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Cell-cycle-specific interaction of nuclear DNA-binding proteins with a CCAAT sequence from the human thymidine kinase gene

(transcription/DNA replication/nucleoprotein complex)

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ABSTRACT **Induction of thymidine kinase parallels the onset of DNA synthesis. To investigate the transcriptional regulation of the thymidine kinase gene, we have examined whether specific nuclear factors interact in a cell-cycle-dependent manner with sequences upstream of this gene. Two inverted CCAAT boxes near the transcriptional initiation sites were observed to form complexes with nuclear DNA-binding proteins. The nature of the complexes changes dramatically as the cells approach DNA synthesis and correlates well with the previously reported transcriptional increase of the thymidine kinase gene.**

Growth factors and oncogenes are intimately involved in the control of the mammalian cell cycle during the G_1 phase (1–4) until the restriction point (5–8), which occurs ≈2 hr prior to the onset of DNA synthesis. After the restriction point, growth factors are no longer required for cells to continue their progression into the S phase. Concomitant with the onset of DNA synthesis, the activities of several enzymes—notably, thymidine kinase (TK) (EC 2.7.1.1) (9–12), thymidylate synthase (EC 2.1.1.45) (11–13), and dihydrofolate reductase (EC 1.5.1.3) (14–16)—increase dramatically. Factors controlling the expression of these enzymes may also control the onset of DNA synthesis.

TK activity exists in proliferating cells but not in resting or terminally differentiated cells (17). Its activity has been associated with the replitase complex during DNA synthesis (18, 19). The TK mRNA level is low or nonexistent during G_1 phase but increases just prior to S phase (20–23) and is responsible, at least in part, for the increase in enzyme activity. In CV1 cells, the rate of TK transcription remains elevated ≈3-fold after a transient 6-fold increase, which occurs just as the cells enter the S phase (22). In A31 cells, there appears to be a 2- to 4-fold increase in transcription, with a >20-fold increase in mRNA level (23). The discrepancy between the increased transcription rates and the increased steady-state level of mRNA during S phase implies that TK regulation occurs at the transcriptional and the posttranscriptional level. Further evidence for a posttranscriptional component comes from studies in which the TK cDNA was linked to a heterologous promoter and TK activity was found to modulate with the cell cycle (22, 24).

The human TK gene has been cloned and, when transfected into mouse LTK^- cells, was found to be properly regulated at the onset of DNA synthesis (25). This finding suggests that a common mechanism controlling TK expression exists in human and mouse cells and that all of the necessary sequence elements for cell cycle regulation are present in the λ TK46 clone. DNA sequence analysis shows that the TK gene spans 13.5 kilobases (kb) and is composed of seven exons that code for a 1430-nucleotide mRNA (26). When a set of plasmids containing deletions in the 5′ region of the human TK gene was transfected into mouse cells, a region between −83 and −10 was reported to be necessary for the transformation of LTK^- cells to LTK^+ (27).

Transcriptional regulation of genes has been shown to occur by way of specific DNA–protein interactions, although the mechanisms of how these interactions regulate gene expression in the context of the cell or organism remains unclear. Transcription factors have been shown to bind to regulatory elements for the human histone H4 gene, which influence the maximal level and the temporal induction of transcription (28, 29). However, DNA–protein interactions at these sites are constitutive throughout the cell cycle (30). Treisman (31, 32) has shown that a region of dyad symmetry in the 5′ flanking region of c-*fos* is required for and mediates the transcriptional response to serum factors. Gel mobility shift experiments have shown that this dyad, as well as two other sites (33), were recognized by specific proteins. *In vitro* mutagenesis experiments on the c-*fos* dyad have shown a strict correlation between nuclear protein binding and *in vivo* inducibility (34). No change in the binding interaction was observed by using extracts prepared from growing, starved, or stimulated cells. In contrast to these findings, epidermal growth factor (EGF) has been shown to induce specific protein binding to the c-*fos* dyad in A431 cells (35). Also, platelet-derived growth factor and v-*sis* conditioned medium, but not EGF, induce binding to a site just upstream to the c-*fos* dyad in 3T3 cells, with constitutive protein binding occurring at the dyad (36). In both cases, the inducible binding appears to persist after the transcriptional response has subsided.

We have examined whether any TK sequences were involved in the transcriptional increase observed at the onset of DNA synthesis by their interaction with nuclear DNA-binding proteins. The 5′ flanking region of TK was dissected into DNA fragments that contained perhaps one or two binding sites. The interactions of two CCAAT sequences and a distal element with nuclear factors were observed. Changes in the binding interaction between the TK promoter and the nuclear factor(s) coincided with the increase in TK transcription.

MATERIALS AND METHODS

Cell Synchrony and Nuclear Extract Preparation. BALB/c 3T3 clone A31 cells were cultured in 10% CO_2 in air at 37°C in Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine (DMEM) and 10% calf serum without antibiotics. Monolayers of cells were plated on 150-mm tissue culture plates at a density of 2.2×10^5 cells per plate and allowed to grow for 3 days. The monolayer cultures were washed twice with DMEM and then incubated with DMEM

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Abbreviations: TK, thymidine kinase; G_1/S, G_1/S boundary.
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supplemented with 0.5% calf serum for 48 hr. The cell density of the quiescent (G_0) monolayer was about 5×10^6 cells per plate. The medium was then removed and the cells were stimulated by the addition of DMEM containing 10% calf serum. The cells were harvested at the indicated times following serum addition.

Nuclear extracts were prepared from the tissue culture cells according to the method of Dignam *et al.* (37) except that the first buffer contained 0.3 M sucrose. The extracts typically contained 2.5–4 mg of protein per ml.

DNA Probes. Genomic DNA from λ TK46 (25) was subcloned into pUC18 or pUC19 and M13mp18 or M13mp19 by standard techniques (38). Subclones were sequenced by standard dideoxy sequence analysis techniques (39). Gel-purified DNA fragments were end-labeled with ^{32}P by the Klenow fragment of DNA polymerase or polynucleotide kinase to a specific activity of 50,000 cpm/ng.

Gel Mobility Shift Analysis. Binding reactions were performed in a buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol for 20–25 min at room temperature. A typical reaction contained 0.5 ng (10,000–25,000 cpm) of end-labeled DNA fragment, 12 μg of nuclear extract, which was added last, and 2.5 μg of poly(dI-dC), in a reaction volume of 25 μl. A 4% polyacrylamide gel (30:1) containing 6.7 mM Tris (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA was preelectrophoresed for 1 hr at 11 V/cm with buffer recirculation. Following binding, the samples were loaded onto the gel and electrophoresis was continued for 1.5 hr. The dried gel was autoradiographed with an enhancing screen at −70°C. For competition binding reactions, the competitor DNA was included at the concentrations given in the figure legends and mixed prior to nuclear extract addition.

Methylation Interference Experiments. The end-labeled DNA fragments were partially methylated (40) and subsequently added to a preparative scale binding reaction, which was scaled up 5- to 10-fold from the standard condition. Methylation interference assays were performed as detailed by Sen and Baltimore (41). Samples were analyzed on 8% polyacrylamide/8 M urea wedge gels.

Oligonucleotides. The oligonucleotides for site 1 (5′ AATTCAGCGGCCGGGCGCTGATTGGCCCCATG 3′ and 5′ GATCCATGGGGCCAATCAGCGCCCGGCCGCTG 3′), site 2 (5′ AATTCGGGGCCGGCTCGTGATTGGCCAGCACG 3′ and 5′ GATCCGTGCTGGCCAATCACGAGCCGGCCCCG 3′), and the region between the "TATA" box and the transcriptional initiation sites (5′ GATCCGCGGTCGGCGCGGGA 3′ and 5′ GATCTCCCGCGCCGACCGCG 3′) were synthesized on an Applied Biosystems model 308 synthesizer at the Dana–Farber Oligonucleotide Facility. Oligonucleotides were denatured in 100 mM NaCl/10 mM Tris, pH 7.5/1 mM EDTA by boiling for 3 min; this was followed by slow cooling to 0°C at 1 mg/ml.

RESULTS

The TK mRNA level is extremely low in quiescent and G_1 cells and increases to maximal levels at the beginning of DNA synthesis. To determine whether these observed changes in mRNA levels coincided with detectable alterations of protein binding to upstream regulatory regions, nuclear extracts were prepared from G_0 cells and G_1/S cells (where G_1/S indicates G_1/S boundary). Several DNA fragments spanning 1 kb of 5′ flanking DNA were used to locate potential regions bound by nuclear factors (Fig. 1*A*). Interaction of nuclear factors with the TK gene upstream region was detected by an electrophoretic mobility shift assay (42, 43). Within the TK 5′ flanking sequences, two regions that bound proteins were detected. One sequence encompassed a proximal promoter region located within 200 bp of the first exon. The other region, composed of a 36-bp poly(dA)-poly(dT) tract, was located about 900 bp upstream and will be discussed in greater detail elsewhere.

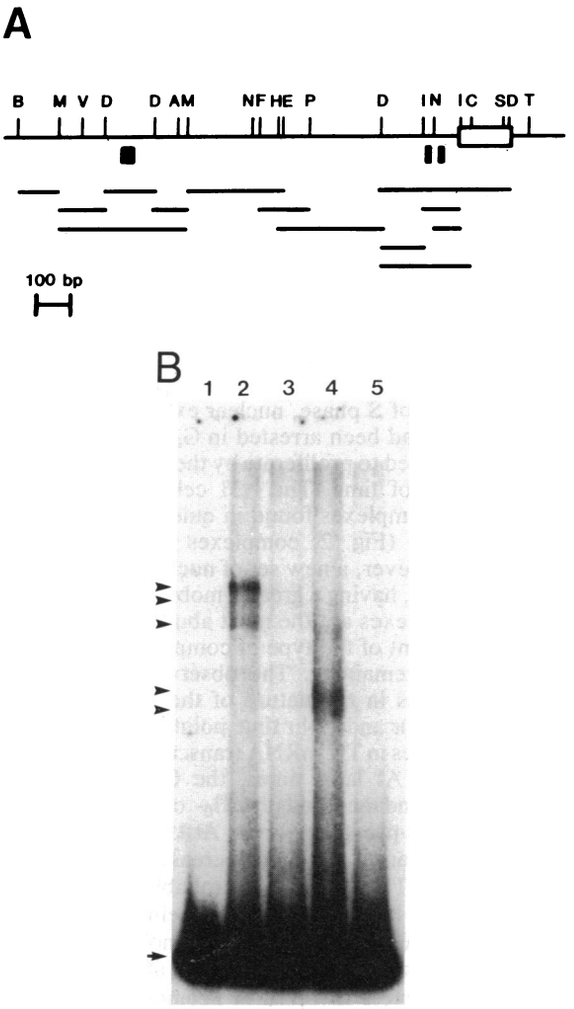

FIG. 1. Nuclear protein binding to the human TK promoter. (*A*) Partial restriction map of the 5′ end of the TK gene. The map depicts data obtained from sequencing the gene (ref. 26; G.B.K., unpublished observations). Restriction enzyme sites are indicated as follows: A, *Alu* I; B, *Bam*HI; C, *Sca* I; D, *Mbo* I/*Dpn* I; E, *Eco*RI; F, *Sph* I; H, *Hin*dIII; I, *Bst*NI; M, *Msp* I; N, *Nco* I; P, *Pst* I; S, *Sma* I; T, *Sst* I; V, *Pvu* II. The first exon is represented by the open rectangle. The 19-bp repeats and the distal element are depicted below the map by the filled rectangles and the filled square, respectively. Lines below the map indicate the DNA fragments used as probes in electrophoretic mobility shift assays to locate DNA regions containing protein-binding sites. (*B*) Electrophoretic mobility shift assay. An end-labeled 228-bp *Mbo* I–*Sca* I fragment was incubated with nuclear extracts prepared from either quiescent (lanes 2 and 3) or G_1/S (lanes 4 and 5) cells; this was followed by electrophoresis through a low ionic strength polyacrylamide gel. In lanes 3 and 5, a 50-fold molar excess of the unlabeled DNA fragment was also added. Lane 1, free DNA fragment. The specific nucleoprotein complexes are indicated by arrowheads and the free DNA is indicated by an arrow.

The proximal region was initially detected by using a 228-bp *Mbo* I–*Sca* I DNA fragment, containing about 30 bp of the first exon and the adjacent 198 bp of 5′ flanking sequences from a TK genomic clone. Fig. 1*B* depicts the nucleoprotein complexes formed with crude nuclear extracts. The nature of the complexes formed with the G_0 and the G_1/S extracts was found to differ dramatically (compare lanes 2 and 4). Competition experiments with an excess of unlabeled DNA fragment demonstrate that these binding interactions are specific (Fig. 1*B*, lanes 3 and 5). Although no

true comparisons of size can be determined due to the nature of the electrophoretic conditions, the complexes formed with the G_0 extract appear to be much larger, or have a different electronic charge, than those formed with the G_1/S extract. This same pattern of binding was observed when the size of the DNA fragment was decreased, with the added benefit of increased band intensity. A 95-bp *Bst*NI–*Bst*NI fragment gave the same general pattern as did a 67-bp *Nco* I–*Bst*NI fragment (data not shown). The 67-bp fragment, which contains only 1 of the 19-bp repeats, gave the clearest results and therefore was used in all subsequent experiments involving this region.

Cell Cycle Modulation of Promoter Binding. To see whether the change in the binding behavior was correlated with previously observed changes in transcription of the TK gene at the beginning of S phase, nuclear extracts were prepared from cells that had been arrested in G_0 by serum starvation and then stimulated to proliferate by the addition of serum for various lengths of time. The A31 cells retain the type of nucleoprotein complexes found in quiescent cells until 9 hr after stimulation (Fig. 2, complexes a–c). At 11 hr after stimulation, however, a new set of nucleoprotein complexes (e and f) appears, having a greater mobility. By 12 hr (G_1/S), these new complexes are the most abundant species, with a very small amount of the type of complexes found in the G_0 or G_1 extracts remaining. The observation that there are dramatic changes in the nature of the nucleoprotein complexes at the 11-hr and 12-hr time points correlates well with reported increases in TK mRNA transcription in A31 (23) and CV1 cells (22). At later times, the G_1/S-type complexes decrease in abundance and the G_0- or G_1-type complexes become the most prevalent forms. At 15 hr, another complex (d) becomes apparent and then decreases in abundance with increasing time. This complex is probably an intermediate in the formation of the larger nucleoprotein complex. Furthermore, the complex having the slowest mobility (a) seen in the G_1 extracts does not appear to reform after the G_1/S.

Specific Sequences Involved in Promoter Binding. To examine the DNA–protein interactions on the 67-bp *Nco* I–*Bst*NI fragment at high resolution we used a methylation interference assay (41, 44, 45). The absence or decreased intensity of a band in the guanine ladder of the DNA from a nucleoprotein complex corresponds to a site of protein binding.

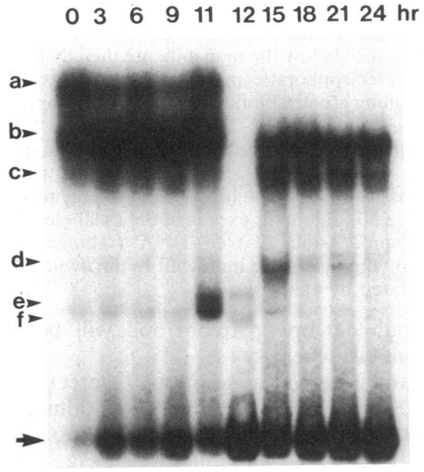

FIG. 2. Cell cycle modulation of factor binding to the TK promoter. An end-labeled 67-bp *Nco* I–*Bst*NI fragment was incubated with 12 μg of nuclear extracts prepared from cells at various times during the G_1 to S transition. The time (hr) is indicated above each lane. Arrowheads indicate the nucleoprotein complexes. An arrow points to the bands for free DNA.

The 67-bp *Nco* I–*Bst*NI fragment was labeled either on the *Bst*NI end (3′ coding strand) or the *Nco* I end (3′ noncoding strand) for use in methylation interference experiments (Fig. 3). The G_0 and the G_1/S extracts show a diminished intensity for two guanines on the coding strand (Fig. 3*A*). These bases comprise part of the inverted CCAAT sequence found in the 19-bp repeat (Fig. 3*C*). Dimethyl sulfate protection of the β-globin CCAAT box (46) shows protection of the same two bases. The protein-binding site was not seen on the noncoding strand (Fig. 3*B*), perhaps due to a paucity of deoxyguanosine bases near the CCAAT sequence. However, some bands resulting from anomalous cleavage of the DNA exhibit a diminished intensity in this region.

Oligonucleotide Competition. The binding site found by the

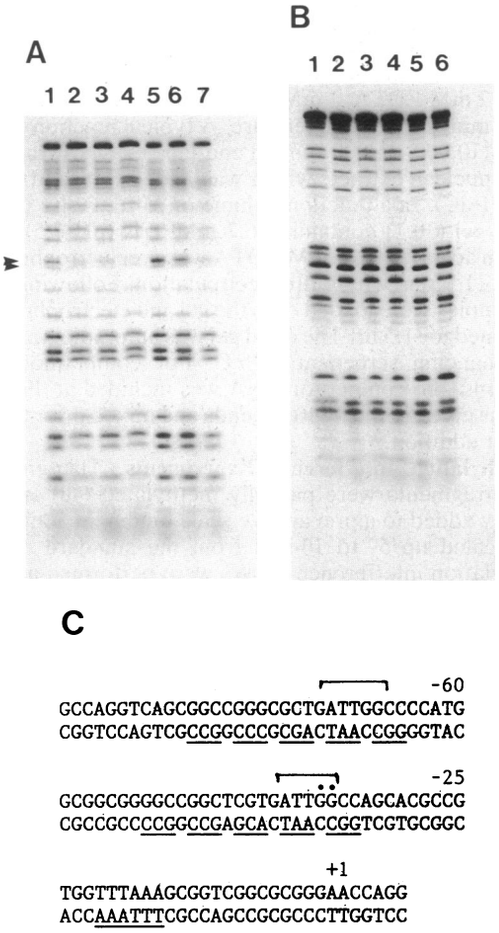

FIG. 3. Methylation interference experiments for the TK promoter. The arrowheads indicate the guanine residues whose methylation by dimethyl sulfate specifically inhibits the binding of a nuclear factor to its cognate sequence. Nucleoprotein complex and free DNA bands were excised from preparative gels and analyzed after piperidine treatment. A guanine ladder was also generated from the fragment alone. (*A*) Methylation interference of the coding strand. The 67-bp *Nco* I–*Bst*NI fragment was end-labeled at the *Bst*NI site on the coding strand. Lanes 1 and 7, guanine ladder; lane 2, complex b; lane 3, complex c; lane 4, complexes e and f; lanes 5 and 6, free DNA from the G_0 and the G_1/S binding reactions, respectively. (*B*) Methylation interference of the noncoding strand. The 67-bp *Nco* I–*Bst*NI fragment was end-labeled at the *Nco* I site on the noncoding strand. Lanes 1–6 are the same as in *A*. (*C*) Summary of methylation interference experiments. The sequence of the promoter region of TK (26) is shown, indicating with dots above the sequence the guanine residues whose methylation interferes with the DNA–protein interaction *in vitro*. The dotted line indicates the 19-bp direct repeats, and the inverted CCAAT sequences are denoted by brackets. The TATA homology is indicated by the solid line.

methylation interference experiments indicates that the inverted CCAAT sequence of the 67-bp *Nco* I–*Bst*NI fragment was involved in the observed binding of nuclear protein. When the methylation interference assay was performed by using the 95-bp *Bst*NI–*Bst*NI DNA fragment, containing both repeats, no decrease in band intensity was apparent, in spite of the fact that the band shift appeared identical (data not shown). Therefore, to determine whether each of the direct repeats were equivalent in terms of binding, oligonucleotides corresponding to each repeat sequence were synthesized and used for competition experiments with the downstream repeat (site 2) contained in the 67-bp *Nco* I–*Bst*NI fragment. Another oligonucleotide, corresponding to the DNA sequence from the TATA homology to the transcription start site, was also prepared. The results shown in Fig. 4 indicate that oligonucleotides for sites 1 and 2 compete for nucleoprotein binding in the G_0 and the G_1/S extracts, but the site 2 oligonucleotide appears to compete more efficiently (compare lanes 2 and 6 with lanes 3 and 7). Neither of these oligonucleotides competes for binding with complex f in Fig. 4, despite the fact that competition of all complexes occurred when the unlabeled 67-bp fragment was used. The competition was specific, as shown by the failure of an oligonucleotide for the sequences just proximal to the transcriptional start site to compete for binding.

DISCUSSION

Several common DNA sequence elements are usually found near the transcriptional initiation site of many genes. In the TK promoter, DNA sequence analysis has demonstrated the presence of a TATA homology, two inverted CCAAT boxes, and G-C boxes (26). From electrophoretic mobility shift experiments, we have shown that the 100 bp immediately upstream of the transcriptional start contains binding sites for nuclear factors. Specific binding was shown to occur at the inverted CCAAT box at −36 and, by inference, to occur at the CCAAT box at −67.

A variety of gene promoters contain CCAAT boxes. This element has been associated with transcriptional activation of

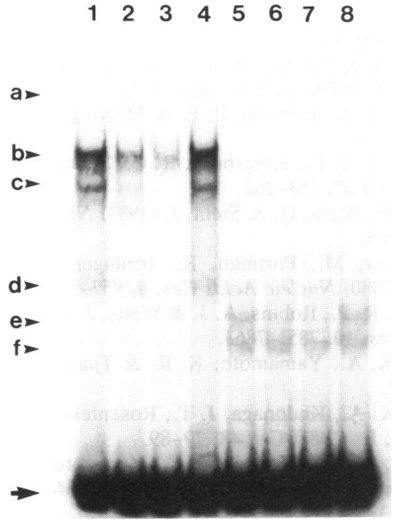

FIG. 4. Oligonucleotide competition of nuclear factor binding. End-labeled 67-bp *Nco* I–*Bst*NI fragment was incubated with either G_0 (lanes 1–4) or G_1/S (lanes 5–8) nuclear extracts in the presence of a 25-fold molar excess of oligonucleotide competitor. Lanes 1 and 5, no competitor; lanes 2 and 6, competition with the site 1 oligonucleotide duplex; lanes 3 and 7, competition with the site 2 oligonucleotide duplex; lanes 4 and 8, competition with the GCGGTCGGCGCGGGA duplex. Bands for the nucleoprotein complexes a–f are indicated. An arrow denotes the bands for free DNA.

genes, functioning either independently or in conjunction with other elements. In the *Xenopus hsp70* gene, a CCAAT box was observed to be required for transcription and maximal induction by heat shock (47, 48). Neither the heat shock element nor the CCAAT box was sufficient for full activation, suggesting that these binding factors may cooperate to enhance transcription. CCAAT boxes function in either orientation, as evidenced by studies on herpes simplex virus TK transcription (49, 50). Inverted and/or tandem CCAAT boxes have also been found in the cell-cycle-regulated histone H4 (51), H2A (52), and H2B (53) genes.

In vitro transcription studies have revealed the coordinate interaction of proteins from HeLa cell nuclear extracts with G-C boxes and a CCAAT box from the herpes simplex virus TK promoter (54). A transcriptional factor has also been reported to bind to the mouse α- and β-globin CCAAT sequences (46). A CCAAT-binding transcription factor (CTF) has been purified from HeLa cell nuclear extracts (55) and found to be indistinguishable from nuclear factor 1 (NF-1). CTF also binds the CCAAT sequence in the *hsp70* gene (56). Another CCAAT-binding protein isolated from rat liver (CBP) (49) appears to be different from CTF, since the two proteins generate different "footprints" on the herpes simplex virus TK promoter and are biochemically distinct. Preliminary experiments suggest that the factor we observe is about 33 kDa, which does not correspond in size to either CTF/NF-1 or CBP (Y.-D. Guo and G.B.K., unpublished observation).

From methylation interference experiments, the CCAAT-binding factor observed in A31 nuclear extracts appears to interact with the TK promoter throughout the cell cycle. However, the nature of the complexes changes dramatically at the G_1/S boundary. This shift in the binding behavior correlates with the increase in the transcription of the TK gene in these cells (23) and in CV1 cells (22). Notably, this shift in binding behavior was not accompanied by a change in a site on the DNA but rather between interactions of proteins. The apparently larger size of the G_0 and G_1 (G_0/G_1) complexes relative to the G_1/S complexes and the number of bands detected suggest that more than one protein is present in these complexes. It appears likely that the G_0/G_1 complexes, or a component of them, dissociate to form the G_1/S complexes. As the cells progress through the cell cycle, the CCAAT-binding protein appears to be the scaffold upon which other proteins reassociate to form the G_0/G_1 complexes (b and c in Fig. 2). No binding sites other than the inverted CCAAT boxes were observed in the proximal promoter region. This modulation of the nucleoprotein complexes suggests that the human TK gene may be transcriptionally repressed until just prior to S phase, when a brief period of derepression occurs, correlating with the spike in TK transcription observed by Stewart et al. (22). Transcription of TK is elevated during S phase (22, 23), and this may correlate with the observation that one of the complexes (complex a in Fig. 2) does not reappear after the G_1/S and that another complex (d in Fig. 2) is more abundantly present. At later times, the ultimate cellular level of TK mRNA and protein appear to be regulated by other means (22, 23).

The mechanism of this postulated derepression of TK transcription could be explained by new synthesis of the CCAAT-binding protein occurring at the beginning of S phase, which displaces the previously bound repressor complex. After a short time, accessory proteins would bind the CCAAT-binding protein, causing repression until another round of DNA synthesis was stimulated. This scenario is unlikely, since previous studies have shown that after the restriction point no new protein synthesis is required for cells to enter S phase (5–8). Another possibility is that the accessory proteins associated with the CCAAT binding protein become modified such that the repressor complex

dissociates at the G_1/S, leaving the CCAAT-binding protein bound to the TK promoter. At this point, transcription could proceed, as CCAAT-binding proteins have been shown to stimulate transcription. This modification would have to involve some reversible change such as a phosphorylation/dephosphorylation cycle, since the G_0/G_1 complexes (b and c in Fig. 2) reappear soon after the G_1/S. A permutation of this hypothesis may be that another protein becomes modified and then associates with the accessory proteins, causing their displacement from the CCAAT-binding protein.

Several regions within the 5′ flanking sequence of the human TK gene contain sequence homology with other genes, but no protein binding to these sites was observed. Perhaps the most surprising finding was that the G-C boxes, which have been shown to bind Sp1 in other genes (54, 57–59), were not bound by factors in the A31 nuclear extracts. Either Sp1 does not exist in these extracts or further purification is required to detect Sp1 binding. Within the 5′ flanking region of the TK gene there is also a sequence identical to a 20-bp region of dihydrofolate reductase, containing a G-C box, to which no binding was detected. Similarly, sequence homology shared by the histone H3 and the TK promoters near the TATA box and the upstream end of the 19-bp repeat show no binding interaction with nuclear extracts. Thus, regions of sequence homology cannot necessarily predict sites for DNA–protein interactions.

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