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Analysis of Sequence-Specific Binding Activity of Cis-Elements in Human Thymidine Kinase Gene Promoter During G1/S Phase Transition

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Expression of thymidine kinase (TK) gene in normal human diploid cells is both cell cycle and age dependent and appears to be transcriptionally regulated. Several studies have indicated that the G1/S control sequence may reside within the region of about 130 bp upstream of the transcription initiation site. We have previously shown that a trans-acting factor, CBP/tk (CCAAT binding protein for TK gene), binds to either one of the two inverted CCAAT boxes in a cell cycle- and age-dependent manner (Pang and Chen, 1993, J. Biol. Chem., *268*:2909–2916). An upstream 25 bp fragment (−109/−84), containing both Yi-like and E2F-like binding sites, has recently been proposed to be essential for the G1/S regulation of human TK gene. To assess the contribution of various cis-elements in human TK promoter to the G1/S regulation, we have examined the binding activity of these cis-elements in the nuclear extracts derived from human IMR-90 cells at low passage number. Our results indicated that no binding activity could be detected using either the 25 bp fragment (−109/−94) or the authentic Yi sequence. However, Yi binding activity was observed in SV-40 transformed IMR-90 cells. In contrast, the 28 bp fragment (−91/−64) that contains the distal inverted CCAAT box exhibited a strong binding in serum-stimulated young IMR-90 cells. The binding of CBP/tk to the 28 bp fragment was abolished by a single base mutation in the CCAAT box. The CBP/tk binding of the 28 bp fragment could not be displaced by either the 25 bp fragment or the authentic Yi element. A deletion of the 5′-flanking region of the 28 bp fragment up to 5 bases also abolished the binding activity. The CBP/tk binding in IMR-90 cells was supershifted by antiserum against NF-Ya, but not by antiserum made against p107, pRb, cyclin A, $p33^{cdk2}$, or $p34^{cdc2}$. Taken together, our results suggest that the G1/S regulatory cis-element in human TK promoter may be confined only to CBP/tk binding sites.

Thymidine kinase (TK, ATP:thymidine 5′-phosphotransferase, EC2.7.1.21) catalyzes the ATP-dependent phosphorylation of thymidine to thymidine 5′-monophosphate. TK activity has been shown to be tightly coupled to DNA synthesis and increases dramatically at the G1/S boundary of the cell cycle (Bello, 1974). Regulation of TK gene expression can occur at transcriptional, post-transcriptional, and translational levels depending on cell types and their physiological states (Sherley and Kelly, 1988; Roehl and Conrad, 1990; Knight et al., 1987; Gross and Merrill, 1988; Lipson et al., 1989). It has been shown that transcriptional regulation plays a key role in normal diploid cells following serum stimulation (Stewart et al., 1987; Travali et al., 1988; Pang and Chen, 1993). The functional promoter and putative cis-regulatory elements of human TK gene have been defined by deletion and mutation analysis using either transiently or stably transfected cells. Arcot et al. (1989) showed that the promoter activity relies primarily on two inverted "CCAAT" boxes and a series of "GC" elements further upstream. Roehl and Conrad (1990) identified the region between 135 and 67 bp upstream of the cap site as G1/S phase regulated. Similarly, Kim and Lee (1991) found a 70 bp region between 133 and 64 bp upstream of the cap site in human TK promoter to be sufficient to confer cell cycle regulation. The cis-elements identified within this 70 bp region includes a GC box, a CCAAT-like sequence, Yi-like and E2F-like sequences, and an inverted CCAAT box. In contrast, Kreidberg and Kelly

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Abbreviations: TK, thymidine kinase; CBP/tk, CCAAT binding protein for thymidine kinase gene; DHFR, dihydrofolate reductase; TS, thymidylate synthase; PCNA, proliferating cell nuclear antigen; DNA pol α, DNA polymerase α; pRb, the retinoblastoma gene product; p34cdc, 34 kDa protein encoded by the cdc2 gene; cdk, cyclin-dependent kinase; p107, a pRb-family protein.

(1986) delimited the functional domain of the TK promoter to within an 83 bp region upstream of the initiation site. Similarly, Lipson et al. (1989) found that the 83 bp promoter element is as strong as longer promoters, suggesting that essential G1/S regulatory elements are within this region. The cis-elements identified in the 83 bp region include only two inverted CCAAT boxes and a TATA box. Sequence-specific binding to DNA fragments containing either one of the inverted CCAAT boxes has been demonstrated to be cell cycle and age dependent (Pang and Chen, 1993). Since methylation interference analysis suggests that the binding is likely due to a CCAAT binding protein, we termed the binding factor as CBP/tk, indicating CCAAT binding protein for TK gene (Pang and Chen, 1993).

In contrast to human TK promoter, the murine TK promoter does not contain either CCAAT or TATA elements (Lieberman et al., 1988). Three protein binding sites sharing a consensus sequence termed Yi have been identified (Dou et al., 1991). An inducible DNA binding activity was observed in mouse A31 cells as the cells crossed the G1/S boundary using the Yi sequence as the probe (Dou et al., 1991). Yi complex contains retinoblastoma-like protein (pRb) and cdc2 kinase (Dou et al., 1992) and may play an important role in murine TK gene regulation. Kim and Lee (1992) have noted that an upstream 25 bp fragment (−109/−84) in human TK promoter contains Yi-like and E2F-like motifs. They further demonstrated that the 25 bp fragment is the target of cyclin A in association with $p33^{cdk2}$ and p107 in hamster K12 cells (Li et al., 1993). This finding together with the reports that the mouse Yi complex contains pRb and $p34^{cdc2}$ (Dou et al., 1992) raise an interesting question on whether the 25 bp fragment or Yi-like sequences alone in human TK promoter may be involved in the regulation of TK gene in human cells. In view of the importance of p107, cyclin A, pRb, and cyclin-dependent kinases in cell cycle-dependent G1/S gene regulation, it seems also relevant to inquire whether trans-acting factor CBP/tk may contain any of these proteins.

The major goal of the present study is to define the key cis-element(s) which may regulate the cell cycle-dependent expression of TK gene in normal human cells. Toward this goal, we report that sequence-specific binding can be demonstrated in human cells only for the 28 bp fragment (−91/−64). Neither the 25 bp fragment (−109/−84) nor the authentic Yi sequence exhibits any cell cycle-dependent binding activities in IMR-90 human cells. Unlike the Yi binding complex in mouse nuclear extracts, the CBP/tk binding complex did not contain p107, Rb, cyclin A, $p34^{cdc2}$, or $p33^{cdk2}$. Taken together, our results suggest that, despite the presence of Yi-like and E2F-like motifs in human TK promoter, the CBP/tk binding site may represent the key cis-element which confers the G1/S regulation of the human TK gene.

MATERIALS AND METHODS

Cell culture

IMR-90 human embryonic lung fibroblasts were obtained from the Coriell Institute for Medical Research (Camden, N.J.) and were maintained in culture as previously described (Chang and Chen, 1988). SV-40 transformed IMR-90 cells, AG03204, were grown as monolayer cultures. Serum deprivation and serum stimulation were carried out as described before (Bradley et al., 1990; Pang and Chen, 1993). Growth arrest was confirmed by measuring [methyl-^{3}H]thymidine incorporation.

Oligonucleotides

The following oligonucleotides: the 28 bp fragment (w.t. 28, 5′-dAGGTC AGCGGCCGGGCGCTGATTGGCCC-3′), the 25 bp fragment (5′-dTGCGGCCAAATG CCCGCCAGGTAGCGGC-3′), the tandem authentic Yi sequence (5′-dGGACGAGGGGGC GGGGATCGAGGGGGCGGGGCGG-3′), 23w (5′-dAGCGGCCGGGCGCTGATTG GCCC-3′), 20w (5′-dCGG CCGGGCGCTGATTGGCC-3′), and 28m (5′-dAGGTC AGCGGCCGGGCGCTGACTGGCCC-3′) were synthesized in our laboratory using a Pharmacia LKB Gene Assembler Plus DNA synthesizer. The synthesized oligonucleotides were purified yb passing through NAP-10 column (Pharmacia, Piscataway, NJ). Complimentary synthetic oligonucleotides were annealed, end labeled with [γ-^{32}P]ATP and T4 kinase, and used for gel mobility shift assay.

Gel mobility shift assays

Nuclear extracts for DNA binding were prepared as described before (Pang and Chen, 1993). To inhibit protease activities, pepstatin A (0.1 μM) and leupeptin (0.1 μM) were always included in the extraction buffer. A typical binding mixture contained 10 μg nuclear extracts, 2 μg poly(dI-dC), 0.2 ng labeled DNA probe in a buffer of 10 mM Tris (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol. The specific activity of labeled DNA prove was >50,000 cpm/ng. The binding was carried out at room temperature for 30 min. The gel mobility shift assay was carried out on a 4% polyacrylamide gel under conditions as previously described (Pang and Chen, 1993). For immunoshift assay, 1 μl of the undiluted antiserum was added to the nuclear extracts in the binding mixture of 20 μl and incubated on ice for 20 min before the addition of oligonucleotide probe.

Materials

All tissue culture media and sera were obtained from GIBCO (Grand Island, NY). [γ-^{32}P]ATP (3,000 Ci/mmol) was from ICN Chemical Radioisotope Division (Irvine, CA). Restriction enzymes and other molecular biological supplies were from Promega (Madison, WI) or Pharmacia. All other chemicals were of reagent grade. Polyclonal anti-Rb and anti-cdc2 antisera were given by Dr. Yuen-Kai T. Fung (University of Southern California, San Francisco, CA). Anti-p107 (polyclonal) antiserum was from Dr. Mark E. Ewien (Dana-Farber Cancer Institute, Boston, MA). Anti-E2F1 (KH95, monoclonal) and anti-cdk2 (polyclonal) were from Drs. Edward Harlow, Kristian Helin, and Nicholas Dyson (MGH Cancer Center, Charlestown, MA). Anti-cyclin A (polyclonal) was from Dr. Tony Hunter (The Salk Institute, San Diego, CA). Anti-T antigen (monoclonal) was from Dr. Harvey Ozer (UMDNJ, Newark, NJ).

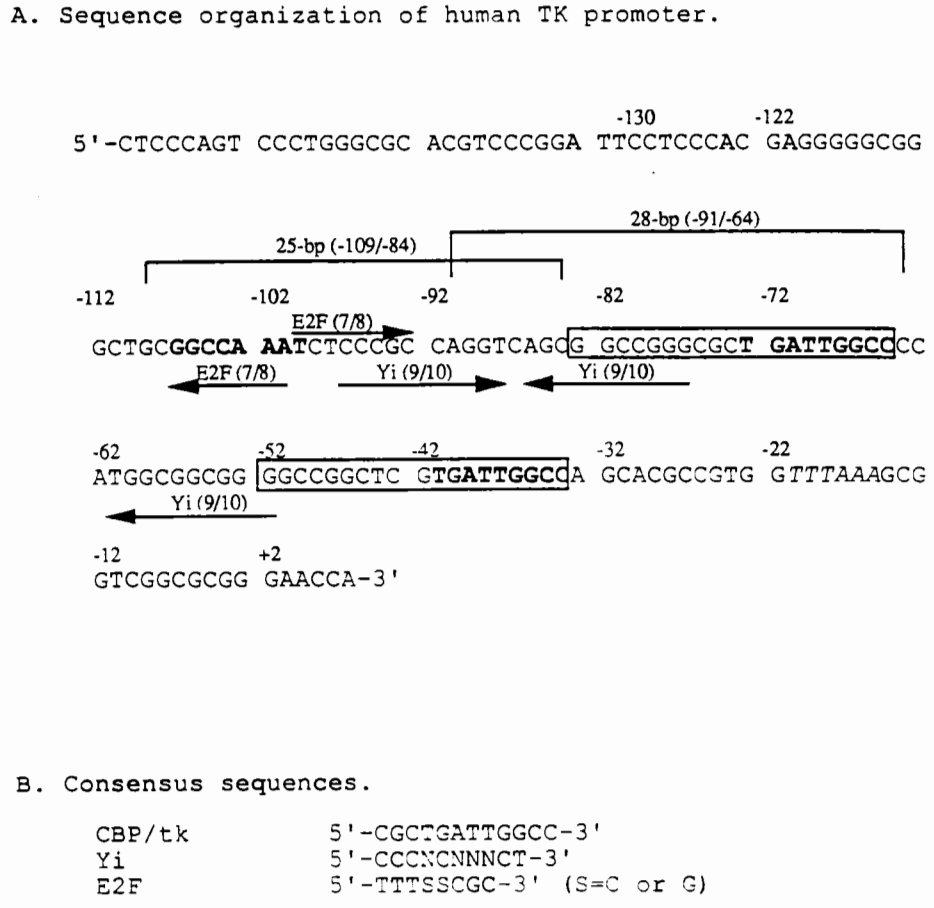


Fig. 1. Cis-elements in the human TK gene promoter. Position +1 is defined in the middle of the major transcription initiation site. The 19 bp repeats containing an inverted CCAAT motif are indicated in the boxes. A CCAAT-like motif (−107 to −100) is indicated in bold letters. Yi binding sites and E2F-like binding sites are indicated by arrows and the numbers in the parentheses indicate the fraction of matches relative to the consensus. The italic region (−21 to −16) is the TATA element. The 28 bp fragment spans −91 to −64, the 67 bp spans from −63 to +4, and the 25 bp fragment spans −109 to −84.

RESULTS

Analysis of cis-elements within the G1/S regulatory domain of human TK promoter

Figure 1 shows the sequence organization of the human TK promoter from −130 to +4. This sequence contains many potential binding sites for known nuclear factors and has been shown to be important for the cell cycle-dependent expression of human TK gene (Arcot and Deininger, 1992). The most notable one is a pair of inverted CCAAT boxes and their 5′-flanking "GC" rich regions. Together, they formed two direct 19 bp repeats as indicated (−83/−65; −52/−34). There exists another sequence GGCCAAATC (−107 to −99) that closely resembles a CCAAT binding motif. E2F is a trans-acting factor which recognizes the sequence with consensus 5′-TTTSSCGC-3′ (S = C or G; Nevins, 1992) and has been shown to be involved in the regulation of many G1/S genes (Nevins, 1992; Farnham et al., 1993). Two sequences bear close resemblance to E2F binding motif, one spans −108 to −101 and the other spans −103 to −96, each contains only one mismatch. Yi binding motif has recently been described in the mouse TK gene promoter with the consensus sequence CCCNCNNNCT (Dou et al., 1991). With only one mismatch allowed, one can identify three Yi-like sequences, one spans −97 to −88, and the other two spanning −86 to −77 and −61/−52 in an inverted direction.

Comparison of binding activity of 28 bp and 25 bp fragments in IMR-90 human cells

Within the region shown in Figure 1, a 28 bp fragment (−91/−64) and a 67 bp fragment (−63/+4) have been excised from the human TK promoter and both fragments bind to CBP/tk in a cell cycle- and age-dependent manner in normal human diploid fibroblasts (Pang and Chen, 1993). The binding sequence within the 67 bp fragment, including the 19 bp repeat, is almost identical to that of the 28 bp fragment (Pang and Chen, 1993). The 25 bp fragment (−109/−84), however, has recently been shown to exhibit a cell cycle-dependent binding activity in hamster cells (Li et al., 1993). Figure 2A shows that the motif arrangements of cis-elements in both the 28 bp fragment and the 25 bp fragment are strikingly similar; both contain a Yi motif (one mismatch each) with CCAAT or CCAAAT at upstream position. Figure 2B compares binding activities

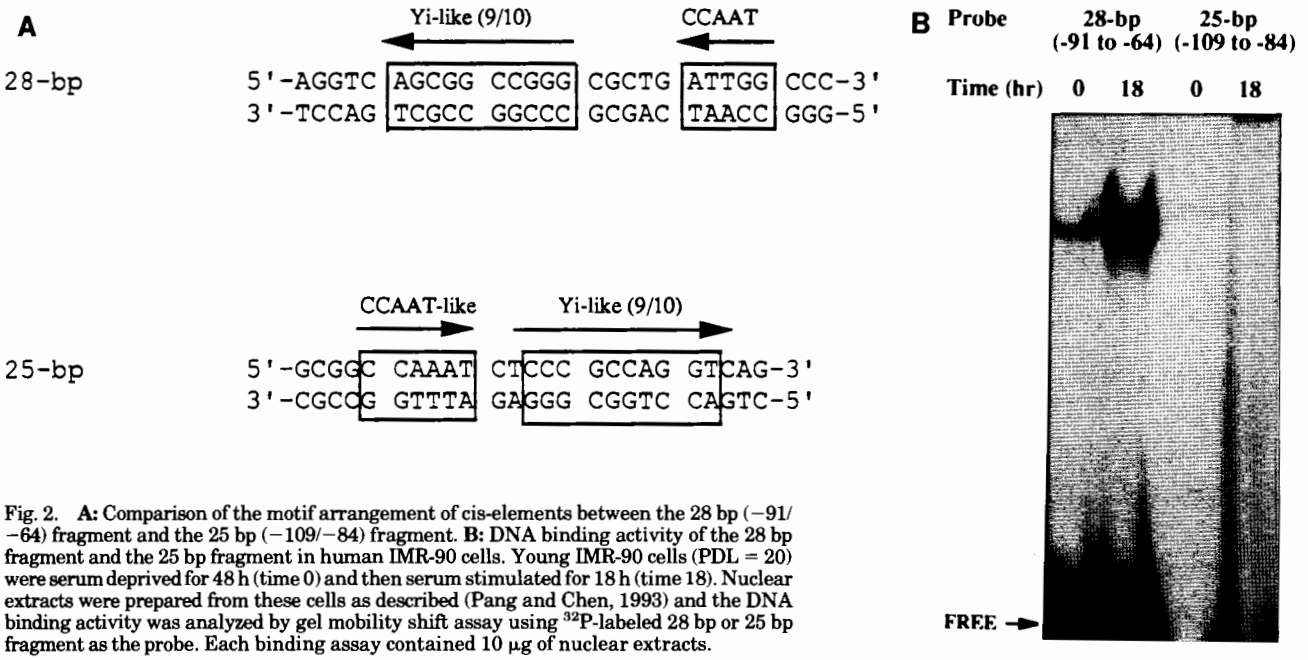


Fig. 2. **A:** Comparison of the motif arrangement of cis-elements between the 28 bp (−91/−64) fragment and the 25 bp (−109/−84) fragment. **B:** DNA binding activity of the 28 bp fragment and the 25 bp fragment in human IMR-90 cells. Young IMR-90 cells (PDL = 20) were serum deprived for 48 h (time 0) and then serum stimulated for 18 h (time 18). Nuclear extracts were prepared from these cells as described (Pang and Chen, 1993) and the DNA binding activity was analyzed by gel mobility shift assay using ^{32}P-labeled 28 bp or 25 bp fragment as the probe. Each binding assay contained 10 μg of nuclear extracts.

of these two fragments in serum-stimulated young IMR-90 cells. Consistent with our previous report (Pang and Chen, 1993), the 28 bp fragment exhibited a prominent serum-dependent binding activity. In contrast, the 25 bp fragment did not show any nuclear protein binding activity either in quiescent human cells (time 0 h) or in serum-stimulated human cells (time 18 h). This result is somewhat unexpected in light of the finding that the 25 bp fragment is a cellular target of cyclin A and p33^{cdk2} in K12 hamster fibroblast cells (Li et al., 1993). Thus, it is possible that in normal human cells, the 25 bp fragment may not be directly involved in protein binding despite the presence of Yi-like and E2F-like sequences.

Competition analysis of possible relations between the 28 bp and the 25 bp fragment

In addition to motif similarities between the 28 bp and the 25 bp fragments, it can be noted that there is an 8 bp overlap between these two fragments. We therefore examined whether the 25 bp fragment may interfere with the 28 bp binding. Since both fragments contain Yi-like motif, we also examined whether the Yi sequence alone may compete with 28 bp binding activity. Figure 3 shows that neither the 25 bp fragment nor the tandem Yi sequence could compete with the 28 bp binding, suggesting that Yi-like motif within the 28 bp fragment or the overlapping region (−91/−84) between these two fragments is not directly involved in CBP/tk protein binding.

Minimal length of the CBP/tk binding sequence

The 28 bp fragment contains an inverted CCAAT box and an inverted Yi-like sequence (Fig. 1). To further assess the contribution of these two motifs to the CBP/tk binding, we have tested several deleted or mutated sequences on the binding activity of the 28 bp fragment using competition analysis. Figure 4 shows

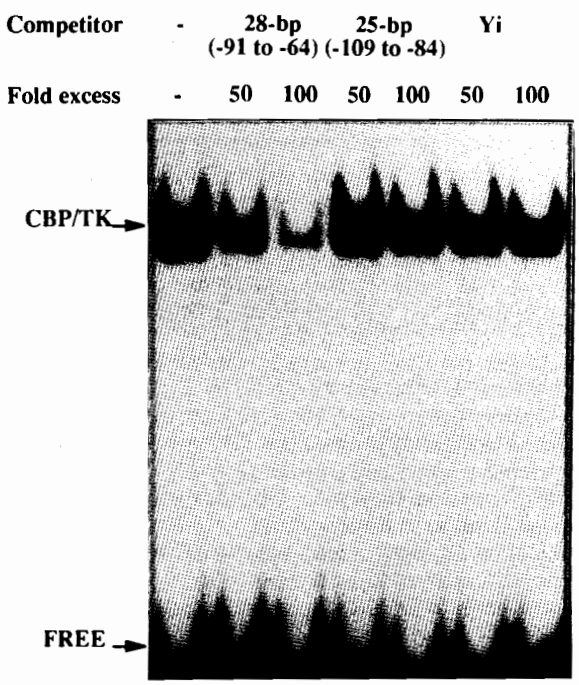
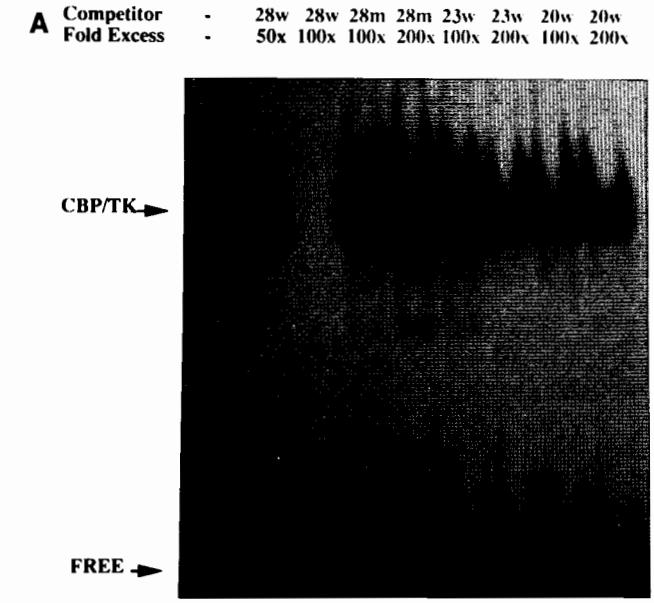


Fig. 3. Competition analysis of the sequence specificity of nuclear protein binding to the 28 bp fragment. The end-labeled 28 bp fragment was incubated with nuclear extracts in the absence or in the presence of 50-fold or 100-fold molar excess of unlabeled 28 bp fragment, 25 bp fragment, or the tandem Yi sequence (39 bp) for 30 min at room temperature before gel mobility shift assay. The oligonucleotide fragments were prepared as described in Materials and Methods.

that a mutant 28 bp oligonucleotide, 28m, containing a single base mutation in the CCAAT box completely, lost the ability to compete with the wild-type 28 bp fragment in CBP/tk binding. The oligonucleotide 20w



B

	-91 -64
28w (wild type 28-bp)	AGGTC AGCGG CCGGG CGCTG ATTGG CCC
23w (wild type 23-bp)	AGCGG CCGGG CGCTG ATTGG CCC
20w (wild type 20-bp)	CGG CCGGG CGCTG ATTGG CC
28m (mutant 28-bp)	AGGTC AGCGG CCGGG CGCTG AcTGG CCC

Fig. 4. **A:** Competition analysis of effects of deletion or mutation on the nuclear protein binding of the 28 bp fragment. The ^{32}P end-labeled 28 bp fragment was incubated with 50-, 100-, or 200-fold molar excess of cold 28 bp fragment (28w), deleted fragments (23w and 20w), or mutated fragment (28m). The binding and gel mobility shift assay was carried out as described in Materials and Methods. **B:** Comparison of the sequences of deleted or mutated 28 bp fragment with the wild-type 28 bp fragment. Lower case letter indicated mutation.

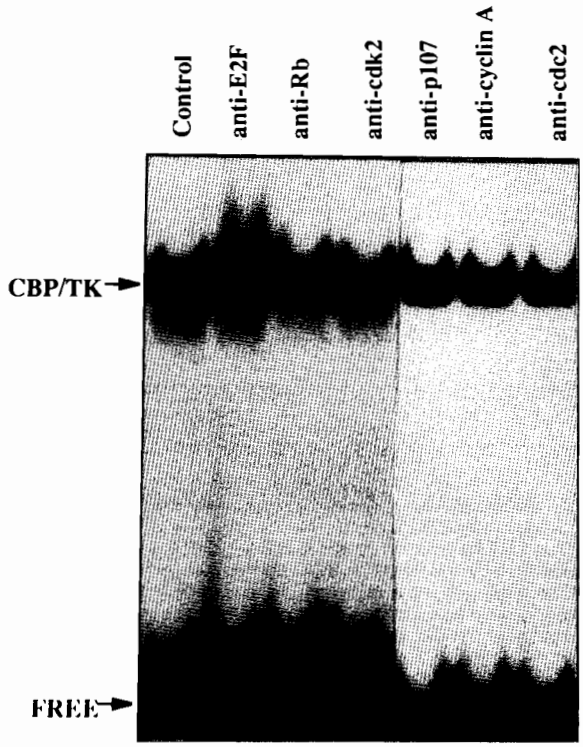


Fig. 5. Immunoshift analysis of the DNA binding affinity of CBP/tk. Nuclear extracts (10 μg) were incubated with 1 μl of undiluted antiserum as indicated in the binding mixture for 20 min on ice before the addition of ^{32}P-labeled synthetic 28 bp fragment. The binding reaction was carried out at room temperature for another 30 min and gel mobility shift assays were performed as described in Materials and Methods. Nuclear extracts were prepared from serum stimulated (20 h) young IMR-90 cells.

that retains the intact 19 bp repeat was found to be inactive in competing with the 28 bp binding even at 200-fold molar excess concentration. In fact, a deletion from the 5′-end of the 28 bp fragment up to 5 bp was sufficient to render the resulting oligonucleotide, 23w, completely inactive in competing with the 28 bp fragment for CBP/tk binding. It is noteworthy that the 23w oligonucleotide still retains the complete Yi-like motif (9/10), albeit truncated at the 5′-end. The combined results indicate that although the inverted CCAAT box appears to be directly involved in CBP/tk binding, the 5′-flanking region extended from the Yi-like motif is required for CBP/tk binding.

Analysis of protein components in CBP/tk binding complex

Immunoshift analysis was used to demonstrate that the murine Yi binding complex contains p107 and $p33^{cdc2}$ (Dou et al., 1992) and that the 25 bp fragment is the target of cyclin A and $p33^{cdk2}$ (Li et al., 1993). A similar approach was employed to investigate the nature of CBP/tk binding complex. Figure 5 shows that CBP/tk binding activity in IMR-90 cells was not affected by antiserum prepared against E2F, pRb, $p34^{cdk2}$, p107, cyclin A, or $p33^{cdc2}$. Control experiment indicates that antiserum made against p107 or $p33^{cdc2}$ caused a supershift of the Yi binding complex in NIH3T3 cells (data not shown). In a separate experiment, we found that antiserum against NF-Ya caused a supershift of the CBP/tk binding complex (Good, L.F., Xie, A.G., unpublished data), suggesting that CBP/tk may be a member belonging to the NF-Y superfamily. Thus, despite the importance of pRb, cyclin A, and associated proteins in G1/S control, they may not play a key role in the cell cycle-dependent regulation of the human TK gene.

Yi binding activity in human cells

Although Yi sequence did not compete with the human CBP/tk binding (Fig. 3), the importance of Yi binding in mouse TK gene expression and the apparent association of Yi complex with cyclins prompted us to directly examine the Yi binding activity in human cells. Figure 6 shows that Yi binding activity was almost undetectable in normal human IMR-90 cells during serum stimulation. Interestingly, high affinity Yi binding activities were detected in AG03204 cells, an SV-40 transformed IMR-90 cell strain. Yi binding activity was also observed in HeLa cells (data not shown).

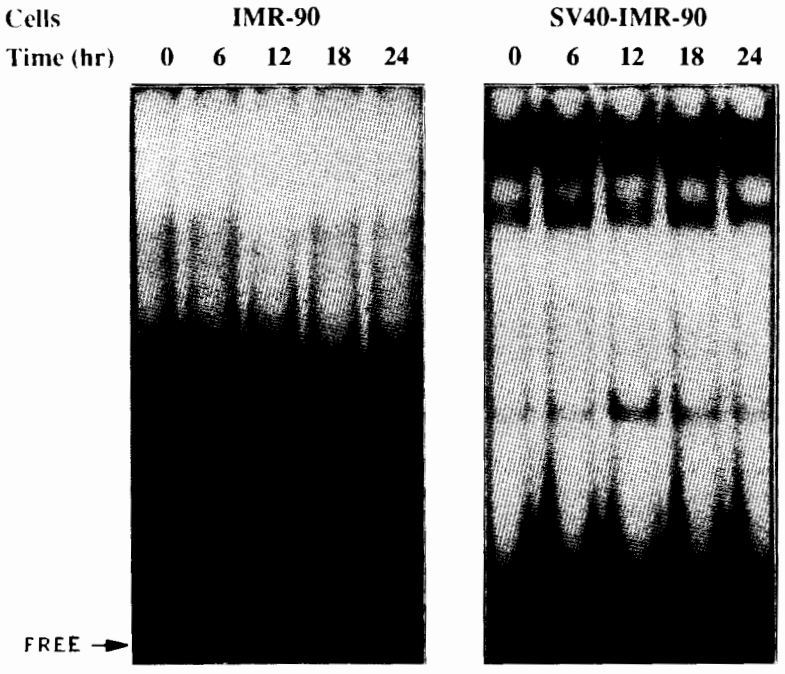


Fig. 6. Yi-like binding activity in young IMR-90 cells and in SV-40 transformed IMR-90 cells (AG03204). Cells were serum deprived for 48 h (time 0) and then stimulated to enter the cell cycle by adding fresh serum. Cells were harvested at various times following serum stimulation and nuclear extracts were prepared as described in Materials and Methods. Gel mobility shift assay was then carried out using ^{32}P-labeled tandem Yi sequence as the probe. Exposure time for the left panel (IMR-90) and for the right panel (SV40-IMR-90) was, respectively, 72 h and 12 h.

In both AG03204 and HeLa cells, we did not detect T-antigen in the binding complex (data not shown). The significance of the induction of Yi binding activities after human IMR-90 cell transformation remains to be investigated. Since Yi binding protein has yet to be purified and identified, we cannot conclusively determine whether or not it is present in normal human cells. However, we have employed a mixing experiment to examine the possibility of the presence of inhibitor which may suppress the Yi binding in normal cells. We found that the Yi binding in HeLa cells was not diminished by the presence of cell extracts derived from normal IMR-90 cells (data not shown), indicating that no inhibitory activity for Yi binding was present in normal human cells.

G1/S regulatory cis-element at the promoter of G1/S genes

The hallmark of cellular aging is the loss of dividing potential when cells become senescent (Cristofalo and Sharf, 1973). Indeed, all the G1/S genes that we and others examined exhibit an age-dependent attenuation of gene expression, suggesting that such attenuation may be a global phenomenon (Pang and Chen, 1994). Since the CBP/tk binding is cell cycle and age dependent, it will be of interest to examine whether the CBP/tk binding site or similar sequence may exist in the promoter of other G1/S genes. Using a 10 bp short sequence containing the inverted CCAAT box as the template, we searched the promoters of many G1/S genes. Table 1 shows that several G1/S genes contain this short CBP/tk binding sequence. Of particular note is that DNA polymerase α contains an identical 10 bp segment in its promoter. We have also searched the Genbank for homologies to the Yi and E2F recognition sites in human G1/S phase genes. We arbitrarily restricted the analyses to those cases where the sites were within 300 nucleotides of a transcription initiation site which were likely to be important for transcriptional control. Authentic E2F and Yi sequence are present in a number of human G1/S genes such as DHFR, PCNA, TS, and DNA pol α. Thus, although there appears to be a global attenuation of gene expression at the G1/S boundary in normal human cells during senescence (Pang and Chen, 1994), the cause for such global attenuation of G1/S genes during aging is unlikely due to the presence of a single master-switch G1/S regulatory cis-element in all these G1/S genes.

DISCUSSION

Genetic analysis has indicated that the human TK promoter region extended up to about 130 bp upstream from the transcription initiation site is important in the cell cycle-dependent expression of TK gene expression (Lipson et al., 1989; Arcot et al., 1989) and in conferring G1/S regulatory properties of chimeric genes (Kim and Lee, 1991). Within this region, there are two E2F-like sequences with one mismatch, three Yi-like sequences with one mismatch, two inverted boxes with a GC-rich flanking region, and a TATA box (Fig. 1). One approach to further delimit the G1/S regulatory cis-element(s) is to determine the binding activity of defined

late G1/S boundary may occur in human IMR-90 diploid fibroblasts during senescence. J. Cell. Physiol., *160*:531–538.

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