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Experimental Gerontology, Vol. 31, Nos. 1/2, pp. 97–109, 1996.
Published 1996 by Elsevier Science Inc.

0531-5565/96 $0.00 + .00

0531-5565(95)02019-5

THE AGE-DEPENDENT BINDING OF CBP/TK, A CCAAT BINDING PROTEIN, IS DEREGULATED IN TRANSFORMED AND IMMORTALIZED MAMMALIAN CELLS BUT ABSENT IN PREMATURE AGING CELLS

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Abstract—CBP/tk, CCAAT Binding Protein for thymidine kinase, has been shown to bind to the distal and proximal CCAAT elements in human TK gene at G1/S boundary in normal human IMR-90 cells after serum stimulation (Pang and Chen, 1993). We now show that the serum-induced binding activity of CBP/tk was inversely related to the population doubling level (PDL) of the normal IMR-90 cells. However, little or almost no CBP/tk binding activity was observed in cells derived from patients with premature aging syndromes (e.g., Werner, Hutchinson-Gilford, and Cockayne syndrome). In contrast, CBP/tk binding activity in SV-40 virus-transformed human cells and in HeLa cells was overexpressed at levels 5- to 15-fold higher than that in normal cells and appeared to be deregulated. The half-life of CBP/tk binding activity in SV-40 transformed cells was at least 10 times longer than that in normal IMR-90 cells, suggesting that posttranslational control may contribute to the deregualtion. CBP/tk binding activity detected in other mammalian cells such as murine NIH3T3, an immortal cell line, did not reveal any cell cycle dependence either. Further characterization of CBP/tk binding complex indicates that the binding complex may contain NF-YA and NF-YB and that the binding activity was sensitive to oxidizing reagents. Taken together, our data showed that the age- and cell cycle-dependent nature of CBP/tk is a function of cell types and that CBP/tk binding activity may be subjected to posttranslational and redox regulation.

Key Words: CCAAT binding protein, normal diploid fibroblasts, transformed cells, progeria, cellular senescence, NF-Y, redox regulation

INTRODUCTION

THE HALLMARK of cellular aging is the loss of dividing potential in senescent normal diploid cells maintained in tissue culture (Hayflick and Moorhead, 1961; Cristofalo and Sharf, 1973; Goldstein, 1990). One possible cause for the loss of dividing potential in senescent cells could be due

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to a global suppression of late G1/S genes such as thymidine kinase (TK[1], ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) (Pang and Chen, 1994). TK catalyzes the ATP-dependent phosphorylation of thymidine to thymidine 5′-monophosphate. TK activity increases dramatically at the G1/S boundary of the cell cycle and appears to be tightly coupled to DNA synthesis in cultured mammalian cells (Bello, 1974). The cell cycle-dependent expression of TK gene is primarily controlled at transcriptional level (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. The essential G1/S regulatory elements within the TK promoter have been identified to be the inverted CCAAT boxes and their flanking regions (Lipson *et al.*, 1989; Roehl and Conrad, 1990; Kim and Lee, 1991; Good *et al.*, 1995). Sequence-specific binding to DNA fragments containing either one of the inverted CCAAT boxes have revealed a cell cycle- and age-dependent binding activity in nuclear extracts derived from IMR-90 cells (Pang and Chen, 1993). Methylation interference analysis and mutant study indicate that the binding is due to a CCAAT-binding protein, and the binding complex was termed CBP/tk, indicating CCAAT binding protein for TK gene (Pang and Chen, 1993; Good *et al.*, 1995).

To further investigate the relationship between CBP/tk and cellular aging, we have examined its binding activity in IMR-90 cells at different PDL and in cells derived from patients with premature aging syndrome. To probe the age-dependent nature of CBP/tk binding, we also examined possible deregulation of CBP/tk binding in transformed human cells and in immortalized murine cells. Unlike normal diploid fibroblasts, CBP/tk binding in transformed human cells, including malignant cancer cells, was constitutiuvely expressed and did not show cell cycle-dependent fluctuations. Moreover, the binding activity in the transformed cells appeared to be overexpressed by manyfolds. One possible contributing cause is that CBP/tk binding activity was much more stable in transformed cells as compared to that in normal cells. This possibility was investigated by comparing the half-life of CBP/tk binding activity in normal and in transformed cells. CBP/tk binding activity was also detected in mouse NIH3T3 cells. However, despite the fact that NIH3T3 is a popular cell line commonly used for cell cycle study, CBP/tk in 3T3 cells was found to be constitutively expressed. Thus, it appears that the cell cycle-dependent nature of CBP/tk binding occurs only in cells that have limited life span in culture and becomes deregulated in both immortalized cells and in transformed cells.

ABBREVIATIONS

CBP/tk, CCAAT binding proteins for thymidine kinase gene; TK, thymidine kinase; PDL, population doubling level; NEM, N-ethyl maleimide; DTT, dithiothreitol.

MATERIALS AND METHODS

Cell culture

Low-passage IMR-90 human embryonic lung diploid fibroblasts (passage 5, PDL = 10) were obtained from the Coriell Institute for Medical Research, Camden, NJ. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and expanded through subculturing to achieve a higher population doubling level (PDL) as previously described (Pang and Chen, 1993). Transformed human cells AG02804C, AG03204, and HeLa

cells, and mouse neuroblastoma cells were also maintained in Dulbecco's medium containing 10% fetal bovine serum. NIH3T3 cells were maintained in Dulbecco's medium containing 15% new-born calf serum. Low-passage human cells derived from premature aging syndrome (AG03141A and AG04410 from Werner syndrome patients, AG01972A and AG06297A from progeria syndrome patients, and AG010400 and AG06244 from Cockayne syndrome patients) were obtained from the Coriell Institute for Medical Research, Camden, NJ, and maintained in culture as described (Chen and Chang, 1986).

Growth stimulation and nuclear extract preparation

Confluent cultures of human cells were serum-deprived for 48 h and growth stimulation was initiated by replenishing the cultures with fresh Dulbecco's medium containing 10% fetal bovine serum. At various times after serum stimulation cells were harvested by trypsinization followed by a centrifugation at 2,000 rpm for five minutes. Nuclear extracts were prepared at 4°C, as described (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. Protein concentration was determined by a modified Lowry method (Bradford, 1976).

Gel mobility shift assay

The 28-bp fragment (5′-dAGGTCAGCGGCCGGGCGCTGATTGGCCC-3′) was synthesized in our laboratory using a Pharmacia LKB Gene Assembler Plus DNA synthesizer. The synthesized oligonucleotides were purified by passing through NAP-10 column (Pharmacia). Complimentary oligonucleotides were annealed, end labeled with [γ-^{32}P]ATP and T4 kinase, and used for gel mobility shift assay. A typical binding mixture contained 10 μg of nuclear extract, 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 probe was > 50,000 cpm per 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. A typical binding assay mixture in a final volume of 25 μL contained 10 μg nuclear extracts, 2.5 μg poly(dI-dC), and 0.5 ng of labeled DNA in a buffer of 10 mM Tris (pH 6.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol. The binding reaction was carried out for 30 min at room temperature. After the reaction, the sample was loaded onto a 4% nondenaturing polyacrylamide gel (30:1) containing 6.7 mM Tris (pH7.5), 3.3 mM sodium acetate and 1 mM EDTA, and electrophoresed at 17 Vcm for two hours. The gel was dried and processed for autoradiography.

Materials

All tissue culture media and sera were obtained from GIBCO, Grand Island, NY. [α-^{32}P]dCTP (3,000 Ci/mmol), [α-^{32}P]dGTP (3,000 Ci/mmol), and [γ-^{32}P]ATP (3,000 Ci/mmol) were from ICN Chemical Radioisotope Division, Irvine, CA. Restriction enzymes and other molecular biological supplies were from Pharmacia, Piscataway, NJ. All other chemicals were of reagent grade. Anti-cdc2, polyclonal antiserum was given by Dr.Yuen-Kai T. Fung, University of Southern California; anti-p107 polyclonal antiserum was given by Dr. Mark E. Ewien, Dana-Farber Cancer Institute; anticyclin A polyclonal antiserum was given by Dr. Tony

Hunter, The Salk Institute; and polyclonal anti-NF-YB antiserum and monoclonal anti-NF-YA (Mab7) antibody were given by Dr. R. Mantovani, University Degli Studi di Milano, Italy.

RESULTS

CBP/tk Binding in Normal IMR-90 Cells and in Premature Aging Human Cells

The serum-induced CBP/tk binding activity in normal IMR-90 cells at different PDL and in cell strains derived from patients with premature aging syndrome was compared by gel mobility shift assay and fluorographic analysis. Figure 1 (left panel) shows that the serum-induced CBP/tk binding activity in normal IMR-90 cells decreased progressively as the PDL of the inculture increased, indicating that CBP/tk binding activity is closely related to replicative potential of the cultures. Quantitation of the binding activity by densitometric tracing indicated an inverse linear relationship between CBP/tk binding activity and PDL of the cultures (data not shown). Such an inverse linear relationship was also previously reported for serum-induced thymidine kinase activity (Chang and Chen, 1988), suggesting that the age-dependent attenuation of thymidine kinase activity is primarily due to the decrease in CBP/tk binding at the thymidine kinase gene promoter. Consistent with the notion that CBP/tk binding activity is

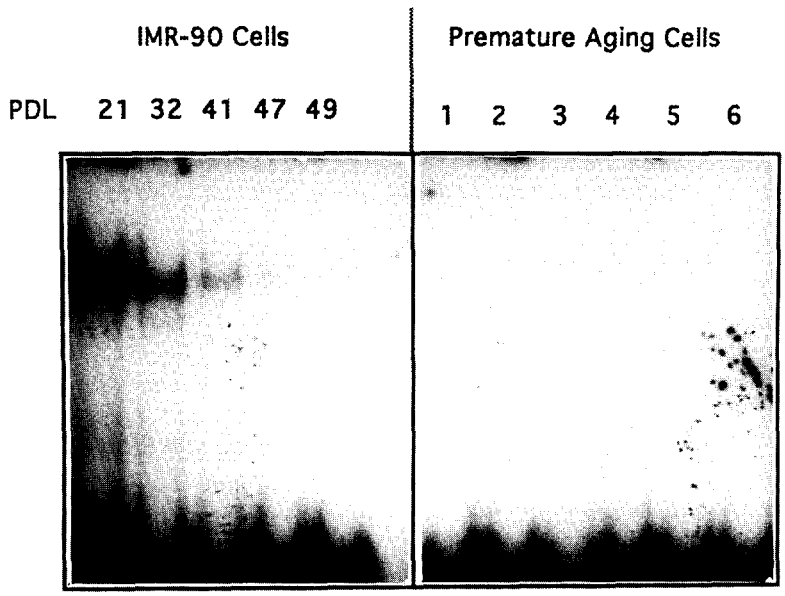

FIG. 1. CBP/tk binding activity in human IMR-90 cells at different PDL (left panel) and in premature aging cells (right panel). Left panel: IMR-90 cells at different PDL were serum deprived for 48 h and then serum stimulated for 20 h and CBP/tk binding activity was measured by gel mobility shift assay. Right panel: six premature aging cell types were studied: Werner syndrome cells (lane 1, AG03141A; lane 4, AG04110); Hutchinson-Gilford syndrome cells (lane 2, AG01972A; lane 3, AG06297A); and Cockayne syndrom cells (lane 5, AG010400; lane 6, AG06244). These cells at low-passage numbers were grown to confluence, serum deprived for 48 h, and then stimulated with fresh serum (10%) for another 20 h. Cells were harvested for nuclear extract preparation. CBP/tk binding activity in nuclear extract was monitored by gel mobility shift assay as described under Materials and Methods.

closely associated with the replicative potential of the culture, we found that fresh serum-containing growth medium could not induce any significant CBP/tk binding activity in cell strains that were derived from patients with Werner syndrome (Fig. 1, right panel, lanes 1 and 4), Hutchison-Gilford syndrome (Fig. 1, right panel, lanes 2 and 3), or Cockayne syndrome (Fig. 1, right panel, lanes 5 and 6).

Overexpression of CBP/tk Binding Activity in Transformed Human Cells

Figure 2 shows a comparison of serum-induced CBP/tk binding activity between normal IMR-90 cells (PDL = 20) and transformed human cells including SV-40 transformed cells and HeLa cells. Densitometric analysis indicated that CBP/tk was expressed at least fivefold greater in transformed cells than in normal cells. The amount of CBP/tk complex in HeLa cells was about 15-fold more than that in young IMR-90 cells. CBP/tk binding complex observed in transformed cells was sensitive to heat treatment (65°C for five min) as we have reported for normal cells (Fig. 2, lane 3 vs. lane 1). We have also compared the sensitivity of CBP/tk binding toward sulfhydryl reagents. The results showed that N-ethyl maleimide (NEM), but not dithiothreitol (DTT), at 10 mM completely abolished the CBP/tk binding in these three cell types (Fig. 2, lane 5 vs. lane 1), suggesting that maintaining cysteine residues at reduced state in CBP/tk may be essential for binding activity.

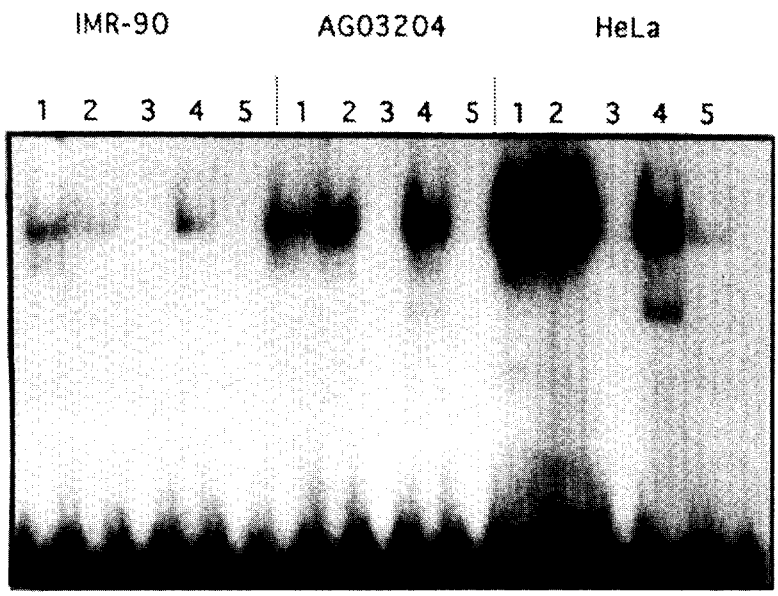

FIG. 2. CBP/tk binding in normal cells (IMR-90), SV40-transformed IMR-90 cells (AG03204), and HeLa cells. Cells were grown to confluence, serum deprived for 48 h, and then stimulated with fresh serum for 20 h. Cells were then harvested for nuclear extract preparation. Gel mobility shift assay was carried out using the labeled 28 bp as the probe, as described in Materials and Methods. Lane 1, control; lane 2, nuclear extract preincubated at 37°C for 5 min; lane 3, nuclear extract preincubated at 65°C for 5 min before binding reaction; lane 4, nuclear extract treated with 10 mM DTT before binding reaction; lane 5, nuclear extract pretreated with 10 mM NEM before binding reaction.

Loss of Cell Cycle Regulation in CBP/tk Binding in SV-40 Transformed IMR-90 Cells

Figure 3 shows that CBP/tk binding activity in young IMR-90 cells (PDL = 21) is serum responsive and cell cycle dependent, as we previously reported (Pang and Chen, 1993). In contrast, CBP/tk binding activity in two SV-40 transformed IMR-90 cell lines, AG02804C and AG03204, appeared to be constitutively elevated throughout the time course of serum stimulation. Thus, unlike IMR-90 cells, both AG02804C and AG03204 cells exhibited high levels of CBP/tk binding at quiescent state. Serum stimulation caused a slight increase in CBP/tk binding in AG03204 cells but had no effect in AG02804C cells over a 24-h period after serum stimulation. The loss of cell cycle dependency of CBP/tk binding suggests that a deregulation of CBP/tk activity may have occurred during or after cell transformation.

Half-Life of CBP/tk Binding Activity in Normal and Transformed Human Cells

The half-life of serum-induced CBP/tk binding in normal IMR-90 cells and in two SV-40 transformed IMR-90 cells activity was compared after their protein synthetic machinery was turned off by cycloheximide. Figure 4 shows that the half-life of CBP/tk binding activity in normal IMR-90 cells was less than 60 min, as we have previously reported (Pang and Chen, 1993). In contrast, the half-life of CBP/tk binding activity in AG03204 cells was about five

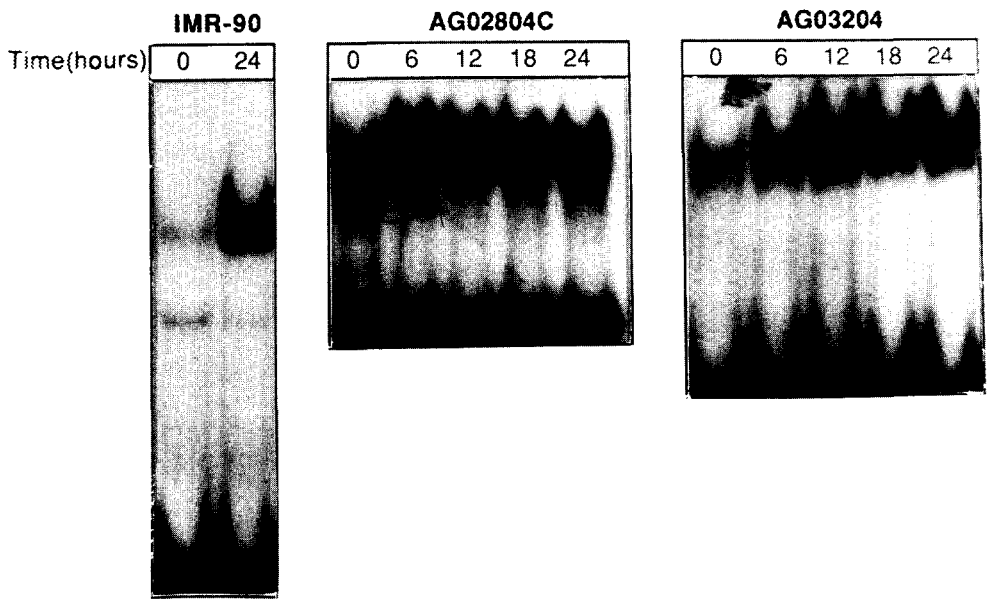

FIG. 3. Loss of cell cycle dependence in transformed human IMR-90 cells. Cells at confluent state were serum deprived for 48 h (time zero), after which cultures were replenished with fresh Dulbecco's medium containing 10% fetal bovine serum. At indicated time points thereafter, cells were harvested and nuclear extracts were prepared. An end-labeled 28 bp fragment was incubated with 10 μg of nuclear extracts for 30 min at room temperature. The binding mixture was applied onto a 4% polyacrylamide gel for a gel mobility shift assay. The autoradiogram for IMR-90 cells were exposed for a period twice longer than that for AG02804C and AG03204 cells.

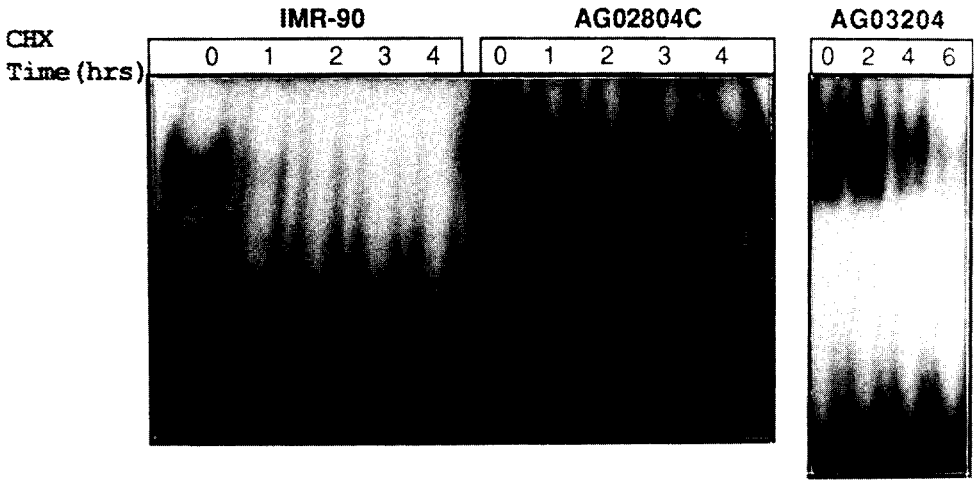

FIG. 4. Stability of CBP/tk binding in IMR-90 cells and AG02804C and AG03204 SV40-transformed cells. Cells at confluence were serum deprived for 48 h and then serum stimulated for 20 h, after which, cultures were treated with cycloheximide (50 μg/ml) (time zero) for various times, as indicated. Cells were then harvested for isolating nuclear extract. The remaining CBP/tk binding activity was measured by gel mobility shift assay as described in Materials and Methods.

hours, whereas in AG02804C cells, CBP/tk binding after cycloheximide treatment did not show any significant decrease in activity over the entire time examined. These results suggest that the increase in the stability of CBP/tk complex was responsible, at least in part, for the deregulation of CBP/tk binding in transformed cells. We have previously shown that CBP/tk activity is very sensitive to protease treatment (Pang and Chen, 1993). It is possible that the very short half-life of CBP/tk activity in normal cells is due to certain proteolytic enzyme activity that is cell cycle regulated. If this is the case, it would suggest that CBP/tk can be regulated at posttranslational level. It is also of interest to note that Yi binding activity in benzo[α]pyrene-transformed BALB/c3T3 cells (BPA31 cells) has a half-life many-fold longer than that in untransformed A31 cells (Bradley *et al.*, 1990).

CBP/tk Binding May Be Subjected to Redox Regulation

The finding that CBP/tk binding could be inhibited by NEM (Fig. 2) suggests that redox regulation may play a role in controlling CBP/tk binding activity in cells. Figure 5 shows that H_2O_2 at submillimolar concentration could abolish CBP/tk binding. The effect of H_2O_2 appeared to be more prominent when added after CBP/tk binding to DNA. H_2O_2 at these concentrations have been shown to activate *trans*-acting factors such as NF-κB (Schreck *et al.*, 1991) and HSTF (Becker *et al.*, 1990).

Characterization of CBP/tk Binding Complex

Several biochemical parameters have been previously used to compare the CBP/tk binding complex in IMR-90 cells with other known CCAAT binding proteins (Pang and Chen, 1993).

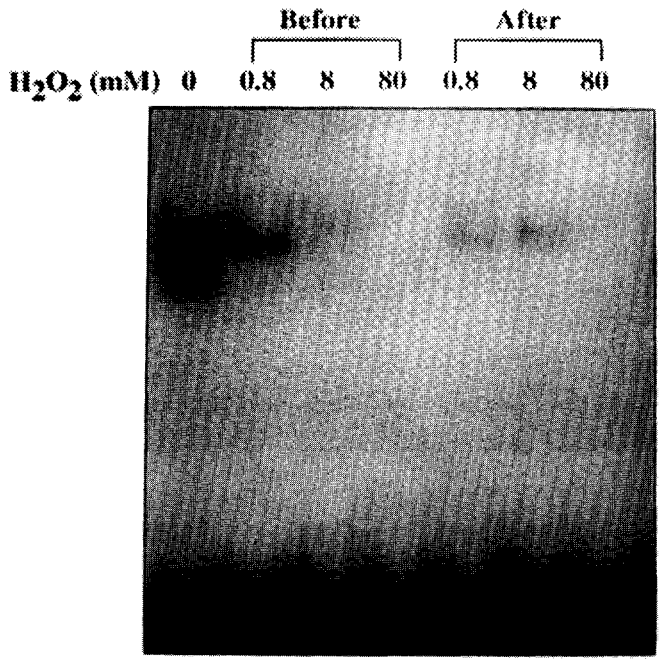

FIG. 5. Effect of H_2O_2 on the CBP/tk binding activity. Nuclear extract (10 μg protein), prepared from serum-stimulated young IMR-90 cells, were incubated in the absence (lane 1, control) or in the presence of H_2O_2 at indicated concentrations for 5 min at room temperature before the addition of the end-labeled 28 bp fragment to the binding mixture (lanes 2–4). Alternatively, H_2O_2 was added to the binding mixture after the reaction has already been initiated for 30 min at room temperature (lanes 5–7). Gel mobility shift assay was performed as described in Materials and Methods.

We found that human CBP/tk differs from mouse NF-Y in heat sensitivity, phenanthroline sensitivity, and urea sensitivity (Pang and Chen, 1993). Detailed binding studies (Arcot *et al.*, 1989) also suggest that CBP/tk binding protein may not be identical to NF-Y. Nevertheless, the presence of the 10 bp Y box (CTGATTGGCT) within the 28 bp fragment together with the earlier work by Dorn *et al.* (1987) suggest that NF-Y proteins could compete for the CBP/tk binding. Alternatively, NF-YA and NF-YB or other NF-Y-like proteins may be a component of CBP/tk complex in human cells. Recently, Chang and Liu (1994) have shown that the partially purified CCAAT binding protein that recognizes the 28 bp fragment in HL-60 cells, presumably CBP/tk, can crossreact with antibodies against NF-YA and NF-YB. Figure 6 shows that antibody against mouse NF-YB, and to a lesser degree, anti-NF-YA antibody, produced significant supershift of CBP/tk binding, suggsting that CBP/tk contained NF-YA and B or factors related to NF-Y. Figure 6 also shows that, unlike Yi binding complex, which is responsible for regulating mouse TK gene activity, CBP/tk does not contain p107, cyclin A or cdc2.

CBP/tk Binding in Murine NIH3T3 Cells

The unique age-dependent nature of CBP/tk binding in normal human cells and the deregulation of this binding activity in transformed human cells prompted us to examine CBP/tk

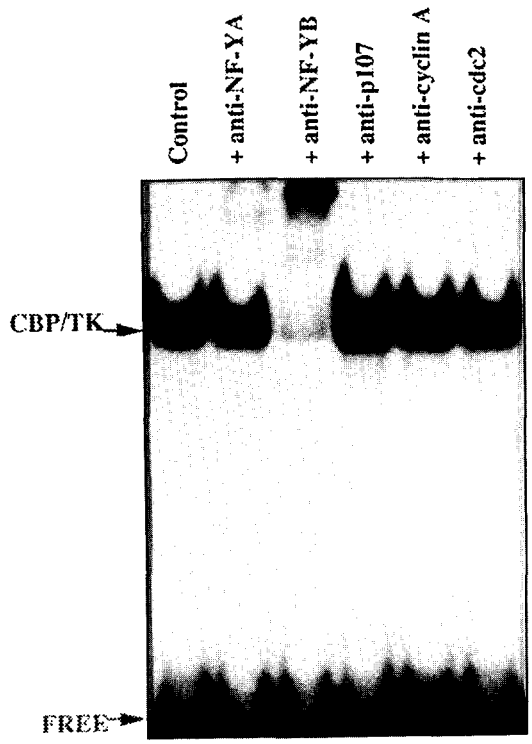

FIG. 6. Immunoshift analysis of the DNA binding affinity of CBP/tk. Nuclear extracts (10 μg protein) from serum-stimulated young IMR-90 cells wereincubated with 1 μl of undiluted antiserum in the binding mixture for 20 min on ice before the addition of end-labeled 28 bp fragment. The binding reaction was carried out at room temperature for 30 min and gel mobility shift assay was performed as described under Materials and Methods.

binding activity in other cell types. Figure 7 shows that CBP/tk binding activity in NIH3T3 cells at quiescent state (lane 2) and at various time following serum stimulation (lanes 3–6). Despite the fact that NIH3T3 cell line is a popular model for cell cycle study and that mouse TK gene in 3T3 cells is cell cycle regulated (Pardee, 1989), the CBP/tk binding activity in 3T3 cells, as measured by 28 bp sequence specific binding, did not exhibit any cell cycle-dependence. The lack of cell cycle-dependence and serum responsiveness for CBP/tk binding was also observed in other murine cell lines including N2a and NB15 mouse neuroblastoma cells (data not shown). Thus, the cell cycle- and age-dependent CBP/tk binding activity appears to be present only in normal human diploid fibroblasts.

DISCUSSION

Thymidine kinase in TK gene expression catalyzes the ATP-dependent phosphorylation of thymidine to thymidine 5′-monophosphate. It has been shown that transcriptional regulation plays a key role in TK gene expression normal diploid cells following serum stimulation

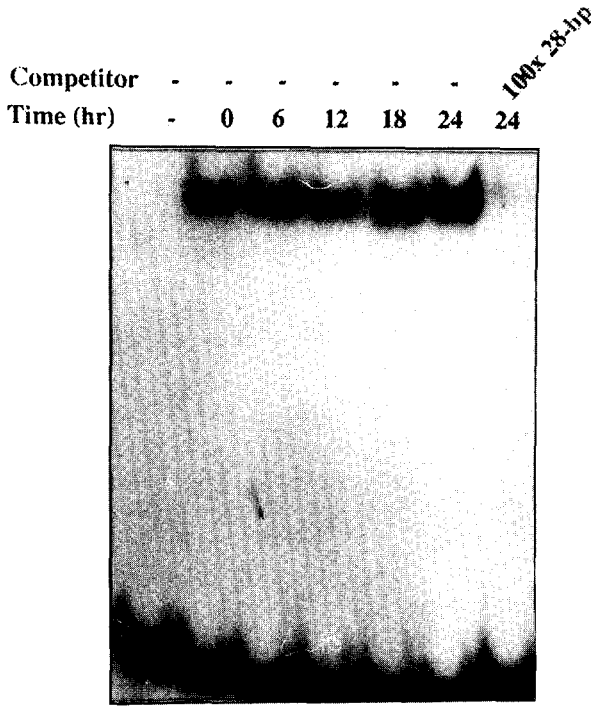

FIG. 7. CBP/tk binding activity in mouse NIH3T3 cells during the course of serum stimulation. Confluent 3T3 cells were serum deprived for 24 h, after which cultures were replenished with fresh Dulbecco's medium containing 15% new born calf serum. Cells were harvested at various times after serum stimulation and nuclear extracts were prepared for gel mobility shift assay as described under Materials and Methods.

(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; Roehl and Conrad, 1990, Kim and Lee, 1991). An upstream region about 130 bp from the cap site in human TK promoter, containing GC box, CCAAT-like sequence, Yi-like, and E2F-like sequences and inverted CCAAT box, has been shown to be sufficient to confer cell cycle regulation. Methylation interference analysis and mutational analysis indicate that the cell cycle-dependent binding activity observed is due to a CCAAT binding protein (Pang and Chen, 1993; Good *et al.*, 1995). We have termed the binding factor as CBP/tk, indicating CCAAT binding protein for TK gene (Pang and Chen, 1993). The present study extended our previous work by showing that there exists an inverse linear relationship between CBP/tk binding and the PDL of the culture and that CBP/tk binding is absent in all premature aging cell strains that we have examined (Fig. 1). The close correlation between CBP/tk binding activity and PDL of the culture strongly suggests that CBP/tk may play an upstream role in regulating a series of sequential events which eventually lead to cell senescence.

Deregulation of cell cycle-dependent *trans*-acting factors has been previously reported. For

example, Holthuis *et al.* (1990) found that HiNF-D, a histone 4 promoter binding factor, is deregulated during cell cycling in tumor-derived or transformed cells. Bradley *et al.* (1990) also reported Yi binding factor for murine thymidine kinase gene exhibits different stability and cell cycle regulation between transformed and nontransformed cells. The change of CBP/tk binding activity from a cell cycle-dependent one in normal cells to a constitutive one in transformed human cells or in tumor cells (Figs. 2 and 3) suggests that such a change may be related to the loss of mortality that is the hallmark of normal diploid cells. Although the cause of deregulation of these *trans*-acting factors is not completely clear, the dramatic difference in the half-life of CBP/tk in normal and transformed cells (Fig. 4) certainly suggsts that posttranslational control of CBP/tk plays a role in regulating its binding activity. Whether such control may involve protease activity is not clear. It can be noted, however, that CBP/tk binding activity in IMR-90 cells has been shown to be very sensitive to protease treatment (Pang and Chen, 1993). In addition to the loss of cell cycle regulation, CBP/tk binding activity in transformed cells was found to be extremely high (Fig. 2). This could be due to (1) a transformation-related increase in CBP/tk biding factor(s), and/or (2) a transformation-related change of cellular composition that favors binding. One possible change associated with cell transformation is a change of overall redox state of cellular milieu (e.g., Chen and McLaughlin, 1985). In this regard, we showed that not only sulfhydryl reagents such as NEM, but also H_2O_2 could inhibit CBP/tk activity, suggesting that this *trans*-acting factor can be subjected to redox regulation. In any event, the fact that CBP/tk is the target for transformation-related deregulation underscores the importance of this binding complex in growth regulation.

The composition of CBP/tk complex has yet to be fully characterized. Studies using competition analysis (Arcot *et al.*, 1989), immunoshift analysis (Fig. 6; also, Chang and Liu, 1994), and partial purification of CBP/tk (Chang and Liu, 1994) suggest that the complex contains NF-YA and NF-YB. However, it is noteworthy that de Crombrugghe and co-workers (Maity *et al.*, 1992) have recently shown that a third factor named CBF-C, tightly associated with CBF-A (equivalent to NF-YA), is required for reconstituting the DNA binding activities of recombinant CBF-A and CBF-B (equivalent to NF-YB). Thus, although it seems certain that CBP/tk contains NF-YA and B, we can not exclude the possibility that it may contain other protein factors. Indeed, we have found that NF-YA and B alone in IMR-90 cells, as measured by Western blot analysis, could not account for the rapid decay of CBP/tk binding activity after the inhibition of protein synthesis (L. Good and K.Y. Chen, unpublished data). Thus, it is possible that CBP/tk binding complex may contain two components: one is NF-Y (both A and B subunit), which provides constitutive binding to Y box within the 28 bp of the human TK promoter; the other is some additional factor(s), which confers a cell cycle-dependent and age-dependent property of CBP/tk in normal diploid fibroblasts. This additional factor is responsible for fine tuning and regulating CBP/tk binding and is likely to be the target for transformation-related deregulation. It should also be noted that NF-Y has a fairly ubiquitous tissue distribution (Hooft van Huijsduijnen *et al.*, 1987) and that many NF-Y–controlled genes are not cell cycle regulated (Dorn *et al.*, 1987). In this regard, it is of interest to note that although mouse TK gene does not contain CCAAT box, a cell cycle-independent CBP/tk activity can be detected in mouse NIH3T3 cells (Fig. 7). Because NIH3T3 cells, although considered normal, are immortal in tissue culture, it is tempting to speculate that the loss of cell cycle-dependent CBP/tk binding may be related to immortalization. In any event, the fact that cell cycle-dependent expression of CBP/tk binding can only be observed in normal diploid cells that have limited life span makes CBP/tk a unique candidate for probing the cause of aging at cellular level.

Acknowledgments—We are grateful to Dr. Yuen-Kai T. Fung, University of Southern California, for anti-cdc2, polyclonal antiserum; Dr. Mark E. Ewien, Dana-Farber Cancer Institute, for anti-p107 polyclonal antiserum; Dr. Tony Hunter, The Salk Institute, for anti-cyclin A polyclonal antiserum; and Dr. R. Mantovani, University Degli Studi di Milano, for polyclonal anti-NF-YB antiserum and monoclonal anti-NF-YA (Mab7) antibody. Their generosity has made this study possible. We also thank Chamu Rathakrishnan for her technical help. This work was supported in part by United States Public Health Service grant RO1 AG03578 awarded by the National Institute of Aging, NIH and a grant from The Charles and Johanna Busch Memorial Fund.

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