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Cell cycle regulated synthesis of stable mouse thymidine kinase mRNA is mediated by a sequence within the cDNA

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Abstract

The cDNA for mouse thymidinekinase (TK) was isolated from a cDNA library in lambda-gt11 and sequenced. It was used as a probe to follow the time course of TKmRNA expression in growth stimulated mouse fibroblasts. Linked to the HSV-TK promoter the cDNA was able to transform LTK$^-$-cells to the TK$^+$ phenotype. The transformed cells expressed the TKmRNA and enzyme activity in a growth dependent fashion suggesting that the regulatory element is localized on the cDNA.

Introduction

Thymidine kinase (EC 2.7.2.21) is an enzyme of the salvage pathway of nucleotide biosynthesis. It catalyses the phosphorylation of thymidine to thymidine-monophosphate. Enzymes with this specificity are present in nearly all organisms (yeast and other fungi being a notable exception); they are, moreover, coded for by many viral genomes (1). Eukaryotes contain separate enzymes in the cytosol and in the mitochondria (2,3). The main enzyme in the cytosol is strongly regulated during the cell cycle. The enzyme level is hardly measurable in resting G_0 or G_1 cells but increases dramatically in cells moving into the S phase. In cells inhibited in growth by reduction of the serum concentration in the medium or by substances such as sodium butyrate (which arrests cells in G_1), TK activity decreases with a half life of only a few hours - 4 hours in the case of serum starved mouse 3T6 cells (4). The increase of enzyme activity in growth stimulated cells does not depend on DNA replication; it occurs even in the presence of inhibitors of replication, such as hydroxyurea or cytosine-arabinoside (4). This is in contrast to the regulation of histone synthesis in the S phase of the

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cell cycle (5, 6) and is in accord with the observation that regulation of TK rather occurs in late G_1 than in S phase.

Despite the strong regulation during the cell cycle, TK does not seem to be essential for the growth of cells in culture, as TK^- cells are readily isolated. They grow well in normal media but can be easily characterized by their inability to grow in a selective medium (HAT) containing hypoxanthine, thymidine and aminopterin, an inhibitor of dihydrofolate reductase which causes a block in the main pathway of thymidine phosphate and purine nucleotide synthesis.

Hence, TK is of interest in several respects. First it is an ubiquitous enzyme, whose physiological role is not yet fully understood. Second its strong regulation facilitates investigations on the mechanism of regulation of enzyme induction in the cell cycle and third, the molecular basis of TK mutations can be studied in cell culture.

We have screened a mouse cDNA library and have isolated several cDNA clones for mouse TK. After inserting the HSV-TK promotor region into plasmids carrying the cDNA, these were capable of transforming LTK^- cells to TK^+. Such transformed cells were found to synthesize TKmRNA in a cell cycle dependent manner, indicating that this cDNA construct carries the signals for regulated TK expression.

Materials and Methods

Screening of cDNA library

A cDNA library produced from mRNA of mouse embryonal cells cloned via Eco RI linkers into lambda gt11 phages was obtained from G. Schütz and S. Ruppert (Center for Tumor Research, Heidelberg). It was screened with a fragment of the cDNA for human TK. Plasmid pTK11 containing cDNA of human TK was kindly provided by H.D.Bradshaw, jr. (7,8). The SmaI-BamHI insert of this plasmid gave a smear if used to probe genomic Southern blots of mouse DNA. We therefore looked for a fragment within this insert yielding a more specific reaction with mouse DNA. Comparing the known sequences of chicken, human and vaccinia virus TK (8-10), a region of relatively

high homology was found. This region was represented by a 354 bp CfoI-DdeI fragment within human TK cDNA. This fragment gave clear and reproducible bands if hybridized to Southern transfers of restricted mouse DNA and was used to screen the mouse cDNA library. Screening was done under standard conditions using a hybridization buffer containing 50% formamide and 43°C. Filters where washed under stringent conditions three times for 20 min each with 2 x SSC (1 x SSC is 0.15 mol/l NaCl and 0.015 mol/l sodium citrate) plus 0.2 % SDS at room temperature, followed by 3 times for 20 min each with 0.2 x SSC plus 0.2 % SDS at 43°C. Phages scoring positive in this assay were rescreened two times and grown up for DNA isolation. The inserts were cut out with EcoRI and inserted into pUC18 for further analysis.

Construction of an expressible cDNA and transformation of LTK^- cells

In order to allow for expression of the cDNA after transfer into LTK^- cells, a BamHI-BglII fragment of a plasmid carrying the Herpes Simplex Virus (HSV)-TK gene (pHSV-106 from BRL) containing the control region of the viral gene (11) was introduced into the BamHI site within the polylinker region of pUC18 ahead of the mouse TK cDNA. LTK^- cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum. About 10^6 cells were transfected with 5μg of plasmid DNA and 15μg of sonicated salmon sperm DNA as carrier in 4 ml of serum free DMEM containing 500 μg/ml of DEAE-dextran. After three hours the transfection medium was replaced by DMEM containing 10% calf serum and after another 20 hours this medium was exchanged against HAT selection medium. HAT resistant colonies were isolated after three weeks, cultivated and frozen. Cells from several colonies were tested for the presence of thymidine kinase activity and for the regulation of TK with the growth conditions as described earlier (12).

Isolation and analysis of RNA

Mouse cells (3T6, LTK^--, LTK^+- and LTK^--cells transformed to TK^+) were grown as described previously adding HAT where appropriate (12). Total cellular RNA was isolated using the guanidinium-isothiocyanate method (13) and $polyA^+$ containing

mRNA selected with Hybond-mAP according to the recommendations of the manufacturer (Amersham, England). RNA was separated on 1% agarose gels in the presence of formaldehyde (14). The 28S and 18S ribosomal RNAs served as size markers. The transfer of the RNA from the gel to nitrocellulose filters was done with 20xSSC over night. Filters were prehybridized and then hybridized with the insert of the mouse TKcDNA labeled by nick translation (15).

DNA sequencing

Nucleotide sequence analysis was performed according to the chain-termination method (16). Radiolabeling of the newly synthesized DNA strand was done via incorporation of deoxy cytosine 5'-(α-[^{35}S]thio)phosphate (Amersham) as described by the vendor (17). Appropriate restriction fragments for sequence analysis created through EcoRI, HindIII, ClaI and partial AvaII digests of the cDNA were then integrated in the cohesive termini of M13mp8. The ends of the ClaI and AvaII partial fragments were filled and ligated to the SmaI site of the vector. Recombinant M13-phages were propagated in E.coli K-12 JM101.

Results

The availability of human TK-cDNA isolated by transfection experiments involving mouse LTK$^-$ cells (7,8), made it possible to screen a cDNA library cloned in lamda gt11 generated from mouse B16 embryonal cell mRNA for the presence of TK sequences. A 354 bp long DdeI/CfoI subfragment of the human TK-cDNA containing a conserved stretch of the TK sequence had to be used in order to obtain a specific signal.

After three cycles of screening, 15 clones with homology to the human probe were isolated. These could be ordered into three groups . The largest insert detected, which was also the most prominent (12/15), was about 1285 bp long, indicating a rather complete cDNA for TK. The other two clones were shorter, missing sequences at the 5'and at the 3'end and consisting of 1000 and 800 bp respectively. Further experiments on the expression of the cloned sequences in mouse cells were therefore done with the longest insert. For better

handling and for restriction analysis all three inserts were subcloned into plasmid pUC18.

Sequence of mouse TKcDNA

The whole sequence of the largest clone was determined. The 1186 bp long sequence is 30 bp longer (extended by 26 nucleotides at the 5'end) than an independently isolated mouse TKcDNA (18) but exhibits some minor differences: a change of a C to a T at nucleotide 718 (our numbering, corresponding to nucleotide 692 in ref.18) creates a new HindIII site in our cDNA but is conservative whereas a conversion of CT at nucleotides 320/321 (our numbering, corresponding to nucleotide 294/295 in ref.18) to TC causes the dissappearance of a SstI site in our cDNA and the transition of leucin to serin in the deduced amino acid sequence. This SstI site is missing in all our cDNA clones, hence it is unlikely to be a cloning artefact. Four additional changes were found in the non translated 3'-part of the cDNA.

Transformation of Ltk$^-$-cells with mouse TKcDNA

It was important to show that the cDNA isolated by us is functional after integration into LTK$^-$-cells. Since transfecting the cDNA as isolated was unsuccessful, we constructed a plasmid containing the known regulatory region of the HSV-TK gene by introducing the BamHI-BglII fragment of pHSV-106, carrying the entire HSV-TK gene, ahead of the coding region of mouse TK cDNA in pUC18. This construct was then used to transform LTK$^-$-cells. Several clones capable of growth in HAT medium were obtained. TK activity was tested in some of these and was found to be about 60 % of that of L-cells from which the LTK$^-$-line was derived. Hence, the cDNA was apparently efficiently expressed under the control of the HSV-TK regulatory region.

Cell cycle regulation of the level of TKmRNA in different cell lines

TK is expressed in a cell cycle dependent manner, increasing in activity in late G_1 to early S phase from a very low level (which is largely due to the presence of the non-regulated mitochondrial TK) to a level that is 50 fold or more above that measured in resting cells (19).

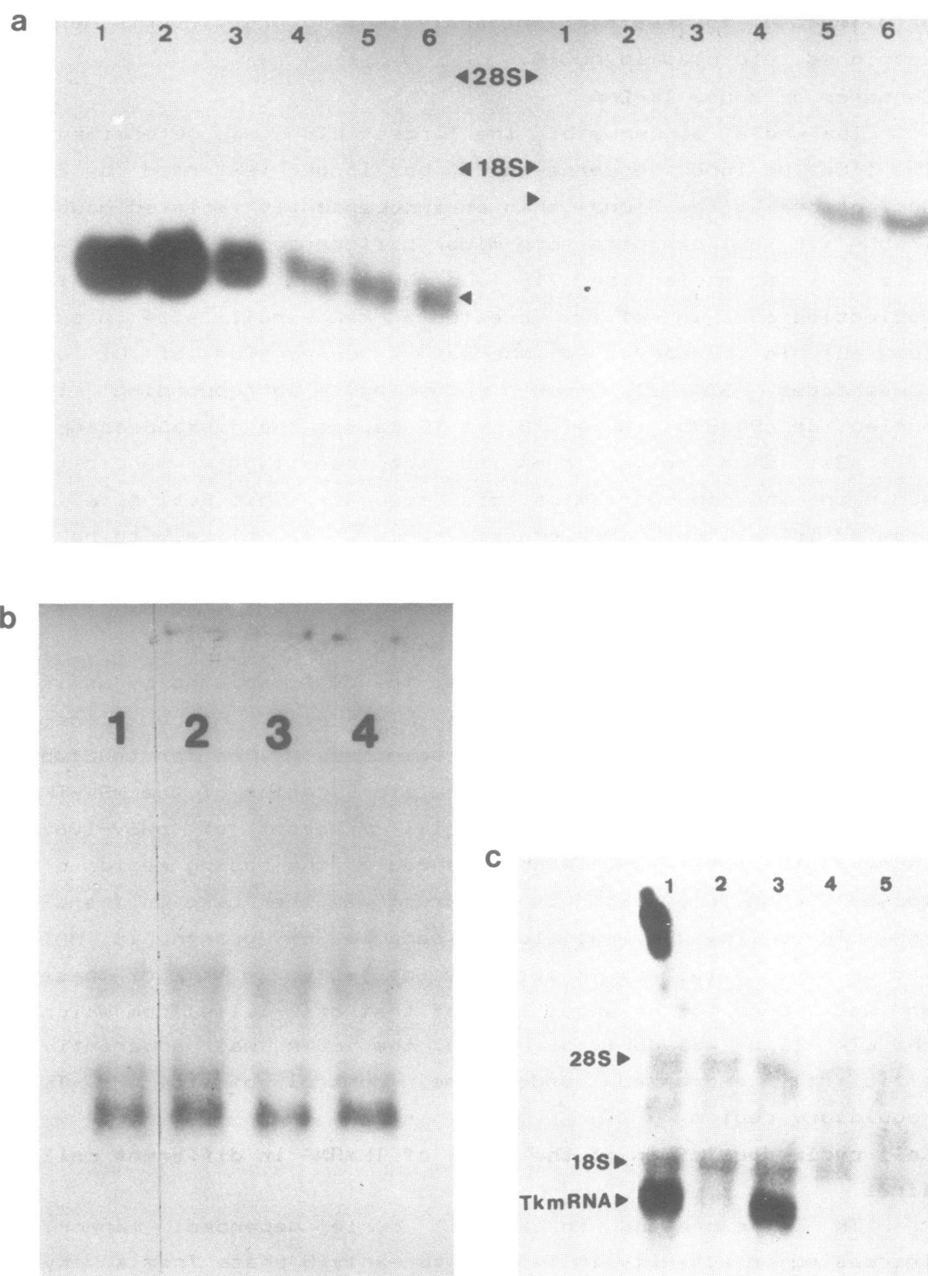

Figure 1. a) Northern blot of cell cycle induction of TKmRNA (right) and metallothionein-mRNA (left). Cells were treated for 24 hrs with 6 mmol/l sodium butyrate to arrest them in G_1 (12).

The drug was then removed by medium change and cytoplasmic RNA isolated at various times thereafter. RNA amounts were determined spectrophotometrically and 40 µg were loaded per slot. After hybridization uniform transfer was ascertained by staining the blot with methylene blue. cDNA probes for hybridization were labeled to about 5 x 10^8 cpm/µg by nick translation (15). Lane 1 : 0 hrs; lane 2 : 4 hrs; lane 3 : 8 hrs; lane 4 : 10 hrs; lane 5 : 13 hrs; lane 6 : 16 hrs after release from the butyrate block.

b) Expression of the viral TK in LTK^- cells transformed to the TK^+ phentype with the HSV-TK gene using the same protocol for blocking with butyrate and stimulating afterwards as described in the legend to fig. 1. Lane 1 : transformants arrested in G_1; lane 2 : 10 hrs ; lane 3 : 13 hours ; lane 4 : 16 hours after release of the block.

c) Expression of TKmRNA in butyrate blocked and in logarithmically growing LTK^- and $3T6TK^-$-cells. Lane 1 : TKmRNA in growing 3T6 as control; lane 2 : $3T6TK^-$ arrested in G_1 ; lane 3 : growing $3T6TK^-$; lane 4 : LTK^- arrested in G_1 ; lane 5: growing LTK^-.

Previous experiments on the cell cycle dependent induction of enzymes involved in DNA precursor production have indicated that this regulation occurs at the level of mRNA synthesis or stabilization. With our cDNA clone we have analyzed the level of mRNA hybridizing to the cDNA. For this purpose we arrested cells in early G_1 by adding sodium butyrate to the medium (12). Arrested cells were then released by removing the drug. At various times after the release, cells were collected and the extracts analyzed for TKmRNA content.

The results are summarized in fig. 1a. Resting cells show very low levels of mRNA. Several hours after release of the block a mRNA with about 1300 bases appears and increases in amount more than 50-fold as expected for TK mRNA. The same RNA can be detected (with the same time course of regulation) regardless of whether total RNA or polyA$^+$ RNA is used. As an example of an inversely regulated mRNA the identical blot was hybridized with metallothionein cDNA (= MTcDNA). A massive decrease (around 10-fold) in the amount of MTcDNA was seen within a few hours after butyrate was withdrawn. Exactly such a cell cycle regulation of the expression of MT has recently been reported (20). As a further control the expression of TKmRNA in LTK^- cells transformed to TK^+ by the HSV-TK gene was analyzed (fig. 1b) under exactly the same experimental

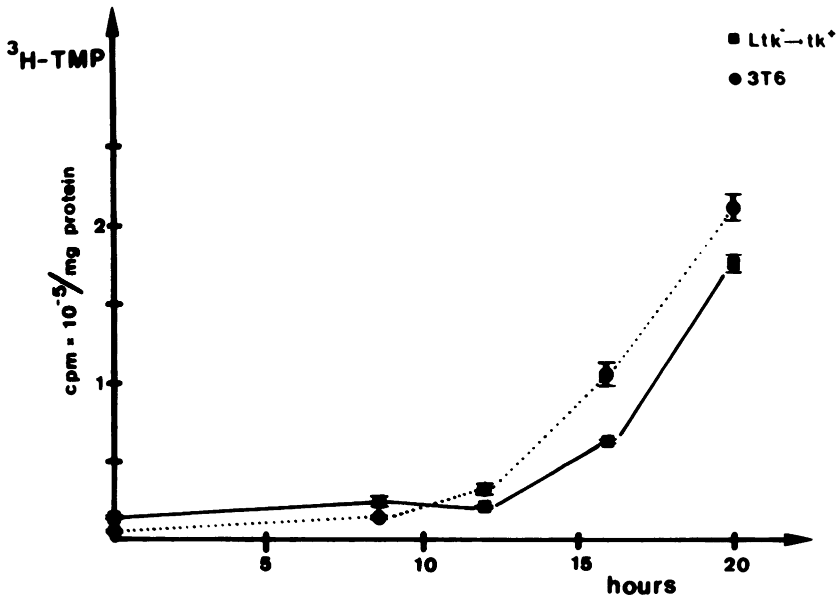

Figure 2. Growth dependent expression of TK in LTK$^-$ cells transformed to the TK$^+$ phenotype: 3T6 mouse fibroblasts (circles) and LTK$^-$-cells transformed to TK$^+$ (squares) were treated with sodium butyrate as described in the legend to fig. 1. TK enzyme activity was measured according to (12) 0, 8, 12, 16 and 20 hours after removal of the inhibitor.

conditions. In agreement with previous reports on enzyme activities in such transformants (21) TKmRNA is expressed constitutively.

Neither in resting nor in growing LTK$^-$-cells a RNA with high homology to TKcDNA was found (fig. 1c). This agrees with the fact that these cells show only back ground level of TK, do not grow in HAT medium and exhibit large deletions in the gene for TK as seen in Southern blots (unpublished).

Interestingly, another TK$^-$-cell line, produced in our laboratory from mouse 3T6 fibroblasts, has a growth regulated mRNA of the appropriate size which hybridizes to the mouse TKcDNA (fig. 1c). It has to be noted, however, that a small deletion would not have been detected in this assay. The cell line (designated 3T6TK$^-$) has only 4% of the normal level of TK activity, does not grow in HAT medium and is very stable: no

spontaneous revertants were found so far. Southern transfers of DNA from this mutant did not show significant differences to those from wild type 3T6-cells (unpublished).

Cell cycle dependent expression of TK enzyme activity in cells transformed by the TKcDNA

Since it was demonstrated (fig. 1b) that transformants produced with HSV-TK do not exhibit cell cycle dependent expression (see also ref.21) we looked for the mode of expression of our cDNA construct in transformed cells.

As shown in fig. 2 expression of TK in the transformed clone was apparently cell cycle dependent, the enzyme activity increasing several hours after removal of the blocking agent. This finding was confirmed using two more TK^+ clones transformed independently with the same cDNA construct (data not shown). Since it can be excluded that the DNA region responsible for regulated expression is localized in the HSV-TK regulatory region (fig. 1b, see also refs. 11,21), it must reside in the isolated cDNA.

Cell cycle dependent expression of TKmRNA in cells transformed by the TKcDNA

In order to investigate whether the LTK^-cell lines transformed to TK^+ by our TKcDNA construct had a cell cycle regulated stable TK mRNA, RNA was isolated from cells either blocked in early G_1 phase by butyrate addition or growing logarithmically. Only a very low level of TKmRNA could be found in two independently derived transformed clones if arrested in growth by butyrate whereas logarithmically growing cells contain large amounts of TKmRNA (fig.3). Taken together these observations imply that the TKcDNA carries the elements necessary for a growth regulated expression of the enzyme.

Discussion

We have isolated three different mouse TKcDNAs varying in length. The largest one was sequenced and analyzed in detail. It has a 56 bp long leader sequence and there are some minor differences compared to a previously published sequence of a mouse TKcDNA (18). When combined with the promoter region of HSV-TK our cDNA could confer the TK^+ phenotype to LTK^- cells.

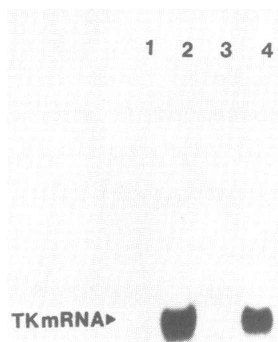

Figure 3. Northern blots of two TK⁺ transformants probed with TKcDNA. Lane 1 : clone 3 arrested; lane 2 : clone 3 growing; lane 3 : clone 4 arrested; lane 4 : clone 4 growing.

Our transformants showed growth-dependent expression of the TKmRNA and enzyme activity (figs. 2 and 3). An upstream promoter region of the mouse TK-gene cannot be responsible for this finding as this is largely missing in our plasmid. The HSV-TK promoter used in the vector construct is shown to be unable to confer cell cycle regulation (fig. 1b, see also refs. 11 and 21). Merril et al. (11) found that the regulation of the chicken TK gene during myoblast differentiation requires sequences downstream of the promoter. They suggested that either exon or intron sequences are involved in this regulation. It is not known whether the same elements control TK expression in this developmental process and in the cell cycle. Data obtained by measuring TK enzyme activity in rat LTK$^-$ cells transfected by several hamster TK-minigenes (22, 23) indicate that TK gene expression in the cell cycle is under the control of a sequence within the coding region of the gene. Our data substantiate and extend these observations by showing that the sequence responsible for growth dependent regulation of mouse TK is located within the mRNA.

As data obtained on an another enzyme similarly regulated during the cell cycle, namely dihydrofolate reductase, suggest that the poly A addition site may be involved in this process (24), it is interesting to note that the poly A addition

signal of mouse TK does not follow the canonical sequence AATAAA (25). Instead there is a putative signal ATTAAA within a longer stretch of a very AT-rich sequence in the appropriate location of the mouse and hamster TK-cDNA and the sequence AATTAAA in the human (8) and chicken (26) gene. Although this sequence does not seem to be necessary for the control of TK expression in differentiating chicken muscle cells (11), its possible involvement in the cell cycle regulation of TK remains to be tested. Interestingly, human TK-cDNA cloned into an Okayama-Berg vector (8) where its expression is under the control of the SV 40 early promoter and which in addition contains an SV 40 poly A addition signal (as well as splice sites) exhibits constitutive expression during the cell cycle (8, 27) although the cDNA also carries the genuine splice site of the human TK gene which seems to be used during RNA synthesis. It is possible that the strong SV 40 promoter overrides the cell cycle regulation in this case which seems not to be so if the HSV-TK promoter is used instead. Cell cycle regulation may thus depend on a subtle interplay between the promoter and a sequence downstream of the promoter region but present within the mRNA. It is furthermore worth to point out that also the expression of mouse histone H4 gene in the S phase of the cell cycle is at least in part regulated by a sequence at the 3'-terminal region (28). Hence similar mechanisms may be responsible for the cell cycle regulation of all these genes.

Acknowledgements

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