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Sequences contained within the promoter of the human thymidine kinase gene can direct cell-cycle regulation of heterologous fusion genes

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ABSTRACT **Recent evidence on the transcriptional regulation of the human thymidine kinase (*TK*) gene raises the possibility that cell-cycle regulatory sequences may be localized within its promoter. A hybrid gene that combines the TK 5′ flanking sequence and the coding region of the bacterial neomycin-resistance gene (*neo*) has been constructed. Upon transfection into a hamster fibroblast cell line K12, the hybrid gene exhibits cell-cycle-dependent expression. Deletion analysis reveals that the region important for cell-cycle regulation is within −441 to −63 nucleotides from the transcriptional initiation site. This region (−441 to −63) also confers cell-cycle regulation to the herpes simplex virus thymidine kinase (HSVtk) promoter, which is not expressed in a cell-cycle manner. We conclude that the −441 to −63 sequence within the human TK promoter is important for cell-cycle-dependent expression.**

One approach to understand the control of cell growth on a molecular level is to identify genes whose expression is modulated during the cell cycle and to study the underlying mechanisms of this regulation. The eukaryotic cell cycle has four distinct phases, G_1, S, G_2, and M (1). There is evidence revealing that several well-studied S-phase-specific genes, such as those encoding the replication-dependent histones, dihydrofolate reductase, and thymidylate synthase, are regulated at multiple control levels (2–4). Recently, it has been shown by DNA-mediated gene transfer that sequences flanking the replication-dependent histone genes can confer transcriptional (5–7) or posttranscriptional control (8) on the heterologous fusion genes, resulting in cell-cycle regulation of their mRNA levels *in vivo*.

Another well-studied cell-cycle-regulated system is the thymidine kinase (*TK*) gene, which encodes a cytosol enzyme of the pyrimidine salvage pathway catalyzing the phosphorylation of thymidine to form thymidine 5′ monophosphate. It has been documented that the activity of the cytosol TK is cell-cycle regulated and the increase in enzyme activity correlates with increases in DNA synthesis (9). In mammalian cells, a 68-kDa protein has been implied in regulation of the TK activity (10). With the isolation of recombinant clones encoding the cellular *TK* genes (11–14), the level of control of mammalian *TK* gene expression during the cell cycle has been vigorously investigated. It has been shown that heterologous mammalian *TK* genes transfected into mouse L cells are regulated in a cell-cycle-dependent manner (13, 15), suggesting that the regulatory sequence is contained within the transfected gene and that different mammalian species may have closely related signals for the cell-cycle control of TK activity. It has been demonstrated in several laboratories that in mammalian cells the TK coding sequence, when fused to heterologous promoters, exhibits cell-cycle-regulated expression (16, 17). The implication is that at least part of the determinants of this regulation are contained within the TK mRNA sequence. With the direct demonstration that the half-life of TK mRNA decreases as S phase cells enter quiescence (18), posttranscriptional regulation of the TK transcripts clearly plays an important role in the cell-cycle-regulated expression of the *TK* gene.

The less well-understood level of control for the *TK* gene is at the step of transcriptional regulation. While it has been demonstrated that TK activity is sensitive to actinomycin D (9), the extreme low levels of the TK transcripts and its transient increase of transcriptional activity occurring only at a narrow period at the G_1 and S border makes it difficult to directly measure its transcriptional rate. Thus, earlier attempts failed to detect an increase in TK transcriptional activity (19). Highly sensitive techniques have been used to demonstrate that *TK* gene expression is controlled at both transcriptional and posttranscriptional levels during the mammalian cell cycle (17, 18). Specifically, at the G_1–S interphase, a 6- to 7-fold increase in transcriptional activity of the *TK* gene has been observed in serum-stimulated cells (17). How this increase is achieved becomes the crucial question. One explanation is that the TK promoter contains a cis-acting element, which is responsive to the cell-cycle regulation, thus influencing the rate of transcription of the *TK* gene. It is also possible that the TK coding sequence contains the cell-cycle-responsive element, which enhances transcription of the TK promoter during the transition from G_1 to S. The focus of our study is to examine whether the TK promoter contains control elements involved in cell-cycle regulation. By fusing the 5′ flanking sequence of the *TK* gene to heterologous transcriptional units, we dissociate any cis-acting regulatory elements contained within the TK coding/structural sequence from the promoter. Here, we demonstrate that a 378-nucleotide (nt) DNA region within the TK promoter can direct cell-cycle regulation.

MATERIALS AND METHODS

Cell Culture. The Chinese hamster cell line K12 is a temperature-sensitive (ts) mutant of Wg1A. Both Wg1A and K12 are derivatives of DON cells, selected for resistance to azaguanine, and have a hypoxanthine phosphoribosyltransferase (HPRT)-negative phenotype (20, 21). Conditions for culturing and synchronization of K12 cells have been described (22, 23). The rate of DNA synthesis during the cell cycle was determined by pulse labeling of the synchronized cells for 30 min with [*methyl*-^{3}H]thymidine as described (23).

DNA Plasmids. To create phTK2W, a 0.92-kilobase (kb) *Eco*RI fragment spanning the human TK promoter region

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Abbreviations: nt, nucleotide(s); HSV, herpes simplex virus; ts, temperature sensitive.

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isolated from the cosmid recombinant pHTKB (14) was subcloned into the *Eco*RI site of pUC8. The orientation of the TK fragment in phTK2W is such that the distal promoter sequence is adjacent to the *Sma* I site of the pUC8 polylinker sequence.

To create pTKN441, a 743-nt *Rsa* I fragment (268-nt pUC8 sequence and 475-nt TK 5′ flanking sequence) was isolated from phTK2W and ligated in the same transcriptional orientation as the neo-transcriptional unit contained in the 4.4-kb *Bam*HI/*Bgl* II fragment from the vector plasmid pNEO3 (5). Similarly, pTKN63 was constructed by fusing the 97-nt *Nco* I/*Rsa* I fragment (Fig. 1*A*) in the same transcriptional orientation as neo. The numbers 441 and 63 denote the 5′ end points of the TK promoter with respect to the transcriptional initiation region set as +1 (24) contained within pTKN441 and pTKN63, respectively.

The plasmid pHSVtk79 was constructed by cleaving pNEO3 with *Eco*RI and *Bam*HI, removing the 600-nt fragment containing the HSVtk 5′ upstream sequence, and religating the resultant 4.5-kb fragment. The number 79 denotes the 5′ end point (*Eco*RI site) of the HSVtk promoter with respect to its transcriptional start site (25). Plasmids pTKN378R and pTKN378W are derivatives of pHSVtk79, in which a 378-nt *Eco*RI/*Nco* I fragment (Fig. 1*A*) from the 5′ flanking sequence of the human *TK* gene was inserted in the same or opposite transcriptional orientation as the neo vector, respectively.

The plasmids pAAD3.7 and pJ were used as hybridization probes for histone H3.2 and actin, respectively; p3A10, an invariant cDNA from hamster, has been described (5, 26).

Gene Transfection. The plasmids were transfected into K12 cells by the calcium phosphate method, and stable transfectants were selected either on the basis of G418 or hypoxanthine/aminopterin/thymidine (HAT) resistance as described (7, 21). Those selected on the basis of HAT resistance were cotransfected with pSV2gpt (27).

RNA Blot Hybridization. Conditions for isolation of cytoplasmic RNA and hybridization of RNA blots have been described (23, 28). The hybridization probe for the neo mRNA is a 1-kb *Bgl* II/*Sma* I fragment previously described (5). All the plasmids or DNA fragments were labeled by the hexamer method (29) to specific activities of $\approx 10^8$ cpm per μg of DNA.

RESULTS

Cell-Cycle Regulation of the *TK–neo* Fusion Gene. The gene encoding the human cellular TK and its flanking sequence have been isolated and sequenced (24). The TK promoter contains a "TATA"-like element ≈20 nt upstream of the transcriptional initiation site (+1) and two inverted CCAAT sequences 20 and 50 nt upstream of the TATA element. Other features of the TK promoter include several potential Sp1 factor binding sites and pairs of inverted repeats. At positions −171 to −164, the sequence ATTTCCAG resembles the octanucleotide sequence ATTTGCAT found in a human histone H4 promoter (30). At positions −19 to −4, the TK sequence matches 15 of 17 nt of a sequence immediately 3′ to the TATA element of a hamster histone H3.2 gene (7).

To determine whether the TK promoter contains determinants for cell-cycle regulation, a fragment containing the TK promoter spanning from 441 nt upstream of the cap site to 34 nt of the 5′ untranslated region was fused in the same transcriptional orientation as the *neo* gene in plasmid pTKN441 (Fig. 1*A*) and transfected into K12 cells, a well-characterized ts G_1 cell-cycle mutant derived from Chinese hamster fibroblasts Wg1A (5, 31, 32). The K12 cell can be synchronized by serum starvation or by its ts mutation. Since the K12 cells have a HPRT-negative phenotype, we can eliminate the bias toward transfectants that expressed *neo* by selecting stable transfectants on the basis of either G418 or HAT resistance. In the latter case, the *TK–neo* fusion gene was cotransfected into K12 cells with pSV2gpt (21).

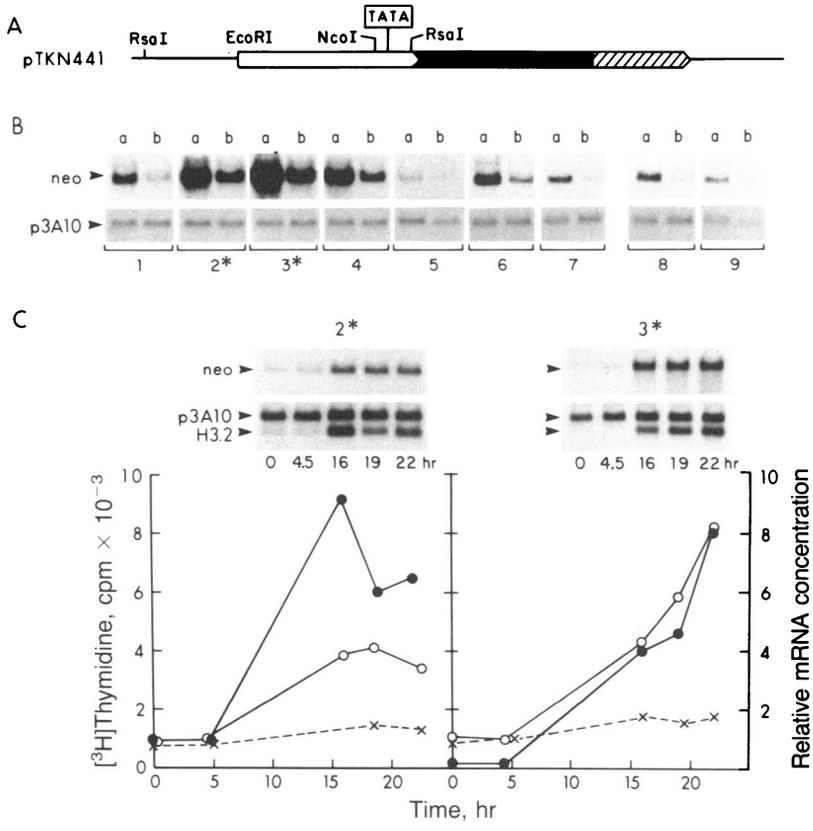

FIG. 1. (*A*) Structure of the plasmid pTKN441. Open bar, human TK 5′ flanking sequence; solid bar, *neo* gene coding sequence; hatched bar, polyadenylylation site; lines, prokaryotic vector sequence. (*B*) Effect of G_1 arrest on neo transcript levels in the individual stable transfectants. Transfectants 1–7 were selected on HAT resistance and 8 and 9 were selected on G418 resistance. Cytoplasmic RNA was extracted from exponentially growing (lanes a) or G_1-arrested (lanes b) cells, and the level of neo and p3A10 mRNA was determined by RNA blot hybridization as described (5). The autoradiograms are shown. Asterisks indicate two individual transformants chosen for more detailed cell-cycle analysis. (*C*) Cell-cycle analysis of the levels of neo, histone H3.2, and p3A10 mRNA in two individual transformants after serum stimulation of synchronized cells. Autoradiograms were quantitated by densitometry to obtain the relative levels of neo (○) and p3A10 (×), which are plotted with the rate of DNA synthesis as measured by incorporation of [^{3}H]thymidine (●).

Individual transfectants were isolated and mass expanded to compare the neo mRNA during exponential growth and G_1-arrested conditions. The latter condition was achieved by shifting the transfected K12 cells to the nonpermissive temperature, 39.5°C, for 24 hr, since K12 cells were arrested in the mid-G_1 phase by the ts mutation (31, 32). As shown in Fig. 1*B*, the levels of the neo transcripts in either the HAT or the neo-selected transfectants harboring the fusion plasmid pTKN441 were 3- to 4-fold higher in exponentially growing cells as compared to the G_1-arrested cells, whereas the transcript levels of a control plasmid, p3A10, were mostly constant. While the sites of integration or the copy number of the integrated gene may contribute to the various levels of neo mRNA, the differential neo mRNA levels in G_1 versus exponentially growing cells were observed in all transfectants examined.

To further determine whether the difference in neo expression reflects cell-cycle fluctuations, cytoplasmic RNA was extracted from two of these transfectants stimulated to traverse the cell cycle after serum stimulation and assayed for the levels of neo, histone H3.2, and p3A10 transcripts. As shown in Fig. 1*C*, the levels of neo mRNA directed by the TK promoter paralleled that of the replication-dependent histone H3.2, whereas the mRNA level of control plasmid p3A10 remained relatively constant. The neo mRNA level at G_1 is low, and as the cells entered the DNA synthetic phase, the levels of neo mRNA increased severalfold correspondingly.

Effect of 5′ Deletion of the TK Promoter. To locate the DNA region in the TK promoter that is important for cell-cycle regulation, a deletion mutant of the *TK–neo* fusion gene, pTKN63, which retained only 63 nt upstream of the cap site, was constructed (Fig. 2*A*). This plasmid still retained the TATA element and one upstream inverted CCAAT element. Upon transfection of pTKN63 into K12 cells and analysis of the neo mRNA levels in G_1 and exponentially growing transfectants, we observed that in 8 of 12 of the transfectants

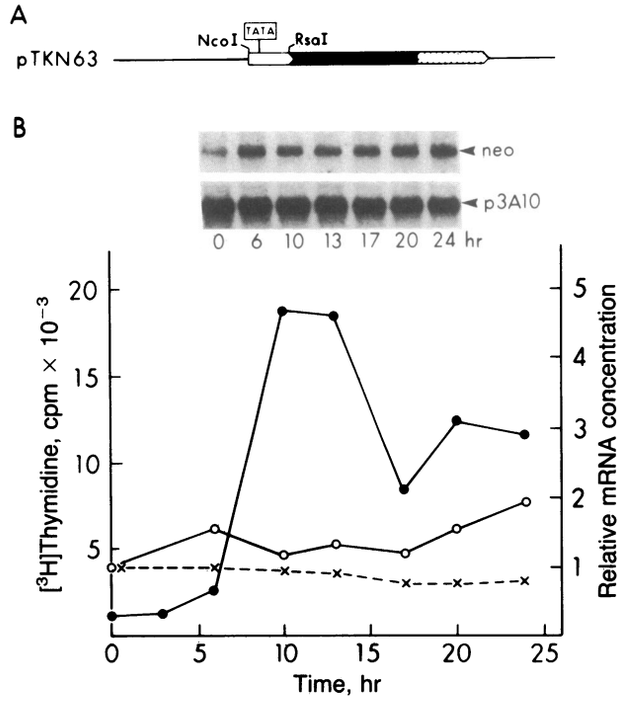

FIG. 2. (*A*) Structure of the plasmid pTKN63. The symbols are described in Fig. 1*A*. (*B*) Cell-cycle analysis of the levels of neo and p3A10 levels in a stable pTKN63 transformant selected by HAT resistance. The analysis was carried out as described in the legend of Fig. 1*C*. The relative levels of neo (○) and p3A10 (×) are plotted with the rate of DNA synthesis (●).

tested, the level of neo transcripts was generally 1/5th to 1/10th that of the pTKN441 transfectants. This result suggests that the deleted DNA sequence is important for high basal level expression; nonetheless, most transfectants still demonstrate a slightly higher (1.5- to 2-fold) level of neo mRNA in exponentially growing versus G_1 cells (data not shown). The expression of neo mRNA levels in a high expressing transfectant during the cell cycle after serum stimulation is shown in Fig. 2*B*. The slight and gradual increase (up to 2-fold) of neo mRNA after serum stimulation was consistently observed for several transfectants examined (unpublished results). However, the strong correlation between neo mRNA level and DNA synthesis observed for the pTKN441 transfectants was absent from the pTKN63 transfectants. Therefore, while it is possible that the 63 nt proximal to the TK cap site contain some DNA sequence responsive to serum stimulation, the sequence accounting for the cell-cycle regulation and high basal level expression appears to reside within the 378-nt *Eco*RI/*Nco* I fragment upstream (Fig. 1*A*).

The 378-nt *Eco*RI/*Nco* I Fragment Can Enhance Transcription of a Heterologous Promoter. To test whether the 378-nt *Eco*RI/*Nco* I fragment contained within the human TK promoter can stimulate transcription of a heterologous promoter, the 378-nt fragment was fused to the herpes simplex virus thymidine kinase (HSVtk) promoter containing only 79 nt of the HSVtk DNA upstream of the TATA element (pHSVtk79). As shown in Fig. 3*A*, the plasmids pTKN378R and pTKTK378W are triple fusion genes in which the human cellular TK 5′ upstream sequence is fused in different transcriptional orientations to the truncated HSVtk promoter and the *neo* structural gene. Individual transfectants selected on the basis of either HAT or G418 resistance were examined for the levels of neo mRNA in exponentially growing and G_1-arrested cells. For comparison, the levels of neo mRNA under the regulation of the truncated HSVtk promoter alone were also examined. The results are shown in Fig. 3*B*. In the case of the pTKN378R transfectants, in which the human TK DNA was fused in the same transcriptional orientation as the HSVtk promoter, a higher level (2- to 4-fold) of neo mRNA was observed in exponentially growing cells than in G_1 cells in the majority of the transfectants analyzed. In the case of pTKN378W transfectants, in which the human TK and HSVtk DNA were fused in opposite transcriptional orientations, similar levels of neo mRNA were observed as in the pTKN378R transfectants. However, unlike the pTKN378R transfectants, the levels of neo mRNA in exponentially growing and G_1 cells were about the same (Fig. 3*B*), implying that the neo mRNA levels in the pTKN378W transfectants are not cell-cycle regulated. In the pHSVtk 79 transformants harboring the plasmid containing only the HSVtk promoter, the neo mRNA level in the majority of the transfectants was either very low or undetectable (Fig. 3*B*). Some transfectants produced aberrant neo transcripts larger than the normal neo transcripts. In addition, these aberrant transcripts were present in similar levels in exponentially growing and G_1 cells. These results, taken together, demonstrate that the 378-nt DNA contained within the TK promoter can stimulate transcription of a heterologous promoter such as that of the HSVtk and direct it to produce the proper size neo transcripts. In addition, the ability of the 378-nt TK fragment to confer regulation of the HSVtk promoter in G_1 and exponentially growing cells is distance and/or orientation dependent.

A 378-nt Fragment of the TK Promoter Can Direct Cell-Cycle Regulation of a Heterologous Promoter. We have previously demonstrated that in transfectants harboring a *neo* fusion gene under the regulation of a HSVtk promoter fragment of ≈800 nt, the level of neo mRNA was easily detectable and followed a gradual increase to ≈2-fold after

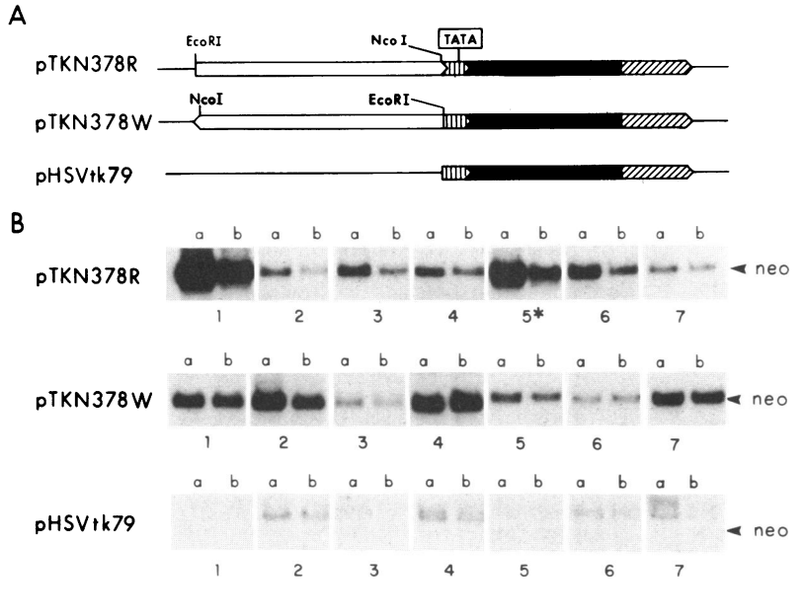

FIG. 3. (*A*) Structures of plasmids pTKN378R, pTKN378W, and pHSVtk79. Vertical bars, HSVtk promoter sequence. All other symbols are as described in Fig. 1*A*. (*B*) Effect of G_1 arrest on neo transcript levels in the individual stable transformants. Lanes a, exponentially growing cells; lanes b, G_1 arrested cells. The expected positions of the neo transcripts (1.5 kb) are indicated. Asterisk indicates the transfectant chosen for more detailed analysis in Fig. 4.

serum stimulation of synchronized cells (7). However, its pattern of expression did not correlate with the DNA synthesis rate but reflected that of growth stimulation by serum. To test whether the 378-nt human TK fragment can confer cell-cycle regulation to the HSVtk TATA element, we examined the neo mRNA profile of several pTKN378R transfectants. For comparisons, the levels of the endogenous p3A10 and actin mRNA were also monitored. An example of such an analysis is shown in Fig. 4. The neo mRNA levels were cell-cycle regulated. Their levels are lowest during G_1 phase. As the cells enter S phase, their levels increased sharply and reached a 5-fold increase over the basal level at G_1. This increase corresponds to the 4- to 7-fold increase in the rate of transcription previously reported for the cellular *TK* gene (17, 18). The peak of neo mRNA accumulation lagged 3 hr behind the peak of DNA synthesis. As the rate of DNA synthesis declined, the levels of neo mRNA also decreased. In contrast, the p3A10 levels were constant throughout the cell cycle. The actin mRNA levels reproducibly demonstrated a slight (1.5-fold) increase 3 hr after the addition of fresh serum. However, by 10 hr, the actin mRNA level reverted back to the basal level and remained relatively constant during the rest of the cell cycle as described (23).

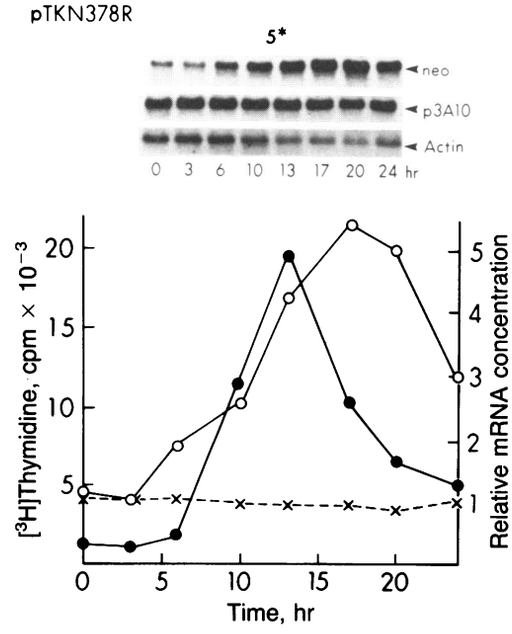

FIG. 4. Cell-cycle analysis of neo, p3A10, and actin mRNA levels in transfectant pTKN378R/5*. The autoradiograms were quantitated and the relative levels of neo (○) and p3A10 (×) are plotted against the rate of DNA synthesis (●) as described in the legend of Fig. 1*C*.

DISCUSSION

Since the isolation of the cellular *TK* gene, its structure and expression have been the topic of intensive investigations. As in the case of the chicken *TK* gene (33), the human *TK* gene contains seven exons (24). While there are conflicting results with regard to the importance of the intron sequence toward efficient formation of TK mRNA (34, 35), there is general consensus that the TK promoter is functional in directing transcription (16, 17, 24, 36). However, the contribution of the TK promoter to cell-cycle regulation has been controversial (16, 37). In fact, earlier studies have shown that TK enzyme expression in differentiating muscle cells of the chicken was mediated primarily through an internal segment of the cellular gene (38). Similar observations were made in rat cells transfected with TK cDNA linked to non-cell-cycle-regulated promoters (16, 17). These results, coupled with the earlier difficulties in detecting transcriptional regulation of the *TK* gene, cast doubt on the importance of transcriptional control and/or the promoter in the *TK* gene system. By direct measurement of the TK transcriptional and mRNA degradational rates, two independent laboratories have now demonstrated that the *TK* gene is transcriptionally regulated (17, 18) and that the regulation of TK expression involved multiple levels of control, as in the case of many S-phase regulated genes.

We have examined the possibility that DNA elements in the TK promoter may contribute to the overall regulation of the *TK* gene expression. By analyzing the activity of the TK promoter fused to a heterologous transcriptional unit, the contribution of any cis-acting control elements contained within the TK transcript sequence will be dissociated from the promoter element. The analysis of a large number of individual transfectants stably integrated with the fusion plasmids allowed a detailed examination of the effect of the TK promoter on transcription during the cell cycle. We have previously demonstrated that TK enzyme activity is cell-cycle regulated in hamster fibroblast K12 and Wg1A cells (39). By using two independent methods for cell synchroni-

zation (K12 ts mutation and serum deprivation), our studies have revealed several unusual features of the human TK promoter function *in vivo*.

First, the human TK promoter including ≈500 nt of the 5′ sequence and its TATA element is effective to direct transcription of a heterologous transcriptional unit in a cell-cycle-regulated manner. From the deletion analysis of the human TK promoter, the most important region for high basal level expression as well as cell-cycle regulation resides within a 378-nt fragment upstream of the TATA sequence.

Second, this cis-acting DNA regulatory element can confer high basal level expression to a heterologous promoter in a distance- and/or orientation-independent manner when fused upstream of the promoter. As such, it has the characteristics of an enhancer. Structurally, it also contains G-C motifs, putative trancriptional factor binding sites (40), and long inverted repeats characteristic of other cellular enhancers (41).

Third, when this 378-nt TK fragment is fused upstream in the same transcriptional orientation to a heterologous promoter (such as the HSVtk TATA element), it confers cell-cycle-regulated expression to the neo transcriptional unit. The level of neo mRNA is detectable but low at the G_1 phase and increases in parallel to DNA synthesis. It is important to note that unlike the cellular TK transcripts, the neo transcripts are not subjected to cell-cycle regulatory mechanisms. This point was demonstrated in a previous study (7) and confirmed here that the level of neo mRNA under the direction of the HSVtk or other non-replication-dependent promoters did not exhibit cell-cycle fluctuation. Interestingly, when this 378-nt fragment is fused in opposite orientation to the heterologous transcriptional unit, the neo mRNA levels are similar in G_1 and exponentially growing cells. Therefore, it appears that the ability of this element to confer cell-cycle regulation to a downstream promoter requires a certain distance and/or orientation.

In summary, our studies strongly support the hypothesis that genetic determinants within the TK promoter are likely to contribute to the cell-cycle-dependent expression of TK, along with sequences within the TK transcript that regulate its degradational rate and possibly other functions. The important functional domain defined by our studies is consistent with the recent observation that the TK promoter plays an important role in the cell-cycle regulation of TK mRNA levels (37). Furthermore, this domain may interact with cellular factors that bind to the CCAAT sequences within the human TK promoter (42). As discussed in detail by Travali *et al.* (37), the discrepancies concerning the importance of the TK promoter in cell-cycle control of the *TK* gene may result from subtle contributions from different viral and cellular promoters used in the various fusion gene constructs. Different cell lines may also regulate TK mRNA levels differently, in that some cell lines may depend more on posttranscriptional than transcriptional regulation of their cell-cycle-regulated genes (8). Our current view is that regulation at both the transcriptional and posttranscriptional levels could account for the 10- to 20-fold increase in TK mRNA during the DNA synthetic phase and that the TK promoter is part of this regulatory mechanism. Further investigations into the TK promoter will need to define the DNA elements involved more precisely and provide the basis for studying their interaction with cell-cycle regulatory factors.

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