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Identification of a 70-Base-Pair Cell Cycle Regulatory Unit within the Promoter of the Human Thymidine Kinase Gene and Its Interaction with Cellular Factors

YONG KYU KIM AND AMY S. LEE*

Department of Biochemistry and the Norris Cancer Research Institute, University of Southern California School of Medicine, Los Angeles, California 90033

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The promoter of the human thymidine kinase gene contains *cis*-regulatory elements responsible for its cell-cycle-regulated expression. We report here that a 70-bp region between −133 and −64 is sufficient to confer cell cycle regulation on a heterologous promoter. The 20-bp region between −64 and −83, which contains an inverted CCAAT motif, is important for transcriptional stimulation of this functional unit. The sequence of this CCAAT motif is nearly identical to the consensus sequence for the transcriptional factor CP1. We also examined the specificity and binding activities of cellular factors interacting with the 70-bp fragment. We showed that the cellular factors binding to the 70-bp region are similar during the G_1, S, and G_2 phases, suggesting that the cell cycle regulatory activity observed must involve processes other than factor binding to the DNA.

The thymidine kinase (TK) gene encodes a cytosolic enzyme of the pyrimidine salvage pathway. This enzyme catalyzes the phosphorylation of thymidine to form thymidine 5′-monophosphate. Its activity is maximal during the onset of DNA synthesis in the mammalian cell cycle (18). In growth-arrested quiescent cells and terminally differentiated postreplicative cells, the TK activity is diminished and the TK gene is transcriptionally repressed (11, 29).

To understand the mechanism whereby the TK gene is temporally regulated during the cell cycle, the levels of control of the TK gene have been investigated. It has been established that the TK gene is regulated at both the transcriptional and the posttranscriptional levels. With the onset of DNA synthesis, there is a severalfold increase in the rate of transcription of the TK gene (6, 32). At the same time, there is a change in the nuclear posttranscriptional processing of *TK* heterogeneous nuclear RNA (12). The steady-state levels of *TK* mRNA also increase sharply as the cells enter the S phase (24). Thus, several mechanisms might contribute to the 10- to 20-fold increase in the *TK* mRNA levels during the DNA synthetic phase of the cell cycle.

The direct observation that the TK gene is transcriptionally activated at the border of the G_1 and S phases suggests that sequences contained within the promoter of the TK gene might direct the increase in its transcription rate during the cell cycle. To establish the existence of such a sequence, we and others fused the *TK* promoter sequence to reporter genes such as the neomycin resistance gene (*neo*) and the chloramphenicol acetyltransferase (CAT) gene and monitored their expression during the cell cycle (17, 33). In both reporter gene systems, cell cycle regulation was observed. These results provide the first evidence that the *TK* promoter sequence is capable of directing cell cycle regulation of heterologous genes. Deletion analysis revealed that the region important for cell cycle regulation is between −441 and −64 bp from the transcriptional start site. We also demonstrated that this 378-bp *TK* promoter fragment has enhancing activity and can confer cell cycle regulation on a heterologous promoter such as that of the herpes simplex virus (HSV) tk gene (17).

The sequence of the human TK promoter has been reported (7). Within the sequence spanning −441 to −64 (Fig. 1A), there is an inverted CCAAT sequence at positions −67 to −71. At positions −113 to −118, −227 to −232, −246 to −252, and −409 to −414, there is a G+C-rich sequence resembling that of the Sp1 consensus site (9). At −164 to −171, a site which resembles the Oct-1-binding site (21) is located, and at −206 to −272, a large inverted repeat sequence centering around the *DraI* site is found. One approach to analyze the control elements within this region is site mutagenesis of all the individual sites. Another approach, which we have taken here, is to identify the minimal sequences within this fragment which can confer cell cycle regulation on an otherwise non-cell-cycle-regulated promoter. In the work described in this report, we identified a 70-bp region which can function as a cell cycle regulatory unit (CCRU). The specificity and binding activities of cellular factors interacting with this 70-bp fragment were examined.

Construction of *TK* promoter fusion genes. Previously, we demonstrated that the human *TK* promoter sequence spanning −441 to +34, fused in the same transcription orientation as the *neo* gene (pTKN441) (Fig. 1B), is capable of conferring cell cycle regulation on *neo*, whereas constructs deleted to −63 (pTKN63) have lost all cell cycle inducibility (17). In addition, the TK promoter subfragment from −441 to −64 is independent of its own TATA element for function. Thus, in pTKN378R, the region from −441 to −64 confers cell cycle regulation on the truncated HSV *tk* promoter in an orientation-dependent manner (17).

To define the minimal sequence requirements within this fragment that can act as a cell cycle regulatory unit, a series of *TK* promoter subfragments containing progressive deletions from the 5′ end but sharing the same 3′ endpoint at −64 were fused to the HSV *tk* promoter linked to the *neo* transcriptional unit (Fig. 1B). The precise 5′ endpoints and orientation of fusion were confirmed by restriction mapping

* Corresponding author.

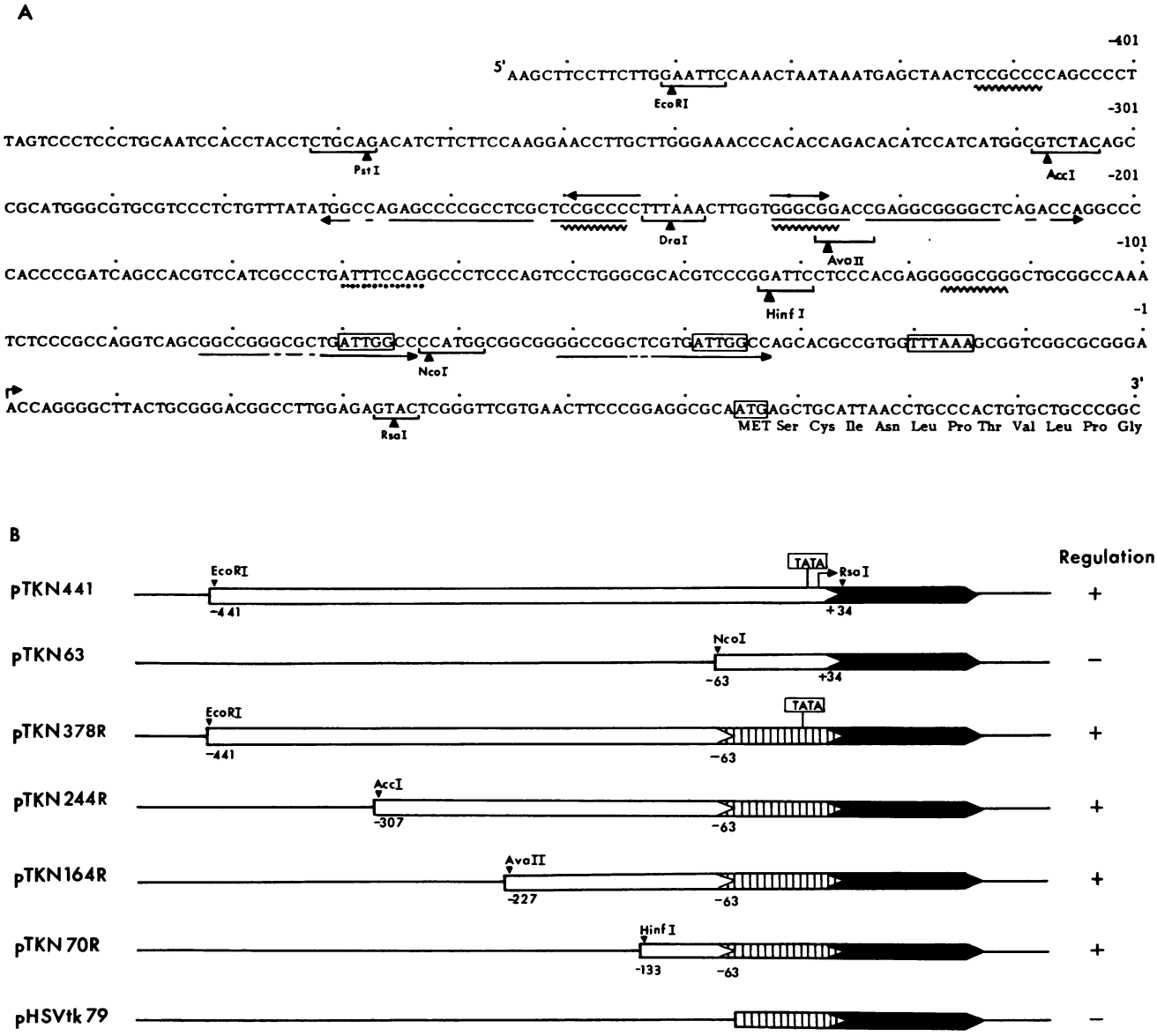

FIG. 1. (A) Promoter of the human TK gene. The DNA sequence has been determined (7). The restriction enzyme sites for *Eco*RI, *Pst*I, *Acc*I, *Dra*I, *Ava*II, *Hin*fI, *Nco*I, and *Rsa*I are indicated. The TATA box, the inverted CCAAT elements, and the translation initiation codon ATG are boxed. Putative Sp1-binding sites are indicated by sawtooth underlining. An Oct-1-binding site has a dotted underline. The inverted and direct repeats are underlined and indicated by pairs of arrows. The major transcriptional start site is numbered as 1 and indicated by a short arrow. (B) Structure of the *TK neo* fusion genes. Symbols: ▭, human *TK* 5′-flanking sequence; ■, *neo* gene coding sequence; ▥, HSV *tk* promoter sequence spanning (−79 to +52); ——, procaryotic vector sequence. The 5′ and 3′ endpoints of the *TK* sequence are indicated.

and DNA sequencing. Plasmids pTKN244R, pTKN164R, and pTKN70R contained 5′ sequences up to −307, −227, and −133, respectively. These triple DNA constructs (human *TK* promoter fragment-HSV *tk* promoter-*neo* coding region) were created to test specifically the ability of the *TK* promoter subfragments to confer cell cycle regulation on a heterologous promoter. Previously, we demonstrated that the HSV *tk* promoter, containing only its TATA element and 50 bp upstream, as in pHSVtk79, was not cell cycle regulated (17).

Cell cycle regulation of the 5′-deleted *TK-neo* fusion genes. To assay promoter activities, the fusion genes were stably transfected into K12 cells as described previously (3, 34). The K12 cell line is a temperature-sensitive G_1 cell cycle mutant line derived from the hamster lung fibroblast line, Wg1A (26). The temperature-sensitive mutation allows K12 cells to be synchronized at G_1 by shifting the cells to the nonpermissive temperature, 39°C. K12 cells also have a hypoxanthine phosphoribosyltransferase-negative ($HPRT^-$) phenotype. Therefore, stable transfectants can be selected on the basis of either G418 or hypoxanthine-aminopterin-thymidine (HAT) resistance. In the latter case, the *TK-neo* fusion genes were cotransfected with pSV2gpt into K12 cells (10). Since selection on the basis of G418 resistance may

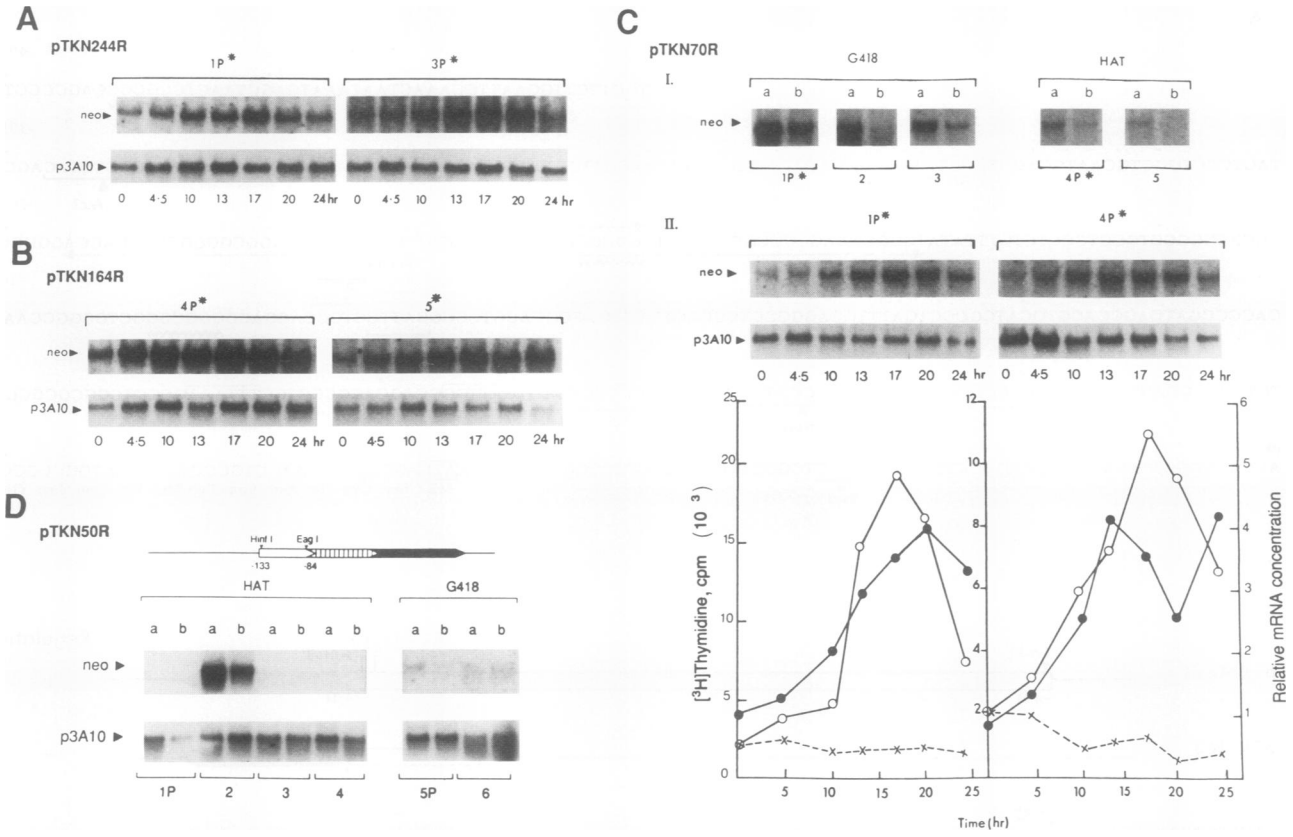

FIG. 2. (A) Cell cycle analysis of *neo* and p3A10 mRNA levels in pooled pTKN244R transfectants selected by G418 (1P) or HAT (3P) after serum stimulation of synchronized cells. (B) Cell cycle analysis of the *neo* and p3A10 mRNA levels in pooled pTKN164R transfectants selected by G418 (4P) or HAT (no. 5). (C) I. Effect of G_1 arrest on *neo* mRNA levels in individual pTKN70R transfectants 2, 3, and 5 and pooled transfectants 1P and 4P selected by G418 and HAT, respectively. Lanes: a, exponentially growing cells; b, G_1-arrested cells. The asterisks indicates the transfectants chosen for analysis in part II. II. Cell cycle analysis of *neo* and p3A10 mRNA levels in 1P and 4P. The autoradiograms were quantitated, and the relative levels of *neo* (○) and p3A10 (×) are plotted against the rate of DNA synthesis (●) as measured by incorporation of [^{3}H]thymidine. (D) Structure of pTKN50R. Symbols: ▭, human *TK* promoter sequence; ▥, HSV *tk* promoter sequence; ■, *neo* gene coding sequence; ——, procaryotic vector sequence. *neo* and p3A10 transcript levels in pooled pTKN50R transfectants selected by HAT (pooled transfectants 1P and individual transfectants 2, 3, and 4), and by G418 (pooled transfectants 5P and individual transfectants 6) are shown. Lanes: a, exponentially growing cells; b, G_1-arrested cells.

result in preferential selection of transfectants expressing high levels of *neo*, the selection on the basis of HAT resistance eliminated this possible bias.

We isolated individual colonies as well as pools of several hundred transfectants. Following mass expansion, the *neo* mRNA levels during exponential growth and under G_1-arrested conditions were compared. The levels of *neo* transcripts in either the HAT- or G418-selected transfectants harboring the fusion plasmid pTK244R were three- to fivefold higher in exponentially growing cells than in cells which had been shifted to 39°C for 16 h (data not shown). To investigate the temporal regulation of *neo* mRNA during the cell cycle, we extracted cytoplasmic RNA from the two pooled transfectants (1P was selected by G418, and 3P was selected by HAT), which had been stimulated to traverse the cell cycle by the addition of fresh serum (Fig. 2A). The mRNA levels of *neo* and of a control plasmid, p3A10, were measured and quantitated. Plasmid p3A10 encodes a hamster transcript which is not cell cycle regulated (17). Concurrently, the rate of DNA synthesis was monitored as previously described (2). As cells entered the DNA synthetic phase around 10 h, the *neo* mRNA level increased by about fourfold and peaked about 17 h after serum stimulation. As DNA synthesis subsided, so did the *neo* mRNA level. The fourfold increase in *neo* mRNA levels during the S phase is consistent with the three- to fivefold increase in the rate of transcription of the TK gene previously reported (32).

Localization of the CCRU to a 70-bp fragment. The deletion constructs pTKN164R and pTKN70R, which contained 164 and 70 bp, respectively, of the *TK* promoter sequence fused to the HSV *tk-neo* transcriptional unit (Fig. 1B), were similarly tested. In the case of pTKN164R, the *neo* mRNA level in pooled (4P) or clonal (no. 5) transfectants selected by HAT showed good correlation with the rate of DNA synthesis throughout the cell cycle (Fig. 2B). Generally, a four- to fivefold increase was observed 17 h after serum release (Fig. 2B). Using a synthetic 24-mer oligonucleotide corresponding to the antisense strand of the *neo* gene, we confirmed the RNA blot results by primer extension analysis (3). Transcription of the hybrid gene initiated at the HSV *tk* cap site and the relative levels of the primer-extended product during the cell cycle were similar to the RNA profile in Fig. 2B (data not shown).

In the pTKN70R transfectants, the *neo* mRNA level was three- to fivefold higher in exponentially growing cells than in G_1-arrested cells (Fig. 2C, panel I). Pools of transfectants,

1P selected by G418 and 4P selected by HAT, showed stringent cell cycle regulation of the *neo* mRNA level after serum release (Fig. 2C, panel II). The *neo* mRNA level increased sharply during the S phase and declined as DNA synthesis subsided. This was in contrast to the p3A10 mRNA level, which fluctuated less than twofold during the cell cycle. Previously, we have shown that the transfectants harboring the fusion plasmid pHSVtk79, which contained only the HSV *tk* promoter sequence, exhibited low levels of *neo* expression (17). The *neo* transcripts were present at similar levels in exponentially growing and G_1 cells. These combined results indicate that a functional CCRU is contained within the 70-bp *TK* promoter subfragment, spanning the region from −133 to −64.

The sequence between −64 and −83 is required for high basal-level expression. To define further the minimal sequence required for the cell cycle regulatory activity, we cleaved the 70-bp *TK* promoter fragment with the restriction enzyme *Eag*I to generate a 50-bp fragment spanning −133 to −84. The proximal CCAAT sequence was eliminated from this subfragment, which was cloned into the HSV *tk-neo* plasmid (17). This construct, pTKN50R (Fig. 2D), was transfected into K12 cells. Pooled and individual transfectants were selected by either HAT or G418 resistance and examined for the *neo* and p3A10 transcript levels. Examples of the RNA analysis are shown in Fig. 2D. We observed that in pooled and individual transfectants, selected by HAT or G418 resistance, the level of *neo* was extremely low or undetectable, with the exception of one individual transfectant selected by HAT resistance (no. 2). In contrast, the levels of p3A10 transcript were comparable to those of the other transfectants described above. These results, together with the intact rRNA profiles observed for all the RNA samples (data not shown), demonstrate that the elimination of the 20-bp sequence between −64 and −83 from the 70-bp fragment results in a dramatic loss of basal-level transcriptional activity for the majority of the transfectants.

Constitutive binding of cellular factors to the 70-bp CCRU. To analyze the interaction of cellular factors with the 70-bp CCRU identified above, total-cell extracts were prepared from synchronized cells traversing through the cell cycle as described previously (25). The DNA probe spanning this region, from −64 to −133, was prepared as follows. The 70-bp *Hin*fI-*Nco*I (Fig. 1B) fragment was subcloned into the *Sma*I site of pUC8. After digestion of this plasmid with *Eco*RI and *Bam*HI, an 80-bp *Eco*RI-*Bam*HI fragment was isolated and labeled with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase I. The interactions between the cellular factors and the DNA fragment were analyzed by gel mobility shift assays. For the binding reaction, 2 μg of protein extract was mixed with 1 ng of labeled probe in a mixture (20 μl) of 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol 0.5 mM EDTA, 6% glycerol, and 1 μg of poly(dI-dC) added as a nonspecific competitor. After incubation for 20 min at room temperature, the samples were loaded onto 6 to 10% polyacrylamide gels. The gels were then dried and autoradiographed at −70°C with intensifying screens.

First, we examined whether DNA-protein complexes were formed. Several bands were apparent (Fig. 3). In this study, we focussed on two complexes, I and II, with I being the most abundant complex in the entire binding pattern. Extracts from G_1-, S-, and G2-phase cells gave very similar binding patterns with some minor fluctuations (Fig. 3). We repeated the above experiments with two other preparations

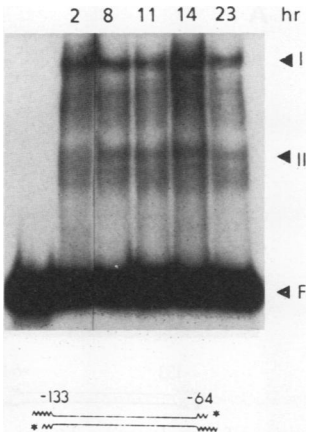

FIG. 3. Gel mobility shift assay with the 70-bp CCRU. End-labeled probe spanning −64 to −133 was mixed with 2 μg of protein extracts from synchronized cells (G_1) 2, (S) 8, 11, 14, and (G_2) 23 h after serum release were used. The positions of complex I, II, and free DNA (F) are shown.

of synchronized cell extracts, and basically the binding activities from the G_1-phase extracts were the same as the extracts prepared from S-phase cells (our unpublished results). From these results, we conclude that the binding activities of cellular factors to this 70-bp region, as assayed in these in vitro assays, are very similar throughout the cell cycle.

Specificity of the protein complexes. Next, we examined whether the complexes formed were specific for the human *TK* promoter sequence by competition assays with homologous or heterologous DNA. For competition assays, the competitor DNA was mixed at various concentrations with the radiolabeled probe prior to the addition of the protein extract. Both complexes I and II were inhibited efficiently by a molar excess of the unlabeled, homologous fragment, whereas a heterologous oligonucleotide (*GRP78* promoter [GRP]) was unable to do so (Fig. 4A). Other heterologous competitors and cell extracts from different time points during the cell cycle were also tested, and similar results were obtained (data not shown). Therefore, complexes I and II were specific for the human *TK* promoter sequence spanning −64 to −133.

To determine which region of this DNA fragment contributes to the formation of complexes I and II, we prepared smaller subfragments of this region. A 26-bp sequence spanning −58 to −83 of the human *TK* promoter (Fig. 1A) was synthesized, with a *Sal*I site (GTCGAC) added at the 5' terminus of the coding strand and a *Xho*I site (GAGCTC) added at the 5' terminus of the noncoding strand. The synthetic oligonucleotides were purified by using SEP-PAK columns (Waters, Milford, Mass.). Reannealed oligonucleotides were prepared by incubating equal amounts of the two strands at 65°C for 2 min and then allowing the solution to cool to 30°C in a solution containing 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.8), and 1 mM EDTA. By using this double-stranded, synthetic oligomer (−58 to −83) as a competitor, complex I was inhibited preferentially. This indicated that complex I is likely to be formed with sequences spanning −58 to −83, while complex II is formed with the sequences between −84 and −133.

This result was confirmed by radiolabeling the synthetic oligomer spanning −58 to −83 and using it as a probe in gel

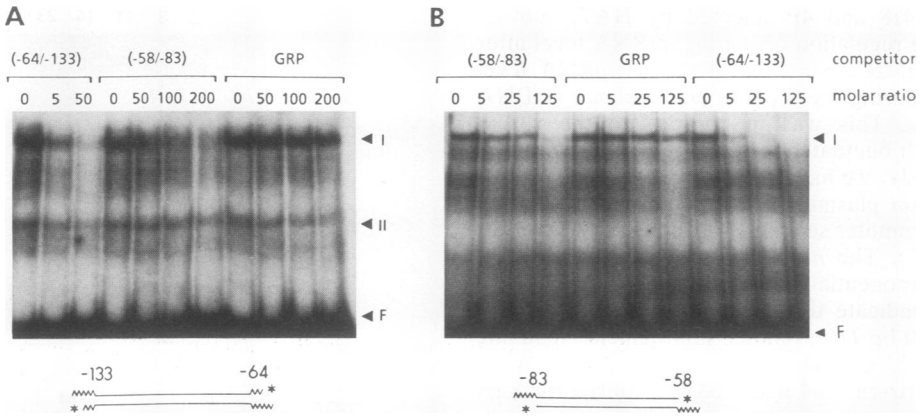

FIG. 4. Competition of complexes by *TK* promoter fragments. (A) End-labeled probe spanning −64 to −133 was mixed with cell extract prepared 14 h after serum release. The complexes formed were subjected to competition with increasing molar excess of the unlabeled, homologous fragment (−64/−133), a 32-bp synthetic oligomer spanning −58 to −83 (−58/−83), or a 41-bp synthetic oligomer spanning −170 to −210 of the *GRP78* promoter (GRP). The sequence of the *GRP* oligomer has been described previously (27). (B) End-labeled probe spanning −58 to −83 was subjected to competition with increasing molar excess of its homologous oligomer (−58/−83), the *GRP* oligomer, or −64/−133. The positions of complex I, II, and free DNA (F) are indicated.

mobility shift assays. The reannealed oligomer with 5′ protruding *Sal*I and *Xho*I ends was labeled with [α-^{32}P]dCTP by using the Klenow fragment of DNA polymerase I and mixed with cell extracts. Only one complex with similar electrophoretic mobility to complex I seen in Fig. 4A was observed (Fig. 4B). This complex was specific to the sequence spanning −58 to −83, as shown by the efficient competition with the unlabeled, homologous oligomer and the failure of a heterologous competitor (GRP) to inhibit the complex. The 70-bp CCRU fragment (−64 to −133) was also highly efficient in inhibiting the complex, even more so than the homologous oligomer (−58 to −83). This could be explained by the fact that a longer oligomer is more efficient than a shorter one in competition assays or that the extra DNA sequences contained within −84 to −133 facilitated the competition of complex I. Although the nature of complex I remains to be determined, we note that with the exception of 1 nucleotide, the 19-nucleotide sequence (−58 to −76), including the inverted CCAAT, is identical to the consensus sequence reported for the CCAAT-binding protein CP1 (Fig. 5).

Conclusions. The expression of TK is under stringent control during the cell cycle. Evidence accumulated so far suggests that both the promoter and the transcriptional unit of the TK gene may contain *cis*-regulatory sequences which are important for S-phase-dependent transcriptional and posttranscriptional regulation of this gene (13, 17, 22, 32, 34).

The sequences responsible for the expression of the TK gene in exponentially growing cells have been analyzed previously. The functional domain of the *TK* promoter was initially delimited to within an 83-bp region upstream of the transcriptional initiation site (20). More recent 5′ deletion analysis showed a gradual decrease in *TK* promoter activity as the sequences were shortened progressively from the 5′ end (1). A region between −139 and −88 showed the most dramatic decrease, of about 2.5-fold. Further 5′ deletion studies in the context of the homologous *TK* promoter revealed that deletion to −83 and −64 resulted in superinducibility of the *TK* transcripts by cycloheximide (23) and that deletion of the *TK* sequence from −135 to −67 resulted in the loss of G_1-S-phase regulation (28). Our results demonstrated that the same region (−133 to −64) is likely to contain a CCRU for the *TK* promoter.

CP1 consensus		T/C	N	N	N	N	N	N	A/G	A/G	C	C	A	A	T	C	A	N	T/C	T/G
human TK	-58	G	C	C	A	T	G	G	G	G	C	C	A	A	T	C	A	G	C	G	-76

FIG. 5. Sequence identity between the CP1 consensus binding site and the human *TK* promoter sequence spanning −58 to −76. The identical bases between the two sequences are connected by dots. The CCAAT motifs are highlighted.

In this report, we demonstrate that a 70-bp fragment derived from positions −133 to −64 of the human *TK* promoter, dissociated from its TATA element and any *TK* transcript sequence (including UTRs and coding regions), is sufficient to confer cell cycle regulation on a non-cell-cycle-regulated, heterologous promoter. There may well be multiple regulatory components present in the human *TK* promoter, such as the other CCAAT sequence proximal to the TATA element (19), the Sp1- and Oct-1-binding sites. However, the 70-bp fragment does not require cooperative interactions with these elements to confer cell cycle regulation. It appears that the regulatory factor(s) which interacts with the 70-bp fragment can mediate cell cycle regulation in the context of other transcriptional machinery normally associated with non-cell-cycle-regulated promoters.

Our results indicate that the region between −64 and −83 appears to be responsible for stimulation of transcription. Our observation is consistent with a previous study that site mutagenesis of the CCAAT motif contained within this region at −70 could result in a loss of promoter activity as assayed by rescue of the *TK* phenotype (23). Whether it is also responsible for the S-phase increases in gene transcription is difficult to determine since the residual transcription is too low to assay accurately. The protein-binding properties of the CCAAT motifs within the *TK* promoter have been investigated. In one study, the nature of the complexes formed with extracts from G_0 BALB/c 3T3 cells differed dramatically from that of the cells at the G_1/S border (19). Our analysis does not include G_0 cells. Since a six- to sevenfold increase in transcriptional activity was observed at the G1/S border in serum-stimulated cells (32), we focused on cells traversing through the cell cycle after serum stimulation. Another study suggested that the factor that binds to

the proximal CCAAT element at −40 may be related to protein nuclear factor Y and that this factor may also have a lower affinity for the CCAAT element at −70 (1). We do not know the identity of the protein that binds to the distal inverted CCAAT element, but we note that this sequence is nearly identical (18 of 19 residues) to the consensus sequence reported for CP1, one of the CCAAT-binding proteins (5). Since most of the cellular and viral promoters containing the CP1 site are not known to be cell cycle regulated (5), we speculate that the primary function of the CP1 site in the TK promoter is to enhance the transcriptional activity of other, S-phase-specific, regulatory elements. Within this 70-bp CCRU, there are sequences which share similarity to the CCAAT motif and are flanked by G+C-rich sequences. Similar arrangements of cell cycle regulatory elements flanked by G+C-rich and CCAAT sequences are found within the regulatory sequence of the histone H3.2 and histone H1 genes, which are known to be cell cycle regulated with kinetics similar to those of the TK gene (3, 8, 30). In addition, the DNA region required for maximal stimulation of transcription of the histone TH2B gene during the S phase contains an CCAAT element (15). It would be interesting to determine whether a common CCAAT-binding or G+C-rich binding protein is involved in the activation of these S-phase-specific genes.

Previously, we have shown that for the histone H2B gene, OTF-1 binds to the Oct-1 site on the promoter region throughout the cell cycle and that its binding activity is increased severalfold during the S phase (16). Using the same extracts and several other independently prepared extracts, we observed, in gel mobility shift analysis of the 70-bp fragment, that the pattern and the binding activities of the multiple DNA protein complexes formed were similar in G_1-, S-, and G_2-phase protein extracts. Thus, these regions probably represent the sites for nuclear factors which are expressed constitutively during the G_1, S, and G_2 phases. Several explanations may account for the cell-cycle-regulated transcriptional activation in the absence of a change in binding pattern or affinity. First, there may be subtle changes in the protein-binding affinities or complex conformation during the cell cycle which were not detectable by this method. However, a recent study has also demonstrated changes in factor-binding activity during the cell cycle, by using the same assay systems (14). Second, cell cycle regulation may be mediated by posttranslational modifications of existing factors constitutively bound to the *TK* promoter. For example, covalent modifications, specifically phosphorylation, have been shown to regulate the activity of the $cdc2^+$ protein, which plays an important role in controlling the commitment of the fission yeast cell to the mitotic cycle and the timing of mitosis (31). Third, the binding proteins detected in our assay may require interaction with other regulatory components. Specific protein-protein interactions have been implicated in transcriptional regulation of a number of gene systems (4). To differentiate among these possibilities it is necessary to isolate the protein factors that are directly interacting with the regulatory domains. Our identification of specific binding complexes within this functional unit provides the essential information for this approach.

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