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Regulation of thymidine kinase protein levels during myogenic withdrawal from the cell cycle is independent of mRNA regulation

Michael K.Gross and Gary F.Merrill*

Department of Biochemistry and Biophysics and Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR 97331, USA

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ABSTRACT

Replication-dependent changes in levels of enzymes involved in DNA precursor biosynthesis are accompanied frequently by changes in levels of cognate mRNA. We tested the common assumption that changes in mRNA levels are responsible for growth-dependent expression of these enzymes using a line of mouse muscle cells that irreversibly withdraws from the cell cycle as part of its terminal differentiation program. Thymidine kinase (TK) mRNA, activity, and protein levels were quantitated in cells transformed with multiple copies of the chicken TK gene. The decline in TK mRNA (both whole cell and cytoplasmic) during myogenesis was poor (2-fold average) and variable (1.2 to 8-fold). In contrast, TK activity always was regulated efficiently (20-fold), even in cells which regulated TK mRNA very poorly. Thus, regulation of TK activity was independent of TK mRNA regulation as myoblasts withdrew from the cell cycle. A TK/ß-galactosidase fusion protein was used to derive an antibody against chicken TK. Immunoblot and immunoprecipitation analyses demonstrated TK protein levels, like TK activity levels, declined to a greater extent than TK mRNA levels. Thus, TK activity likely was regulated by a mechanism involving either decreased translation of TK mRNA or increased degradation of TK protein in committed muscle cells.

Introduction

During non-S phase portions of the cell cycle, the activities of several enzymes involved in DNA precursor biosynthesis decline. A similar reduction in replicative enzyme activities also is observed in growth-arrested quiescent cells or terminally differentiated postmitotic cells. The depression of DNA biosynthetic activities in nonreplicating cells is widely assumed to be due to reduced levels of the encoding mRNA. Numerous studies have demonstrated a positive correlation between levels of a specific replicative enzyme activity and its encoding mRNA (1-12).

Although the studies cited above confirm that growth-coincident changes in replicative enzyme activity are accompanied by shifts in the level of cognate mRNA, such correlative observations do not establish causality. Furthermore, most comparisons of changes in activity and mRNA levels have been qualitative. Rarely have mRNA regulation studies critically addressed the question of whether observed changes in mRNA can account quantitatively and temporally for observed changes in activity.

A direct test of the model that steady state mRNA levels are the primary determinant of replicative enzyme activity would be possible if experimental methods of preventing the decline in mRNA were developed. If the model is correct, preventing the change in mRNA should block the change in enzyme activity. Unfortunately, the more likely routes for altering the pattern of mRNA have not been successful.

Replacing the promoters of cellular thymidine kinase (TK) or dihydrofolate reductase (DHFR) genes with the promoters of the adenovirus major late and herpesvirus TK genes does not result in constitutive expression of DHFR (13) or TK (6) mRNA. Specific genetic manipulations that consistently allow escape from S phase-dependent expression have not been reported.

Myogenic cell lines derived from mouse skeletal muscle (14) are a useful system for studying S phase-dependent expression of replicative enzymes. When mitogenic activity is withdrawn from the culture medium, exponentially growing myoblasts irreversibly withdraw from the cell cycle and commit to terminal differentiation. Complete conversion to a population of postreplicative myocytes occurs within a single cell generation time. Myocytes are biosynthetically active, fusing to form myotubes and elaborating many of the proteins required for muscle structure and function (15, 16).

During the transition from proliferative myoblast to postreplicative myocyte, TK activity rapidly disappears. TK activity also is regulated in TK^- myoblasts transformed with cloned chicken or human TK sequences (1, unpublished observation), but not in myoblasts transformed with herpesvirus TK sequences. Interestingly, TK activity continues to be regulated when the chicken or human TK promoter and 3' nontranslated region are replaced with the herpesvirus TK or SV40 virus early promoter and polyadenylation signals (1, unpublished observation). Either the supplied heterologous promoters or 3' sequences are themselves cell cycle regulated or the *cis* acting information responsible for TK regulation is associated with the protein coding region of the gene. In the latter case, TK could be regulated either transcriptionally by an intragenic control element, or posttranscriptionally by information carried within the encoded RNA or protein. Merrill et al. (1) and Gross et al. (2) showed TK mRNA levels and TK gene transcription were regulated in myoblasts transformed with the intact chicken TK gene, but did not analyze myoblasts transformed with promoter-switched or 3'-switched constructs. Other studies utilizing similar TK genes, but different cell lines and methods of generating proliferative and nongrowing cell populations, generally have confirmed (6, 7, 17, 18) the hypothesis that the body of the TK gene contains the *cis* acting information for growth regulation; although recently, a role for the transcriptional promoter has also been reported (18, 19).

Quantitative measurements of TK mRNA levels in differentiating muscle cell transformants (2) provided the first clue that the loss of TK activity is not due solely to a decline in TK mRNA. In contrast to the stringent regulation of TK activity (usually greater than 10-fold), the regulation of TK mRNA was more relaxed (usually less than 4-fold). At this juncture, the contribution of mRNA decline to activity decline became questionable. One could argue that the relationship between mRNA and protein is sigmoidal and therefore a smallfold decline in TK mRNA could result in a largefold decline in TK activity. Alternatively, changes in mRNA levels as well as translational or posttranslational mechanisms could share in the overall regulation of activity levels. Finally, if TK mRNA was not a limiting determinant of TK activity levels, the smallfold decline in mRNA would not contribute at all to the decline in activity.

To test the causal relationship between changes in mRNA levels and activity levels we exploited the fact that in individual experiments, muscle cell transformants exhibited wide differences in the efficiency with which they regulated TK mRNA levels. TK mRNA and activity levels in proliferating and

postreplicative cells were precisely quantitated in several dozen experiments. No correlation between between ability to regulate mRNA and ability to regulate activity was detectable. Even cells that regulated TK mRNA levels extremely poorly (a 1.2-fold decline in postreplicative cells) regulated TK activity very tightly (a 20-fold decline). These results indicated that changes in steady state mRNA levels were not a significant determinant of TK enzyme activity levels.

The disappearance of TK activity in the continued presence of TK mRNA in nonreplicating cells could be due to: 1) inefficient translation of TK mRNA; 2) increased degradation of TK protein; 3) maintenance of TK protein in an inactive state. To investigate these possibilities, an antibody to TK protein was derived and used to quantitate steady state TK protein levels. Immunological assays established that TK protein levels, like activity levels, declined more than mRNA levels. Our results indicated postreplicative cells were less efficient at generating TK protein from a given quantity of TK mRNA. Either the mRNA was inefficiently translated, or the nascent protein was rapidly degraded. In seeking a mechanistic basis for depressed DNA precursor biosynthesis in nonreplicating cells, future studies should focus on translational or posttranslational control processes.

MATERIALS AND METHODS

Cell Culture and Transformation

Mouse muscle cells were grown and induced to differentiate by mitogen deprivation for 18 hours as described elsewhere (1), except that bovine brain fibroblast growth factor was used in place of chicken embryo extract as the source of mitogen. Muscle cells were cotransformed with pCHTKfl and pKNeo using calcium phosphate precipitation and selection in G418. The plasmid pCHTKfl contained the full length chicken TK gene; it extended from a synthetic BamH1 site located 775 bp upstream from the translation start codon to a synthetic EcoR1 site located 2130 bp downstream from the translation stop codon (20). A TK^- myoblast strain derived from the MM14D line (21) was used in all experiments in which TK activity was monitored. Some experiments, in which only TK mRNA or protein levels were measured, were done with a $HPRT^-$ derivative of MM14D.

Assays of TK activity and TK mRNA

TK activity in soluble extracts was measured and normalized to DNA content as described by Merrill *et al.* (1). Total RNA was isolated by extraction in guanidinium isothiocyanate and ultracentifugation through CsCl as described previously (2). Production of synthetic RNA probe and standards, and absolute TK mRNA quantitation via RNase mapping were described in detail elsewhere (2). Laser densitometry was used to compare signal intensities in standard and sample lanes of RNase quantitation gels. Northern analysis, using formaldehyde gels , was as described previously (2).

Isolation of Nuclear and Cytoplasmic RNA

All subcellular fractionation procedures were carried out at 4^{o} C. Cultured myoblasts were rinsed and harvested in phosphate buffered saline (approx. $25x10^6$ cells in 1 ml). Cells were centrifuged 10 minutes at 500 rpm in a tabletop centrifuge. After aspirating the supernatant, the pellet was loosened by low speed vortexing and resuspended in 5 ml RSB (10 mM Tris pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$) by

gentle swirling. Cells were centrifuged again and the pellet loosened by low speed vortexing. The pellet was resuspended in RSB containing 100 U/ml RNasin (Promega), 10 mM DTT, and 0.5% NP40, and vortexed vigorously for 20 seconds. After 5 minutes, cells were disrupted with 5 strokes of a hand operated dounce homogenizer. Samples were centrifuged 20 minutes at 2000 rpm in a tabletop centrifuge. Cytoplasmic total nucleic acid (TNA) was isolated immediately from the supernatant (as below). The pellet was resuspended in 2 ml of RSB, centrifuged, and the new pellet resuspended in 2 ml RSB. Nuclear TNA was isolated from this fraction. TNA was isolated from fractions by adjusting to 1xTES (10 mM Tris pH 7.5, 5 mM EDTA, 1% SDS) and 0.2 mg/ml Proteinase K, and incubating one hour at 55°C. Samples were adjusted to 250 mM NaCl, phenol /chloroform extracted, and ethanol precipitated. DNA content in each fraction was determined fluorometrically (22). TNA samples were treated with DNase and precipitated in 2 M ammonium acetate and 40% isopropanol to remove oligonucleotides.

Production and Purification of anti-TK Antibody

Due to its rareness, we anticipated difficulty in isolating TK protein from vertebrate cells. Therefore, to generate enough TK antigen for immunizations, we used a bacterial expression vector. The parental expression plasmid pMLB1113 was obtained from M. L. Berman and had a polylinker located between the promoter/operator (P/O) sequences and lac Z coding region, and also contained an overexpressing lac repressor gene (I^q). To construct pMLB1113TK/ß-gal, a TK cDNA fragment extending from an EcoR1 linker 45 bp downstream of the start codon to a Pvu2 site 39 bp upstream of the stop codon was inserted into the EcoR1 and Hind3 (filled in) sites of the polylinker region. The plasmid had a continuous open reading frame starting with 7 codons from the polylinker (fMTMITNS), followed by codons 16-212 of TK, 3 codons from the polylinker (SLA), and codons 7-1025 of ß-galactosidase. A second expression plasmid pMLB1113ΔN15rTK was constructed by first inserting the genomic EcoR1/H3 fragment containing the coding region of TK into the polylinker and then replacing the EcoR1/Bgl2 genomic fragment with a cDNA fragment extending from an EcoR1 linker 45 bp downstream of the start codon to the Bgl2 site in the seventh exon. This plasmid had an open reading frame with 7 codons from the polylinker (fMTMITNS) followed by codons 16-223 and the stop codon from TK. It encodes a protein identical in sequence to native chicken TK except that 7 heterologous amino acid residues replace the native 15 amino-terminal residues. Plasmids were transformed into DH5Δlac (derivative of DH1 (23) obtained from M.L. Berman).

Bacteria transformed with pMLB1113TK/ß-gal expressed large quantities of a protein with the mobility expected for the 139 kD TK/ß-gal fusion protein when induced with IPTG (isopropyl-ß-D-thio-galactopyranoside). Soluble extracts from bacteria transformed with either pMLB1113TK/ß-gal or pMLB1113ΔN15rTK had very high levels of TK activity. On a per unit DNA basis, IPTG-induced bacteria transformed with pMLB1113TK/ß-gal had 10^3-fold more activity than uninduced bacteria and 10^7-fold more activity than vertebrate cells. Since TK/ß-gal was difficult to solubilize, TK activity in soluble extracts probably underestimated the total amount of fusion protein produced. We were not able to compare specific activities of TK/ß-gal or ΔN15rTK with the native TK protein since the actual concentration of TK protein in each extract was unknown. However, we note the 15 N-terminal and 12 C-terminal amino acids of the native TK protein that were missing in the fusion protein were not essential for catalysis. Also,

either enzymatic activity did not require posttranslational modifications of the protein, or bacteria were capable of carrying out such modifications.

TK/ß-galactosidase (TK/ß-gal) was isolated from overnight cultures grown in 2xYT (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) containing 0.2 mM IPTG. Total bacterial protein was electrophoresed on denaturing (24) preparative gels. Gels were surface stained with Coomasie blue and the fusion protein band excised and electroeluted. Yield was approximately 1 mg per 50 ml culture, as determined by the colorometric assay of Bradford (25). A 1:1 emulsion of TK/ß-gal sample with Freunds complete adjuvant was injected intradermally in 15 sites (17 µg/site) on the backs of two male New Zealand White rabbits. Five weeks later rabbits were given intramuscular injections in the hind legs with antigen (0.25 mg/rabbit) emulsified with Freunds incomplete adjuvant (1:1). After 10 days immune serum was collected twice weekly for 3 weeks by ear bleeds.

Anti-TK antibody was purified from sera by double affinity chromatography. The methods of Carroll and Laughon (26) were used to partially purify ß-gal and TK/ß-gal from soluble and insoluble fractions, respectively, and to couple each protein to sepharose-4B (about 1 mg protein/ml column matrix). All antibodies against ß-gal were removed from immune serum by multiple passages through a 30 ml column of ß-gal-sepharose-4B. Eluent was applied to a 10 ml column of TK/ß-gal-sepharose-4B and anti-TK antibodies eluted with 4 M guanidine hydrochloride. The eluate was dialysed two days against phosphate buffered saline, concentrated by ultrafiltration, and stored as aliquots at -70°C.

Western Blot Analysis

To analyse TK protein content, cells were harvested with collagenase, centrifuged, resuspended in growth medium for counting, centrifuged, and resuspended in serum free medium. Aliquots of $5x10^6$ cells were collected by centrifugation and frozen at -70°C. Pellets were resuspended in 100 µl of TK extraction buffer (27) and sonicated twice for 5 seconds on ice. Sonicates were clarified by centrifugation, and 40 µl was electrophoresed on SDS polyacrylamide (15%) minigels (24). Gels were soaked 10 min in transfer buffer (25 mM Tris-OH, 190 mM glycine, 20% methanol, 0.1% SDS) and proteins electroblotted to nitrocellulose (presoaked 4 hours in dH_2O) overnight at 150 mA constant current. Blots were baked, blocked 1-4 hours with 25 mg/ml fraction V bovine serum albumin in 1xTTBS (20 mM Tris pH 7.5, 0.5 M NaCl, 0.5% Tween-20), and probed 24 hours with affinity purified anti-TK antibody in blocking solution. Blots were washed 5 min with 1xTBS (1xTTBS without Tween-20), twice for 5 min with 1xTTBS, and 5 min with 1xTBS. [^{125}I]protein A ($2x10^5$ cpm/ml) in blocking solution was applied to blots for 1 hour and the wash sequence repeated. [^{125}I]protein A was freshly prepared by the method of Haas and Bright (28). Dried blots were exposed to Kodak XAR-5 film with intensifying screens. Longer exposures (up to 2 months) without intensifying screens were used to obtain sharp bands.

Radiolabeling and Immunoprecipitation

For [^{35}S]Met radiolabeling, cells were adapted to medium made with Ham's F12/DMEM (GIBCO), because a Met-free formulation of this basal medium was available. Cultures containing about 2×10^6 proliferating or committed cells were rinsed twice with Met-free medium and incubated in 1 ml Met-free medium containing 10^{-6}M insulin and 150-250 µCi/ml [^{35}S]Met (14-24 nM; 1.0 Ci/µmol)(New England

Nuclear). In pulse/chase experiments, labeled cultures were rinsed twice with basal medium and incubated in 10 ml Ham's F12/DMEM containing 10^{-6}M insulin and 120 mM extra Met.

Clarified cell extracts from radiolabeled cells were prepared as for TK activity measurements (27). Immunoprecipitations were carried out on ice. Cell extracts (50-100 µl) were incubated 1 hr with a titred amount of antibody (0.25 µl/10^6 cells) and 30 min with 0.2 volumes of a 1:1 slurry of pre-washed Protein A agarose in TK extraction buffer. Immune complexes adsorbed to the agarose were collected by centrifugation (20 sec at 11,000 x g) and washed five times with phosphate buffered saline. Washed pellets were denatured in 40 µl loading buffer, and 28 µl was applied to SDS polyacrylamide (15%) minigels (24) Gels were soaked 1 hr in fix (10% MeOH, 10% TCA, 30% HOAc), 1 hr in water, and 30 min in Fluoro-Hance (Research Products International), and dried overnight between cellophane membranes (Bio-Rad). Dried gels were exposed to XAR-5 film at -70° using an intensifying screen.

Incorporation of [^{35}S]Met into total soluble protein was determined by dotting aliquots (2-10 µl) of Protein A immunoprecipitation supernatants onto Whatman GFC filters pre-wet with 10% trichloroacetic acid, 2% sodium pyrophosphate (TCA solution). Filters were bathed several minutes in TCA solution and then were rinsed on a vacuum filter holder with TCA solution followed by 95% EtOH. Dried filters were digested with 0.2 ml Soluene (Packard) and counted in 2 ml Omnifluor (New England Nuclear).

RESULTS

Variable and Poor Regulation of TK mRNA

Fig.1A shows TK mRNA regulation during myoblast differentiation in six representative independent transformant pools. TK$^-$ myoblasts were cotransformed with pCHTKfl and pKNeo, and stable transformant pools selected in G418. Absolute TK mRNA levels were measured in proliferating and committed populations by quantitative RNase mapping (2). Qualitatively, TK mRNA levels declined in all six transformant pools (compare P and C lanes). However, quantitative comparison of proliferative and committed TK mRNA levels, using the standard curve of TK pseudo-mRNA (left lanes), revealed significant variation in the fold decline. Examination of our entire TK mRNA regulation data set on TK$^-$ or HPRT$^-$ myoblasts transformed with the chicken TK gene (24 determinations), showed that TK mRNA was regulated as little as 1.2-fold and as much as 8-fold (data not shown). The variation was not due to imprecision in the RNase quantitation method as repeated analyses on identical RNA preparations showed relatively little variation.

To find out if the smallfold decline of TK mRNA in some transformants was due to saturation of a regulatory mechanism by high initial levels of TK mRNA, regulation data from 12 experiments was collected and arranged according to proliferative TK mRNA levels (Fig.1B). Transformants expressing low initial message levels, similar to levels found in proliferative tissues *in vivo* (1-20 copies/cell; ref. 2), were no more effective at clearing out TK mRNA during terminal differentiation than transformants initially expressing two orders of magnitude more message. Hence poor regulation of TK mRNA in some transformant pools was not due to saturation of a regulatory mechanism.

The variation in TK mRNA regulation was not an artifact of different transformations since TK mRNA regulation was measured three times in independent growths of a monoclonal transformant and

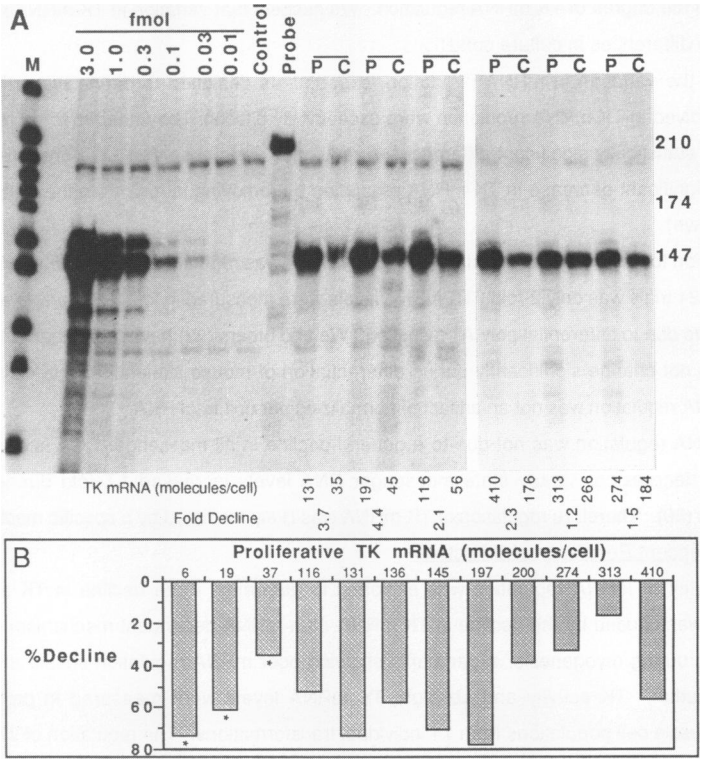

Figure 1. [A] Representative RNase protection assay of absolute TK mRNA levels in proliferative and committed muscle cell transformants. A 147 bp fragment is protected when a complimentary, 210 base RNA probe spanning the sixth intron acceptor site is hybridized to TK mRNA and subsequently digested with RNase. The 12 right hand lanes each contain 20 μg of RNA from either proliferative (P) or committed (C) populations of six independently derived transformant pools. The six lanes at left contain dilutions of synthetic TK pseudo-mRNA that were used to establish a standard curve from which absolute mRNA levels in test samples were determined (numerals below each lane). Absolute levels in the right panel were established from a different standard curve (not shown). The fold decline is the quotient of proliferative divided by committed message levels. The control lane contains 20 μg of yeast RNA.

[B] Data from 12 independent experiments were arranged in order of increasing proliferative TK mRNA concentration (numbers on top). Percent decline refers to the reduction in TK mRNA 18 hours after inducing differentiation. Polyclonal transformants were used in all experiments except those labeled with asterisks, which designate experiments on a monoclonal transformant.

significantly different regulation was observed in each trial (asterisks in Fig. 1B). Chromosomal integration sites were not relevant since polyclonal transformant pools were used in most experiments. Several other possible sources of variation were considered: passage number after transformation; FGF batch; harvesting protocol; committed cell contaminants in the proliferative population; proliferative cell contaminants in the committed population. None provided a consistent explanation for observed

differences in the degree of TK mRNA regulation. We suspect that variation in TK mRNA regulation was due to subtle differences in culture conditions.

Due to the variation in mRNA regulation, experiments designed to define *cis* acting regulatory elements involved in TK mRNA regulation were exceedingly difficult. They needed to be repeated many times before statistically significant differences in regulation were observed. We observed a slight but statistically significant decrease in TK mRNA regulation by removing introns from the transformed gene (data not shown).

In addition to being regulated variably, TK mRNA also was regulated poorly. The average decline in TK mRNA in 24 trials was only 2-fold. TK mRNA levels were measured in total RNA preparations, thereby avoiding errors due to differential poly A^+ selection. We and others (29) have found that the ratio of RNA to DNA does not change significantly during differentiation of mouse skeletal muscle cells. Therefore, poor TK mRNA regulation was not an artifact of normalizing per unit total RNA.

TK mRNA regulation was not due to a general decline in all messenger RNA in committed cells. Rather than decreasing, steady state messenger RNA levels increased 1.5-fold during muscle cell differentiation (30). Therefore regulation of TK mRNA was likely mediated by a specific mechanism.

mRNA-Independent Regulation of TK activity

Variability of mRNA regulation was exploited to determine if the decline in TK activity during myogenesis was caused by the decline in TK mRNA. If a mRNA-dependent mechanism controlled TK activity levels during myogenesis, experiments showing poor mRNA regulation should also show poor activity regulation. TK activity and absolute TK mRNA levels were measured in proliferative and committed muscle cell populations from 14 individual transformations. The regulation of TK activity was compared in experiments which showed different degrees of TK mRNA regulation. Fig. 2A shows that regardless of what percent of the original TK mRNA remained in committed cells in a given experiment, the percent of the original TK activity that remained was always lower. The average decline in activity (20-fold) was an order of magnitude greater than the average decline in mRNA (twofold). Moreover, the magnitude of the decline in activity did not depend on how well mRNA was regulated. For example, in experiment 1 only 20% of TK mRNA remained in committed cells and in experiment 14 almost all (80%) TK mRNA remained, yet in both experiments less than 3% of TK activity remained. Despite having an ample supply of TK mRNA, committed cells did not have significant TK activity.

Several models involving alternative splicing of TK mRNA could account for the lack of TK activity in committed cells. For example, committed cells could produce an alternatively spliced TK mRNA, which was detected by the RNase quantitation probe, but was inefficiently translated. Conversely, proliferative cells could express low amounts of a very efficiently translated alternatively spliced TK mRNA which committed cells do not express. To test these types of models, RNA from proliferative and committed muscle cell transformants were analyzed on northern blots (Fig. 2B). In every transformant analyzed, the major band visible had the mobility expected for the 2.1 kb messenger RNA encoding TK and the intensity of the 2.1 kB band decreased in committed cells. No new types of TK mRNA were observed in either proliferative or committed cells. Hence, within the resolution of a northern blot assay, alternative splicing did

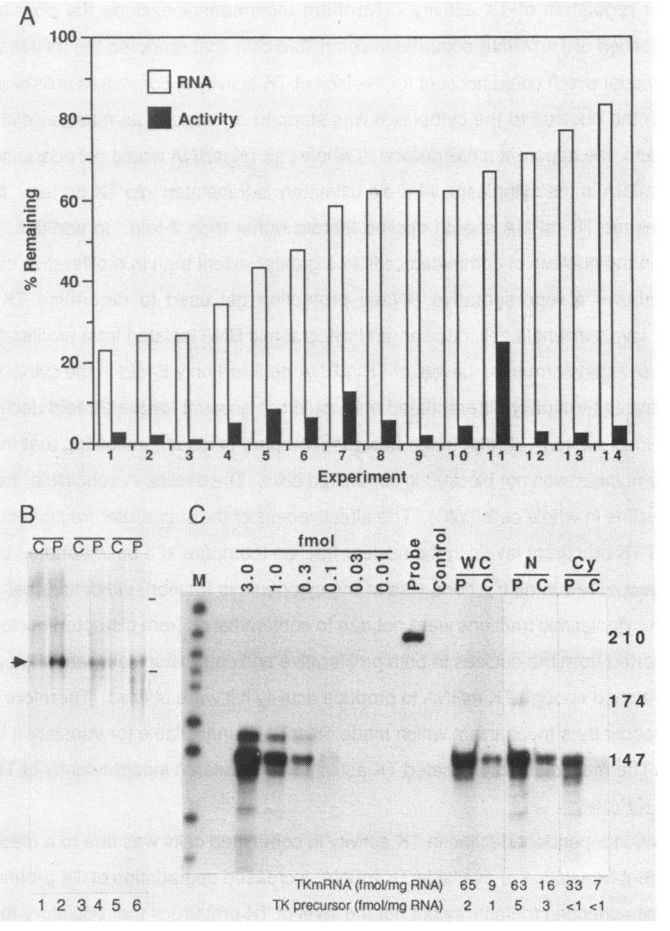

Figure 2. [A] Regulation of TK activity and its independence from TK mRNA regulation. Proliferative and committed levels of TK activity and TK mRNA were measured in parallel in several TK$^-$ muscle cell lines polyclonally cotransformed with pCHTKfl and pKNeo. The percent of proliferative levels remaining in committed cells is plotted for each experiment. Experiments were organized by efficiency of TK mRNA regulation. All experiments had proliferative TK activity levels above 0.5 pmol TdR/min/μg DNA.

[B] Northern blot confirmation of TK mRNA size homogeneity during differentiation. Proliferative (P) and committed (C) cell RNA from three muscle lines cotransformed with pCHTKfl and pKNeo were analyzed. Blots were probed with a nick-translated 2.3 kb Kpn1/Hind3 fragment of pCHTKfl. The cell lines used and quantity of RNA assayed were: H$^{cfl:neo3}$pool, 10 μg (lanes 1-2); TK$^{cfl:neo3}$pool, 12 μg (lanes 3-4); TK$^{cfl:neo1}$pool, 20 μg (lanes 5-6). Arrow indicates 2.1 kb TK mRNA; bars indicate 5.1 and 1.9 kb rRNA markers.

[C] TK mRNA regulation in nuclear and cytoplasmic compartments of muscle cells. Whole Cell (WC), nuclear (N), and cytoplasmic (Cy) RNA from proliferative (P) and committed (C) cultures of a polyclonal muscle cell transformant line carrying multiple copies of the chicken TK gene was analyzed by RNase protection. The line, H$^{cfl:neo1}$pool, was derived by cotransformation of HGPRT$^-$ myoblasts with pCHTKfl and pKNeo. Standard curve, probe, and control lanes were as described in Fig. 1. TK precursor RNA protected a 174 base fragment and TK mRNA protected a 147 base fragment.

not account for regulation of TK activity. Northern blots cannot exclude the possibility that a small covalent modification of TK mRNA occurred in committed cells that rendered the mRNA untranslatable.

Another model which could account for the lack of TK activity in committed cells was that transport of TK mRNA from the nucleus to the cytoplasm was stopped or reduced as muscle cells differentiated. If this were the case, the apparent small decline in whole cell TK mRNA would not adequately reflect a large decline in TK mRNA in the cytoplasm, where it ultimately is translated into TK protein. If this model were correct, cytoplasmic TK mRNA should decline 20-fold rather than 2-fold. In addition, TK mRNA levels might build up in the nucleus of committed cells to a greater extent than in proliferative cells.

Fig. 2C shows a representative RNase protection gel used to determine TK mRNA and TK precursor RNA levels in whole cell, nuclear, and cytoplasmic RNA isolated from proliferative or committed mouse muscle cell transformants. Levels of TK mRNA declined only 5-fold in the cytoplasm (compare Cy lanes) as myoblasts terminally differentiated and could not account for the 20-fold decline in TK activity. TK mRNA declined similarly (4-fold) in the nucleus (compare N lanes), indicating that the transport of TK mRNA from the nucleus was not blocked in committed cells. The decline in subcellular fractions was similar to the the decline in whole cells (WC). The effectiveness of the subcellular fractionation was confirmed by the enriched TK precursor levels in the nuclear fraction (compare 174 base bands). Less than 0.1% of the total DNA recovered in all fractions was in the cytoplasmic fraction, indicating that the observed TK mRNA levels in cytoplasmic fractions were not due to contamination from disrupted nuclei. TK mRNA was similarly transported from the nucleus in both proliferative and committed cells, and the cytoplasm of committed cells contained enough TK mRNA to produce activity if it were utilized. Therefore, regulation of TK activity did not occur by a mechanism which made TK mRNA unavailable for translation by sequestering it in the nucleus. The results above indicated TK activity was regulated independently of TK mRNA.

Regulation of TK Protein

The mRNA-independent decline in TK activity in committed cells was due to a mechanism involving either less efficient translation of available TK mRNA, increased degradation of TK protein, or posttranslational processes affecting the activity but not the level of TK protein. If the regulatory mechanism involved only posttranslational activity modulation, then TK protein levels should change no more than the smallfold change in TKmRNA levels. If, on the other hand, the regulatory mechanism involved either differential translation or protein degradation, then TK protein should decline to the same extent as TK activity during myogenesis. To distinguish between these models, an antibody against chicken TK was generated using a bacterially-produced chicken TK/ß-galactosidase fusion protein as antigen. The antibody was used to assay relative TK protein levels in proliferative and committed muscle cell transformants.

Probably due to its rarity, detection of TK protein by immunoblotting was difficult. Western blots in which maximal, non-overloading amounts of extract were assayed ($2x10^6$ cell equivalents) failed to give a detectable TK signal when probed with unfractionated antiserum and horseradish peroxidase-conjugated second antibody. To improve sensitivity and reduce background, anti-TK antibody was purified by affinity chromatography, and [^{125}I]protein A was used as visualization reagent instead of an enzyme-

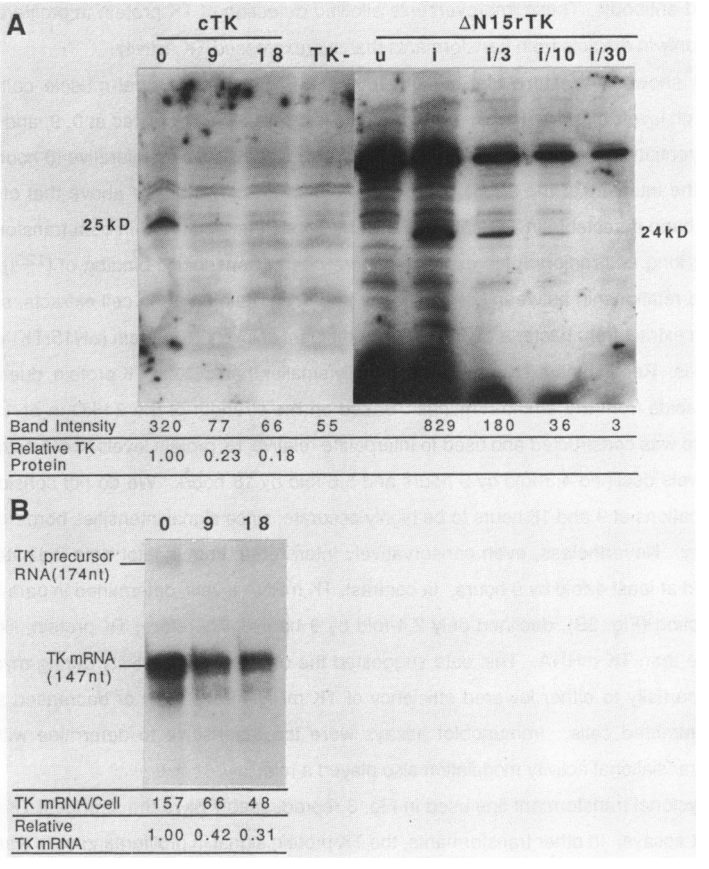

Figure 3. [A] Western blot of TK protein during muscle cell differentiation. Protein from polyclonal muscle cell transformants carrying 50 copies of the chicken TK gene (cTK) was harvested at zero (0), nine (9), and eighteen (18) hours after inducing differentiation, and $2x10^6$ cell equivalents were immunoblotted as described in Materials and Methods. (The line used, $H^{cfl:neo}1$pool, was derived by transforming HGPRT$^-$ myoblasts with pCHTKfl and pKNeo at a 200:1 ratio). Protein from nontransformed myoblasts (TK$^-$) was used as a control for nonspecific binding. Successive threefold dilutions of bacterial extracts containing recombinant TK protein (ΔN15rTK) were included to allow calculation of the fold decline in sample lanes. Protein from an uninduced bacterial culture (U) was included as a control. The blot was probed with purified anti-TK antibody and [^{125}I]protein A. Band intensity was determined densitometrically by scanning each lane twice with a narrow laser beam and weighing the peak with a mobility corresponding to TK protein. The contribution of a faint 25 kD nonspecific band present in TK$^-$ extracts was subtracted from cTK band intensities. Relative TK protein in cTK samples was interpolated from a standard curve of band intensity versus the dilution coefficient for ΔN15rTK samples. [B] At the same time that cultures were harvested for immunoblotting, parallel cultures were harvested for quantitation of TK mRNA levels by RNase protection. For each timepoint, 10^6 cell equivalents of RNA (10 μg) were analyzed. Arrows designate expected mobilities of fragments protected by TK mRNA (147 nt) and TK precursor RNA (174 nt). Indicated values for TK mRNAs/cell were interpolated from a range of TK pseudo-mRNA standards run in parallel lanes (not shown).

linked second antibody. These improvements allowed detection of TK protein in proliferating myoblast extracts, but only in extracts from transformants that overexpressed TK activity.

Fig. 3A shows a Western blot of soluble protein from a polyclonal muscle cell transformant expressing high levels of TK activity in proliferating cells. Cells were harvested at 0, 9, and 18 hours after inducing differentiation. A band of the correct size (25 kD) was visible in proliferative (0 hour) cell extracts. By 9 hours, the intensity of the 25 kD band had declined to a level barely above that of a nonspecific co-migrating band detectable in TK^- cells. The many bands that appeared in both transformant and TK^- extracts after long autoradiographic exposures were due to nonspecific binding of [^{125}I]protein A. To determine the relationship between band intensity and TK protein levels in cell extracts, serial threefold dilutions of an extract from bacteria expressing recombinant chicken TK protein (ΔN15rTK) were analyzed in parallel lanes. Recombinant TK protein was slightly smaller than cellular TK protein, due to the deletion of 15 amino acids from the amino-terminus. Based on the strength of the 24 kD in ΔN15rTK lanes, a standard curve was constructed and used to interpolate relative TK protein levels in cell extracts. Relative TK protein levels declined 4.3-fold by 9 hours and 5.6-fold by 18 hours. We do not consider TK protein level determinations at 9 and 18 hours to be highly accurate, since signal intensities bordered at the limits of detectability. Nevertheless, even conservatively interpreted, immunoblot data indicated TK protein levels declined at least 4-fold by 9 hours. In contrast, TK mRNA levels, determined in parallel cultures by RNase protection (Fig. 3B), declined only 2.4-fold by 9 hours. Therefore, TK protein, like TK activity, declined more than TK mRNA. This data suggested the decline in TK activity during myogenesis was due at least partially to either lowered efficiency of TK mRNA translation or decreased stability of TK protein in committed cells. Immunoblot assays were too insensitive to determine with confidence whether posttranslational activity modulation also played a role.

The polyclonal transformant line used in Fig. 3 reproduceably gave the strongest TK protein signal in immunoblot assays. In other transformants, the TK protein signal in proliferating cells was weaker, and therefore, the absence of a TK signal in committed cells was less informative in terms of quantifying the fold decline in TK protein. However, we report qualitatively, that in all other transformants tested (n=6) we never observed persistence of TK protein in committed cells.

An alternative method of determining TK protein levels in proliferative and committed muscle cells was to metabolically label cells with [^{35}S]Met and to quantitate TK-specific radioactivity by immunoprecipitation and gel electrophoresis. By using sufficiently long labeling times, an estimation of TK protein steady-state levels was possible. In addition to being more sensitive, immunoprecipitation assays could potentially reveal the rate of synthesis and degradation of TK protein in proliferating and committed cells.

Two multicopy, polyclonal, muscle cell transformant lines were pulsed or pulse/chased with [^{35}S]Met as described in Figs. 4A and B. In proliferating cells, radiolabeled TK protein was detectable using [^{35}S]Met pulses as brief as 15 minutes. The intensity of the TK specific band increased less rapidly as pulse length was extended, indicating that labeling equilibrium was being approached. The halflife of TK protein was determined by measuring the rate of decline in TK band intensity when labeled cells were chased with unlabeled methionine. Both transformant populations yielded halflife values of about 45

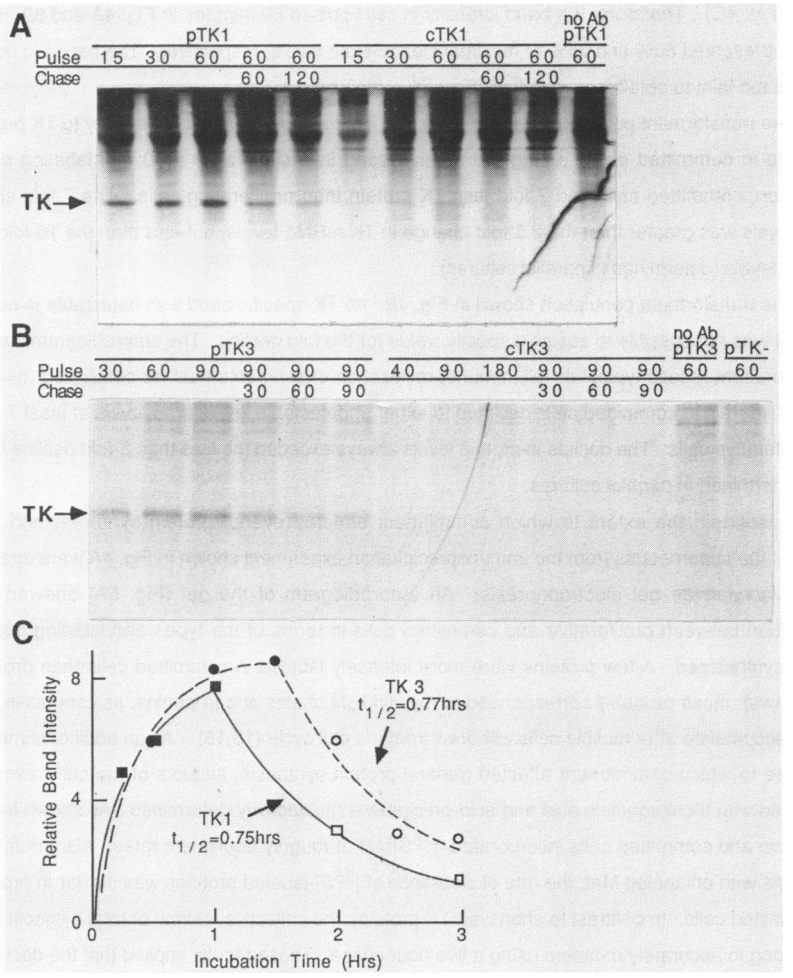

Figure 4. Incorporation of [^{35}S]Met into TK protein in proliferating and committed muscle cells. Autoradiograms show immunoprecipitation data from two polyclonal transformant populations, TK$^{cfl:neo1}$ pool (TK 1 in panel [A]) and TK$^{cfl:neo3}$ pool (TK3 in panel [B]), derived by cotransforming TK$^-$ myoblasts with pCHTKfl and pKNeo at a 30:1 ratio. Prefixes "p" and "c" denote proliferative and committed cells, respectively. Cells were pulsed with [^{35}S]Met and chased with unlabeled Met for the indicated number of minutes. In "no Ab" lanes extracts from proliferative transformants were not incubated with antibody. In the "pTK$^-$" lane extracts from proliferative nontransformed TK$^-$ cells were incubated with antibody. In [A], labeling medium contained 250 μCi/ml, 2.6 x 10^6 cell equivalents were analyzed per lane, and autoradiography was for 3 days. In ([B], labeling medium contained 150 μCi/ml, 3.8 x 10^6 cell equivalents were analyzed, and autoradiography was for 5 days. Panel [C] summarizes densitometric measurements of TK band intensity in proliferating cells: solid line shows data from autoradiogram in [A]; broken line shows data from autoradiogram in [B]; solid and open symbols represent data from pulsed and pulse/chased dishes, respectively. Decay curves represent the best fit line satisfying the equation $N=N_0e^{-.693t/t1/2}$. In both experiments, determinations done on parallel dishes confirmed TK activity declined more than tenfold and TKmRNA declined less than threefold during commitment.

minutes (Fig. 4C). Therefore, the band intensity in cells pulsed 60 minutes in Fig. 4A and 90 minutes in Fig. 4B represented 60% and 75% of maximal steady-state levels, respectively. The bands in committed cells were too faint to obtain accurate halflife measurements.

In the transformant population shown in Fig. 4A, a band corresponding in mobility to TK protein was detectable in committed cells. Using the relative band intensities after a 60 min labeling period for comparison, committed cells had 7-fold less TK protein than proliferating cells. The 7-fold change in protein levels was greater than the 2.5-fold change in TK mRNA levels, but less than the 10-fold change in activity levels (determined in parallel cultures).

In the transformant population shown in Fig. 4B, no TK-specific band was detectable in committed cells; so it was not possible to assign a specific value for the fold decline. The autoradiograms in Figs 4A and B represent results typical of all immunoprecipitation experiments thus far conducted (n=4). In all cases, TK protein in committed cells declined to either undetectable levels or to levels at least 7-fold less than proliferative cells. The decline in protein levels always exceded the less than 3-fold decline in mRNA levels, determined in parallel cultures.

To ascertain the extent to which commitment affected overall protein synthesis and stability, aliquots of the supernatants from the immunoprecipitation experiment shown in Fig. 4A were analyzed by SDS polyacrylamide gel electrophoresis. An autoradiogram of the gel (Fig. 5A) showed general conservation between proliferative and committed cells in terms of the types and labeling intensity of proteins synthesized. A few proteins were more intensely labeled in committed cells than proliferating cells (arrows); these probably corresponded to myosin light chains and troponins, as contractile proteins begin to accumulate after muscle cells withdraw from the cell cycle (15,16). As an additional measure of the degree to which commitment affected general protein synthesis, aliquots of the cell extracts were precipitated with trichloroacteic acid and acid-precipitable radioactivity determined. As shown in Fig. 5B, proliferative and committed cells incorporated [^{35}S]Met at roughly equivalent rates. Also, during chase incubations with unlabeled Met, the rate of clearance of [^{35}S]-labeled proteins was similar in proliferative and committed cells. In contrast to shortlived TK protein, the collective lifetime of total cytosolic proteins was too long to accurately measure using a two hour chase. These results implied that the decline in TK protein levels evident in immunoprecipitation assays was reasonably specific. Also, the decline in [^{35}S]-labeled TK protein in committed cells was not due to a nonspecific effect of mitogen-depletion on amino acid transport or the specific radioactivity of the tRNA^{Met} pool.

In both immunoblot and immunoprecipitation experiments, TK protein levels declined to a greater extent than TK mRNA levels during myogenic withdrawal from cell cycle. In general the decline in activity levels was 10-fold or greater; the decline in mRNA levels was 3-fold or less; the decline in protein levels was at least 7-fold. From these results we conclude nonreplicating cells were less efficient at generating TK protein from a given quantity of TK mRNA. Either the mRNA was translated less efficiently or the synthesized protein was degraded more rapidly. Within the detection limits of our immunological assays, protein levels did not change as much as activity levels. Formally, the disparity between the size of the change in protein levels and activity levels suggests the existence of a posttranslational mechanism

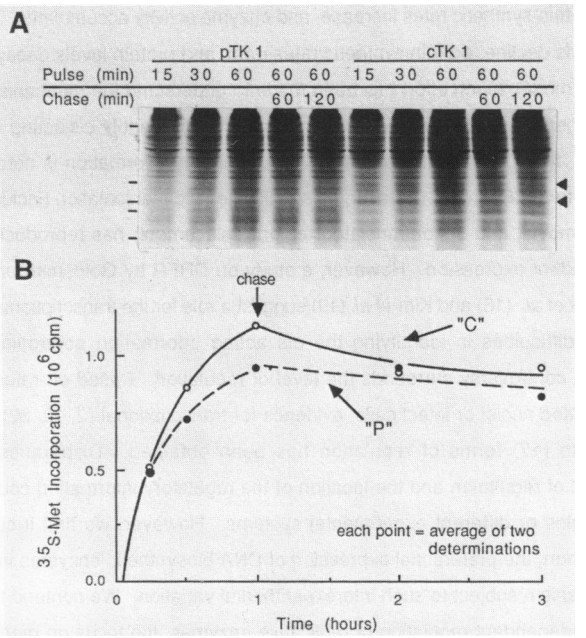

Figure 5. Incorporation of [^{35}S]Met into total soluble protein in proliferating and committed muscle cells. Aliquots of the Protein A supernatant from the immunoprecipitation samples used in Fig. 4A were either run on denaturing SDS polyacrylamide gels and autoradiographed [A] or total protein was precipitated with 10% TCA and radioactivity in the precipitate determined by scintillation counting [B]. Bars at left indicate molecular weight markers (from the top: 66, 24, 20.1, 18.4, and 14.3 kD, respectively). Arrows at right indicate bands that increase in intensity with committment.

controlling the catalytic efficiency of TK enzyme. However, due to difficulties in detecting TK protein in committed cells, the 7-fold decline in protein levels reported here is a minimum estimate. Thus, although our data establish the existence of either a translational or degradational mechanism governing TK protein levels, the possible existence of an ancillary mechanism affecting the catalytic rate of TK protein is conjectural.

DISCUSSION

Growth-dependent expression of genes encoding replicative enzymes has usually been ascribed to changes in steady state mRNA levels. In fibroblastic cells released from growth arrest, changes in mRNA levels can account quantitatively and temporally for increases in DHFR (3), thymidylate synthetase (5), and TK (4) activity levels. Similarly, in several tissues of the developing chicken embryo, declines in TK mRNA levels can account for declines in activity levels (2). Although these correlative studies do not establish causality, they nonetheless are consistent with the simple model that replicative enzyme levels are governed by the abundance of cognate mRNA. As cells enter S phase, mRNAs encoding replicative

enzymes appear, protein synthetic rates increase, and enzyme activity accumulates. After completing replication, mRNA levels decline, protein synthetic rates drop, and protein levels decay. Given the central role of mRNA in this model, much effort has been made to understand the mechanism governing mRNA levels. *In vitro* mutagenesis studies were launched in an effort to identify *cis* acting regulatory elements (1, 6, 7, 13, 18, 31). Most investigations indicate the regulatory information is not associated with the transcriptional promoter (1, 6, 7, 13). Thus far, no specific genetic manipulation (including promoter replacement, intron removal, and 3' nontranslated region replacement) has reproducibly allowed escape from S phase-dependent expression. However, a study on DHFR by Goldsmith *et al.* (31) and recent work on TK by Travali *et al.* (18) and Kim *et al.* (19) suggest a role for the transcriptional promoter .

In addition to difficulties in identifying the *cis* acting information controlling genes encoding replicative enzymes, controversy surrounds the level of regulation. Based on rates of RNA precursor incorporation by isolated nuclei or intact cells, evidence for transcriptional (2, 12, 32), posttranscriptional (13, 33, 34), or both (17) forms of regulation has been obtained. Disparaties and difficulties in establishing the level of regulation and the location of the regulatory information could be due to use of different methodologies or different experimental systems. However, we find it curious that a highly conserved phenomenon, the preferential expression of DNA biosynthetic enzymes in replicating cells, is mediated by a mechanism subject to such interexperimental variation. We contend that in attempting to understand S phase-dependent regulation of replicative enzymes, the focus on mechanisms controlling mRNA levels may be misplaced.

Our results argue against the conventional model that mRNA levels are a limiting determinant of TK activity levels. In earlier work we noted the decline in TK mRNA during muscle cell differentiation in culture could not account fully for the observed decline in enzyme activity (2). We suggested that a transcriptionally mediated decline in TK mRNA levels may contribute to the decline in TK activity, but a translational or posttranslational mechanism must also be operative. In the present study, we exploited intrinsic variability in the degree to which TK mRNA levels were regulated to establish that regulation of TK activity was independent of regulation of TK mRNA. The results in Fig. 2 provide the most vivid support for our conclusion. In all cases, TK mRNA was regulated poorly compared to TK activity. In general, TK mRNA in committed cells was 50% of proliferative levels (a 2-fold decline). In contrast, TK activity in committed cells was 5% of proliferative levels (a 20-fold decline). More importantly, no correlation existed between the degree of mRNA regulation and the degree of activity regulation. In all cases, substantial TK mRNA remained in committed muscle cells and yet was not expressed as TK activity. We cannot exclude the possibility there might be some subtle structural alteration of most of the TK RNA in the transformed cells such that only a minor fraction of the RNA is competent to serve as message, and that the level of the competent fraction declines 20-fold during differentiation. However, this explanation is complex, invoking the presence of a constitutively-expressed incompetent message and a facultatively-expressed competent message. A simpler explanation is that TK mRNA is poorly translated in postreplicative cells or TK protein is degraded or inactivated. Recent results by Travali et al (18) are consistent with our interpretation. These investigators showed that in heat shocked fibroblasts transformed with a chimeric

gene consisting of a heat shock promoter and human TK coding region, TK activity was maximal during S phase, even though TK mRNA was highest in G1.

The twofold reduction in TK mRNA we measured in postreplicative muscle cells was small compared to the multifold reduction others have observed in growth-arrested fibroblastic cells. Fibroblasts arrested by a variety of techniques, such as contact inhibition (34), serum deprivation (4, 35), or drug inhibition (6), typically show a 20-fold or greater decline in TK mRNA compared to exponentially growing cells or cells released from inhibition and allowed to enter S phase. Growth-arrested fibroblastic cells also show large-fold declines in the mRNAs encoding DHFR (9, 13) and thymidylate synthetase (36). One likely explanation for the disparity in mRNA regulation between muscle cell and fibroblast studies takes into account the ontogeny of the two cell types. Muscle cells placed under growth-arresting conditions initiate a developmentally-determined program, characterized by intense biosynthetic activity (16). Fibroblasts placed under growth-arresting conditions may withdraw into a nonphysiological state, characterized by diminished synthetic capabilities and increased degradative activities. Serum-starved fibroblasts possess few polyribosomes, compared with growing cells (37); perhaps the shift to monosomes is indicative of a general destruction of many mRNAs in growth-arrested fibroblasts. In both myoblasts and fibroblasts, TK gene transcription is repressed in nongrowing cells (2, 17). After transcription declines, TK mRNA levels may decay rapidly in growth-arrested fibroblasts and decline more slowly in differentiating muscle cells. With time, TK mRNA levels eventually decline to barely detectable levels in differentiated muscle cells *in vivo* (2) and in culture (unpublished observation). Clearly, a mechanism for regulating TK mRNA levels exists; our argument is that in cells that withdraw from the cell cycle as part of a differentiation program, the slowly occuring decay in mRNA levels is not responsible for the rapid disappearance of enzyme activity.

Fibroblastic cells synchronized by minimally interruptive methods, such as mitotic selection or centrifugal elutriation, show only a modest decline in TK mRNA (35), DHFR mRNA (38), or TS mRNA (36) during non-S phase portions of the cell cycle. Thus, the twofold decline in TK mRNA levels we see in postreplicative muscle cells is similar in magnitude to the decline seen during G1 in a noninterrupted cell cycle. This correspondence reinforces our opinion that the largefold change in mRNA levels observed in cells synchronized by release from growth-arrested conditions is misrepresentative of the mechanism normally governing TK activity levels.

Faced with evidence that the smallfold changes in TK mRNA levels were not responsible for the largefold changes in TK activity, we investigated the possibility that the catalytic rate of the protein was modulated posttranslationally. Several types of posttranslational mechanisms were envisionable. TK activity might depend on an as yet undiscovered regulatory subunit or coenzyme that disappears in nonreplicating cells. Alternatively, activity might be affected by numerous types of covalent modifications: cleavage; glycosylation; acylation; ribosylation; phosphorylation. We were particularly interested in the latter possibility because many growth factor receptors and oncogenes have protein kinase activity (for review see 39), and the activity of certain enzymes are known to be governed by protein kinases (40).

If the mRNA-independent decline in TK activity was due solely to posttranslational events affecting the catalytic rate of TK protein, we would expect TK protein levels to change little during commitment,

commensurate with the smallfold change in mRNA levels. Instead, direct immunological measurement of TK protein levels indicated TK protein declined to a greater extent than TK mRNA in committed cells. The magnitude of the decline in protein indicates the existence of either a translational mechanism controlling the synthesis of TK protein or a degradational mechanism controlling the stability of TK protein.

The minimum detection limits of our immunological assays prevented us from determining whether the decline in TK protein was great enough to account fully for the decline in TK activity. Therefore, although the existence of a translational or degradational mechanism affecting protein levels was established, we did not eliminate the possibility that posttranslational activity modulation contributes to the overall mechanism regulating TK activity levels. We realize that if the decline in TK protein in committed cells is due to a degradational mechanism, posttranslational modifications may play a role in targeting TK protein for destruction.

While our results were being readied for publication, a highly relevant study by Sherley and Kelly (41) was published. Using human fibroblasts synchronized by centrifugal elutriation, these investigators showed 15-fold changes in TK activity levels during the cell cycle, but only 3-fold changes in TK mRNA levels. The relative changes in mRNA and activity strongly resemble our determinations on muscle cells. Using an antibody derived against purified human TK, they showed by immunoblotting that the decline in TK activity was accounted for fully by a decline in TK protein; again, consistent with our findings on muscle cells. Finally, through a series of labeling experiments with [^{35}S]Met, they showed that cyclical expression of TK protein was due to increased translation of TK mRNA during S and G2, and increased degradation of TK protein during early G1. If these results can be extrapolated to the muscle system, postreplicative muscle cells contain neglible TK activity because preexisting TK protein was degraded as the cells withdrew from the cell cycle in G1, and no further TK protein is being synthesized because TK mRNA is not translated in G1 cells.

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*To whom correspondence should be addressed

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