Positive and Negative Regulation of the Human Thymidine Kinase Promoter Mediated by CCAAT Binding Transcription Factors NF-Y/CBF, dbpA, and CDP/cut

Edmund C. Kim, Julie S. Lau, Stephen Rawlings, and Amy S. Lee

Department of Biochemistry and Molecular Biology and the Norris Comprehensive Cancer Center, University of Southern California School of Medicine, Los Angeles, California 90033-0800

Abstract
The proximal CCAAT element located 38 bp upstream of the transcription initiation site contributes to the human thymidine kinase (htk) promoter activity, because site-directed mutagenesis of a 10-bp region containing this CCAAT motif (TKC1) reduced the promoter activity by 55%. Through binding site competitions and antigenic cross-reactivity, the major factor that binds TKC1 from both HeLa and hamster nuclear extracts is identified as NF-Y/CBF. In serum-stimulated cells, the binding of NF-Y/CBF to TKC1 increased gradually, reaching a plateau at the S phase. In cell transfection assays, a dominant-negative mutant of NF-Y/CBF inhibited the htk promoter in a dosage-dependent manner, providing direct evidence that NF-Y/CBF is required for maximal htk promoter activity. Recently, it has been demonstrated that the site occupied by NF-Y/CBF also binds the serum-inducible dbpA and dbpB. We show here that recombinant dbpA interacts with the htk promoter, and overexpression of dbpA can stimulate htk promoter activity mediated through TKC1. In contrast, CDP/cut, the CCAAT displacement protein with known repressor property, binds the htk promoter through both the proximal and distal CCAAT elements. Our discovery that CDP/cut binds the htk promoter primarily in quiescent cells and that overexpression of CDP/cut inhibits htk promoter activity provides an explanation for the reported dramatic increase in htk promoter activity in serum-starved cells when both CCAAT elements were mutated. Thus, a combination of suppression in quiescent cells and activation in serum-stimulated cells mediated through various CCAAT-binding proteins may account in part for the induction of htk promoter activity as quiescent cells reenter the cell cycle.

Introduction
In response to serum stimulation, quiescent cells enter the cell cycle by transcriptionally activating a set of replication-dependent genes (1). One of the best-studied gene systems whose expression is stringently controlled during the cell cycle is the thymidine kinase gene encoding for an enzyme in the salvage pathway of TTP formation. Extensive mutational analysis has revealed that the htk promoter contains several important functional elements that include the two inverted CCAAT motifs, located at positions −38 and −70, and the core of the CCRU, positioned at −110 and −84 (2–5). Several lines of evidence show that the CCRU is critical for the G1-S-phase stimulation of transcription of the htk promoter: (a) site-directed mutagenesis of the sequence between −109 and −84 resulted in the loss of the G1-S-phase increase in transcription (6); (b) complexes containing cyclin A, p107, and cdk2 were detected binding to the CCRU in serum-stimulated cells (6); and (c) stimulation of the htk promoter by overexpression of cyclin A and cyclin E required the CCRU (7). Recently, an abundant 40-kDa protein distinct from the known E2Fs has been identified as a binding factor to the CCRU (8).

The role of the two inverted CCAAT motifs immediately upstream of the initiation start site of the htk promoter has also been investigated through mutational analysis (2, 3, 9, 10). Collectively, these studies demonstrate that both the proximal and the distal CCAAT motif contribute to the htk promoter activity. However, mutation or deletion of either CCAAT motif still retains htk promoter G1-S-phase regulation in serum-stimulated cells (9, 10), suggesting that the regulatory factor(s) that binds to the CCAAT motifs is not obligatory for the rapid increase in the transcription rate of the htk gene when cells enter into the S phase. Nonetheless, the CCAAT motifs are important for the overall promoter strength of the htk promoter, and their binding factors may act as coactivators for the htk promoter. In support of this, the nuclear protein interacting with the distal CCAAT motif of the htk promoter (referred to below as TKC2) has been implicated in the serum-dependent activation of the htk gene in normal human fibroblasts (11). By biochemical characteriza-

Received 8/12/97; revised 10/8/97; accepted 10/17/97.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by NIH Grant GM31108 (to A. S. L.).
2 To whom requests for reprints should be addressed, at Department of Biochemistry and Molecular Biology, University of Southern California/Norris Comprehensive Cancer Center, Los Angeles, CA 90033-0800. Phone: (213) 764-0507; Fax: (213) 764-0024.

3 The abbreviations used are: htk, human thymidine kinase; CCRU, cell cycle regulatory unit; EMSA, electrophoretic mobility shift assay; CHO, Chinese hamster ovary; NE, nuclear extract; SNE, simplified NE; β-gal, β-galactosidase; poly(dI-dC), poly(dI-dC); poly(deoxyinosinic-deoxycytidylic acid); GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase.
tions, the heteromeric CCAAT binding protein, NF-Y, whose subunit A expression is serum-stimulated in human diploid fibroblasts, has been identified as the binding factor to the distal CCAAT element of the htk promoter (11). Nonetheless, no functional assays in vivo have been reported to test the requirement of NF-Y or other CCAAT binding proteins in htk promoter expression.

NF-Y, also referred to as CBF (12), is evolutionarily conserved with the Saccharomyces cerevisiae HAP2 and HAP3 (13). Recently, it has been demonstrated that the site occupied by NF-Y/CFB also binds dbpA and dbpB, the latter of which is commonly referred to as YB-1 (14). The dbps belong to the Y-box protein family, which is one of the most evolutionarily conserved nucleic acid-binding proteins defined in bacteria, plants, and animals (15). Y-box proteins exhibit an interesting property: they can bind to single-stranded templates of cellular and viral promoters and have the ability to repress as well as stimulate transcription of various promoters in different cell types (16–18). Interestingly, analysis of the cell cycle expression of a Y-box protein referred to as RYB-a after serum stimulation of quiescent cells revealed that the transcript level of RYB-a increased dramatically after serum stimulation, peaking at the G_{1}–S-phase border, and was sustained throughout the S phase (19). When the expression of the RYB-a transcript was deregulated through the addition of genistein, a tyrosine kinase inhibitor, the induction of the histone H2B transcript at the S phase was completely suppressed. Thus, Y-box proteins can play an important role in G_{1}–S-phase progression.

In this report, we focus on the functional contribution of the proximal CCAAT element (referred to as TKC1) to htk promoter activity and the identification of transcription factors that interact with it and confer regulation in vivo. What are the regulatory proteins for TKC1? Can they mediate activating as well as repressing mechanisms? Through binding site competitions and antigenic cross-reactivity, we identified that NF-Y/CFB is the major binding factor for TKC1. Thus, both TKC1 and TKC2 share the same CCAAT binding factor. To establish a functional role of NF-Y/CFB, we demonstrate through the use of a dominant-negative mutant of NF-Y (20) that not only does NF-Y/CFB bind to TKC1, it is required for maximal htk promoter activity in vivo. The positively activating role of NF-Y correlates with the binding profile of NF-Y to TKC1, which increases gradually as the cells are released from serum starvation and reaches a plateau at the S phase.

With the establishment that NF-Y is a regulatory protein for htk promoter activity, we tested whether the Y-box proteins known to bind to an NF-Y site have any affinity for TKC1, which bears striking sequence similarity to a Y-box protein–interactive site in the MHC class II DRA promoter (14). If there is binding, does it act as an activator or a repressor in the context of the native htk promoter? We show here that the transcript level of Y-box proteins increases when quiescent cells enter the cell cycle, and in cotransfection experiments, dbpA can transactivate the htk promoter containing a functional TKC1. Furthermore, using recombinant dbpA in EMSAs, we observed that it has a high affinity for the single-strand template encoding the sense strand of TKC1.

The induction of the htk promoter activity on serum stimulation can also involve repression mechanisms that suppress htk promoter activity in quiescent cells. Consistent with this hypothesis, a large increase in htk promoter activity was observed in quiescent cells when both TKC1 and TKC2 were mutated (10). This intriguing result strongly suggests that a repressor may bind to TKC1 and TKC2 in quiescent cells. We propose here that the CCAAT displacement protein CDP, also known as cut (21), may serve such a role in the htk promoter. CDP has been shown to repress transcription through active repression or competition for NF-Y/CFB and Sp1 binding sites (22). Its binding to CCAAT motifs in the promoter regions of replication-dependent histone genes has been reported recently (23, 24). Interestingly, in several of the promoters reported to bind CDP, the formation of the CDP-containing complex requires the presence of at least two CCAAT motifs in close proximity (21, 23). We show that a CDP-containing complex could be detected when a htk promoter subfragment containing both TKC1 and TKC2 was used in EMSAs. Our finding that CDP binding activity was primarily detected in quiescent cells and that CDP conferred an inhibitory effect on htk promoter activity in cotransfection experiments lends support to our hypothesis that CDP acts as a repressor for htk promoter activity in quiescent cells.

Results

The Proximal CCAAT Motif Is a Major Regulatory Element of the htk Promoter. Regulatory domains of the htk promoter include two inverted CCAAT elements at −38 and −70 (referred to as TKC1 and TKC2, respectively), a 70-bp CCRU, and two GC-rich motifs flanking the CCAAT sequences (Fig. 1). In this study, we examined the functional significance of TKC1 and the transcription factors that interact with this CCAAT site most proximal to the TATA element. Previously, we have shown that 474 bp of the htk promoter, when fused to a reporter gene and transfected into K12 cells, a well-characterized temperature-sensitive Chinese hamster cell cycle mutant cell line, retain their high basal activity and G_{1}–S-phase inducibility (5). Whereas deletion or mutation of TKC1 did not affect the G_{1}–S phase transcriptional increase of the htk promoter (9, 10), site-directed mutagenesis of TKC1 resulted in a 55% decrease in the overall htk promoter activity in exponentially growing hamster cells (Fig. 1). Thus, TKC1 is required for maximal htk promoter activity.

A Common Nuclear Factor, NF-Y/CFB, Binds TKC1 from Human and Chinese Hamster Cells. To determine whether the same factors interact with TKC1 in human and hamster cells, NEs were prepared from HeLa and CHO cells, and EMSAs were performed. The sequences of the TKC1 and random sequence synthetic oligomers used in the binding reactions and competition experiments are shown in Table 1. Using NEs from either cell line, a major specific complex (C1) was formed with the labeled TKC1 probe. The electrophoretic mobility of C1 formed with HeLa NE was identical to the one formed with CHO cells (Fig. 2). This complex could not be competed with a heterologous oligomer (Ran #1) but could be competed with increasing molar excess of TKC1. Using an antibody specific for NF-Y/CFB in EMSAs, we observed that the NF-Y antibody, but not the
preimmune serum, was able to inhibit the formation of the C1 complex using either the HeLa or CHO NE (Fig. 3). Therefore, by criterion of antigenic cross-reactivity, NF-Y/CFB is a component of the TKC1 complex in both human and hamster cells.

If NF-Y/CFB binds to TKC1, and because NF-Y/CFB has been shown to bind TKC2, TKC2 should be an effective competitor for the C1 complex. Reciprocally, TKC1 should also be an effective competitor for the complex formed with TKC2. In EMSAs, the complexes formed using HeLa NE and either radiolabeled TKC1 or TKC2 as probe ran identically in EMSAs (Fig. 4A). As predicted, TKC1 and TKC2, but not a random sequence oligomer, were equally effective in inhibiting the formation of the C1 complex.

**Table 1** Sequences of synthetic oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>TKC1</td>
<td>aatttcgcccgtctggtaaggccgcaagcttcgctggctccatag</td>
</tr>
<tr>
<td>TKC2</td>
<td>gtcgacctggccccggcgctggccagagcttcgctggctccatag</td>
</tr>
<tr>
<td>CBF</td>
<td>tcagacgttctccagactgagcgctggccagcttcgctggctccagct</td>
</tr>
<tr>
<td>MutCBF3A</td>
<td>tcagacgttctccagactgagcgctggccagcttcgctggctccagct</td>
</tr>
<tr>
<td>LY</td>
<td>ctagctttgagacagagaaatct</td>
</tr>
<tr>
<td>MY</td>
<td>ctagctttgagacagagaaatct</td>
</tr>
<tr>
<td>TKC2</td>
<td>ctagctttgagacagagaaatct</td>
</tr>
<tr>
<td>MuTKC2</td>
<td>ctagctttgagacagagaaatct</td>
</tr>
<tr>
<td>AP1</td>
<td>agcttttaacagaggtatgacgctagct</td>
</tr>
<tr>
<td>MutAP1</td>
<td>ctagcttttaacagaggtatgacgctagct</td>
</tr>
<tr>
<td>Random #1</td>
<td>ctagcttttaacagaggtatgacgctagct</td>
</tr>
<tr>
<td>Random #2</td>
<td>ctagcttttaacagaggtatgacgctagct</td>
</tr>
</tbody>
</table>

* The CAAT motifs are boxed, and bold italic print indicates mutated bases substituted in the wild-type site. The lowercase letters at the end of the oligomers indicate poly linker DNA sequence.
Recently, it was shown that the GA-rich motif flanking the CCAAT element of the α2(I) collagen promoter (25) is also important for NF-Y/CBF binding, such that mutation of the GA motif would diminish its ability as a NF-Y/CBF competitor (26). In EMSAs, we observed that the wild-type NF-Y/CBF site was as efficient as TKC1 in competition assays for the C1 complex (Fig. 4B). Mutation of the GA-rich motif (mutCBFGA) weakened its potency as a competitor. Therefore, by criteria of binding site competition and antigenic cross-reactivity, NF-Y/CBF is the binding factor for both TKC1 and TKC2.

Kinetics of NF-Y/CBF Binding to TKC1 After Serum Stimulation. Previously, it has been reported that human tumor cells exhibited an elevated binding level to TKC2, such that the TKC2 binding activity was serum independent in human tumor cells but was serum dependent in normal human diploid fibroblasts (27). Subsequently, it was determined that the expression of the A subunit of NF-Y was serum dependent in human diploid fibroblasts, accounting for the increased NF-Y/CBF binding to the TKC2 16 h after serum stimulation (11). Despite these important observations, a detailed binding profile of NF-Y/CBF to either TKC1 or TKC2 after serum stimulation of quiescent cells has not been reported. To address this issue, SNEs were prepared from serum-stimulated Chinese hamster fibroblasts (K12 cells) at 2-h intervals, and EMSAs were performed using equal amounts of protein from each time point and TKC1 as probe (Fig. 5A). For comparisons, EMSAs were also performed with two other probes, one containing the collagenase AP1 binding site (Fig. 5B) and another containing a mutated AP1 binding site within a hamster H3 promoter subfragment (Fig. 5C), referred to as mutAP1 (Table 1). The binding activity of the abundant but nonspecific complex X with the mutAP1 probe was used as a control to normalize for the amount of protein and the binding competency of each protein sample throughout the cell cycle. Our results indicate that on the addition of fresh medium containing serum to quiescent cells, the level of CBF binding to TKC1 remained unchanged for the first 4 h and then increased gradually, reaching a plateau at the S phase, with about a 2-fold increase in binding activity (Fig. 5D). In contrast, the binding activity of AP1 rapidly increased by about 5-fold after serum stimulation and quickly subsided.

Inhibition of NF-Y/CBF Down-Regulates htk Promoter Activity in Vivo. To determine whether NF-Y/CBF, in addition to binding to the CCAAT motifs of htk promoter, is actually contributing to the overall promoter activity of the htk promoter, functional assays were performed in vivo. Because NF-Y/CBF is a heteromeric transcription factor consisting of at least three subunits (28), its transcription activation activity cannot be easily reconstituted in vivo. To circumvent this problem, a dominant-negative mutant of NF-Y referred to as NF-YA29 (20) was used in transient transfection assays to test if NF-Y/CBF is required for maximal htk promoter activity. NF-YA, in which three amino acids in the DNA binding domain have been mutated, forms a complex with NF-YB, rendering it functionally inactive as a transcription activator (20). In these experiments, phkt(474R)CAT or pSV2CAT was cotransfected with either NF-YA29 or pSV2gpt, a similar vector containing a heterologous gpt gene, and a β-gal expression vector was included as an internal control for transfection efficiency. An example of the results is shown in Fig. 6. The NF-Y dominant-negative mutant inhibited htk promoter activity in a dosage-dependent manner (Fig. 6). At the highest dosage tested, the htk promoter activity was reduced 4.3-fold, as compared to a 1.4-fold reduction observed for the control SV40 promoter.
Stimulation of htk Promoter Activity by dbpA. The binding of NF-Y/CFB to the TKC1 site raises the question of whether Y-box proteins are also able to interact with TKC1. In comparing the TKC1 sequence with a Y-box protein interactive site (LY) in the DRA promoter, striking similarity was noted in both the 5' and 3' flanking sequences of the CCAAT motif (Table 1). Using HeLa NEs and TKC1 as probe, the htk-specific C1 complex formed can be efficiently competed by LY, but not when the CCAAT motif of LY was mutated (MY; Fig. 7). The expression of rat Y-box-binding protein has been shown to be serum inducible in BALB/c 3T3 fibroblast cells (19). In hamster fibroblast K12 cells, the endogenous mRNA level of both dbpA and YB-1 expression is also serum stimulable (Fig. 8A). The low level overall of dbpA transcripts in K12 cells suggests that they are suitable recipient cells for the examination of the effect of overexpression of dbpA on htk promoter expression. To test this, the reporter plasmid phtk(474R)CAT and the expression vector for dbpA (SFFV-dbPA) were cotransfected into K12 cells. As a control, identical sets of cells were transfected with the expression vector (SFFVneo) instead of SFFV-dbPA. The relative htk promoter activities, as measured by CAT assays (Fig. 8B), were determined after normalization of the transfection efficiencies with the β-gal activities in each of the cell extracts (Fig. 8C). In cells overexpressing dbpA, a 2.4-fold stimulation of the htk promoter was observed. When the CCAAT motif of TKC1 was mutated [phtkLS(−43/−33)CAT] or pSV2CAT was used, no induction was observed.

To test whether dbpA can bind TKC1, bacterially expressed GST-dbPA was used in EMSA (Fig. 9). Our results showed that recombinant dbpA, in the absence of other mammalian nuclear factors, was able to bind TKC1. Because dbpA can also bind single-stranded templates, we tested whether it has preferential affinity for the sense or antisense strand of TK1C (Table 1). Our results indicated that in EMSAs, molar excess of the sense strand of TK1C was able to partially inhibit the formation of the dbpA complex (Fig. 9). At the concentration of oligomers tested, the antisense strand of TK1C was not effective as a competitor.

Association of CDP with the htk Promoter in Quiescent Cells. Through site-directed mutagenesis of TKC1 and TKC2, it was reported that on simultaneous mutation of both elements, a large increase in the basal promoter activity of htk in serum-deprived cells was observed (10). However, a decrease in promoter activity was observed if either TKC1 or TKC2 was mutated. These results strongly suggest that a repressor, whose binding to the htk promoter can only be eliminated when both TKC1 and TKC2 are mutated, acts to inhibit htk promoter activity when the cells are serum-starved. One such candidate for the repressor is the CCAAT displacement protein (21), a 180–190-kDa protein that has been shown to bind to promoter subfragments with two or more CCAAT motifs. To test whether CDP interacts with the htk promoter, SNEs were prepared from serum-starved cells and cells stimulated to progress through the cell cycle. The quality of each protein sample was tested using the mutAP1 probe as described in Fig. 5. EMSAs were performed using a synthetic oligomer (TK2C) containing both TKC1 and TKC2 (Table 1). In using this relatively long probe, multiple protein-containing complexes were observed (Fig. 10A). The band patterns were similar at all time points, with the exception of a set of faint bands (complex a) that migrated with the slow-
were mutated (mutTK2C), the NF-Y/CFB consensus binding site, and a random oligomer (Table 1 and Fig. 10C). The results indicated that complex a was specifically competed by TK2C. None of the other competitors was effective in eliminating complex a. It was notable that the addition of the NF-Y/CFB site seemed to alter the mobility of complex b, which is likely to contain multiple protein components. Collectively, these results showed that CDP binds TK2C through the CCAAT motifs, and its binding is detected primarily in quiescent cells.

Because CDP has been shown to be a potent transcription inhibitor, we tested its effect on htk promoter activity by cotransfecting an expression plasmid of CDP or its vector with phtk(474R)CAT. The results, as shown in Fig. 10E, demonstrated a 3.5-fold reduction in htk promoter activity when the CDP expression plasmid was used. In contrast, the expression of CDP reduced the SV40 promoter activity by only about 1.3-fold.

**Discussion**

Mutational analysis on the htk promoter (2, 4–6, 10) established that the proximal and distal CCAAT elements contribute to the promoter strength of the htk promoter. In this study, we show that both elements bind the multimeric NF-Y/CFB, a CCAAT-binding protein ubiquitously present in many cell types. In addition to the htk promoter, NF-Y/CFB has also been shown to interact with other cell cycle-regulated promoters. For example, it has been reported that the S-phase-G2-specific transcription of the human cdc25 gene is mediated by the periodic repression of NF-Y/CFB that serves as an activator (29). In the case of the cell cycle-regulated transcription of the R2 gene of mouse ribonucleotide reductase, a proximal CCAAT element specifically binding NF-Y/CFB is required for continuous activity of the R2 promoter through the S phase (30). Despite the strong correlation data on the binding of CBF/NF-Y to the CCAAT elements of promoters of cell cycle-regulated genes including that of htk, direct functional tests of the involvement of NF-Y/CFB in the regulation of these genes are lacking. This could be attributed to the difficulty in the reconstitution of the NF-Y/CFB activity in vivo. Because this CCAAT-binding protein consists of at least three subunits and all three are required for DNA binding (31), cotransfection assays to test for function are extremely difficult. To circumvent that problem, we demonstrated here that a dominant-negative mutant of NF-Y/CFB, when cotransfected into mamalian cells with a reporter gene driven by the htk promoter, is able to inhibit its promoter activity. These results established that NF-Y/CFB is one of the rate-limiting transcription factors for the overall htk promoter activity.

Previously, NF-Y/CFB has been linked to the induction of the htk promoter in quiescent cells after serum stimulation and to the differential regulation of the htk promoter in normal human diploid cells versus HeLa cells. This is based on the important finding that in non-transformed diploid human cells, when stimulated with serum, NF-Ya is expressed, and a full complement of the NF-Y subunits allows it to form a stable complex on NF-Y/CFB sites (11). The serum dependence of the NF-Y binding activity in normal human diploid fibroblasts

---

**Fig. 5.** Binding activity of complex C1 after serum stimulation of synchronized K12 cells. One μg of SNE prepared from synchronized K12 cells was mixed with 500 ng of poly(dIdC) and with either 1 ng of radiolabeled TK1 (A), AP1 (B), or mutAP1 (C) as probes. The EMSA was resolved on a 4% polyacrylamide gel. Top, the time (h) after serum release is indicated. In D, the relative C1 and AP1 binding activities were normalized against complex X and plotted against the time (h) after serum release.
Fig. 6. Effect of the dominant-negative NF-Y on promoter activities. Each reporter construct [10 μg of phtk(474R)CAT or pSV2CAT] was cotransfected into K12 cells with either 1 or 2 μg of NF-YA29 or a similar vector containing a heterologous gpt gene [pSV2gpt] as indicated at the top. The transfections were performed in duplicate. The autoradiograms are shown. Bottom, the average percentage of CAT conversion for each set of reactions is shown.

Fig. 7. Competition of complex C1 by the DRA promoter Y-box protein binding site. The EMSAs were performed as described in the Fig. 2 legend. The competitors used were TKC1, wild-type Y-box protein binding site LY, mutated site MY, and random oligomer (Ran#1; Table 1) at 10-, 20-, and 40-fold molar excess. The positions of C1 and free probe (F) are indicated.

is due to the decrement of NF-Ya but not NF-Yb expression after serum deprivation. Further, such serum dependence is absent in a variety of human tumor cell lines (11, 27). Here, we present a detailed analysis of the cell cycle binding profile of NF-Y/CFB to the most proximal CCAAT element of the htk promoter. Our results indicated a gradual increase in binding of NF-Y/CFB to the htk CCAAT element when quiescent cells entered the cell cycle after serum stimulation, beginning in mid-G1, and persisted through the S phase. This binding profile lends support to the role of NF-Y/CFB as an accessory activator of the htk promoter contributing to maximal htk promoter activity during S-phase. One proposed mechanism for NF-Y/CFB action is through the stabilization and recruitment of upstream DNA binding transcription factors (32). In the case of the htk promoter, factors that bind to the CCRU are likely candidates for NF-Y/CFB recruitment.

Fig. 8. Stimulation of htk promoter activity by cotransfection of dbpA in K12 cells. A, transcript levels of dbpA and YB-1 in serum-released K12 cells. Ten μg of cytoplasmic RNA samples prepared at various times (h) after serum stimulation of K12 cells were subjected to RNA blot analysis using dbpA, YB-1, and glyceraldehyde-3-phosphate dehydrogenase cDNAs as probes. The autoradiograms are shown. B, 5 μg of phtk(474R)CAT, phtkLS(-43/-33)CAT, or pSV2CAT were cotransfected with either 10 μg of the dbpA expression vector or the vector SFFV-neo (V). The experiments were performed in duplicate. The autoradiograms for the CAT assays are shown. C, summary of three independent cotransfection experiments. The relative CAT activities and their SDs are indicated.

Our investigation into the role of the Y-box proteins in htk promoter regulation mediated by the proximal and possibly also the distal CCAAT element addresses the critical issue of target genes for this highly evolutionarily conserved tran-
scription factor that is serum-stimulable. Although it remains to be determined whether the Y-box protein binding to the htk promoter is of sufficient stability to be detected in the TKC1 complex, we showed that recombinant dbpA, a Y-box protein, can bind to the htk promoter that shares striking sequence similarity to the DRA promoter site for Y-box protein and NF-Y/CFB (14). Overexpression of dbpA activates the htk promoter primarily through the proximal CCAAT element of the htk promoter, because mutation of TKC1 while retaining an intact TKC2 eliminates the dbpA stimulation. This suggests that although TKC1 and TKC2 bind similar factors, they may not be functionally equivalent (10). Earlier studies with different promoters indicate that Y-box proteins can bind to single-stranded DNA templates and, in cooperation with other transcription factors, modulate gene activity (14, 33). Our finding provides an additional example that dbpA has an affinity for the sense strand of TKC1 and raises the possibility that in the case of the htk promoter, the simultaneous induction of CBF/NF-Y binding activity and the expression of Y-box proteins after serum stimulation could result in the synergistic activation of the htk promoter mediated by their common CCAAT binding sites.

Our discovery that the CCAAT displacement protein, CDP/ cut, binds to the htk promoter in quiescent cells provides an explanation for the dramatic increase in htk promoter activity when both the most proximal and distal CCAAT motifs were mutated (10). Because CDP has been found to exhibit strong repressing activities in multiple cell systems either through active repression or competition with site occupancy (21, 22, 34), in the htk system, one possibility is that in quiescent cells, CDP binding to the promoter interferes with the activating activity of NF-Y/CFB by occupying the same CCAAT sites. As in the case of other promoters down-regulated by CDP, the htk promoter contains multiple CCAAT elements in close proximity. Interestingly, the CDP binding site has re-
cently been linked to the transcription regulation of various histone genes, which are also growth regulated (23, 24). Furthermore, CDP itself may associate with different retino blastsoma protein family members (24). Previously, we demonstrated that the htk promoter can bind cyclin A, p107, and cdk2 in S-phase cells after serum stimulation (6). Thus, CDP and NF-Y/CBF could be common regulatory factors shared among replication-dependent genes such as histone and htk. As such, they may be part of a common mechanism for their coordinated suppression in quiescent cells and maximal expression in S-phase cells induced by serum stimulation.

Materials and Methods

Cell Culture. Conditions for culturing and the synchronization of the Chinese hamster fibroblast K12 cell line have been described previously (35).

Plasmids. The construction of phtk(474)CAT and the pCH110 plasmid, which contains the β-gal gene under the control of the SV40 promoter, has been described previously (6). Plasmid pHKLS(−43 to −33)CAT was created by PCR-directed site mutagenesis. The mutated sequence spanning −43 to −33 of the wild-type promoter is TGTCAGATTC, and it contains a Spel site; subsequently, this was verified by restriction digestion and sequencing. The construct for the expression vector of dbpA has been described previously (18). The pS2Vgpt expression plasmid has been described previously (36). The NF-Y/CBF dominant-negative construct, referred to as NF-YA29 (a gift of Peter Edwards, UCLA, Los Angeles, CA), was described previously (20). The expression plasmid for CDP and its vector control, under the control of the CMV promoter (a gift of Ellis Neufeld, Harvard Medical School, Boston, MA), have been described previously (21).

NEs. NEs from HeLa, CHO, and K12 cells were prepared as described previously (35). Preparation of SNES from exponentially growing and synchronized K12 cells has been described previously (35).

Transfections and CAT Assays. Transient transfections into exponentially growing K12 cells were performed using the calcium phosphate method as described previously (37), with slight modifications. Each transfected contained the reporter gene, the β-gal expression vector pCH110 (3 μg), and the HeLa carrier DNA (3 μg). Assaying for transfection efficiency was performed using the β-gal assay. Extracts containing equal β-gal activities were assayed for CAT activity and quantitated by either a PhosphorImager (Molecular Dynamics) or by an AMBS radioactive imaging system (AMBS Systems). Each of the transfections was repeated independently two to four times.

EMSSAs. Table 1. assay the sequences of the oligonucleotides used. Probes were prepared by annealing the oligonucleotides and labeling the duplex with [α-32P]dATP and [α-32P]dCTP using the Klenow enzyme. For the analysis of TKC1 and TKC2 complexes, 1–2 ng of probe (about 5 × 10^6 cpm) were added to 1 μg of HeLa or CHO NE with 1 μg of poly[d(C·D)] in a 20-μl volume containing 20 mM HEPS (pH 7.9), 40 mM KCl, 2.5 mM MgCl2, and 5% glycerol. Competitor binding site oligonucleotides were added to the reactions at the concentrations indicated in the figure legends. Reactions were incubated at room temperature for 20 min; for the antibody reactivity, rabbit anti-CBF A peptide (a gift of Sankar Maiti, M. D. Anderson Cancer Center, Houston, TX), guinea pig anti-CBP (gift of Ellis Neufeld), or their respective preimmune sera were added after allowing the reaction to proceed for 15 min. The preparation of the anti-CBF and anti-CBP antibody directed specifically against their respective CCAAT-binding protein has been described previously (38, 39). The preparation of bacterially expressed GST-dbPA has been described previously (18). For the formation of the dbPA complex, the binding reaction contained 1 ng of probe, 500 ng of poly[d(C·D)], and 2 μl of purified GST-dbPA.

Preparation of CCAAT RNA antisequences were performed as described previously (40). The probes used were cDNA fragments for dbPA and YB-1 (18) and glyceraldehyde-3-phosphate dehydrogenase (41).

Acknowledgments

We thank Ellis Neufeld for the CDP antibody and expression plasmid, Sankar Maiti for the CBF-A peptide antibody, and Peter Edwards for expression plasmid NF-YA29. We thank Victor Wong and Yanhong Zhou for excellent technical assistance. Helpful discussions and assistance from Binayak Roy, Gadi Gazit, and Frank Wu are greatly appreciated.

References