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METHYLATION-SENSITIVE PROTEIN-DNA INTERACTION AT THE CELL CYCLE REGULATORY DOMAIN OF HUMAN THYMIDINE KINASE PROMOTER

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SUMMARY: We have investigated the DNA-protein complex formation in nuclear extracts of human cells using the sequence of cell-cycle regulatory unit (CCRU) of human thymidine kinase (TK) promoter. It appeared that a distinct DNA-protein complex was present in three human tumor cell lines and that the CCAAT box within the sequence of CCRU was a necessary element for complex formation. Upon 4 days of serum deprivation, this DNA-protein complex remained unchanged in HeLa cells, but the expression of TK mRNA was decreased. Furthermore, DNA methylation of the HhaI site of the CCRU sequence of the TK promoter greatly reduced the binding activity of nuclear proteins from different human tumor cell lines. On the basis of these data, we proposed a possible role for DNA methylation in the regulation of TK transcription during late G1/S phase progression of the cell cycle.

Thymidine kinase (TK) is a cytosolic enzyme that catalyzes the transfer of the terminal phosphate of ATP to the 5' hydroxyl group of thymidine to form dTMP. The level of TK activity is highly dependent on the growth state and cell cycle phase in mammalian cells. It is one of the best studied enzyme systems that is closely associated with cell proliferation. The regulation of TK expression has been revealed at the multiple control levels such as transcriptional rate (1, 2, 3), post-transcriptional processing (4, 5, 6), translational efficiency (7, 8), and post-translational stability (9). Recently, studies on the cell cycle using murine cells have shown that formation of the transcriptional complex, Yi, with the mouse TK promoter sequence contributes to the transcriptional activation of the TK gene in the late G1/S phase (10, 11).

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Transfection experiments that analyzing the heterologous human TK promoter in hamster cells have demonstrated that the upstream sequence of the TK promoter, spanning between -64 and -133 bp, is responsible for G1/S control during the cell cycle (12). This sequence has, therefore, been termed cell cycle regulatory unit (CCRU). Furthermore, the protein-binding site in this region required for the G1/S regulated transcription has been identified by site-directed mutagenesis experiments in the same cell system (13). However, it has also been shown that the binding activity of the nuclear protein to the 70 bp TK CCRU sequence remains constant throughout the period of growth stimulation in hamster cells (13). Hence, it has been postulated that post-translational modification or protein-protein interaction are possible mechanisms responsible for activating the transcription of TK gene during the G1/S phase (12,13).

In the present report, we investigated the DNA-protein complex formation in nuclear extracts isolated from different human tumor cell lines with the sequence of the 70-bp TK CCRU, using mobility shift electrophoresis analysis. We found that a specific DNA-protein complex formation occurred in HeLa, HL-60 and KG-1 cells and that the CCAAT box within the sequence of CCRU was a necessary element for complex formation. Although the DNA-protein binding patterns formed in these three human tumor cell lines were different from that observed in hamster cells, this distinct complex also remained unaltered in HeLa cells after serum starvation, suggesting that the cellular factor interacting with the CCRU sequence is not affected by growth arrest. Many studies on the regulation of gene expression have shown that CpG methylation of the promoters contributes to the repression of many genes in cultured cell lines (14, 15). Along this line, the effect of DNA methylation on the binding of nuclear protein to 70bp TK CCRU was examined. We found that DNA binding activity of the nuclear protein in the three human tumor cell lines was greatly reduced when the HhaI site near 5' upstream of the CCAAT box of the CCRU sequence was methylated. According to these data, we propose methylation of the TK promoter region in the genomic DNA during the cell cycle as another possible mechanism regulating TK gene activity.

MATERIAL AND METHODS

Cell Cultures HL-60, HeLa, KG-1 cells were obtained from American Type Culture Collection, and grown in RPMI-1640 (GIBCO) supplemented with 10% heat inactivated fetal bovine serum (Hyclone) in a humidified atmosphere of 95% air, 5% CO_2. The confluent monolayer cultures and cell suspensions (5x 10^5 cells/ml) were harvested for nuclear extract preparations.

Preparation of Probes for Gel Mobility Shift Assays The 70-bp HinfI/NcoI fragment, spanning from -64 to -133 of TK promoter region , was subcloned into the EcoRV site of pBluescript SK vector (Stratagene). After digestion of this plasmid with EcoRI and HindIII, an 86 bp EcoRI/ HindIII fragment was isolated and labeled with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase I. The 70-bp NcoI/HinfI fragment of TK promoter contains one HpaII and one HhaI site , which are next to each other. The subcloned plasmid described above was methylated by HpaII methylase and HhaI methylase (Bio-Lab), separately. The specifically methylated plasmid, resistant to the corresponding HpaII or HhaI digestion, was digested by EcoRI and HindIII, followed by isolation and radiolabeling of the 86-bp fragment

Preparation of Nuclear Extracts and Gel Mobility Shift Assays Nuclear extracts were prepared by the method of Dignam et al. (16). Final protein concentration, measured by the method of Bradford(Bio-Rad), was approximately 2.5 mg/ml. The gel mobility shift reactions each contained 0.025 pmol of radiolabeled probe (40,000 cpm), 0.5 μg of poly(dI:dC), 10μg of nuclear extract, 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 non-radioactive 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 non-denaturing 4% polyacrylamide gels 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 volt for 1.5 h. The gels were dried and analyzed by autoradiography.

Northern Blot Total cellular RNA was prepared from cells using the guanidine thiocyanate procedure described by Chomczynski and Sacchi (17). Polyadenylated RNA was enriched by affinity chromatography on an oligo(dT) cellulose column (18). RNAs separated by formaldehyde gel electrophoresis were subsequently transferred to nitrocellulose paper (Schleicher & Schuell), and hybridized to hTK cDNA probe (6).

RESULTS AND DISCUSSION

The CCRU sequence located between -64 and -133 of the human TK promoter was used to investigate the interaction between this DNA sequence and nuclear proteins isolated from human tumor cell lines. By gel mobility shift analysis (Figure 1), nuclear proteins present in KG-1, HeLa, and HL-60 cells recognized sequences between -64 and -133 of human TK promoter and formed a specific complex, which could be competed by homologous sequence but not by other DNA fragment purified from pBluescript SK vector. The binding pattern was clearly different from the binding complexes of the nuclear proteins isolated from hamster cells, where two distinct well separated complexes were detected (12). This

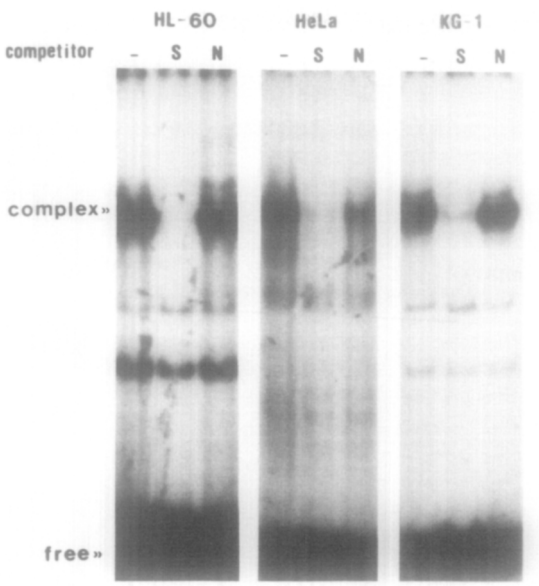

Figure 1. Gel mobility shift assay with TK 70-bp CCRU in different human tumor cell lines. The mobility shift assay was carried out in nuclear extracts from exponentially growing HL-60, HeLa, and KG-1 cells with the end-labeled probe spanning -64 to -133 of the TK promoter. The specificity of the binding complex is indicated by the competition assay with a 20-fold molar excess of unlabled DNA fragment. The specific competitor (S) was the sequence of -64 to -133 of the TK promoter. The nonspecific competitor (N) was the 110bp fragment purified from the the HpaII digest of pBluescript SK vector.

difference in binding pattern between human and hamster cells may suggest that a divergent nuclear protein binding activity has occurred during evolution.

The sequence of TK 70-bp CCRU contains one inverted CCAAT box (-67/-71) and a Yi like binding domain (-109/-84) (13). The CCAAT box within this sequence has been described as being responsible for the high basal transcriptional activity of hTK promoter in hamster cells (12). Since human tumor cells exhibited a binding pattern distinct from that of hamster cells with the TK 70 bp CCRU probe, we next determined whether the CCAAT box could contribute to complex formation. A radioactive probe spanning -75 to -133 was prepared by HhaI digestion of TK 70 bp CCRU to delete the CCAAT box located between -64 to -74 for the binding assay. Figure 2A indicates that the nuclear proteins from neither HeLa nor HL-60 cells could form a complex with this sequence. Furthermore, the unlabeled -75/-133 sequence (CCAAT deleted) could not compete for nuclear protein binding to the radiolabeled TK 70-bp CCRU probe (Figure 2B). According to these results, we concluded that binding of the nuclear

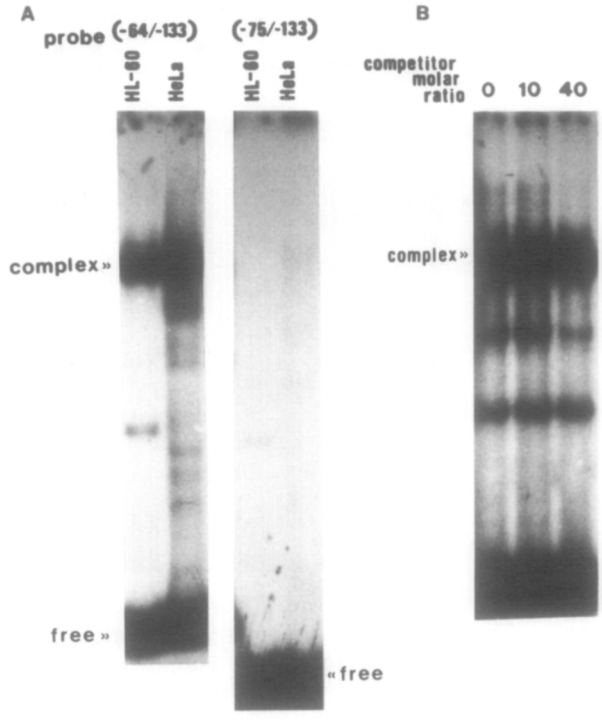

Figure 2. The role of CCAAT box in the TK CCRU sequence on binding activity. (A) The sequence spanning -75 to -133 was prepared by digestion of TK 70-bp CCRU by HhaI to delete the CCAAT box and end-labeling was used for the binding assay and compared to the TK 70-bp CCRU probe without CCAAT deletion (-64/-133). (B) The binding complex formed between the HL-60 nuclear proteins and the TK 70-bp CCRU probe (-64/-133) was competed by the unlabeled sequence spanning -75 to -133 described above in 0, 10- and 40 -fold molar excess.

protein to CCAAT box is a necessary step for mediating DNA-protein complex formation.

In this study, we also compared the complex formation in both proliferating HeLa cells and in cells that had been growth-arrested by serum deprivation for 4 days. As shown in Figure 3, the binding property of the nuclear protein was not changed by growth arrest, but that the expression of TK mRNA was decreased after serum deprivation, an indication that the complex is constitutively formed in HeLa cells. This result was consistent with the observation with hamster cells (12).

Numerous studies on DNA methylation have suggested that methylation of the promoter region can greatly change the transcriptional activity of the downstream coding gene. Since the 5' upstream of CCAAT box of 70-bp TK CCRU contains one HhaI and one HpaII site adjacent to

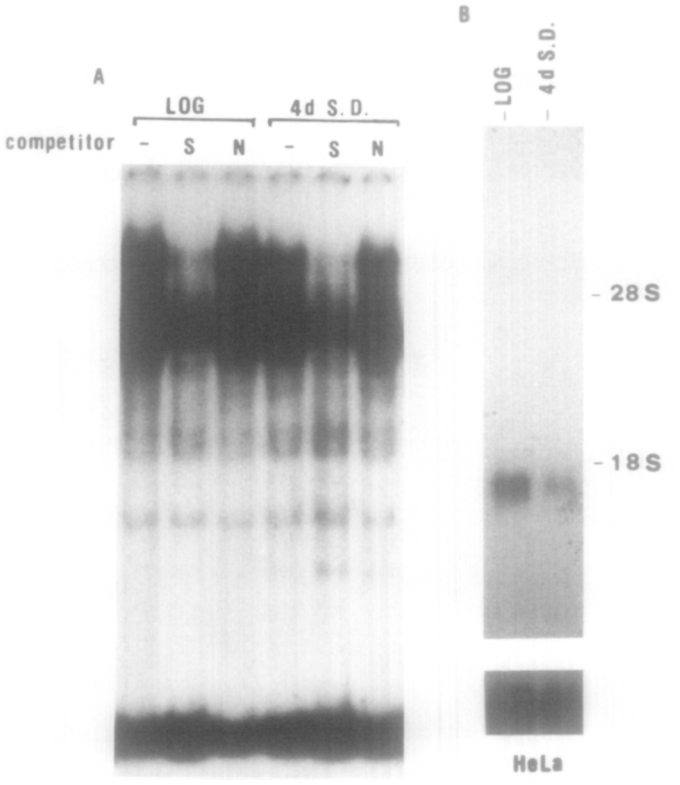

Figure 3. The effect of serum deprivation on the expression of TK mRNA and TK CCRU binding complex in HeLa cells. (A) The specific TK CCRU binding assay was carried out in the nuclear extracts prepared from HeLa cells at the log phase and the cells that had been serum-deprived for 4 days. The specificity of the binding complex was indicated by the competition assay as described in Figure 1. (B) Poly(A+) RNAs (2μg) from the log phase and serum-deprived (4 days) HeLa cells were analyzed by Northern blot and hybridized with ^{32}P-labeled pTK11. The same blot was later rehybridized with a β-actin probe.

each other as shown in Figure 4A, we employed specific methylase to methylate these two sites to test the effect of specific methylation on the complex formation. As shown in Figure 4B, HhaI methylation of this sequence greatly reduced the binding activity, while HpaII methylation, which is 3 bp upstream of the HhaI methylation site, caused only slight reduction in complex formation. We have tried to use Southern blot to determine the methylation pattern of the TK promoter of the genomic DNA in proliferating and in growth-arrested HeLa cells. However, the results (data not shown) were unclear, since HhaI or HpaII digestion of TK promoter region generated several DNA fragments smaller than 80 bp, which did not allow stringent washing in order to remove nonspecific hybridization. Nevertheless, our data presented here clearly indicate that the maintenance of DNA in an unmethylated form at the HhaI site, but not

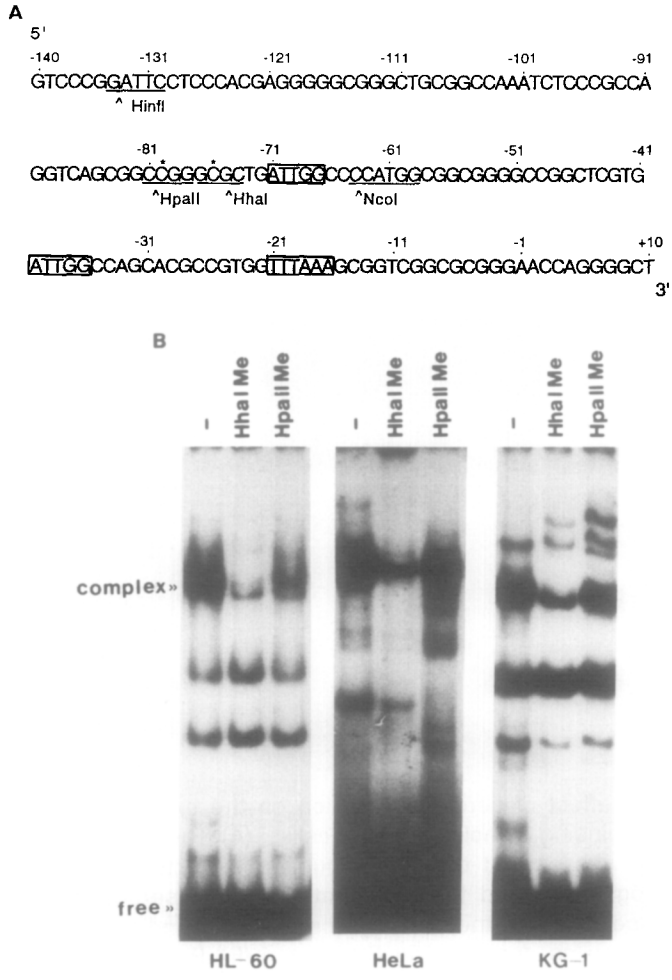

Figure 4. The effect of specific DNA methylation on the 70-bp TK CCRU binding activity. (A) The DNA sequence spanning -140 to +10 of the human TK promoter (19). The restrictive enzyme sites for HinfI, NcoI, HhaI, HpaII are indicated. The TATA box and the inverted CCAAT elements are boxed. The specific methylation sites are indicated by a star. (B) The ^{32}P-radiolabeled HhaI, HpaII methylated and unmethylated TK 70-bp CCRU sequence was prepared separately as described in Material and Methods and used for the DNA binding assays performed with the nuclear extract from HeLa, HL-60 , and KG-1.

the HpaII site, near the CCAAT box is important for the binding potential of nuclear protein. The question of whether the decrease in nuclear protein binding to the methylated HhaI site of CCRU is due to a direct interference of methylation with the DNA-complex formation or to the presence of the methyl-CpG binding protein remains to be established. Nevertheless, these results suggest that the methylation status of the TK promoter during the cell cycle may serve as a good candidate in controlling the transcriptional activity of the TK gene. It is possible

that during the early stages of DNA replication the CpG sites within the TK CCRU sequence becomes less methylated, leading to a more stable transcriptional complex formation. As a result, the high level of TK gene transcription can continue during S phase. Contrarily, during growth-arrest, the TK promoter may be stably hypermethylated, which in turn would hinder transcriptional complex formation. At this stage, further analysis by other techniques is needed to define the role of DNA methylation on the regulation of TK transcription during the cell cycle.

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