Journal of Cellular Biochemistry 75:300–309 (1999)

NF-Y-Mediated *Trans*-Activation of the Human Thymidine Kinase Promoter Is Closely Linked to Activation of Cyclin-Dependent Kinase

Zee-Fen Chang,[1]* Duen-Yi Huang,[1] and Su-Fen Hu[2]

[1]Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

[2]Department of Biochemistry, Chang Gung University, Tao-Yuan, Taiwan, Republic of China

Abstract Transcriptional activation is important for the elevated expression of human thymidine kinase (hTK) in tumor cells. Here, we used TK(−133/+33)-luciferase reporter gene construct and bandshift assay to study the *cis*-elements involved in transcriptional activation of the hTK promoter. We found that two CCAAT boxes at −71/−67 and −40/−36 and Sp1 binding site located at −118/−113 were critical for maximal expression of the hTK promoter activity. As Sp1-mediated activation of the hTK promoter was not detectable for the promoter construct with double mutations at two CCAAT boxes, we proposed that NF-Y binding to the hTK promoter sequence is a requisite step for the functional interaction with Sp1. Here, we further showed that the hTK promoter activity was reduced in HeLa cells transfected with p16 or p21, both of which are inhibitors of cyclin-dependent kinases (CDKs). Inhibition of the hTK promoter activity by p16 could be abrogated by overexpression of cyclin A, indicating that the cyclin A activating event is more directly involved in transcriptional activation of the hTK promoter. We thus proposed that NF-Y-mediated activation of the hTK promoter is closely linked to the activation of CDK2/cyclin A pathway. J. Cell. Biochem. 75:300–309, 1999. © 1999 Wiley-Liss, Inc.

Key words: human thymidine kinase promoter; cyclin-dependent kinase

Thymidine kinase (TK) is a crucial enzyme in the salvage pathway of thymidine triphosphate formation, which is important for the process of DNA replication. The level of TK activity is known to be increased at the G1/S phase of the cell cycle in the normal cells [Bello, 1974; Johnson et al., 1982]. Compelling evidence has shown that transcriptional activation play a critical role in the induction of TK activity at the G1/S phase [Stuart, 1985; Coppock and Pardee, 1987; Lipson et al., 1989]. It has been further revealed that the sequence between −133 and −64 relative to the transcription initiation site is involved in its cell-cycle-dependent regulation [Roehl and Conrad, 1990; Kim and Lee, 1991]. In contrast to normal cells, tumor cells often overexpress TK constitutively [Kit, 1976; Hallek et al., 1992; Hengstschlager et al., 1994, 1998]. We have previously shown that the hTK promoter is differently regulated in HeLa and normal human fibroblasts [Chang et al., 1995]. However, a detailed mechanism responsible for deregulation of the hTK promoter activity in tumor cell line is still unclear.

The putative *cis*-elements within the regulatory region (−133/−64) of the hTK promoter found by computer homology search include several Sp1-binding sites, one inverted CCAAT box, two Yi-like sequence and several E2F-like binding sequences [Roehl et al., 1993]. NF-Y has been shown to bind to the CCAAT box and play an important role in this promoter [Arcot et al., 1989; Chang and Liu, 1993; Mao et al., 1995]. However, the roles of other putative *cis*-elements are still not well clarified. For example, the DNase I footprinting patterns of the hTK promoter obtained from different experiments varied in the Sp1 binding sequences in the regulatory region [Arcot and Deininger, 1992; Kim and Lee, 1992]. In addition, the role

Grant sponsor: Chang Gung University; Grant number: CMRP436; Grant sponsor: National Science Council, Taiwan, ROC; Grant number: NSC-86-2314-B-002-346.

*Correspondence to: Dr. Zee-Fen Chang, Institute of Biochemistry, College of Medicine, National Taiwan University, No. 1 Jen Ai Road 1st Section, Taipei, Taiwan, Republic of China.

Received 3 November 1998; Accepted 26 April 1999

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of E2F's involvement was also unclear. In a cotransfection experiment, E2F1 was shown to stimulate the hTK promoter activity in quiescent mouse REF52 cells [Johnson et al., 1993]. However, the other report showed only slightly increased level of TK mRNA in REF52 cells which was serum-starved followed by infection with a recombinant adenovirus containing the E2F-1 cDNA, while the genes encoding thymidylate synthase, proliferating cell nuclear antigen, ribonucleotide reductase, and DNA polymerase α were clearly induced [DeGregori et al., 1995]. Therefore, the roles for Sp1 and E2F in the regulation of the hTK promoter remain unclear. In this report, the critical *cis*-elements required for maximal activation of the hTK promoter were determined in HeLa cells, where the hTK promoter stays highly active as described previously [Chang et al., 1995].

Because the hTK promoter is stringently controlled in normal cells in a cell cycle dependent manner, we also investigated the role of cell cycle regulator in the activation of the hTK promoter. We have previously shown that cyclin A can act as a limiting factor for the hTK promoter activity in normal cells [Chang et al., 1995]. Likely, the S phase activation of the hTK promoter is probably linked to the cell cycle events through the cyclin/CDK complexes. CDKs are a family of proteins composed of a regulatory cyclin subunit associated with a catalytic kinase subunit. The activity of these enzymes can be regulated by transient associations with cyclins, reversible phosphorylation reactions and binding of a class of regulators known as CDK inhibitors [Nigg, 1995; Peter and Herskowitz, 1994; Elledge and Harper, 1994]. There are two classes of CDK inhibitor in mammalian cells, Inks and Cip/Kips. The tumor suppressor p16, targeting cyclin D/CDK4 or CDK6, belongs to the Inks class of inhibitors [Serrano et al., 1993, 1995; Lukas et al., 1995]. The Cip/Kip family member p21 in vitro inhibits most CDK/cyclin complexes [Xiong et al., 1993]. As the elevated level of TK expression and deregulation of CDKs are often found in malignant cells, it is interesting to define the temporal relationship between hTK transactivation and CDK activation. For this reason, here we examined the effects of p21 and p16 in combination with cyclin A on the hTK promoter activity in HeLa cells.

MATERIALS AND METHODS

Reagents

Two double-stranded oligodeoxynucleotides corresponding to the Sp1 consensus sequence ATTCGATCGGGGCGGGGCGACC and the E2F consensus sequence ATTTAAGTTTCGCGCCCTTTCTCAA were purchased from Promega (Madison, WI) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Cell Culture

HeLa and HL-60 cells were obtained from American Type Culture Collection (Rockville, MD), and maintained in Dulbecco's Modified Eagle medium (DMEM) containing 10% FBS and RPMI medium containing 10% heat-inactivated FBS, respectively.

Transfection and Reporter Gene Assay

Cells in 35-mm dish were incubated with a mixture of plasmid DNA and lipofectamine (GIBCO BRL/Life Technologies, Gaithersburg, MD) at a ratio of 1:6 in 1 ml of DMEM for 6 h. The medium was then replaced with fresh DMEM containing 10% fetal bovine serum and incubated further for 24 h. Cells were washed three times with phosphate-buffered saline and lysed with an addition of 100 µl of reporter lysis buffer (Promega). Cell lysates were centrifuged for 10 min at 10,000*g* to remove the debris. Ten ml of the supernatant were added with 100 µl of luciferase assay buffer [Schwartz et al., 1990]. The luminescence was measured in a Packard liquid scintillation counter. Plasmid RSV-CAT was included in all transfection experiments as an internal control for transfection efficiency.

Reporter Plasmid Construction

The DNA fragment containing −133 to +33 sequence of the hTK promoter was cloned to a luciferase vector, pGL-2-Basic (Promega) in a sense direction to obtain p(−133/+33)TK-Luc plasmid as described previously [Chang et al., 1995]. To generate mutation at distal or proximal CCAAT box of TK promoter, PCR based oligonucleotide-directed mutagenesis was carried out by using p(−133/+33)TK-Luc plasmid as a template. Oligonucleotides A and C were the primers used for PCR to generate the mutated distal CCAAT fragment, which was digested by *Nco*I and *Kpn*I. This mutated DNA fragment was substituted for the wild-type (*Nco*I/*Kpn*I) fragment in p(−133/+33)TK-Luc

to obtain pDCM plasmid. Oligonucleotides B and C were the primers used for PCR to generate mutated proximal CCAAT fragment, which was digested by *Bal*I and *Kpn*I. This mutated DNA fragment was substituted for the wild-type (*Bal*I/*Kpn*I) fragment in p(−133/+33)TK-Luc to obtain pPCM plasmid. Oligonucleotide A: 5′CCGCCATGGGGCCCTTCAGCGCC3′ spanning from −56 to −78 of non-coding strand of TK promoter, in which underlined 'CT' represents a substitution for the conserved AA in the distal CCAAT box. Oligonucleotide B: 5′TGCTGGCCAGTCACGAGCCGGC3′ spanning from −30 to −51 of non-coding strand of TK promoter, in which underlined 'G' represents a substitution for the A in the proximal CCAAT box. Oligonucleotide C: 5′TTGGGTACCGGGCCCCCCCTCG3′ is a polylinker sequence on the coding strand of p(−133/+33)TK-Luc plasmid. Plasmid with mutations at both proximal and distal CCAAT boxes was generated by substituting a Nco/Kpn DNA fragment from pDCM plasmid with pPCM plasmid at position between −133 and −64 to obtain pD/P. The constructs, pM102 was generated as follows. A synthetic oligomer containing sequence spanning from −133 to −58 of the coding strand with a mutated sequence at −106 to −102 (CCAA → AGAT) was annealed with its complementary-strand oligomer followed by *Nco*I/*Kpn*I digestion. The resultant DNA fragment was cloned into a 5,690 bp *Nco*I/*Kpn*I digested DNA fragment from p(TK−133/+33)Luc to generate pM102. For construct pSpM, a synthetic oligomer containing sequence spanning from −133 to −77 of the coding strand with a mutated sequence at −118 to −113 (GGGCGG → ATGCAT) was annealed with its complementary-strand oligomer followed by *Eag*I/*Kpn*I digestion. The resultant DNA fragment was cloned into a 5,690 bp *Eag*I/*Kpn*I digested DNA fragment from p(TK−133/+33)Luc to generate pSpM. All reporter constructs were confirmed by DNA sequencing.

Preparation of Nuclear Extracts and Gel Mobility Shift Assays

Nuclear extracts were prepared from HL-60 cells as previously described [Dignam et al., 1983]. The bandshift reactions each contained 0.025 pmol of radiolabeled probe (40,000 cpm), 0.5 mg of poly(dI:dC), 30 µg of nuclear protein, and variable amounts of unlabeled competitor (when applicable) in a final concentration of 50 mM NaCl, 30 mM KCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. In the competition experiment, radioactive and nonradioactive DNA fragments were mixed prior to addition of nuclear extract. Reactions were incubated at room temperature for 25 min and subsequently analyzed by electrophoresis through nondenaturing 4% polyacrylamide gels at 4°C in a buffer containing 15 mM Tris, pH 8.2, 15 mM boric acid, and 1.2 mM EDTA. After prerunning the gel for 1 h, electrophoresis was performed at 170 V for 1.5 h. The gels were dried and analyzed by autoradiography.

Immunoblotting

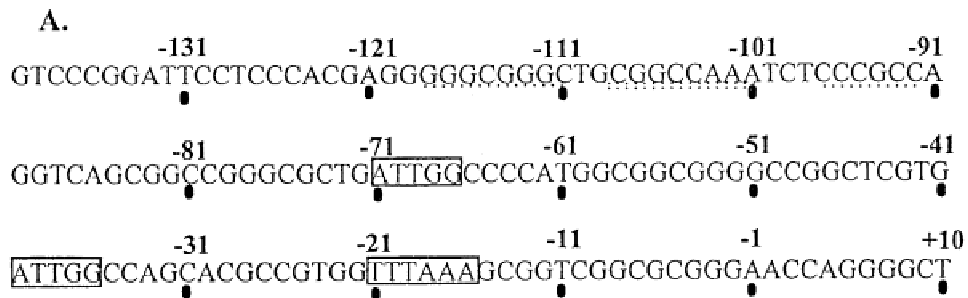
Thirty-five µg of protein extracted by RIPA buffer were separated on 12% SDS-PAGE followed by electrophoretic transfer to PVDF membrane (Millipore, Bedford, MA). After blocking with 5% powdered milk, the membrane was incubated with antiserum for 4 h, and treated for 2 h with alkaline phosphatase (AP) conjugated goat anti-rabbit IgG antibody (Promega). The AP color development was performed according to the manufacturer's instruction. The antibodies against p21$^{Waf1/Cip1}$, p16, and cyclin A were obtained from Santa Cruz Corporation and diluted at 1:2,000 for immunodetection.

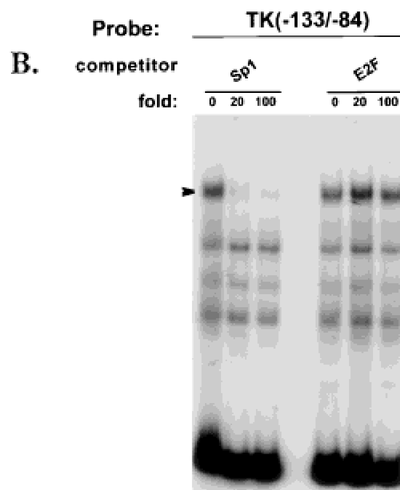
RESULTS

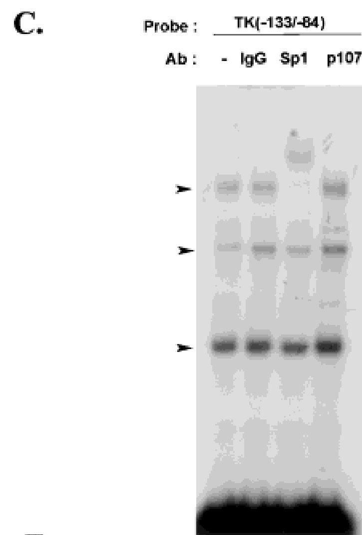
Identification of Sp1 as the Major Factor Binding to the −133/−84 Sequence of the hTK Promoter

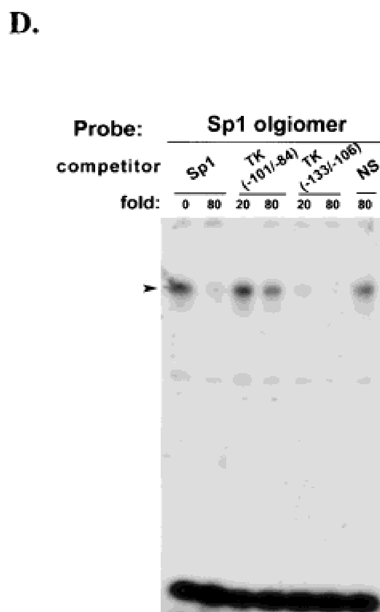
The hTK promoter region between −133 and −84 contains E2F-like sequence (−109/−101) and Sp1-binding sequence (−118/−113, −97/−92) as shown in Figure 1A. It has been demonstrated that the sequence between −109 and −84 of the hTK promoter is responsible for the TK *trans*activation observed at the G1/S transition period in CHO fibroblasts, which were stably transfected with the hTK promoter fused with CAT-reporter gene [Kim and Lee, 1992]. To verify whether transcription factors Sp1 and E2F can interact with TK promoter sequence region between −133 and −84, we first performed the bandshift assay with the radiolabeled oligonucleotides containing sequence spanning from TK(−133 to −84; Fig. 1B). Multiple complexes could be formed with the nuclear extracts in this assay. Two complexes indicated by arrow were specifically competed by the un-

labeled homologous DNA fragment. When oligonucleotides corresponding to E2F and Sp1 consensus binding sequence in the excessive amounts were added in the bandshift assays, it appeared that only one major DNA-protein complex was competed out by Sp1 consensus oligonucleotide. In contrast, none of the complexes was affected by the presence of E2F consensus









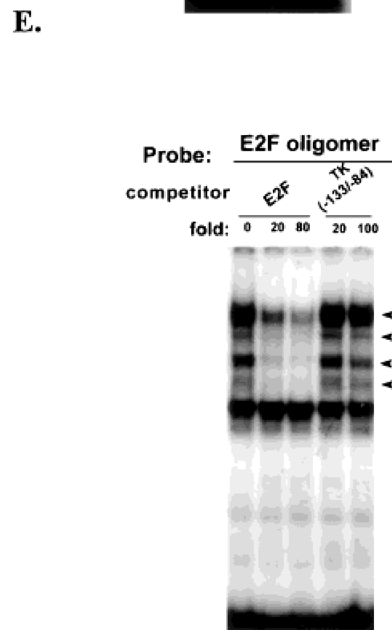


Figure 1. (Legend on p. 304.)

sequence (Fig. 1B). The complex with faster mobility could not be competed by the DNA fragment TK (−133/−64) carrying mutation at (−94/−84), indicating the involvement of the −94/−84 sequence in this complex formation (data now shown). The identity of Sp1 binding to TK(−133/−84) probe was further confirmed by adding antibody specifically against human Sp1 in the bandshift assay. In agreement with results from the competitive bandshift experiment, only the major complex could be specifically supershifted by anti-Sp1 antibody (Fig. 1C), confirming the presence of Sp1 in the major complex. When a consensus Sp1 oligomer was used as a probe for bandshift assay, only one DNA-protein complex was formed and specifically eliminated by an excess of unlabeled homologous oligonucleotide. This radiolabeled complex could be abrogated by unlabeled oligonucleotide containing the TK −133 to −106 sequence, but not by oligonucleotide containing the −101 to −84 sequence or nonspecific DNA fragments (Fig. 1D). This indicated that Sp1 binding to the hTK promoter is probably localized at −118/−113 position, but not at −97/−92. When E2F oligonucleotide derived from adenovirus E2 promoter was used as the probe for the bandshift assay, specific multiple complex formation could be clearly detected (Fig. 1E). However, an excess of unlabeled −133/−84 fragment in the binding reaction could not compete for specific E2F complex formation, indicating that this regulatory region of the hTK promoter does not interact with E2F under our in vitro assay condition.

Fig. 1. Interaction of the −133/−84 sequence of the hTK promoter with nuclear factors. **A:** The DNA sequence spanning from −140 to +10 of the hTK promoter. The putative CCAAT and TATA sites are shown in box, GC sequence underlined by single dot, and E2F site underlined by double dot. **B:** The ^{32}P-labeled TK(−133/−84) fragment was incubated with nuclear extract from HL-60 cells for the bandshift assay in the presence of 20- and 100-fold molar excess of double-stranded oligonucleotides corresponding to Sp1, or E2F consensus sequences as indicated. The double-stranded E2F and Sp1 consensus sequences were described in Materials and Methods. The arrowhead indicates the complex that could be specifically competed by the unlabeled TK(−133/−84) DNA fragment. **C:** TK(−133/−84) probe was incubated with 30 μg of nuclear protein from HL-60 cells in the absence or presence of 1 μg of IgG affinity-purified from normal rabbit, polyclonal antibody against human Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA) or p107 (Santa Cruz Biotechnology). **D:** The bandshift assays using ^{32}P-labeled Sp1 oligomer as a probe were performed in the absence or presence of unlabeled homologous competitors at a 80-fold molar excess, double-stranded oligonucleotides containing sequence −101/−84, −133/−106 of the hTK promoter at 20- and 80-fold molar excess and HpaII digested pBluescript KSII (2 pmole) as the nonspecific competitior. **E:** The bandshift assays using ^{32}P-labeled E2F oligomer as a probe were performed in the absence or presence of unlabeled homologous competitors and the DNA fragment of −133/−84 of the hTK promoter in the excessive amounts as indicated.

Effect of Various Mutations on the hTK Promoter Activity

To more precisely examine the role of the Sp1 binding site at −118/−113, sequence with homology to the E2F element at −106/−102, and two inverted CCAAT boxes at −71/−67 (distal) and −40/−36 (proximal) in mediating hTK transcription in cells, we generated a series of mutations in specific sequences of the hTK promoter (+33/−133) within the luciferase reporter construct pTK(+33/−133)LUC. Five mutant constructs, pCPM, pCDM, pP/D, pM102, and pSpmut, were generated, each containing mutation at proximal, distal and both CCAAT boxes, and sequences at −106/−102, −118/−113, respectively. Because of the low transfection efficiency in HL-60 cells, here we used HeLa cells for the transient transfection experiment. These constructs were individually cotransfected with RSV-CAT into HeLa cells and compared their promoter activity relative to the wild type promoter. We found that mutation at −106/−102, with homologous sequence to E2F element, has little effect on the promoter activity. In contrast, mutation at Sp1 binding site, −118/−113, caused about 50% of reduction of the hTK promoter activity (Fig. 2).

Previous studies in different cell lines have already demonstrated that the distal CCAAT box plays an essential role for transactivation of the hTK promoter [Arcot et al., 1989; Mao et al., 1995]. Transfection assays in HeLa cells showed that each mutation at proximal or distal CCAAT box yielded near 50% reduction of the promoter activity, suggesting that both CCAAT boxes are functional and are involved in activating the hTK promoter in HeLa cells (Fig. 2). Double mutations at these two CCAAT boxes abolished the hTK promoter activity (Fig. 2). As the promoter carrying double mutations at two inverted CCAAT boxes still retains an intact Sp1 binding site at −118/−113, this result suggests that Sp1 binding at −118/−113 alone cannot activate the transcription of the hTK promoter.

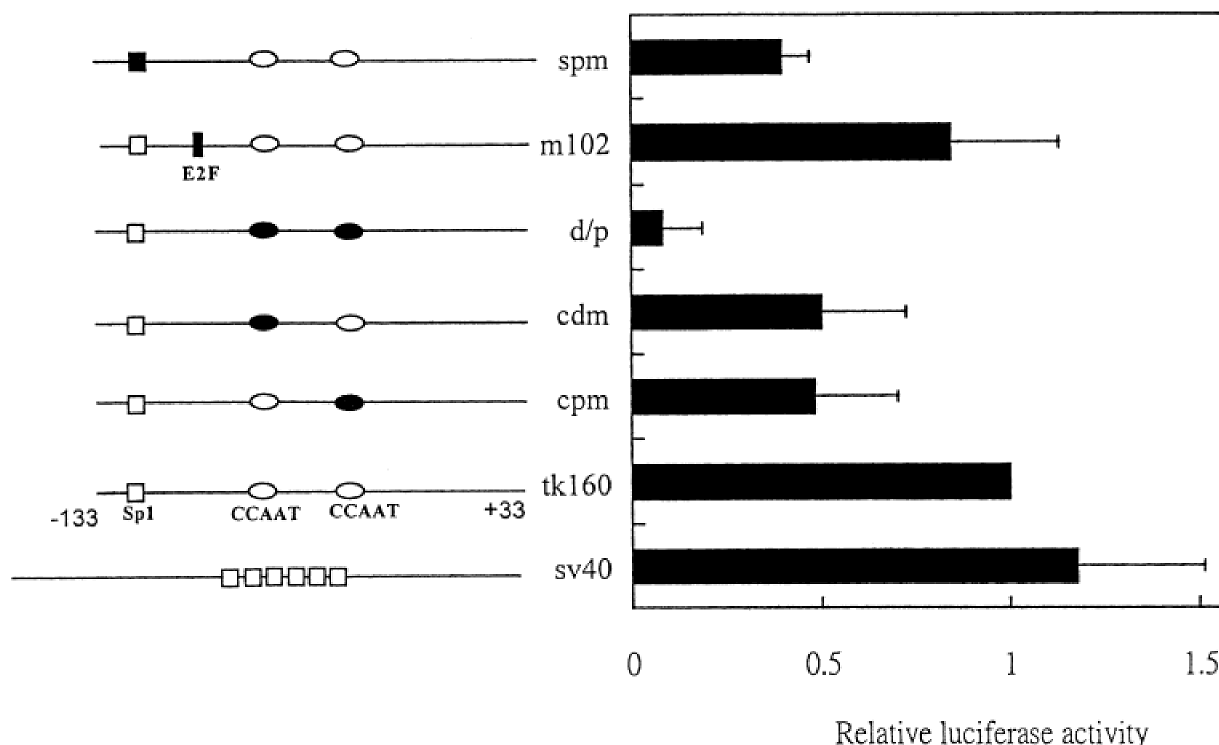


Fig. 2. Activities of the hTK promoter carrying various mutation in HeLa cells. **Left:** Schematic diagram of the wild-type hTK promoter (−133 to +33) and five mutated promoters fused to the luciferase reporter gene created as described in Materials and Methods. The Sp1 binding site is shown as square, the putative E2F site as rectangle, and the CCAAT box as ellipse; dark shapes marked all mutations. HeLa cells (2×10^5) were transfected with pSV40-Luc (Promega, Madison, WI), p(−133/+33)TK-Luc (TK160), or mutated plasmid (1 μg) together with equal amount of the RSV-CAT plasmid, which contained the CAT (chloramphenicol acetyltransferase) gene under the control of the Rous sarcoma virus long terminal repeat. **Right:** Luciferase activity was normalized by CAT activity and expressed as percentage of that from the wild-type p(−133/+33)TK-Luc construct. Values are the average of six independent experiments and error bars represent the standard deviation of the mean.

NF-Y's Binding Contributes to the Major DNA-Protein Interaction in the Cell-Cycle Regulatory Region of the hTK Promoter

Because Sp1-mediated activation of the hTK promoter required the occupancy of the CCAAT box, we then investigated the relative contribution of Sp1 and NF-Y when binding to the promoter. We used a longer DNA probe covering −133/−64 sequence for bandshift assays to analyze the relationship between Sp1 and NF-Y when binding to the hTK promoter. Using nuclear extract of HL-60 cells, one major DNA-protein complex formed in a diffuse and broad band, and another minor DNA-protein complex with the slowest mobility could be clearly seen in the bandshift assay (Fig. 3). Increasing amounts of Sp1 competitor in the DNA binding reactions could specifically reduce the formation of this DNA-protein complex a, indicating the presence of Sp1 in complex a (Fig. 3). When the bandshift assays were performed in the presence of increasing amounts of CCAAT oligonucleotide, formation of complexes a also became abolished. Evidently, complex a is a result of the concomitant binding of NF-Y and Sp1 to the −133/−64 segment. Moreover, the prominent DNA-protein complex b formation was abrogated in the presence of CCAAT competitor, indicating a strong interaction between factor with the CCAAT binding site. Taken together, NF-Y's binding contributes to the major DNA-protein interaction in this region.

Involvement of Cyclin-Dependent Kinase in Transcriptional Activation of the hTK Promoter

As transcriptional activation of the hTK promoter in normal cells is a cell cycle regulatory event, we further addressed the question of whether the general cell cycle regulators can modulate the activity of the hTK promoter. For this purpose, we cotransfected expression vector of human p16 or p21 under the control of the

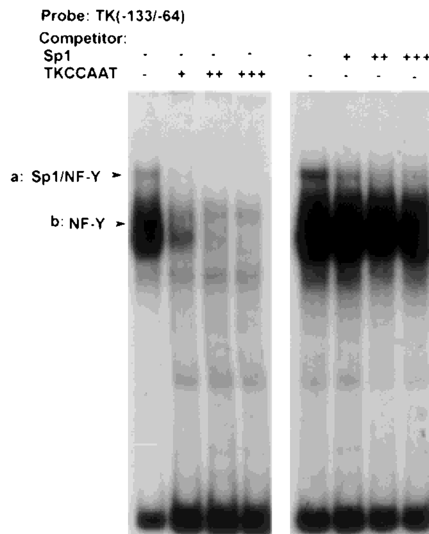


Fig. 3. Binding of NF-Y and Sp1 to the sequence of TK(−133/ −64). DNA fragment of TK(−133/−64) was ^{32}P-labeled and incubated with 30 μg of nuclear proteins from HL-60 cells for the bandshift assays. All assays were performed in the presence of increasing amounts of Sp1 competitor or TK-CCAAT oligonucleotides corresponding to the −86/−64 sequence of the human TK promoter (0-, 20-, 100-fold of molar excess) as indicated.

cytomegalovirus early promoter [Xiong et al., 1992; Serrano et al., 1995] with the hTK promoter reporter plasmid into HeLa cells. It appeared that ectopic expression of p16 and p21 in Hela cells could significantly decrease the reporter activity expressed from pTK(+33/ −133)Luc, indicating that activation of the hTK promoter is indeed related to the CDKs' activities (Fig. 4A). Reporter activity expressed from the hTK promoter was significantly increased when pCMVp16 was cotransfected with increasing amounts of pCMV-cyclin A (Fig. 4A). Clearly, inhibition of the hTK promoter activity by p16 could be overcome by overexpressing cyclin A in HeLa cells. In contrast, cotransfection of cyclin A expression vector could not recover p21-mediated inhibition of the hTK promoter activity in HeLa cells (Fig. 4A). We reasoned this as a result of the universal inhibitory effect by p21 on most of CDKs. The cell lysates were further analyzed by Western blot to ensure that the ectopic expressed levels of cyclin A were similar in the cells cotransfected with pCMV p16 and pCMVp21, respectively (Fig. 4B). Because p21 was highly expressed in the transfected cells, it is also possible that the amount of cyclin A is not enough to sequester p21 that overexpressed in the cells, and relieves the p21-mediated inhibition. Nevertheless, the ecotopic expression of cyclin A overrides p16-mediated inhibition of the TK promoter, implicating a more direct role of cyclin A in activation of the TK promoter.

DISCUSSION

In an attempt to elucidate the molecular processes governing *trans*activation of the hTK promoter, we determined the *cis*-elements contributory to the maximal activity of the hTK promoter in HeLa cells, and the role of CDK regulators in its activation in tumor cell HeLa. Here, we established three points: (i) the cooperation between NF-Y binding at two inverted CCAAT boxes and Sp1 binding at −118/−113 is probably the most important key factor for maintaining the maximal transcriptional potential for this promoter in HeLa cells; (ii) the event of NF-Y binding at one or the other inverted CCAAT box is a necessary step for Sp1-mediated activation of the hTK promoter; and (iii) transcriptional activation of the hTK promoter can be modulated by regulating the CDK activity.

Our bandshift assay and mutation analysis in HeLa cells clearly indicated that Sp1 binding at −118/−113 could contribute to 50% of the promoter activity. Yet, the mutated promoter containing the double mutations at two inverted CCAAT boxes located at −71/−67 and −40/−36 no longer expressed the hTK promoter activity in HeLa cells. Since the Sp1 binding site was still intact for this mutated promoter, it is evident that the functional role of Sp1 in modulating the hTK promoter requires NF-Y binding at two inverted CCAAT boxes as a prerequisite step. It has been described that NF-Y binding to the HLA-DRA promoter can facilitate in vivo recruitment of other upstream DNA binding transcription factors [Wright et al., 1994], suggesting the assembly role of NF-Y in proximal promoter complex formation. Data from the bandshift assay in this study have shown that in vitro Sp1 can bind to the hTK promoter region −133/−64 independently. Therefore, it is possible that the active transcriptional assembly requires NF-Y binding to the CCAAT box, at which Sp1 binding to the promoter region can positively modulate the assembly.

As for the role of E2F in the regulation of the human TK promoter, data obtained from our bandshift assays did not reveal any clear evidence for the presence of E2F binding within

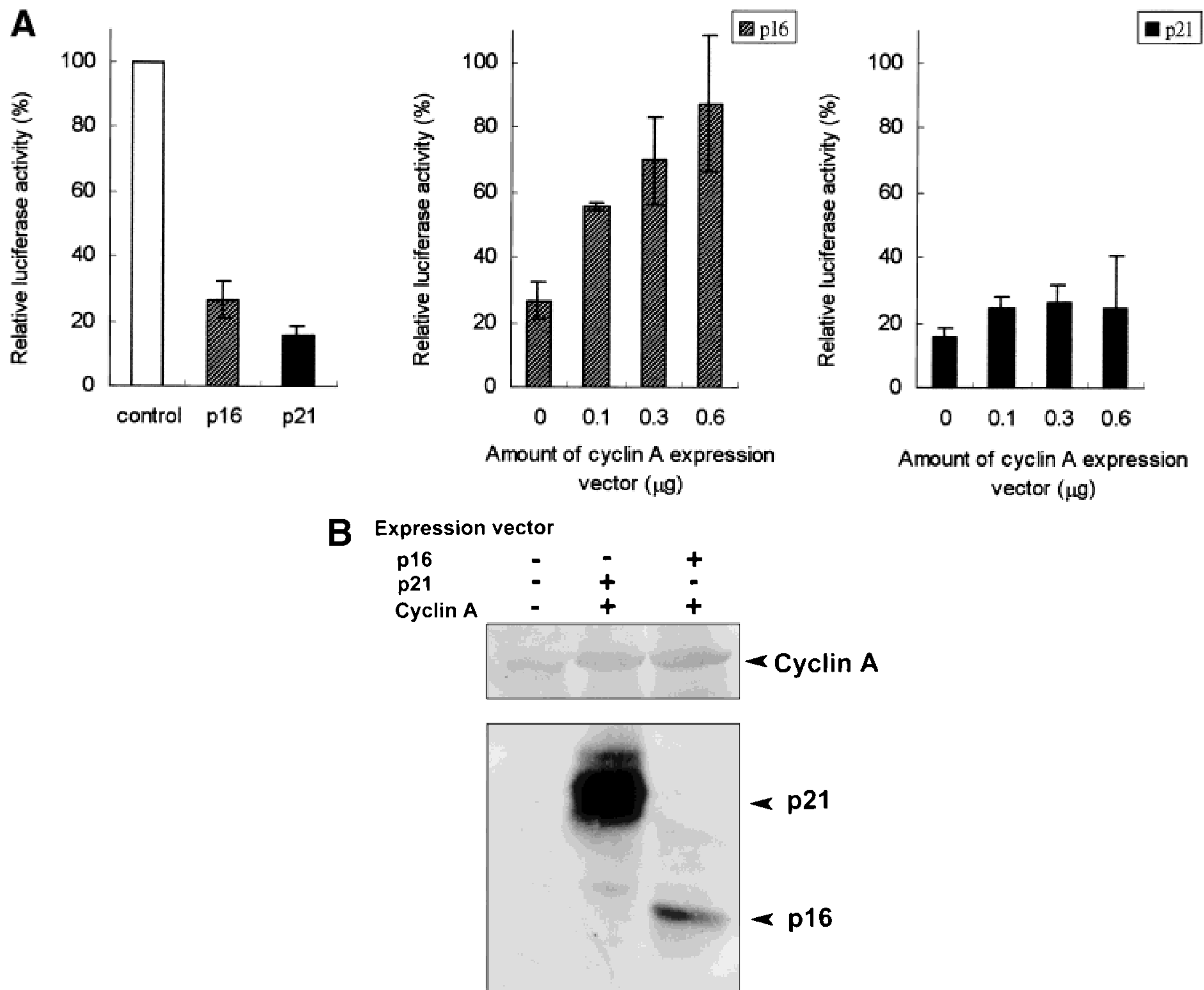


Fig. 4. Cyclin A abrogates p16, but not p21, mediated inhibition of the hTK promoter activity. **A:** Reporter plasmid p(−133/+33)TK-Luc (1 μg) was transfected into HeLa cells (2×10^5) together with 0.3 μg control pCDNA3, pCMVp16, or pCMVp21 expression vector. The above cotransfection experiments were also performed in combination with different amounts pCMVcyclin A as indicated. Values are the average of two independent experiments. The luciferase activities were measured in duplicate. Numbers were divided by the values obtained from cells transfected with the reporter plasmid p(−133/+33)TK-Luc plus control vector only. **B:** Western blot analysis of cyclin A p16 and p21 for cell extract (35 μg of protein) from HeLa cells transfected with control pCDNA3 only, pCMV cyclin A (0.3 μg) in the presence of 0.3 μg of pCMVp16 or pCMVp21.

the region of −133/−84 in the nuclear extracts, even though a putative E2F-like binding sites (−107/−101, 5GGCCAAA3) are present in the sequence. Furthermore, the hTK promoter carrying mutation at −106/−102 did not cause significant effect on the promoter activity in HeLa cells. Thus, E2F binding site at −107/−101 is not a *cis*-element for this promoter. By the in vivo footprinting analysis, it has been shown that the E2F-like sequence at −97/−89 is constantly protected in normal human fibroblasts during growth stimulation [Tommasi and Pfeifer, 1997]. Although we found that the −94/−84 sequence can be involved in another DNA/protein complex formation shown in Figure 1, the protein present in this complex has not been identified. Mutation at −94/−84 can result in 30% increase of the hTK promoter activity, indicating the presence of a repression mechanism (data not shown). Overall, it still remains to determine which nuclear factor is indeed responsible for this binding.

Our laboratory has previously shown that ectopic expression of cyclin A can act as a positive modulator for the hTK promoter in normal human IMR-90 HDFs, but not in HeLa cells [Chang et al., 1995]. Since the basal activity of the hTK promoter expressed in HeLa cells was much higher than that in normal IMR-90 HDFs, we hypothesized that elevated level of cyclin A

in HeLa cells might constitute a cellular environment for the hTK promoter to stay fully activated. Here, we showed that p21 and p16 when overexpressed in HeLa cells inhibited the hTK promoter activity, further confirming the nature of CDK-related activation of the hTK promoter. Because p16-mediated inhibition of the hTK promoter activity was abrogated by ectopic expression of cyclin A, the cyclin A-mediated activation event seems to be more directly involved in the transactivation. The direct target of p16 is cyclin D/CDK4, which is a G1 phase kinase, and cyclin A/CDK2 complex is well known to be activated in the S phase, at which timing TK promoter is also activated [Pines and Hunter, 1991; Pagano et al., 1992; Nigg, 1995]. Therefore, it is quite possible that the excess amount of p16 in the cell blocks the S phase entry, leading to down regulation of both cyclin A/CDK2 and TK activation events. Taken together, we proposed that NF-Y cooperates with Sp1 in transcriptional activation of the hTK promoter, and this transcriptional assembly may be modulated by the activity of CDK2. In tumor cells the highly elevated level of CDK activity may constitute a cellular environment for maximal transcriptional activation of the hTK promoter.

ACKNOWLEDGMENTS

We thank Drs. David Beach and Yue Xiong for providing expression vectors of p16 and p21, respectively.

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