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tk Enzyme Expression in Differentiating Muscle Cells Is Regulated Through an Internal Segment of the Cellular *tk* Gene

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Thymidine kinase (tk) enzyme expression is shut down when cultured skeletal muscle cells terminally differentiate. This regulation is mediated by a rapid and specific decline in the abundance of cellular *tk* mRNA. *tk*-deficient mouse myoblasts were transformed to the *tk*-positive phenotype by using both the cellular *tk* gene of the chicken and the herpesvirus *tk* gene. Myoblasts transformed with the cellular *tk* gene effectively regulate tk enzyme activity upon terminal differentiation. Conversely, myoblasts transformed with the herpesvirus *tk* gene continue to express tk enzyme activity in postreplicative muscle cells. A regulated pattern of expression is retained when the promoter of the cellular *tk* gene is replaced by the promoter of the herpesvirus *tk* gene. Moreover, the cellular *tk* gene is appropriately regulated during terminal muscle differentiation when its 3′ terminus is removed and replaced by the terminus of the viral *tk* gene. Thus, the element of the cellular *tk* gene sufficient to specify its regulation is entirely intragenic.

During terminal differentiation of skeletal muscle, proliferating myoblasts withdraw irreversibly from the cell cycle and fuse to form multinucleated myotubes. The transition to a postmitotic state is accompanied by the accumulation of gene products required for muscle function (8) and by the decline of proteins involved with cell proliferation (23, 25, 34). Research on the mechanisms that account for the rapid accumulation of muscle-specific gene products has been pursued extensively. However, relatively little attention has been given to the corresponding problem of the selective decline in the expression of replication-associated proteins. The latter problem may have relevance extending beyond muscle cells. Before expressing the various phenotypes characteristic of the differentiated state, cells of many lineages withdraw from the cell cycle (12, 24, 41, 43, 50) or become committed to withdraw after completing a predetermined set of terminal divisions (49). The selective loss of replicative function is thus a general feature of terminal differentiation.

Mechanisms that mediate the loss of replication-associated proteins during terminal differentiation may also be involved in regulating the periodic expression of this class of proteins during the cell cycle. Cell cycle-dependent changes in enzymatic activity have been reported for thymidine kinase (tk) (26, 45), deoxycytidine kinase (4), thymidylate synthetase (32), ribonucleotide reductase (47), dihydrofolate reductase (48), ornithine decarboxylase (20), and DNA polymerase (15). Despite the breadth of these studies, the molecular basis for the transient changes of these enzymes is understood only for dihydrofolate reductase (*dhfr*). In this system, cell-cycle dependent expression of *dhfr* occurs through a mechanism involving altered stability of nuclear *dhfr* RNA (22). It is unclear, however, whether this type of regulation represents a general mechanism for coordinating periodic changes in replication-associated enzymes during the cell cycle. Furthermore, it is untested whether similar postranscriptional regulation occurs during terminal differentiation.

Isolation of recombinant clones bearing cellular *tk* genes (3, 21, 38) permits a genetic approach to the problem of how replication-associated enzymes are regulated during terminal differentiation or during the cell cycle. If a cloned *tk* gene is expressed in a replication-dependent manner after transfection into a suitable host cell, the mechanism of regulation can be investigated by systematic in vitro modification of the gene before its transfection.

We have found that tk enzyme activity disappears rapidly when wild-type mouse myoblasts are induced to differentiate. A similar decline in tk activity occurs when myoblasts transformed with cloned copies of the chicken *tk* gene are induced to differentiate. In contrast, tk activity remains at constitutively high levels during the differentiation of muscle cells transformed with the herpesvirus *tk* gene. The availability of these two *tk* genes, one that exhibits appropriate regulatory properties and one that does not, has allowed us to carry out a series of experiments that localize the segment of the cellular *tk* gene that affects its regulation during terminal differentiation.

MATERIALS AND METHODS

Cell culture conditions. Muscle cells were grown on gelatin-coated culture dishes at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO_2. Muscle cell growth medium consisted of Ham F10 nutrients supplemented with 0.8 mM $CaCl_2$, 15% horse serum, 3% chicken embryo extract (17), and 1% antibiotics solution (10 U of penicillin G per ml, 0.5 mg of streptomycin sulfate per ml). Horse serum was prescreened for support of muscle cell growth at clonal densities. For selection and maintenance of transformed muscle cell lines, growth medium was supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 10^{-5} M thymidine (HAT). Muscle cells were passaged by dissociation with 0.01% collagenase in calcium-free, magnesium-free saline. At densities exceeding 10^5 cells per 10-cm dish, mouse myoblasts rapidly deplete their growth medium of mitogens and begin to terminally differentiate. Therefore, to maintain homogeneously proliferating populations at a high cell density, growth medium was replenished daily, and the cell cultures were rocked at about one cycle per min. To induce differentiation, cultures were rinsed twice with Ham F10 nutrients and incubated in a defined mitogen-depleted medium consisting of Ham F10 nutrients supplemented with

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0.8 mM $CaCl_2$, 10^{-6} M insulin, and 10^{-7} M dexamethasone. The wild-type myoblast line used in these experiments is a derivative of the MM14 cell line (24). The tk^- subline was derived by ethylmethylsulfonate mutagenesis and selection, using 5′-bromodeoxyuridine plus visible light (30).

Cell transformations. Approximately 3×10^5 tk^- myoblasts were added to a 10-cm culture dish. Growth medium was replenished the next day. Four hours later, cells were exposed to 1 μg of linearized recombinant plasmid DNA plus 19 μg of calf thymus carrier DNA, using the calcium-phosphate precipitation method (11) as modified by Corsaro and Pearson (6). The culture medium was replaced 4 h after exposure to DNA. On the next day, each culture was split into three 10-cm dishes containing HAT growth medium. HAT medium was replenished daily for the first 2 days and then at 3-day intervals. HAT-resistant colonies appeared within 10 days at a frequency of 3 to 50 transformants per 3×10^5 cells originally plated. A majority of the transformants exhibited a myogenic phenotype, as determined by their ability to form multinucleated myofibers in response to mitogen depletion. To confirm that HAT-resistant clones were bona fide transformants, their DNA was analyzed by blot transfer (44). All clones tested contained the appropriate transfected *tk* gene. In the case of herpesvirus *tk* transformation, experiments using the viral-specific tk inhibitor iododeoxycytidine confirmed that the tk enzyme being expressed was of viral origin.

Assay of tk enzyme activity. Cultures were initiated by adding 5×10^4 myoblasts to 6-cm dishes containing growth medium. Two days later cultures were either harvested for analysis of proliferative cell levels of tk enzyme activity or given mitogen-depleted medium to induce muscle cell differentiation. At various times after mitogen depletion, cultures were harvested for analysis of tk enzyme activity as a function of differentiation. In all populations tested, withdrawal from the cell cycle was complete and commitment to differentiation was maximal 18 h after mitogen depletion. To assay tk enzyme activity, cells were rinsed with Tris-buffered saline (pH 7.2) and scraped from the dish in 0.2 ml of extraction buffer (25 mM Tris-hydrochloride [pH 7.8], 1.6×10^{-5} M thymidine, 10 mM KCl, 1 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM ATP). Cells were sonicated twice for 5 s at maximum power with a Sonifier cell disruptor (Heat Systems-Ultrasonics, Inc.). Samples were withdrawn for DNA determination by the Hoecht 33258 dye-binding method (19). The remaining cell extract was centrifuged for 15 min in an Eppendorf microfuge. Supernatant samples (60 μl) were mixed with 20-μl samples of tk reaction buffer (600 mM Tris-hydrochloride [pH 7.8], 20 mM $MgCl_2$, 20 mM ATP, 5 mM NaF, 30 mM creatine phosphate, 12 U of creatine kinase per ml, 0.1 mCi of [^{3}H]thymidine per ml) and placed in a 37°C water bath. After timed intervals of 5, 10, and 15 min, 25-μl samples of the reaction cocktail were withdrawn, mixed with 7.5-μl samples of 25 mM EDTA, and transferred to 1-cm^2 squares of DEAE filter paper. After drying, filters were washed three times with 1 mM ammonium formate, once with distilled water, and once with 95% ethanol. Filters were digested with 0.2 ml of Soluene (Packard Instrument Co.), and bound radioactivity was determined by liquid scintillation spectrophotometry, using Omnifluor (New England Nuclear Corp.).

Northern blot analysis of RNA. Muscle cell cultures containing 2.5×10^6 proliferating or differentiated muscle cells were harvested in 4 ml of TES buffer (10 mM Tris-hydrochloride [pH 7.5], 5 mM EDTA, 1% sodium dodecyl sulfate) containing 0.2 mg of proteinase K per ml. After digestion for 1 h at 50°C, lysates were extracted with phenol and chloroform, precipitated with ethanol, and resuspended in water. The RNA content was estimated by subtracting the DNA concentration (determined fluorometrically) from the concentration of total nucleic acids (determined spectrophotometrically at an absorbance at 260 nm). Samples were digested with DNase I that had been pretreated with iodoacetate. DNase I-digested samples were again extracted with phenol and chloroform, precipitated with ethanol, and resuspended in water to a concentration of 2 mg of RNA per ml. Samples were denatured with glyoxal, electrophoresed, and transferred to nitrocellulose (46). Hybridizations were carried out in 5× SSC (0.15 M NaCl plus 0.015 M sodium citrate), 25 mM sodium phosphate (pH 6.5), 0.02% bovine serum albumin, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 250 μg of salmon sperm DNA per ml, and 50% formamide. Radiolabeled probe was prepared by nick translation of the 1,400-base-pair *KpnI-BglII* internal fragment of the chicken *tk* gene (31). Filters were washed twice with 2× SSC–0.5% sodium dodecyl sulfate, followed by three 15-min rinses in 0.1× SSC–0.5% sodium dodecyl sulfate at 55°C. Filters were exposed to Kodak X-ray film at −70°C with the aid of an intensifying screen.

RESULTS

Regulation of tk enzyme activity in wild-type myoblasts and muscle cell transformants. Before initiating studies to determine whether tk enzyme expression from an exogenously supplied gene is regulated during muscle cell differentiation, expression of the endogenous *tk* gene of wild-type myoblasts was analyzed. As shown by the decline in the [^{3}H]thymidine pulse-labeling index in Fig. 1A, wild-type myoblasts began to withdraw from the cell cycle ca. 2.5 h after they were induced to differentiate by mitogen depletion. By 12.5 h, or roughly one generation time, virtually all myoblasts had withdrawn from the cell cycle. This is an irreversible process; restoration of mitogen-rich medium does not induce withdrawn myoblasts to reenter the cell cycle (24). Figure 1A also shows that myoblast withdrawal is closely paralleled by a decline in tk enzyme activity. By 12.5 h after mitogen depletion, tk activity had declined to less than 5% of the proliferative cell level. The decline in tk activity is not due to a general inhibitory effect of mitogen depletion on cellular gene expression; shortly after withdrawing from the cell cycle, myoblasts fuse to form multinucleated myotubes and begin to accumulate muscle-specific proteins (25, 30). The effect of mitogen depletion on cell fusion is shown in Fig. 2. The tk^- myoblast cells used in the transfection experiments outlined in the following sections also withdrew from the cell cycle and initiated differentiation in response to mitogen depletion. However, the kinetics of withdrawal and differentiation were delayed slightly relative to the parental wild-type myoblasts. This may be attributed to the 18-h cell doubling time of the tk^- subline (30).

A different pattern of *tk* expression occurred when myoblasts transformed with the herpesvirus *tk* gene were induced to differentiate (Figure 1B). As with wild-type myoblasts the [^{3}H]thymidine labeling index declined and the fusion index increased upon mitogen withdrawal, indicating that viral transformants exit from the cell cycle and initiate differentiation. However, unlike wild-type myoblasts, viral *tk* transformants maintained high levels of tk enzyme during and after the differentiation process. High levels of viral tk activity persisted even 60 h after induction, when muscle cultures consisted almost exclusively of multinucleated myofibers. The small (35%) decline in viral tk activity seen in

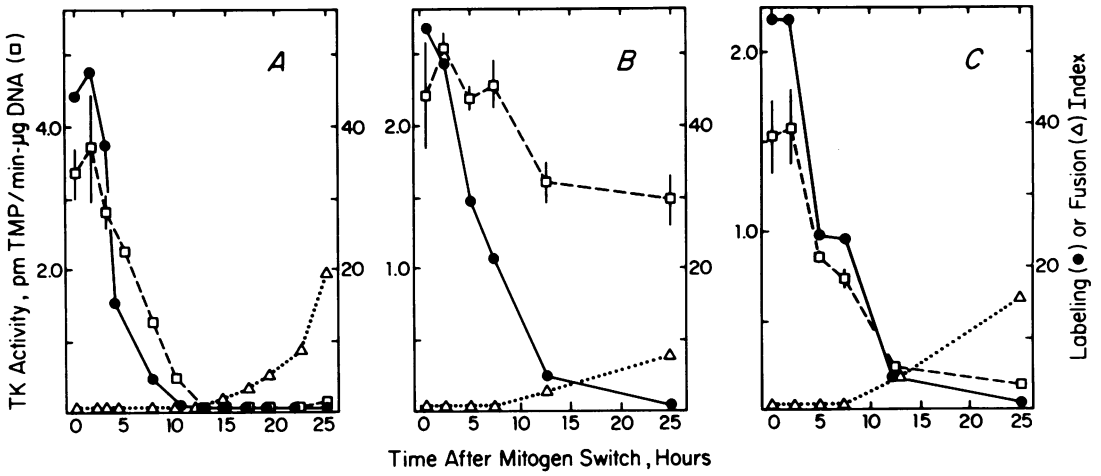

FIG. 1. Effect of mitogen depletion on DNA synthesis, myoblast fusion, and tk enzyme activity in wild-type and transformed mouse myoblast cells. Cultures were initiated at 5×10^4 cells per 6-cm dish in complete growth medium (see the text). After 2 days, cultures were shifted to mitogen-depleted medium. At indicated times thereafter, cultures were exposed to [^{3}H]thymidine for 1 h and fixed for determination of percent labeled nuclei (labeling index) and percent nuclei in multinucleated myofibers (fusion index). At the time of fixation, parallel cultures were harvested for tk enzyme assays. Cells used in the experiment were (A) the wild-type MM14 muscle cell line expressing the endogenous mouse *tk* gene, (B) the herpesvirus *tk* transformed muscle cell line, TK^{v}14A, and (C) the chicken *tk* transformed muscle cell line, TK^{c}11C. Vertical error bars for tk activity represent the range of duplicate determinations.

Figure 1B was not consistently observed in differentiating herpesvirus *tk* transformants. As shown in the first set of entries in Table 1, the direction and magnitude of change in tk enzyme activity that accompanies differentiation of viral *tk* transformants were somewhat variable. Even within the same transformed cell line, some variability was encountered (cf. values for TK^{v}14A in Fig. 1B and Table 1). However, none of the myoblast cell lines derived by transfection with the herpesvirus *tk* gene displayed the sharp decline in tk activity observed in differentiating wild-type myoblasts. We refer to the persistent maintenance of high tk activity during differentiation as a constitutive pattern of gene expression.

In contrast to the results obtained with cell lines transformed with the viral *tk* gene, myoblasts transformed with cloned copies of the chicken *tk* gene exhibited a rapid and dramatic reduction in tk enzyme activity during differentiation (Fig. 1C). As shown in the second set of entries in Table 1, a sharp decline in tk activity occurred concomitant with differentiation in all chicken *tk* gene transformants thus far analyzed. On the basis of these observations we assume that expression of the transfected chicken *tk* gene is subject to the same regulatory mechanism that accounts for the diminished expression of the *tk* gene endogenous to wild-type mouse muscle cells. We refer to the loss of tk activity during differentiation, characteristic of wild-type myoblasts and chicken *tk* gene transformants, as a regulated pattern of expression.

Regulation of tk enzyme expression is governed by information downstream from the transcriptional promoter of the cellular *tk* gene. As an initial approach toward resolving which metabolic step accounts for the disappearance of tk activity in postmitotic muscle cells, we constructed and assayed a set of reciprocal promoter-switched *tk* genes (Fig. 3). One such recombinant consists of the chicken *tk* mRNA-coding sequence fused downstream from the herpesvirus *tk* promoter (HSV_p/CH_{st}). The reciprocal recombinant consists of the herpesvirus *tk* mRNA-coding sequence fused downstream from the chicken *tk* promoter (CH_p/HSV_{st}). The derivation of these respective recombinant *tk* genes is outlined in the accompanying report (31).

If the disappearance of cellular tk enzyme activity were due to a change in the rate of transcription governed by promoter sequences of the chicken *tk* gene, myoblasts transformed by the CH_p/HSV_{st} recombinant should exhibit regulation of tk activity even though the mRNA and enzyme being produced are viral. This conclusion would be further

TABLE 1. tk enzyme activity in proliferating and differentiated muscle cells transformed with viral, chicken, or promoter-switched *tk* genes[a]

Transfected gene	Transformed clone	tk activity (pmol of TMP/min per μg of DNA)		
		Proliferating	Differentiated	% Remaining
HSV	3A	1.53	1.27	83
	4A	5.97	15.39	258
	9A	7.50	7.70	103
	14A	1.83	1.67	91
	21A	4.19	3.31	79
	22A	3.06	1.50	49
Chicken	1A	7.04	0.75	11
	2A	2.98	0.30	10
	3A	11.25	0.13	1
	4A	16.72	0.24	1
	11A	2.12	0.12	6
	11C	2.69	0.14	5
CH_p/HSV_{st}	1A	7.83	4.94	63
	2C	3.06	3.62	118
	3A	3.18	2.19	69
HSV_p/CH_{st}	2A	3.60	0.31	8
	2D	3.20	0.48	10

[a] Cell extracts from proliferating and postmitotic myoblast cultures were prepared and assayed for tk activity as described in the text. In all cases the length of time used to induce differentiation was sufficient to cause greater than 95% withdrawal from the cell cycle as determined by the [^{3}H]thymidine pulse-labeling index.

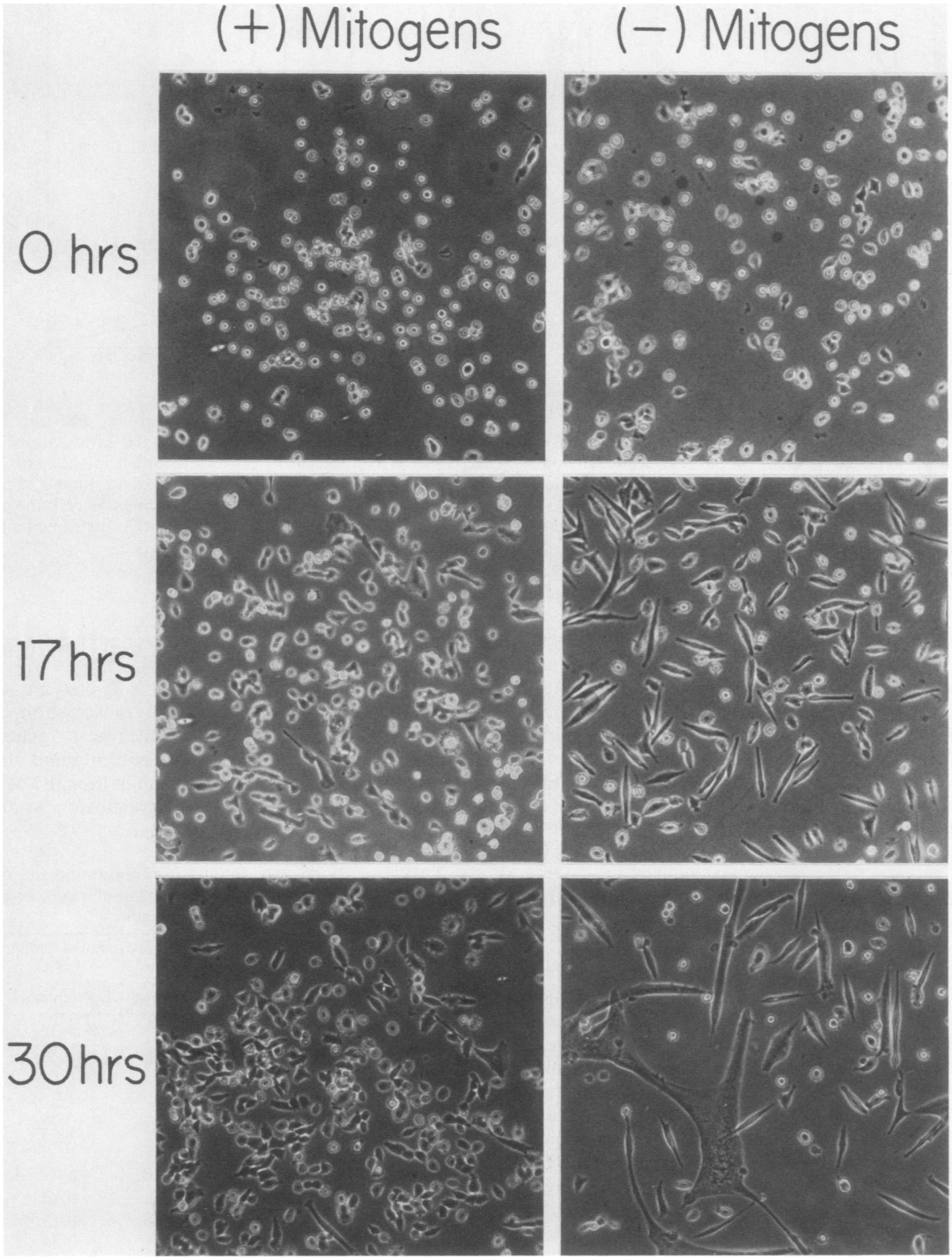

FIG. 2. Photomicrograph of mouse muscle cells cultured in the presence or absence of mitogens. Cultures of wild-type myoblasts were initiated as described in Fig. 1. Two days later, cultures were fed either fresh growth medium [(+) Mitogens] or mitogen-depleted medium [(−) Mitogens]. A single microscopic field was selected and photographed by using phase-contrast optics at the indicated times after feeding. Note that in the culture given mitogen-depleted medium, cells have elongated and are beginning to fuse by 17 h; by 30 h myotube formation is extensive. Indirect immunofluorescent staining with antiserum specific to muscle myosin confirms that all of the cells are actively synthesizing muscle-specific gene products 30 h after mitogen depletion (data not shown).

substantiated if myoblasts transformed with the HSV_p/CH_{st} recombinant were to show constitutively high levels of tk enzyme activity upon differentiation. The opposite set of results would indicate either that regulation of cellular tk enzyme activity was due to a posttranscriptional process or that regulation of the rate of synthesis of the primary transcript was governed by sequences downstream from the cellular *tk* promoter.

The pattern of tk enzyme expression in muscle cells transformed with promoter-switched *tk* genes is shown in

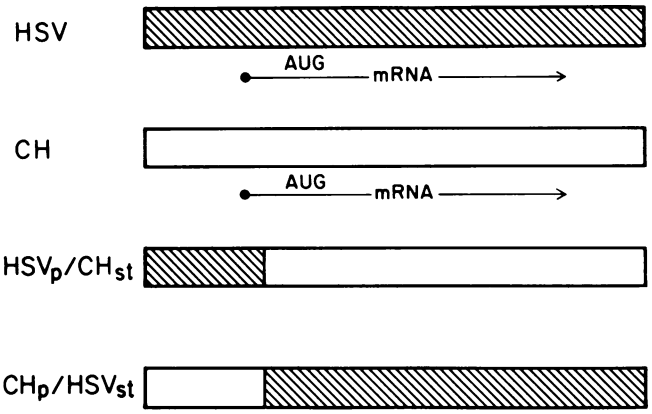

FIG 3. Structures of herpesvirus and chicken *tk* genes and promoter-switched derivatives. Diagram shows intact herpesvirus (HSV) *tk* gene (hatched) and intact chicken (CH) *tk* gene above promoter-switched recombinants. Black dots designate putative transcription start sites of the respective RNA molecules. AUG triplets designate putative translation start sites. Recombination points for both promoter-switched genes were positioned between the respective transcription and translation start sites. To construct the HSV_p/CH_{st} recombinant, the viral promoter was fused at its natural *Bgl*II site to the synthetic *Bam*HI linker present at the terminus of the *tk* deletion mutation Δ5'-2182 as described in the accompanying report (31). To construct the CH_p/HSV_{st} recombinant, a 3' deletion mutation of the chicken *tk* gene terminating with a *Bam*HI linker attached to the guanine residue 6 base pairs upstream from the translation start codon (31) was ligated to a promoterless HSV *tk* gene at the viral *Bgl*II site.

Table 1. All myoblast cell lines transformed with the HSV_p/CH_{st} recombinant displayed the same reduction in tk enzyme activity upon differentiation, as exhibited by myoblasts transformed with the intact chicken *tk* gene. In contrast, all myoblasts transformed with the CH_p/HSV_{st} recombinant showed the constitutive pattern of *tk* expression characteristic of lines transformed with the intact herpesvirus *tk* gene. Taken together, these results indicate that the reduction of cellular tk enzyme activity which occurs during terminal differentiation of muscle cells is not governed by DNA sequences associated with the cellular *tk* promoter. Instead, it appears that the element of the cellular *tk* gene that governs regulated expression is associated with sequences downstream of the promoter.

Regulation of tk enzyme expression is governed by information upstream from the 3' terminus of the cellular gene. Having found that sequences associated with the chicken *tk* promoter fail to dictate appropriate differentiation-induced regulation, attention was turned to sequences downstream from the protein-coding segment. Our approach was to determine whether deletion or replacement of sequences at the 3' end of the chicken *tk* gene results in loss of regulation. Initially, the pattern of tk enzyme expression was determined in muscle cells transformed with either deletion mutant Δ3'-144, which lacks only the final 144 base pairs of the parental chicken *tk* clone, or deletion mutant Δ3'-739, missing virtually all sequences encoding the 3' nontranslated portion of the mRNA (31). As Table 2 shows, muscle cells transformed with either of these 3' deletion mutants exhibited the same reduction in tk activity upon differentiation as cells transformed with the intact gene (Table 1). Table 2 also shows that tk activity was appropriately regulated in myoblasts transformed with deletion mutants to which the 3' terminus of the herpesvirus *tk* gene was ligated. The derivation of these recombinant templates is outlined in the accompanying report (31).

Evidently, replacement of the putative chicken tk polyadenylation signal with a polyadenylation signal from a gene that is not regulated during terminal differentiation is not sufficient to confer a constitutive pattern of *tk* expression. We conclude from these experiments that the reduction of tk activity which occurs during muscle cell differentiation is not governed by sequence information located 3' to the protein-coding region of the cellular *tk* gene.

Reduction of cellular *tk* mRNA during differentiation. The genetic experiments outlined in the two preceding sections identify a region of the cellular *tk* gene that is sufficient for regulated expression. These experiments do not, however, resolve the metabolic step at which cellular tk activity is regulated. The decline in enzyme activity we observed during terminal muscle cell differentiation could result from repressed *tk* gene transcription. Alternatively, the regulated pattern may result from either a posttranscriptional or post-translational control mechanism. In vitro experiments in which cytoplasmic extracts from proliferating and differentiated cells are mixed give no evidence of a trans-acting inhibitor of tk enzyme activity in differentiated cells (data not shown). A negative result of this type does not, however, exclude the possibility that posttranslational destabilization or degradation of tk enzyme occurs in vivo.

An alternative approach toward resolving which metabolic process accounts for the disappearance of tk activity is to compare the stable *tk* mRNA levels in proliferative and differentiated muscle cells. A transformant muscle cell line carrying multiple copies of the chicken *tk* gene was analyzed by Northern transfer hybridization for the presence of chicken *tk* mRNA before and after differentiation. RNA from proliferating myoblasts of this cell line contained an RNA species 1,600 ribonucleotides in length that is complementary to the chicken *tk* gene (Fig. 4). In contrast, RNA prepared from differentiated muscle cells of the same cell line contained no such RNA species. Thus, the reduction in tk enzyme activity that accompanies terminal differentiation of muscle cells apparently involves a mechanism that controls the production of stable *tk* mRNA.

TABLE 2. tk enzyme activity in muscle cells transformed with 3' deletions of the chicken *tk* gene[a]

Transfected gene	Transformed clone	tk activity (pmol of TMP/min per μg of DNA)		
		Proliferating	Differentiated	% Remaining
3'-139	1A	1.92	0.30	16
	1C	1.69	0.00	0
3'-734	4A	1.77	0.05	3
	4D	1.24	0.02	2
3'-139tx	1A	1.28	0.15	12
	1B	17.16	0.42	2
	1C	3.48	0.15	4
3'-734tx	1A	1.21	0.42	35
	1B	3.00	0.44	15
	1C	78.79	5.73	7

[a] Cell extracts were prepared from proliferating or differentiated muscle cell transformants as described in the text. The suffix tx refers to deletion mutants to which a DNA fragment containing the herpesvirus polyadenylation signal was ligated.

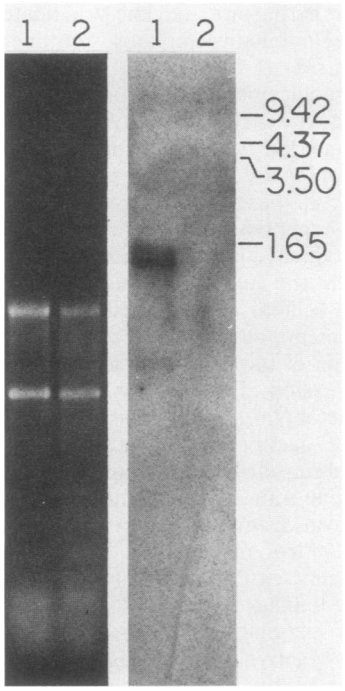

FIG. 4. Northern transfer analysis of chicken *tk* mRNA from proliferating and differentiated muscle cells. Total cellular RNA was prepared from actively proliferating myoblasts or from terminally differentiated myofibers. The cell line used was a multicopy chicken *tk* gene transformant. In the panel shown on the left, 10-μg samples of native RNA were electrophoresed and stained with ethidium bromide to confirm the integrity and concentration of each RNA preparation. The panel on the right shows an autoradiographic exposure of a Northern transfer of the same RNA samples. In the latter case, RNA was denatured with glyoxal before electrophoresis. Lanes 1 contain RNA from proliferating muscle cells, and lanes 2 contain RNA from differentiated cells. The RNA sample prepared from proliferating muscle cells contains a 1,600-ribonucleotide species that is homologous to the chicken *tk* DNA probe. No such species is present in the sample prepared from differentiated muscle cells. Molecular weight markers are glyoxalated *Hin*dIII fragments of bacteriophage lambda DNA and 28S and 18S ribosomal RNA.

DISCUSSION

The experiments presented in this report were designed to identify the component of the cellular *tk* gene responsible for regulating expression of tk enzyme during terminal muscle cell differentiation. Our initial experiments focused on the transcriptional promoter of the cellular *tk* gene. Numerous studies have implicated sequences upstream of structural genes in the control of gene expression. For example, in all previous instances in which derivatives of the herpes simplex virus (HSV) *tk* gene were placed downstream from promoter regions of inducible genes, an inducible pattern of expression was conferred upon the HSV *tk* gene product. Such experiments have shown that promoter sequences play an important role in heavy metal induction of the mouse metallothionein gene (28, 36), glucocorticoid induction of transcription from the long terminal repeat of mouse mammary tumor virus (5), heat-induced expression of the HSP-70 heat shock gene of *Drosophila melanogaster* (37), and temporal specific expression of a herpesvirus immediate-early gene (39).

In contrast to these precedents, our experiments indicate that a very different mechanism may be operative in regulating cellular *tk* gene expression during differentiation. First, the HSV *tk* structural gene fails to show a regulated expression pattern when placed downstream from the chicken *tk* promoter. Second, the mRNA-coding element of the chicken *tk* gene continues to show regulated expression when placed downstream from the HSV *tk* promoter.

We next asked whether sequences 3′ to the protein-coding region of the cellular *tk* gene are necessary for appropriate regulation of tk activity. There are considerably fewer instances in which sequences downstream from the coding region of a gene have been implicated in the control of gene expression. Two particularly relevant examples, because they involve gene products preferentially expressed in actively replicating cells, are the control of yeast histone gene transcription by an element located 3′ to the histone H2B gene (35) and the dependence of dihydrofolate reductase regulation on the type of polyadenylation signal present at the 3′ terminus of transfected *dhfr* genes (16).

A mechanism involving the regulated use of a polyadenylation signal does not appear to be operative in the control of cellular tk enzyme expression in differentiating muscle cells. Virtually all of the 3′ nontranslated region of the chicken *tk* gene can be deleted, and yet a regulated pattern of expression is retained. Furthermore, when a polyadenylation signal from a gene capable of constitutive expression during muscle differentiation is attached to the most-truncated 3′ chicken *tk* deletion mutation, the level of tk enzyme activity is effectively regulated as a function of terminal differentiation. Our findings do not exclude a mechanism similar to the yeast histone model. Osley and Herefored (35) identified a control sequence downstream of the histone H2B gene that is closely associated with a putative DNA replication origin. This downstream control sequence is required for the periodic expression pattern of histone gene expression in cycling yeast cells. If such a model were to apply to the chicken *tk* gene, then the control element must lie within the gene itself.

The experiments we have presented in this report indicate that information sufficient for differentiation-induced regulation is located fully within the transcribed region of the chicken *tk* gene. Taking this finding into account, several different models can be formulated to explain how cellular *tk* mRNA levels are regulated as a function of differentiation. First, it is possible that *tk* gene expression is regulated at the transcriptional level by a control element located within the transcription unit. Intragenic sequences have been shown to play a positive role in controlling the expression of 5S ribosomal RNA, the virus-associated RNA of adenovirus, and many different tRNAs, all of which are transcribed by RNA polymerase III (2, 9, 14, 42). Moreover, the tissue-specific expression pattern of heavy- and light-chain immunoglobulin genes, which are transcribed by RNA polymerase II, is positively mediated through a control element located within the major intervening sequence (1, 10, 40). It should also be noted that transcription of the cellular *tk* gene could be regulated by an internal control signal that acts in a negative manner to block expression in noncycling cells.

A second mechanism that could account for the regulation of cellular tk enzyme expression in differentiated muscle cells involves destabilization of mature *tk* mRNA. Specific changes in mRNA stability are known to occur in several differentiating systems (7, 27, 33) and during temperature-sensitive cell cycle arrest in yeasts (13).

A third explanation of our results also involves regulation at a posttranscriptional level. It is possible that the cellular *tk* gene is actively transcribed in differentiated muscle cells but that mature myofibers are unable to appropriately process

the nuclear precursor. One obvious difference between the chicken *tk* gene, which is regulated in differentiated muscle cells, and the HSV *tk* gene, which is not, is that the former contains introns (18, 31), whereas the latter does not (29). Thus, it may be that the decline in *tk* mRNA levels which accompanies terminal differentiation reflects the inability of postmitotic muscle cells to splice cellular *tk* transcripts. If this is the case, however, an element providing specificity must exist because it is known that differentiated muscle cells are fully capable of splicing introns from precursors for muscle-specific mRNAs (51).

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