Overexpression of Thymidine Kinase mRNA Eliminates Cell Cycle Regulation of Thymidine Kinase Enzyme Activity*

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Expression of thymidine kinase (TK) enzyme activity and mRNA is strictly $S$ phase-specific in primary cells. In contrast, DNA tumor virus-transformed cells have enhanced and constitutive levels of TK mRNA during the whole cell cycle. Their TK protein abundance, however, still increases at the $G_1$-$S$ transition and stays high throughout $G_2$ until mitosis. Therefore, post-transcriptional control must account for the decoupling of TK mRNA from protein synthesis in $G_2$. To characterize the underlying mechanism, we studied the consequences of TK mRNA abundance on the cell cycle-dependent regulation of TK activity in nontransformed cells. Constitutive as well as conditional human and mouse TK cDNA vectors were stably transfected into mouse fibroblasts, which were subsequently synchronized by centrifugal elutriation. Low constitutive TK mRNA expression still resulted in a fluctuation of TK activity with a pronounced maximum in $S$ phase. This pattern of cell cycle-dependent TK activity variation reflected the one in primary cells but is caused by post-transcriptional control. Increasing overexpression of TK transcripts after hormonal induction compromised this regulation. At the highest constant mRNA levels, regulation of enzyme activity was totally abolished in each phase of the cell cycle. These data indicate that post-transcriptional regulation of TK is tightly coupled to the amount of mRNA; high concentrations apparently titrate a factor(s) required for repressing TK production during $G_1$, and presumably also $G_2$.

During growth stimulation and the cell cycle of normal cell types, thymidine kinase (TK), EC 2.7.1.21 enzyme activity is strongly increased, mainly by transcriptional activation of the gene just prior to the onset of DNA synthesis (Stuart et al., 1985; Coppack and Pardee, 1987; Pardee, 1989). In normal cells, both TK mRNA and activity decline in the $G_2$ phase to the levels observed in $G_0$ (for review see Wintersberger et al. (1992) and Hengstschläger et al. (1994b)). It has been proposed that the $G_2$-specific release of the transcription factor E2F from the complex with tumor suppressor proteins like retinoblastoma gene product (pRb) and p107 is responsible for the rise in transcription of TK and other genes involved in DNA precursor metabolism (Kim and Lee, 1992; Li et al., 1993; Ogris et al., 1993; Mudrak et al., 1994; Hengstschläger et al., 1994c). Moreover, cells infected with DNA tumor virus, like polyoma virus, SV40 virus, adenovirus, or human papilloma virus do not exhibit inactivation of the TK promoter during $G_1$ and $G_2$. In these transformed cells, a constant level of free E2F in the cell cycle due to the permanent disruption of E2F-pRb complexes by the action of viral transactivator proteins like large T, E1A, or E7 results in an elevated and constitutive expression of TK transcripts. The deregulation of TK mRNA appearing in transformed as well as neoplastic cells is accompanied by a rise in TK activity, which is induced at the onset of $S$ phase and remains high throughout $G_2$ (Hengstschläger et al., 1994a, 1994b, 1994c). During mitosis, the abundance of TK polypeptide is rapidly decreased by proteolysis in HeLa cells (Sherley and Kelly, 1988; Kauffman and Kelly, 1991). Residues near the C terminus of human TK were held responsible for this phenotype, because corresponding deletions led to constitutive stabilization of the protein (Kauffman et al., 1991).

In addition, post-transcriptional control of TK gene expression has been reported during growth stimulation of serum-starved cells (Ito and Conrad, 1990; Kauffman et al., 1991; Mikulits and Müllner, 1994) in the cell cycle (Sherley and Kelly, 1988; Kauffman and Kelly, 1991; Hengstschläger et al., 1994b; Mikulits and Müllner, 1994) and throughout terminal differentiation (Gross and Merrill, 1988, 1989; Knöfler et al., 1993). Translational repression was suggested to reduce the rate of TK protein synthesis in a variety of cell types at the quiescent state, and furthermore, this mode of TK inactivation does not involve changes in TK protein turnover. In cycling HeLa cells, a strong increase in the efficiency of TK translation has been demonstrated to account for the induction of TK activity prior to DNA replication (Sherley and Kelly, 1988). On the contrary, there has also been a report on stabilization of TK protein after growth stimulation of transfected rat cells (Carozza and Conrad, 1994).

In the current examination we demonstrate that distinct constant levels of TK mRNA are attainable during the cell cycle either by stable transfection of cells with constitutive expression vectors or by different periods of induction on the TK target gene using conditional gene expression. The highest amount of TK transcripts observed after hormone induction of transfected normal cells exceeded the top level observed in tumor cells. Ectopic overexpression of TK mRNA in all phases of the cell cycle totally abolished the post-transcriptional regulation of TK enzyme activity due to the titration of the corresponding cellular function(s). This presents evidence that the post-transcriptional control of TK activity in normal as well as...
transformed cells depends on the expression levels of TK transcripts by a specific regulatory mechanism.

EXPERIMENTAL PROCEDURES

Cell Culture—The permanent mouse fibroblast cell line 3T6 (ATCC CCL 96), which can still be contact-inhibited, and polyoma virus-transformed COP-8 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (CS) and antibiotics (30 mg/ml penicillin and 50 mg/ml streptomycin sulfate). Normal Ltk- (ATCC CCL 1.3) and NIH 3T3tk- mouse fibroblasts received Dulbecco's modified Eagle's medium supplemented with 10% fetal CS or 10% charcoal-stripped (to remove endogenous glucocorticoids) fetal CS (Samuels et al., 1979) and antibiotics, respectively. All cells were grown at 37 °C and 7.5% CO2, and routinely screened for the absence of mycoplasma.

Cloning and Transfection—Full-length mouse TK (mTK) cDNA was cloned as a EcoRI-EcoRI fragment (Hoffbauer et al., 1987) into the eucaryotic pcD expression vector (Okayama and Berg, 1983) to create pcD-mTK. Full-length human TK (hTK) cDNA (Bradshaw and Deininger, 1984) was either cloned into pcD plasmid (pcD-HTK) or expressed under the control of the dexamethasone-inducible promoter mouse mammary tumor virus long terminal repeat (MMTV-LTR) (Kühnel et al., 1986; Buetti and Kühnel, 1986; Buetti, 1994) to get MMTV-hTK (Mikulits and Müllner, 1994). pcD-TK expression constructs were stably transfected into NIH 3T3tk- cells, whereas the hormone-dependent hTK expression vector (MMTV-hTK) was stably transfected into NIH 3T3tk- cells using the calcium phosphate co-precipitation technique (Gorman, 1985). Selection for stable transfections was done either in hydroxyurea/aminopterin/thymidine (HAT) medium (Littlefield, 1964) alone or in HAT medium supplemented with 30 μM of the glucocorticoid antagonist dexamethasone (Sigma). pcD-mTK analysis of pcD vector transfectants, 50–100 HAT resistance clones were pooled and expanded. For experiments with the hormone-inducible MMTV-hTK plasmid, single colonies were picked from transfected NIH 3T3tk- cells and expanded into mass cultures. The abundance of hTK target transcripts obtained by the induction of MMTV-LTR with 1 μM of agonist dexamethasone (Sigma) for 4 h in each isolated clone was determined by Northern blot analysis with the highest hTK mRNA induction was used for the recultivation experiments described in the following paragraphs.

Cell Cycle Fractionation and Recultivation of Cells—Separation of logarithmically growing cells into distinct cell cycle phases was accomplished by centrifugal elutriation in a Beckman J-2–21 M centrifuge and a J-E-6B rotor with the separate separation chamber (Beckman). Fractionation was done by stepwise increasing the pump speed (Cole-Parmer Masterflex pump) from the initial flow rate of 14 ml/min to a maximum of 25 ml/min. The elutriations were performed at a constant rotor speed of 2000 rpm and a constant temperature of 20 °C as described (Hengstschläger et al., 1994a, 1994b). The elutriation medium consisted of 0.5% MC, 0.1% CS in phosphate-buffered saline. The purity of each fraction was determined by analyzing DNA profiles in a PAS-II flow cytometer (Partec). DNA was stained with 6 μg 4,6-diamidino-2-phenylindole dihydrochloride. The percentages of cells in the various cell cycle phases were calculated using a software package from the same manufacturer. For recultivation, elutriated fractions enriched for cells in specific cell cycle phases were resuspended into Petri dishes using the preconditioned medium before elutriation and incubated for 1 h to allow attachment of the adherent cells. Immediately after plating, recultivated cells received 30 μg/ml 3H-thymidine for 16 h to label DNA and 15 μg/ml 35S-methionine for 2 h to label protein. In addition, the cells were incubated in Dulbecco's modified Eagle's medium with 10% fetal CS alone or induced with 1 μM dexamethasone for 1 or 4 h, respectively. Subsequently, cells were collected for RNA extraction and measurement of TK enzyme activity. Cell cycle progression of recultivated cells was ascertained by flow cytometry.

Northern Blot Analysis—Total cytoplasmic RNA was extracted according to a procedure described by Fawcett et al. (1983). After treatment with RNAse, 1 μg of RNA from each sample was separated on 1% formaldehyde-agarose gels and transferred to nylon membranes (Genescreen, DuPont NEN). After UV fixation, filters were hybridized sequentially with 32P-labeled probes (Feinberg and Vogelstein, 1983) specific for mouse or human TK (Hoffbauer et al., 1987; Bradshaw and Deininger, 1984) and 35S-microglobulin (Danil et al., 1983) sequences for normalization. After washing, hybridized filters were exposed to x-ray films at ~70 °C using intensifying screens. Signals on the autoradiographs were scanned and quantified with an Image Quant densitometer (Molecular Dynamics).

Absolute Determination of TK mRNA Levels—Full-length mouse and human TK mRNA was transcribed from linearized pGEM3Zf(-) plasmids with T7 RNA polymerase (Promega) as described (Müllner et al., 1989) and quantified fluorometrically. Total cytoplasmic RNA from 2 million cells each was separated on a 1% formaldehyde-agarose gel. In the same gel, samples with different amounts of in vitro synthesized mTK or hTK transcripts were included. After transfer, the filters were further processed as described in the procedure for Northern blot analysis. Signals on the autoradiographs specific for endogenous mTK and hTK as well as mouse and human T7 TK transcripts were densitometrically scanned and quantified. Finally, the number of TK mRNA molecules/cell was calculated by comparing the obtained TK-specific signals in vivo with the signals from in vitro synthesized TK transcripts.

Immunoblotting—300 μg of protein from total cytoplasmic extracts were separated under denaturing conditions on 14% SDS-polyacrylamide gels followed by electrophoretic transfer onto nitrocellulose membrane (Protran, Schleicher & Schuell, Dassel, Germany). Reversible staining of the filter with acidic Ponceau S solution (Merck, Darmstadt, Germany) was used to visualize the molecular weight markers. The membrane was blocked with 1% low fat dry milk and incubated overnight in the same solution containing 1:500 dilution of affinity purified mouse TK antiserum (Knöfler et al., 1993). A 1:1000 dilution of sheep antiserum to mouse TK (Sigma) was used as secondary antibody to develop the Western blot. Detection of the specific TK protein signal was performed by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham).

Thymidine Kinase Assay—Cytoplasmic extracts were prepared as described (Sherley and Kelly, 1988). After trypsinization and washing with phosphate-buffered saline, cells were pelleted at 200 x g and lysed in a buffer containing 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 160 mM KCl, 1.5 mM MgCl2, 3 mM Μ-mercaptoethanol, 50 mM 2-α-d-ribofuranosyluracil, and 0.8 mg/ml digitonin. TK enzyme activity in vitro was measured by the conversion of radioactive thymidine to thymidine monophosphate (Wawra et al., 1981). The reaction buffer consisted of 10 mM NaF, 80 mM Tris-HCl (pH 7.5), 5 mM ATP, 2.5 mM dithiothreitol, 5 mM MgCl2 and 0.125 μCi of [methyl-3H]thymidine (5 Ci/mM; DuPont NEN). All samples were spotted onto DEAE-cellulose filters, washed extensively with 4 mM ammonium formiate, and counted. TK activity was normalized to total protein concentration using the Bio-Rad protein assay reagent (Bradford, 1976).

RESULTS

Regulation of TK Expression during the Ongoing Cell Cycle of Normal versus Transformed Cells—We separated logarithmically growing 3T6 cells (nontransformed murine fibroblasts) and COP-8 cells (polyoma virus-transformed mouse fibroblasts) according to their different cell cycle phases by centrifugal elutriation. The separation quality between these two cell lines was comparable as judged by the DNA content and by the DNA distribution from the different fractions indicated (Fig. 1A). Two opposite patterns of TK expression were observed during the cell cycle of normal versus DNA tumor virus-transformed cells. In 3T6 fibroblasts, TK mRNA was up-regulated 5-fold, and TK protein and enzyme activity increased 8-fold at the G1-S transition. In G0, these values return to a low level similar to that observed in G1 (Fig. 1). This type of regulation was also found in the cell cycle of primary cells as well as other nontransformed mammalian cell lines (Hengstschläger et al., 1994a, 1994b, 1994c). In contrast, during the cell cycle of polyoma virus-transformed COP-8 mouse fibroblasts, there is almost no fluctuation of TK mRNA and only a moderate 4-fold induction of TK protein as well as enzyme activity at the G1-S boundary, which remain elevated throughout the G2 phase until mitosis (Fig. 1). These data indicate that TK protein expression and enzyme activity are decoupled from TK mRNA expression in the G1 phase of transformed COP-8 cells. Similar alterations in TK cell cycle regulation with constitutive RNA expression and reduced fluctuations of protein expression and enzyme activity were described for other DNA tumor virus-transformed cells as well as tumor-derived cell lines but never in normal cycling cells (Hengstschläger et al., 1994a, 1994b). As
Overexpression of TK mRNA Eliminates TK Activity Regulation

What might be the molecular basis for this altered “transformation-specific” regulation of mTK activity in “normal” cells, whereas the hTK transfectants did not exhibit this phenotype?

Fig. 1. Cell cycle distribution of TK mRNA, protein, and enzyme activity in normal 3T6 cells versus transformed COP-8 cells. Logarithmically growing cells were separated into fractions of distinct cell cycle position by centrifugal elutriation. A, the cells in each fraction were analyzed for DNA distribution by flow cytometry with the best fractions in G1, S, and G2 enriched to at least 96, 65, and 63% purity, respectively. B, TK protein expression was determined by immunoblotting as described under “Experimental Procedures.” C, TK activity is given in picomoles of TMP formed per mg of protein per hour. D, TK mRNA expression was normalized to the level of the constitutive $\beta_{2}$-microglobulin mRNA.

indicated by previous investigations, deregulation of TK mRNA in transformed cells is brought about by the constant setting free of transcription factor E2F due to the action of viral trans-activators like polyoma virus large T antigen (Ogris et al., 1993; Mudrak et al., 1994; Hengstschläger et al., 1994c).

Furthermore, we demonstrate that the absolute quantitative amount of TK activity correlates with the amount of TK mRNA and protein expression; S phase-specific TK activity in COP-8 cells is twice as high compared with 3T6 fibroblasts (Fig. 1C).

TK mRNA expression is constant throughout the cell cycle of COP-8 cells and remarkably higher than in 3T6 cells (Hengstschläger et al., 1994b and Fig. 3). In addition, we analyzed the content of TK protein in various positions of the cell cycle and compared it with the amount of enzyme activity. For both, normal 3T6 and virally transformed COP-8 cells, the levels of TK protein exactly parallel the levels of TK activity (Fig. 1, B and C). Furthermore, Western blot analysis indicates a higher TK protein abundance during the cell cycle of transformed cells than is found in normal cells (data not shown). From this clear correlation we exclude that post-translational modification of TK protein provides cell cycle-dependent regulation of TK activity, which is in agreement with prior studies in the cell cycle (Sherley and Kelly, 1988; Hengstschläger et al., 1994b) during differentiation (Gross and Merrill, 1988; Knöfler et al., 1993) and after growth stimulation (Ito and Conrad, 1990).

Taken together, these data provide evidence that a post-transcriptional mechanism must account for the maintenance of low TK enzyme activity in the presence of high mRNA levels during the G2 phase of DNA tumor virus-transformed cells. For that reason we wanted to ascertain whether this post-transcriptional mechanism is also active during the cell cycle of normal, nontransformed cells and determine its regulatory capacity upon overexpression of TK mRNA.

Post-transcriptional Regulation of TK Activity in the Cell Cycle of Normal Cells—One of our first goals was to study contributions from post-transcriptional mechanisms to the control of TK expression in the absence of interference from tran-
The answer could not be a cross-species difference in regulation because the human cDNA in the mouse genetic background was more efficiently controlled than the murine construct. Also, changes of protein stability did not seem a likely explanation, because data from this and other groups had demonstrated that the half-life of mouse and human TK does not significantly vary between different normal and transformed cell lines (Sherley and Kelly, 1988; Knöfler et al., 1993; Hengstschläger et al., 1994b). Post-translational modification by phosphorylation (Chang et al., 1994) could also be ruled out as a major factor, because we never detected any discrepancy between levels of protein and enzyme activity (Knöfler et al., 1993; Hengstschläger et al., 1994b), Mikulits and Müllner (1994), and this report).

Therefore we reasoned that the explanation might lie in the fortuitous differences of RNA levels in our pools of transfectants. In other words, we thought to have "titrated" the regulatory mechanism by sufficient levels of TK mRNA without any change in the transformation status of the cells. In order to address this possibility, we devised a strategy that allowed expression of varying levels of hTK mRNA throughout the entire cell cycle within a single individual cell clone by using an inducible promoter system. We preferred this approach over the selection of different clones with high versus intermediate versus low levels of expression, because we had observed in previous experiments that there is only a poor correlation between transfected TK gene dosage and mRNA production in randomly picked clones (see also "Discussion").

As a first step, an expression vector carrying hTK cDNA under control of the hormone-inducible MMTV-LTR promoter was transfected into mouse NIH 3T3tk- cells. The selection for stable cell lines in HAT medium was done in the presence of the glucocorticoid antagonist 17α-methyltestosterone and with serum cleared of endogenous glucocorticoids by charcoal stripping. This treatment lowers the basal activity of the MMTV promoter severalfold (Mikulits et al., 1995). The persistent combination of HAT selection and low level promoter activity was intended to yield clones, which under induction conditions by addition of dexamethasone would produce especially high levels of TK mRNA. Out of 6 individually analyzed clones, the one with highest TK activity was chosen for all further experiments.

Absolute Quantitation of Cellular TK mRNA Abundance—To facilitate comparison of TK mRNA expression levels in the various cell types, we devised a method to determine the absolute number of TK transcripts on a per cell basis (Fig. 3). First, cytoplasmic RNA was isolated from an exactly known number of cells and separated in denaturing formaldehyde gels. Within the same gel, a precisely defined amount of in vitro transcribed hTK or mTK RNA at an appropriate dilution was included to serve as a comparison. The copy number of the latter material was calculated by converting intensity of the original fluorometric measurements versus molecular weight of the transcripts into the corresponding number of molecules. Finally, the number of TK transcripts/cell was estimated by comparison of signal intensity in the autoradiographs from the cellular samples with the in vitro generated samples. The results of these experiments are presented in Fig. 3.

As expected, the constitutive levels of hTK and mTK mRNA in both transfection approaches described above with the pcD expression vectors differed about 10-fold; mTK mRNA expression was enhanced in comparison with the steady state level of hTK mRNA in continuously cycling Ltk- cells. In line with our hypothesis, the data suggested that elevated expression of mTK transcripts (pcD-mTK; 250 copies) during G1 phase of the cell cycle should result in a smaller magnitude of TK activity induction at the G2/S boundary, as was indeed the case (compare to Fig. 1C).

To put these data into perspective, we next compared the amounts of TK mRNA in the transfectants to those of S phase synchronized 3T6 and COP-8 cells. We detected that the "malignant": COP-8 cells (transformed by polyoma virus) expressed 1200 copies of TK mRNA/cell, more than twice as much as the normal 3T6 cells. Although 3T6 fibroblasts exhibited a considerable level of endogenous TK transcripts (600 copies), this was restricted to the S phase only, whereas the cells had 5-fold less TK mRNA (i.e., about 130 copies, data not shown) during G1 and G2.

In contrast, COP-8 cells maintained the high transcript levels throughout the cell cycle. Therefore, integrated over the duration of an entire cycle, expression of TK mRNA in 3T6 is only about 20% of that found in COP-8 cells. These observations strengthened the hypothesis that high level expression of TK transcripts might indeed interfere with cell cycle-dependent regulation of TK enzyme activity (compare to Fig. 1C), most notably during the S-G2 transition but also during the progression from G1 into S phase.

In the NIH 3T3tk- donor transfected with the inducible MMTV-LTR construct, TK mRNA levels were elevated by the dexamethasone treatment as anticipated. Although the basal level of transcription resulted in about 90 TK mRNA molecules/cell, within 1 h of induction this number was rising to 430 and reached 3100 copies/cell after 4 h of hormone treatment, corresponding to a more than 30-fold induction in mRNA production. The maximal transcript concentration in this transfectant by far exceeded the TK mRNA amounts found in any cell line tested for expression of the endogenous gene (of which COP-8 is the highest), whereas the uninduced levels were well below those found in cells with the normal regulatory phenotype. Therefore, we indeed had a tool to test the consequences of expressing different levels of TK mRNA in various cell cycle phases within the same cell.

Conditional TK Expression in Recultivated Normal Cells and

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2 W. Mikulits, unpublished data.
Overexpression of TK mRNA—With the NIH 3T3 tk<sup>-</sup> done expressing the hormone-inducible TK construct described above, we were able to directly verify our hypothesis that the posttranscriptional regulation mechanism depends on the steady state level of TK mRNA. Technically it proved necessary to use a combination of centrifugal elutriation, recultivation of synchronized cell populations, and optimized dexamethasone induction.

First, logarithmically growing NIH 3T3 tk<sup>-</sup> MMTV-hTK cells were separated according to their cell cycle phase by centrifugal elutriation. DNA profiles of individual fractions were determined by flow cytometry, and the percentages of cells in G<sub>1</sub>, S, and G<sub>2</sub>-M were calculated (Fig. 4). In the particular experiment shown, the highest enrichment for cells in G<sub>1</sub>, S, and G<sub>2</sub>-M was 93, 63, and 62%, respectively. Second, synchronized cell populations were recultivated for several hours in conditioned medium to provide optimal conditions for TK gene induction. This procedure definitively is essential for avoiding artifacts in the quantitation of unstable gene products (like TK, cyclins, and transferrin receptor) resulting from the stress during fractionation. Most cell types resume cell cycle progression within 30 min after replating. Finally, the response to dexamethasone over time was applied as a tool to express different amounts of hTK mRNA in the cell cycle. As shown previously, hormone treatment of 3T3 mouse fibroblasts does not influence level and cell cycle regulation of endogenous TK (Hengstschläger et al., 1994b). Moreover, dexamethasone-inducible gene expression offers the benefit of rapid induction kinetics, reaching the highest target mRNA level within 3–4 h (Mikulits et al., 1995). The short period until total induction of hTK transcription enabled us to study the consequences of TK enzyme activity in cells that remained highly synchronous during hormone treatment (Fig. 4). In addition, the use of the glucocorticoid antagonist 17α-methyltestosterone allowed us to lower the basal activity of the MMTV-LTR promoter during recultivation until the start of the agonist treatment for 1 or 4 h, respectively. The anticancer hormone by itself does not affect cell growth rates (Mikulits et al., 1995). In summary, these methods let us to study conditional TK gene expression with only minimal perturbation of normal cellular processes.

As expected, hTK mRNA expression was virtually independent of cell cycle position and only depended on the different conditions of antagonist/agonist incubation (Fig. 5). In absolute terms, transcript concentrations closely corresponded to the copy numbers observed in the logarithmically growing transfectants, i.e. on average 100 molecules/cell in the absence of hormone (which is due to MMTV-LTR promoter leakiness), about 500 copies after 1 h of induction, and 3000 copies after 4 h of dexamethasone treatment (compare to Fig. 3).

In NIH 3T3 tk<sup>-</sup> MMTV-hTK cells expressing the low TK mRNA level characteristic for basal promoter expression, TK activity was up-regulated at least 4-fold at the G<sub>1</sub>-S transition (Fig. 5A). This value is a lower level estimate, because the fraction with the highest activity only consisted of 43% of cells in S (compare to Fig. 4). When the recultivated cells were treated for 1 h with dexamethasone, a 5-fold higher constant hTK transcript level was reached and the corresponding TK activity was still induced at the G<sub>1</sub>-S transition, albeit at the reduced factor of 2.5 (Fig. 5B). Enzyme activity and mRNA expression at this stage corresponded roughly to the level in 3T6 fibroblasts during S phase. Again, as mentioned above, the reduced up-regulation of TK may be either due to the less than perfect synchronization or, more interestingly, to the maintenance of high TK mRNA concentrations in G<sub>1</sub>. This was corroborated by the data from cells that had been incubated for 4 h with the inducing hormone after recultivation. In this case, the highest levels of hTK mRNA could be sustained in the different cell cycle positions, reflecting an amount of transcripts 3-fold higher than that in continuously growing transformed COP-8 cells and an even 30-fold rise as compared with noninduced NIH 3T3 tk<sup>-</sup> transfectants. As we had speculated, this overexpression of hTK transcripts totally eliminated cell cycle regulation of TK activity (Fig. 5C). As for the mRNA, the amount of enzyme activity exceeded that of transformed COP-8 cells (1408 pmol/mg/h versus 1079; see also Hengstschläger et al. (1994b)). Interestingly, it seems that TK activity can only be expressed up to a certain threshold level, which is at best 2-fold higher than in COP-8 cells, even upon massive overexpression of TK mRNA, corroborating a large series of earlier transfection experiments. This may be due to massively mutagenic effects of imbalances in DNA precursor pools.

As mentioned above, elevated expression of TK mRNA from the endogenous gene was already previously described in DNA tumor virus-transformed cells (Hengstschläger et al., 1994a, 1994b, 1994c). Our result that the absolute abundance of TK mRNA in the polyoma virus-transformed COP-8 cells is more than twice as high as compared with a normal cell type like 3T6 cells perfectly matches to data from these previous studies (Hengstschläger et al., 1994c). It had been suggested that the

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3 H. Dolznig, H. Beug, and E. W. Müllner, manuscript in preparation.
Overexpression of TK mRNA Eliminates TK Activity Regulation

Several previous reports had already described discrepancies between the levels of TK mRNA and protein, which were either attributed to transpositional repression (Gross and Merrill, 1988, 1989; Knöfler et al., 1993; Mikulits and Müllner, 1994) or changes of protein stability (Sherley and Kelly, 1988; Kauffman and Kelly, 1991; Kauffman et al., 1991; Carozza and Conrad, 1994). In this contribution, we wanted to address the question of whether the factor(s) responsible for this regulatory phenotype are present at limiting concentrations (indicating specificity for TK) or abundant cellular functions (pointing to a more general phenomenon).

TK-deficient cell lines were stably transfected with mouse and human TK expression vectors under the control of either constitutive or hormone-inducible heterologous promoters. The one hand we used the pCD plasmid containing the constitutive early SV40 promoter element (Okayama and Berg, 1983), alternatively we employed an expression construct with hTK cDNA under the control of the dexamethasone-responsive MMTV-LTR (Kühnel et al., 1986; Buetti and Kühnel, 1986; Buetti, 1994). Immortalized but nontransformed mouse fibroblasts devoid of any endogenous TK activity were chosen as recipients. This strategy avoided interference from transcriptional regulation of the resident TK gene promoter, which is known to be growth and cell cycle-dependent (Stuart et al., 1985; Coppock and Pardee, 1987; Pardee, 1989). After synchronization by centrifugal elutriation, the transfectants were analyzed for cell cycle regulation of TK mRNA and enzyme activity. Whereas no fluctuation of mRNA during the cell cycle was detectable by this procedure, regulation of TK expression critically depended on the level of mRNA. At low concentrations the enzyme was maximally expressed in S phase, whereas overexpression of TK transcripts abolished this cell cycle-dependent phenotype and led to constitutive activity. These data clearly indicate the presence of a factor(s) required for repressing TK production during G1, which can be titrated by high mRNA concentrations, suggesting a mechanism highly specific for TK and obviously conserved between human and mouse (see Fig. 5).

Our results strongly indicate that the phenotype of posttranscriptional regulation of TK is strictly correlated to TK mRNA content; deviation from the normally cell cycle-dependent pattern is not a consequence of cellular transformation. Several earlier reports had shown conclusively that viral transactivators like the large T antigens (SV40 and polyoma virus), E1A (adenovirus), or E7 (human papilloma virus) lift the restriction of transcription from the endogenous TK promoter on S phase (Ogris et al., 1993; Mudrak et al., 1994; Hengstschläger et al., 1994b), resulting in constitutive TK mRNA production. The common effect of these viral proteins is a disruption of the complex between the tumor suppressor protein pRb and the transcription factor(s) E2F. This may be a strategy to improve conditions for viral DNA replication. In the case of TK, the increased mRNA levels induce a phenotype that differs from that of normal cells but is still not completely deregulated (compare with Fig. 1, polyoma virus-transformed COP-8 cells). Although TK enzyme activity stays elevated throughout S and G2, it drops to a much lower level during the passage through mitosis and entry into the following G1 phase. A similar pattern had been observed in a wide variety of virally transformed cells, including papilloma virus-transformed HeLa cells, SV40-transformed mouse SVMK cells and others (Sherley and Kelly, 1988), Hengstschläger et al. (1994a, 1994b), and this report).

This is most likely due to the action of a mitotic protease that degrades TK protein if it is still expressed at the G2-M transition (Sherley and Kelly, 1988; Kauffman and Kelly, 1991). Because all the virally transactivators mentioned above have

Fig. 5. Conditional expression and overexpression of hTK mRNA and enzyme activity in the cell cycle of NIH 3T3tk- cells. Recultivated cells from different cell cycle positions were processed for RNA analysis and TK assay. The evaluation of TK mRNA and enzyme activity was done as described in the legend to Fig. 2. A, recultivated NIH 3T3tk-MMTV hTK cells without hormone treatment. B, recultivated transfectants treated for 1 h with 1 μM dexamethasone. C, induction for 4 h with 1 μM dexamethasone. The peaks of TK activity correspond to 177 ± 13 (no hormone treatment), 445 ± 23 (induction for 1 h with hormone), and 1408 ± 96 pmol/mg/h (4 h dexamethasone), respectively. No TK activity was detectable in untransfected wild type NIH 3T3tk- cells. Absolute levels of TK transcript corresponded to 100, 500, and 3000 mRNA copies/cell, respectively (compare with legend of Fig. 3).

DISCUSSION

In this study, we examined molecular mechanisms contributing to post-transcriptional regulation of cytosolic TK enzyme activity during the cell cycle of apparently normal, nontrans-
pleiotropic effects on a multitude of cellular processes, we had to exclude that the changes in regulation of TK expression, including the repression of TK—translation during G_s were the result of transformation rather than the unmasking of an inherent regulatory capacity that had been there a priori. Therefore, TK-deficient, nontransformed mouse fibroblasts were transfected with constitutive expression vectors for either human or mouse TK, and their cell cycle regulation was studied (Fig. 2). Thereby we could prove (i) that repression of TK activity during G_s is a phenotype that indeed can be found in normal cells (see also Mikulits and Müller, 1994) and (ii) that higher levels of TK mRNA alone (in the TK transfectants) can induce a pattern of regulation that resembles the one of transformed cell types (compare Figs. 1 and 2B). This also clearly indicated that the protease degrading TK during mitosis is not a transformation-specific function. Nevertheless, TK may not be a major substrate for this protease because in normal cells a marked decline of TK protein and activity occurs already in late S and G_2 due to a shut-off of the endogenous promoter (Ito and Conrad, 1990) in S phase and a rather short half-life of the protein (Hengstschläger et al., 1994b).

At this stage of work we still had to be concerned that some of our results might be explained by variations in the genetic background of the stable transfectants. Particular integration sites within the genome are well known to influence the final concentration of a given gene product. This is definitely true for expression of TK; the correlation between gene dosage, mRNA production, and enzyme activity in individually selected clones is quite poor (Mikulits et al., 1995). This notion was also confirmed by the differences in TK mRNA as well as activity between the pools of clones transfected with either pcD-hTK or pcD-mTK (see Fig. 2 and 3). To alleviate this potential problem, we decided to reanalyze the regulation pattern in a single cell done, where highly variable TK expression levels would be attained by the use of a conditional promoter construct (Kühnel et al., 1986; Buetti and Kühnel, 1986; Buetti, 1994). The measurements at various time points during the cell cycle at low, medium, and high concentrations of hTK mRNA nicely confirmed our previous assumptions (see Fig. 4 and 5). Although a pronounced S phase-specific regulation of TK enzyme activity was observed at low mRNA concentrations, this phenotype was less obvious at medium mRNA abundance and obliterated in the presence of high transcript levels (Fig. 6).

What do these results imply for the specificity of a regulatory factor(s) on TK mRNA? Did the overexpression compromise general cellular functions or indeed titrate a defined control mechanism? High level overexpression via strong promoters (e.g., from cytomegalovirus) of important regulators (like transcription factors) has been described to produce unwanted and even deleterious phenomena in the recipient cells (Gill and Ptashne, 1988; White et al., 1988). Therefore, we have to put the level of TK mRNA produced from MMTV LTR after hormonal induction into perspective. An average fibroblast cell contains about 1.5 pg of cytoplasmic mRNA, roughly equivalent to $1.5 \times 10^6$ molecules. Of these, about 150 are copies of TK mRNA (over the whole cell cycle in a logarithmically growing normal cell), putting endogenous TK mRNA in the 1:10 000 abundance class of rare mRNAs. After hormonal induction from the transfected MMTV-LTR construct, about 3000 copies are produced, 20 times more than in the normal cells. Now TK transcripts were in the moderate high abundance class of 1:500, still well below highly expressed mRNAs like the one for $\beta$-actin, which has a prominence of about 1:100. Therefore, it is unlikely (although this is no definitive proof) that 3000 molecules of TK mRNA overwhelmed a factor that might be required for general translation initiation. On the other hand, this can happen with cytomegalovirus promoter-driven constructs, which may easily surpass the 1:100 ratio observed for the highest abundant endogenous mRNAs. Consequently, we propose the existence of a mechanism dealing specifically with post-transcriptional regulation of TK mRNA. The titration of factor(s) inhibiting TK expression indicates that the factor(s) act(s) in the translational process. The requirement for a feedback loop like that in the autoregulation of dihydrofolate reductase or thymidylate synthase on their respective mRNAs can be excluded (Chu et al., 1991, 1993a, 1993b).

As far as the molecular mechanism is concerned, our data are most easily explained by a repressor of translation that acts in G_s (Gross and Merrill, 1988, 1989; Mikulits and Müller, 1994). An S phase-specific activator of translation is incompatible with the loss of regulation at high mRNA concentrations. Titration of such an activator would still have attained S phase-specificity of TK production at a maximal level, independent of further rise in mRNA, whereas we observed a good correlation between the increase in mRNA and TK activity. We cannot formally rule out the possibility that there may be an increase in TK protein stability at the G_s transition, as was recently reported for Rat 1 cells (Carozza and Conrad, 1994), although in previous reports from this and other laboratories no S phase-specific change in TK protein stability could be detected (Sherley and Kelly, 1988; Hengstschläger et al., 1994b; Mikulits and Müller, 1994). This apparent discrepancy may in part be explained by differences in the experimental protocols, which employed either stimulation of serum-starved cells (Ito and Conrad, 1990; Carozza and Conrad, 1994) or synchronization of continuously cycling cells by centrifugal elutriation (Sherley and Kelly, 1988; Kauffman and Kelly, 1991; Hengstschläger et al., 1994b; Mikulits and Müller, 1994). One can speculate that differences between the first cycle after restimulation and the consecutive ones may account for this disagreement. In addition, recent studies have demonstrated that human TK polypeptide gets phosphorylated (Chang and Huang, 1993; Chang et al., 1994). This post-translational modification, however, is no absolute requirement for enzymatic activity (Chang and Huang, 1993) and the cell cycle-dependent variation in phosphorylation status remains to be determined. From the work of Kelly and co-workers, it appears that the structural determinant required for regulation resides within the region corresponding to the 40 C-terminal amino acid residues of human TK protein or mRNA (Kauffman and

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4 P. Stiegler, unpublished data.
5 M. Knöfler, submitted for publication.
During mitosis in order to return to lower levels in the next G1 amount of TK protein is rapidly decreased by degradation transcription of TK mRNA. Still, in this situation the elevated activity becomes virtually constitutive at a high level through-out the entire cell cycle.

Integrating our data from this report into the body of evidence from previous studies, we propose the following working hypothesis to account for S phase-specific post-transcriptional up-regulation of TK activity (see also Fig. 6). First, TK protein can be translationally induced at the G1-S transition and repressed during S-G2 in normal growing cells as suggested by the strict dependence of the mechanism on the level of TK mRNA (Mikulits and Mullner, 1994). Second, in transformed cells, translational control is less pronounced in G1-S and eliminated in S-G2, possibly due to the increased and constitutive transcription of TK mRNA. Still, in this situation the elevated amount of TK protein is rapidly decreased by degradation during mitosis in order to return to lower levels in the next G1 phase of the cell cycle (Sherley and Kelly, 1988; Kauffman and Kelly, 1991). Only by artificial overexpression of TK mRNA to higher concentrations, both regulatory pathways are no longer capable of dealing with the gene product, and as a result, TK activity becomes virtually constitutive at a high level throughout the entire cell cycle.

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