Different Regulation of Thymidine Kinase during the Cell Cycle of Normal Versus DNA Tumor Virus-transformed Cells*

(Received for publication, January 5, 1994)

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We compared the cell cycle regulation of thymidine kinase (TK) after centrifugal elutriation in normal human and mouse cells (primary cells, diploid fibroblasts) with its expression in cells transformed with different DNA tumor viruses. Normal cells showed a rise of TK enzyme activity near the G1/S boundary, which peaked in S phase, and in G2 returned approximately to the level of G1. Conversely, in cells derived from viral transformation, TK activity remained high throughout S and G2 phases, although it was induced to a comparable extent at the onset of DNA replication. In addition, transformed cells exhibited much more enzyme activity during all phases of the cell cycle. The observed differences in expression were due neither to different rates of protein turnover nor to differences in enzyme stability. Enzyme activity was always totally paralleled by the protein level. In all normal cells, the pattern of TK mRNA variation during the cell cycle was similar to that of enzyme activity. In all transformed lines, however, mRNA levels were higher and did not fluctuate throughout the cell cycle. Recently we showed (Ogris et al., 1993) that the E2F binding site present in the TK promoter is a target for trans activation of the TK gene by polyoma virus large T antigen. Using cells expressing this antigen under the control of a hormone-inducible promoter, we were able to switch TK cell cycle expression from the normal to the transformed status. Obviously, DNA tumor viruses suppress transcriptional down-regulation of the endogenous DNA precursor pathway enzyme TK during the eukaryotic cell cycle, maybe to improve conditions for their own replication.

Thymidine kinase (ATP:thymidine 5'-phosphotransferase; EC 2.7.1.21) is an enzyme of the pyrimidine nucleotide salvage pathway, which occurs mainly in two forms in normal eukaryotic tissue (Show et al., 1979). The cytosolic enzyme dominates in replicating cells but is absent in resting ones (Littlefield, 1966; Bello, 1974). The second enzyme is of mitochondrial origin and causes the much lower and constant level of activity in resting cells (Adler and McAuslin, 1974).

The level of cytosolic thymidine kinase (TK)† is almost undetectable in quiescent (G0) cells. After growth stimulation, the activity remains very low throughout G1 but increases at least 30-fold as cells enter the S phase (Bello, 1974; Wawra et al., 1981; Johnson et al., 1982). Several laboratories have shown that this increase in activity is not due to stabilization or activation of the enzyme. After addition of cycloheximide at G1, S, or G2 phase, a half-life of about 5 h was observed. These results suggest that alterations in enzyme activity reflect periodic changes in the rate of enzyme synthesis rather than changes in the rate of enzyme degradation (Bello, 1974; Johnson et al., 1982).

The molecular basis of this regulation was investigated in a number of studies (for a recent review see Wintersberger et al. (1992)). Several lines of evidence indicate that the TK gene is transcriptionally regulated in serum-stimulated cells. Although no differences in the chromatin structure of the TK gene are detectable after the onset of replication, the steady state TK mRNA level parallels the increase in enzyme activity (Groudine and Casimir, 1984). Also in simian virus 40-infected cells the induction of TK enzyme activity follows the increase in mRNA level; the virally infected cells ultimately accumulate more TK mRNA than do the serum-stimulated cells (Stuart et al., 1985).

Nuclear run-on transcription assays have shown that the rate of transcription of TK increases at the G1/S boundary following serum stimulation (Stewart et al., 1987; Coppock and Pardee, 1987; Lieberman et al., 1988). However, run-on transcription assays do not conclusively show that transcriptional control can account totally for the observed induction of either mRNA or enzyme activity. In fact post-transcriptional events also play an important role in the regulation of TK; the half-life of TK mRNA is three times longer during S phase than in G0 cells (Coppock and Pardee, 1987), and changes in nuclear processing of TK pre-mRNA between G1 and S phase have been reported (Gudas et al., 1988). During terminal differentiation of muscle cells, TK enzyme activity disappears despite the continuous presence of TK mRNA. This loss of TK activity has been shown to be associated with a decrease in the translational efficiency of TK mRNA (Gross and Merrill, 1988, 1989).

Recently published results from our laboratory (Knöfler et al., 1993) indicate that translational control contributes to endogenous TK gene inactivation in various cell types during growth arrest and differentiation.

While TK regulation at the transition from the resting to the growing state has been investigated in many studies, TK expression during the cell cycle (G1, S, G2, M) is less well characterized. Using centrifugal elutriation, different regulation pat-
terns were observed. In a lymphoblastoid cell line, TK activity increases 8–9-fold at the G1/S boundary, and in G2 phase the activity declines to about the same level as in G1. (Piper et al., 1980). In the leukemia cell lines KG-1 (myeloblastic), HL-60 (promyelocytic), and U937 (monoblastic) TK activity is induced during S phase but remains at this elevated level during G1 and M phase (Chiba et al., 1989). The latter observations are in agreement with the analysis of TK activity during the cell cycle of elutriated HeLa cells (Sherley and Kelly, 1988). In contrast, the steady state level of TK mRNA does not change during HeLa cell cycle. This kind of regulation has been shown to be due to an increased efficiency of translation of TK mRNA during DNA replication and to the fact that stability of TK protein dramatically decreases upon cell division (Sherley and Kelly, 1988). In chicken embryo fibroblasts and in a chicken T-cell line, TK mRNA level is 5–10-fold enhanced at the beginning of replication (Thompson et al., 1985). Contrary to the HeLa model, this suggests that a major point of the cell cycle control of TK expression lies prior to translation.

In an effort to integrate this disparate series of observations into a common model, we characterized the regulation of TK throughout the cell cycle of various cancer and normal eukaryotic cell types, human cell types, fetal fibroblasts (ATCC CCD-27) and adult fibroblasts and skin of a female mouse, pregnant for 138 days. Tissues were homogenized in a Trizol™-RNA isolation kit and total RNA was isolated by phenol extraction and ethanol precipitation. RNA samples were reverse-transcribed into cDNA using an M-MLV reverse transcriptase. The TK cDNA fragment was amplified by PCR using primers flanking the TK coding region. The amplified fragments were cloned into a plasmid vector and sequenced. The TK cDNA sequences were aligned with the published TK sequences using the ClustalW multiple sequence alignment program. The TK cDNA sequences were also compared to the TK mRNA sequences from various cell lines using the BLASTN program.

**Cell Cycle Phase Separation—Centrifugal elutriation of randomly growing cells was performed as described (Hengstschläger and Wawra, 1993). The elutriation system consisted of a Beckman J2–21 M centrifuge and a JE-6B rotor equipped with a standard separation chamber (Beckman Instruments). The rotor was kept at a speed of 2000 rpm. The temperature was kept at 20 °C, and medium flow was controlled by means of a flowmeter. The TK enzyme was eluted from the elutriated cells by washing with phosphate-buffered saline supplemented with 0.9 mM/liter CaCl2, 0.5 mM/liter MgCl2, and 2% calf serum. Consecutive fractions of 150–200 ml were collected at increasing flow rates. Cell cycle distribution was determined using a PAS-II flow cytometer (Partec, Münster, Germany) after the DNA was stained with 6 μg/ml 4,6-diamidino-2-phenylindole dihydrochloride (see Hengstschläger et al., 1993)). Early G1 fractions typically consisted of about 85% G1 cells. The most representative fractions for S and G2/M phase on average contained 75% S phase cells and 77% G2/M phase cells, respectively. In cases where the separation did not fulfill these requirements, the cells were discarded, and the experiment was repeated with a fresh preparation of cells.

**Thymidine Kinase Assay—** Cytoplasmic extracts were prepared as described by Sherley and Kelly (1988). Cells were trypsinized, washed with phosphate-buffered saline, and pelleted by centrifugation at 200 x g. Cell lysis was performed using a buffer consisting of 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 160 mM KCl, 1.5 mM MgCl2, 3 mM β-mercaptoethanol, 50 mM e-amino-n-caproic acid, and 0.8 mg/ml digitonin. Thymidine kinase activity in vitro was measured by the conversion of radioactive thymidine to thymidine monophosphate as described previously (Wawra et al., 1981). The reaction buffer consisted of 0.1 mM Tris-HCl (pH 6.5), 10 mM MgCl2, 150 mM KCl, 3 mM β-mercaptoethanol, 2 μg thymidine, and 4 μCi of [3H]thymidine (5 Ci/mM; DuPont NEN). Samples were incubated at 37 °C; 15-μl aliquots were spotted on DEAE-cellulose filters, which were washed and counted. Thymidine kinase activity was normalized to total protein concentration and determined using the Bio-Rad protein assay reagent with bovine serum albumin as a standard.

**RNA Extractions and Northern Blotting—** Total cytoplasmic RNA was prepared according to a method described by Favaloro et al. (1980). RNA was isolated after cell lysis in an isothiocyanate buffer containing the nonionic detergent Nonidet P-40 and after the removal of nuclei by membrane filtration. 20–40 μg of RNA of each sample were separated in 1% formaldehyde-agarose gel and transferred to Biotrans nylon membranes (ICN, Costa Mesa, CA). After UV fixation, filters were hybridized sequentially with [32P] radiolabeled probes specific for mouse and human TK and β-microglobulin sequences. Autoradiographs were scanned and quantitated with a Quick Scan densitometer (Helena Laboratories, Beaumont, TX).

**Immunoblotting—** Western blotting and detection of mouse thymidine kinase protein with a specific antisera was performed as described previously (Knüller et al., 1993). 200–300 μg of protein of total cytoplasmic extracts were separated under denaturing conditions on 12% polyacrylamide gels. After transfer to nitrocellulose membranes, the membranes were blocked with 5% low fat dry milk, and incubated overnight in the same solution containing a 1:5000 dilution of mouse TK antibody (Knüller et al., 1994). A 1:1000 dilution of sheep anti-rabbit IgG (Sigma) was employed as a secondary antibody prior to developing the immunoblot with goat anti-sheep IgG coupled to alkaline phosphatase (Sigma; 1:10.000 dilution). The substrate for alkaline phosphatase was 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim), and nitro blue tetrazolium (Boehringer Mannheim) was used for staining. Photographic negatives were taken, and densitometric quantitation was carried out using an ImageQuant densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

**Pulse-Chase and Immunoprecipitation—** Logarithmically growing 376 or CEP cells were rinsed with phosphate-buffered saline and incubated in 90-mm Petri dishes in 2 ml of methionine-free medium with 10% dialyzed fetal calf serum and 400 μCi of [35S]methionine (1100 Ci/mM; DuPont NEN). The labeled cells were elutriated; well separated G1, S, and G2/M fractions were recultivated in medium containing unlabeled methionine. After 0, 90, 180, and 270, and 360 min, cytoplasmic extracts were prepared in a hypotonic buffer described above (Sherley and Kelly, 1988), and nuclei and membrane debris were removed by centrifugation. About 900–1200 μg of protein in 300 μl of this buffer were incubated with 5 μl of preimmune serum and protein A-Sepharose beads to reduce background from unspecifically absorbed proteins. The supernatants were incubated with 15 μl of antiserum for 90 min at 4 °C. Afterward, complexes were precipitated by the addition of 20 μl of 1:1 suspension of protein A-Sepharose beads (Pharmacia LKB Biotechnol-
TK Regulation of Normal versus Transformed Cells

RESULTS

Cell Cycle Regulation of TK Activity in Normal and Transformed Cells—To investigate the expression of TK during the normal eukaryotic cell cycle we elutriated various nontransformed cell types. In the course of a number of analyses (reviewed in Wintersberger et al. (1992)), it has been shown that the TK promoters of the mouse and human genes have very little in common. The human TK promoter has a TATA-like sequence and two reverse CCAAT boxes, at least one of which seems to be required for regulated gene expression. The mouse promoter lacks both of these regulatory elements. To find out if these different promoters cause differences in the cell cycle expression pattern, we analyzed TK enzyme activity and steady state mRNA levels in elutriated primary fibroblasts of both species (see "Materials and Methods" for origin and preparation of the primary cells). We were also interested in whether fetal and adult fibroblasts display the same cell cycle regulation of TK. Two other normal references in our analysis were phytohemagglutinin-stimulated human lymphocytes and the permanent but not virally transformed line of contact-inhibited mouse fibroblasts, 3T6.

Most recently we showed that TK can be up-regulated by expression of polyoma virus LT antigen via dissociation of E2F from the Rb complex (Ogris et al., 1993). The capacity of setting the transcription activator E2F free has also been assigned to the LT antigen of simian virus 40, the E1A protein of adenovirus, and the E7 protein of papilloma virus (reviewed in Hamel et al. (1992)). A specifically higher amount of free E2F during the whole eukaryotic cell cycle could lead to an aberrant regulation of this enzyme. Therefore, we examined the expression of TK in cells transformed with papilloma virus, adenovirus, SV40, or polyoma virus and synchronized by the method of centrifugal elutriation. The analysis of the cell line Y79, which does not produce any retinoblastoma gene product (Horwitz et al., 1990), was used in order to test whether the differences we observed between virally transformed and normal cells might involve Rb.

Total TK activities measured in extracts from normal and virus-transformed human and mouse cells are presented in Table I. The results indicate that TK activity in transformed cells is generally higher than in normal cells. As the activities given in Table I reflect the expression of TK in randomly growing cells, several explanations are possible. The increased TK enzyme activity in transformed cells may be caused by 1) a higher amount of proliferating cells in the analyzed population, 2) a longer S phase relative to the duration of their cell cycle, 3) a higher induction of TK activity in S starting from a low level in G1, or 4) a consistently higher TK expression throughout the cell cycle. Cytofluorometric analysis of log nethymically growing normal and transformed cells indicated that there are no significant differences in the DNA distributions (amount of S phase cells and duration of the S phase) between the different cell types (data not shown). To test possibilities 3 and 4 as well as to study TK cell cycle expression in detail, we used counterflow centrifugal elutriation. Elutriation is an ideal method for the separation of cell cycle phase-specific populations without prior perturbation of metabolic functions. Cells are separated on the basis of size and density; this method, therefore, does not allow mitotic cells to be distinguished from those from G2.

Figs. 1 and 2 and Table I summarize the results of elutriations from several normal cell types and DNA tumor virus-transformed lines, showing TK enzyme activity and mRNA content during the cell cycle. As the DNA distributions in Fig. 2 indicate, the method provides good separation. Normally we collected between 8 and 12 fractions. It is important to state that the separation quality of all analyzed cell types is comparable, which makes it unlikely that variations in expression patterns are due to fractionation artifacts.

During the cell cycle of DNA tumor virus-transformed cells, TK enzyme activity was consistently higher than in normal cells (compare Table I, Figs. 1 and 2). In all cell types studied, TK activity was induced 6–14-fold at the G1/S boundary, and in transformed cells, enzyme activity in G2 phase did not return to a low level similar to G1, as observed in normal cells. As already mentioned there are distinct differences between the mouse and human TK promoters. We could, therefore, expect different cell cycle regulation patterns for this DNA precursor pathway enzyme in the two species. Our data, however, show that there is no variation in TK regulation, which can be assigned to differences between mice and man. Studying fetal and adult fibroblasts we also could exclude possible age-dependent differences in cell cycle regulation of TK.

Cell Cycle Regulation of TK mRNA Content—In each fraction assayed for TK activity, we also determined steady state TK mRNA content. To avoid problems from cross-hybridizations, only homologous hybridizations were done: murine cytoplasmic RNA with murine TK and β2-microglobulin cDNAs and human RNA with the human probes. The TK mRNA level was normalized to β2-microglobulin expression and presented in arbitrary units. The values given in Figs. 1 and 2 are, therefore, not comparable in absolute terms. On the other hand, these experiments allow the detection of differences in the patterns of mRNA distribution throughout the cell cycle, which was the primary aim of this study.

We always found variations of steady state TK mRNA during the cell cycle of normal cells. The induction at the G1/S boundary was paralleled by the increase of enzyme activity. Cells exhibiting a large increase of activity when replication begins

| Table I
<table>
<thead>
<tr>
<th>Thymidine kinase activities of various cells</th>
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<tr>
<td><strong>Cells</strong></td>
</tr>
<tr>
<td>Normal fetal fibroblasts</td>
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<tr>
<td>Normal adult fibroblasts</td>
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<tr>
<td>Phytohemagglutinin-stimulated lymphocytes</td>
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<tr>
<td>HeLa (papilloma virus-transformed cells)</td>
</tr>
<tr>
<td>293 (adenovirus-transformed cells)</td>
</tr>
<tr>
<td>Y79 (retinoblastoma cells)</td>
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<tr>
<td>Murine</td>
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<tr>
<td>Normal fetal fibroblasts</td>
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<tr>
<td>Normal adult fibroblasts</td>
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<tr>
<td>3T6 (permanent mouse fibroblasts)</td>
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<tr>
<td>SVMK (SV40 virus-transformed cells)</td>
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<td>COP-8 (polyoma virus-transformed cells)</td>
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Note: A, these cells show a rise of TK activity near the G1/S boundary, and in G2, the activity is about the same as in G1. B, TK activity also increased in late G1/S but remained high during S and G2. In these lines, the enzyme was much more active during all phases of the cell cycle compared with cells with cell cycle regulation A.
TK Regulation of Normal versus Transformed Cells

FIG. 1. TK activity and TK mRNA during the cell cycle of various cell types. Cells were separated by centrifugal elutriation into fractions representing different cell cycle positions as indicated at the top and bottom (G1, S, and G2); the different fractions were analyzed for TK activity and steady state TK mRNA content. TK activity is represented by solid lines; TK mRNA (dashed lines) is given in relative units normalized to $\beta_2$-microglobulin mRNA content (the highest value was set as 100%). 1, phytohemagglutinin-stimulated human lymphocytes; 2, human normal adult fibroblasts; 3, mouse normal fetal fibroblasts; 4, HeLa (papilloma virus-transformed human cells); 5, 293 (adenovirus-transformed human cells); 6, SVMK (SV40 virus-transformed mouse kidney cells).

also show higher amounts of mRNA at this point.

In contrast, the analyzed transformed cells never displayed cell cycle-dependent changes on the level of TK mRNA, although TK enzyme activity was still drastically induced near the G1/S transition. This observation indicates post-transcriptional cell cycle regulation in transformed cells, as had previously been shown for the papilloma virus-transformed HeLa cells (Sherley and Kelly, 1988).

TK Protein Content during Cell Cycle—Wherever it has been studied, throughout the eukaryotic cell cycle (Sherley and Kelly, 1988), during differentiation (Gross and Merill, 1988; Knöller et al., 1993), or after serum stimulation (Ito and Conrad, 1990), changes in the levels of TK activity reflected similar changes in the levels of TK polypeptide. Studying the cell cycle regulation of enzyme activity in normal and DNA tumor virus-transformed cells, we observed two different patterns. Do these different regulations involve changes in the amount of enzyme protein or are they caused simply by modulation of enzyme specific activity?

We recently described the production and characterization of an antiserum specific for mouse TK (Knöller et al., 1993). This serum was raised by inoculation of rabbits with a bacterially expressed mouse TK. Using this polyclonal antiserum, we determined the relative amount of TK protein in synchronized 3T6 mouse fibroblasts (Fig. 3a) and polyoma virus-transformed mouse fibroblasts (Fig. 3b). To obtain sufficient material, the cells were only fractionated into five aliquots. 300-μg cytoplasmic protein extracts of the cell cycle fractions were analyzed. Both in normal and in virally transformed cells there was an excellent correlation between the levels of TK protein and the levels of enzyme activity. The blots presented in Fig. 3 clearly demonstrate that, in transformed cells, TK protein is higher throughout the cell cycle than in their normal counterparts.

FIG. 2. TK activity versus mRNA content during the cell cycle of elutriated 3T6 mouse fibroblasts and the permanent cell line COP-8 (polyoma virus-transformed mouse fibroblasts). Logarithmically growing 3T6 cells (a) and COP-8 cells (b) were separated by elutriation centrifugation. For each fraction, DNA distribution was quantitated by flow cytometry after staining with 4,6-diamidino-2-phenylindol dihydrochloride; the resulting patterns were aligned along the ordinate. TK activity (solid line) is given in picomoles of TMP formed per mg of protein per h. TK mRNA (dashed line) was normalized to $\beta_2$-microglobulin mRNA and expressed in relative units; the highest value was set as 100.

The level of polypeptide as well as enzyme activity stays at the high level of S phase until mitosis. These experiments make it unlikely that the differences in TK cell cycle regulation between normal and transformed cells are caused by modifications of enzyme specific activity.

TK Stability during Cell Cycle—Up to this point we have presented evidence that, in normal cells, TK activity decreases during G2 relative to the high level present in S phase. The question arose if this pattern of regulation is caused by a lower stability of enzyme activity or protein. Therefore, we determined the decay of enzyme activity in the presence of the translation inhibitor cycloheximide in cell cycle phases of various cell types (Table II). The stability of enzymatic activity was found comparable during the cell cycle of both normal and transformed cells; viral transformation apparently does not influence this property.

To extend the data obtained for the half-life of TK activity, protein stability was determined in 3T6 and COP-8 cells by in vivo pulse-chase experiments with radiolabeled methionine. Labeled cells were separated in populations of cell cycle-specific phases; G1, G2, fractions of both cell lines were chased in medium containing unlabeled methionine for 0, 90, 180, and 360 min. At these time points, TK protein was precipitated by the TK-specific polyclonal antiserum. TK protein was determined after separation in denaturing polyacrylamide gels (Fig. 4). Again we could not detect any significant variations in TK protein stability during the cell cycle either in normal cells or in...
polyoma virus-transformed counterparts.

Elevated TK mRNA and Activity Caused by Expression of Polyoma LT Antigen—Obviously, TK activity is up-regulated throughout all phases of the cell cycle in DNA tumor virus-transformed cells. The only significant difference from normal cells that we have observed so far is the loss of cell cycle-dependent changes of steady state mRNA levels. In a recently published work of our laboratory (Ogris et al., 1993), it has been shown that the large T antigen of polyoma virus has the capacity to transactivate TK mRNA expression in serum-starved mouse fibroblasts. Polyoma virus LT antigen activates the TK promoter via Rb and the transcription factor E2F.

Our data indicate that in normal cells G1 and G2 phases display low steady state levels of TK mRNA. After DNA tumor virus transformation, the TK mRNA level is high during the whole cell cycle, which seems to lead to a consistently higher enzyme activity. We wanted to know if it is possible to switch from one type of TK cell cycle regulation to the other only by expressing polyoma LT within the same cell.

To study these questions we used the cell line 3T3LT (Ogris et al., 1992). These cells are mouse fibroblasts conditionally expressing polyoma virus large T antigen from the dexamethasone-responsive mouse mammary tumor virus promoter.

In order to determine if dexamethasone incubation alone causes any variations in TK cell cycle regulation, we first elicited normal 3T3 mouse fibroblast cultures after incubation with 1 μmol/liter of this hormone. Studying TK enzyme activity and TK mRNA (normalized to GAPDH) we observed the typical regulation of normal cells (see Fig. 5).

3T3 fibroblasts stably transfected with the LT antigen gene downstream of the mouse mammary tumor virus promoter were then separated according to the phase of cell cycle by centrifugal elutriation after a 24-h incubation with dexamethasone. When 3T3LT cells were exposed to this hormone, significant amounts of polyoma LT were expressed, free E2F accumulated, and TK promoter activity was up-regulated (Ogris et al., 1993). In Fig. 5 it is shown that the expression of polyoma large T antigen indeed leads to an elevated (3-4-fold) and unregulated mRNA expression during the cell cycle of these mouse fibroblasts. The enzyme activity is also higher (similar to the mRNA level) than in the counterparts not expressing LT.

Finally 3T3LT cells were analyzed in the same way but without preincubation with dexamethasone. The normal cell cycle regulation of thymidine kinase was observed (see Fig. 5).

These results clearly demonstrate that expression of viral transforming genes can cause not only up-regulation of endog-
Fig. 5. Cell cycle regulation of TK activity and mRNA after specific expression of polyoma virus large T antigen. Logarithmically growing 3T3 mouse fibroblasts and 3T3-LT fibroblasts conditionally expressing polyoma virus LT antigen from the dexamethasone (DEX)-responsive mouse mammary tumor virus promoter (3T3LT) were separated by centrifugal elutriation after a 24-h incubation with dexamethasone (1 μmol/liter). Randomly growing 3T3LT cells were elutriated without any preincubation with the hormone dexamethasone. The cell cycle phase-specific fractions were analyzed for TK activity and steady state TK mRNA. TK enzyme activity given in picomoles of TMP formed per mg of protein per h; B, Northern blot hybridization of TK mRNA; C, Northern blot hybridization of glyceraldehyde-phosphate dehydrogenase mRNA.

enous TK but also lead to an indicative change of the expression pattern throughout the cell cycle.

DISCUSSION

In this report we examined the behavior of TK during the cell cycle of normal cells in comparison with virally transformed cells. As deoxyribonucleoside triphosphate biosynthesis is closely connected with DNA replication, enzymes like TK are mainly expressed during the S phase of the eukaryotic cell cycle. The molecular basis for the periodic expression of TK activity during the cell cycle has been defined in elutriated fibroblasts (Sherley and Kelly, 1988). Studying this papilloma virus-transformed line, these authors showed that a large induction of TK activity at the G1/S boundary is not paralleled at the steady state mRNA level. Using pulse-labeling methods, they observed that the efficiency of translation of TK mRNA increased about 10-fold as cells began DNA replication; the protein stability was constant during GI, S, and G2 phases but dramatically decreased upon cell division (in metaphase). From recent work in our laboratory (Ogris et al., 1993) it is known that TK can be transcriptionally up-regulated by expression of polyoma virus large T antigen via dissociation of E2F from the RB complex even in serum-starved cells. Polyoma virus large T, the large T antigen of simian virus 40, the E1A protein of adenovirus, and the E7 protein of papilloma virus have been shown to share the capacity to activate E2F by setting it free from the E2F site (reviewed in Hamel et al., 1997). Subsequently we wanted to determine the basis for the different cell cycle regulations. Besides HeLa cells, we also characterized the regulation in cell lines transformed with other DNA tumors viruses that encode proteins known to interact with Rb. All of these transformed cells exhibited the "HeLa type" regulation: high and unregulated mRNA and elevated enzyme activity during all phases of the cell cycle. A strong argument for the mechanism of this deregulation comes from the retinoblastoma line Y79, which does not produce any retinoblastoma gene product (Horwitz et al., 1990). This cell line has not been virally transformed and, interestingly, displays the regulation of TK, which has so far only been observed in DNA tumor virus-transformed cells. This clearly indicates that this deregulation is not induced by the viral capacity to transform the cell but by the capacity to dissociate E2F from the Rb complex. It should be noted that our results suggest that the human and murine TK promoters share an E2F site (or sites), which is the target for transactivation.

We have already reported that polyoma LT antigen is able to transactivate TK mRNA expression via E2F (Ogris et al., 1993). In this study we demonstrate that inducible expression of this viral oncogene alone is sufficient to cause an elevated level of steady state TK mRNA throughout the cell cycle of 3T3 fibroblasts. Consequently, this elevation is accompanied by a transition to the "HeLa TK phenotype" in these otherwise normal cells.

The observed deregulation of TK expression may cause an imbalance in the pool of DNA synthesis precursors. It has been shown that cytoplasmic TK is involved in the fine regulation of the precursor pool for DNA synthesis (McKenna and Yasseen, 1982). This imbalance might lead to a reduced fidelity of DNA replication and, thus, could account for the higher mutation rate and the chromosome instability observed in cells transformed by these DNA tumor viruses. It is well known that deregulation of the activities of thymidine kinase, ribonucleotide reductase, dCMP deaminase, or CTP synthase causes substantial irritation of the nucleotide pools, which leads to a 200-1000-fold increase in the spontaneous mutation rate (reviewed in Reichard, 1988). It is attractive to assume that
deregulation of deoxynucleotide synthesis is one basis of the cell immortalizing and transforming capacity of DNA tumor viruses.

One possible reason for TK deregulation after transformation could be the general effort of these viruses to prolong the period of optimal conditions for their own replication by securing a high rate of supply with phosphorylated DNA precursors. Up-regulation of TK alone would not be sufficient for that purpose. Along with this concept, it is interesting to note that genes for other enzymes involved in DNA synthesis or precursor production like DNA polymerase α (Pearson et al., 1991) or dihydrofolate reductase (Blake and Azizkhan, 1989) carry binding sites for E2F in their promoters. We recently could show (Mudrak et al., 1994) that these enzymes are transactivated by a polyoma virus LT antigen like TK.

Acknowledgments—We thank Claudia Denk, Ingrid Mudrak, and Thomas Sauer for providing excellent technical assistance. The cytofluorometer was a donation from the Jubiläumsfonds der Österreichischen Nationalbank.

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