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Role of the Promoter in the Regulation of the Thymidine Kinase Gene

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To identify the regulatory elements of the human thymidine kinase (TK) gene, we have established stable cell lines carrying different chimeric constructs of the TK gene. Our results can be summarized as follows. (i) When the TK coding sequence is under the control of the calcyclin promoter (a promoter that is activated when G_0 cells are stimulated by growth factors), TK mRNA levels are higher in G_1-arrested cells than in proliferating cells; (ii) when the TK coding sequence is under the control of the promoter of heat shock protein HSP70, steady-state levels of TK mRNA are highest after heat shock, regardless of the position of the cells in the cell cycle; (iii) the bacterial CAT gene under the control of the human TK promoter is maximally expressed in the S phase; (iv) the TK cDNA driven by the simian virus 40 promoter is also maximally expressed in the S phase; and (v) TK enzyme activity is always at a maximum in the S phase, even when the levels of TK mRNA are highest in nonproliferating cells. We conclude that although the TK coding sequence may also play some role, the TK promoter has an important role in the cell cycle regulation of TK mRNA levels.

Thymidine kinase (TK) is a member of a group of enzymes involved in the synthesis of cellular DNA. The activity of TK, as well as that of other enzymes of the DNA-synthesizing machinery, increases sharply at the G_1-S boundary of the cell cycle and remains elevated throughout the S phase (4, 26, 38). Steady-state levels of TK mRNA also increase sharply as the cells enter the S phase (8, 27, 37, 42). Because of its close association with the enzymes of cellular DNA synthesis, the TK gene has been used as a model to study the regulation of expression of an important group of genes, which could act as primary elements in the control of cell cycle progression.

Kreidberg and Kelly (23) used deletion analysis to define the functional promoter of the TK gene to an 83-base-pair region upstream of the mRNA CAP site. However, workers in other laboratories have reported that cell cycle regulation of expression of the TK gene is controlled by its own coding sequence (18, 24, 29, 36). In the present paper, we report the following findings. (i) When the TK coding sequence is placed under the control of two different cellular promoters, the promoters regulate TK mRNA levels during the cell cycle, such that maximal amounts of TK mRNA occur outside the S phase. (ii) The bacterial CAT gene driven by the human TK promoter is maximally expressed in the S phase. (iii) We confirm that the TK cDNA coding sequence, under the control of simian virus 40 (SV40) promoter, is cell cycle regulated with maximal expression in the S phase. (iv) TK enzyme activity increases only at the G_1-S boundary, regardless of the time of maximal expression of TK mRNA. For convenience, in the remainder of the paper we refer to a TK chimeric gene that is expressed at the correct time (such as the endogenous gene, in the S phase) as cell cycle regulated. If the chimeric gene is not expressed at the correct time, we refer to it as deregulated, even though its expression may be regulated in a different manner. The results presented here indicate that the 5′ flanking sequence of the human TK promoter plays an important role in cell cycle regulation of TK mRNA, but that TK enzymatic activity is based largely on translational controls.

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MATERIALS AND METHODS

Cell lines. BALB/c3T3 cells are grown routinely in our laboratory, as previously described (34). 3T3pSp cells were derived from BALB/c3T3 by cotransfection (35) with pSpTK (see below) and a selectable marker, pRSVneo (see below). The cotransfected cells were selected in G418. Cells were also derived from BALB/c3T3 by transfection with pTK-CAT-neo (described below), followed by selection in G418. One clone, 3T3KL-1, and a mixture of clones, 3T3KL-m, were grown and used for these studies.

The other cell lines were derived from TK^-ts13 (35), a TK^- mutant of ts13 cells (40), which are a G_1-specific temperature-sensitive mutant of the cell cycle, originally derived from BHK cells and therefore of Syrian hamster origin. All derivatives of ts13 cells are grown at 34°C; the nonpermissive temperature is 39.6°C. TK^+ts13 cells were derived from TK^-ts13 cells by transfection with pTKR2-Cal (see below) and selection in glycine-hypoxanthine-aminopterin-thymidine (gHAT) medium. SVtK^+ts13 cells were also derived from TK^-ts13 cells by transfection with pTK11 (3) and selection in gHAT medium.

Heat shock was carried out by incubating cells at 42°C for 5 h. We had previously determined that under these conditions, quiescent BALB/c3T3 can still be serum stimulated, although the entry into the S phase is slightly delayed. Unless otherwise stated, the cells were collected and RNA was extracted at the termination of the heat shock.

Plasmids. pTK11 contains a full-length cDNA of human TK. It was isolated from an Okayama-Berg library (32) by Bradshaw and Deininger (3). Construction of pTKR2-Cal was previously described (12). This plasmid contains a fragment of the human TK cDNA under the control of the calcyclin promoter, which is activated when G_0 cells are stimulated by growth factors (5, 12). The TK fragment was

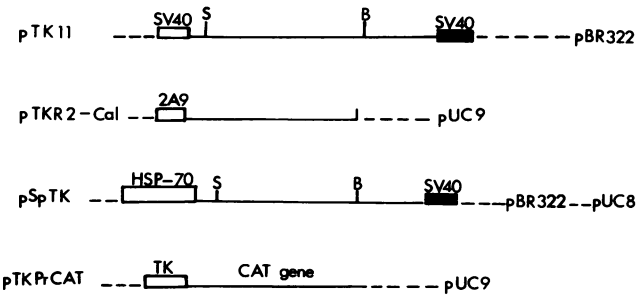

FIG. 1. Structure of the most important recombinant plasmids used in these experiments. Symbols: ▭, promoters and their origins; ▬, polyadenylation signals (in both cases, when the polyadenylation signal is from SV40 it is the late polyadenylation signal); ----, vector; ——, coding sequences. Abbreviations: S, *Sca*I restriction site in the TK cDNA; B, *Bam*HI restriction site in the same TK cDNA (see also Materials and Methods).

derived from pTK11 and lacks 28 nucleotides of the 5′ noncoding region and 75 nucleotides of 3′ untranslated sequence. Both the TK and SV40 polyadenylation signals present in pTK11 were deleted by isolation of this fragment. pSpTK contains the full-length human TK cDNA controlled by the *Drosophila* HSP70 promoter. It was constructed by inserting a *Pst*I fragment of pTK11 into the *Pst*I site of pSp7, a pUC8 vector containing the HSP70 promoter (9, 31). The correct orientation of the TK cDNA, relative to the promoter, was selected by restriction analysis. The *Hin*dIII-*Rsa*I fragment of the human TK gene 5′-flanking sequences (13) in an M13 vector was the kind gift of Prescott Deininger, Louisiana State University. An *Eco*RI fragment deleting 16 nucleotides from the 5′ end of this fragment was subcloned into pUC9 and selected by restriction analysis. The construction of a plasmid containing a promoterless CAT gene was previously described (12). A *Bam*HI fragment of this plasmid containing the CAT cDNA was inserted 3′ to the human TK 5′ flanking sequences in pUC9. The proper orientation of the CAT gene was selected by restriction analysis with *Pvu*II. This plasmid and pRSVneo (14) were digested with *Nde*I and then ligated. The recombinant plasmid pTK-CAT-neo was selected by colony hybridization (28). Other plasmids used to isolate probes for histone H3 (33) and v-*myc* (10) have been described elsewhere.

Product analysis. RNA was extracted by the method of Chomczynski and Sacchi (7), and RNA blots (10 μg per lane) were made by the method of Thomas (41). Inserts of TK, histone H3, and v-*myc* were isolated from the plasmids described above and used as probes for hybridization with RNA blots. The probes were labeled by the random priming method (11). S1 nuclease protection analysis was performed as described previously (43) by using a 564-base-pair *Nco*I fragment from pTK11, which includes the 5′ region of the TK cDNA and 292 base pairs of the Okayama-Berg vector (32). The lengths of the protected fragments were calculated to be 245 base pairs for TK^+ts13 cells and 272 base pairs for 3T3pSp cells. The CAT assay was done by the method of Gorman et al. (15). TK enzyme activity was determined by the method of Liu et al. (27).

RESULTS

The recombinant plasmids used in these experiments are described in Materials and Methods, and the important features of the most relevant ones are summarized for convenience in Fig. 1. These plasmids were used to obtain stably transformed cell lines. The choice of the two cellular promoters was based on the following considerations: (i) the calcyclin promoter is most active in G_1 cells (5) and is the controlling element (12) of a gene that is also growth regulated, like the TK gene, but at a different point in the cell cycle; (ii) the *Drosophila* HSP70 promoter is a strongly inducible promoter, which can be regulated by manipulations that are independent from cellular growth; and (iii) we have chosen the *Drosophila* rather than the human HSP70 promoter because the *Drosophila* promoter is not inducible by serum, whereas the human promoter responds to serum stimulation (44). In some of the plasmids, the polyadenylation signals from SV40 were eliminated, for reasons that are explained in the Discussion. The human TK cDNA under the control of two cellular promoters is not cell cycle regulated. Two cell lines were studied: one cell line was from BALB/c3T3 cells transfected with the pSpTK construct and a selectable marker (3T3pSp cells), and the other was one in which TK^-ts13 cells were transfected with pTKR2-Cal and directly selected in gHAT medium (TK^+ts13 cells). The steady-state levels of TK mRNA in these two cell lines were investigated under different growth conditions (Fig. 2). To monitor the extent of cell proliferation in these cell lines, we simultaneously hybridized the RNA blot both to a probe for TK and one for histone H3. We also monitored the extent of cellular proliferation by autoradiography with [^{3}H]thymidine, which gave the same results as the histone H3 probe (data not shown). In the TK^+ts13 cells, in which the human TK coding sequence was under the control of the calcyclin promoter, the steady-state levels of TK mRNA did not increase in actively proliferating cells (Fig. 2, lanes a and c with high histone H3 expression). In fact, the expression of TK mRNA was higher in serum-deprived cells (lanes b and d with low histone H3 expression). This would be expected if the calcyclin promoter (G_1 specific) controlled mRNA expression, since these cells arrest in the G_1 phase rather than in the G_0 phase when serum deprived (see below). The human TK cDNA was not cell cycle regulated in the 3T3pSp

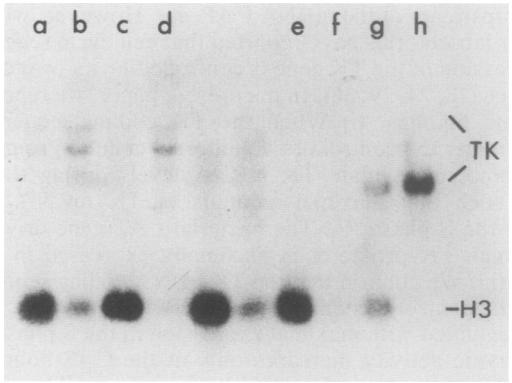

FIG. 2. Expression of TK mRNA in tK^+ts13 cells and 3T3pSp cells. A Northern blot of RNA extracted from these cells was simultaneously hybridized to a TK probe from pTK11 and to a histone H3 probe. Lanes a to d are RNAs from tK^+ts13 cells, and lanes e to h are RNAs from 3T3pSp cells. Lanes: a, exponentially growing cells at 34°C; b, serum-deprived cells at 34°C; c, exponentially growing cells shifted to 39.6°C for 24 h; d, serum-deprived cells at 39.6°C; e, exponentially growing cells; f, quiescent cells; g, exponentially growing cells heat shocked for 5 h; h, quiescent cells heat shocked for 5 h. The two lanes that are not marked are a repetition of lanes a and b.

cells, where it was under the control of the HSP70 promoter (Fig. 2) (22). In these cells, heat shock caused an increase in the amount of TK mRNA, even in G_0 cells (lane h). This increase was quite marked, because under the conditions used, the TK mRNA from the endogenous mouse gene was not visible (lane e), although it could be detected if the Northern (RNA) blots were overexposed. Since the mRNA levels of histone H3 are cell cycle regulated and reach a maximum in the S phase, the levels of histone H3 expression clearly indicate the proliferative state of these cells. Thus, Fig. 2 shows a dissociation in the expression of endogenous histone H3 and human TK under the control of two different cellular promoters.

Although in 3T3pSp cells a single TK mRNA was observed, in TK^+ts13 cells (Fig. 2) several bands were present that hybridized to a TK probe. This is probably due to readthrough transcription, since the calcyclin-TK construct lacks a polyadenylation signal (see Materials and Methods). Therefore, an S1 nuclease analysis of the RNAs shown above was used to confirm the results of Fig. 2 and to determine whether the transcriptional initiation site was correct.

S1 nuclease analysis of RNA (Fig. 3) from exponentially growing (lane 3) and serum-deprived (lane 4) TK^+ts13 cells confirmed that there was an increase in the levels of TK mRNA in serum-deprived cells. After 24 h of incubation at the restrictive temperature of 39.6°C, TK^+ts13 cells expressed somewhat lower amounts of human TK (lanes 6 and 7) than did cells incubated at 34°C. In 3T3pSp cells that were not heat shocked, no TK mRNA could be detected (lane 8). Heat shock induces a marked expression of TK mRNA in G_0 cells (lane 9) which was higher than in heat-shocked exponentially growing cells (lane 10). The specificity of the S1 nuclease probe for human TK mRNA was confirmed by failure to detect TK mRNA in BALB/c3T3 cells stimulated

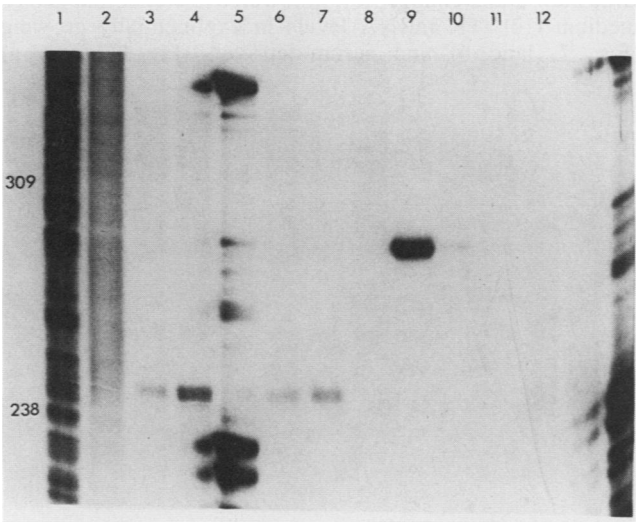

FIG. 3. S1 nuclease analysis of the mRNA from TK^+ts13 and 3T3pSp cells. The same RNAs as described in the legend to Fig. 2 were analyzed by an S1 nuclease assay with a human TK probe (see Materials and Methods). The relevant lanes are the following: lane 3, TK^+ts13 cells exponentially growing at 34°C; lane 4, the same cells serum deprived for 24 h; lane 6, TK^+ts13 cells exponentially growing and shifted to 39.6°C for 24 h; lane 7, the same cells serum deprived for 24 h; lane 8, exponentially growing 3T3pSp cells; lane 9, 3T3pSp cells quiescent and heat shocked for 5 h; lane 10, 3T3pSp cells exponentially growing and heat shocked for 5 h; lane 11, BALB/c3T3 cells serum stimulated for 24 h.

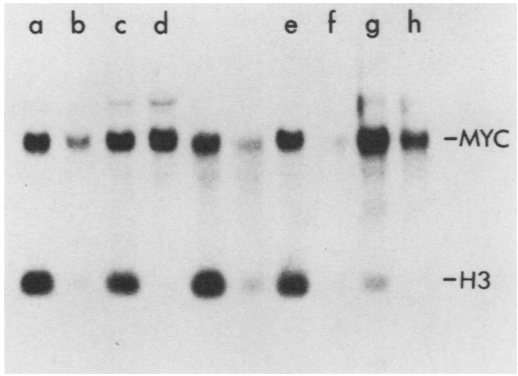

FIG. 4. Expression of c-*myc* and histone H3 in tK^+ts13 cells and 3T3pSp cells. The filter shown in Fig. 2 was rehybridized to a c-*myc* probe and to a histone H3 probe. The lanes are exactly the same as in Fig. 2.

with serum (lane 11). By Northern blots, TK mRNA is detectable in BALB/c3T3 cells from 16 to 24 h after serum stimulation (data not shown). These experiments indicate that the expression of human TK cDNA controlled by either the calcyclin or the HSP70 promoter is not cell cycle regulated.

TK^+ts13 cells are growth arrested in the G_1 phase. Although the calcyclin promoter was activated when G_0 cells were stimulated to proliferate (5, 12), TK^+ts13 cells expressed the calcyclin promoter-controlled TK even when they were serum deprived (Fig. 2 and 3). TK^-ts13 cells, from which TK^+ts13 cells are derived, are cells that grow very vigorously even in low-serum conditions. When they are placed in serum-deprived medium they growth arrest, but, like many other cell lines that grow so vigorously, they arrest in the G_1 phase rather than in the G_0 phase. To confirm this possibility, we have examined the expression of c-*myc* in these cells under different growth conditions. As previously reported, c-*myc* mRNA is not detectable in G_0 cells, but it is detectable in G_1 cells or in G_0 cells stimulated to proliferate by growth factors (17, 19, 21). It is also detectable in serum-starved transformed cells (6). Therefore, the filter shown in Fig. 2 was rehybridized to probes for c-*myc* and histone H3 (Fig. 4). c-*myc* was clearly expressed in TK^+ts13 cells even when they were serum deprived, whether they were at 34°C (lane b) or at the nonpermissive temperature of 39.6°C (lane d), indicating that under these conditions TK^+ts13 cells are in the G_1 phase and not in the G_0 phase. c-*myc* was not expressed in quiescent 3T3pSp cells (lane f), but was clearly expressed in exponentially growing 3T3pSp cells (lane e). It is interesting that c-*myc* was also markedly expressed when quiescent cells were heat shocked at 42°C (lane h). A probe for β-actin was used to confirm that similar amounts of RNA were transferred to the filter (not shown).

Expression of TK cDNA in heat-shocked 3T3pSp cells. In the experiment to study expression of TK cDNA in heat-shocked 3T3pSp cells, 3T3pSp cells were made quiescent and then divided into two groups. The first group was simply serum stimulated. The second group was heat shocked for 5 h at 42°C before serum stimulation. The expression of TK mRNA was then investigated at various times after serum stimulation (Fig. 5). Mouse TK mRNA in the 3T3pSp cells simply stimulated for 24 h with serum is barely detectable; it is seen as a faint band that migrates faster than the human TK band (lane c). The human TK band is clearly detectable in lanes d to f, in which the RNA comes from cells heat

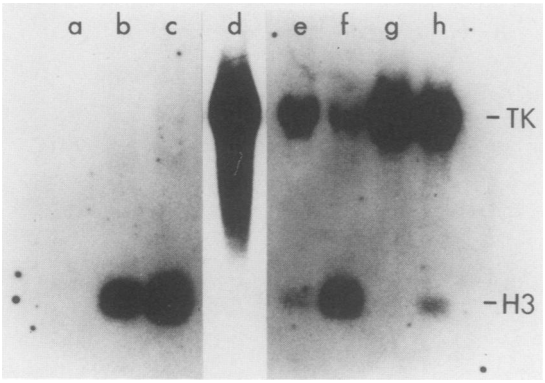

FIG. 5. Expression of TK mRNA and histone H3 mRNA in 3T3pSp cells. The RNA blot was simultaneously hybridized to a TK probe and a histone H3 probe. Lanes: a, quiescent cells; b, cells serum stimulated for 16 h; c, cells serum stimulated for 24 h; d, quiescent cells heat shocked for 5 h; e, cells heat shocked for 5 h and then serum stimulated for 16 h; f, cells heat shocked for 5 h and then serum stimulated for 24 h; g, cells serum stimulated for 16 h (heat shocked for the last 5 h); h, cells serum stimulated for 24 h (heat shocked for the last 5 h).

shocked for 5 h at 42°C and then serum stimulated. The level of expression of TK mRNA under these conditions was higher in quiescent heat-shocked cells (lane d) than in cells heat shocked and serum stimulated for 16 or 24 h (lanes e and f) or, conversely, serum stimulated and then heat shocked in the S phase (lanes g and h). Again, the histone H3 probe monitors the proliferative state of the cells under these conditions. This experiment clearly indicates that the expression of the human TK gene in 3T3pSp cells, in which the TK gene is under the control of the *Drosophila* HSP70 promoter, depends on the promoter and not on the cell cycle position of the cell.

TK activity in 3T3pSp cells. We measured TK activity in 3T3pSp cells that were heat shocked and then serum stimulated. Enzyme activity was determined at 0, 12, 18, and 24 h after serum stimulation, and the results are shown in Table 1. TK activity reached a maximum at 24 h when the cells were in the S phase. It was, instead, very low at 0 and 12 h, i.e., before the cells entered the S phase, although the steady-state levels of TK mRNA were higher at those points than at 24 h after heat shock and serum stimulation (Fig. 5). The results therefore clearly indicate that TK enzyme activity is dependent on the position of the cell in the cell cycle, regardless of the time at which the maximum expression of TK mRNA is reached. It should be noted that the pSpTK construct produces a functional protein, since it makes TK^- cells return to a TK^+ phenotype (growth in gHAT medium [data not shown]).

TABLE 1. TK enzyme activity in 3T3pSP cells serum stimulated with or without heat shock[a]

Treatment and time (h) after serum stimulation	TK activity (cpm/μg of protein)[b]
No heat shock	
0	3.4
12	2.0
18	12.7
24	79.2
Heat shock	
0	3.4
12	4.0
18	30.6
24	83.0

[a] 3T3pSp cells were made quiescent under low-serum conditions and then stimulated with 10% fetal calf serum for the times indicated. Some cultures were heat shocked (5 h at 42°C) prior to serum stimulation.

[b] TK enzyme activity was determined by the method of Liu et al. (27).

The human TK promoter makes the bacterial CAT gene cell cycle regulated. Stable cell lines were made that carried a chimeric gene in which the TK promoter (13) was driving the bacterial CAT gene as a linked reporter. The TK promoter-CAT construct was part of a larger plasmid that also carried the selectable marker pRSVneo (see Materials and Methods). The cells were selected with G418, and we derived both a single clone and a mass culture of cells selected at random. Both the clone (3T3KL-1) and the mass culture (3T3KL-m) were made quiescent in 1% serum, serum stimulated, and harvested at various times for measurements of CAT activity (Fig. 6). It is evident that CAT activity in these BALB/c3T3-derived cells markedly increased during the S phase (between 16 and 30 h after serum stimulation). Since the CAT gene itself is not cell cycle regulated (30, 34), these data indicate that the human TK promoter confers cell cycle regulation to sequences it precedes.

Cell cycle regulation of the human TK cDNA driven by the SV40 promoter. Stewart et al. (36) have shown that cell lines carrying pTK11, in which the SV40 promoter drives the human TK cDNA sequence, have steady-state levels of TK mRNA that are cell cycle regulated. In the previous experiments, we found that the same cDNA sequence under the control of cellular promoters is not cell cycle regulated. We therefore decided to investigate whether in our cells (which are different from the cells used by Stewart et al. [36]) pTK11 itself is cell cycle regulated. These experiments were carried out with $SVTK^+ts13$ cells, which are derived from TK^-ts13 cells by transfection with pTK11, the plasmid described by Bradshaw and Deininger (3), followed by selection in gHAT medium (12). TK mRNA levels in exponentially growing (Fig. 7, lane b) and serum-deprived (Fig. 7, lane a)

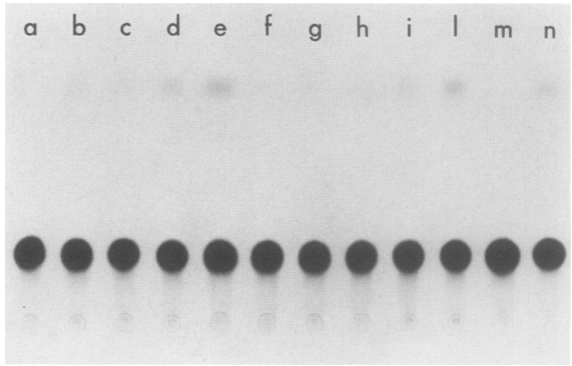

FIG. 6. Expression of the CAT gene in 3T3KL cells. 3T3KL cells are stable cell lines obtained by transfection with a plasmid that contains the *neo* resistance gene, as well as a human TK promoter driving the bacterial CAT gene. 3T3KL-1 (lanes a to e) are a cloned cell line. 3T3KL-m cells are a mixture of clones (lanes f to l). Lane m is the negative control, and lane n is the positive enzyme control. The cells were made quiescent and then stimulated with serum. Lanes a and f are quiescent cells. Lane b and g are cells stimulated for 6 h; lanes c and h are cells stimulated for 16 h; lanes d and i are cells stimulated for 20 h; and lanes e and l are cells stimulated for 28 h.

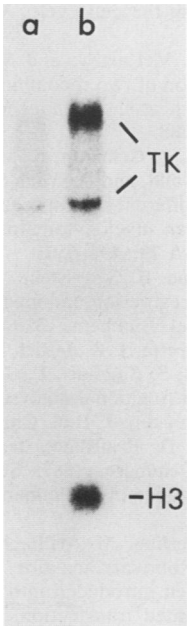

FIG. 7. Expression of the TK and histone H3 genes in SVtK$^+$ ts13cells. These cells are derived from tK$^-$ts13 cells and carry the pTK11 human TK cDNA under the control of an SV40 promoter. Lanes: a, cells serum deprived for 24 h; b, cells exponentially growing at 34°C.

SVTK$^+$ts13 cells were determined. As in previous experiments, we monitored the proliferative state of the cells with the histone H3 probe. Unlike other constructs, with pTK11 there is a correlation between the expression of histone H3 and the expression of TK, which is more elevated in proliferating cells than in serum-deprived cells. We have therefore confirmed that in cells derived from TK$^-$ts13 and containing the SV40 promoter, the human TK cDNA is cell cycle regulated.

DISCUSSION

If we wish to know the mechanism(s) by which the TK gene (2, 13, 23, 25) is regulated and to identify the proteins that regulate its expression, it is necessary to identify the regulatory sequences of the gene as clearly as possible. In the present paper we report results that are seemingly at variance with those reported several times in the literature, in which the cell-cycle-dependent expression of the TK gene appeared to be regulated by its own coding sequence. The results of our experiments can be summarized as follows. (i) When placed under the control of two different cellular promoters, the calcyclin promoter and the *Drosophila* HSP70 promoter, the human TK cDNA sequence is not cell cycle regulated. It is, however, still cell cycle regulated when placed under the control of the SV40 promoter. (ii) The bacterial CAT gene is cell cycle regulated; i.e., CAT activity reaches a peak in the S phase when it is placed under the control of the human TK promoter. (iii) TK enzyme activity still is limited to S-phase cells, even in cells such as 3T3pSp cells in which the expression of the TK gene after heat shock reaches a maximum in G_0.

The trivial explanation that the two cellular promoters are stronger than the SV40 promoter and therefore override the cell cycle regulation is not tenable. We have compared the calcyclin promoter and the SV40 promoter in their ability to transform TK$^-$ts13 cells to the TK$^+$ phenotype and have found, as reported by Ferrari et al. (12), that the SV40 promoter is 10-fold more efficient than the calcyclin promoter.

The question, therefore, is how to reconcile the previous reports with our own data. In the first of these reports, Lewis and Matkovitch (24) found that the expression of the Chinese hamster TK gene was related to the proliferative state of the cell even when it was placed under the control of the herpes simplex virus (HSV) TK promoter. These authors, however, measured only the TK enzyme activity of proliferating or nonproliferating cells. The present paper demonstrates that TK activity does indeed follow the pattern of cell proliferation regardless of the steady-state levels of TK mRNA. Thus, there is no contradiction between the two reports. Another possible explanation can be found in the paper by Artishevsky et al. (1), who found that serum can increase expression from an HSV TK promoter, although not in a cell-cycle-regulated fashion. Merrill et al. (29) came to the same conclusions as Lewis and Matkovitch (24) by comparing proliferating mouse myoblasts and differentiated myocytes transfected with a chicken TK gene controlled by the HSV TK promoter. Most of their data were based on TK enzyme activity, but they also showed a Northern blot in which TK RNA expressed from the HSV TK promoter was detectable in proliferating myoblasts but not in differentiated cells.

Two other investigators also examined the steady-state levels of TK mRNA and found that the expression of TK mRNA was clearly cell cycle regulated when the TK cDNA was under the control of viral promoters. In the first investigation, by Hofbauer et al. (18), the mouse TK cDNA coding sequence was under the control of an HSV TK promoter. The steady-state levels of TK mRNA were cell cycle regulated. However, these investigators made the cells quiescent with butyrate and then restored proliferation by removing the butyrate. It is well known that butyrate itself can either inhibit or stimulate the expression of certain genes (16, 45). More convincing are the experiments of Stewart et al. (36), who made rat cell lines in which the human TK cDNA coding sequence was controlled by either the SV40 promoter or the human TK promoter. No difference could be detected between the two, and both constructs were cell cycle regulated, with maximal steady-state levels of mRNA during the S phase. The discrepancy between the experiments of Stewart et al. (36) and our own does not depend on the cell type, because we also observed cell-cycle-regulated expression of TK mRNA when the TK cDNA was under the control of the SV40 promoter. Such cell cycle regulation disappeared, however, when the SV40 promoter was replaced by either the calcyclin or the HSP70 promoter. The fact that we used cellular promoters rather than the viral promoters used in the other three investigations is the primary difference between this and the previous reports. The suggestion by Stewart et al. (36) that the SV40 promoter may be regulated in an S-phase-specific manner is unlikely, because in our laboratory we have shown that the SV40 promoter is more expressed in 1% serum than in 10% serum (39). It is possible that the viral promoters are neutral and leave the TK cDNA sequence to regulate its own cell-cycle-dependent expression.

Another possibility to explain the discrepancy between the data of Stewart et al. (36) and our own, besides the nature of promoters, is the polyadenylation signal that was used. With the SV40 promoter, Stewart et al. (36) used the late polyadenylation signal of SV40. Kaufman and Sharp (20)

reported the growth-dependent regulation of the dihydrofolate reductase gene. They noticed, however, that this growth-dependent regulation of the dihydrofolate reductase gene occurred only when the gene was inserted in a plasmid that contained the late polyadenylation signal of SV40. If the plasmid contained the early polyadenylation signal of SV40, the growth-dependent regulation of dihydrofolate reductase disappeared. For this reason, the SV40 polyadenylation signal was removed from the calcyclin promoter-TK construct used in our experiments (pTKR2-Cal). In this plasmid, the 3′ noncoding sequences of pTK11 were also removed, but Merrill et al. (29) have reported that these sequences do not participate in the regulation of the TK gene. The pSpTK construct still contains the late polyadenylation signal of SV40, and yet is not cell cycle regulated. Thus, the most plausible explanation of the different results lies in the difference between viral and cellular promoters.

The present experiments therefore reopen the question of which regulatory elements of the TK gene play the major role in its cell-cycle-dependent expression. In our experiments, whatever is upstream of the coding sequence appears to be most important for regulating cell-cycle-dependent expression. However, the experiments from other laboratories, and especially those of Stewart et al. (36), still offer the possibility that the cDNA coding sequence may participate in this process. The role that each of these sequences plays in cell cycle regulation can be determined only when appropriate constructs are made and transfected into cells for more detailed studies. For the moment, though, we can say that the role of the 5′ flanking sequence is at least as important as that of the cDNA coding sequence in the expression of TK mRNA levels, whereas enzyme activity is probably regulated by the coding sequence, at a translational or posttranslational level.

ACKNOWLEDGMENT

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LITERATURE CITED

1. **Artishevsky, A., S. Wooden, A. Sharma, E. Resendez, Jr., and A. S. Lee.** 1987. Cell cycle regulatory sequences in a hamster histone promoter and their interactions with cellular factors. Nature (London) **328**:823–827.
2. **Bradshaw, H. D., Jr.** 1983. Molecular cloning and cell cycle specific regulation of a functional human thymidine kinase gene. Proc. Natl. Acad. Sci. USA **80**:5588–5591.
3. **Bradshaw, H. D., Jr., and P. L. Deininger.** 1984. Human thymidine kinase gene. Molecular cloning and nucleotide sequence of a cDNA expressible in mammalian cells. Mol. Cell. Biol. **4**:2316–2320.
4. **Brent, T. P., J. A. V. Butler, and A. R. Crathorn.** 1965. Variations in phosphokinase activities during the cell cycle in synchronous populations of HeLa cells. Nature (London) **207**: 176–177.
5. **Calabretta, B., R. Battini, L. Kaczmarek, J. K. deRiel, and R. Baserga.** 1986. Molecular cloning of the cDNA for a growth factor-inducible gene with strong homology to S-100, a calcium-binding protein. J. Biol. Chem. **261**:12628–12632.
6. **Campisi, J., H. E. Gray, A. B. Pardee, M. Dean, and G. Sonenshein.** 1984. Cell cycle control of c-myc but not c-ras expression is lost following chemical transformation. Cell **36**: 241–247.
7. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
8. **Coppock, D. L., and A. B. Pardee.** 1987. Control of thymidine kinase mRNA during the cell cycle. Mol. Cell. Biol. **7**:2925–2932.
9. **Craig, E. A., B. J. McCarthy, and S. C. Wadsworth.** 1979. Sequence organization of two recombinant plasmids containing genes for the major heat-shock induced protein of D. melanogaster. Cell **16**:575–588.
10. **Dalla Favera, R., E. P. Gelmann, S. Martinotti, G. Franchini, T. S. Papas, R. C. Gallo, and F. Wong-Staal.** 1982. Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA **79**:6497–6501.
11. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
12. **Ferrari, S., B. Calabretta, J. K. deRiel, R. Battini, F. Ghezzo, E. Lauret, C. Griffin, B. S. Emanuel, F. Gurrieri, and R. Baserga.** 1987. Structural and functional analysis of a growth-regulated gene, the human calcyclin. J. Biol. Chem. **262**:8325–8332.
13. **Flemington, E., H. D. Bradshaw, Jr., V. Traina-Dorge, V. Slagel, and P. L. Deininger.** 1987. Sequence, structure and promoter characterization of the human thymidine kinase gene. Gene **52**:267–277.
14. **Gorman, C., G. Merlino, M. Willingham, I. Pastan, and B. Howard.** 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA mediated transfection. Proc. Natl. Acad. Sci. USA **79**:6777–6781.
15. **Gorman, C., L. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1054.
16. **Gorman, C. M., and B. H. Howard.** 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. Nucleic Acids Res. **11**:7631–7648.
17. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) **311**:433–437.
18. **Hofbauer, R., E. Mullner, C. Seiser, and E. Wintersberger.** 1987. Cell cycle regulated synthesis of stable mouse thymidine kinase mRNA is mediated by a sequence within the cDNA. Nucleic Acids Res. **15**:741–751.
19. **Kaczmarek, L., B. Calabretta, and R. Baserga.** 1985. Expression of cell cycle dependent genes in phytohemagglutinin stimulated human lymphocytes. Proc. Natl. Acad. Sci. USA **82**:5375–5379.
20. **Kaufman, R. J., and P. A. Sharp.** 1983. Growth-dependent expression of dihydrofolate reductase mRNA from molecular cDNA genes. Mol. Cell. Biol. **3**:1598–1608.
21. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell specific regulation of the c-myc gene by lymphocyte mitogens and platelet derived growth factor. Cell **35**:603–610.
22. **Klemenz, R., D. Hultmark, and W. J. Gehring.** 1985. Selective translation of heat shock mRNA in Drosophila melanogaster depends on sequence information in the leader. EMBO J. **4**: 2053–2060.
23. **Kreidberg, J. A., and T. J. Kelly.** 1986. Genetic analysis of the human thymidine kinase gene promoter. Mol. Cell. Biol. **6**: 2903–2909.
24. **Lewis, J. A., and D. A. Matkovitch.** 1986. Genetic determinants of growth-phase-dependent and adenovirus V-responsive expression of the Chinese hamster thymidine kinase gene are contained within thymidine kinase mRNA sequences. Mol. Cell. Biol. **6**:2262–2266.
25. **Lin, P. F., S. Y. Zhao, and F. H. Ruddle.** 1983. Genomic cloning and preliminary characterization of the human thymidine kinase gene. Proc. Natl. Acad. Sci. USA **80**:6528–6532.
26. **Littlefield, J. W.** 1966. The periodic synthesis of thymidine kinase in mouse fibroblasts. Biochim. Biophys. Acta **114**:398–403.
27. **Liu, H.-T., C. W. Gibson, R. R. Hirschhorn, S. R. Rittling, R. Baserga, and W. E. Mercer.** 1985. Expression of thymidine kinase and dihydrofolate reductase genes in mammalian ts mutants. J. Biol. Chem. **260**:3269–3274.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory,

Cold Spring Harbor, N.Y.
29. **Merrill, G. F., S. D. Hauschka, and S. L. McKnight.** 1984. TK enzyme expression in differentiating muscle cells is regulated through an internal segment of the cellular TK gene. Mol. Cell. Biol. **4**:1777–1784.
30. **Miwa, T., and L. Kedes.** 1987. Duplicated CArG box domains have positive and mutually dependent regulatory roles in expression of the human α-cardiac actin gene. Mol. Cell. Biol. **7**:2803–2813.
31. **Morris, T., F. Marashi, L. Weber, E. Hickey, D. Greenspan, J. Bonner, J. Stein, and G. Stein.** 1986. Involvement of the 5′ leader sequence in coupling the stability of a human H3 histone mRNA with DNA replication. Proc. Natl. Acad. Sci. USA **83**: 981–985.
32. **Okayama, H., and P. Berg.** 1983. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. Mol. Cell. Biol. **3**:280–289.
33. **Plumb, M., J. Stein, and G. Stein.** 1983. Coordinate regulation of multiple histone mRNA during the cell cycle in HeLa cells. Nucleic Acids Res. **11**:2391–2410.
34. **Rittling, S. R., and R. Baserga.** 1987. Functional analysis and growth factor regulation of the human vimentin promoter. Mol. Cell. Biol. **7**:3908–3915.
35. **Shen, Y.-M., R. R. Hirschhorn, W. E. Mercer, E. Surmacz, T. Tsutsui, K. J. Soprano, and R. Baserga.** 1982. Gene transfer: DNA microinjection compared with DNA transfection with a very high efficiency. Mol. Cell. Biol. **2**:1145–1154.
36. **Stewart, C. J., M. Ito, and S. E. Conrad.** 1987. Evidence for transcriptional and post-transcriptional control of the cellular thymidine kinase gene. Mol. Cell. Biol. **7**:1156–1163.
37. **Stuart, P., M. Ito, C. Stewart, and S. E. Conrad.** 1985. Induction of cellular thymidine kinase occurs at the mRNA level. Mol. Cell. Biol. **5**:1490–1497.
38. **Stubblefield, E., and G. Muller.** 1965. Thymidine kinase activity in synchronized HeLa cell cultures. Biochem. Biophys. Res. Commun. **20**:535–538.
39. **Surmacz, E., L. Kaczmarek, O. Ronning, and R. Baserga.** 1987. Activation of the ribosomal DNA promoter in cells exposed to insulin-like growth factor I. Mol. Cell. Biol. **7**:657–663.
40. **Talavera, A., and C. Basilico.** 1977. Temperature-sensitive mutants of BHK cells affected in cell cycle progression. J. Cell. Physiol. **92**:425–436.
41. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
42. **Thompson, C. B., P. B. Challoner, P. E. Neiman, and M. Groudine.** 1985. Levels of c-myc oncogene mRNA are invariant throughout the cell cycle. Nature (London) **314**:363–366.
43. **Weaver, R. F., and C. Weissman.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5′ termini of 15S β-globin mRNA precursor and mature 10S β-globin mRNA have identical map coordinates. Nucleic Acids Res. **7**:1175–1193.
44. **Wu, B. J., and R. I. Morimoto.** 1985. Transcription of the human hsp 70 gene is induced by serum stimulation. Proc. Natl. Acad. Sci. USA **82**:6070–6074.
45. **Yuan, Z.-A., R. R. Hirschhorn, and R. Baserga.** 1985. Effect of butyrate on the expression of microinjected or transfected genes. J. Biol. Chem. **260**:3778–3783.