimulation of U6 promoter activity by SB202190 is dependent on the presence of octamer sequence

To establish the U6 promoter driven luciferase is transcribed by RNA Pol III, the effect of α -amanitin was studied. Results presented in Figure 1B shows the effect of different concentrations of α -amanitin on U6 and CMV promoter, a promoter transcribed by RNA

polymerase II. Differential sensitivity of U6 promoter and CMV promoter to α -amanitin suggests that the U6 promoter is transcribed by RNA Pol III (Kunkel et al., 1986).

Cells transfected with U6 or CMV promoter luciferase reporter vector were treated with increasing concentrations of p38 kinase inhibitor SB203580 and luciferase activity was determined. Results show that p38 kinase inhibitor activates U6 promoter in a concentration dependent manner and the CMV promoter activity is not affected by the inhibitor (Fig 1C). *cis*-acting element of the U6 promoter responsive to the p38 kinase inhibitor-stimulated up-regulation was explored using a series of deletion mutants (Fig. 1A). Deletion of sequences between -264 to -200 bp from the U6 promoter (pU6-200) led to significant loss of promoter function and deletion of additional sequence did not affect the promoter activity (Fig. 1D). In addition, SB202190 treatment did not alter the activity of the U6 promoter lacking the upstream sequences (Fig. 1D), indicating that promoter region between -264 to -200 bp that encompasses the octamer sequence contains the element responsive to SB202190 mediated up-regulation. Deletion of additional sequences from -200 bp of the U6 promoter did not significantly alter the promoter activity. pU6-50 lacking PSE showed significantly reduced but detectable activity which is in contrast to the results reported earlier (Carbon et al., 1987; Mattaj et al., 1988; Lobo and Hernandez, 1989). However, the pU6-50 promoter activity was not responsive to p38 kinase inhibitor.

Octamer sequence is located at -221 to -214 of U6 promoter and is a likely candidate associated with SB202190-stimulated activity. To investigate the hypothesis that the p38 kinase inhibitor stimulatory activity is octamer-dependent, U6 promoter with mutated octamer sequence was tested (OctM in Fig. 1E). This mutant showed no stimulatory response to SB202190 treatment (Fig. 1F). Next, octamer sequence inserted adjacent to position -200, -150, -100 and -50 bp of U6 promoter was tested for SB202190 induced activity (Fig. 1F). Oct-200 showed SB202190-stimulated activity, albeit to a lesser extent (Fig. 1F). No stimulatory effect was observed in Oct-150, Oct-100 and Oct-50, indicating the stretch of 100 bp sequence present between -200 to PSE and TATA box are also important for the SB202190-mediated stimulation of octamer-dependent activity (Fig. 1F) (Zhao et al., 2001). Taken together, these results strongly suggest that octamer sequence is responsible for the SB202190-stimulated activity.

3.2. SB202190-treated nuclear extract showed increased level of octamer binding activity

Binding of Oct-1 to octamer sequence has been well established and this binding is necessary for optimal transcription from various promoters (Jawdekar and Henry, 2008; Zhao et al., 2001). To establish the presence of Oct-1 in our cell lysate, IR-dye labeled U6 promoter octamer DNA was mixed with nuclear extract and analyzed by EMSA (Fig. 2A). A slow moving DNA band was seen (Fig. 2A, lane 2), intensity of this band was reduced significantly when 200x concentration of unlabeled octamer DNA was added (lane 3) and this binding was not affected when 200x concentration of mutant octamer DNA was added (lane 4). When an Oct-1 antibody was present in the assay, a supershift band was observed (lane 5) but not when control antibody was added (lane 6), demonstrating the presence of the Oct-1 and its specific binding to the octamer sequence.

We have shown that U6 promoter activity was stimulated by SB202190 and octamer sequence is critical for this up-regulation (Fig. 1). To test the hypothesis that p38 kinase inhibitor-treated cells have higher levels of Oct-1 DNA binding activity, EMSA for Oct-1 binding with limiting amounts of nuclear extract from untreated or SB202190-treated cells was performed. Results show that SB202190-treated nuclear extract had 40 to 50% more octamer sequence binding activity (Fig. 2B). The increased Oct-1 DNA binding activity to octamer probe as seen in Figure 2B was from cellular intrinsic Oct-1. We wanted to further investigate whether similar DNA binding profile also can be observed by over-expression of

exogenous Oct-1. We also used an additional p38 kinase inhibitor, SB203580, to treat the cells and found that similar augmentation of up to 25% of binding level when Oct-1 was over-expressed in p38 kinase inhibitor-treated 293FT cells (Fig. 2C). Together, increased level of Oct-1 DNA binding activity to octamer sequence correlated with the increased level of U6 promoter transcription (Fig. 1) in p38 kinase inhibitor-treated cells.

3.3. p38 kinase inhibitor enhanced transcription of U6 promoter in Oct-1 over-expressed cells

Next, we investigated whether the over-expressed Oct-1 up-regulates U6 promoter when treated with p38 kinase inhibitor. Jurkat cells were transfected with different concentrations of Oct-1-expressing vector and the level of Oct-1 was quantitated. As shown in Fig. 3B, the level of Oct-1 present in Oct-1 transfected cells were proportional to the Oct-1 transfected plasmid, and the treatment with p38 kinase inhibitor did not alter the Oct-1 levels significantly. Increased level of Oct-1 led to increased transcription from the U6 promoter and p38 kinase inhibitors further stimulated the U6 promoter activities (Fig. 3A). However the increase in transcriptional level was not proportional to the Oct-1 protein expression level, suggesting that other factors that control transcription might be limiting under these conditions.

3.4. p38 kinase inhibitor reversed the oxidative stress- or PMA-induced down-regulation of U6 promoter activity

Results in Fig. 1 and 3 suggest that the transcription from U6 promoter is negatively regulated by p38 kinase implying that the activation of p38 kinase activity would inhibit U6 promoter. Hydrogen peroxide has been shown to activate p38 kinase via stress-responsive mitogen-activated protein kinase pathway (Guyton et al., 1996; Wang et al., 1998; Tibbles and Woodgett, 1999). Results presented in figure 4A show that the U6 promoter activity was lower in cells treated with hydrogen peroxide and the hydrogen peroxide induced inhibition of the U6 promoter activity could be restored by pre-treating the cells with SB203580. Similar results were obtained when Oct-1-overexpressed cells were treated with hydrogen peroxide and p38 kinase inhibitor (Fig. 4A).

Also, the treatment of Jurkat cells with PMA, a known protein kinase C activator which can trigger downstream MAPKs activation (Mauro et al., 2002), resulted in inhibition of U6 promoter activity (Fig. 4B, lane 2 and 6) and this inhibition could be reversed by p38 kinase inhibitors (Fig. 4B, lane 3, 4 and 7, 8).

3.5. Over-expression of p38 α kinase down-regulated U6 promoter activity which can be subsequently restored by p38 kinase inhibitors

To further examine whether p38 kinase specifically regulates U6 promoter via Oct-1, a plasmid expressing p38 α kinase along with pCMV6-Oct-1-FLAG (pOct-1) was co-transfected into Jurkat cells. Over expression of p38 α kinase down-regulated U6 promoter (Fig. 5A, lane 3) and PMA-treated cells over-expressing p38 α kinase showed further down-regulation of U6 promoter activity (lane 4) and treatment with p38 kinase inhibitors increased the U6 promoter activity (lane 5). The expression of p38 α kinase was confirmed by Western blot using anti-DDK(FLAG) antibody (Fig. 5B) which can be differentiated from endogenous p38 kinase. PMA treatment stimulated the phosphorylation of both exogenous and endogenous p38 kinase (Fig. 5B, lane 4) and the phosphorylation was inhibited by SB203580 (Fig. 5B, lane 5). These results support the concept that the p38 kinase negatively regulates U6 promoter activity through Oct-1.

3.6. Endogenous U6 RNA was increased in SB202190-treated Jurkat cells

We further investigated whether p38 kinase regulates cellular U6 RNA level. It is known that multiple full-length U6 snRNA genes in the human genome contain the octamer sequence in their promoter regions (Domitrovich and Kunkel, 2003). By real-time quantitative PCR (qRT-PCR) assay, we found that the endogenous U6 RNA level was increased about 50% in SB202190-treated Jurkat cells (Fig. 6), suggesting that the expression of the U6 snRNA is negatively regulated by p38 kinase.

4. Discussion

Oct-1 is ubiquitously expressed and specifically binds to octamer sequence in mammalian cells. A consensus octamer sequence of 5'-ATTTGCAT-3' is recognized by Oct-1 POU DNA binding domains regardless of the orientation of the octamer sequence (Mittal et al., 1996; Zhao et al., 2001). The class III snRNA genes transcribed by RNA Pol III contain three major sequence elements in their promoter. snRNA U6 promoter contains an octamer sequence at DSE around -220 where Oct-1 binds and recruits SNAPc binding to PSE located around -60. The efficient transcriptional activation is achieved by a cooperative binding of Oct-1 and SNAPc with a positioned nucleosome occupied about 150 bp between DSE and PSE (Zhao et al., 2001; Stunkel et al., 1997; Boyd et al., 2000), subsequently recruiting TATA-box binding protein to a TATA box at around -30 on the promoter region. In this study, we have observed that deleting the octamer sequence dramatically reduced U6 promoter activity. Our results also suggested that location of octamer sequence is critical for U6 promoter activity. By reducing the distance between DSE and PSE to 150 bp or less severely reduced the promoter activity. This results confirms that the distance between PSE and DSE is important for an appropriate cooperative binding between Oct-1 and SNAPc or for a possible nucleosome binding which may contribute to appropriate positioning of transcriptional activation through Oct-1, SNAPc and RNA Pol III interaction (Stunkel et al., 1997; Zhao et al., 2001).

We have shown that the cellular U6 RNA level is augmented by about 50% in the presence of p38 kinase inhibitor. Also, in the presence of SB202190 or SB203580, U6 promoter activity was stimulated up to 1.8-fold in transiently transfected cells. This stimulated activity was octamer-dependent as deletion or mutation of octamer sequence totally abolished the stimulated activity, strongly suggesting the transcription factor Oct-1 is involved in this stimulation. A Staf/ZNF143-binding site is found adjacent to DSE in U6 promoter region (Myslinski et al., 2006). However, Oct-1 binding site mutant (Oct-M) that has the intact Staf/ZNF143 site is not stimulated by p38 kinase inhibitor, suggesting that the Staf/ZNF143 site is not involved in this up-regulation (Fig. 1F). This conclusion was further supported by the observation that the p38 kinase inhibitor-treated cells showed specific and increased level of Oct-1 binding activity and the U6 promoter activity was further stimulated when Oct-1 was over-expressed in the presence of p38 kinase inhibitors. The increased level of Oct-1 binding ability to octamer probe was not proportional to the increase in promoter activity indicating additional factors are also involved in this regulation (Jawdekar and Henry, 2008).

Pyridinyl imidazole compounds, including SB202190 and SB203580, are specific ATP-competitive inhibitors to p38 kinase (Lee et al., 1994; Lee et al., 2000; Ashwell, 2006). With p38 kinase signaling blocked by these cell permeable inhibitors, the downstream substrates, such as MAPK activated-protein kinases (MAPKAPKs) and transcription factors, are not activated by p38 kinase (Ono and Han, 2000; Kyriakis and Avruch, 2001). Even though we cannot rule out "off-target" effect of these compounds on other kinases, Davies et al. (2000) have shown that these compounds are highly specific inhibitor of p38 kinase and have very little effect on other kinases tested.

One common characteristic of p38 kinase and Oct-1 is both are cellular stress sensors (Wang et al., 1998; Torres, 2003; Tantin et al., 2005; Zhao et al., 2000; Meighan-Mantha et al., 1999; Bulavin et al., 2001; Pedraza-Alva et al., 2006). Upon the stress stimuli, p38 kinase is activated and elicits a wide range of signaling for cell survival. On the other hand, Oct-1-mediated histone H2B and U2 RNA gene expression are reduced with ionizing radiation treatment (Meighan-Mantha et al., 1999; Schild-Poulter et al., 2003). Oct-1 is phosphorylated under genotoxic or oxidative stress and shows reduced binding to histone H2B promoter under ionizing radiation exposure (Kang et al., 2009). In addition, we found that U6 promoter activity was down-regulated in the presence of hydrogen peroxide. These findings indicate that Oct-1-mediated transcriptional activity is decreased when p38 kinase is activated under stress conditions. We further showed that over-expressed p38 α kinase down-regulated U6 promoter activity in untreated or PMA-treated cells and such inhibition was restored by p38 kinase inhibitors. Although ERK and JNK signaling pathways are also activated under stress signals (Torres, 2003), we found U6 promoter activity unchanged when Jurkat cells were treated with ERK or JNK kinase inhibitor, U0126 or L-JNK-I peptide inhibitor, respectively (unpublished results).

The transcriptional activity of transcription factors can be negatively regulated by protein kinases (Hunter and Karin, 1992). For instance, signal transducers and activators of transcription (STAT)-dependent transcription is negatively regulated by ERK and phosphoinositide 3-kinase (PI3K). It has been demonstrated that the interferon- γ activated site (GAS)-containing luciferase reporter vector is up-regulated (GAS is the sequence element for STATs activation) by ERK or PI3K inhibitor in human melanoma cells (Krasilnikov et al., 2003). In addition, a synthetic vitamin K analog induces ERK activation that represses c-Myc DNA binding activity as well as c-Myc transcriptional activity, and this inhibition is reversed by ERK inhibitor (Wang et al., 2006). Phosphorylation of c-Myc by p21 protein (Cdc42/Rac)-activated kinase 2 also shows similar negative regulation on c-Myc transcriptional activity (Huang et al., 2004).

Our findings strongly indicate that Oct-1 is negatively regulated by p38 kinase and the transcription from U6 promoter is negatively regulated by p38 kinase. Increased level of U6 RNA in p38 kinase inhibitor-treated cells further supports this conclusion. Whether Oct-1 is a substrate of p38 kinase is not known but it is a possibility. MAPK signaling to protein substrates have specific determinants known as MAPK-docking sites (D domain) for MAPK recognition (Sharrocks et al., 2000; Tanoue and Nishida, 2003; Bardwell, 2006). These sequence motifs on substrates including transcription factors determine the specificity and efficiency of MAPK activation. For instance, activating transcription factor 2 and myocyte enhancer factor-2A are p38 kinase substrates and contain the docking domains for p38 kinase phosphorylation (Bardwell, 2006; Barsyte-Lovejoy et al., 2002). A MAPK-docking site is specified with a consensus sequence [K/R]₂₋₃-X₁₋₆-[L/I/ ϕ]-X-[L/I/ ϕ] (where ϕ is a hydrophobic residue) (Tanoue and Nishida, 2003; Bardwell, 2006). Basic residues followed by a LXL triplet and a hydrophobic region are critical for ERK phosphorylation whereas the [L/I/ ϕ]-X-[L/I/ ϕ] motif is not always required for p38 kinase targeting (Barsyte-Lovejoy et al., 2002). Based on this signature, we have found two putative p38 kinase docking sites in Oct-1 (GenBank ID: AAX43959). The first site is located in POU-specific domain with the peptide sequence of H₂N-K²⁹³TFKQRRIKLGFTQGDVGLA³¹²-COOH, and contains the positive residues lysine and arginine followed by an IKL triplet and a series of hydrophobic residues. The second site is located in POU-homeo domain with the sequence of H₂N-L³⁷⁶SRRRKRTSIETNIRVALE³⁹⁵-COOH which composes of a cluster of positively charged arginine and lysine residues and a stretch of hydrophobic amino acids. Notably, Ser³³⁵ and Ser³⁸⁵ are located at upstream or downstream of these putative docking sites and are accessible for phosphorylation to regulate Oct-1 DNA binding activity (Kang et al., 2009).

It has been shown that different kinases are able to phosphorylate Oct-1. Segil et al. have shown that protein kinase A (PKA) phosphorylates serine³⁸⁵ of Oct-1 *in vitro* and prevents Oct-1 binding to the octamer sequence (Roberts et al., 1991; Segil et al., 1991). DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) phosphorylates Oct-1 on multiple serine/threonine residues scattered throughout the N-terminal glutamine-rich domain to promote cell survival and Oct-1 stabilization (Schild-Poulter et al., 2003; Schild-Poulter et al., 2007). Ku DNA-binding complex within DNA-PK is able to enhance Oct-1 phosphorylation (Schild-Poulter et al., 2007; Schild-Poulter et al., 2001). Also, Oct-1 is found to be post-translationally modified in herpes simplex virus 1 infected cells and shows a decreased binding to octamer sequence (Advani et al., 2003). PKA, protein kinase C and casein kinase II are suggested to phosphorylate Oct-1 or Oct-2 and regulate the DNA binding ability (Pevzner et al., 2000; Grenfell et al., 1996). All these data show that Oct-1 is prone to modification by different kinases and this may alter the binding of Oct-1 to octamer sequence.

The moderate up-regulation of U6 snRNA and promoter activity by p38 kinase inhibitors observed in this study reflects the fundamentally crucial role of U6 snRNA in cellular metabolism and that could be regulated by p38 kinase, but may also implicitly suggest the involvement of additional factor in concert with Oct-1 (Domitrovich and Kunkel, 2003; Jawdekar and Henry, 2008). Multiple lines of evidence show that Oct-1 is dispensable for cell viability and proliferation (Sebastiano et al., 2010; Wang et al., 2004; Shakya et al., 2009). However, Tantin et al. (2005) have proposed that Oct-1 could modulate the expression of genes in response to cellular stress because cells lacking Oct-1 were hypersensitive to stress signals. Thus, with the results presented here, we conclude that p38 kinase negatively regulates Oct-1 DNA binding and transcriptional activity on U6 promoter and propose that this regulation is achieved potentially through phosphorylation and subsequently modulates cellular stress response and cell survival (Tantin et al., 2005).

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Abbreviations

PMA	phorbol 12-myristate 13-acetate
snRNA	small nuclear RNA
RNA Pol III	RNA polymerase III
PSE	proximal sequence element
DSE	distal sequence element
SNAPc	snRNA activating-protein complex
MAPK	mitogen-activated protein kinase
ERKs	extracellular signal-regulated kinases
JNKs	c-Jun amino-terminal kinases

RPML-1640	Roswell Park Memorial Institute medium 1640
HEPES	4-[2-hydroxyethyl]piperazine-1-ethanesulfonic acid
DMEM	Dulbecco's modified eagle medium
G-418	Geneticin
bp	base pair
PCR	polymerase chain reaction
cDNA	DNA complementary to RNA
DMSO	dimethylsulfoxide
PBS	phosphate-buffered saline
EDTA	ethylenediaminetetraacetic acid
PAGE	polyacrylamide gel electrophoresis
Bis-Tris	bis[2-hydroxyethyl]-imino-tris[hydroxymethyl]-methane
MES	2-[N-morpholino]-ethanesulfonic acid
SDS	sodium dodecyl sulfate
EMSA	electrophoretic mobility shift assay
IR	infrared
IgG	immunoglobulin G
TBE	Tris-Borate-EDTA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
$\Delta\Delta C_t$	comparative cycle threshold
qRT-PCR	real-time quantitative PCR
MAPKAPKs	MAPK activated-protein kinases
STAT	signal transducers and activators of transcription
PI3K	phosphoinositide 3-kinase
GAS	interferon- γ activated site
PKA	protein kinase A
DNA-PK_{cs}	DNA-dependent protein kinase catalytic subunit

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Research Highlights

- p38 kinase inhibitors, SB202190 and SB203580, enhanced transcription of U6 promoter
- Stimulation of U6 promoter activity by SB202190 is dependent on octamer sequence
- SB202190-treated nuclear extract showed increased level of octamer binding activity
- p38 kinase inhibitor restored down-regulation of U6 promoter by stress, PMA or p38 α
- Cellular U6 RNA was augmented in SB202190-treated Jurkat cells

Fig. 1.

SB202190 stimulated DSE-dependent U6 promoter activity. (A) Schematic illustration of the full length (pU6-264) and deletion mutants (pU6-200, -150, -100, -50) of U6 promoter (B) Validation of U6 promoter transcription by RNA Pol III. pU6-264 or pCMV-luc was transfected into 293FT cells and after 5 h treated with different concentrations of α -amanitin. Cells were harvested after 48 h for luciferase assay. (C) Jurkat cells were transfected with 2.5 μ g pU6-264 or pCMV-luc. The next day, cells were treated with different concentrations of SB203580 and then harvested after 18 h. (D) Jurkat cells were transfected with indicated plasmids. After 24 h, cells were treated with SB202190 or DMSO (untreated). Luciferase activity was assayed after 18 h. (E) Schematic illustration of octamer mutants of U6 promoter. Octamer sequence was mutated from ATTTGCAT to ACGTGCAG in OctM and octamer sequence was inserted at -200, -150, -100 and -50 bp position to obtain Oct-200, Oct-150, Oct-100 and Oct-50 respectively. Octamer sequence is in the reverse orientation (5'-ATGCAAAT-3') in Oct-150, Oct-100 and Oct-50. (F) Relative luciferase activities from transfection of U6 promoter (pU6-264) and indicated mutants in Jurkat cells.

A

EMSA components	1	2	3	4	5	6
unlabeled octamer (200X)	-	-	+	-	-	-
unlabeled mt. octamer (200X)	-	-	-	+	-	-
IRDye-700 labeled octamer	+	+	+	+	+	+
Nuclear Extract (untreated)	-	+	+	+	+	+
Oct-1 antibody	-	-	-	-	+	-
Normal mouse IgG antibody	-	-	-	-	-	+

Fig. 2. p38 kinase inhibitor-treated nuclear extract showed higher binding activity to octamer sequence. (A) Oct-1 specifically bound to octamer sequence. Untreated nuclear extract (5 µg) was incubated with IRDye-labeled octamer sequence-containing DNA probe (lanes 1~6). Black arrow: Oct-1 supershift. ▲: non-specific binding. (B) Upper panel: Oct-1 associated with octamer was determined in EMSA in presence of excess probe and limiting amounts (0.25 µg or 0.5 µg) of nuclear extract. Untreated or SB202190-treated nuclear extracts were used in EMSA in seven replicates. Lower left panel shows the Oct-1 binding levels in inhibitor treated cells and the lower right panel shows relative increase in Oct-1 binding activity calculated as described in Materials and Methods. *: $p < 0.05$ (C) pCMV6-Oct-1-FLAG (5 µg) was transfected to 293FT cells to over-express Oct-1. Untreated or SB203580-, SB202190-treated nuclear extract (5 ng) was mixed with labeled octamer probe in EMSA (upper panel) and the relative increase in Oct-1 binding activity was calculated (lower panel).

Fig. 3.

Intrinsic Oct-1 or exogenous Oct-1 stimulated U6 promoter activity in the presence of p38 kinase inhibitors. (A) pU6-264 plasmid (2.5 µg) was co-transfected with different amount (1, 2.5, 5 µg) of pCMV6-Oct-1-FLAG vector to Jurkat cells. Same vector without *oct-1* gene was used to make up the total DNA to 7.5 µg in each of the transfections. (B) Indicated nuclear extracts (10 µg) were applied in Western blot. Oct-1 expression was detected by anti-DDK(FLAG) antibody and histone H4 was used as loading control.

Fig. 4.

Oxidative stress- or PMA-induced down-regulation of U6 promoter activity can be reversed by p38 kinase inhibitors. (A) Jurkat cells were transfected with pCMV6-Oct-1-FLAG (0 or 2.5 μ g). The next day, cells were treated with hydrogen peroxide (25 μ M) or SB203580 2 h prior to hydrogen peroxide treatment. Cells were harvest after 18 h. (B) Jurkat cells were transfected with or without FLAG-tagged Oct-1-expressing vector (pOct-1, 2.5 μ g). The next day, cells were treated with or without p38 kinase inhibitors 2 h prior to PMA treatment. Cells were harvested after 18 h. Same vector without *oct-1* gene was used to make up the total DNA to 5 μ g in each of the transfections All transfection includes pU6-264 (2.5 μ g) to a total DNA of 5 μ g. SB80: SB203580, SB90: SB202190, *: $p < 0.05$.

Fig. 5.

U6 promoter activity was down-regulated by over-expressed p38α kinase and can be restored by p38 kinase inhibitor. (A) Jurkat cells were co-transfected with pU6-264 and pOct-1 with p38 kinase expressing vector (1 μg). The next day, cells were treated with SB203580 2 h prior to the addition of PMA. (B) Whole-cell lysate (25 μg) were applied in Western blot and Oct-1 was detected by anti-DDK(FLAG) antibody and p38α kinase were detected by anti-DDK or anti-p38 kinase antibody. Phospho-p38 kinase was detected by anti-phospho-p38 kinase. β-actin was used as loading control. SB80: SB203580. SB90: SB202190. endo-p38: endogenous p38 kinase. Antibody used in each Western blot is annotated in parenthesis.

Fig. 6.

Endogenous U6 RNA was augmented in p38 kinase inhibitor-treated Jurkat cells. qRT-PCR was performed for U6 RNA level and for β -actin mRNA as internal control. Duplicate qRT-PCR reactions were performed for each cDNA sample. Fold change values were calculated using $\Delta\Delta C_t$ (comparative cycle threshold) values from three independent experiments. Similar results were obtained when GAPDH was used as internal control.