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S-phase-specific regulation by deletion mutants of the human thymidine kinase promoter

(serum stimulation/cell cycle/mRNA/temperature-sensitive cells/cycloheximide)

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ABSTRACT **The levels of thymidine kinase (TK; EC 2.7.1.21) mRNA were determined in nine established cell lines derived from TK⁻ts13, a temperature-sensitive mutant cell line that arrests in late G_1 phase of the cell cycle at the restrictive temperature. The derivative cell lines carried either a cDNA or a minigene of human TK under the control of TK promoters of different lengths. A tenth cell line carried a human TK cDNA under the control of a simian virus 40 promoter. Two different assays were used to determine the S-phase-specific regulation of human TK mRNA levels in quiescent cells stimulated to proliferate. Results from these two assays indicated that (*i*) the first two introns of the human TK gene had no effect on the S-phase-specific regulation of TK mRNA levels, although the presence of introns increased the amount of TK mRNA; (*ii*) similar amounts of TK mRNA were present in cells containing constructs with an 83-base-pair (bp) promoter as with other TK promoters comprising up to ≈4000 bp of 5′ flanking sequence; (*iii*) a 456-bp promoter was fully S-phase-regulated, whereas the 83-bp promoter was only partially regulated; (*iv*) a 63-bp promoter was much less regulated than an 83-bp promoter; and (*v*) the crucial element in the 20-bp fragment comprising bp −83 to −64 has been localized, by site-directed mutagenesis, to the CCAAT element at −70.**

Thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) is one of several enzymes involved in cellular DNA synthesis. The amount of TK polypeptide and activity, as well as that of other enzymes involved in cellular DNA synthesis, increases sharply at the G_1/S boundary of the cell cycle and remains high throughout S phase (1–4). Steady-state levels of TK mRNA also increase sharply as the cells enter S phase from a G_0 state (5–7), although in exponentially growing HeLa cells TK mRNA levels are similar in different phases of the cell cycle (4). This diversity between G_0 cells stimulated to proliferate and exponentially growing cells is not unique to the TK gene: the DNA polymerase α gene is also constitutively expressed in cycling cells but is inducible by growth factors in G_0 cells (8).

The regulation of the amount of TK is complex and occurs at transcriptional, posttranscriptional, and translational levels (4, 6, 9–11). The mechanisms by which TK mRNA levels are regulated are currently unresolved. Several reports state that cell cycle expression of the TK gene is controlled exclusively by its coding sequence (12–15). However, more recent reports indicate that another major regulatory element in the S-phase-specific regulation of the human TK gene is located in the 5′ flanking sequence (16–18). This paper examines elements in the 5′ flanking sequence of the human TK gene that contribute to the regulation of TK mRNA levels in G_0 cells stimulated to proliferate. We used two methods to test the S-phase-specific regulation of TK mRNA levels. In the first, we used a temperature-sensitive (ts) mutant of the cell cycle that arrests in G_1 at the restrictive temperature. Serum stimulates the expression of early cell cycle genes (19, 20), but not that of late G_1/S-phase genes such as those encoding TK or histones, at the restrictive temperature (5, 20, 21). Since all the factors necessary to activate transcription are present in medium used to stimulate the cells, lack of mRNA expression in a G_1-specific ts mutant blocked at the restrictive temperature indicates that the promoter is regulated as that of a late G_1/S-phase gene. The second method used was to study the levels of TK mRNA in cells treated with cycloheximide (Chx). Low concentrations of Chx that only partially inhibit protein synthesis completely suppress the expression of TK mRNA (7). In contrast, even high concentrations of Chx do not inhibit, and often increase, the mRNA levels of early growth-regulated genes like c-*fos* and c-*myc* (22–26). Thus, response to Chx also indicates whether a promoter is regulated as a G_1/S boundary gene.

In this investigation we only determined the levels of TK mRNA in G_0 cells stimulated to proliferate. Therefore, our results simply indicate which elements of the human TK promoter mediate S-phase-specific expression of TK mRNA levels with normal posttranscriptional regulation (4, 10).

MATERIALS AND METHODS

Cloning of TK 5′ Flanking Sequences. A λEMBL-3 phage library of human genomic DNA was provided by Louise C. Showe (Wistar Institute). Approximately 3.5×10^5 plaques of this library were screened for TK 5′ flanking sequence (27) with an *Eco*RI [base pair (bp) −442] to *Rsa* I (bp +32) fragment of TK promoter obtained from Prescott L. Deininger (Louisiana State University Medical Center); positions of restriction sites are given relative to the transcription initiation (cap) site (see Fig. 1*a*). One clone was isolated that contained ≈10 kilobases (kb) of TK genomic DNA and ≈7 kb of 5′ flanking sequence. An 8-kb segment of this clone extending from a *Sal* I site (−4263) to the first *Kpn* I site (+3798) was subcloned into plasmid pUC18 to yield p5′TK8KS.

Plasmid Construction. The plasmids shown in Fig. 1*b* were constructed from p5′TK8KS and pTK11. The latter is an Okayama–Berg vector containing the human TK cDNA (28). The 5′ flanking sequence was truncated at a *Sal* I (−4263), *Bam*HI (−1210), *Hin*dIII (−456), or *Eag* I (−63) site. Minigenes were constructed with a fragment containing the 3′ 88% of TK cDNA isolated from pTK11 digested with *Mlu* I and *Xho* I. Plasmids without introns were constructed from minigenes with a *Sma* I–*Mlu* I fragment of pTK11. Plasmids p−0.065N5′TKTKi, pTKMUT1, and pTKMUT2 were constructed from p−4.2S5′TKTKi and oligodeoxynucleotides

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Abbreviations: TK, thymidine kinase; ts, temperature-sensitive; Chx, cycloheximide; gHAT medium, medium containing glycine, hypoxanthine, aminopterin, and thymidine; SV40, simian virus 40.

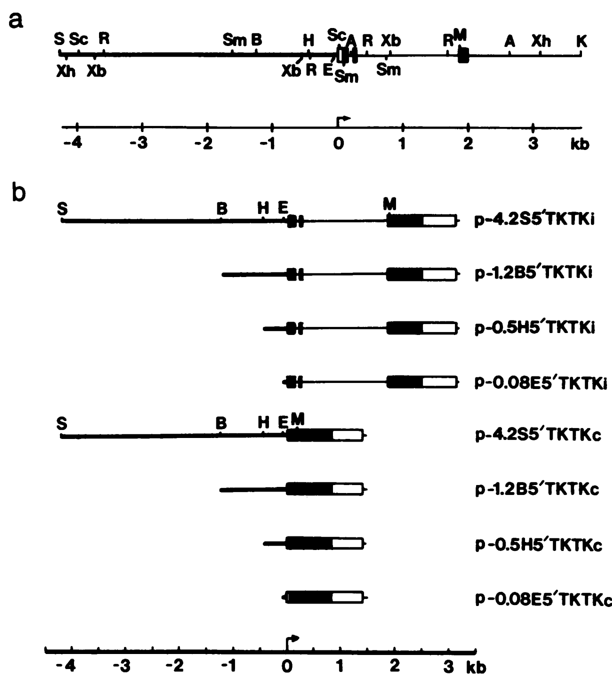

FIG. 1. (*a*) Restriction map of the human TK sequence in p5'TK8KS. The heavy line represents 5' flanking sequence and the thin line identifies introns. The exons are indicated by boxes, with the unfilled box representing untranslated sequence. Restriction enzyme sites are indicated: A, *Apa* I; B, *Bam*HI; E, *Eag* I; H, *Hin*dIII; K, *Kpn* I; M, *Mlu* I; R, *Eco*RI; S, *Sal* I; Sc, *Sca* I; Sm, *Sma* I; Xb, *Xba* I; Xh, *Xho* I. Distance in kilobases (kb) is indicated on the scale and is relative to the 5' cap site (↱). (*b*) Constructs used to generate cell lines carrying different promoters. Symbols are as in *a*.

were synthesized with an Applied Biosystems model 380B synthesizer (Fig. 2).

Cell Lines. TK^-ts13 Syrian hamster fibroblasts (29), a TK-deficient cell line derived from ts13 cells (30), were transfected in suspension (29) with the constructs described above. The transformants were selected at 34°C in gHAT medium (glycine, hypoxanthine, aminopterin, thymidine medium) supplemented with 10% calf serum. The established cell lines are a mixture of several hundred clones and were always maintained in gHAT medium at the permissive temperature. For experiments, the cells were made quiescent by serum deprivation for 4 days.

RNA Extraction and RNA Blots. Total RNA was extracted from cells by the method of Chomczynski and Sacchi (31), and RNA gel blots were prepared by standard procedures (32). Radioactive probes were prepared by the random priming method (33). To monitor the relative amounts of RNA in each lane, we used a cDNA probe, clone L7, of an unidentified mRNA whose expression is not growth-regulated.

RESULTS

We isolated from a human genomic library a phage with a 17-kb insert that contained ≈10 kb of human TK DNA and ≈7 kb of 5' flanking sequence. An 8-kb fragment of this insert subcloned in pUC18 (p5'TK8KS) served as the basis for construction of plasmids used in this study. A restriction map of the insert from this subclone (Fig. 1*a*) agrees with that previously published (34). It extends from a *Sal* I restriction site at ≈4250 bp upstream of the cap site to a *Kpn* I site 3798 bp downstream of the cap site and contains the first three exons and the first two introns, plus most of the third intron, of the human TK gene.

Using 5' flanking sequence of this insert we constructed eight plasmids (Fig. 1*b*) with four progressively shorter promoters: 4263, 1210, 456, and 83 bp 5' to the cap site. The last is the same as that described by Kreidberg and Kelly (35). Each of these promoters was used to drive transcription either of a minigene of TK, which included the first two exons and the first two introns, completed by cDNA sequence, or of TK cDNA without introns. A ninth plasmid, p−0.06N5'TKKi, contained a 63-bp promoter driving the minigene. As a control, we used pTK11 (28), which consists of the simian virus 40 (SV40) promoter driving human TK cDNA and was isolated from an Okayama–Berg library (36).

Establishment and Growth of Cell Lines. TK^-ts13 cells were transfected with one of the eight constructs outlined in Fig. 1*b* or with pTK11, and transformants were selected in gHAT medium, which does not support the growth of TK^-ts13 cells (29). Because the TK promoter is weak, we purposely elected direct selection in order to obtain mixed populations in which the construct was active in every cell. There were no appreciable differences in the rate of growth of the different cells lines established with the 9 different constructs, indicating that all promoters were sufficiently active in 10% calf serum to provide the necessary amount of TK for growth in gHAT.

TK mRNA Levels in Cell Lines Carrying the Human TK Minigenes. The cell line TK^-ts13 is a TK-deficient mutant derived from line ts13 (30), a ts cell cycle mutant that arrests in late G_1 at the nonpermissive temperature (37). TK^-ts13 cells also arrest in G_1 at the nonpermissive temperature, 39.6°C. We therefore determined TK mRNA levels in the various transformants under four different conditions: (*i*) serum deprivation, (*ii*) stimulation with serum at the permissive temperature (34°C), (*iii*) stimulation with serum at the restrictive temperature (39.6°C), and (*iv*) stimulation with serum, but with the addition of Chx (10 μg/ml) 4 hr after stimulation. TK mRNA is not detectable in nontransfected TK^-ts13 cells (16). Thus, only mRNA from the transfected constructs is detectable in RNA blots. The concentration of Chx used by Coppock and Pardee (7) to inhibit the appearance of TK mRNA was 100 ng/ml, applied 8 hr after stimulation. Preliminary experiments (data not shown) indicated that TK^-ts13 are less sensitive to Chx than BALB/c

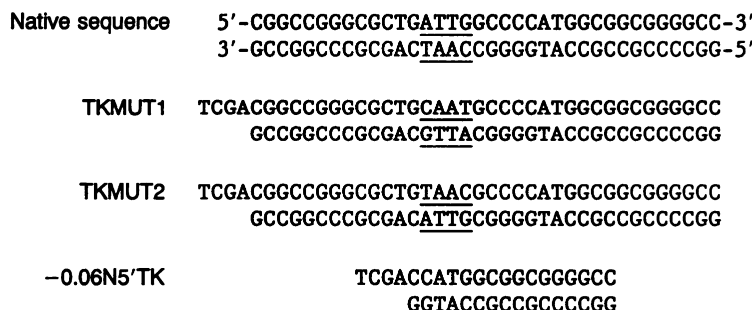

FIG. 2. Sequences of synthetic oligodeoxynucleotides. Underlined bases in TKMUT1 and TKMUT2 are different from the native sequence.

3T3 cells, and therefore we used 10 μg/ml, 4 hr after serum stimulation.

Serum-deprived cells containing the constructs shown in Fig. 1b had little or no TK mRNA (Fig. 3). Stimulation with 10% serum at 34°C increased TK mRNA levels in all cell lines. Stimulation with 10% serum at 39.6°C resulted in no detectable TK mRNA. When cells were stimulated with 10% serum in the presence of Chx, the levels of TK mRNA were obviously lower than in the absence of Chx. However, the inhibitory effect of Chx was limited to the three cell lines carrying constructs with the longer promoters. In the cells with the 83-bp promoter, Chx did not inhibit the expression of TK mRNA. The RNA blots were also hybridized with a probe for histone H3 mRNA, which is expressed specifically in S phase (20, 21, 38). As expected, there was a good correlation between the expression of TK mRNA and the expression of histone H3 mRNA. The low level of H3 mRNA in lane D of the p−0.08E5′TKTKi cell line further emphasizes the lack of Chx inhibition of TK mRNA expression. The relative strength of the various promoters is discussed below.

Expression of TK mRNA in Cell Lines Carrying the TK cDNA. Similar experiments were performed with cells lines containing the various-length promoters expressing a TK cDNA (intronless) rather than the TK minigene with two introns. The results were essentially identical to those obtained with the minigene and showed that with the three longer promoters, TK and histone H3 mRNA were abundant only in cells stimulated with serum at 34°C. In the cells with the 83-bp promoter, TK mRNA expression was not inhibited by Chx (data not shown).

Comparison of the Intensity of Expression of the TK mRNA in S-Phase Cells. To determine whether there were differences in the activities of the promoters, and whether any differences depended on the presence or absence of the first two introns, an RNA blot was prepared from cells stimulated with serum for 24 hr at 34°C (Fig. 4). Although the experimental conditions were identical for each cell line, the number of cells in S phase at 24 hr could vary between cell lines. Such differences may be normalized by comparing the ratio of TK mRNA to the endogenous, S-phase-specific histone H3 mRNA. This ratio was greater when TK mRNA was transcribed from the minigene than when it was transcribed from the cDNA. The length of the promoter, however, did not seem to greatly affect the level of TK mRNA. Densitometry showed only minor differences in the TK/H3 ratios for the various promoters, regardless of whether minigenes or cDNAs were used. Differences varied by ≤50%, which is compatible with experimental error. By comparison, the intensity of the TK band from cells stimulated at 34°C was often >20 times as intense as the TK band from quiescent cells.

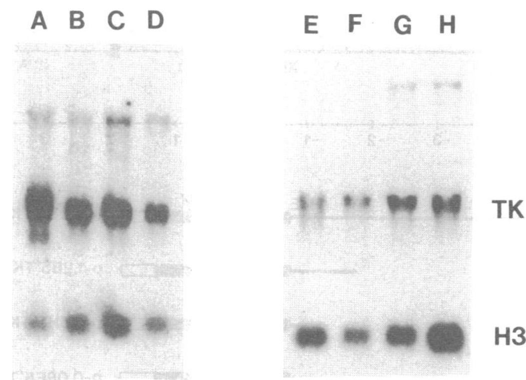

FIG. 4. RNA blots of S-phase cells from eight cell lines carrying different promoters driving either the TK minigene (lanes A–D) or the TK cDNA (lanes E–H). Cells were stimulated at the permissive temperature for 24 hr and the RNA was isolated and blotted. Lanes: A and E, 83-bp promoter; B and F, 456-bp promoter, C and G, 1.2-kb promoter; D and H, 4.2-kb promoter. Hybridization was with human TK and histone H3 probes.

The 63-bp Promoter. The 63-bp promoter of the human TK gene was reported to be weak and without cell cycle regulation (17). Since the 83-bp promoter was still strong (Fig. 4) and sensitive to the G_1 ts block (Fig. 3) it became imperative to test the 63-bp promoter under our experimental conditions. Our data (Fig. 5, autoradiograph intentionally overexposed) with a 63-bp promoter driving a TK minigene confirm the results of Kim et al. (17): the 63-bp promoter is weak and has largely lost S-phase specificity. It was actually superinduced by Chx, and the intensity of the TK band from cells stimulated at 39.6°C was the same as for serum-deprived cells (compare with Fig. 3).

Site-Directed Mutagenesis of the CAAT Region. Since the sequence between bp −83 and −63 seems to be crucial, we synthesized two promoters in which the CAAT region at −70 was mutated (Fig. 2). In the wild-type promoter, the −70 CAAT is an inverted CCAAT on the noncoding strand (18). In the first mutation, CAAT was transferred to the coding strand in the forward orientation (TKMUT1); in the second, the inverted CAAT was transferred to the coding strand, maintaining the reverse orientation (TKMUT2). TK^{-}ts13 cells were transfected with each plasmid and colonies were stained with crystal violet 10 days after selection had begun. Only a few colonies survived selection after transfection with pTKMUT1 or the −63 promoter. In contrast, pTKMUT2 was

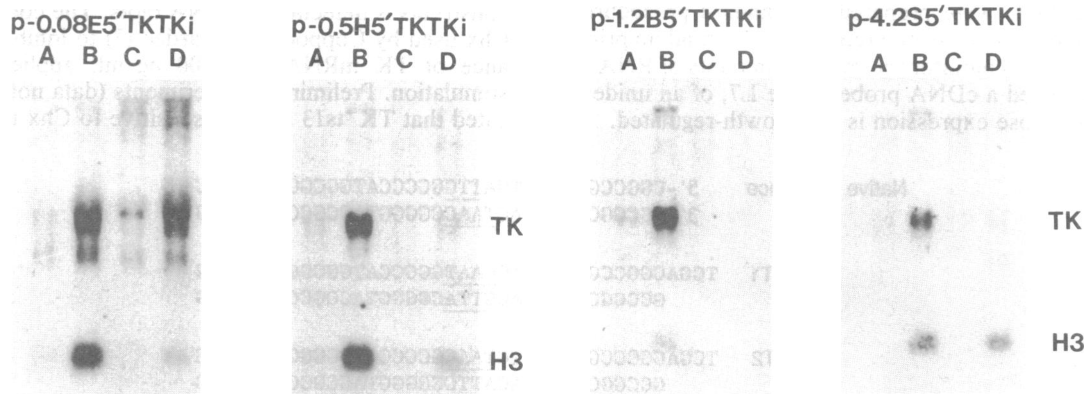

FIG. 3. RNA blots of TK mRNA in cell lines carrying the human TK minigene under the control of promoters of different lengths. Lanes: A, serum-deprived cells; B, cells stimulated for 24 hr with 10% serum at 34°C; C, cells stimulated with 10% serum for 24 hr at 39.6°C; D, cells stimulated with 10% serum for 24 hr but with the addition of Chx (10 μg/ml) 4 hr after stimulation. The various promoters are indicated above each group of lanes (see Fig. 1b). The RNA blots were hybridized with both a TK probe and a histone H3 probe.

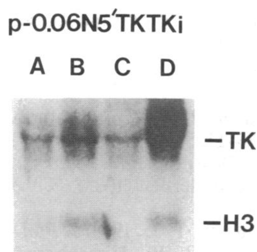

FIG. 5. RNA blot from a cell line carrying a minigene expressed by a 63-bp promoter. Lanes: A, serum-deprived cells; B, cells stimulated with 10% serum for 24 hr at 34°C; C, cells stimulated at 39.6°C; D, cells stimulated at 34°C with the addition of Chx (10 μg/ml) after 4 hr. The blot was hybridized with probes for TK and histone H3.

more efficient at converting cells to the TK^+ phenotype (Fig. 6).

Level of TK mRNA in a Cell Line Carrying pTK11. As mentioned above, pTK11 contains the human TK cDNA driven by the SV40 promoter. We used this plasmid to demonstrate that transcription in general is not inhibited when TK^-ts13 cells are placed at the restrictive temperature.

The cell line carrying pTK11 was treated in exactly the same way as the other cell lines (Fig. 7). TK mRNA was detected in serum-deprived cells, and the levels of TK mRNA increased when cells were stimulated with serum at 34°C. However, it increased even further when the cells were stimulated with 10% serum at 39.6°C or in the presence of Chx. These data clearly indicate that transcription from some promoters continues vigorously, even when TK^-ts13 cells are placed at 39.6°C.

The exceptionally strong expression of TK mRNA by the SV40 promoter at the nonpermissive temperature or in the presence of Chx required that the autoradiograph be underexposed. As a result, hybridization with a probe for histone H3 is not visible in Fig. 7, although it could be seen in the original. That the restrictive temperature and Chx do not inhibit transcription in general was also confirmed by rehybridizing the RNA blot of Fig. 3 with a cDNA probe of an unidentified mRNA, clone L7, whose expression is not growth factor- or cell cycle-regulated. Since there were only negligible differences in the intensity of hybridization in the

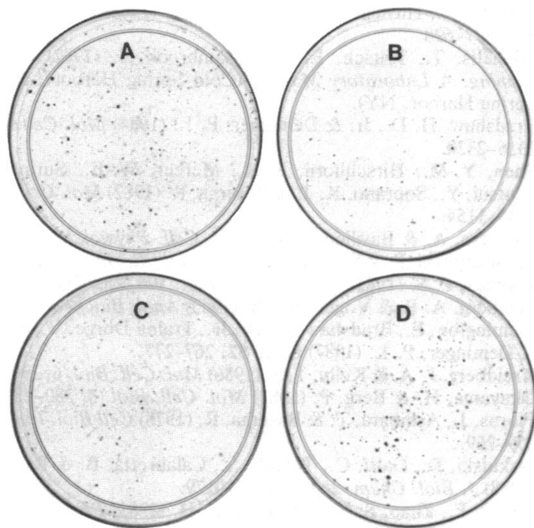

FIG. 6. Activity of promoters altered by site-directed mutagenesis. TK^-ts13 cells were transfected with plasmids (6 μg) containing a TK minigene expressed by an 83-bp promoter (*A*), a 63-bp promoter (*B*), TKMUT1 (*C*), or TKMUT2 (*D*). After 10 days of selection, colonies were stained with crystal violet.

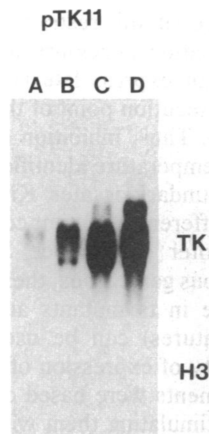

FIG. 7. RNA blot of TK^-ts13 cells carrying the pTK11 construct. In these cells the TK cDNA was under the control of the SV40 promoter. Lanes: A, serum-deprived cells; B, cells stimulated at 34°C in 10% serum for 24 hr; C, same, but at 39.6°C; D, same, except that Chx (10 μg/ml) was added 4 hr after stimulation. Hybridization was with human TK and histone H3 probes.

various lanes, this also showed that the amounts of RNA transferred to the filter were comparable in the various lanes and that the transcription of non-S-phase-regulated genes is totally unaffected by either the restrictive temperature or Chx.

DISCUSSION

In order to know the mechanism by which the TK gene (28, 34, 35, 39) is regulated, it is necessary to identify regulatory sequences of the gene. Travali *et al.* (16) showed clearly that the promoter of the human TK gene plays a major role in its regulation. These results were confirmed by Kim *et al.* (17) and, in some respects, by Arcot *et al.* (18). Introns can also affect gene expression. For example, deletion of the first intron of the Chinese hamster dihydrofolate reductase (DHFR) gene greatly decreased its ability to transform $DHFR^-$ CHO cells to a $DHFR^+$ phenotype (40). Although introns are not essential for efficient expression of TK mRNA (41), it was possible that they could have affected the S-phase-specific regulation of the TK gene. To test this and to further delineate regulatory elements in the promoter, we examined the expression of a human TK minigene, containing the first two introns, or a TK cDNA under the control of human TK 5′ flanking sequence with five progressive deletions. The most important findings of this investigation are as follows. (*i*) A 63-bp promoter is not S-phase-regulated, whereas an 83-bp promoter is still partially regulated; this localizes important regulatory elements to 20 bp. (*ii*) A mutation at the −70 inverted CCAAT of the TK promoter dramatically reduces expression from the 83-bp promoter, indicating that this is a crucial element in regulation. (*iii*) The 83-bp promoter is unresponsive to Chx, indicating that there is a dissociation between elements regulated by the ts block and those regulated by Chx. (*iv*) Although the cDNA is sufficient for detectable expression of TK mRNA, the presence of the first two introns markedly increases mRNA levels. (*v*) The SV40 promoter-driven TK construct is stimulated by serum and is completely refractory to inhibition by the ts block or Chx. Therefore, it cannot be used to demonstrate the cell cycle regulation of TK mRNA levels by its coding sequence (16). These findings are discussed below.

We have studied the regulation of TK mRNA levels in serum-deprived cells stimulated to proliferate and have used two different assays to determine its S-phase-specific regulation: a G_1-specific ts mutant of the cell cycle and the ability of Chx to inhibit the synthesis of TK mRNA. Both of these assays are indicative of genes that are expressed at the G_1/S boundary. Cell cycle genes expressed in the early part of G_1 are not inhibited in G_1-specific ts mutants of the cell cycle stimulated at the restrictive temperature (19, 20), and such genes are not inhibited by Chx; on the contrary, mRNA levels of early cell cycle genes are actually increased by Chx

(22–26). G_1-specific ts mutants blocked at the restrictive temperature do not contain the information necessary for cellular DNA synthesis (42) and do not express late G_1/S-phase genes (5, 16, 20, 21, 38). The execution point of the ts13 mutation is located in late G_1 (37). Thus, inhibition of gene expression at the nonpermissive temperature identifies transcription that occurs at the G_1/S boundary or later. Kim *et al.* (17) also used this assay, with a different ts mutant cell line, to confirm that the TK promoter can induce S-phase-specific regulation of a heterologous gene. Thus, these two assays (sensitivity to temperature in ts mutants and sensitivity to Chx at normal temperatures) can be used effectively to test the S-phase specificity of expression of a gene (see also ref. 17). All our experiments were based on making the cells quiescent and then stimulating them with serum. We acknowledge that the regulation of TK mRNA levels may be different in continuously proliferating cells, like HeLa cells (4), and that in other situations, such as during myogenic differentiation, regulation may actually occur almost exclusively at the translational level (11). However, TK mRNA is undetectable in G_0 fibroblasts or fibroblast-like cells and increases only at the G_1/S boundary (5–7, 38).

Although we do not know the mechanism of action of Chx on the TK gene, the results are still intriguing. In the first place, it is hard to believe that Chx exerts its present effect by affecting mRNA stability: if this were so, all promoters should be equally affected. That only the smaller promoters are unresponsive to Chx seems to indicate an element immediately upstream whose activity is inhibited by Chx. Since, at these concentrations, many other promoters (early growth-regulated genes, SV40, L7, and probably others) are totally unaffected, Chx could be profitably used to identify regulatory proteins for the TK promoter.

The validity of our results is confirmed by the invariant expression of the gene for cDNA clone L7, which indicates that changes of TK expression did not result from the presence of different amounts of RNA or a general failure of transcription at the restrictive temperature or in the presence of Chx. The latter point is also demonstrated by the fact that the SV40 promoter is quite active at 39.6°C, thereby confirming that G_1 ts mutants of the cell continue to transcribe genes whose expression is regulated before the ts block (19).

Kim *et al.* (17) found that the sequences necessary for cell cycle regulation of the human TK gene could be localized to a 378-bp *Eco*RI–*Nco* I fragment of the TK 5′ flanking sequence. They also observed that a 63-bp promoter seemed to have lost its cell cycle regulation and was expressed at only a very low level, about 20% the level observed for the promoter including nucleotides −441 to −63 relative to the cap site (17). The observation that an 83-bp promoter is as strong as longer promoters, whereas a 63-bp promoter is not (ref. 17 and present data), strongly implies that the 20 bp from −83 to −63 are important for the expression of the human TK gene. Indeed, proteins binding to the −70 CCAAT element have already been described (9, 18), and our results demonstrate that this element is crucial to TK expression. Also intriguing is the dissociation between the 83-bp and 456-bp promoters *vis a vis* their inhibition by either the ts block or Chx. This dissociation suggests a hierarchy of elements in the promoter region that are responsive to different regulatory molecules. This interpretation is compatible with the fact that TK expression is regulated by at least two different growth factors (38). Finally, these results should set to rest the theory that, in fibroblasts, the regulation of the expression of the human TK gene depends on the coding sequence (14, 15).

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