Nucleic Acids Research, 1998, Vol. 26, No. 21 **4989–4995**

Growth-regulated antisense transcription of the mouse thymidine kinase gene

Hedwig Sutterluety+, Stefan Bartl, Angelika Doetzlhofer, Harald Khier, Erhard Wintersberger and Christian Seiser*

Institute of Molecular Biology, University of Vienna, Vienna Biocenter, Dr Bohr-Gasse 9, A-1030 Vienna, Austria

Received May 18, 1998; Revised August 26, 1998; Accepted September 15, 1998 DDBJ/EMBL/GenBank accession no. AJ010394

ABSTRACT

The expression of the salvage pathway enzyme thymidine kinase (TK) is very low in resting mammalian cells, but increases dramatically when growth-stimulated cells enter S phase. The 30-fold rise in TK mRNA levels in response to growth factors is due to a well-characterized transcriptional activation and less defined post-transcriptional mechanisms. A minigene containing the murine TK promoter and the TK cDNA showed a 3-fold increase in TK mRNA levels after growth induction in stably transfected mouse TK-deficient L fibroblasts. Introduction of the first three TK introns resulted in a 10-fold regulation of TK expression which was predominantly due to repressed TK mRNA levels in serum-deprived cells. Removal of intron 3 from this construct or replacement of the TK promoter by a constitutive SV40 promoter led to a reduced, but still significant increase in TK mRNA levels during the onset of proliferation. These results indicate that both the TK promoter and specific TK introns contribute independently to the growth-dependent regulation of TK mRNA expression. To examine the regulatory mechanisms in more detail we analyzed TK transcription rates and steady-state levels of nuclear transcripts from an SV40 promoter-driven minigene that contains introns 2 and 3 of the TK gene. Using a set of single-stranded probes we detected TK-specific antisense transcription that was up-regulated in resting cells. Similarly, antisense transcription of the endogenous TK gene in Swiss 3T3 cells rose during serum deprivation while sense transcription was regulated in the opposite way. Luciferase reporter assays revealed the presence of a putative antisense promoter in intron 3 of the murine TK gene. These results suggest a negative role for intron-dependent antisense transcription in the regulation of TK mRNA expression in mouse fibroblasts.

INTRODUCTION

Thymidine kinase (TK) belongs to a group of enzymes such as dihydrofolate reductase, thymidylate synthase, DNA polymerase α and proliferating cell nuclear antigen that are involved in DNA synthesis and precursor production. The expression of these genes is low in resting cells but increases to high levels when cells traverse the G1/S boundary. Multiple regulatory mechanisms ensure the exclusive expression of these enzymes in replicating cells.

For instance TK mRNA levels are regulated in part by a growth-dependent rise in transcription rate. It was demonstrated that upstream elements that are recognized by protein complexes containing E2F, pRB, cdk2 and SP1 are crucial for the transcriptional activation of the TK gene (1–4). However, the 3–5-fold rise in TK transcription rate measured by run-on experiments is not sufficient to explain the 20–50-fold increase in TK mRNA levels after growth stimulation (5–7). Therefore, a part of this induction has been attributed to post-transcriptional events, but the exact molecular mechanisms are poorly understood. The fact that TK transcript levels were constant during growth induction when TK cDNA was expressed from a heterologous promoter argues against regulated mRNA stability and points toward a nuclear post-transcriptional mechanism (8). Pardee and colleagues (9) found that the level of nuclear TK precursors rose dramatically when growth-induced BALB/c 3T3 cells progressed from G1 into S phase. Sequential intron excision from these TK pre-mRNAs was more efficient in S phase cells (9) and occurred in a preferred order (10). Previously, we have identified two DNase hypersensitive sites within intron 2 of the murine TK gene (11). The presence of these sequences in chloramphenicol acetyltransferase reporter genes positively modulated the activity of the TK promoter, but their role in the growth-dependent induction of TK expression was unclear.

In the present study, we attempted to investigate the mechanism(s) responsible for post-transcriptional regulation of TK mRNA expression. This was carried out by investigating the expression of TK minigenes under control of the original TK promoter or the constitutive late SV40 promoter in stably transfected TK-deficient L cells (LTK$^-$ cells). Our results demonstrate that the TK promoter and TK introns 2 and 3 are independent determinants of growth-specific regulatory mechanisms. Furthermore we show that the ratio between sense transcription and antisense transcription changes from higher sense transcription in cycling Swiss 3T3 fibroblasts to predominant antisense transcription in serum-deprived cells. These data indicate that regulated antisense transcription might contribute to the accumulation of TK transcripts in growth-induced fibroblasts.

*To whom correspondence should be addressed. Tel: +43 1 4277 61770; Fax: +43 1 4277 9617; Email: cs@mol.univie.ac.at

+Present address: Friedrich Miescher Institute, Basel, Switzerland

MATERIALS AND METHODS

Construction of plasmids

A series of TK minigenes were constructed by combining regions of the murine TK cDNA (12) and genomic sequences from TKcosmid A (13) that carries the entire mouse TK gene. M0 was derived from plasmid pE/A-CAT (14) by replacing the chloramphenicol acetyltransferase gene with the murine TK cDNA. This minigene comprises the TK cDNA fused to the *Eco*RI–*Asp*700 fragment of the murine TK gene known to contain full promoter activity. Plasmid M1+2+3 was constructed by digesting M0 with *Nco*I (cutting at position –173 in the promoter and at position 278 of the cDNA within exon 4). The short fragment was replaced by the corresponding genomic *Nco*I fragment, the resulting minigene M1+2+3 contains introns 1–3. Triple ligation of the genomic *Apa*I–*Hae*II (nt 172 in exon 3) fragment with the *Hae*II–*Cla*I fragment of TK cDNA and the large fragment of the *Apa*I + *Cla*I-digested minigene M1+2+3 led to minigene M1+2. This minigene contains introns 1 and 2. For construction of minigene MSV0 the blunted mouse TK cDNA was cloned into the *Sma*I site of eukaryotic expression vector pSVL (Pharmacia) downstream of the constitutive SV40 late promoter. The pSVL vector contains the SV40 VP1 intron for better expression. The minigene MSV2+3, which contains in addition introns 2 and 3, was constructed by replacing the *Apa*I (nt 144 in exon 2)–*Cla*I (nt 344 in exon 4) fragment of MSV0 by the corresponding genomic *Apa*I–*Cla*I fragment. Luciferase reporter constructs containing TK intron 3 were obtained by cloning of the respective PCR products fragment 1 (nt 1–515), fragment 2 (518–1013), fragment 3 (1025–1482), fragment 4 (1490–1953) and fragment 5 (1953–2478) in antisense direction into pGL2 (Promega).

Cell culture

Mouse LTK$^-$ fibroblasts and REF52 cells were cultured in 7.5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics (30 mg/l penicillin, 50 mg/l streptomycin sulfate).

Cells were growth arrested by reduction of the serum concentration to 0.2% for 3 days and stimulated by addition of fresh medium containing 20% FCS. The growth state of cells was routinely controlled by cytofluorometric determination of DNA content.

Transfection into LTK$^-$ cells

Logarithmically growing cultures of LTK$^-$ fibroblasts were transfected by the calcium phosphate method and selected in HAT (hypoxanthine/aminopterine/thymidine) medium as described (8,15). After 2 weeks pools of 100–200 colonies were collected and propagated for further analysis.

Cytosolic RNA isolation and northern blot analysis

Cytosolic RNA isolation and northern blot analysis were performed as described (7). Equal amounts of RNA (20 µg) were separated on a 1.2% denaturing agarose gel containing formaldehyde and transferred to GeneScreen plus nylon membranes (Du Pont-New England Nuclear). To assure the transfer of high molecular weight RNA the gel was treated with 50 mM NaOH for 15 min and subsequently neutralized with 0.3 M sodium acetate. RNA was cross-linked to membranes using a Stratalinker (Stratagene). The 1.2 kb mouse TK cDNA (12), the 600 bp *Pst*I fragment of mouse TK intron 2 (11), the 600 bp *Kpn*I fragment of TK intron 3 (13) and the 300 bp *Hha*I–*Eco*RI fragment of mouse β_2-microglobulin cDNA (16) were used as probes.

Isolation of nuclei

Nuclei were isolated as described (7). Cells were harvested, washed and suspended in lysis buffer containing 60 mM KCl, 30 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM HEPES, pH 7.5, 14 mM β-mercaptoethanol, 0.15 M sucrose, 0.25 mM EGTA, 1.0 mM EDTA and 0.1% NP-40 and incubated on ice for 5 min. The suspension was layered over a cushion of 12.5% sucrose and nuclei were pelleted at 1500 *g* at 4°C for 8 min. For run-on assays, nuclei were stored in glycerol buffer (20 mM Tris, pH 8.0, 0.5 mM EDTA, 75 mM NaCl, 0.85 mM DTT and 50% glycerol) at a concentration of 1×10^5 nuclei/µl.

Preparation of nuclear RNA

The nuclei were washed twice in RSB/K buffer (10 mM Tris–HCl, pH 7.9, 20 mM NaCl, 10 mM $MgCl_2$, 100 mM KCl) and resuspended in HSB buffer (10 mM Tris–HCl, pH 7.4, 500 mM NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$) supplemented with 200 U DNase I (Boehringer) and 100 U RNasin (Promega). After 30 min incubation at room temperature, the nuclei were lysed in an equal volume of extraction buffer containing 10 mM Tris–HCl, pH 7.4, 20 mM EDTA, 1% SDS. Proteins as well as the remaining DNA were removed by two extractions with phenol/NETS (50% v/v phenol, 10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.2% SDS) for 10 min at 55°C. The aqueous phase was transferred to a new Eppendorf tube and precipitated with 2 vol 96% ethanol. RNA was quantified and its quality checked by ethidium bromide staining on denaturing 0.8% agarose gels.

Nuclear run-on transcription assays

Transcription reactions were performed for 20 min in the presence of [α-^{32}P]CTP (800 Ci/mmol) as described previously (7). Equal amounts of transcripts (usually 1×10^7 c.p.m.) were hybridized to single-stranded DNA probes immobilized on 0.45 µm BA 85 nitrocellulose filters (Schleicher & Schuell) for 3 days at 42°C. Hybridization was performed in the buffer described for northern blot hybridization (above). Filters were washed in 2× SSC, 1% SDS at room temperature and twice in 0.2× SSC, 0.2% SDS at 55°C for 10 min. Subsequently filters were incubated for 20 min in 10 mM Tris–HCl, pH 7.5, 0.3 M NaCl, 10 µg/ml RNase A. Filters were rinsed and exposed to X-ray films at –70°C.

The following fragments were subcloned in both orientations in M13 derived vectors and used as single-strand DNA probes: The 700 bp *Eco*RI–*Hin*dIII fragment of the mouse TK cDNA (12) that recognizes exons 1–6 of the TK gene, the 500 bp *Hin*dIII–*Eco*RI fragment of the mouse TK cDNA (12) as exon 7 probe, the 600 bp *Pst*I fragment of mouse TK intron 2 (11), the 600 bp *Kpn*I fragment of TK intron 3 (13) and the 600 bp *Hha*I–*Eco*RI probe of β_2-microglobulin (16) and a 1.5 kb cDNA fragment specific for the subunit R2 of murine ribonucleotide reductase (17).

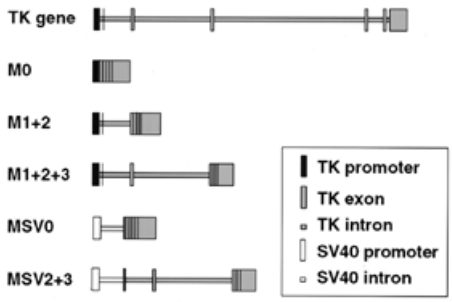

Figure 1. Structures of TK gene and TK minigenes. The TK gene is expressed from a cosmid described as TKcosmid A in Seiser *et al*. (13). The intron-less TK minigene M0 consists of the 440 bp *Eco*RI–*Asp*700 genomic fragment containing the mouse TK promoter and the murine TK cDNA (14). Minigene M1 contains the same elements as M0 plus the TK intron 1 while the construct M1+2 includes both TK introns 1 and 2. M1+2+3 combines the mouse TK gene up to exon 4 with the residual TK cDNA. MSV0 was derived from the eukaryotic expression vector pSVL (Pharmacia). The minigene includes the mouse TK cDNA expressed from the constitutive SV40 late promoter. It also contains SV40 intron VP1 and an SV40 polyadenylation signal. MSV2+3 was constructed by cloning TK introns 2 and 3 into minigene MSV0. The cosmid was microinjected into LTK$^-$ cells, while the minigenes were transfected by calcium phosphate precipitation. TK$^+$ cells were selected in HAT medium and ~100 clones were pooled for further analysis.

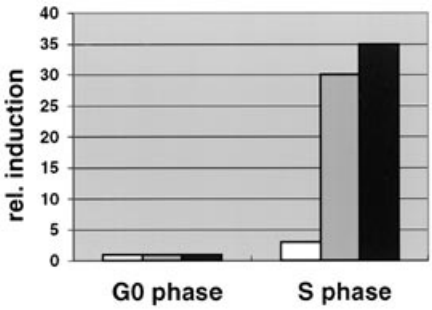

Figure 2. Regulation of TK expression in L cells containing the complete TK gene. LTK$^-$ cells stably transfected with the entire TK gene were arrested by serum reduction for 3 days and then restimulated for 12 h with fresh medium containing 20% FCS. Arrested cells (G0 phase) and stimulated cells (S phase) were analyzed for TK enzyme activity, TK mRNA expression and TK transcription rate. Protein extracts were prepared and TK enzyme activity was determined as described in Materials and Methods. Enzymatic activities were plotted relative to the 0 h value set as 1 (black bars). Cytosolic RNA was extracted from arrested and stimulated cells and equal amounts (20 µg) of RNA were resolved by electrophoresis and blotted onto a nylon membrane. Hybridization of northern blot was performed with ^{32}P-labeled probes for TK and β_2-microglobulin. Signals from both hybridizations were determined by densitometric scanning and normalized to the β_2-microglobulin signal. TK mRNA levels were depicted relative to the value of arrested cells (taken arbitrarily as 1, gray bars). To determine TK transcription rates nuclei were isolated from arrested and induced cells. Equal amounts of *in vitro* labeled transcripts were hybridized to single-stranded M13 plasmids immobilized on nitrocellulose. The plasmids contained the 5′ *Eco*RI–*Hin*dIII fragment of the mouse TK cDNA, the 300 bp *Hha*I–*Eco*RI fragment of mouse β_2-microglobulin cDNA or no insert at all (M13). Control hybridization to single-stranded DNA showed no background. Values for TK transcription rates are shown relative to the β_2-microglobulin signal as described above (white bars).

Protein extraction and thymidine kinase enzyme assay

Cytoplasmic protein extraction was performed as described by Sherley and Kelly (18). Protein concentrations were determined using the BioRad protein assay as described by the manufacturer. Thymidine kinase activity in cell extracts was measured as described (8).

Transient transfection and luciferase assay

Transient transfection of REF52 fibroblasts by calcium phosphate co-precipitation and luciferase activity assays were done as described (19). β-Galactosidase activity (as a control for transfection efficiency) was assayed using the Luminescent β-Galactosidase Detection kit from Clontech.

The mouse TK intron 3 sequence was determined by standard methods and deposited in the EMBL nucleotide sequence database (accession no. AJ010394).

RESULTS

Growth-dependent regulation of TK gene expression in transfected LTK$^-$ cells

Initial studies were carried out to determine the suitability of the LTK$^-$ system for investigating growth-dependent regulation of TK mRNA. For this purpose, we analyzed the expression of TK in LTK$^-$ fibroblasts stably transfected with the entire murine TK gene (Fig. 1, top panel). Cells were arrested by reduction of the serum concentration in the medium to 0.2% for 72 h and stimulated to proliferate with medium containing 20% FCS. FACS analysis and measurement of DNA synthesis by thymidine incorporation showed that cells reach S phase after 8–12 h serum stimulation (data not shown). Measurements of TK enzyme activity in extracts prepared from resting and S phase cells demonstrated that regulation of TK expression was indistinguishable from cells harboring an endogenous TK gene. As depicted in Figure 2 the enzymatic TK activity increased 35-fold when cells harboring the TK cosmid enter S phase. To examine TK mRNA levels during growth induction we prepared cytosolic RNA from resting and stimulated cells and analyzed it by northern blot hybridization. TK-specific transcripts were very low in serum-starved cells but reached 30-fold levels after 12 h serum induction (Fig. 2). To assess the contribution of transcriptional mechanisms to this rise in TK gene expression in transfected LTK$^-$ cells, we performed run-on assays with nuclei of arrested and restimulated cells. Equal amounts of labeled transcripts were hybridized to immobilized single-stranded M13 plasmids containing the 5′ *Hin*dIII fragment of the murine TK cDNA, the mouse β_2-microglobulin cDNA or no insert. As shown in Figure 2 TK transcription rate was 3-fold induced when cells reached S phase. Additional run-on experiments with nuclei isolated after shorter induction periods revealed that transcriptional activation was at no time >3-fold (data not shown). These results are in good agreement with data of several groups which showed that the induction of TK expression is due in part to a 3–5-fold increase in transcription rate (5–7). As a control we also included a plasmid bearing a cDNA fragment of the murine ribonucleotide reductase M1 subunit. As expected the transcription rate of this S phase-specific gene is also induced (6-fold), while an M13 plasmid without insert gave no signal at all (data not shown). Taken together the data clearly demonstrate that LTK$^-$ cells contain all the factors essential for the regulation of TK mRNA expression.

Specific introns contribute to the repression of TK mRNA levels in resting cells

Subsequently, we proceeded to investigate the role of specific TK introns in the growth-dependent expression of TK mRNA. TK minigenes were constructed by replacing parts of the TK gene with the corresponding cDNA fragments (Fig. 1). The first set of minigenes contained the authentic TK promoter linked to TK genes with no intron (M0), the first 2 introns (M1+2) and the first 3 introns (M1+2+3) as depicted in Figure 1. Two independent pools of stably transfected cells were investigated for each construct and essentially yielded the same results. Cytosolic RNA was extracted from growth-arrested cells and cells stimulated for different periods of time and analyzed by northern blot hybridization. TK mRNA transcribed from the minigene M0, already detectable in resting cells, increased 4-fold after 6 h serum stimulation when cells reached S phase and was slightly reduced at 12 h induction (Fig. 3A). The timing and extent of the increase in TK mRNA levels was nearly identical in cells transfected with a minigene containing the first 2 introns (M1+2) (Fig. 3A). Cells expressing the minigene M1+2+3, in contrast, showed a more pronounced rise of TK-specific transcripts during growth stimulation. TK mRNA levels were very low in resting cells but increased after 3 h stimulation and reached 10-fold levels after 6 h serum treatment. These results suggest that intron 3 plays a significant role in the regulation of TK mRNA expression. The 4-fold induction of TK transcripts from intron-less minigenes or minigenes comprising only the first 2 introns most probably reflects a similar rise in TK transcription (Fig. 2). The difference in TK mRNA regulation between M0 and M1+2+3 expressing cells is mainly due to significantly lower levels of TK mRNA in the resting state. This would suggest that the presence of intron 3 is important for the reduction of minigene M1+2+3 expression in G0 cells.

The regulatory effect of specific TK introns is independent of the TK promoter

Consequently, we replaced the TK promoter by the growth-independent SV40 late promoter to determine if the intron-specific effect also occurs when the TK minigene is expressed from a heterologous promoter. These constructs also contain an SV40 intron located upstream of the TK minigenes to improve their expression. TK mRNA transcribed from a minigene without TK introns (MSV0, Fig. 1) was clearly detectable in resting fibroblasts and its expression was even slightly reduced in serum-stimulated cells (Fig. 3B). Introduction of TK introns 2 and 3 into the minigene (MSV2+3, Fig. 1), in contrast, resulted in a clear reduction of TK-specific transcript levels in resting cells (Fig. 3B). TK mRNA expression rose within the first 3 h serum stimulation and reached ~3-fold levels at 12 h. These data confirm that the repression of TK mRNA levels in resting cells is associated with specific intron sequences. Moreover, it became clear that the TK promoter is dispensable for the intron-dependent effect. These results raise the question of which post-transcriptional mechanism is responsible for the regulation of TK transcripts encoded by the MSV2+3 minigene.

Processing of TK-specific nuclear transcripts in MSV2+3 transfected cells

In an initial attempt to reveal the mechanisms involved in the intron-dependent regulation of TK transcript levels, we investigated

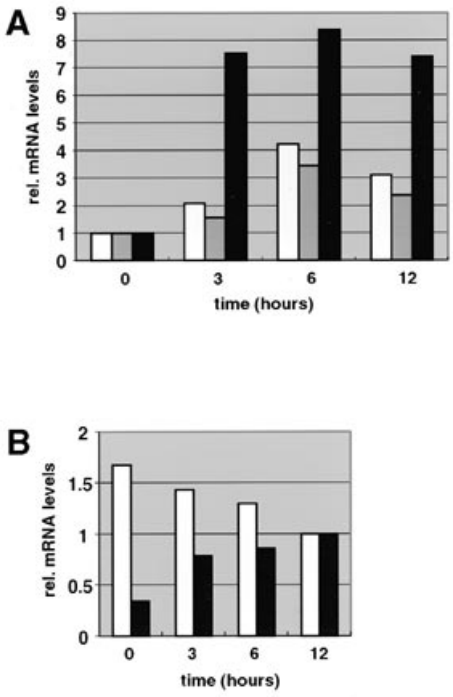

Figure 3. Growth-dependent expression of TK minigenes in stably transfected L fibroblasts. (**A**) TK minigenes under the control of the TK promoter. Cells expressing TK from minigenes M0 (white bars), M1+2 (gray bars) and M1+2+3 (black bars) were arrested by serum withdrawal and restimulated with 20% FCS for the duration indicated in hours. Cytosolic RNA was prepared and analyzed by sequential hybridization with a TK cDNA probe and a β_2-microglobulin fragment. Signals were quantified by densitometric scanning and relative amounts of TK mRNA were normalized to the corresponding signals for β_2-microglobulin and plotted. In each cell line the TK mRNA level at 12 h induction was defined as 100. (**B**) TK minigenes under control of the SV40 late promoter. Serum-arrested cells expressing minigenes MSV0 (white bars) and MSV2+3 (black bars) were restimulated for 0, 3, 6 and 12 h. Cytoplasmic RNA was analyzed by northern blot hybridization with radiolabeled probes for TK and β_2-microglobulin. TK mRNA levels were quantified and plotted as described under (A).

if the efficiency of processing and export of nuclear TK transcripts changes upon growth stimulation of MSV2+3 transfected fibroblasts. Cells were arrested for 72 h and restimulated with fresh medium containing 20% FCS for 1, 3 and 6 h. RNA was extracted from isolated nuclei, resolved on a denaturing gel and transferred to a nylon filter. The efficient transfer of equivalent amounts of nuclear RNA was confirmed by methylene blue staining of the membrane (not shown). Hybridization of the northern blot with a radiolabeled TK cDNA probe revealed that higher molecular weight TK precursors and mature TK mRNA were detectable at low levels in the nuclei of serum-deprived cells and accumulated after serum stimulation (Fig. 4A). Rehybridization with probes specific for TK introns 2 and 3 showed ordered splicing without evidence for accumulation of unspliced precursors. Furthermore, the absence of high levels of mature TK transcripts in the nuclei of resting cells argues against regulation of minigene MSV2+3 at the level of nuclear export.

TK-specific antisense transcription from minigene MSV2+3

Next, we analyzed TK sense and antisense transcription from the MSV2+3 minigene by run-on assays. After serum deprivation for

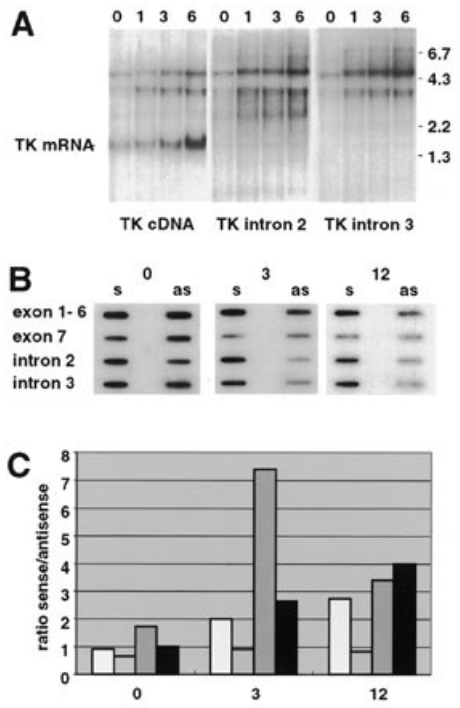

Figure 4. Nuclear TK RNA precursors and TK transcription rates in MSV2+3 transfected cells. Cells were synchronized by serum reduction for 3 days. At time 0 and at designated periods (indicated in hours) after restimulation with 20% FCS-containing medium, nuclei were isolated and used for transcription assays and for analysis of nuclear TK transcripts. (**A**) Nuclear RNA was isolated as described in Materials and Methods. Equal amounts of RNA were analyzed on a northern blot by hybridization with a ^{32}P-labeled TK cDNA probe. (**B**) Run-on transcription assay. Nuclear transcripts were elongated in the presence of [^{32}P]CTP. TK sense (s) and antisense (as) transcription rates were measured by hybridization to single-stranded plasmids containing the 5′ *Eco*RI–*Hin*dIII fragment (exons 1–6) and the 3′ *Hin*dIII–*Eco*RI fragment (exon 7) of the murine TK cDNA, a subfragment of TK intron 2 and a subfragment of TK intron 3. (**C**) Values for each time point of the run-on transcription assay obtained by densitometric scanning of TK-specific transcription signals were plotted as ratio of sense to antisense signals. The relative values are shown for exons 1–6 (white bars), exon 7 (light gray bars), intron 2 (dark gray bars) and intron 3 (black bars).

72 h, cells were growth stimulated and nuclei were isolated at 0, 3 and 12 h serum induction. Equal amounts of radiolabeled elongated transcripts were hybridized to immobilized single-stranded M13 plasmids. An *Eco*RI–*Hin*dIII subfragment of the mouse TK cDNA (exons 1–6) encompassing the first 6 exons and a small part of exon 7 served for examination of the total TK transcription rate. In addition, strand-specific probes for intron 2, intron 3 and exon 7 were used to investigate transcription elongation. Single-stranded M13 plasmids containing sense probes of all TK fragments served as probes for the detection of antisense transcription. As shown in Figure 4B general TK sense transcription from the late SV40 promoter as measured with the probe for exons 1–6 was basically constant after serum stimulation of resting cells. In contrast, TK antisense transcription was high in serum-deprived cells and significantly decreased during the induction period. A growth-dependent change in the ratio between sense and antisense transcription was also observed with probes for TK intron 2, TK intron 3 and, although less pronounced, for exon 7 (Fig. 4C). These results suggest that antisense transcription from the MSV2+3 minigene might play a role in the regulation of TK mRNA expression levels. This mechanism is linked to the presence of TK introns 2 and 3, since regulated antisense transcription was absent in cells expressing minigene MSV0 (data not shown).

Regulated TK antisense transcription in Swiss 3T3 fibroblasts

To confirm our findings concerning growth arrest-induced antisense transcription we decided to analyze TK transcripts in Swiss 3T3 cells that contain a functional and growth-regulated TK gene (S.Bartl, Diploma thesis, University of Vienna). Exponentially growing Swiss 3T3 fibroblasts were growth arrested and harvested after different periods of time. Nuclei were isolated and TK transcription rates were measured by run-on assays. As shown in Figure 5A, deprivation of growth factors resulted in a decrease in TK sense transcription and a simultaneous increase in TK antisense transcription. Similar to the observations with MSV2+3 cells we failed to detect significant arrest-specific induction of antisense transcription with the exon 7 probe. Signals from TK sense and antisense transcription detected with the probe for exons 1–6 were determined by densitometric scanning and the ratio of sense to antisense values were plotted for each time point (Fig. 5B). Sense transcription was clearly higher than antisense transcription during the first 6 h but antisense transcription became predominant after 12 h of serum. In perfect agreement with these findings we observed a 4-fold rise of TK sense transcription during serum stimulation of resting Swiss 3T3 fibroblasts while antisense transcription simultaneously dropped to very low levels (data not shown). These data confirm our results with the MSV2+3 expressing cell lines and demonstrate that part of the endogenous TK gene is transcribed in the antisense direction in a growth-regulated manner.

Antisense transcription from TK intron 3

In order to detect a potential antisense promoter, the entire intron 3 of the murine TK gene was cloned and the nucleotide sequence was determined. The transcriptional activity of five intron 3 sub-fragments each covering ~500 bp (Fig. 6A) was analyzed in luciferase assays in resting and serum-stimulated REF52 fibroblasts after transient transfection. A luciferase reporter under control of the mouse TK promoter (4) was included as control. As shown in Figure 6B, fragment 3 conferred significant transcriptional activity to a luciferase reporter in resting fibroblasts. This promoter activity was ~3-fold reduced in growth-stimulated cells. Fragment 3 contains a TATA box-like sequence and putative binding sites for the transcription factors SP1 and TFIIIA (Fig. 6C). The other subfragments of TK intron 3 showed low and unregulated promoter activity (fragments 1 and 4) or no activity at all (fragments 2 and 5). These results support the idea that sequences within TK intron 3 can act as an antisense promoter in resting fibroblasts.

DISCUSSION

Thymidine kinase belongs to the group of enzymes involved in the nucleotide salvage pathway. They function to reutilize nucleosides obtained from the degradation of DNA. It was suggested that these enzymes have an additional, regulatory role by fine tuning the dNTP pools in the cells (20). Perturbations of

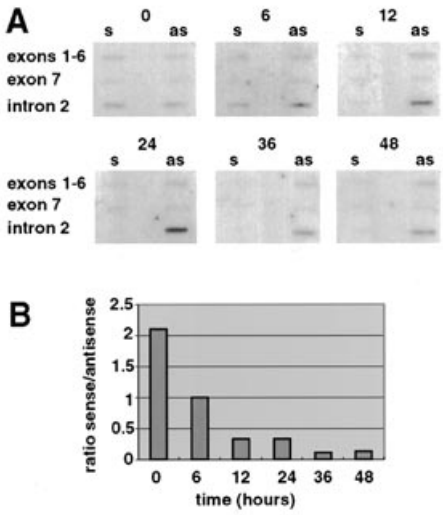

Figure 5. Growth factor-dependent sense and antisense transcription from the TK gene in Swiss 3T3 fibroblasts. Exponentially growing Swiss 3T3 cells were deprived of growth factors by reduction of the FCS concentration in the culture medium to 0.2%. Nuclei were isolated at different time points (indicated in hours) and analyzed in run-on assays. (**A**) Equal amounts of radiolabeled transcripts were analyzed for TK sense (s) and antisense (as) transcription by hybridization to single-stranded plasmid probes containing 5′ *Eco*RI–*Hin*dIII fragment (exons 1–6) and the 3′ *Hin*dIII–*Eco*RI fragment (exon 7) of the TK cDNA and the TK intron 2 probe. (**B**) Values for each time point obtained by densitometric scanning of TK-specific transcription signals (exons 1–6) were plotted as ratio of sense to antisense signals.

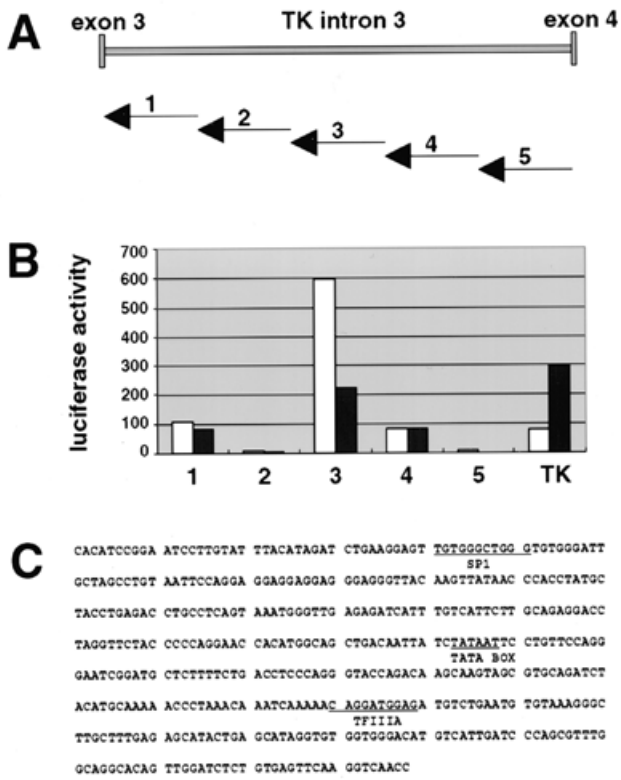

Figure 6. Antisense promoter activity of intron 3 sequences. (**A**) The nucleotide sequence of intron 3 of the murine TK gene was determined and five subfragments (1–5) were cloned in the antisense direction into the luciferase reporter pGL. (**B**) REF52 fibroblasts were transiently transfected with each of the five intron 3 reporter constructs or a luciferase plasmid containing the murine TK promoter (TK) together with pCMVβGal (as control for transfection efficiency). Twenty four hours after transfection cells were arrested by serum deprivation for 48 h (white bars) and restimulated culture medium containing 20% FCS for 20 h (gray bars). Luciferase activity was normalized to β-galactosidase activity. Data are representative for three experiments. (**C**) Nucleotide sequence of subfragment 3 is shown in the antisense direction. A TATA box-like sequence and putative binding sites for the transcription factors SP1 and TFIIIA are indicated.

the balanced levels of deoxynucleotides were shown to result in an increase in mutation rate (21). Therefore it might be important for the mammalian cell to prevent inappropriate expression or overexpression of salvage enzymes. Consequently, the expression of these proteins is regulated at different molecular levels.

In this report, we investigated the post-transcriptional mechanism that suppresses TK mRNA expression in resting mouse fibroblasts. TK mRNA from a minigene consisting of the TK cDNA coupled to an SV40 promoter was constitutively expressed as shown previously in several reports. Replacement of the viral promoter by the murine TK promoter led to 3-fold suppression of TK transcripts in resting cells reflecting the growth factor-dependent regulation of the mouse TK promoter. These data are consistent with results from TK transcription assays in different mammalian cell systems (Fig. 2; 5–7). Introduction of the first two introns had no effect, but additional insertion of TK intron 3 led to an additional 3-fold down-regulation of TK mRNA levels in serum-deprived cells. While the presence of intron 3 is crucial for the observed post-transcriptional regulation of M1+2+3 and MSV2+3, it is not yet clear if intron 2 is required for this process. Two potential protein binding sites were previously detected within this intron, but none of these recognition sequences was able to confer growth regulation to the reporter genes (11).

Intron-dependent regulation in response to growth stimulation has been reported for a number of genes such as proliferating cell nuclear antigen (22), thymidylate synthase (23), 4F2 antigen (24), interleukin 2 (25), prostaglandin synthase (26), β-actin (27) and eukaryotic initiation factor 2α (28). In the case of thymidylate synthase, the intron-mediated growth regulation was shown to be dependent upon the presence of specific sequences within the promoter (29). In contrast, we demonstrated in this report that the post-transcriptional regulation of TK mRNA expression occurs independently of the TK promoter. TK introns 2 and 3 were able to exert their regulatory function under the control of the constitutive SV40 promoter.

A major finding of this report is the observed TK antisense transcription that was induced in resting LMSV2+3 cells and in resting Swiss 3T3 cells. In both cell lines enhanced antisense transcription was correlated with down-regulation of TK mRNA expression. The fact that the endogenous gene and the MSV2+3 transgene are both transcribed in antisense argues against the possibility of ‘accidental’ read-through transcription of an adjacent downstream gene. This argument is also supported by the finding that TK exon 7 showed little or no increase in antisense transcription in resting cells. The latter observation also suggests that the putative antisense transcription start site is located within the TK gene. Since removal of intron 3 led to loss of the post-transcriptional component of growth regulation it is likely that the potential antisense promoter is located within

intron 3. In accordance with this idea a portion of this intervening sequence can mediate antisense transcription to a luciferase reporter in G0 fibroblasts.

Gene regulation by naturally occurring antisense transcription has been observed both in prokaryotes and in eukaryotes (30–32). While prokaryotic antisense transcription acts usually on the translational level, eukaryotic antisense regulation is thought to occur primarily in the nucleus. Regulation of primary sense transcripts by overlapping antisense transcripts has been proposed as a mechanism for a number of eukaryotic genes (28,33–38). The antisense transcripts seem to exert their regulatory role in several possible ways. Double-stranded RNA can interfere with correct splicing of the sense transcript (37) or is recognized by specific RNases (39) or RNA-modifying enzymes (33,40). Since TK antisense transcription is perfectly correlated with the repression of TK mRNA in resting cells it is very likely that it plays a role in the regulation of TK expression. The determination of regulatory elements within TK intron 3 and the identification of potential *trans*-acting factors will be the next step towards the understanding of post-transcriptional events during the growth stimulation of TK.

ACKNOWLEDGEMENTS

We thank T.Sauer for performing the FACS analysis and part of the nucleotide sequence analysis and Dr M.Hengstschlaeger for providing the ribonucleotide reductase cDNA. We are grateful to Dr A.Infante, F.M.Yeong and Dr H.Rotheneder for critically reviewing this manuscript. This work was supported in part by the Austrian Science Foundation (grant P11179-GEN to C.S. and grant P10873-GEN to E.W.), the Austrian National Bank, the Austrian Ministry of Science (grant 70.003/2-Pr/4/95) and the Herzfelder Foundation.

REFERENCES

1 Ogris,E., Rotheneder,H., Mudrak,I., Pichler,A. and Wintersberger,E. (1993) *J. Virol.*, **67**, 1765–1771.
2 Dou,Q.P., Markell,P.J. and Pardee,A.B. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 3256–3260.
3 Li,L.J., Naeve,G.S. and Lee,A.S. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3554–3558.
4 Karlseder,J., Rotheneder,H. and Wintersberger,E. (1996) *Mol. Cell. Biol.*, **16**, 1659–1667.
5 Stewart,C.J., Ito,M. and Conrad,S.E. (1987) *Mol. Cell. Biol.*, **7**, 1156–1163.
6 Coppock,D.L. and Pardee,A.B. (1987) *Mol. Cell. Biol.*, **7**, 2925–2932.
7 Seiser,C., Teixeira,S. and Kühn,L.C. (1993) *J. Biol. Chem.*, **268**, 13074–13080.
8 Sutterluety,H., Bartl,S., Karlseder,J., Wintersberger,E. and Seiser,C. (1996) *J. Mol. Biol.*, **259**, 383–392.
9 Gudas,J.M., Knight,G.B. and Pardee,A.B. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 4705–4709.
10 Gudas,J.M., Knight,G.B. and Pardee,A.B. (1990) *Mol. Cell. Biol.*, **10**, 5591–5595.
11 Rotheneder,H., Grabner,M. and Wintersberger,E. (1991) *Nucleic Acids Res.*, **19**, 6805–6809.
12 Hofbauer,R., Müllner,E., Seiser,C. and Wintersberger,E. (1987) *Nucleic Acids Res.*, **15**, 741–752.
13 Seiser,C., Knofler,M., Rudelstorfer,I., Haas,R. and Wintersberger,E. (1989) *Nucleic Acids Res.*, **17**, 185–195.
14 Weichselbraun,I., Ogris,E. and Wintersberger,E. (1990) *FEBS Lett.*, **275**, 49–52.
15 Wigler,M., Silverstein,S., Lee,L.S., Pellicer,A., Cheng,Y. and Axel,R. (1977) *Cell*, **11**, 223–232.
16 Daniel,F., Morello,D., Le-Bail,O., Chambon,P., Cayre,Y. and Kourilsky,P. (1983) *EMBO J.*, **2**, 1061–1065.
17 Thelander,L. and Berg,P. (1986) *Mol. Cell. Biol.*, **6**, 3433–3442.
18 Sherley,J.L. and Kelly,T.J. (1988) *J. Biol. Chem.*, **263**, 375–382.
19 Bartl,S., Taplick,J., Lagger,G., Khier,H., Kuchler,K. and Seiser,C. (1997) *Mol. Cell. Biol.*, **17**, 5033–5043.
20 Reichard,P. (1988) *Annu. Rev. Biochem.*, **57**, 349–374.
21 Wilkinson,Y.A. and McKenna,P.G. (1989) *Leukemia Res.*, **13**, 615–620.
22 Ottavio,L., Chang,C.D., Rizzo,M.G., Travali,S., Casadevall,C. and Baserga,R. (1990) *Mol. Cell. Biol.*, **10**, 303–309.
23 Ash,J., Ke,Y., Korb,M. and Johnson,L.F. (1993) *Mol. Cell. Biol.*, **13**, 1565–1571.
24 Lindsten,T., June,C.H., Thompson,C.B. and Leiden,J.M. (1988) *Mol. Cell. Biol.*, **8**, 3820–3826.
25 Gerez,L., Arad,G., Efrat,S., Ketzinel,M. and Kaempfer,R. (1995) *J. Biol. Chem.*, **270**, 19569–19575.
26 Xie,W.L., Chipman,J.G., Robertson,D.L., Erikson,R.L. and Simmons,D.L. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 2692–2696.
27 Ng,S.Y., Gunning,P., Liu,S.H., Leavitt,J. and Kedes,L. (1989) *Nucleic Acids Res.*, **17**, 601–615.
28 Silverman,T.A., Noguchi,M. and Safer,B. (1992) *J. Biol. Chem.*, **267**, 9738–9742.
29 Li,Y., Li,D., Osborn,K. and Johnson,L.F. (1991) *Mol. Cell. Biol.*, **11**, 1023–1029.
30 Knee,R. and Murphy,P.R. (1997) *Neurochem. Int.*, **31**, 379–392.
31 Wagner,E.G. and Simons,R.W. (1994) *Annu. Rev. Microbiol.*, **48**, 713–742.
32 Inouye,M. (1988) *Gene*, **72**, 25–34.
33 Kimelman,D. and Kirschner,M.W. (1989) *Cell*, **59**, 687–696.
34 Murphy,P.R. and Knee,R.S. (1994) *Mol. Endocrinol.*, **8**, 852–859.
35 Dolfini,S., Consonni,G., Mereghetti,M. and Tonelli,C. (1993) *Mol. Gen. Genet.*, **241**, 161–169.
36 Noguchi,M., Miyamoto,S., Silverman,T.A. and Safer,B. (1994) *J. Biol. Chem.*, **269**, 29161–29167.
37 Munroe,S.H. and Lazar,M.A. (1991) *J. Biol. Chem.*, **266**, 22083–22086.
38 Chang,Y., Spicer,D.B. and Sonenshein,G.E. (1991) *Oncogene*, **6**, 1979–1982.
39 Nellen,W. and Lichtenstein,C. (1993) *Trends Biochem. Sci*, **18**, 419–423.
40 Kumar,M. and Carmichael,G.G. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 3542–3547.