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Post-transcriptional repression of thymidine kinase expression during cell cycle and growth stimulation

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Abstract

In vertebrates, endogenous thymidine kinase (TK) gene expression is strictly growth-dependent. Here we report that in continuously cycling Ltk$^-$ mouse fibroblasts, stably transfected with a vector expressing human TK cDNA from a constitutive promoter, enzyme activity rises 8-fold at the G_1/S phase transition and declines again in G_2. The mechanism did not involve changes in protein stability. When hTK was put under the control of a hormone-inducible promoter, production of high mRNA levels following addition of dexamethasone did not result in any enzyme activity in resting NIH-3T3tk$^-$ cells. After growth stimulation with serum, TK activity rose together with the onset of DNA synthesis only in the simultaneous presence of the hormone.

Key words: Post-transcriptional control; Thymidine kinase, Inducible gene expression; Cell cycle; Elutriation centrifugation

1. Introduction

The stringent linkage of thymidine kinase (TK; EC 2.7.1.21) gene expression with continuous growth has raised considerable interest in the underlying mechanisms. Transcriptional as well as post-transcriptional events have been held responsible for the maximum enzyme activity coinciding with DNA replication during the S-phase of the cell cycle (see [1] for review). Promoter elements of human and mouse TK genes, as well as *trans* acting DNA binding proteins, required for growth-dependent transcription have been described in considerable detail ([2–5] and references in [1]). Post-transcriptional events have been found to repress TK translation despite the presence of high mRNA levels, for example during the differentiation of myoblasts [6,7] or teratocarcinoma cells [7], as well as during growth arrest [7] or growth stimulation [8,9]. In addition, TK protein stability was suggested to decrease during mitosis in HeLa cells [10,11]. To complicate matters further, TK is overexpressed at the transcriptional as well as at the post-transcriptional level in cells transformed by viral transactivating proteins [12,13] as compared to their normal counterparts.

In the present study we devised model systems allowing the quantitation of the contribution of post-transcriptional events for TK gene expression without interference from transcriptional regulation in apparently non-transformed fibroblasts. To that purpose, we monitored human TK (hTK) production from a constitutive expression vector throughout the unperturbed cell cycle. In addition, we made use of hormone-inducible constructs that allow the induction of TK mRNA at any given time, irrespective of cellular growth rates. The results obtained indicate that, even in the absence of transcriptional control, post-transcriptional events are completely sufficient to ensure proper regulation of TK during growth stimulation as well as in continuously cycling cells.

2. Materials and methods

2.1. Cloning

The SV40 late promoter (*Eco*RI–*Pvu*II fragment) from the eukaryotic expression vector pSVL (Pharmacia) was replaced by the hormone-inducible mouse mammary tumour virus long terminal repeat (MMTV-LTR) (*Bam*HI–*Bam*HI fragment) [14,15]. Subsequently, the full-length cDNA for human TK was cut from the original constitutive pcD hTK expression vector [16] as a 1.6 kb *Bam*HI–*Bam*HI fragment and inserted into the *Sma*I site of the inducible construct.

2.2. Cell culture, transfection and growth stimulation

Ltk$^-$ (ATCC CCL 1.3) and NIH 3T3tk$^-$ mouse fibroblasts (obtained from Meinrad Busslinger, IMP, Vienna) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). For stable transfection, 300,000 logarithmically growing cells were treated overnight with 4 μg expression vector DNA plus 6 μg salmon sperm carrier DNA using the calcium phosphate co-precipitation technique [17]. 2 days later hypoxanthin-aminopterin-thymidine [18] was added to the medium to select for cells with the TK$^+$ phenotype. All experiments were performed with a pool of 50–100 expanded transformants. Growth arrest was achieved by reducing the serum concentration to 0.2% for at least 3 days. Re-stimulation was achieved with medium containing 10% charcoal-stripped serum, and entry into S phase was monitored cytofluorometrically. Where appropriate, dexamethasone was added to a final concentration of 1 μM to induce the transfected MMTV promoter. Human primary lymphocytes were isolated from heparinized peripheral blood by the Ficoll-Hypaque gradient method. After stimulation with phytohemaglutinin, cells were harvested after reaching logarithmic growth.

2.3. Elutriation

For cell cycle analysis, cells were fractionated in a JE-6B rotor (Beckman) at a constant speed of 2,000 rpm by the stepwise increasing of the pump rate from the initial flow rate of 14 ml per min as described [13].

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After analysing DNA distribution by flow cytometry to asses the purity of each fraction, samples were further processed for RNA analysis or TK assay.

2.4. RNA isolation and analysis

Cytoplasmic RNA was prepared by the method of Favaloro et al. [19] except that macaloid was omitted from the lysis buffer. After separation in 1% formaldehyde agarose gels, RNA was transferred to nylon membranes. After UV-fixation, filters were hybridized with ^{32}P-labelled probes specific for human or mouse TK [16, 20], mouse histone H4 [21] and, as an unregulated control, mouse β2-microglobulin [22].

2.5. TK assay

Enzyme activity from cell extracts was determined in vitro by quantitating the conversion of [^{3}H]thymidine to thymidine monophosphate as described [23].

3. Results and discussion

3.1. Post-transcriptional induction of TK activity during the cell cycle

In normal cells, TK expression during the cell cycle is highly S-phase specific. For example, in primary, phytohemaglutinin-stimulated human lymphocytes synchronised by centrifugal elutriation, we observed an 8-fold increase of TK mRNA as well as enzyme activity at the transition from G_1 to S (Fig. 1A). In late G_2 fractions, mRNA and activity levels returned to the low levels observed in early G_1. Recent work has suggested that transcription factor E2F is of critical importance for cell cycle and growth-dependent expression of mammalian TK [4,5]. However, S-phase specificity of transcription can be disrupted upon cell transformation by viral or other transactivators [4,12,13]. Therefore, in order to investigate post-transcriptional components of TK regulation independent of transformation state and transcriptional interference, we initially chose to analyse TK activity during the cell cycle of Ltk$^-$ cells transfected with the constitutive pcD hTK expression vector [16].

As expected, TK mRNA expression was constant throughout the cell cycle, whereas enzyme activity still rose about 8-fold at the G_1/S transition (Fig. 1B) and declined 5-fold during G_2. Similar results were obtained for Ltk$^-$ cells transfected with a mouse TK expression construct (data not shown). This suggests that post-transcriptional control is not only active during the G_1 phase of the cell cycle but in G_2 as well, despite the continuous presence of high mRNA levels. Such an effect could not be observed in papilloma virus-transformed human HeLa cervical carcinoma cells [10], which further validates our transfection approach using apparently non-transformed cells. S-phase-specific TK induction was not due to altered protein stability (Fig. 1C): when

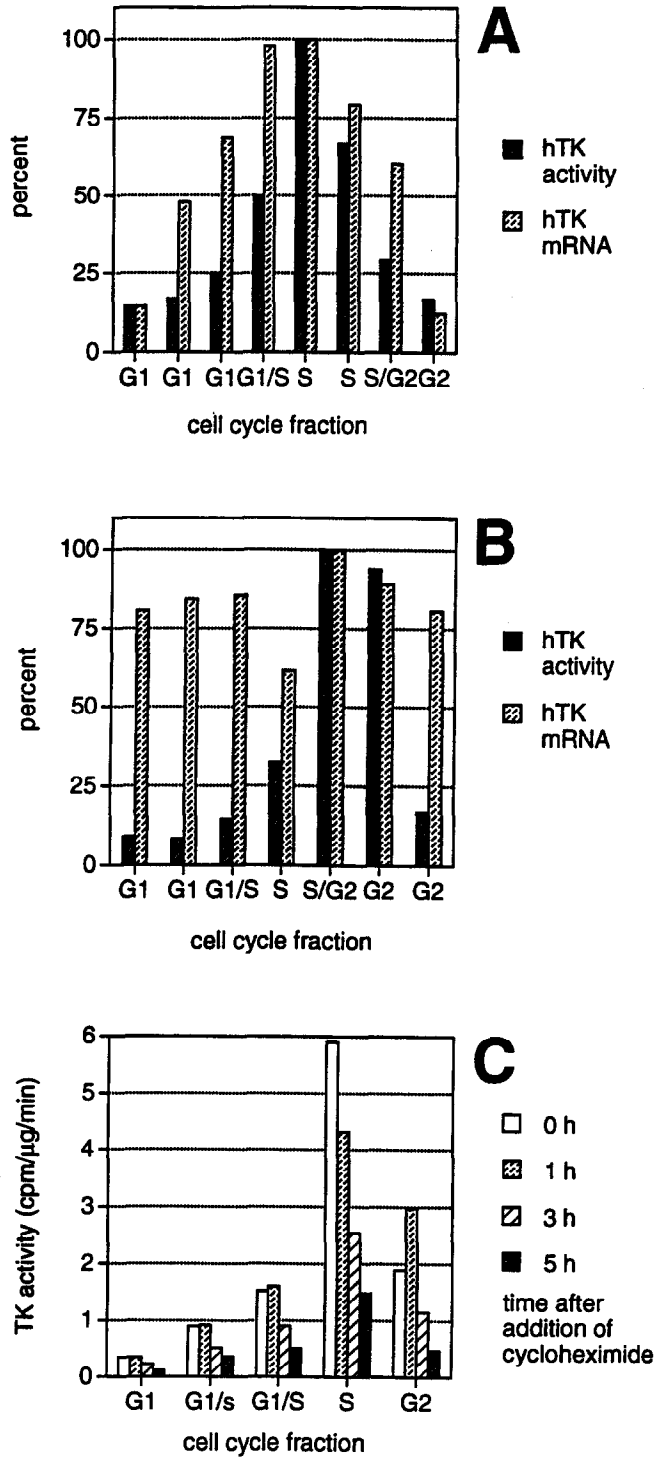

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Fig. 1. hTK stability and activity vs. mRNA content during the cell cycle. Phytohemagglutinin-stimulated human primary lymphocytes or Ltk$^-$ cells stably transfected with expression vector pcD hTK, were separated into fractions of different cell cycle position by centrifugal elutriation. In the particular experiments shown, the best G_1, S, and G_2 fractions were enriched to at least 97, IRL press, Oxford, Washington, DC, 65, and 55% purity, respectively, as determined by flow cytometry. hTK mRNA levels were normalised to endogenous β2-microglobulin mRNA [22] by densitometry and, as for TK enzyme activity, expressed in relative units with the highest value set to 100% to facilitate comparison. (A) Endogenous hTK mRNA and enzyme activity in primary lymphocytes; (B) hTK mRNA and enzyme activity in Ltk$^-$ cells transfected with the constitutive pcD hTK expression vector; (C) Ltk$^-$ cells carrying pcD hTK plasmids from the indicated cell cycle phases were re-seeded after elutriation in the presence of cycloheximide for 0, 1, 3 and 5 h at which time points cell extracts were harvested for TK assay.

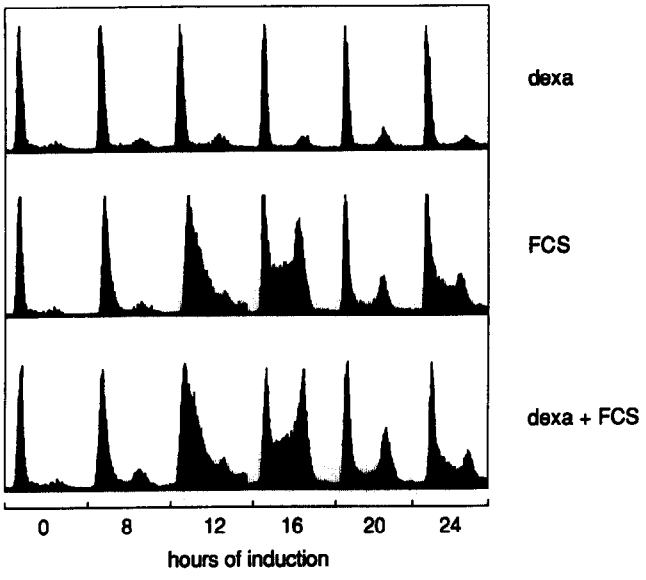

Fig. 2. Growth stimulation of serum-arrested NIH 3T3tk⁻ cells. DNA content was determined by flow cytometry after staining with 4,6-diamidino-2-phenylindol-dihydrochloride (DAPI) at the indicated times of treatment with dexamethasone and/or FCS.

cells in different phases of the cell cycle were re-cultivated after elutriation in the presence of the protein synthesis inhibitor cycloheximide, enzyme activity decreased with the same kinetics in cells in G_1, S and G_2 phases, indicating a half-life of about 3.5 h throughout the whole cycle (3.8 in G_1, 3.8 in S, 3.1 in G_2). These data are in accordance with previous results from our laboratory [7], where we have shown in pulse-chase experiments followed by immunoprecipitation that stability of endogenous mouse TK is not altered in a variety of differentiating or arresting cells. In the same report we could also demonstrate by Western blotting that there is no discrepancy between enzyme activity and amount of protein, ruling out a major contribution of post-translational protein modifications to the regulatory phenotype, although TK has been reported to be phosphorylated in human HL60 cells [24]. Recently, we have extended our work [7] from differentiation and growth arrest to cell cycle regulation of TK expression in a large group of primary as well as transformed human and mouse cells [13]. Again, no evidence was found for differences in TK protein stability between G_1, S and G_2 phases of the cell cycle by either pulse-labelling, pulse-chase or cycloheximide experiments, and no disparity between levels of protein and activity was detectable. All these observations suggest that the underlying mechanism involves control of TK mRNA at the level of translation. Similar conclusions were reached from the regulation of chicken TK transfected into mouse cells [6]

3.2. *Inducible hTK mRNA expression in transfected NIH 3T3tk⁻ cells*

To characterise the contribution of post-transcriptional TK repression in resting cells, in an independent set of experiments we stably introduced hTK cDNA under the control of the glucocorticoid-responsive LTR promoter from MMTV into NIH 3T3tk⁻ mouse fibroblasts. Again, following the same rationale as in the preceding paragraph, this approach should separate the transcriptional from the post-transcriptional regulation capacity, as the endogenous TK gene is not significantly transcribed in arrested cells.

In the following we used serum stripped of naturally occurring glucocorticoids by pre-treatment with char-

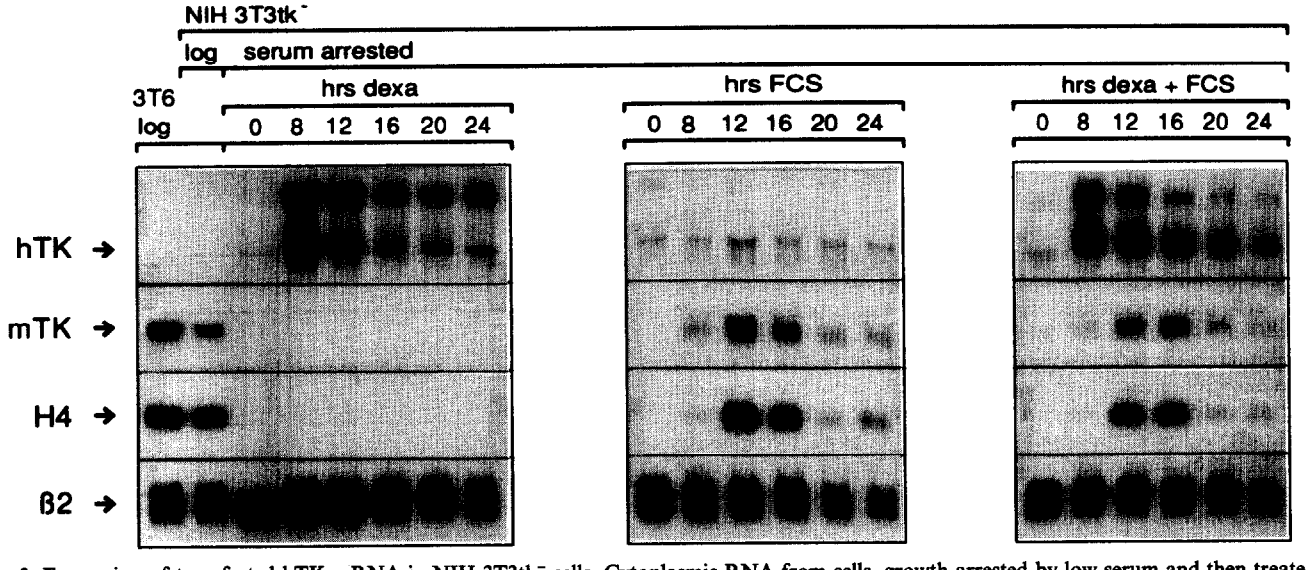

Fig. 3. Expression of transfected hTK mRNA in NIH 3T3tk⁻ cells. Cytoplasmic RNA from cells, growth arrested by low serum and then treated with either dexamethasone alone or in combination with serum for the times indicated, was hybridized sequentially with radiolabelled probes for hTK, the endogenous non-functional mTK [20], histone H4 [21] and the constitutive β2-microglobulin mRNA [22]. The upper band in the hTK panels results from unspliced transcripts.

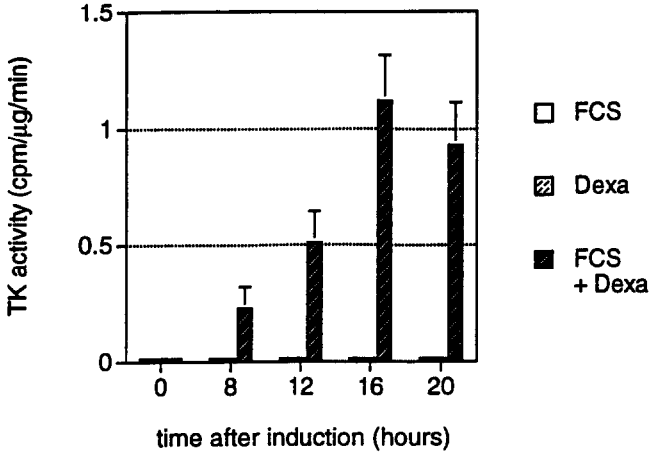

Fig. 4. TK enzyme activity in NIH 3T3tk⁻ cells transfected with the inducible hTK expression vector. Activity was determined from 20 µg of cell extract in a total volume of 50 µl assay mixture.

coal to minimise unquantifiable synergistic effects (data not shown). As an initial control we demonstrated that low serum-arrested NIH 3T3tk⁻ cells are not growth stimulated by dexamethasone alone (Fig. 2) but progress into S-phase when treated with stripped serum either alone or in combination with the synthetic hormone. In the pool of cells transfected with the inducible hTK construct, dexamethasone by itself led to a massive 8-fold induction of transcription as compared to the weak basal level (Fig. 3) in the absence of any concomitant enzyme activity (Fig. 4). When addition of hormone was combined with serum, however, the induced TK activity peaked at the same time when the majority of cells were in S-phase (compare to Fig. 2). In a further control it was evident that stripped serum alone would not induce enzyme activity (Fig. 4). Fortuitously, NIH 3T3tk⁻ cells express an endogenous non-functional mouse-specific TK mRNA. Therefore a mTK probe and one for the also strictly S-phase-specific histone H4 mRNA were used to demonstrate, on an additional level, that the arrested cells express growth-dependent genes only when stimulated by serum alone or in combination with dexamethasone, whereas the hormone by itself sufficed to induce mRNA production from the transfected hTK vector (Fig. 4).

Taken together, our results indicate that in the absence of any endogenous transcriptional control, post-transcriptional mechanisms can efficiently repress TK activity in arrested cells as well as in all periods of the cell cycle except the S-phase. Work from other groups and in this laboratory [6–8,13] suggest that the underlying mechanisms involves translational control, possibly at the level of elongation. There is, however, also clear evidence that TK protein is rapidly degraded during mitosis if it is still present at this late stage of the cell cycle [10,11], which seems to be the case particularly in transformed cells [13]. This leaves us with the question of the physiological significance of the phenomena observed. Although the post-transcriptional regulation we observed for human TK in mouse cells obviously works across species barriers, it should be noted, however, that virally encoded TKs (for example of herpes simplex virus type) are constitutively expressed in transfected or transformed target cells. Therefore, when transforming viruses not coding for their own TK induce transcription of the host TK gene, the ability of infected cells to repress inappropriate translation of endogenous TK mRNA may be part of an evasive strategy to limit the invading virus supply of precursors required for replication of its genetic information. This interpretation is strengthened by the observation that several other enzymes involved in nucleotide precursor metabolism, like thymidylate synthase, deoxycytidine kinase or dihydrofolate reductase [25–27], may be subject to similar types of post-transcriptional control.

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