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Molecular cloning and characterization of the human p19^{INK4d} gene promoter

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Abstract p19^{INK4d}, a member of the INK4 family of cyclin-dependent kinase (CDK) inhibitors, negatively regulates the cyclin D–CDK4/6 complexes, which promote G1/S transition by phosphorylating the retinoblastoma tumor-suppressor gene product. To investigate the mechanism of transcriptional regulation of the p19^{INK4d} gene, we characterized the 5′-flanking region of the human p19^{INK4d} gene. The cap-site hunting method revealed that the transcription starts at −16 nucleotide (nt) upstream of the initiation codon. The 5′-flanking region of the human p19^{INK4d} gene was ligated to a luciferase reporter gene and possessed functional promoter activity. Luciferase assay with a series of truncated 5′-flanking regions indicated that the region from −81 to −2 nt could drive the transcription of the p19^{INK4d} gene. Several Sp1 and activating protein 2 binding sites are located within the region from −81 to −2 nt. Mutation of the second Sp1 binding site from −33 to −25 nt decreased the promoter activity. Collectively, it was demonstrated that the human p19^{INK4d} gene is under the control of TATA-less promoter and the Sp1 binding site is involved in the transcription. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: p19^{INK4d}; Promoter; Luciferase assay

1. Introduction

In the eukaryotic cell cycle, several positive and negative factors regulate the cell cycle progression. Among the positive factors, the key molecules are a family of protein kinases each of which comprises a regulatory subunit, or cyclin, and a catalytic subunit, termed a cyclin-dependent kinase (CDK). A number of reports have suggested that cyclin D–CDK4/6 and cyclin E–CDK2 play important roles in promoting the transition from the G1 phase to the S phase by the phosphorylation of the retinoblastoma protein (pRB). One further level of control has recently become apparent, namely, the expression of CDK inhibitors [1]. Two families of CDK inhibitors have already been identified in mammalian cells, with different modes of action. One group, comprised of related proteins known as p21$^{Cip1/Waf1}$, p27^{Kip1} and p57^{Kip2}, inhibits a broader spectrum of cyclin/CDK complexes [2–5]. The second family of CDK inhibitors is also called INK4 family proteins. The four members of this family, designated p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}, bind directly to CDK4/6 and are therefore specific inhibitors of the cyclin D-dependent kinases [6–8].

The four proteins of the INK4 family possess a similar structure dominated by several ankyrin repeats and bind to CDK4/6 with similar affinity, but they are reported to have different biological roles. p16^{INK4a} functions as a tumor suppressor. Inactivation of the p16^{INK4a} gene through gene deletions, point mutations or transcriptional silencing by methylation of the promoter is one of the most frequent defects contributing to oncogenesis [9–12]. Mice deficient in p16^{INK4a} develop normally and are highly cancer prone [13,14]. Disruption of p15^{INK4b} does not influence development of mice, but leads to frequent lymphoproliferative disorders [15]. Mice lacking p18^{INK4c} have deregulated epithelial cell growth, resulting in the frequent development of various types of neoplasia [15,16]. p19^{INK4d}-deficient mice exhibit marked testicular atrophy associated with increased apoptosis of germ cells, suggesting an important role for p19^{INK4d} in testis development [17].

A possible molecular basis for these biochemically indistinguishable molecules to carry out distinct biological functions appears to lie in differences in the transcriptional regulation. In fact, the INK4 proteins are expressed in distinct, tissue-specific patterns [18]. Furthermore, their transcription is known to respond differentially to diverse stimuli. Induction of p15^{INK4b} in response to transforming growth factor β, or upregulation of p16^{INK4a} with increasing population doublings or following some oncogenic stimuli, or increased expression of p18^{INK4c} associated with the antiproliferative action of interleukin-6, are among examples of such differential transcriptional regulation [11,12,19]. In the present study, to investigate the mechanism of the transcriptional regulation of the p19^{INK4d} gene, we cloned the 5′-flanking region of the human p19^{INK4d} gene and analyzed its promoter region.

2. Materials and methods

2.1. Cell culture

The human T lymphoblastic cell line Jurkat (a kind gift from Dr. J.I. Fujisawa, Kansai Medical University) was maintained in RPMI 1640 medium with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2.

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Abbreviations: AP-2, activating protein 2; SRY, sex determining region Y

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2.2. Cloning of the 5′-flanking region

Genome screening was performed using a human genomic leukocyte library in the λPS1 phage (Mo Bi Tec, Göttingen, Germany). Phage plaques (1×10^7) of this library were screened with a ^{32}P-labeled probe encoding a part of the human p19^{INK4d} gene. This probe was amplified by PCR based on the data in GenBank no. AF044171, with the forward (5′-AGCTCGTAGTAAGGGCCAATGAATGTTCT-3′) and the reverse (5′-ACAAACTCGCTACTGTGTAGCGGCA-3′) primers. The positive phage plaques were purified through three rounds of rescreening, and the phage DNA was converted to plasmid DNA by transduction into a Cre-recombinase-expressing bacterial strain. The genomic DNA fragments were digested with the appropriate enzymes and analyzed by Southern blotting.

2.3. Determination of the transcriptional start site

To identify the transcriptional start site, the cap-site hunting method with human testis cap-site cDNA (Nippon Gene, Toyama, Japan) was used according to the manufacturer's instructions. Briefly, r-oligo was ligated to the 5′-end of cap-removed mRNA and the linked mRNA was used to synthesize the first-stranded cDNA by reverse transcriptase in the presence of oligo-dT primers. The first round of PCR was performed using a sense DNA primer complementary to r-oligo (1RDT; 5′-GATGCTAGCTGCGAGTCAAGTC-3′) paired with the target gene-specific antisense primer 3 (TGP3; 5′-GTGTCCAGGAATCCAGTGCG-3′; based on the data in GenBank no. NM_001800). Aliquots of the first PCR reaction were used as the template in the second round of PCR reaction (nested PCR). Nested PCR was performed using sense DNA primer complementary to r-oligo (2RDT; 5′-CGAGTCAAGTCGACGAAGTGC-3′) paired with the nested target gene-specific antisense primers 1 or 2 (TGP1, 5′-GTCTTGCCGAAGCGGTTGAG-3′; TGP2, 5′-TGCTGCCAAACATCATGACC-3′). The PCR products were cloned into TA vector and sequenced using a DNA sequencer (Applied Biosystems Inc.).

2.4. Construction of the reporter plasmid

An approximately 3.8 kb fragment derived from the positive phage DNA was subcloned into luciferase reporter plasmid pGVB2 (Nippon Gene, Tokyo, Japan). This human p19^{INK4d}-luciferase fusion plasmid was termed p19 (−3814/−2). The deletion mutants of p19 (−3814/−2), termed p19 (−265/−2) and p19 (−81/−2), were generated using a Mungbean-Exonuclease III system from the Kilo-sequence Deletion kit (Takara, Tokyo, Japan). The reporter plasmids with mutations in the activating protein 2 (AP-2) binding site −75/−65 (Mutation 1), the Sp1 binding site −39/−31 (Mutation 2) and the Sp1 binding site −33/−25 (Mutation 3) were generated by site-directed mutagenesis [20] using the Quick Change Site-Directed Mutagenesis kit (Stratagene). The top strands of the oligonucleotides were as follows, with mutations indicated by underlines; Mutation 1, 5′-CGCTAGCCATCT-3′; Mutation 2, 5′-GGGTTTTGG-3′; Mutation 3, 5′-GGATGGGTA-3′. All of the generated constructs were confirmed by sequencing.

2.5. Transient transfection and luciferase activity

Reporter constructs were co-transfected with pRL-TK (Promega) to standardize by *Renilla* luciferase activity. Jurkat cells (1×10^6 cells) were transfected with a total of 5.1 μg DNA (5 μg reporter plasmid and 0.1 μg pRL-TK) by the DEAE-dextran method [21]. Cells were harvested 48 h after transfection. The luciferase activity of each cell lysate was measured using the Dual-Luciferase Reporter Plasmid System (Promega). The firefly luciferase activity of each sample was normalized by its *Renilla* luciferase activity and the fold activation was obtained by setting the value of the empty vector control as 1.0. All of the transfection assays were carried out in triplicate.

3. Results and discussion

3.1. Molecular cloning of human genomic fragment containing the p19^{INK4d} promoter

To investigate the mechanism of the transcriptional regulation of the p19^{INK4d} gene, we cloned a genomic DNA fragment containing the human p19^{INK4d} promoter region. A part of the human p19^{INK4d} genomic DNA fragment was obtained by PCR. Using this probe, 1×10^7 phage plaques of a human leukocyte genomic library were screened to obtain the genomic DNA fragment containing the 5′-flanking region of

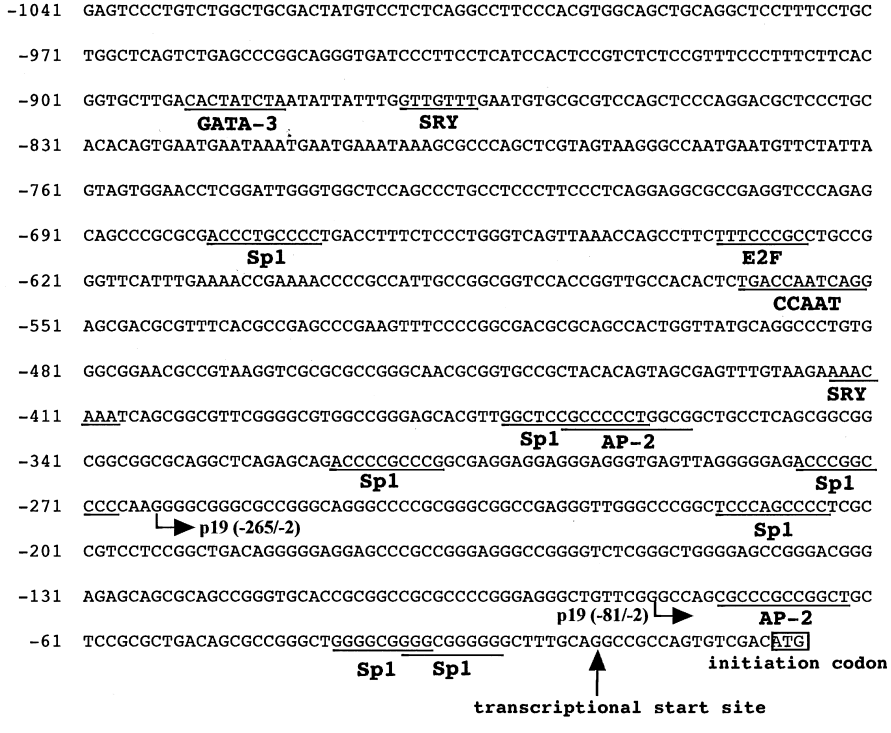

Fig. 1. Nucleotide sequence of the 5′-flanking region of the human p19^{INK4d} gene. The transcriptional start site of the human p19^{INK4d} gene was determined by the cap-site hunting method and is indicated by the arrowhead. The various potential transcriptional factor binding sites are indicated. The nucleotide number was counted from the first base of the initiation codon. The arrows indicate the 5′-end of the deletion mutants of the p19^{INK4d} promoters shown in Fig. 3.

the human p19^{INK4d} gene. Four positive clones were identified and phage DNAs were converted to plasmid DNAs by transduction into a Cre-recombinase-expressing bacterial strain. The plasmid DNAs were digested with several restriction enzymes and analyzed by Southern blotting using the same probe. These fragments contained the 5′-flanking region, exon 1 and intron 1 of the human p19^{INK4d} gene (data not shown).

3.2. Sequence analysis of the human p19^{INK4d} promoter

A 3.8 kb fragment containing the 5′-flanking region of the human p19^{INK4d} gene was subcloned into pGVB2 and sequenced. A computer search using MatInspector V2.2 at the TRANSFAC WWW site [22] for potential regulatory elements in the promoter region revealed that there could be multiple transcriptional factor binding sites such as Sp1, AP-2, CCAAT, sex determining region Y (SRY), GATA-3 and E2F (Fig. 1) which might transcriptionally regulate the p19^{INK4d} gene expression. Analysis of the region surrounding the transcriptional start site failed to reveal the presence of a TATA box.

3.3. Transcriptional start site of the human p19^{INK4d} promoter

To identify the transcriptional start site, the cap-site hunting method was performed with human testis cap-site cDNA. In the first amplification, no specific PCR products were detectable using 1RDT as the sense primer complementary to r-oligo and TGP3 as the target gene-specific antisense primer (data not shown). The resulting cDNA extending to the cap site was then amplified by nested PCR in the presence of 2RDT as the sense primer complementary to r-oligo and either TGP1 or TGP2 as the target gene-specific antisense primer. The amplified products were electrophoresed on agarose gels and stained with ethidium bromide (Fig. 2). Products of approximately 220 and 250 bp were obtained using 2RDT/TGP1 and 2RDT/TGP2, respectively. Two specific PCR products were cloned and sequenced. The results of sequencing analysis demonstrated that all 15 selected clones terminated at the same nucleotide, −16 nt from the initiation codon (Fig. 1), indicating that this nucleotide is the major transcriptional start site of the human p19^{INK4d} promoter.

3.4. Promoter activity and deletion analysis of the human p19^{INK4d} promoter

To analyze the promoter activity of the 5′-flanking region of the p19^{INK4d} gene, several deletion mutants were generated by the Mungbean-Exonuclease III system. A series of the 5′-deletion constructs were subcloned into the luciferase reporter plasmid pGVB2 and transfected into the human T lymphoblastic cell line Jurkat. The expression of p19^{INK4d} gene in this cell line was confirmed by Northern blot analysis (data not shown). The full-size human p19^{INK4d} promoter construct, p19 (−3814/−2), conferred luciferase activity six-fold higher than that of the parental vector (Fig. 3). The deleted constructs of p19 (−265/−2) and p19 (−81/−2) showed luciferase activity three-fold higher than that of the parental vector (Fig. 3). These results suggest that the region between −81 and −2 nt might include the transactivating elements of the human p19^{INK4d} gene.

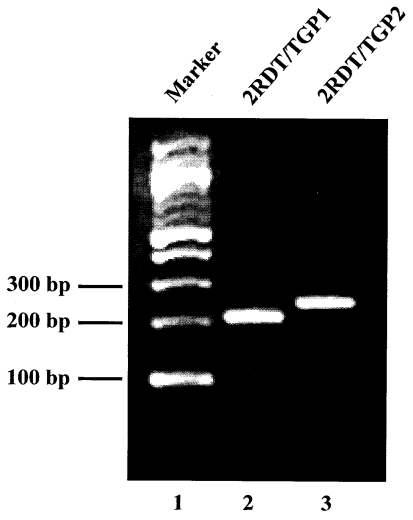

Fig. 2. Gel electrophoresis of PCR products of oligo-capped p19^{INK4d} mRNA. Human testis cap-site cDNA was used as a PCR template to identify the transcriptional start site of the human p19^{INK4d} gene. The first round of PCR was performed using 1RDT as the sense DNA primer complementary to r-oligo and TGP3 as the gene-specific antisense primer. An aliquot of the initial PCR reaction served as the template for nested PCR. The resulting cDNA extending to the cap site was amplified by nested PCR using 2RDT as the nested sense DNA primer complementary to r-oligo and either TGP1 or TGP2 as the nested gene-specific antisense primer. The nested PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. Lane 1, 100 bp DNA ladder (molecular size marker); lane 2, nested PCR product using 2RDT/TGP1; lane 3, nested PCR product using 2RDT/TGP2.

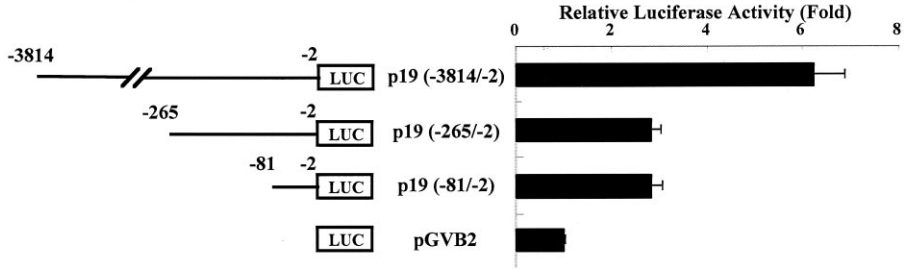

Fig. 3. Deletion mutants of the human p19^{INK4d} promoter-luciferase constructs. The human full-size p19^{INK4d} promoter-luciferase construct, p19 (−3814/−2), was generated by subcloning a 3.8 kb DNA fragment in front of the luciferase reporter gene in pGVB2. The 5′-deletion mutants were generated using the Mungbean-Exonuclease III system. Jurkat cells were transfected with the different reporter gene constructs together with the pRL-TK vector, which served as an internal control. The firefly luciferase activity of each sample has been normalized by its *Renilla* luciferase activity, and this value has subsequently been compared with the mean value of the empty pGVB2 to acquire the fold induction of each construct. Data are shown as means ± S.D. ($n = 3$).

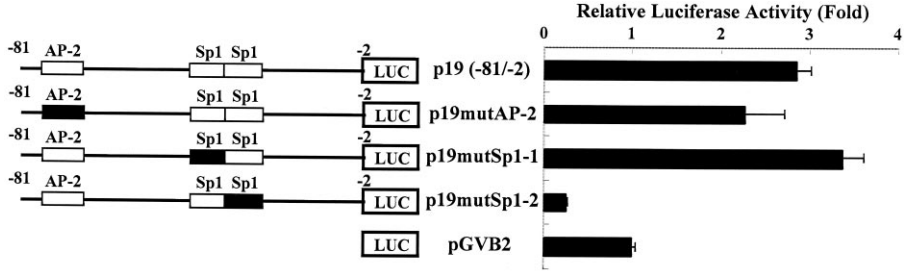

Fig. 4. Mutational analysis of the human p19^{INK4d} promoter-luciferase constructs. Three different mutants, shown on the left, are identical to p19 (−81/−2), except for the mutation indicated. These mutants were generated by site-directed mutagenesis. Jurkat cells were transfected with the different reporter gene constructs together with the pRL-TK vector, which served as an internal control. The firefly luciferase activity of each sample has been normalized by its *Renilla* luciferase activity, and this value has subsequently been compared with the mean value of the empty pGVB2 vector to acquire the fold induction of each construct. Data are shown as means ± S.D. ($n = 3$).

3.5. Mutational analysis of the human p19^{INK4d} promoter

Potential binding sites for Sp1 and AP-2 are located in the region between −81 and −2 nt (Fig. 1). To clarify the contribution of these sites to the transcription, we introduced mutations in each of these binding sites using the deleted construct, p19 (−81/−2). We mutated the AP-2 binding site at −75/−65 (p19mutAP-2), the Sp1 binding site at −39/−31 (p19mutSp1-1) and the Sp1 binding site at −33/−25 (p19mutSp1-2). The mutations at the AP-2 and Sp1 (−39/−31) sites did not affect the promoter activity, but the mutation at the Sp1 (−33/−25) site significantly reduced the promoter activity (Fig. 4). These results suggest that the second Sp1 binding site, −33/−25, is involved in the transcription of the p19^{INK4d} gene.

3.6. Future aspects of transcriptional regulation of the p19^{INK4d} gene

The results of the present study indicate that the human p19^{INK4d} gene is under control of TATA-less promoter, and that the transcription starts at −16 nt upstream of the initiation codon, and that Sp1 binding site at −33/−25 is involved in the transcription. The result of Fig. 3 shows that the promoter activity of the region between −81 and −2 nt is about half of that of the full-size promoter region (−3814/−2). Furthermore, mutational analysis in Fig. 4 indicates that the Sp1 (−33/−25) site is essential for the promoter activity of the region −81 to −2 nt. Therefore it is considered that the Sp1 (−33/−25) site contributes to about half of the p19^{INK4d} promoter activity, suggesting the Sp1 (−33/−25) site as one of the essential elements of the p19^{INK4d} promoter. Sp1 is ubiquitously expressed in a wide variety of tissues and hence regulates numerous genes including common house keeping genes [23]. This is consistent with the previous report that p19^{INK4d} gene is widely expressed in most cell lines and mouse normal tissues [18,24].

The computer search using MatInspector V2.2 at the TRANSFAC WWW site [22] for potential regulatory elements in the promoter region revealed that there could be multiple transcriptional factor binding sites such as Sp1, AP-2, CCAAT, SRY, GATA-3 and E2F (Fig. 1) which might transcriptionally regulate p19^{INK4d} gene expression. In particular, we had much interest in the E2F binding site. Phosphorylation of pRB by cyclin D–CDK4/6 and cyclin E–CDK2 at the G1/S phase results in the release of E2F transcriptional factors, which activate numerous genes associated with G1/S phase progression. A previous report has shown that the expression of p19^{INK4d} gene oscillates through the cell cycle, with the levels of mRNA increasing as cells entered the S phase [8]. To determine if the p19^{INK4d} gene promoter is transactivated by E2F, transient transfections were performed by co-transfecting the E2F1 effector plasmid with the full-size human p19^{INK4d} promoter construct, p19 (−3814/−2). However, E2F1 did not significantly alter the luciferase activity (data not shown). This result suggests that the E2F binding site in the p19^{INK4d} promoter might not be functional.

The p19^{INK4d} gene is controlled by a post-translational mechanism such as ubiquitination [25]. However, several reports have shown an increase of p19^{INK4d} mRNA induced by several cytokines and neuronal differentiation, suggesting that the p19^{INK4d} gene is also transcriptionally regulated in some cases [26–31]. Our present work of molecular cloning and characterization of the p19^{INK4d} gene promoter may provide a powerful means for investigating the regulatory mechanism of these diverse stimuli.

Inactivation of p16^{INK4a} has been extensively reported in almost all human cancer cells [9–12]. In contrast, genetic alterations of p19^{INK4d} gene are rare events in human tumors, although alterations of p19^{INK4d} gene in osteosarcoma, and a loss of p19^{INK4d} protein in testicular cancer have been reported [32–34]. As a member of the INK4 family, p19^{INK4d} is known to possess a function similar to that of p16^{INK4a} [8]. This functional similarity to p16^{INK4a} suggests that p19^{INK4d} may function as a replacement for p16^{INK4a} in case p16^{INK4a} is inactivated. Therefore transcriptionally regulated agents of p19^{INK4d} gene may contribute to new strategies for the prevention or therapy of malignancies which we have termed 'gene-regulating chemoprevention or chemotherapy' [35,36]. Our study of the human p19^{INK4d} promoter may provide information useful for these strategies.

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