Molecular cloning and characterization of the human p27^{Kip1} gene promoter

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Abstract p27^{Kip1} is an inhibitor of multiple cyclin-dependent kinases (cdk), and can arrest the cell-cycle progression by inhibiting the phosphorylation of the retinoblastoma gene family products. Tumor formation in p27^{Kip1} knockout mice clearly shows that p27^{Kip1} plays an important role in inhibiting tumor formation and progression. To investigate the mechanism of transcriptional p27^{Kip1} gene expression, we isolated the genomic DNA fragment of the 5′ flanking region of the human p27^{Kip1} gene and characterized its promoter region. The human p27^{Kip1} promoter is TATA-less, and the sequence is highly homologous to the murine p27^{Kip1} promoter sequence. In the promoter assay, deletion from −774 to −435 relative to the initiating codon resulted in a 15–20-fold reduction of the p27^{Kip1} promoter activity, suggesting that the elements for basal promoter activity exist in this highly conserved 340 bp region, where putative CTF and ATF sites are conserved.

Key words: p27^{Kip1}; Promoter; Cdk inhibitor

1. Introduction

In the eukaryotic cell cycle, several positive and negative factors regulate the cell-cycle progression. Among the positive factors, the key players 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 suggest that cyclin D-cdk4, cyclin D-cdk6 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}, p27^{kip1} and p57^{kip2}, appears to function as broad specificity inhibitors 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, p16, p18 and p19, bind directly to cdk4 and cdk6 and are therefore specific inhibitors of the cyclin D-dependent kinases [6–8].

Although the precise roles of p27^{Kip1} are far from clear, its level declines as cells are stimulated to enter the cycle, and increases when cells are arrested by transforming growth factor or by contact inhibition [9]. p27^{Kip1} was cloned as a binding protein with cyclin E-cdk2 [3] or cyclin D-cdk4 [4]. p27^{Kip1} inhibits the activity of most cyclin–cdk complexes, and in addition, is capable of inhibiting the phosphorylation of cyclin–cdk complexes by CAK (cdk-activating kinase) [10]. p27^{Kip1} therefore functions as a negative regulator of G1/S progression.

Tumor-specific mutations of the p27^{Kip1} gene are rare [11], whereas several cell cycle regulators such as p16, p53 and RB are frequently mutated in several cancers and have been shown to be tumor-suppressor genes. However, p27^{Kip1}-deficient mice were observed to have increased body size, multiple organ hyperplasia, retinal dysplasia, and the formation of the pituitary tumors [12–14], which is in part similar to the case of the RB heterozygous knockout mice [15]. In addition, cohort studies of breast and colorectal carcinoma patients showed that a lower expression of the p27^{Kip1} protein correlated with poorer survival [16–18]. These results clearly indicate that p27^{Kip1} plays an important role in inhibiting tumor formation and tumor progression. The expression of p27^{Kip1} has been shown to be controlled by a post-translational mechanism [19,20]. However, recent reports showed that p27^{Kip1} mRNA is induced by vitamin D3 in U937 cells [21] and by neuronal differentiation [22], suggesting that transcriptional regulation of the p27^{Kip1} gene might be also important in cellular differentiation. In the present study, to investigate the mechanism of the transcriptional regulation of the p27^{Kip1} gene, we cloned the 5′ flanking region of the human p27^{Kip1} gene and analyzed its promoter region.

2. Materials and methods

2.1. Cell culture

The human osteosarcoma cell lines Saos2 and U2OS (kind gifts from Dr. R. Takahashi, Kyoto University and from Dr. E. Hara, Kyoto Prefectural University, respectively) and the human cervical carcinoma cell line C33A (purchased from the American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. In the U2OS cells, the p53 and RB genes are intact [23], and in the Saos2 [23,24] and C33A [25] cells, both the p53 and RB genes are mutated.

2.2. Cloning of the human p27^{Kip1} promoter fragment

A part of the human p27^{Kip1} cDNA containing exon 1 was obtained from the human p27^{Kip1} cDNA inserted in the plasmid pBluescript SK-p27FL (a kind gift of Dr. J. Massague, Memorial Sloan-Kettering Cancer Center, USA) by digestion with *Eco*RI and *Pst*I, and was used as a probe to screen a human leukocyte genomic library. Using this probe, about 10^6 phage plaques of a human leukocyte genomic library in the EMBL3 SP6/T7 phage vector (Clontech, Palo Alto, CA) was screened to obtain the genomic DNA fragments con-

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Abbreviations: PEA3, polyoma virus enhancer A binding protein 2; CTF, CAAT-box binding transcriptional factor; PEBP2, polyoma virus enhancer binding protein 2; AP2, activating protein 2; NFκB, nuclear factor κB; ATF, activating transcriptional factor

The nucleotide sequences reported in this paper have been submitted to the DNA Data Bank of Japan (DDBJ).

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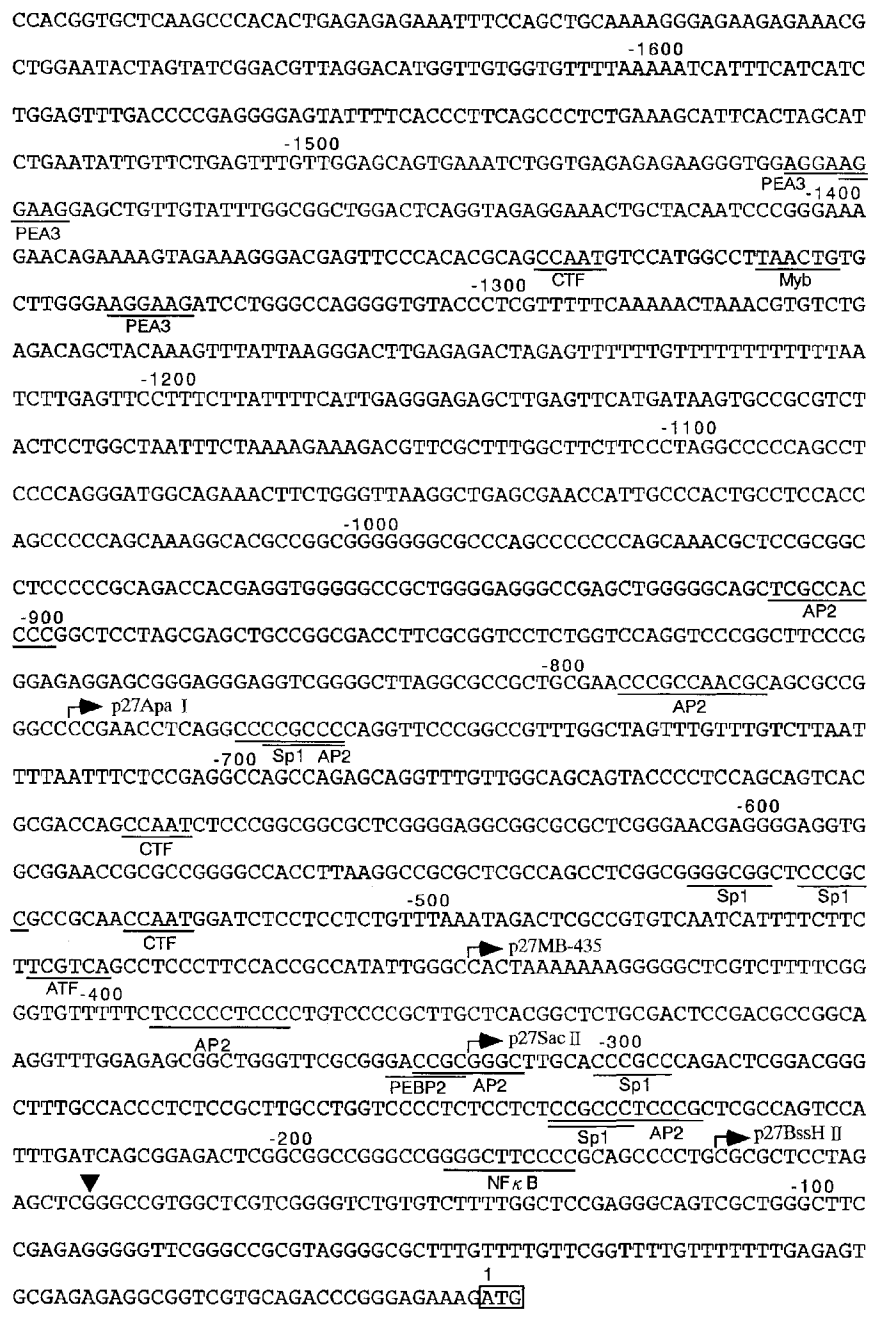

Fig. 1. Nucleotide sequence of the 5′ flanking region of the human $p27^{Kip1}$ gene. The transcriptional start site of the human $p27^{Kip1}$ gene was determined by primer extension and is indicated by the arrowhead (▼). The consensus sites of various transcriptional factors are indicated and underlined. The nucleotide number was counted from the translational start site. The arrows indicate the 5′ end of the deletion mutants of $p27^{Kip1}$ promoters shown in Fig. 3.

taining the 5′ region of the human $p27^{Kip1}$ gene. Hybridization was performed in a buffer containing 5×SSC, 50% formamide, 1% SDS, 5×Denhards solution, and 0.1 μg/μl denatured and sonicated fish sperm DNA at 42°C for over 16 h. The positive phage plaques were expanded and the DNA was purified. The genomic DNA fragments were digested with the appropriate enzymes and analyzed by Southern blotting.

2.3. Plasmid preparation

An approximately 4.8 kb *Xho*I fragment derived from the positive phage DNA was subcloned into pBluescript SK+ (Stratagene, La Jolla, CA). This construct was used for sequencing, and an approximately 3.5 kb fragment between −3568 and −12 from the start site of translation of the human $p27^{Kip1}$ gene was subcloned into the luciferase reporter plasmid pGVB2 (Nippon Gene, Tokyo, Japan). This human $p27^{Kip1}$-luciferase fusion plasmid was termed p27PF. To generate the deletion mutants of p27PF, p27PF was digested separately with *Kpn*I, *Apa*I, *Sac*II and *Bss*HII. These enzyme sites were blunted with Klenow or T4 DNA polymerase and then self-ligated. These plasmids were termed p27KpnI, p27ApaI, p27SacII and p27BssHII, respectively. Another deletion mutant termed p27MB-435 was generated using a Mungbeans–Exonuclease III system, the Kilo-sequence Deletion Kit (Takara, Tokyo, Japan). The series of all of the generated constructs were confirmed by sequencing.

2.4. DNA sequencing and primer extension analysis

The sequence was determined by the dideoxynucleotide chain termination method using a USB sequenase version 2.0 DNA sequencing

kit (Amersham, Buckinghamshire, UK). The primers used for sequencing were T3, T7 and synthetic oligonucleotides derived from the genomic DNA sequence. To determine the transcriptional start site, two 20-bp oligonucleotides, 0-128 (5′-CCCAGCGACTGCCCTCGGAG-3′), and 0–234 (5′-CCCTCTCGGAAGCCCAGCGA-3′), complementary to the regions from −120 to −101 and from −108 to −89, respectively, were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Toyobo, Osaka, Japan). This extension primer was hybridized to 20 μg of total RNA isolated from human osteosarcoma MG63 and Saos2 cells at 42°C. The annealed primers were extended with 200 U of Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) for 50 min at 42°C. The resultant products were analyzed on a 6% polyacrylamide denatured gel in parallel with a sequencing reaction generated with the same extension primer.

2.5. Transient transfection

C33A cells (5×10^5 cells) and U2OS cells (3×10^5 cells) were seeded into 6-cm-diameter tissue culture dishes. After 24 h, 2 μg of p27PF and 1 μg of RSVβ-gal, containing the β-galactosidase gene under the control of the Rous sarcoma virus promoter (for normalizing the transfection efficiency) were co-transfected into cells using the calcium phosphate co-precipitation method [26], and the luciferase activity was measured after 48 h.

2.6. Luciferase assay

The luciferase activity of each cell lysate was measured as described previously [27], and was normalized by β-galactosidase activities in the cell lysates. All the transfection assays were carried out in triplicate. Each experiment was repeated at least twice.

3. Results and discussion

3.1. Molecular cloning of human genomic DNA fragment containing the p27^{Kip1} promoter

To investigate the mechanism of the transcriptional regulation of the p27^{Kip1} gene, we cloned a genomic DNA fragment containing the human p27^{Kip1} promoter region. A part of the human p27^{Kip1} cDNA fragment containing exon 1 was obtained by digestion with *Eco*RI and *Pst*I from the human p27^{Kip1} cDNA-inserted plasmid SK-p27FL, and was used to screen a human leukocyte genomic library. Using this probe, about 10^6 phage plaques of a human leukocyte genomic library was screened to obtain the genomic DNA fragments containing the 5′ flanking region of the human p27^{Kip1} gene. One positive clone was identified and the phage DNA was purified. The positive phage DNA was digested with several restriction enzymes and analyzed by Southern blotting using the same probe. This fragment contained the 5′ flanking region, exon 1 and intron 1 of the human p27^{Kip1} gene (data not shown).

3.2. Sequence analysis of the human p27^{Kip1} promoter

A 4.8 kb fragment digested by *Xho*I was subcloned into pBluescript SK+ and sequenced. This fragment contained the 5′ flanking region, the first exon and the first intron. The sequence of the 5′ untranslated region of the genomic DNA fragment was identical to the sequence of the human p27^{Kip1} cDNA probe. The computer search using GENETYX software (Software Development Co, Tokyo, Japan) for potential regulatory elements in the promoter region revealed that there could be multiple transcription factor binding sites such as Sp1, PEA3, CTF, Myb, PEBP2, AP2, NFκB and ATF (Fig. 1) which might transcriptionally regulate p27^{Kip1} gene expression. Analysis of the region surrounding the transcriptional start site failed to reveal the presence of a TATA box.

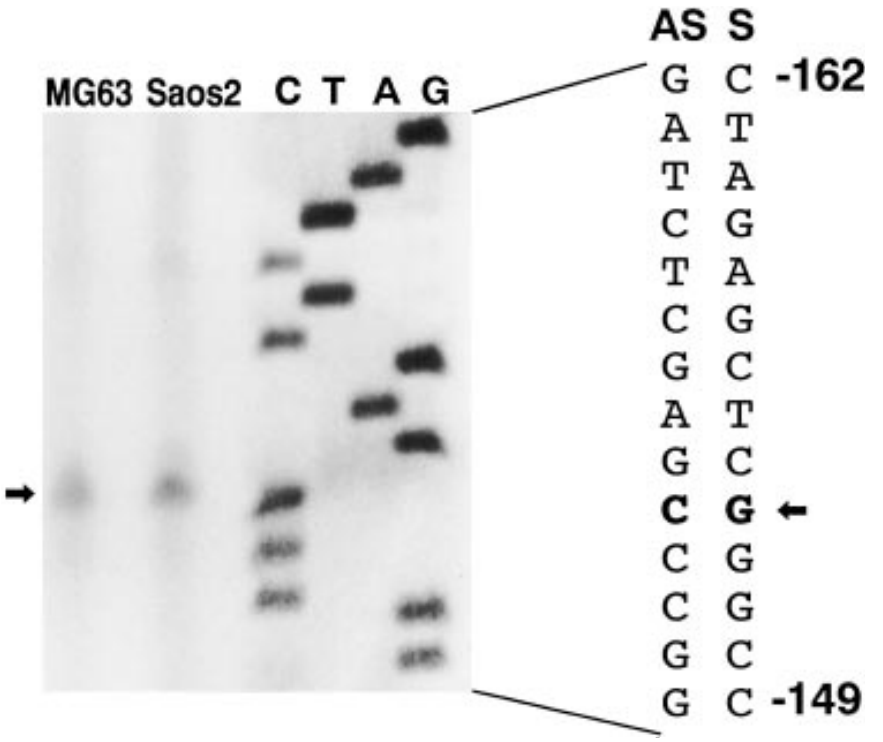

Fig. 2. Primer extension mapping of the human p27^{Kip1} gene. Primer extension was performed as described in Section 2. Extension products from primer O-128 are shown using total RNA from MG63 and Saos2 cells as the template. In this figure, the antisense sequence ladder is shown. The arrow indicates the C-residue (G-residue in sense strand) as a putative transcriptional start site. S; sense strand, AS; antisense strand.

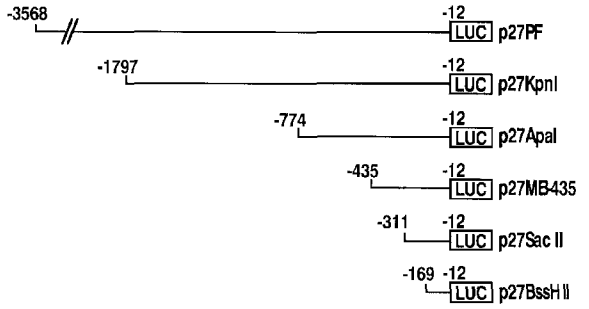

Fig. 3. Deletion mutants of the human $p27^{Kip1}$ promoter-luciferase constructs. The human full-size $p27^{Kip1}$ promoter-luciferase construct, p27PF, was generated by subcloning the DNA fragment from the *Xho*I site (−3568) to *Sma*I site (−12), in front of the luciferase reporter gene in pGVB2. The 5′ deletion constructs were generated by using restriction enzyme sites or the Mungbeans–Exonuclease III system.

3.3. Transcriptional start site of the human $p27^{Kip1}$ promoter

To identify the transcriptional start site, a primer extension analysis was performed. We used two distinct primers annealing to the different sequence of the first exon. As templates, two kinds of mRNA of human osteosarcoma MG63 and Saos2 cells were used. The two primers extended to the same G-residue at the position −153 from the translational start site reproducibly in mRNA from these two cell lines (Fig. 2), where we suspect the major transcriptional start site is. In the upstream region, there could be two more transcriptional start sites at −225 and −247 (data not shown).

3.4. Promoter activity and deletion analysis of the human $p27^{Kip1}$ promoter

To determine the core element for the human $p27^{Kip1}$ promoter activity, several deletion mutants were generated by using restriction enzyme sites and also by the Mungbeans–Exonuclease III system. A series of the 5′ deletion constructs was subcloned into the luciferase reporter plasmid pGVB2 (Fig. 3) and transiently transfected into C33A and Saos2 cells. The RSVβ-galactosidase plasmid was used as an internal control. The promoter activities of the full-size human $p27^{Kip1}$ promoter construct, p27PF, and the deleted constructs of p27KpnI and p27ApaI were not significantly different. However, the promoter activity of the p27MB-435 construct was markedly decreased, by about 15–20-fold compared to that of the p27ApaI construct in the C33A and Saos2 cells (Fig. 4a,b) and U2OS cells (data not shown). This decrease in promoter activity suggests that the essential transcriptional factor binding sites or enhancer sites for the human $p27^{Kip1}$ promoter activity could exist in this region.

3.5. Comparison of the human and mouse $p27^{Kip1}$ promoter sequences

The comparison of the human and murine $p27^{Kip1}$ promoters by a computer analysis using GENETYX software revealed that the sequence of the human $p27^{Kip1}$ promoter is highly homologous to the murine $p27^{Kip1}$ promoter (Fig. 5) [28]. The sequence of the region between −774 and −435, which includes the region for basal promoter activity (Fig. 4a,b), matches the murine sequence by 85%, suggesting that the consensus and essential transcription factors could act on this region. Putative transcriptional factor binding sites in this region are three consensus Sp1 sites, an AP2 site, two CTF

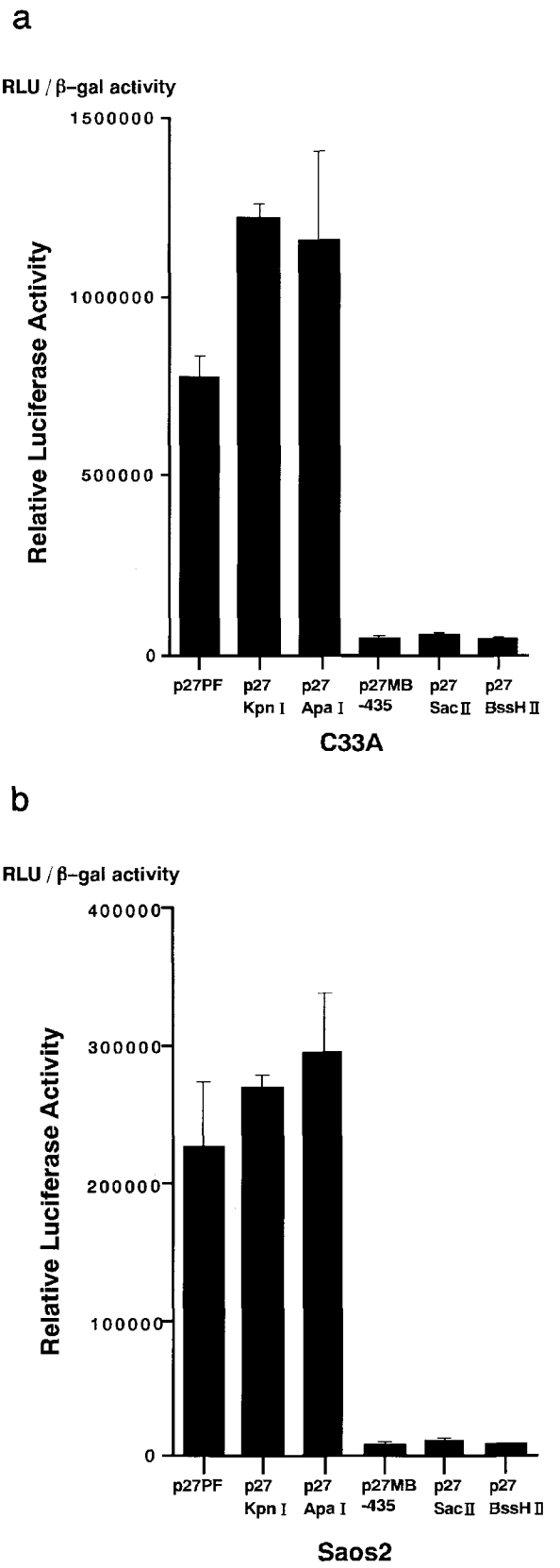

Fig. 4. Basal promoter activities of the human $p27^{Kip1}$ promoter constructs. Transfection was performed as described in Section 2 with the various 5′-deleted human $p27^{Kip1}$ promoter-luciferase constructs using C33A (a) and Saos2 cells (b). The luciferase activity was normalized by β-galactosidase activity driven by co-transfected RSV-gal.

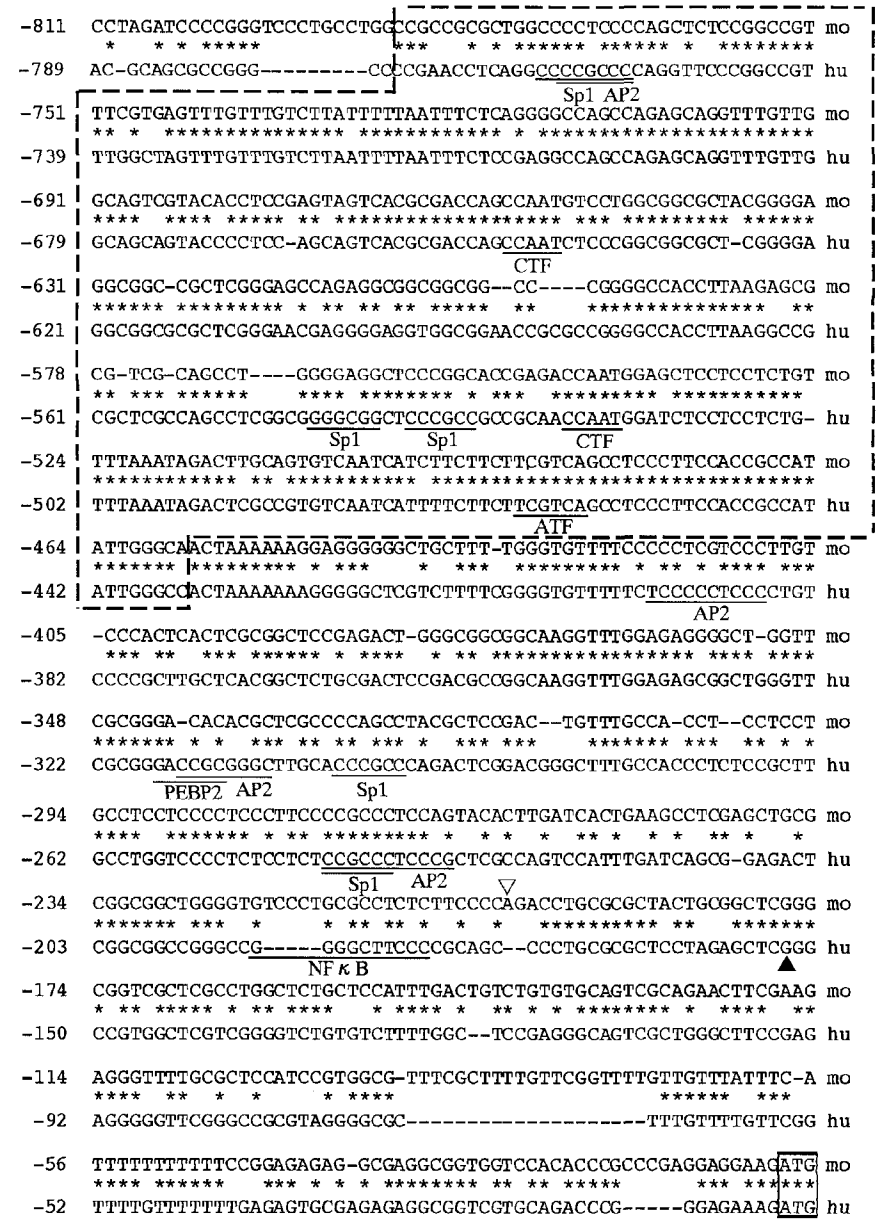

Fig. 5. Sequence homology between the human and mouse p27^{Kip1} promoters. A comparison of DNA sequence using GENETYX software was performed between the human (indicated as 'hu') and mouse (indicated as 'mo') promoters of the p27^{Kip1} gene. The putative basal activity area from −774 to −435 is surrounded by the dotted line. The transcriptional start sites of the human (▲) and the mouse (▽) p27^{Kip1} genes are shown by the arrowheads. The consensus sites for various transcriptional factors are indicated and underlined.

sites, and an ATF site (Fig. 5). Among these, two CTF and ATF sites are conserved, but other unknown conserved sites might be also important for the basal p27^{Kip1} promoter activity.

3.6. Future aspects of transcriptional regulation of p27^{Kip1} gene

p27^{Kip1} gene is known to be controlled by a post-translational mechanism such as ubiquitination [20] and by binding to E1A oncoprotein [29]. However, several reports showed an increase of p27^{Kip1} mRNA induced by vitamin D3 in U937 cells [21] and by neuronal differentiation [22], and decrease in p27^{Kip1} mRNA by anti-CD3 in T-lymphocytes [28], suggesting that the p27^{Kip1} gene is also transcriptionally regulated in some cases. In addition, recent studies have shown the importance of the p27^{Kip1} gene in the point of sensitization of tumor cells to an anti-cancer agent or cancer prognosis factor [30,18–20]. Therefore, transcriptionally regulated agents of the p27^{Kip1} gene may contribute to new strategies for the prevention or therapy of malignancies which we have termed 'gene-regulating chemoprevention or chemotherapy' [31]. Our study of the human p27^{Kip1} promoter may provide information useful for these strategies.

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