〈Original Article〉

Identification of transcriptional regulatory elements of the poly(ADP-ribose) polymerase-1 gene in neural stem/progenitor cells

Suguru Kurokawa[1], Akiko Okuda[2], Yoko Kondo[1],
Seigo Tanaka[1] and Masanori Takehashi[1*]

Summary Poly(ADP-ribose) polymerase 1 (PARP1) has plays multiple roles in the cellular responses to DNA damage and the regulation of several nuclear events. Mouse neural stem/progenitor cells (NSPCs) express higher levels of PARP1 than mouse embryonic fibroblasts (MEFs). Our previous study proposed that the abundant PARP1 contributes to the proliferation and self-renewal of NSPCs through the suppression of p53 activation. However, the molecular mechanisms involved in the regulation of *PARP1* expression in NSPCs remain to be elucidated. In the present study, to identify the transcription factor involved in *PARP1* transcription in mouse NSCPs, we performed a luciferase reporter assay and found two transcriptional regulatory elements upstream of the mammalian conserved promoter region. Database analysis revealed that the elements overlap with putative SP1 and Zics binding sites. These findings suggest a possibility that these transcription factors are associated with a transcriptional regulation of mouse *PARP1* gene in NSPCs.

Key words: PARP1, Neural stem/progenitor cells, Transcriptional regulatory elements, Reporter assay

[1]Laboratory of Pathophysiology and Pharmacotherapeutics, Faculty of Pharmacy, Osaka Ohtani University, 3-11-1 Nishikiori-kita, Tondabayashi, Osaka 584-8540 Japan
[2]Niigata University Graduate School of Health Sciences, 2-746 Asahimachidori, Chuo-ku, Niigata, 951-8518 Japan

*Corresponding author: Masanori Takehashi, Laboratory of Pathophysiology and Pharmacotherapeutics, Faculty of Pharmacy, Osaka Ohtani University, 3-11-1 Nishikiori-kita, Tondabayashi, Osaka 584-8540 Japan
Tel/Fax: +81-721-24-9427
E-mail: takeham@osaka-ohtani.ac.jp

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1. Introduction

Poly(ADP-ribose) polymerase 1 (PARP1) catalyzes the poly(ADP-ribosyl)ation of proteins by using NAD^+ as a substrate and transfers[1]. PARP1 plays multiple roles in the cellular response to DNA damage and is involved in the regulation of DNA repair, replication, transcription, and chromatin modification[2]. Neuronal cells injured by ischemia and reperfusion are committed to die to a certain extent via necrosis following the excessive activation of PARP1; the depletion of NAD^+ by PARP1 after severe DNA damage results in ATP consumption in an attempt to replenish NAD^+, leading to an energy crisis and necrotic cell death. Previously, we reported that one of the principal roles of PARP1 is in the induction of mitochondrial impairment that ultimately leads to neuronal apoptosis after cerebral ischemia[3], indicating that PARP inhibitors could be applied to therapeutic intervention for cerebral infarction. We also reported that neural stem/progenitor cells (NSPCs) in the mouse brain express more PARP1 protein than mouse embryonic fibroblasts (MEFs), and abundant levels of poly(ADP-ribosyl)ated proteins are found in a steady state in NSPCs[4]. PARP inhibitors induce apoptosis and suppress cell cycle progression at the G1/S and/or G2/M phase in NSPCs. Poly(ADP-ribosyl)ation contributes to the proliferation and self-renewal of NSPCs through the suppression of p53 activation[4]. More recently, we reported that treatment with the PARP inhibitor inhibits cyclin B expression at the mRNA level and suppresses G2/M to G1 progression in NSPCs[5].

Limited research has been conducted on the regulation of *PARP1* transcription. *PARP1* gene promoters have been identified and cloned in fruit fly[6], human[7], rat[8], and mouse[9]. Although the Drosophila promoter contains a functional TATA box, the three mammalian promoters lack a consensus TATA box core element around the -30 to -50 region from the transcriptional start site (TSS)[10]. For rodents, promoter activity depends on several Sp1 binding sites[11] and on the NF1 family of transcription factors[12]. Promoter-binding sites for transcription factors Sp1, AP-2[13], YY1[14], ETS1[15], and E2F4-RBL2-HDAC1-BRM complex[16] could be localized for the human *PARP1* gene.

Similar to NSPCs, mouse pluripotent stem cells, such as embryonic stem (ES) cells and induced pluripotent stem cells, express higher levels of PARP1[17]. PARP1 is less abundant in differentiated monocytes than in proliferating hematopoietic stem/progenitor cells[16]. However, the molecular mechanisms that regulate *PARP1* expression in stem cells remain to be elucidated.

In the present study, to identify the candidate transcription factor associated with *PARP1* transcription in mouse NSCPs, we identified transcriptional regulatory elements in the promoter by using a luciferase reporter assay.

2. Materials and Methods

Separation and passage of NSPCs

NSPCs were obtained from Slc:ICR mouse embryos (embryonic day 13.5) and cultured as previously described[4]. All experimental protocols conformed to the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and all experiments were approved by the Animal Experiment Committee of Osaka Ohtani University (No. 1012).

Plasmid constructions

Mouse genomic DNA extraction and purification were performed by QIAamp DNA Mini kit (Qiagen, Valencia, California) according to the manufacturer's protocol. Mouse *PARP1* (-4537/+60) was amplified by PCR and cloned into pGL4.24 vector (Promega, Madison, Wisconsin) using XhoI and NcoI sites (forward 5'-GAGACTCGAGAGGCATGGAGGACACCTT -3', reverse 5'- CCGCCATGGTCCTTCTCGTGCTGC -3'), resulting in pGL4.24-PARP1. TSS based on NM_007415.2 (NCBI Reference Sequence) was

designated as position +1 in this study. Position +59 is just upstream of start codon of *PARP1* mRNA. Plasmids were extracted and purified using the Fast Gene Xpress plasmid PLUS kit (NIPPON Genetics, Tokyo, Japan). Site-directed DNA mutagenesis was performed to generate a series of 5'-truncation mutants, deletion mutants, or base substitution mutants (Table 1). The pGL4.24-PARP1 plasmid DNA was amplified by inverse PCR using Phusion-polymerase (New England Biolabs, Ipswich, Massachusetts), and the amplified product was 5'-phosphorylated by T4 polynucleotide kinase (Toyobo, Osaka, Japan) and then self-ligated. The correctly mutated sequence was confirmed by DNA sequencing analysis.

Luciferase reporter assay

pRL-SV40 and each cloned pGL4.24 plasmid were co-transfected in NSPCs using the NEON transfection system (Thermo Fisher Scientific, Waltham, Massachusetts). Briefly, 1×10^5 cells were resuspended in 10 μL of R buffer containing 3 μg of each cloned pGL4.24 and 60 ng of pRL-SV40. Electroporation was carried out at 1400 V with a 10 ms pulse width for 3 pulses. Transfected cells were seeded on 24-well plates at 1.0×10^5 cells/well. After 24-h of incubation, firefly and *Renilla* luciferase activity was determined with using a Dual Luciferase Assay Kit (Promega), and luminescence was measured using a Lumat LB 9507 (Berthold technologies, Bad Wildbad, Germany). Firefly luciferase reporter activity from each cloned pGL4.24 was normalized to *Renilla* luciferase activity from pRL-SV40. pGL4.24 without the minimal promoter was generated by site-directed mutagenesis and used as the control plasmid (designated pGL4 in this study).

Statistical analysis

The data are expressed as mean ± standard

Table 1 Primer sequences for plasmid construction

plasmid name	Forward primer	Reverse primer
-1941/+59	5′-AGAGCTCGAGACAATGACATGGCGAAAG-3′	5′-GCTAGCGAGCTCAGGTACCGGCCAGTTAG-3′
-1718/+59	5′-TTCCAAGTAAATGCAACGGTTCCTAAGTTG-3′	5′-GCTAGCGAGCTCAGGTACCGGCCAGTTAG-3′
-191/+59	5′-CAGAGGCAGAGACCAGCGTGCACAG-3′	5′-GCTAGCGAGCTCAGGTACCGGCCAGTTAG-3′
-174/+59	5′-GTGCACAGGCGCAGTCGCGCTGGGAC-3′	5′-GCTAGCGAGCTCAGGTACCGGCCAGTTAG-3′
-162/+59	5′-AGTCGCGCTGGGACCCAACCTCCCAAGGACA-3′	5′-GCTAGCGAGCTCAGGTACCGGCCAGTTAG-3′
-124/+59	5′-AAGCCCCGCCCTATGAGCTGAGGC-3′	5′-GCTAGCGAGCTCAGGTACCGGCCAGTTAG-3′
-51/+59	5′-AGTAATCTATCCTGAGCGGAGGCGGC-3′	5′-GCTAGCGAGCTCAGGTACCGGCCAGTTAG-3′
-1/+59	5′-ACACGTTAGCGGAGCGCACCAGC-3′	5′-GCTAGCGAGCTCAGGTACCGGCCAGTTAG-3′
Δ-218/-174	5′-GTGCACAGGCGCAGTCGCGCTGGGAC-3′	5′-GAATTCAATCTGACAGGCGCCACCCCTTTTGTCGG-3′
Δ-218/-162	5′-AGTCGCGCTGGGACCCAACCTCCCAAGGACA-3′	5′-GAATTCAATCTGACAGGCGCCACCCCTTTTGTCGG-3′
Δ-218/-149	5′-CCCAACCTCCCAAGGACACGTGCTG-3′	5′-GAATTCAATCTGACAGGCGCCACCCCTTTTGTCGG-3′
Δ-218/-116	5′-CCCTATGAGCTGAGGCCCCGCCC-3′	5′-GAATTCAATCTGACAGGCGCCACCCCTTTTGTCGG-3′
Δ-218/-93	5′-TCCGCGCGCGAGCTTGGG-3′	5′-GAATTCAATCTGACAGGCGCCACCCCTTTTGTCGG-3′
Δ-218/-51	5′-AGTAATCTATCCTGAGCGGAGGCGGC-3′	5′-GAATTCAATCTGACAGGCGCCACCCCTTTTGTCGG-3′
Δ-218/-1	5′-ACACGTTAGCGGAGCGCACCAGC-3′	5′-GAATTCAATCTGACAGGCGCCACCCCTTTTGTCGG-3′
Δ-218/+42	5′-GCAGCACGAGAAGGAGGATGGCGGAGGCC-3′	5′-GAATTCAATCTGACAGGCGCCACCCCTTTTGTCGG-3′
Δ-149/-136	5′-GACACGTGCTGAAGCCCCGCCCTATG-3′	5′-AGGAATTCTCCCAGCGCGACTGCGCCTGTG-3′
Δ-143/-125	5′-AAGCCCCGCCCTATGAGCTGAGGC-3′	5′-CACTTTGTGTTGGGTCCCAGCGCGACTGC-3′
Δ-135/-117	5′-CCCTATGAGCTGAGGCCCCGCCC-3′	5′-CCTGAACAAGGCTTGGGAGGTTGGGTCC-3′
Δ-124/-109	5′-GCTGAGGCCCCGCCCTCCGC-3′	5′-GTTTAAACCAGCACGTGTCCTTGGGAGG-3′
Δ-115/-94	5′-TCCGCGCGCGAGCTTGGG-3′	5′-ATTTAAATGCGGGGCTTCAGCACGTGTCC-3′
-143/-137mut	5′-GACACGTGCTGAAGCCCCGCCCTATG-3′	5′-CACTTTGTGTTGGGTCCCAGCGCGACTGC-3′
-135/-126mut	5′-GACACGTGCTGAAGCCCCGCCCTATG-3′	5′-CCTGAACAAGGCTTGGGAGGTTGGGTCC-3′
-124/-117mut	5′-CCCTATGAGCTGAGGCCCCGCCC-3′	5′-GTTTAAACCAGCACGTGTCCTTGGGAGG-3′
-118/-109mut	5′-GCTGAGGCCCCGCCCTCCGC-3′	5′-ATTTAAATGCGGGGCTTCAGCACGTGTCC-3′
-108/-101mut	5′-CCCGCCCTCCGCGCGCGAGC-3′	5′-CCTGCAGGTCATAGGGCGGGGCTTCAGC-3′
-100/-94mut	5′-TCCGCGCGCGAGCTTGGG-3′	5′-ATTTAAATGCCTCAGCTCATAGGGCGGG-3′
-149/-145mut	5′-CCCAAGGACACGTGCTGAAGCC-3′	5′-AGGAATTCTCCCAGCGCGACTGCGCCTGTG-3′

error of the mean (SEM). Student's *t*-test was used to assess differences between two groups. Results were considered statistically significant at $p < 0.05$.

3. Results and Discussion

In our previous study, the PARP1 protein was found to be more abundant and to demonstrate higher enzyme activity in mouse NSPCs than in MEFs[4]. NSPCs were also confirmed to express much higher levels of *PARP1* mRNA than MEFs by RT-PCR (data not shown).

In mouse NSPCs, the ability of the 5'-upstream region of the *PARP1* gene to function as a promoter was assessed by luciferase reporter assay. A series of constructs with a truncated the 5' end was fused to a promoterless firefly luciferase gene of the pGL4.24 vector without the minimal promoter. The 5' ends began at positions -1941, -1718, -191, -174, -162, -124, -51, and -1 (Fig. 1A). Even if the positions from -1941 to -162 were truncated, there was no significant difference between the luciferase reporter activity indicated by the -1941/+59 plasmid. However, truncation of the sequence from -162 to -124 significantly decreased reporter activity (-162/+59 vs. -124/+59 in Fig. 1A). Furthermore,

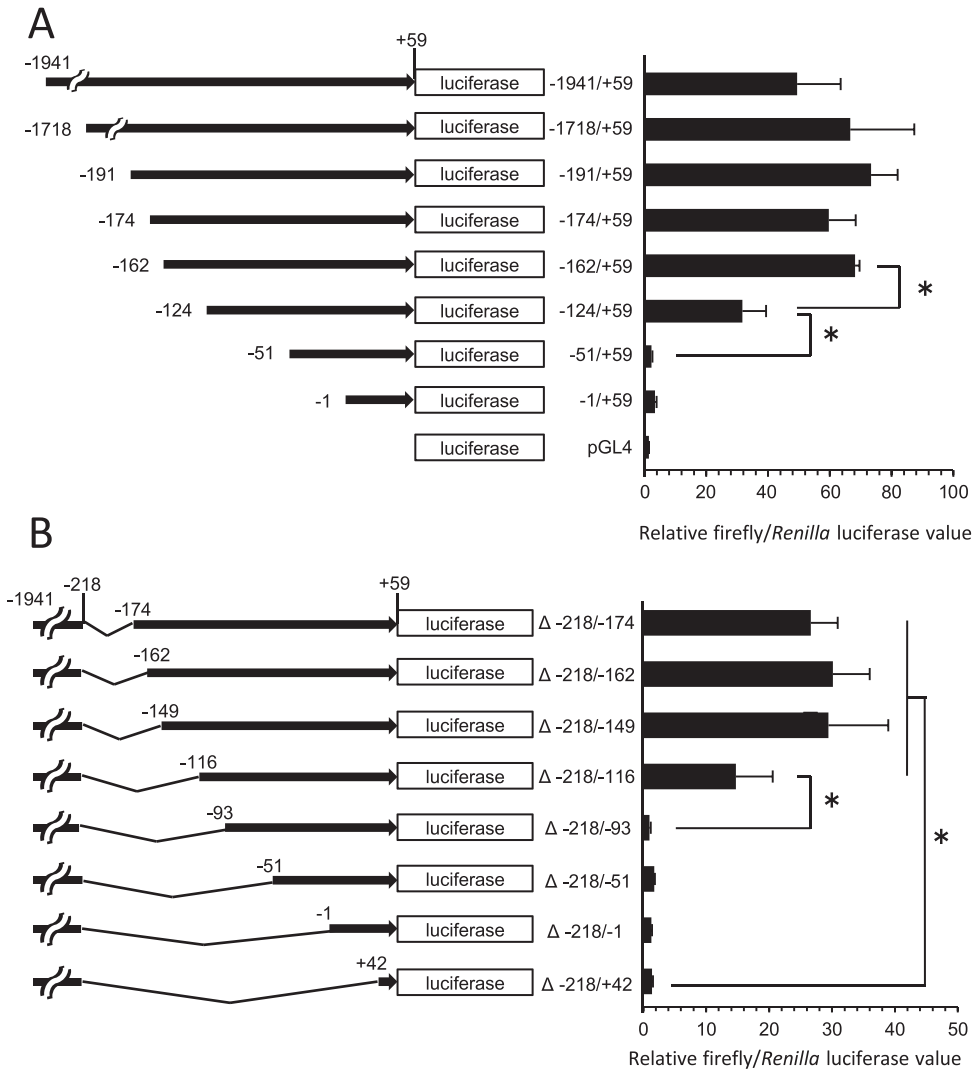

Figure 1 Transient transfection analysis of mouse *PARP1* promoter. A schematic representation of each of the constructs used in this study is provided on the left side of the figure. Firefly/*Renilla* luciferase activity ratio was measured for 24 h after transfection in NSPCs. Data represent the mean ± SEM (n = 3). *$p < 0.05$ by comparison between two plasmids using Student's *t*-test.

truncation of the sequence from -124 to -51 conspicuously decreased the reporter activity to the level of the control plasmid (-124/+59 vs. -51/+59 in Fig. 1A). To confirm these results, a series of deletion mutants were generated starting from position -218 of -1941/+59 plasmid. The 3' ends of deletion were at positions -174, -162, -149, -116, -93, -51, -1, and +42 (Fig. 1B). The luciferase reporter activity did not significantly differ between Δ-218/-174, Δ-218/-162, and Δ-218/-149 (Fig. 1B). However, deletion of the sequence from -149 to -116 showed a tendency to decrease the reporter activity (Δ-218/-149 vs. Δ-218/-116 in Fig. 1B). Furthermore, deletion of the sequence from -116 to -93 conspicuously decreased the reporter activity (Δ-218/-116 vs. Δ-218/-93 in Fig. 1B). Taken together, these results suggest the presence of critical elements for promoting *PARP1* transcription in the sequence from -149 to -93.

To analyze this region (from -149 to -93) in detail, we generated a series of deletion mutants that partially overlapped (Fig. 2A). The luciferase reporter activity of these deletion mutants was relatively evaluated. Our results suggest that the sequence from -115 to -94 is essential to promote

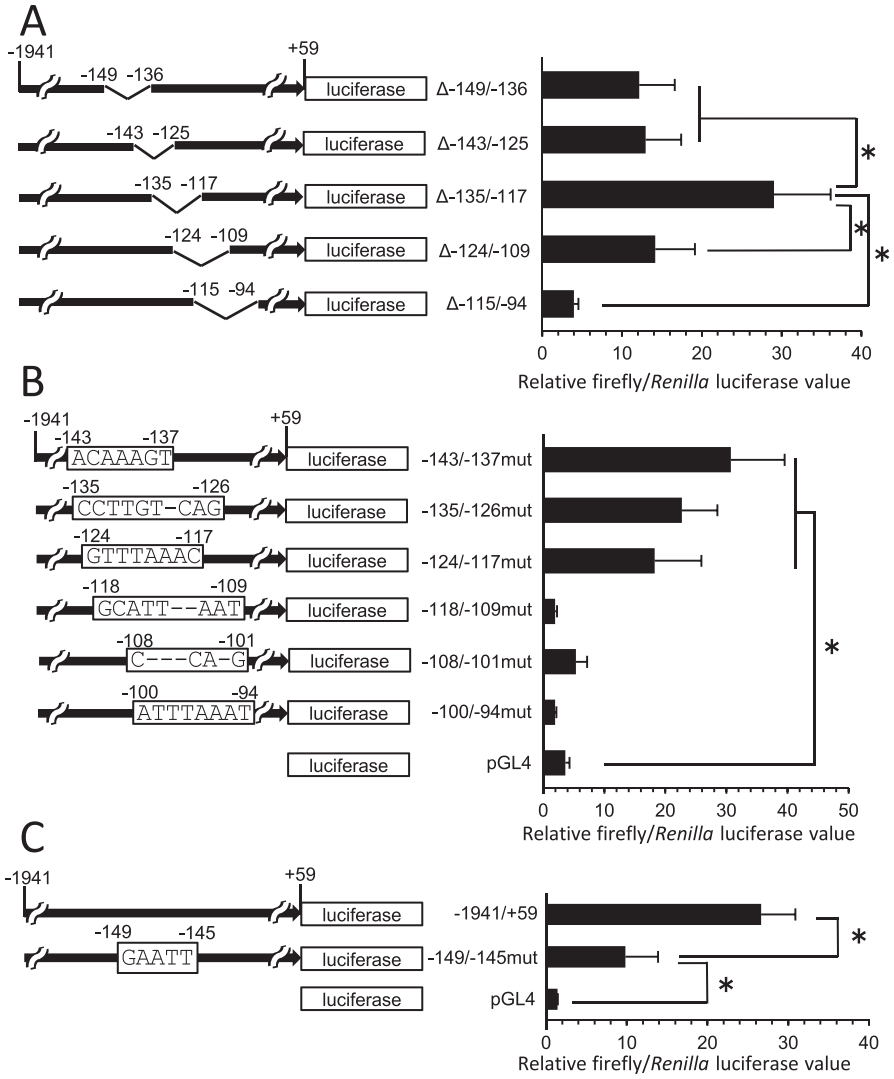

Figure 2 Transient transfection analysis that focused on the -149/-93 region. A schematic representation of each of the constructs used in this study is provided on the left side of the figure. Firefly/*Renilla* luciferase activity ratio were measured for 24 h after transfection in NSPCs. Data represent the mean ± SEM (n = 3). *$p < 0.05$ by comparison between two plasmids using Student's *t*-test. Only base substitution was described in a box (B, C).

reporter activity. The sequence from -149 to -136 is thought to enhance the reporter activity (Fig. 2A). To define the respective contribution of these elements to mouse *PARP1* promoter activity, we introduced a series of base substitution mutations in each element (Fig. 2B and 2C). The reporter activity with three mutations (-118/-109mut, -108/-101mut, and -100/-94mut) in element -115/-94 was similar to that of the control plasmid (Fig. 2B), confirming assay of deletion mutants (Δ-115/-94 in Fig. 2A). In the other element -149/-136, mutation of element -143/-137 did not show an apparent effect on promoter activity (Fig. 2B), while mutation of element -149/-145 significantly decreased promoter activity as compared to the wildtype sequence (Fig. 2C). These results suggest that element -149/-145 among the -149/-136 sequence is necessary to enhance mouse *PARP1* promoter activity.

In addition to our results, Fig. 3 shows the DNA sequence alignment of mouse *PARP1* proximal promoter region. The region surrounding the TSS, from -78 to +4 in Fig. 3, is almost completely conserved in all three mammalian species[10]. In addition to or instead of the TATA box, many promoters contain a consensus sequence called an initiator (Inr) element that overlaps the TSS[18]. The mouse *PARP1* promoter also contains the Inr element, which frequently overlaps TSSs from a database search of the DBTSS (https://dbtss.hgc.jp).

In this study, we identified two elements upstream of the conserved region that influence mouse *PARP1* transcription. We searched known transcription regulatory elements in the DNA sequence of Fig. 3 by using PROMO[19,20] and found several putative transcription factors. Among these several candidates, the element -115/-94 overlaps with two putative SP1 binding sites (-119 to -111 and -99 to -94), suggesting the possibility that SP1 contributes to the PARP1 expression in NSPCs through binding to the element -115/-94. It is known that SP1 is involved in transcriptional regulation rat *PARP1*, but the regulatory element varies between cell types[11]. In contrast, the element -149/-145 overlaps with the putative Zics binding sites. Members of the Zic family of transcription factors are essential for maintaining pluripotency of ES cells[21] and are

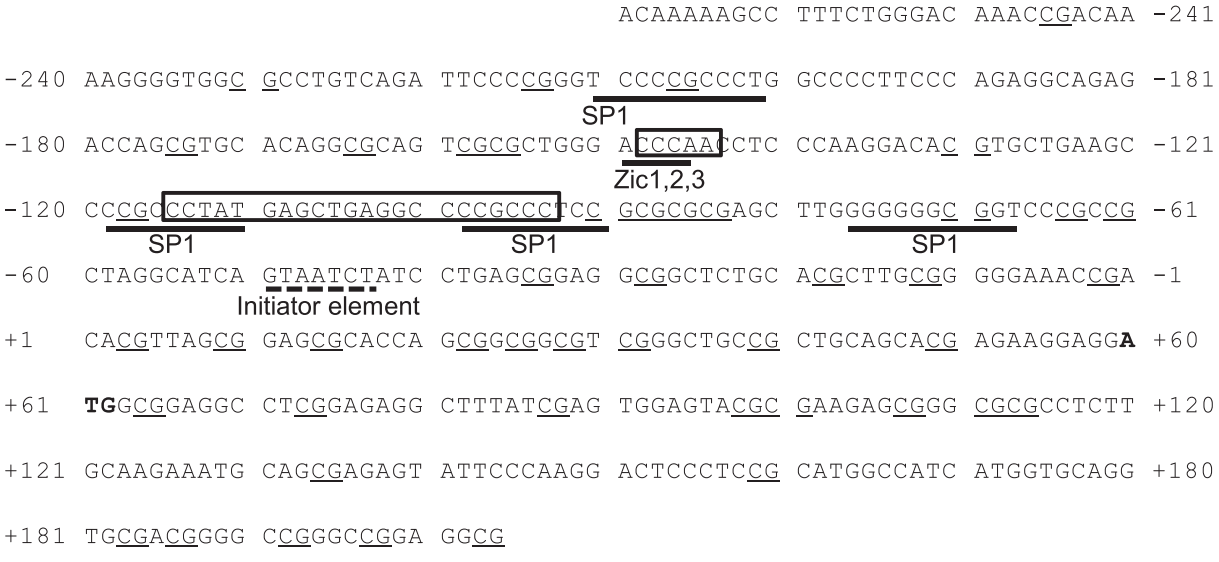

Figure 3 DNA sequence alignment of the mouse *PARP1* proximal promoter region. This region is GC-rich. The sequence from -78 to +4 is almost completely conserved in mammalian species. The two identified transcriptional regulatory elements in NSPCs are indicated in the box. The element -115/-94 overlaps with two putative SP1 binding sites, and the element -149/-145 overlaps with putative Zics (Zic1, Zic2, and Zic3) binding sites. The initiator element is shown as a dotted line. CG shows the CpG site. TSS based on NM_007415.2 was designated as position +1.

expressed in pluripotent stem cells during very early mouse development[22]. In our preliminary experiment, *SP1* and *Zics* mRNAs were expressed at higher levels in mouse NSPCs than in MEFs. These findings suggest a possibility that these transcription factors are associated with a transcriptional regulation of mouse *PARP1* gene in NSPCs. Further investigation is required to confirm this possibility.

Conflicts of interest

The authors have no conflicts of interest.

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