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Replication associated protein and enzyme activity levels increase as cells enter S-phase of the cell cycle and diminish as cells leave S-phase. Accordingly, replication associated functions decrease as myoblasts withdraw from the cell cycle to terminally differentiate. In an effort to understand signals effecting growth associated expression of genes, the molecular mechanism controlling declining thymidine kinase (TK) activity levels during myogenic withdrawal from the cell cycle was investigated. Initially, the hypothesis that TK was regulated at the level of mRNA was investigated both *in vivo* and in an *in vitro* myoblast cell culture system. Qualitatively, TK mRNA declined by a transcriptional mechanism. However, quantitative comparison of the decline in TK mRNA and TK activity revealed TK activity was regulated by a mRNA-independent mechanism. Consequently, the hypothesis that TK activity was regulated by a posttranslational mechanism was tested. Antibodies against TK protein were derived and used to demonstrate, *via* immunoblot and immunoprecipitation experiments, the existence of a translational or protein degradational mechanism. The possible contribution of posttranslational modulation of TK activity could not be rigorously eliminated.

A second approach to understanding the mechanism of decline of TK activity during myogenic withdrawal from the cell cycle involved further localization of intragenic cis-acting regulatory elements. Regulation of TK activity was monitored in myoblasts transformed with intron deletion mutants of TK. Introns were inconsequential to regulation of TK activity. Thus, cis-acting regulatory elements mediating the decline in TK activity were within the protein coding region, consistent with the translational or protein degradational level of regulation. Quantitative evaluation of TK mRNA regulation in myoblasts transformed with promoter switch, 3' replacement, and intron deletion mutants also localized cis-acting elements mediating the transcriptional decline in TK mRNA to the protein coding region. However, the controversy surrounding the

nature of the heterologous promoters used, the smallfold and variable decline in TK mRNA, the possibility of redundant control elements, and the unusual location of the transcriptional regulatory element cast doubt on this conclusion. Two general mechanisms for controlling TK mRNA levels were proposed.

The available set of intron deletion mutants was used to test the popular hypothesis that introns are essential for expression of mRNA. Quantitative evaluation of TK mRNA expression in mouse fibroblasts transformed with full length TK genes or intron deletion mutants revealed no significant difference in expression. Thus introns were inconsequential to expression of TK mRNA in fibroblasts.

Thymidine Kinase mRNA and Protein Levels During Myogenic Withdrawal from the Cell Cycle: Identification of an mRNA-Independent Regulatory Mechanism

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CONTRIBUTIONS OF AUTHORS

Mark S. Kainz, the laboratory technician, performed the nuclear run-on assays described in chapter2 and constructed some of the intron deletion mutants described in chapter 5.

Gary F. Merrill, the principle investigator of the laboratory and my thesis adviser, provided essential guidance in the collection of data for, and the writing of the entire thesis.

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CHAPTER 1: Introduction

I. Cooperation Amidst Competition

A basic tenet of life on earth is reproduction. All living organisms on earth, whether prokaryotes and eukaryotes, would multiply their numbers in an exponential fashion if unlimited resources were provided. Of course, selection pressure is normally exerted by a limitation of resources. Cells are the common subunit of all earthly life and indeed, unicellular organisms normally divide exponentially until the available resources are used up. However, cells within multicellular organisms have adopted a cooperative survival strategy whereby somatic cells lose their ablity to reproduce to enhance the survival chances of a closely related germ cell line. Although competition for survival is a much touted paradigm in biology, a more important paradigm may be how biological entities, whether organisms in a community or cells in an organism, cooperate to survive. Understanding the mechanisms of cellular cooperation in multicellular organisms is of fundamental importance to biology.

A. Growth Control in Development: Terminal Differentiation

A critical step in cooperation of cells in multicellular organisms is the decision of proliferating somatic cells, at some stage in development, to cease dividing during terminal differentiation. For example, in leg muscle of chicken embryos at 7 days of incubation, labeling with [^{3}H]thymidine and counting labeled nuclei or mitotic figures indicates that 70% of the cells are rapidly dividing (Marchok and Harmon, 1967). Twenty days later, less than 3% of the cells in the leg muscle are dividing. Similarly, nerve, cartilage, blood, and other cells stop dividing at the stage in development during which they acquire extreme cell specialization. Thus, terminal differentiation is the process whereby cells lose the ability to divide and acquire a specialized role in the body. If the cells all continued to divide, no body pattern would emerge and eventually the cells would die, being unable themselves to procure resources necessary for survival. The molecular mechanism of growth control during terminal differentiation is not understood.

B. Loss of Growth Control: Oncogenesis

An intrinsic problem of terminal differentiation is the necessity to maintain a nonreplicating state of cells in an environment that provides ample nutrients and energy for cell division. The cells of multicellular organisms limit their division by their molecular design rather than by nutrient or energy supply. Critical alterations in the molecular design of growth regulating mechanisms can result in the loss of growth control. Oncogenesis is the process whereby a cell loses growth control and results in a population of proliferating cells that crowds properly placed, quiescent cells either locally (benign tumors) or globally (malignant tumors). Loss of growth control in only a single cell can result in cancer, and perhaps death, in a multicellular organism. For this reason much effort and funding has gone into understanding the molecular mechanism of growth regulation.

II. Studying Growth Control

Two fundamental approaches have been used to determine the molecular mechanism of the mitogenic signalling cascade used to regulate growth in cultured cells. The forward approach involves changing the growth phenotype of cells by an agent such as an oncogene, carcinogen, or mitogen and trying to discover the early molecular components in the mitogenic cascade which ultimately bring about the altered growth phenotype. The reverse approach discerns a growth correlated gene expression phenotype and establishes molecular mechanistic links backwards along the mitogenic cascade to the primary signal(s). Together, these two approaches should provide a detailed model of how a few primary growth signals can bring about the vast array of differences between proliferating and quiescent cells, or between stem cells and terminally differentiated cells.

A. The Forward Approach: Oncogenes

Certain retroviruses cause tumors in appropriate host animals. These RNA or DNA tumor viruses can cause neoplastic transformation of cultured cells. Normally, cultured cells proliferate exponentially until they form a confluent monolayer on the dish, at which time cell division ceases and the cells enter a quiescent (G1 or G0) state. Early studies found that Rous sarcoma virus (RSV) has the ability to allow quiescent

fibroblasts to regain their ability to divide, thus forming foci of cells on the dish (for review see Bishop, 1978). Further analysis established the v-src gene of the viral genome was responsible for neoplastic transformation (Wyke *et al.*, 1974). Using a cDNA of v-src, Stehelin *et al.*(1976) showed that the chicken genome contained a gene, c-src, which was closely related to the viral oncogene and was phylogenetically conserved. The cellular-oncogene was thought to participate in regulating growth in normal cells because an altered form of it, the viral oncogene, could induce abnormal cell proliferation.

This hypothesis was subsequently confirmed by studies in which DNA from a tumor cell lines was used to transform a nonmalignant cultured cells to a tumorigenic phenotype (Murray *et al.*,1981; Krontiris and Cooper, 1981). The cellular homolog of H-ras, a known viral oncogene, was found to be the gene responsible. Sequencing revealed that the non-tumorigenic c-ras gene found in normal cells differed from the tumorigenic c-ras gene found in tumor cells (Tabin *et al.*,1982; Reddy *et al.*, 1982; Taparowski *et al.*,1982). Thus, the general idea that a native proto-oncogene is "activated" to become an oncogene was first established. Activation involves a crucial change in the expression or structure of the proto-oncogene and can occur by a variety of means including retroviral transduction, chemical or physical environmental insults, transposition to a different chromosomal location, and proviral insertion.

Because proto-oncogenes are phylogenetically conserved and cause disruption of growth control when "activated", they are likely to be important to the molecular mechanism of cellular growth control. Between 20 and 30 distinct oncogenes have now been isolated based on their homology with oncogenes derived from retroviruses, DNA tumor viruses, and tumor cells. In many cases corresponding proto-oncogenes have been identified. The protein products of oncogenes (or proto-oncogenes) fall into several classes. Each class represents a different type of protein involved in the complex mitogenic signalling cascade. Oncogenes (or proto-oncogenes) encode growth factors (sis, TGF-alpha, TGF-beta), receptors (erb A, erbB, fms, neu, ros), protein kinases (abl, erbB, fms, fps/fes, neu, ros, sea, src, yes, mil/raf, mos), G proteins (ras), DNA binding proteins (myb, myc, p53, jun), transcription factors (Adenovirus E1A, SV40 large T, plt, fos, jun), and replication factors (SV40 large T, plt) (see Kahn and Graf, 1986). Thus, the study of oncogenes has led to the discovery of some of the molecular components constituting the early mitotic signalling cascade, namely those components which transmit a growth signal from outside of the cell to the inside. However, the molecular mechanisms by which these early signalling components interact still needs to be characterized more rigourously. Future studies along these

lines will be very productive because the genes of key components have been identified.

In any biological cascade, the number of effects or interactions becomes larger as it proceeds. In the mitogenic cascade, more oncogenes have been discovered that code for protein kinases, than for receptors, than for growth factors. The role of the many critical protein kinase elements in the mitotic signalling cascade is as yet unknown. In addition, it is not clear which protein kinases should be studied and which aspect of each to study as each protein kinase is likely to have several interactions in the next "step" of the cascade. Similarly, oncogenes involved in DNA binding, transcriptional regulation, and DNA replication are likely to serve multiple roles in the cell in the next "step" of the signalling cascade, and it is not clear which role to study. An alternative approach is to start with a known growth correlated phenotype and work backwards to try to establish a mechanistic connection to the earlier components of the mitogenic cascade that have been elucidated by studying oncogenes.

B. The Reverse Approach: Replication Associated Genes

The reverse approach is based on the knowledge that certain gene products associated with DNA replication are expressed at reduced levels in cells which are no longer dividing. Intuitively, since DNA is no longer replicated the enzymes involved in replication are no longer needed and are therefore shut down by some mechanism(s). The object of the reverse approach is to establish mechanistic connections with previous steps in the mitogenic signalling cascade.

1. Enzyme activities Correlated With Cell Proliferation *In Vivo*

Early studies discovered a correlation between the rate of cell proliferation and certain enzyme activities. As embryonic development proceeds, the time required for doubling the number of cells in an organism decreases continuously. Hence there is a slowing of the average doubling time of the cells in the organism. Concommittant with the slowing of cell proliferation, enzymatic actvities such as DHFR (Silber *et al.*, 1962), dCMP and adenosine deaminase (Roth and Buccino, 1963), TK (Weinstock and Dju, 1967), TMP kinase (Scholl *et al.*, 1968), and DNA polymerase (Stockdale, 1970) decrease. Elevated levels of such enzymes in regenerating liver and in rapidly proliferating tumor cells confirmed the correlation between cell proliferation and these enzymatic activities. However, little progress was made discerning the mechanisms

which govern growth-correlated expression because one could not isolate or purify these enzymes or their genes. At the time the investigator had to guess at a probable cause which could be mimiced or altered artificially *in vivo*, and test it. Of the many possible models, few were readily testable.

2. New Methods

The development of cell culture systems, recombinant DNA technology, and gene transfer methods greatly facilitated studies of molecular mechanisms of growth control. Culturing eukaryotic cells outside of the organism creates a simpler system which permits manipulation of the cellular environment and isolation of homogeneous populations of identical cells. Thus, dividing (growing) and growth arrested (quiescent) populations of a cell type can be compared directly, allowing molecular differences correlated with growth state to be observed.

Recombinant DNA techniques allowed isolation of genes that are growth related. Thus, specific probes for genes or growth related gene products can be made easily. Earlier biochemical studies of gene expression and regulation were limited to highly (12000 mRNAs/cell) or moderately (300 mRNAs/cell) expressed genes, which constitute less than 5% of the genes in the cell and generally encode abundant structural proteins. Use of modern recombinant techniques allows one to study the other 95% of genes (expressed at low levels of approximately 15 mRNAs per cell) (Alberts *et al.*, 1983) which are likely to encode crucial regulatory components of the cell. Recombinant techniques allow restructuring of a gene to create mutant genes, whose function can be tested in a host cell after gene transfer.

Gene transfer methods allow an exogenous gene to be inserted into the genome of cells that do not normally express it. If the exogenous gene is expressed and/or growth regulated appropriately, then mutated genes can be transferred into the same host cell line and the effect of the mutations on expression and/or regulation monitored. This general method is termed reverse genetics.

a. Cell Culture Methods To Study Growth Control

A variety of methods exist for establishing homogeneous populations of "growing" and "quiescent"cells. Cultured cells can be growth arrested, isolated in different phases of the cell cycle, or induced to undergo terminal differentiation.

The most commonly used methods of growth arrest are serum starvation and

contact inhibition. In serum starvation experiments the growing population is cultured in high serum (usually 10%) and the quiescent population is cultured for the last few days in low (0.1%) or no serum. Sometimes a serum starved population is treated with fresh serum (10%) and monitored as the population begins to grow again. A serious problem with this method is that cell death often occurs in low serum. Hence, one cannot be sure if growth regulation or cell death (or recovery from near death) is being monitored. Contact inhibition studies involve seeding cultures at low density and measuring a parameter as the cells to grow to confluence, at which time they become quiescent. Such studies more closely mimic the *in vivo* situation, yet the cessation of growth is not synchronous and cultured cells are frequently tumor cells which do not contact inhibit well.

Since quiescence involves stalling the cell in the G1 phase of the cell cycle and proliferation involves reentry into S-phase, differences in enzyme activity or protein levels between G1 and S phase of the continuous cell cycle in proliferating cells are thought to resemble differences between quiescent and proliferating cells. Because cultured cells at low density are asynchronously dividing, meaning that the population of cells is in all phases of the cell cycle, one must first obtain homogeneous populations in G1 and S phase. Two fundamental types of methods exist for obtaining homogeneous populations of cells in particular stages of the cell cycle, synchronization or sorting. Synchronization methods utilize various drugs or temperature sensitive mutations to block cells in a particular stage of the cell cycle. The block is maintained for one generation time so that all cells are synchronized at a particular stage. A parameter is then monitored after the block is removed and the cells synchronously proceed through the next cell cycle. Different methods of blocking include amino acid deprivation, butyrate, high thymidine concentrations (Bootsma *et al.*, 1964), thymidine-hydroxyurea, hydroxyurea alone, nitrous oxide, aphidicolin, and colcemid (Stubblefield and Murphree, 1967). The major problem with synchronization methods is the difficulty in distinguishing between artifacts of the particular drug and real cell cycle events (Lloyd *et al.*, 1982).

Sorting methods allow homogeneous cell cycle populations to be obtained with relatively little perturbation and are therefore the methods of choice. Sorting methods include mitotic selection and centrifugal elutriation. Mitotic selection involves agitating a cell culture to selectively release cells undergoing mitosis, which are rounded and therefore release relatively easily. The mitotic cells are then seeded into fresh cultures, which are synchronous and can be monitored as they progress through the cell cycle. Centrifugal elutriation relies on the fact that the size of a cell increases as it traverses the

cell cycle from G1 to S to G2. A low speed centrifugation sorts cells into size fractions (i.e. cell cycle fractions) in which measurements can be made directly.

Culture methods which cause cells to undergo terminal differentiation seem to mimic *in vivo* growth termination. As cells cease to divide *in vivo*, they often begin to serve a very specialized role in the body (i.e. nerve, muscle, blood cells). To serve this role, the cells acquire a specialized set of proteins. The precursor cells to a specialized, terminally differentiated cell type can sometimes be cultured and caused to undergo terminal differentiation *in vitro*. Parameters in the proliferative precursor population can then be compared to the quiescent, terminally differentiated population to make inferences about the process of growth control. This type of system is useful because the induction of specialized proteins in differentiated cells can be monitored to show that the "resting" cells are biosynthetically active (healthy) and that the *in vitro* differentiation event mimics a similar event *in vivo*. Thus, events controlling growth in this type of system are more likely to resemble events controlling growth *in vivo*. Terminally differentiating culture systems are availabe for erythrocytes, B and T lymphocytes, nerve cells, and muscle cells. My research has made extensive use of the latter system.

Using the various culture techniques described above, investigators were able to measure growth correlated changes of replication associated enzyme activities or proteins *in vitro*. Serum starvation and contact inhibition studies initially demonstrated that activities of TK (Eker, 1965; Johnson *et al.*, 1982; Kit *et al.*,1965; Littlefield, 1965; Postel and Levine, 1975), DHFR (Johnson *et al.*, 1978; Wiedeman and Johnson, 1979), DNA polymerase activity (Howard *et al.*, 1974), DNA polymerase protein (Thommes, 1986), and TS (Conrad, 1971; Conrad and Ruddle, 1972; Navaglund *et al.*,1980) are maximal during mid-log phase and decline as cells reach confluence and withdraw from the division cycle. Using synchronized cultures, S-phase dependent expression of TK activity (Bootsma *et al.*, 1964; Littlefield, 1966; Mittermayer *et al.*, 1968; Stubblefield and Mueller, 1965; Stubblefield and Murphree, 1967; Stubblefield and Murphree, 1968; Kit and Jorgenson, 1972; Schneider *et al.*, 1983; Lui *et al.*, 1985), thymidylate kinase activity (Brent *et al.*, 1965), TS activity (Rode *et al.*, 1980; Storms *et al.*,1984; Greenwood *et al.*, 1986), DNA polymerase activity (Schneider *et al.*, 1985), topoisomerase 1 and 2 activity (Tricoli *et al.*, 1985), ribonucleotide reductase activity (Turner *et al.*, 1968; Murphree *et al.*, 1969; Engstrom *et al.*, 1985), DNA ligase activity (White *et al.*, 1986), deoxycytidylate deaminase activity (Kit and Jorgenson, 1972), deoxycytidine kinase activity (Brent, 1971), ortnithine decarboxylase activity (Landy-Otsuka and Scheffler, 1980), and histone protein (Spaulding *et al.*, 1966; Stein

and Borun, 1967; D'Anna *et al.*, 1982) levels were observed. The minimally disruptive methods of mitotic selection and centrifugal elutriation have been used to examine the S-phase dependent expression of TK activity (Schlosser *et al.*, 1981; Bello, 1974), TK protein (Sherley and Kelley, 1988), TS activity (Storms *et al.*,1984), ribonucleotide reductase activity (Kucera *et al.*, 1983), dCMP deaminase activity (Gelbard *et al.*, 1969), DHFR activity and protein (Mariani *et al.*, 1981), and histone protein (Robbins and Borun, 1967; Stein and Borun, 1972; Wu *et al.*, 1981; Chanabas *et al.*, 1983). Histone protein levels were found to increase during S-phase of an ongoing cell cycle in the macronucleus of a protozoan (Prescott, 1966). Growth correlated regulation of activity or protein levels during terminal differentiation in culture was demonstrated for TK (Merrill *et al.*, 1984a; Borelli *et al.*, 1987), DNA polymerase (O'Neill and Strohman, 1968), and adenosine deaminase (Valerio, 1985).

The growth correlated regulation of enzymatic activities, proteins, or mRNAs observed in culture depends on the method used for establishing homogeneous populations of "growing" and "quiescent" cells. Although the results are often qualitatively the same, quantitative differences in the degree of regulation are observed by using different methods (see below).

b. Recombinant DNA Technology: Isolation of Replication Associated Genes and Level of Regulation

Recombinant DNA technology has allowed the genes encoding replication-associated proteins to be isolated and characterized. TK genes were the first to be isolated because they confer a selectable phenotype (Perucho *et al.*, 1980). TK genes from chicken (Perucho *et al.*, 1980), mouse (Lin *et al.*, 1985), human (Bradshaw, 1983; Lin *et al.*, 1983; Bradshaw and Deininger, 1984; Lau and Kan, 1984; Stuart *et al.*, 1985) , hamster (Lewis *et al.*, 1983), and vaccinia virus (Hruby and Ball,1982; Weir *et al.*, 1982) have been isolated. Sequences of chicken (Merrill *et al.*, 1984a), mouse (Lin *et al.*, 1985), human (Bradshaw and Deininger, 1984; Flemington *et al.*,1987), hamster (Lewis, 1986), and vaccinia virus (Hruby *et al.*, 1983; Weir and Moss, 1983) TK genes show significant similarity. In contrast, the TK gene of herpes virus (Wigler *et al.*, 1977; McKnight, 1980) is distinctly different.

Other replication-associated genes have been isolated and sequenced as well. Mouse (Crouse *et al.*, 1982), human (Masters and Attardi, 1983, Anagnou *et al.*, 1984, Yang *et al.*,1984, Chen *et al.*,1984; Srimatkandada *et al.*,1983), and hamster (Carothers *et al.*,1983, Milbrandt *et al.*, 1983) DHFR genes were found to be extremely long genes

(30 kb) containing large introns. The TS gene has been isolated from mouse (Geyer and Johnson, 1984; Deng *et al.*, 1986), humans (Takeishi *et al.*,1985), and yeast (Taylor *et al.*, 1987). Murine ribonucleotide reductase (Thelander and Berg, 1986) and adenosine deaminase (Yeung *et al.*, 1983; Yeung *et al.*, 1985) have also been isolated and sequenced. Numerous histone genes have been isolated and characterized (for review see Stein *et al.*, 1984). The wealth of isolated, sequenced, replication-associated genes is the starting material for a detailed examination of the molecular mechanism(s) which control replication-associated expression. As a first step, molecular probes were made from the cloned genes and used to determine the level of regulation.

The level of regulation refers to the step in the synthesis of a gene product that changes from one cell state to another (proliferative and quiescent states, for example). Distinctions between different levels of regulation are limited by our current knowledge of the mechanism of macromolecular synthesis in a cell. For example, the central dogma of molecular biology is: DNA "makes" RNA "makes" protein. In the context of a system which regulates levels of a particular protein, this crude mechanistic description of how a protein is made suggests two levels of regulation, namely regulation at the level of RNA "making" or protein "making". Experimentally, the distinction between these two mechanisms could be made by determining whether RNA levels change with protein levels. If RNA levels do not change, then regulation cannot be at the level of RNA "making" and is likely to be at the level of protein "making". If RNA levels change with protein levels, regulation is likely, but not certain, to be at the level of RNA "making" and not at the level of protein "making" (i.e. RNA "making" and protein "making" could be independently regulated). In reality, the current model describing the of production of an enzymatic activity in a cell is much more complex and therefore many more distinctions need to be made before the precise level of regulation can be identified. Regulation can occur at the level of transcription (McKnight and Palmiter, 1979; Groudine *et al.*, 1981; Derman *et al.*, 1981; Banerji *et al.*,1983; Khoury and May, 1977 ; Nevins *et al.*,1979; Hager and Palmiter, 1981), precursor RNA stability (Narayan and Towle, 1985, Leys *et al.*, 1984), nuclear mRNA stability (no definite example found), stalled processing (Warner *et al.*, 1985), alternative processing (Alt *et al.*, 1980; Early *et al.*, 1980; Anamara *et al.*, 1982; Capetanaki *et al.*, 1983; Breitbart *et al.*, 1985), nuclear transport (no definite example found), cytoplasmic mRNA stability (Graves *et al.*, 1987, Mullner and Kuhn, 1988), translation (Aziz and Munro, 1986; Endo and Nadal-Ginard, 1987; Ballinger and Pardue, 1983; Thireos *et al.*, 1984; Logan and Shenk, 1984), protein stability, or by postranslational modifications of protein (phosphorylation,

methylation, ubiquitination, etc.). In addition, as will become apparent in this thesis, regulation can occur at more than one level.

Since specific macromolecular probes have become available, investigations were undertaken to determine if fluctuations in growth related activities or protein levels were accompanied by similar changes in the corresponding mRNAs. Qualitatively, mRNA levels of TK (Groudine and Casimir, 1984; Thompson *et al.*, 1985; Stuart *et al.*, 1985; Liu *et al.*, 1985a,b; Stewart *et al.*, 1987; Hofbauer *et al.*, 1987; Coppock and Pardee, 1987; Gross *et al.*, 1987; Consenza *et al.*, 1988; Gudas *et al.*, 1988; Travali *et al.*, 1988), DHFR (Kellems *et al.*, 1979; Hendrickson *et al.*, 1980; Leys and Kellems, 1981; Mullner *et al.*, 1983; Kaufman and Sharp, 1983; Liu *et al.*, 1985; Farnham and Schimke, 1985 and 1986; Schmidt and Merrill, 1988), TS (Jehn *et al.*, 1985; Ayusawa *et al.*, 1986; Imam *et al.*, 1987; Greenwood *et al.*, 1987), and histone (Heintz *et al.*, 1983; Plumb *et al.*, 1983a,b; Baumbach *et al.*, 1983; DeLisle *et al.*, 1983; Plumb *et al.*, 1984; Alterman *et al.*, 1984) change as the corresponding activity or protein levels. Studies which use mitotic selection or centrifugal elutriation to monitor changes in mRNA during the continuous cell cycle either observe no qualitative changes in mRNA levels or the fold-changes observed are lower (TK: Sherley and Kelly,1988; Gross *et al.*,1988; Thompson *et al.*, 1985; DHFR: Farnham and Schimke,1986; TS: Imam *et al.*, 1987; Histone: Thompson *et al.*, 1985; Imam *et al*, 1987). Rigorous quantitative studies have not been done to insure that the change in mRNA accounts for the change in activity or protein. The exception is this thesis, which describes a quantitative study of TK activity, protein, and mRNA regulation during myoblast terminal differentiation. The results indicate that TK mRNA levels do not account for changes in TK protein and TK activity levels. This conclusion is corroborated in recent studies by Sherley and Kelly (1988).

Because changes in mRNA levels are thought (perhaps incorrectly) to account for changes of growth related protein (or activity), studies have been undertaken to determine if mRNA levels of replication-associated genes were controlled by transcriptional or postranscriptional mechanisms. Experiments in a variety of systems have lent support to either mechanism. TK mRNA is thought to be regulated by a transcriptional (Gross *et al.*,1988; Travali *et al*, 1988; Kim *et al.*, 1988), a postranscriptional mechanism (Groudine and Casimir, 1984; Coppock and Pardee, 1987; Gudas *et al.*, 1988), or both (Stewart *et al.*, 1987). Similarly, studies on DHFR have also led to both results. The groups of Schimke (Farnham and Schimke,1985) and Johnson (Santiago *et al.*, 1984) observe transcriptional regulation whereas the groups of Kellems (Leys and Kellems, 1981; Leys *et al.*, 1984), Johnson (Collins *et al.*, 1983), and Kaufman (Kaufman and Sharp, 1983) observe posttranscriptional

regulation. For TS, posttranscriptional (Ayusawa *et al.*, 1986) and a combiation of transcriptional and posttranscriptional mechanisms (Jehn *et al.*, 1985) has been invoked. These differences in results may be due to different experimental techniques used for analysing transcription rates or for obtaining proliferative and quiescent cell populations. Alternatively, mRNA levels may be determined by both transcriptional and postranscriptional mechanisms and knowledge of the relative contributions of each mechanism awaits a more quantitative investigation. Such an investigation may require a fundamentally new technique of measuring transcription rates which is less expensive, difficult, dangerous, and variable than the nuclear run-on technique currently in use.

Studies on histone expression have generally supported a model involving both a transcriptional mechanism and a posttranscriptional mechanism to account for the transient increase in mRNA levels as cells replicate their DNA (Alterman *et al.*, 1984; Artishevsky *et al.*, 1984; Baumbach *et al.*, 1984; DeLisle *et al.*, 1983; Lycan *et al.*, 1987; Sittman *et al.*, 1983; Plumb *et al.*, 1983; Heintz *et al.*, 1983).

c. Gene Transfer Techiques: *Cis* Acting Elements and *Trans* Acting Factors

A third, crucial methodology which has allowed the field to move in yet another direction is gene transformation. Gene transfer is the process whereby foreign DNA is transferred into a host cell. Gene transformation refers to situations where the transferred gene is expressed. In some cases the expression of the transformed gene in the cell is regulated with alterations in the tissue culture environment such as hormone levels, divalent cations, etc, or by changes in growth state. The ability to successfully express and regulate foreign genes transferred into cultures cells and the ability to reconstruct genes by recombinant DNA technology has allowed reverse genetics to be done.

Reverse genetics involves the directed reconstruction of a genotype by recombinant DNA technology, followed by transfer of the mutant gene into cultured cells and determination of a potentially altered phenotype. In contrast, classical genetics involves discovering an altered phenotype and then trying to discern the alteration in genotype. The critical advantage of reverse genetics is that an active, systematic search of mutations in the genotype can be conducted to find the information required for a particular phenotype, such as the replication associated expression of a gene. Alteration of a critical part of the gene will lead to an altered

phenotype when the mutant gene is inserted into the cell and tested. The critical part on a gene which, when mutated, alters its expression or regulation is termed the *cis* acting information (or element).

The likely location of the *cis* acting elements depends strongly on the level of regulation. If regulation is transcriptional, then *cis* acting elements are likely to reside in the 5' nontranscribed region of the gene (McKnight and Kingsbury, 1982; Chandler *et al.*, 1983; Gruss *et al.*, 1981; Pelham and Bienz, 1982; Mayo *et al*, 1982; Guarente *et al.*, 1982). Postranscriptional *cis* acting regulatory elements must reside on the RNA. *Cis* acting elements involved in determining mRNA stability are usually found in the 3' nontranslated region (Shaw and Kamen, 1986; Mullner and Kuhn 1988; Stauber *et al.*, 1986; Mosca and Pitha, 1986; Jones and Cole, 1987; Rahmsdorf *et al.*, 1987; Simcox *et al.*, 1985) Translational *cis* acting control elements are likely to reside in the 5' nontranslated region (Mueller and Hinnebusch, 1986; Hultmark *et al.*, 1986). Postranslational elements must reside on the protein. Hence detailed knowledge of the level of regulation is a good starting point for studies designed to determine *cis* acting regulatory elements. Such studies involve making appropriate deletions in the gene, tranferring the deleted genes into cells, and testing wether the mutated genes are regulated in a manner differring from appropriately regulated intact gene.

TK was among the first genes used to successfully transform cultured cells because it confers both a negatively and positively selectable phenotype. Cell lines which lack the TK gene (TK $^-$) can be derived by repeatedly treating cells with bromodeoxyuridine (BUdR) and visible light (Merrill *et al.*,1980). TK genes can then be transferred into these TK$^-$ cells and transformants selected in hypoxanthine-aminopterin-thymidine (HAT) medium. Early gene transformation experiments with TK showed that TK activity from the transformed genes was appropriately regulated with changes in growth state (Sclosser *et al.*, 1981). Since then, TK mRNA levels have been shown to be growth-regulated in transformants (Hofbauer *et al.*, 1987; Merrill *et al.*,1984; Gross *et al.*, 1987; Stewart *et al.*, 1987). Other systems have only recently exploited available DHFR$^-$ (Chasin and Urlaub, 1980; Urlaub *et al.*, 1983)and TS$^-$ (Ayusawa *et al.*, 1981) cell lines to do reverse genetics. Growth regulated expression of transformed DHFR (Gasser *et al.*, 1982; Kaufman and Sharp, 1983; Goldsmith *et al.*, 1986) genes has been demonstrated. In the case of DHFR, the use of minigenes, constructed by fusing 5' and 3' flanking regions with a cDNA fagment of the protein coding region, was instrumental in developing a functional gene which was small enough (i.e. lacking the large introns) to be manipulated and transformed. Expression of transformed TS genes has also been achieved (Kaneda *et al.*, 1987) although

growth regulation of the transformed gene has not yet been demonstrated. Because histone - cell lines are not available, histone genes used for transformation must be slightly altered so their mRNAs can be distinguished from those produced by the endogenous genes. Nevertheless, transformed histone genes are properly growth regulated at the level of mRNA (Alterman *et al.*, 1985; Capasso and Heintz, 1985; Luscher *et al.*, 1985; Artishevsky *et al.*, 1985,1987; Stauber *et al.*, 1986; Morris *et al.*, 1986; Seiler and Paterson, 1987).

The ability to obtain proper regulation of transformed gene expression and the more detailed, although perhaps misleading, knowledge of the level of regulation has spurred efforts to determine the *cis* acting elements involved in replication-associated expression of genes. Using promoter switch and 3' terminal exchange mutants, Merrill *et al.* (1984b) localized the *cis* acting elements controlling growth regulated TK activity of the chicken TK gene to the internal part of the gene. This result was confirmed by later studies on the chinese hamster TK gene (Lewis and Matkovich, 1986). Using a mouse TK cDNA driven by the constitutive HSV TK promoter, Hofbauer *et al.* (1987) demonstrated that sequences in the cDNA are sufficient to confer growth regulation. This observation was recapitulated by Stewart *et al.* (1987) using an SV40 promoter to drive the human TK cDNA. On the other hand, experiments by Travali *et al.* (1988) and Kim *et al.* (1988) demonstrate that the promoter of human TK, when fused to heterologous genes, is sufficient to direct growth regulation. Similar studies on the DHFR gene have concluded that *cis* acting growth-regulatory information controlling DHFR mRNA resides in the 3' nontranslated region (Kaufman and Sharp, 1983) and *cis* acting information controlling DHFR protein levels resides in the 5' nontranscribed region (Goldsmith *et al.*, 1986). Neither of these studies is very convincing. No progress has been made in determining the *cis* acting regulatory elements in TS by reverse genetics, although in this regard it is interesting that the growth regulated mouse TS mRNA lacks a 3' nontranslated region (Jehn *et al.*, 1986). *Cis* acting regulatory elements of the histone genes have been localized to the 5' end (Seiler *et al.*, 1987; Morris *et al.*, 1986; Artishevsky *et al.*, 1985; Artishevsky, 1987) or the 3' end (Luscher *et al.*, 1985; Stauber *et al.*, 1986) by use of reverse genetics. Studies which identify an element in the 5' flanking or 5' nontranslated regions, implicate it in transcriptional control (Seiler *et al.*, 1987; Artishevsky *et al.*, 1985 and 1986) or mRNA stability (Morris *et al.*, 1986), respectively. An *in vitro* transcription system which preferentially transcribes a histone gene in S-phase extracts (Heintz and Roeder, 1984) has also been used to define *cis* acting regulatory elements in the 5' flanking region (Hanley *et al.*, 1985). The group which has localized a *cis* acting element at the 3' end

has found it in the same location as an element required for 3' processing of the histone mRNA (Stauber *et al.*, 1986).

Trans acting factors are the proximal molecular signals mediating regulation or expression of a gene by their interaction with the appropriate *cis* acting elements. Detailed knowledge of the *cis* acting regulatory information and the level of regulation are valuable in identifying and isolating the *trans* acting factors mediating regulation or expression of a gene product. The level of regulation determines wether the *trans* acting factor interacts with DNA, precursor RNA, mRNA, or protein. Knowledge of the *cis* acting information allows one to design an assay specific for binding of the factor, which, for example, can be used to characterize fractions in a classical isolation procedure. If the *trans* acting factor is a protein, its regulation can be studied by the same strategy used for the original gene product, that is by isolating the gene, determining the level of regulation, using reverse genetics to determine the *cis* acting regulatory information, and then identifying and isolating another *trans* acting factor. This method can be applied repeatedly until a primary signal is discovered. Most examples of *trans* acting factors are related to transcriptionally regulated systems. Since the research described in this thesis does not deal with *trans* acting factors, only a brief review of progress in isolating *trans* acting factors involved in replication associated gene expression is presented.

Very few putative *trans* acting factors controlling replication associated expression have been identified. Using a gel retention assay, Knight *et al.*(1987) have demonstrated that a protein found only in S-phase extracts binds the human TK promoter sequences. Histone genes transfected into mammalian cells at a high copy number were able to supress regulated expression of the endogenous gene, leading to the hypothesis that *trans* acting transcription factors specific to histones exist (Capasso *et al.*, 1985). Perhaps more convincing is a gel retardation study which shows proteins binding in a S-phase specific manner to a hamster H3 promoter sequence which confers cell cycle regulation to a neomycin resistance gene (Artishevsky *et al.*, 1987).

The object of the reverse approach is to establish mechanistic connections from the regulated gene product up the mitogenic signalling cascade, to the primary signal that changes its expression. The underlying assumption of this approach is that certain categories of growth regulated genes will be controlled by the same primary signals that control growth. Although substantial progress has been made in isolating and characterizing expression patterns of S-phase dependent genes, the precise level of regulation, the *cis* acting regulatory information and the *trans* acting factors involved

often remain controversial or not determined.

III. Scope of Thesis

A. Background

The research described in this thesis on the growth regulation of chicken TK during terminal differentiation of myoblasts in culture grew out of initial studies by Merrill *et al.* (1984 a,b), which involved determination of the structure of the chicken TK gene, transformation and expression of the gene in cultured cells, and observation of regulated TK activity levels during terminal differentiation of transformed TK^- myoblasts. The rationale for studying thymidine kinase was multifaceted. TK was one of the first genes to be isolated and cloned and was at the time one of the only genes available for this type of research. Second, TK was one of the first genes that could be successfully transformed into and expressed in cultured cells lacking the endogenous gene because it serves as both a negative and positive selectable marker. Myoblast cell lines lacking endogenous TK expression, but retaining the ability to terminally differentiate in culture had been derived by Merrill *et al.* (1980). Lastly, TK was known to be regulated in a growth dependent manner (Schlosser *et al.*,1981) in transformants. For the four reasons above, TK was amenable to reverse genetics.

Myoblasts undergoing terminal differentiation were chosen as the system for studying growth dependent TK expression because a TK^- line was available and because this system more closely resembled the *in vivo* situation of a cell undergoing cessation of division than other commonly used disruptive methods.

A 3 kb genomic fragment containing most of the cTK gene was cloned by Perucho *et al.*, (1980). This fragment can confer HAT resistance to TK^- mouse L cells. The functional boundaries of the chicken TK gene were established by creating nested sets of deletion mutants at the 5' and 3' ends of this fragment and determining which deletion mutants could confer resistance to HAT (Merrill *et al.*, 1984a). This fragment can also transform TK^- myoblast cell lines. Northern analysis revealed that a 2.1 kb TK mRNA was produced from the transformed gene (Merrill *et al*,1984a). TK activity and TK mRNA declined as transformed mouse myoblasts terminally differentiated (Merrill *et al.*, 1984b). Thus, the 3 kb fragment contained sufficient *cis* acting information for growth regulated expression of TK. TK activity was also regulated in myoblasts transformed with promoter switch mutants and 3' replacement mutants, indicating that the *cis* acting regulatory information was in the protein coding region or introns (Merrill *et al.*, 1984b).

B. Summary of Chapters

Because TK mRNA declined during myogenesis, my initial research on this project was to determine if TK was regulated at the transcriptional or posttranscriptional level. In addition, it was necesary to see if results on TK regulation obtained in transformed myoblasts reflected the *in vivo* situation. Chapter 2 describes this research, which was published in volume122 of Developmental Biology (1987). Nuclear run-on analysis determined that the decline in TK mRNA was accompanied by a decline in transcription of the TK gene. A sensitive, quantitative method was developed to determine TK mRNA levels. This method was used to demonstrate that TK mRNA levels decline with growth rate in four tissues of the developing chicken embryo. Reexaminatioin of TK mRNA regulation in transformed myoblasts was also undertaken with the aid of this sensitive and quantitative technique. Unexpectedly, the decline in TK mRNA could not account fully for the decline in TK activity. Therefore another level of regulation, either translational or posttranslational, was invoked.

Compilation of a large data set on the regulation of TK mRNA and TK activity expressed from the full length gene demonstrated the regulation of TK mRNA was smallfold and variable and the decline in TK activity was independent of the decline in TK mRNA levels. TK activity was therefore regulated either at the level of translation, protein degradation, or posttranslational modification. In order to distinguish between these models an anti-TK antibody was generated and used to determine that TK protein levels were regulated. Thus, TK activity declines due to a translational or protein degradational mechanism. These studies are described in chapter 3 and have been accepted for publication in Nucleic Acids Research in 1988. Polysome distribution studies of TK mRNA currently being conducted to determine if a translational or degradational mechanism is operative are not included in the thesis.

Because TK mRNA and TK activity are regulated independently, each mode of regulation is likely to be mediated by a unique set of *cis* acting information. Chapter 4 presents data which localize *cis* acting information mediating TK activity and TK mRNA regulation to the protein coding region. Promoter switch and 3' replacement mutants were transformed into myoblasts and tested for TK activity and TK mRNA regulation. No mutant gene consistently abolished regulation of TK activity (Merrill *et al.*,1984b) or TK mRNA. At this juncture we reasoned that the *cis* acting elements responsible for TK activity and TK mRNA regulation resided in the introns, since it seemed unlikely that the protein coding region should also contain regulatory information. Therefore, a precise

set of intron deletion mutants was constructed and tested for TK activity and TK mRNA regulation. No intron deletion mutant altered the regulated TK activity phenotype. Hence, *cis* acting information mediating TK activity regulation was thought to reside in the protein coding region, a location consistent with the translational or degradational level of regulation. Similarly, no intron deletion mutant could consistently abolish the regulation of TK mRNA, although a slight alleviation of TK mRNA regulation was observed in intron deletion mutants. Interpreted directly, these results indicated *cis* acting information mediating transcriptional TK mRNA regulation was also located in the protein coding region. However the latter result required equivocation for four reasons: 1) the smallfold and variable decline in TK mRNA levels which made it difficult to interpret changes in regulatory phenotype without numerous repeats of a very costly experiment; 2) controversy surrounding the growth regulatory properties of several heterologous promoters used calls into question the promoter switch experiments used to determine the promoter was inconsequential to TK mRNA regulation; 3) the possibility of redundant regulatory elements in different parts of the gene could not be eliminated; and 4) transcriptional regulatory elements in the protein coding region have never been noted previously. Therefore, two more general mechanisms for TK mRNA regulation were proposed to reconcile our data with published work.

Because we had a complete set of precise intron deletion mutants, we used them to answer a fundamental question in molecular biology, namely: Are introns essential for expression of mRNA? Early studies by Gruss and Khoury using chimeric genes indicated that introns were essential for expression of mRNA in mammalian cells. However, other studies with yeast and plant genes, which utilized precise intron deletion mutants, indicated that introns were inconsequential to expression of mRNA. We conducted a detailed comparison of expression from the wild-type gene and intron deletion mutants transformed into mouse L cells. An internal control gene was used to demonstrate that introns were inconsequential to the expression of TK mRNA in L cells. This research was described in chapter 5 and has been published in volume 7 of Molecular and Cellular Biology (1987).

CHAPTER 2:

The Chicken Thymidine Kinase Gene is Transcriptionally Repressed During Terminal Differentiation; the Associated Decline in TK mRNA Cannot Account Fully for the Disappearance of TK Enzyme Activity

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Abstract

Thymidine kinase is representative of a class of enzymes involved in DNA precursor biosynthesis that decline as cells withdraw from the cell cycle. If TK activity is regulated exclusively by the availability of messenger RNA, changes in enzyme activity levels should not precede or excede changes in TK mRNA levels. This prediction was tested in several tissues during chicken embryogenesis and in differentiating muscle cells in culture. A sensitive method of determining absolute TK mRNA levels was developed. A synthetic complimentary RNA probe spanning an intron acceptor site in the chicken TK gene was hybridized with cellular RNA or synthetic colinear TK RNA of known concentration. After RNase digestion and gel electrophoresis, the intensity of the protected fragment was used to calculate absolute TK mRNA levels. As few as 0.02 molecules of TK mRNA per cell could be measured accurately. Depending on the tissue type, 8 day embryos contained between 3 and 12 TK mRNAs per cell. Proliferating mouse muscle cells transformed with the chicken TK gene contained between 30 and 150 TK mRNAs per cell. Both *in vivo* and *in vitro*, TK mRNA levels declined as cells withdrew from the cell cycle during differentiation. *In vivo*, the decline in TK activity never preceded or exceded observed changes in TK mRNA. However, in the cell culture system, TK activity consistently declined to a greater extent than TK mRNA. Thus, a translational or posttranslational mechanism must also be operative in controlling TK activity levels. Estimation of transcription rates in nuclei isolated from proliferating and differentiated muscle cell transformants indicated that the TK gene was transcriptionally repressed in postreplicative cells.

Introduction

A common motif in the development of higher organisms is the cessation of DNA replication as cells terminally differentiate. Prior to accumulating tissue-specific gene products characteristic of the differentiated state, cells of many lineages either reversibly or irreversibly withdraw from the cell cycle. Cells thus have a mechanism for selectively shutting down their replicative apparatus. One aspect of this mechanism is the process by which postreplicative cells coordinately suppress the expression of enzymes involved in DNA precursor biosynthesis and polymerization. Elucidation of the molecular basis for the replication-dependent expression of this class of gene products would contribute to our overall understanding of how cell proliferation is regulated during normal development.

Only rarely have changes in the levels of replicative enzymes been demonstrated during development *in vivo* (Stockdale, 1970; Scholl, 1968; Silber *et al.*, 1962). Far more frequently, the replication-dependent expression of this class of gene products has been investigated using cultured cells. For example, numerous studies using synchronized cells have established that levels of thymidine kinase (TK), dihydrofolate reductase (DHFR), thymidylate synthetase (TS), and other replicative enzymes are transiently elevated during S phase (Navaglund *et al.*, 1980; Mariani *et al.*, 1981; Schlosser *et al.*, 1981; Bradshaw, 1983; Storms *et al.*, 1984; Farnham and Schimke, 1986; Liu *et al.*, 1985). In addition, these enzymes have been shown to be more abundant in proliferating cells than in serum starved or contact inhibited cells (Conrad and Ruddle, 1972; Johnson *et al.*, 1978; Johnson *et al.*, 1982; Lewis and Matkovitch, 1986). The development of schemes for genetically selecting for or against expression of certain replicative enzymes has facilitated molecular cloning of the corresponding genes and allowed investigation of the regulatory mechanism by *in vitro* mutagenesis. For example, the *cis* acting regulatory information involved in the reduction of cytoplasmic TK activity in differentiating myoblast transformants was localized by *in vitro* mutagenesis to a region within or very close to the protein coding region of the chicken TK gene (Merrill *et al.*, 1984b).

To better understand the molecular mechanisms governing growth dependent expression of replicative enzymes, it is important to identify the level of regulation; thus establishing whether the *cis* and *trans* acting regulatory elements are acting on the DNA, RNA, or protein. Paradigms exist for several types of control. Changes in enzyme levels have been variously attributed to posttranslational effects on protein activity or

stability (vanBlerkom, 1985), differential efficiency of translation from a constant level of mRNA (Storti *et al.*, 1980), posttranscriptional effects on RNA processing or stability (Leys and Kellems, 1981), or differential rates of gene transcription (McKnight and Palmiter, 1979). As an initial means of distinguishing among the above possibilitities, one approach is to determine whether changes in mRNA level can account temporally and quantitatively for observed changes in enzyme level.

Quantitative comparison of specific replicative enzyme and mRNA levels during terminal differentiation would provide a test of the simple model that replicative enzymes are regulated exclusively by the cellular concentration of mRNA. Using proliferating mouse cells containing differentially amplified DHFR genes, Alt et al. (1978) showed that DHFR enzyme levels are directly proportional to DHFR mRNA levels. We have determined that a similar linear relationship exists between TK enzyme and TK mRNA levels in proliferating mouse cells transformed with chicken TK genes (unpublished observation). If the decline in TK activity with withdrawal from the cell cycle is mediated exclusively at the mRNA level, the magnitude of the decline in mRNA must exceed (or at least equal) the decline in enzyme. Furthermore, to be causal, the reduction in mRNA must precede (or at least coincide with) the decline in enzyme level. If enzyme declines earlier than or to a greater extent than mRNA, a translational or posttranslational component to the regulatory mechanism must be invoked.

In this report, we describe a sensitive method for measuring absolute levels of the rare TK messenger RNA. SP6-generated TK pseudo-mRNA was used to establish a standard curve in a quantitative assay based on RNase protection (Melton *et al.*, 1984). We use this method to demonstrate that the steady state level of chicken TK mRNA declines during *in vitro* skeletal muscle differentiation and during *in vivo* muscle, heart, liver and brain embryonic development. The magnitude and timing of the decline in TK mRNA in all *in vivo* cases was sufficient to explain observed changes in TK enzyme activity. However, during *in vitro* muscle differentiation, TK enzyme activity declined earlier and to a greater extent than TK mRNA, indicating that a translational or posttranslational mechanism must also be operative. To investigate the molecular basis for the decline in TK mRNA level, nuclear run-off transcription assays were done using nuclei from proliferating and committed muscle cell transformants. Our results indicate that the cellular TK gene is transcriptionally repressed as cells withdraw from the cell cycle during terminal differentiation.

Materials and Methods

Cell Culture and Transformation Conditions

Mouse muscle cells were grown as described elsewhere (Merrill *et al.*, 1984b). To induce differentiation, cultures were rinsed twice with Ham's F10 and incubated in a defined mitogen-depleted medium consisting of Ham's F10 supplemented with 0.8 mM $CaCl_2$, 10^{-6} M insulin, and 10^{-7} M dexamethasone. The TK^- subline used for transformation was derived from the MM14 cell line (Linkhart *et al.*, 1980) as described by Merrill *et al.*, 1980. Muscle cells were transformed using the calcium phosphate precipitation method (Graham and van der Eb, 1973) with modifications (Merrill *et al.*, 1984b). The TK-containing plasmid used in transformations was either pCH-TK5, containing a 3.0 kb Hind3 fragment encoding chicken TK (Perucho *et al.*, 1980), or pCHTKfl, a plasmid containing the full length chicken TK gene (Merrill and Tufaro (1986). The pKNeo plasmid used for co-transformation was obtained from D. Hanahan. Co-transformant were selected in G418 (Gibco) at 400 µg/ml and then maintained in G418 at 100 µg/ml.

Assay of TK Enzyme Activity.

At various times after induction, cultures were harvested for analysis of TK activity as described previously (Merrill *et al.*, 1984b). To determine TK activity in tissues, intact organs were dissected from white leghorn chick embryos immediately after sacrifice. The muscle "organ" was the leg between the hip and knee joint from which most bone and skin was removed. Organs were weighed and then homogenized on ice in 10 volumes of TK extraction buffer (Merrill *et al.*, 1984b) with a polytron (Kinematica GmbH) for 30 s at maximum power. Aliquots of the homogenate were spun 15 min in a microfuge and the TK activity in the supernatant was determined as described by Merrill *et al.*, (1984b). A fluorimetric assay involving Hoecht 33258 dye binding (Labarca and Paigen, 1980) was used to determine the DNA concentration in the tissue and cell culture homogenates before centrifugation.

Isolation of RNA

RNA was prepared from cultured cells by a method employing Proteinase K and DNase digestion. Cells were scraped from 10 cm dishes in 0.4 ml 1x TES (10 mM

Tris-HCl [pH 7.5], 5 mM EDTA, 1% SDS) containing 200 µg proteinase K per ml and were digested 30 min at 50°C. Digests were brought to 250 mM NaCl, extracted with phenol/chloroform followed by chloroform, and precipitated with ethanol. Precipitates were resuspended in DNase buffer (20 mM HEPES [pH 7.8], 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$) and digested for 30 min at 37°C with RNase-free DNase (Promega-Biotec) at 0.1 U/µl. Digestions were stopped by adding EDTA to 50mM, extracting with phenol/chloroform followed by chloroform, and precipitating with ethanol. Following resuspension in water, remaining DNA oligomers were by adjusting samples to 2 M ammonium acetate and 38% isopropanol, allowing precipitate to form for 30 min at room temperature, and centrifuging for 15 min using a microfuge. Pellets were washed with 70% ethanol and resuspended in water.

RNA was isolated from tissues using the method of Glisin *et al.* (1974) with modifications. Immediately after excision, organs were homogenized in at least five volumes of denaturant (4 M guanidinium isothiocyanate, 5 mM sodium citrate [pH 7.0], 0.1 M 2-mercaptoethanol, 0.5% sarkosyl) using a polytron (Kinematica GmbH) at maximum setting for 30 s. Homogenates were sonicated for 1 min to shear DNA and centrifuged for 10 min at 1000 x g to remove small amounts of debris. After addition of CsCl (0.4 g/ml), homogenates were layered on 0.3 volume pads of 5.7 M CsCl, 0.1 M EDTA and centrifuged at 85,000 x g for at least 18 hours in a swinging bucket rotor. After sequentially removing supernatant and pad, each RNA pellet was resuspended in 1X TES, brought to 250 mM NaCl, immediately extracted with phenol/chloroform followed by chloroform, and precipitated with ethanol.

To represent TK mRNA levels on a per cell basis, it was neccessary to establish total RNA content per cell. For this purpose, total nucleic acid was prepared from individual organs by homogenization in 10 volumes 1xTES, removal of 400 µl for digestion for 1 hr with proteinase K (200 µg/ml), extraction with phenol/chloroform followed by chloroform, and precipitation with ethanol. Following resuspension of pellets in water, total nucleic acid content was determined by absorbance at 260 nm, and DNA content was determined by Hoechst staining (Labarca and Paigen, 1980). RNA content was calculated by subtraction. Using this method, RNA content per cell (i.e. RNA content per genome equivalent of DNA) was invariantly 2.6 pg in all chicken muscle preparations throughout development and 6.5 pg in mouse cells during differentiation *in vitro*. These values were used in representing TK mRNA copy number on a per cell basis as shown in Figs. 2.2A and 2.3C. For the other *in vivo* tissues analyzed, RNA content per cell sometimes varied during development. In these tissues

TK mRNA levels were individually normalized for RNA content per cell at each developmental time point as shown in Table 2.1.

Preparation of Synthetic RNAs

DNA templates (see Fig. 2.1) were linearized to give the desired transcript end, extracted with phenol/chloroform, precipitated with ethanol, washed twice with 70% ethanol, and dried. Each transcription reaction contained 2 μg template, 4 μl of 5x transcription buffer (200 mM Tris-HCl [pH 7.5], 30 mM $MgCl_2$, 10 mM spermidine), 0.4 μl freshly thawed 0.5 M dithiothreitol, 0.7 μl of 30 U per μl RNasin ribonuclease inhibitor (Promege-Biotec Inc.), 4 μl of freshly thawed 5X rNTPs (GTP,CTP,ATP, 2.5 mM each), and 1 U SP6 polymerase (Promega-Biotec Inc.). Reactions designed to generate non-radioactive transcripts included UTP at a final concentration of 0.5 mM whereas those designed to generate high specific activity, full length probes included 30 μM unlabeled UTP and 50 μCi (3.3 μM) [^{32}P]UTP (New England Nuclear). All reactions were assembled from components at room temperature and were brought to a final volume of 20 μl with water. After incubation at 40°C for 1 hr, 30 U of RNasin, 25 μg of tRNA, and 1 U of RNase-free DNase were added. After 15 min at 37°C, unincorporated nucleotides were removed by gel filtration on a 10 ml Sephadex G-50 column equilibrated with 1x TES. The eluant fraction containing the probe was extracted with phenol/chloroform followed by chloroform, and precipitated with ethanol.

RNase Protection Assay

Sample RNA (up to 50 μg) was mixed with high specific activity probe (approximately 1 fmol of probe per μg RNA). In order to duplicate digestion conditions in standards and unknowns, all samples were adjusted to equivalent amounts of RNA with yeast total RNA. Similarly, controls containing undigested probe were supplemented with yeast RNA, as well as 0.1 fmol of TK pseudo-mRNA. RNA mixtures were lyophilized to dryness and immediately resuspended in 30 μl of hybridization mix (80% freshly thawed deionized formamide, 40 mM PIPES [pH 6.7], 0.4 M NaCl, 1 mM EDTA). Samples were hybridized 15-24 hours at 55°C and 300 μl of RNase digestion buffer (10 mM Tris-HCl [pH 7.5], 5 mM EDTA, 0.3 M NaCl) containing 40 μg/ml RNase A and 2 μg/ml RNase T1 was added. Digestions were for one hour at 37°C, conditions empirically determined to give an optimal protected signal. Digestions were terminated

by addition of 10 µl of 10% SDS and 2.5 µl of a 20 mg/ml proteinase K stock and continued incubation at 37°C for 15 min. This was followed by vortexing with a half volume of phenol, addition of 20 µg tRNA, vortexing with a half volume of chloroform, and removal of the aqueous phase to a fresh tube. Samples were then extracted with one volume of chloroform and precipitated with 2.5 volumes of 100% ethanol. Precipitates were washed twice with 70% ethanol and resuspended in 2 µl water. The samples were mixed with 8 µl of deionized formamide containing tracking dyes, heated to 65°C for 15 min, and loaded on 0.3 mm thick, prerun, prewarmed, sequencing gels (9% polyacrylamide, 8 M Urea, 0.5xTBE [45 mM Tris-OH, 45 mM Boric acid, 1 mM EDTA]). Gels were dried and autoradiographed with the aid of an intensifying screen. The protected bands on the autoradiograph were scanned with a Zeineh model SL-504-XL soft laser densitometer (Biomed Instruments Inc.). Peaks were cut out and weighed to determine band intensity. Band intensity was plotted against input pseudo-mRNA, and the resulting standard curve was used to determine absolute levels of TK mRNA in experimental samples.

Determination of TK Transcription Rates in Isolated Nuclei

Nuclei were isolated from proliferating and differentiated muscle cells as described by Groudine and Casimir (1984). All manipulations were performed on ice or at 4°C. Scraped up cells were disrupted by vortexing in 10 volumes of RSB (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 5 mM $MgCl_2$) including 0.5% NP-40. Nuclei were pelleted by centrifuging 20 min at 2000 x g, washed in RSB, and resuspended in nuclei freezing buffer (50 mM Tris-HCl [pH 8.3], 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol) at a DNA concentration of 2 mg/ml, determined by the method of Labarca and Paigan (1980).

Transcription of nuclei and subsequent RNA isolation were performed using a modification of the procedure of McKnight and Palmiter (1979). For transcription, nuclei equivalent to 275 µg of DNA were incubated for 10 min at 26°C in a 0.5 ml reaction containing 30% glycerol, 30 mM Tris-HCl [pH 8.3], 2.5 mM dithiothreitol, 1 mM $MgCl_2$, 70 mM KCl, 0.5 units/µl RNasin (Promega-Biotec), 0.4 mM each of ATP, GTP, and CTP, and 500 µCi [^{32}P]UTP (800 Ci/mmol, New England Nuclear). The reaction was terminated by incubation with 100 µg/ml proteinase K in 1xTES for 60 min at 55°C. Following extraction with phenol/chloroform and chloroform, the aqueous phase was precipitated with ethanol. The precipitate was washed with 70% ethanol, resuspended

in water, adjusted to 1x DNase buffer (50 mM Tris-HCl [pH7.5], 10 mM $MgCl_2$, 2mM $CaCl_2$), and incubated with 10 units of DNase I for 60 min at 37°C. Following DNase digestion, the reaction was adjusted to 1% SDS, 5 mM EDTA and 100 µg/ml proteinase K, incubated at 55°C for 60 min, and extracted with phenol/chloroform and chloroform. The aqueous phase was adjusted to 30 mM $Na_4P_2O_7$ and precipitated at 4°C with cold 10% trichloroacetic acid. The precipitate was collected by centrifugation, washed 3 times with cold 5% trichloroacetic acid, 10 mM $Na_4P_2O_7$, and resuspended in 200 µl 0.25 M Tris-HCl [pH 8], 5 mM EDTA. Incorporation of [^{32}P]UTP into RNA was determined by liquid scintillation counting. The remainder was precipitated with ethanol.

Single-stranded M13 phage DNAs were immobilized on nitrocellulose filter discs using procedures described by McKnight and Palmiter (1979). Each disc contained 0.5 pmol of immobilized DNA. A 945 bp Sst1/Bgl 2 insert from the chicken TK gene was cloned into mp18 or mp19. This insert corresponds to 33% of the primary TK transcript and was purposefully used because it lacks sequences near the 5' and 3' end of the TK gene that tend to hybridize with ribosomal RNA. Singlestranded mp19TK DNA is complementary to TK RNA and was used to detect transcription from the TK encoding strand. Singlestranded mp18TK is colinear with TK RNA and was used to detect transcription from the opposite strand of the TK gene. A third disc contained parental mp19 DNA and was used to detect nonspecific hybridization of labeled RNA to the filters. Filters were prehybridized for 1 hr at 55°C in 100 µl of hybridization buffer (50% formamide, 5xSSC, 50 mM $NaPO_4$ [pH 6.5], 1x Denhardt's, 250 µg/ml salmon sperm DNA). Filters in hybridization buffer were overlaid with 0.2 ml mineral oil. RNA to be hybridized was heated 15 min at 65°C in water, adjusted to 1X hybridization buffer (total volume of 100 µl), maintained at 65°C for 15 min, transferred to prehybridized filters, and overlaid with 0.2 ml mineral oil. Hybridizations were for 16-24 hrs at 55°C. The filters were washed twice for 15 min at room temperature in washing buffer (20 mM $NaPO_4$ [pH 6.5], 50 mM NaCl, 1 mM EDTA, 0.1% SDS) and twice for 1 hr at 65°C in washing buffer. Filters were then washed twice for 5 min at room temperature in 2xSSC (0.3 M NaCl, 30 mM sodium citrate), digested with 0.1 µg/ml RNase A in 2xSSC for 5 min at 37°C, and washed twice for 15 min at 37° in washing buffer. Filters were air dried and exposed at -80°C to Kodak XAR-5 film to determine the effeciency of the washing procedure. If necessary the filters were retreated with RNase as above. Filters were dissolved in soluene and quantified by liquid scintillation counting in omnifluor.

Results

Absolute Quantitation of TK mRNA

Fig. 2.1 outines essential features of the RNase protection assay used to determine absolute TK mRNA levels. The protein coding region of the chicken TK gene is interrupted by six introns. To generate a ^{32}P-labeled RNA probe complementary to TK mRNA, a genomic Pst1/Bgl2 restriction fragment, spanning the sixth intron acceptor site, was inserted into the SP65 transcription vector. Linearization of this template with Hind3 followed by transcription in the presence of [^{32}P]UTP, low concentrations of unlabeled UTP, and excess concentrations of the other three nucleotides, yielded a 214 base RNA probe suitable for RNase mapping. The ratio of labeled to unlabeled UTP in the reaction was optimized to make a probe of the highest specific activity while minimizing premature termination due to lack of UTP. From the known sequence, 147 bases of this probe was expected to be complementary to mature TK mRNA. To generate a synthetic RNA that was colinear with TK mRNA, a chimeric genomic DNA/cDNA fragment from EcoRI to Hind3, which spans the entire protein coding region, was cloned into SP65. Linearization of this template with Bgl2 and transcription in the presence of high concentrations of all four nucleotides yielded 10 μg of RNA, as measured by absorbance at 260 nm. The integrity of the synthetic colinear RNA was confirmed by gel electrophoresis. Although the synthetic colinear RNA lacked native 5' and 3' termini, it was identical to TK mRNA in the region to which the radioactive probe hybridizes. It is henceforth referred to as pseudo-mRNA.

To generate a standard curve for quantitating TK mRNA in an experimental sample, various quantities of pseudo-mRNA were hybridized to the complementary probe and then treated with RNase to remove nonhybridized sequences. Protected fragments were sized on denaturing polyacrylamide gels and autoradiographed. The intensity of each protected band was determined by laser densitometry, and a standard curve relating intensity to input pseudo-mRNA was constructed. To assess the reproducibility and precision of the assay, a sample containing 0.1 femtomoles of pseudo-RNA was quantitated in six separate experiments and was found to give a mean value of 0.100 fmol with a 0.011 fmol standard deviation.

The assay was extremely sensitive. Using 50 μg of total cellular RNA, messages as rare as 0.02 copies per cell could be accurately quantitated. Because of the sensitivity of the assay, the enrichment provided by oligo-d(T) selection of polyadenylated RNA

was not neccessary. Use of total RNA in the quantitation assay was preferable because errors due to differential enrichment during oligo-d(T) selections were eliminated. It also assured that all messages in the cell were analyzed. Oligo-d(T) selection would exclude mRNAs with short polyA tracts or none at all.

TK Enzyme and mRNA Regulation during *in vitro* Myoblast Differentiation

Proliferating myoblasts in culture withdraw from the cell cycle and commit to terminal differentiation when shifted to mitogen depleted medium (Linkhart *et al.*, 1980). TK activity declines soon after induction in mouse myoblasts expressing either their endogenous TK gene or transfected copies of the chicken TK gene (Merrill *et al.* 1984b). To determine whether observed changes in TK activity could be attributed to a decline in TK mRNA, total RNA was isolated from chicken TK transformants at various times after induction and analysed by the RNase protection assay described above. An autoradiograph of a representative RNase mapping gel is shown in Fig. 2.2B. As expected, nondigested probe (lane 9) gave a prominent signal at 214 bases, corresponding to the full length probe. The presence of shorter fragments, probably corresponding to transcripts that had terminated prematurely during the transcription reaction, did not interfere with the quantitation assay. A control digestion containing only yeast RNA (lane 10) gave no protected signal. Similarly, RNA from nontransformed mouse cells, or mouse cells transformed with plasmids encoding genes other than chicken TK, never gave a protected signal using the chicken TK probe (data not shown). Total RNA from proliferating myoblast cultures (lane 11) gave a strong signal at 147 bases, corresponding to mature TK mRNA. In addition, a weak signal moving slightly faster than the nondigested probe was observed occasionally. This weak signal was probably due to incomplete RNase digestion of nonhybridized probe. Lanes 12-16 contain RNA from muscle cells at various times after inducing differentiation. By 25 hours after induction (lane 16), the strength of the 147 base signal had declined several-fold. To calculate absolute TK mRNA levels, the intensity of the 147 base signal in experimental samples was compared to a range of pseudo-mRNA standards (lanes 2-8).

The calculated absolute levels of TK mRNA during differentiation are shown in Fig. 2.2A (squares). TK mRNA, initially 31 copies/cell, declined to less than 8 copies/cell by 25 hours after induction. Fig. 2.2A also shows results obtained from parallel cultures

carried through the experiment to monitor the [^{3}H]thymidine labeling index (circles) and TK enzyme activity (triangles). TK activity declined to a greater extent than TK mRNA. Clearly a mechanism exists for reducing steady state TK mRNA levels as cells withdraw from the cell cycle during terminal differentiation. However, the four fold decline in TK mRNA cannot account fully for the 13 fold decline in TK activity. This suggests that there is an additional translational or posttranslational component to the overall mechanism governing TK activity levels.

TK Regulation during *in vivo* Muscle Development

Since our *in vitro* system was comprised of mouse cells transformed with a chicken gene, it seemed prudent to establish that the pattern of TK regulation documented above also ocurred during muscle development *in vivo*. Myoblasts cannot be distinguished from non-myogenic cells *in vivo*. However, in mature muscle over 85% of the nuclei are in clearly distinguishable syncytia (Marchok and Herrmann, 1967). Therefore we assumed that embryonic muscle is rich in myogenic cells and that phenotypic changes in the total cell population was reflective of changes ocurring in the myogenic subpopulation.

Accurate measurement of proliferative rates *in vivo* is difficult. Using an analysis based on [^{3}H]thymidine incorporation into nuclear DNA and autoradiography of thin sections, Marchok and Herrmann (1967) estimated that in day 8 chicken leg muscle 55% of the nuclei are proliferative and at day 24 this figure drops to 10%. As TK enzyme is required for entry of thymidine into the DNA precursor pathway and our aim was to investigate the relationship between TK enzyme and mRNA level as a function of proliferative state, we estimated the proliferative state of tissues by measuring the mass and DNA content of thigh muscle during development. As shown in Fig. 2.3A, both wet weight and DNA content increased less rapidly at later stages of development, suggesting a slowing of proliferative rate with embryonic age. The first derivative plot of DNA values best shows the change in proliferative rate during development (Fig. 2.3B). DNA content per thigh muscle increased 420% between day 8 and 10. In contrast, DNA content increased only 20% between day 22 and 24. Overall there was a steady drop in the rate of DNA accumulation throughout later development. Fig. 2.3C shows that TK activity in thigh muscle homogenates decreased progressively during development. As shown in the autoradiograph in Fig. 2.3D and represented quantitatively in Fig. 2.3C (squares), absolute TK mRNA levels also declined

progressively during development. Both TK activity and TK mRNA exhibited a 13 fold decline between day 8 and 20. There was no need to invoke a translational or posttranslational mechanism for developmental regulation of muscle TK activity *in vivo*. The data is consistent with regulation of TK activity by a mechanism affecting only TK mRNA levels. It conflicts with the results of *in vitro* determinations which suggested that the decline in TK enzyme activity during differentiation was at least partially mediated by a translational or posttranslational mechanism.

TK Regulation During *In Vivo* Development of Liver, Heart and Brain

The analysis of TK activity and TK mRNA in muscle *in vivo* failed to reveal evidence for translational or postranslational control of TK activity. We therefore investigated the relationship between TK enzyme and TK mRNA levels in three additional organs of the developing chicken embryo. Liver, heart and brain were selected because they were relatively easy to dissect *en masse* even at early stages of development. Four parameters were measured: proliferative rate, TK enzyme activity, total RNA content and TK mRNA level. Results of these analyses are summarized in Table 2.1 (see also appendix 1).

Proliferative rate (% change in DNA per organ per 48 hr interval) declined during development in all three tissues. The timing and magnitude of the change in proliferative rate varied with each particular organ type. The growth rate of liver declined 88% between days 8 and 18 and rose slightly thereafter. The growth rate of heart declined only 71% between days 8 and 18 but continued to decline until hatching. The initial growth rate in brain was much lower than in any of the other tissues and declined only two-fold between days 8 and 22. This was not surprising since the brain is already well developed by day 8.

Quantitative comparisons of TK enzyme and TK mRNA levels in all three organ systems were consistent with a model whereby TK enzyme activity was governed solely by a mechanism affecting TK mRNA levels. In all cases, declines in TK enzyme activity during development were preceded by equal or greater declines in TK mRNA levels. For example, at 18 days postfertilization liver TK mRNA levels reached a nadir of 0.2 molecules per cell, 96% lower than initial 8 day levels. On the other hand, liver TK activity declined only 54% by day 18 and required several additional days to reach minimal levels. A similar pattern was observed in heart, where throughout development the decline in heart TK mRNA always preceded and exceeded the decline in heart TK

activity.

The data in Table 2.1 (Appendix 1) shows that TK activity usually failed to decline to the same extent as TK mRNA. This result was not unexpected. A decline in mRNA often affects protein levels only after a lag; the length of the lag being dependent on protein half life. Furthermore, in proliferating cells, mRNA levels must support a rate of protein synthesis sufficient to keep pace with both protein turnover and cell doubling. On the other hand, in nondividing cells, mRNA levels need only support a rate of protein synthesis sufficient to compensate for protein turnover. Thus a substantial decline in TK mRNA during withdrawal from the cell cycle need not be accompanied by as great a decline in TK enzyme. We emphasize that the reverse is not true. If enzyme had declined to a greater extent than mRNA, the model that mRNA decline was the sole cause of enzyme decline would be untenable.

The data for brain was unusual in several respects. At day 8, the 65% proliferative rate in brain was low compared to the 175% rate in heart and 215% rate in liver. Yet 8 day brain gave high levels of TK activity . Even more puzzling, brain TK activity remained high throughout development, even though TK mRNA levels declined more than 98% between days 8 and 24. One explanation was that an alternatively spliced TK mRNA which our assay did not detect was producing the high enzyme levels at later stages. This was unlikely since the probe was complementary to a portion of the protein coding region of the gene. Nevertheless, this possibility was investigated by Northern blot analysis of oligo(dT)-selected brain RNA using a hybridization probe spanning the entire protein coding region of the gene. (Use of poly A+ RNA was neccessary to minimize nonspecific hybridization of probe to rRNA.) As shown in Fig. 2.4, only the usual 2.1 kb species of cytoplasmic TK mRNA was observed at all times during development. In addition to precluding the existence of an alternatively spliced mRNA, the Northern analysis also attests to the integrity of the RNA preparations from brain. A second explanation was that a thymidine phosphorylating activity other than cytoplasmic TK was present at high levels in brain. Perhaps brain expressed unusually large amounts of mitochondrial TK. We tested this possibility using iododeoxycytidine and deoxycytidine triphosphate, analogs which inhibit the mitochondrial but not the cytoplasmic isozyme of TK (Kit et al., 1973). Using sensitivity to 0.2 mM drug concentrations as a criteria, brain was found to contain extraordinarily high levels of mitochondrial TK. The mitochondrial enzyme accounted for 50% of total TK activity in brain at day 8 and 70% at day 24. In contrast, mitochondrial TK accounts for less than 5% of total TK activity in rapidly growing cell cultures (Kit and Leung, 1974; Ellims et al.,

1981; our unpublished data). Thus, the most likely explanation for the anomolously high levels of TK activity in brain is unusually high expression of mitochondrial TK. Brain consumes ATP at a high rate and is rich in mitochondria. Perhaps high levels of TK activity are required for the biogenesis of this organelle.

TK Gene Transcription Rate in Muscle Nuclei of Young and Old Embryos

The decrease in TK mRNA during differentiation could be due to repressed transcription of the TK gene. To test this possibility, the rate of TK gene transcription was determined in nuclei isolated from proliferating and committed mouse myoblast transformants expressing multiple copies of the chicken TK gene. Isolated nuclei were allowed to continue transcription in the presence of [^{32}P]UTP, and specific transcripts were quantified by filter hybridization as described under Materials and Methods. To establish that the method was quantitative the amount of radioactivity added to each hybridization was varied over a 30-fold range. In all cases, the hybridization signal was directly dependent on the amount of labeled RNA added to the hybridization reaction. The top row of Fig. 2.5A shows hybridization to filters containing single-stranded DNA complementary to TK RNA. The hybridization signal was significantly greater using nuclei from proliferating cells, indicating that that the RNA polymerase density on the TK gene was greater in proliferating cells than committed cells. The middle row of Fig. 2.5A shows hybridization to filters containing single stranded DNA colinear with TK RNA. Although hybridization to the colinear DNA was weaker than to complementary DNA, it was still greater than to parental M13 phage DNA (Fig. 2.5A, bottom row), suggesting that some transcription was occurring on the opposite strand of the TK gene.

Radioactivity bound to the filters was quantitated by liquid scintillation counting. Fig. 2.5B shows the bound radioactivity plotted as a function of the input radioactivity to each hybridization mixture. Data was corrected for nonspecific hybridization by subtracting the radioactivity bound to filters containing only parental mp19 phage DNA. Transcription from the TK-encoding strand was 12.8 ppm in proliferating cells (solid line, open squares) and 1.0 ppm in committed cells (solid line, open circles). Transcription from the opposite strand of the TK gene was 4.9 ppm in proliferating cells (broken line, solid squares) and 2.4 ppm in committed cells (broken line, solid circles). The repressed TK transcription rate in committed cells was not attributable to a general decline in all pol II transcription. Using a related mouse myoblast subclone and identical

culture conditions to induce commitment, Jaynes *et al.* (1986) showed that the gene encoding muscle creatine kinase was transcriptionally activated 9-fold by 18 hours after induction. Also, in transiently expressing muscle cells, transcription from the herpesvirus TK and murine leukemia virus LTR promoters was stimulated two-fold after inducing commitment (S. Hauschka, personal communication). Thus the observed 12-fold repression of chicken TK transcription was not the result of a general inhibition of all transcription.

The specificity of the filter hybridization assay was confirmed by measuring the chicken TK transcription signal in nuclei from transformed versus nontransformed mouse cells. Only nuclei from mouse cells transformed with the chicken TK gene gave a hybridization signal above nonspecific levels (data not shown). Also, inclusion of alpha-amanitin during the transcription reaction eliminated the specific hybridization signal in transformants. We attempted to measure the TK gene transcription rate in nuclei isolated from day 8 and day 22 embryonic muscle, but encountered two problems. First, the observed TK transcription signal, about 50 ppm in both 8 day and 22 day nuclei, was unreasonably strong for cells containing only a diploid equivalent of TK genes. Second, total [^{32}P]UTP incorporation was reduced 8-fold in nuclei from 22 day muscle, creating problems in interpreting the relative transcriptional signal.

As noted earlier for single copy transformants, multicopy transformants showed a much greater decline in TK activity than TK mRNA level. As calculated from the data shown in Fig. 2.3C, proliferating multicopy transformants (lanes Px and Py) contained 150 TK mRNAs per cell, whereas committed transformants (lanes Cx and Cy) contained 42 TK mRNAs per cell. Cultures harvested in parallel with the experiment described in Fig. 2.5 gave a proliferative TK activity level of 5.52 pmTMP/min/μg DNA and a committed TK activity level of 0.08 pmTMP/min/μg DNA. The 70-fold decline in TK activity, in the face of only a 3.5-fold decline in TK mRNA, again indicates that translational or posttranslational processes contribute to the net decline in TK activity during differentiation.

Discussion

The absolute levels of an mRNA encoding a replicative enzyme have been measured directly for the first time in cells without gene amplification. Depending on the tissue type, rapidly growing early embryonic cells contained between 3 and 12 molecules of the messenger RNA encoding thymidine kinase. For example, muscle

from day 8 embryos contained 3.2 TK mRNAs per cell equivalent of DNA (Fig. 2.3C). In culture, proliferating mouse myoblasts transformed with single copies of the chicken TK gene contained 31 TK mRNAs per cell (Fig 2.2A). The higher level in culture may be due partly to a faster growth rate. *In vivo*, muscle DNA content increased 400% between day 8 and 10, suggesting a cell doubling time of 24 hours. In the muscle cell transformant, the cell doubling time was 17 hours. A previous estimate of 15,000 copies per cell for DHFR mRNA in cells containing 500 gene copies (Leys *et al.*, 1984) gives a value of 30 DHFR mRNAs per gene in cultured cells. Thus, on a per gene basis, both TK and DHFR mRNAs are expressed at about the same efficiency in proliferating transformed cells.

Quantitative comparison of TK enzyme and mRNA during *in vitro* differentiation negated the simple model that TK activity is determined exclusively by the level of cognate message. In both single copy (Fig. 2.2A) and multi-copy (Fig. 2.3C) transformants, the level of TK mRNA dropped four-fold as myoblasts withdrew from the cell cycle during differentiation. Over the same interval, TK activity declined to a much greater extent. For example, by 18 hours after induction, TK activity declined 70-fold in multi-copy transformants. Thus, the decline in mRNA cannot account fully for the decline in activity. Differentiation also must reduce either the translational efficiency of TK mRNA, or the activity or stability of TK protein. In the *in vivo* tissues we analyzed, observed declines in TK activity could be fully accounted for by declines in TK mRNA level. However, the *in vitro* evidence for translational or posttranslational control should not be discounted. *In vivo* systems, being less homogeneous and less managible, may simply have failed to reveal an important additional level of control. Analysis of translational or posttranslational processes affecting TK enzyme activity will require isolation of an antibody specific to cytoplasmic TK.

The decline in TK mRNA levels during both *in vivo* and *in vitro* differentiation implies that there is a specific mechanism for regulating the steady state levels of mRNAs encoding proteins involved in DNA precursor biosynthesis. On the basis of nuclear run-off determinations, our results indicate that the TK gene is transcriptionally repressed as muscle cells withdraw from the cell cycle. Although we have not ruled out a parallel affect on TK RNA stability, the observed 13-fold decline in TK gene transcription rate by 18 hours after induction can account fully for the 4-fold change in TK mRNA level, if we assume that TK mRNA has a reasonably short half life of a few hours. In other words, after transcription is repressed, pre-existing TK mRNA may simply decline with an unchanged half life.

Our results conflict with the nuclear run-off determinations of Groudine and Casimir (1984), which indicated that replication-dependant expression of chicken TK mRNA in chicken cells was regulated primarily by a posttranscriptional mechanism. The chicken TK transcriptional signal reported by Groudine and Casimir was strong, about 10% as strong as the globin transcriptional signal in erythrogenic cells. In nuclei from 8 and 22 day embryonic muscle, we also detected a strong and unchanging chicken TK hybridization signal of about 50 ppm. However, in multi-copy myoblast transformants expressing 150 chicken TK mRNAs per cell (50-fold higher levels than 8 day embryonic muscle), the chicken TK transcription signal was only 13 ppm. In single copy myoblast transformants, expressing 30 TK mRNAs per cell, the chicken TK transcription signal was barely detectable (unpublished result). Furthermore, in using Northern blots or primer extension assays to monitor TK mRNA levels, we have frequently observed nonspecific hybridization of probe to abundant RNA species present in chicken cells or tissues. We therefore consider it likely that chicken nuclei produce an abundant transcript that cross hybridizes with filter-immobilized TK sequences, thus giving a misleadingly high transcription signal.

The regulation of DHFR mRNA levels has been the subject of similar controversy. Santiago *et al.* (1984) concluded that serum starved cells which are induced to proliferate via serum addition increase DHFR mRNA by increasing transcription of the gene. Farnham and Schimke (1985) reached the same conclusion when studying DHFR regulation in cells synchronized by mitotic shake-off. In contrast, Leys *et al.* (1984) concluded that cells which are contact inhibited and then induced to proliferate via replating increase DHFR mRNA by stabilizing DHFR transcripts in the nucleus. Their conclusions, in turn, are supported by the results of Kaufman and Sharp (1983), who showed that DHFR cDNA minigenes are regulated mainly at a posttranscriptional level. The different results obtained by these groups may depend on the means by which growth arrest was achieved or proliferation was induced. We sought to avoid this source of variation by studying a system which undergoes a change in proliferative state as part of a differentiation program. An alternative explanation for the opposite conclusions drawn from studies on this class of gene products is that differences in methodology could influence the results of transcription rate determinations. A drawback of standard nuclear run-off analyses is that the specificity of the hybridization signal is never unequivocal. If the RNase protection assay described above can be adapted to quantitate labeled transcripts produced by intact cells ar isolated nuclei, the size of the protected fragment would serve as an additional criterion of specificity.

The *cis* acting information involved in the regulation of replicative enzymes has been localized only roughly. Luscher *et al.* (1985) and Morris *et al.* (1986) presented data suggesting that mouse histone H1 and human histone H3 are regulated by information contained within the 3' and 5' nontranslated regions of the respective mRNAs. We showed previously that replacement of the chicken TK promoter with heterologous promoters did not result in loss of a regulated pattern of TK enzyme expression in myoblast transformants (Merrill et al., 1984b). Similar results were obtained by Lewis and Matkovitch (1986) for the chinese hamster TK gene. We now realize that posttranslational regulation of chicken TK enzyme may have hidden an affect of promoter replacement on transcriptional regulation. We have subsequently confirmed that TK mRNA level, as well as enzyme level, is appropriately regulated when the native chicken TK promoter is replaced with the herpesvirus TK promoter or the Moloney murine leukemia virus LTR promoter (unpublished result). Either these heterologous promoters are themselves dependent on the replicative state of the cell or their transcriptional activity is subject to control by information contained within the chicken TK coding region. Experiments to further localize the *cis* acting control region must measure TK mRNA or TK transcriptional activity directly. The mRNA quantitation assay described herein should permit phenotypic analysis of deletion mutants in the protein coding region and thereby allow more precise localization of the *cis* acting regulatory information. Identification of the *cis* acting information would facilitate identification of *trans* acting factors that recognize this structure.

Acknowledgements

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Fig. 2.1. Description of RNase protection assay used to quantitate TK mRNA levels. The chicken TK gene is located within a 3.6 kb genomic fragment bounded by a natural Eco R1 site on the left and a synthetic Eco R1 site on the right. Six introns are removed from the primary transcript to give a 2.1 kb mRNA. A 174 bp restriction fragment extending from a non-unique Pst1 site to the Bgl2 site was cloned into SP65. Linearization of this template with Hind3 and transcription in the presence of [^{32}P]UTP yields a 214 base RNA probe complementary to 147 bases of exonic sequence in the native TK mRNA. This probe also contains 32 bases of upstream sequence and 6 bases of downstream sequence derived from the vector, as well as 30 bases of intronic sequence. To produce a pseudo-mRNA, a 1550 bp Eco R1/Hind3 fragment (constructed by fusing 5' genomic sequences to a partial TK cDNA) was cloned into SP65. Linearization of this template with Bgl2 and transcription in the presence of unlabeled ribonucleotides yields an 843 base pseudo-mRNA. The pseudo-mRNA contains 10 bases of upstream vector sequence. Hybridization of the radioactive probe with either pseudo-mRNA or native TK mRNA, followed by RNase treatment, would be expected to yield a 147 base protected fragment. Abbreviations used in the restriction map are: S, Sst1; R, EcoR1; K, Kpn1; Ps, Pst1; Bg, Bgl 2; H, Hind3. Open rectangles represent the 5' and 3' nontranslated portion of the message. Hatched rectangles represent the protein-coding region. Arrows on the vectors represent the position and polarity of the SP6 promoter with respect to the insert.

Fig. 2.1

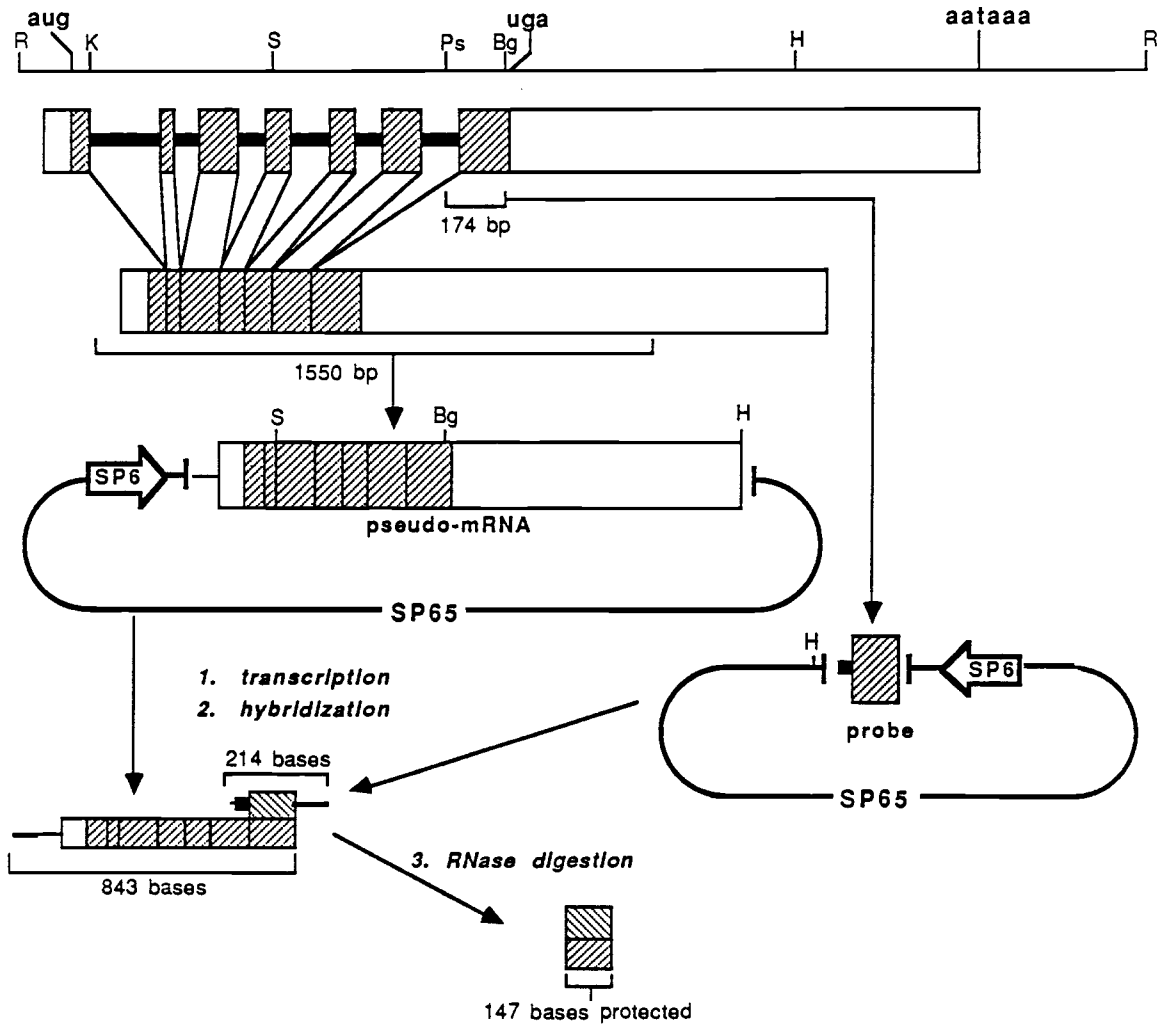
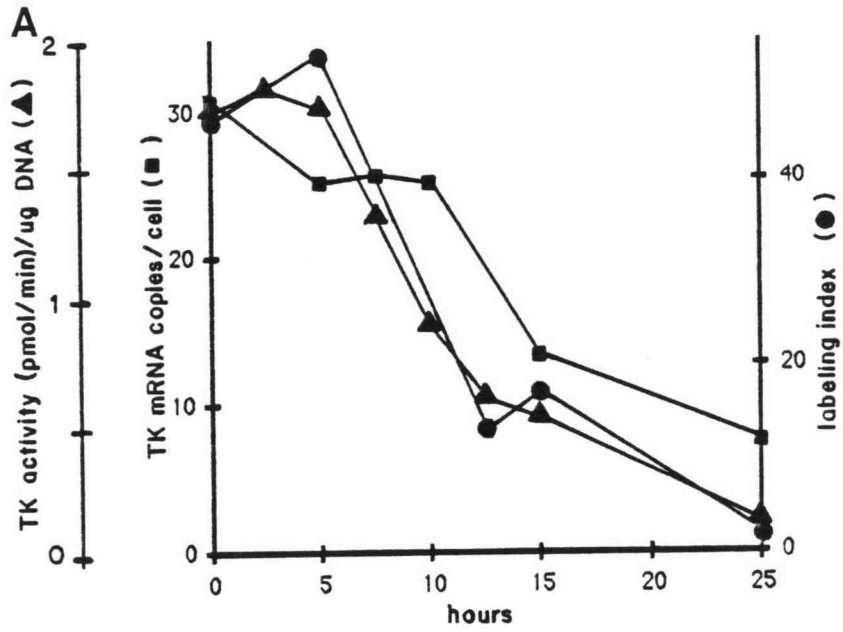

aug
uga
aataaa
R
K
S
Ps
Bg
H
R
174 bp
1550 bp
S
Bg
H
SP6
pseudo-mRNA
SP65
1. transcription
2. hybridization
H
SP6
probe
SP65
214 bases
843 bases
3. RNase digestion
147 bases protected

Fig. 2.2. Labeling index, TK activity, and TK mRNA levels during *in vitro* myoblast differentiation. Cultures of mouse myoblasts transformed with a cloned chicken TK gene were switched to mitogen depleted medium to induce differentiation after reaching a density of about 10^5 cells per 10 cm dish. (A) shows the effect of mitogen withdrawal on labeling index, TK activity and TK mRNA levels. At indicated times: three cultures were harvested and pooled for determination of TK mRNA copy number (i.e., TK mRNA molecules per genome equivalent of DNA); two cultures were harvested and assayed separately for TK activity; a single culture was incubated with [^{3}H]thymidine for 1 hr and fixed for autoradiographic determination of labeling index ($\geq$ 500 nuclei were scored in randomly selected microscopic fields). (B) shows the gel autoradiograph used to quantitate TK mRNA levels: lane 1, molecular weight markers (Msp1 digested pBR322); lanes 2-8, pseudo-mRNA standards (1.1, 0.37, 0.11, 0.037, .026, .011 and .007 fmol, respectively); lane 9, nondigested probe (only 2% of the mock-digested sample was loaded to prevent over exposure of the film); lane 10, 25 μg of yeast RNA; lanes 11-16, 25 μg of total RNA isolated from muscle cells at 0, 5, 7.5, 10, 15, and 25 hours after induction.

Fig. 2.2



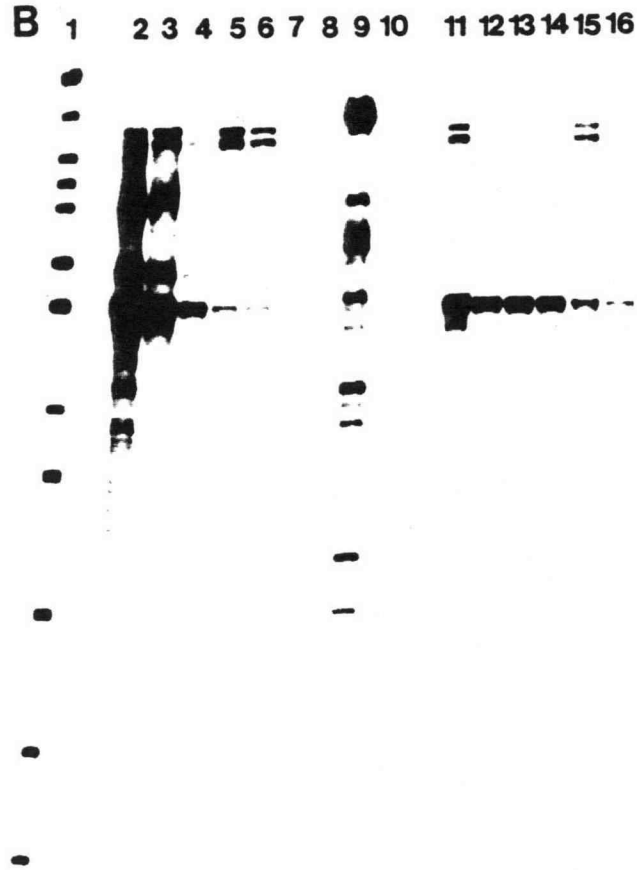


Fig. 2.3. Growth rate, proliferative rate, TK activity, and TK mRNA levels in chick leg muscle during embryogenesis. (A) shows wet weight values (seven determinations per time point) and DNA content (3 or 4 determinations from two experiments per time point) as a function of embryo age. (B) shows a first derivative plot generated from the smooth curve drawn for DNA content in panel A; each point represents the percent change in DNA content over a two day interval and is plotted in the middle of the interval. (C) shows TK activity and TK mRNA levels (determined as described under Material and Methods). (D) shows the gel autoradiograph used to quantitate TK mRNA levels: lanes 1-5, pseudo-mRNA standards (0.003, 0.01, 0.03, 0.1 and 0.3 fmol, respectively); lane 6, non-digested probe (only 2% of the sample was loaded on the gel); lane 7, 50 µg yeast RNA; lanes 8-16, 50 µg of total RNA isolated from chicken thigh muscle on days 8, 10, 12, 14, 16, 18, 20, 22 and 24 postfertilization, repectively; lane 17, molecular weight markers (Msp1 digested pBR322).

Fig. 2.3

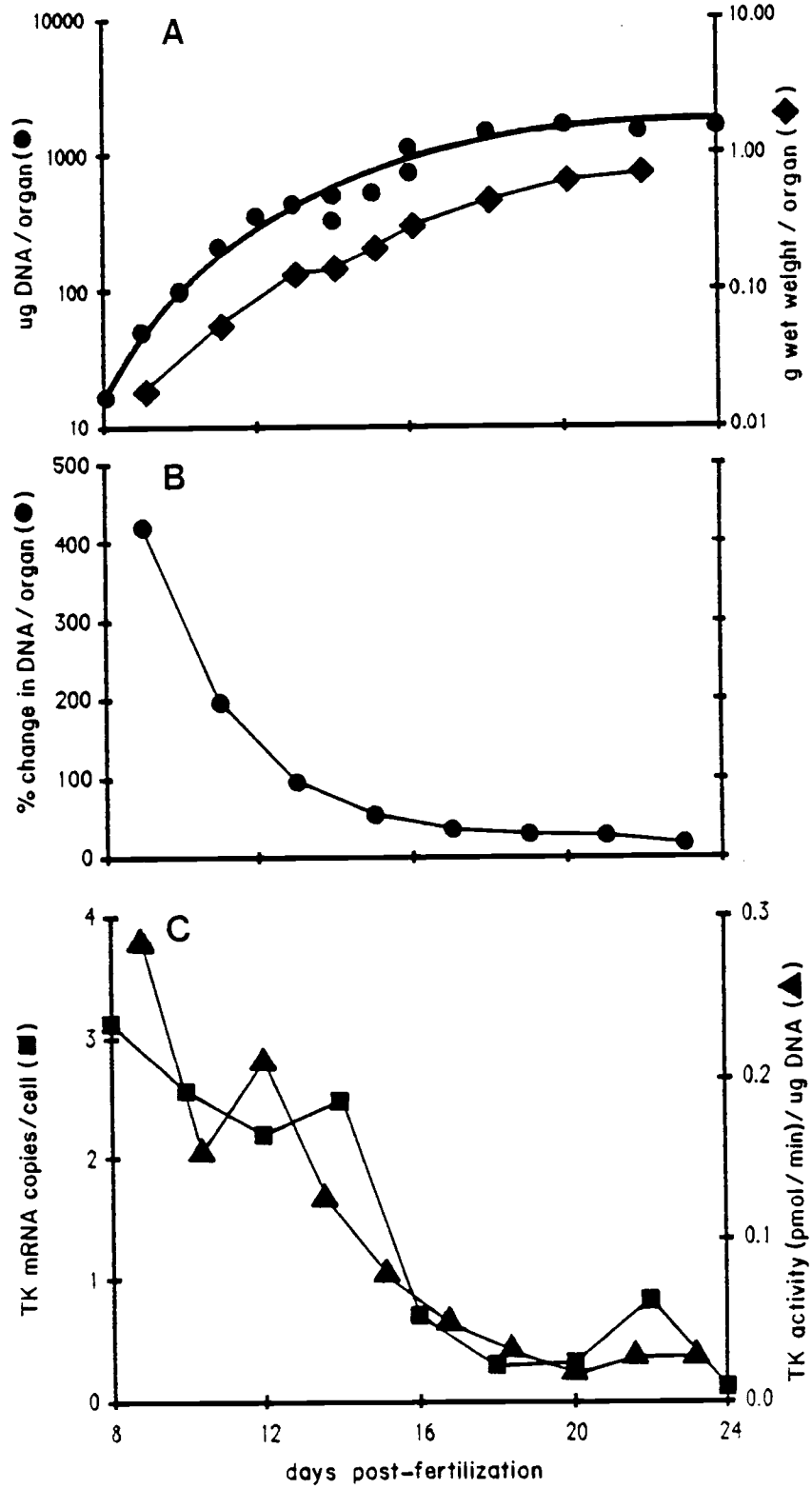

A
10000
1000
100
10
ug DNA / organ (●)
10.00
1.00
0.10
0.01
g wet weight / organ (◆)
B
500
400
300
200
100
0
% change in DNA / organ (●)
C
4
3
2
1
0
TK mRNA copies/cell (■)
0.3
0.2
0.1
0.0
TK activity (pmol / min) / ug DNA (▲)
8
12
16
20
24
days post-fertilization

Fig. 2.3

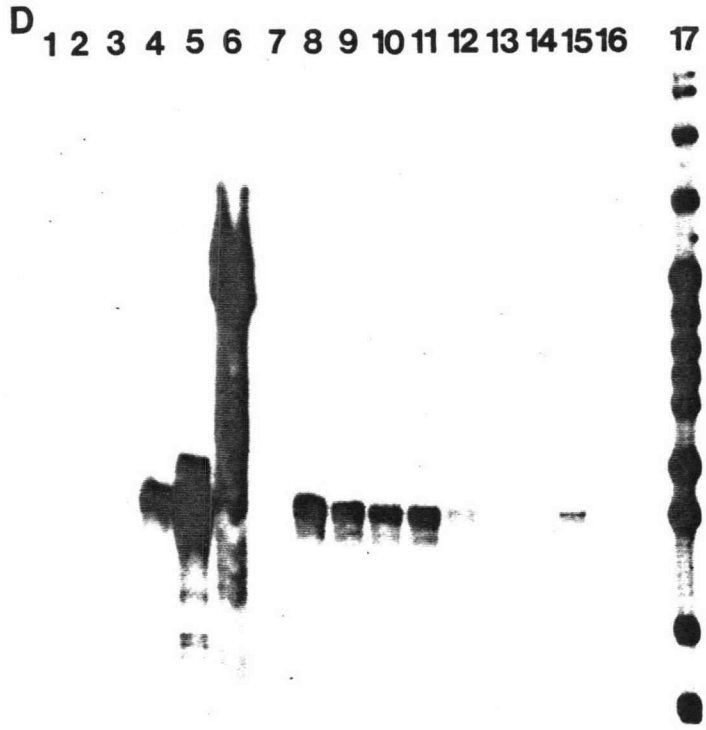


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Fig. 2.4. Northern blot analysis of cytoplasmic chicken TK mRNA in brain during embryogenesis. Lanes 1-7 contain 4.5 μg of oligo[dT]-selected RNA from brain at 10, 11, 13, 15, 17, 19, and 22 days postfertilization, respectively. The blot was hybridized with a riboprobe complementary to TK mRNA in the region between the Sst1 and Bgl2 restriction sites (see Fig. 2.1). Electrophoresis, transfer and hybridization conditions were as described elsewhere (Merrill *et al.*, 1984a), except that, prior to autoradiography, washed blots were exposed to RNase A (10 μg/ml) for 20 min at 20°C and given a final 30 min wash at 65°C.

Fig. 2.4

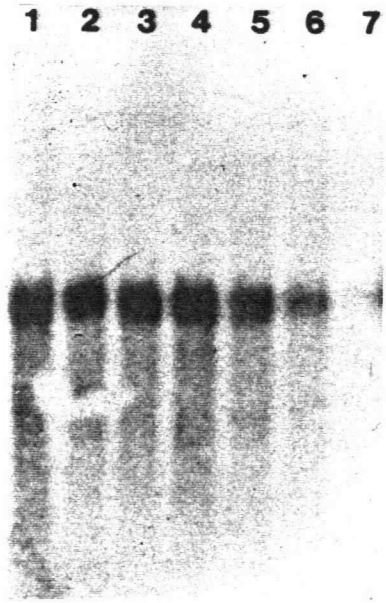
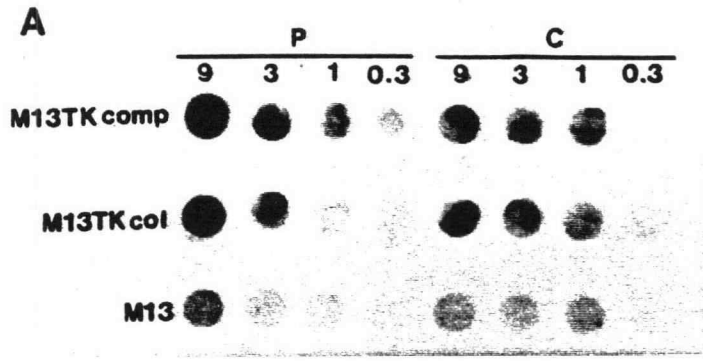
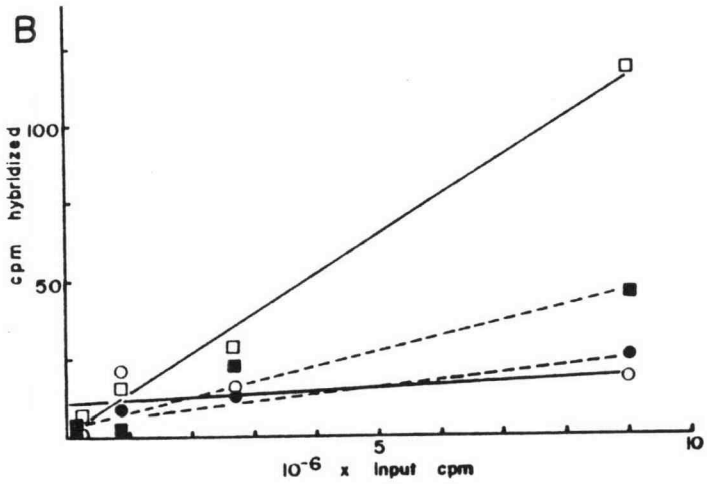


Fig. 2.5. TK gene transcription rate and steady state level of TK mRNA in mouse myoblasts transformed with multiple copies of the chicken TK gene. A polyclonal transformant population with multiple copies of the chicken TK gene was obtained by selection in G418 following exposure to 30 μg pCHTKfl and 0.3 μg pKNeo. Nuclei from proliferating and committed cells were isolated and transcribed as described under Materials and Methods. An 18 hr incubation in mitogen-depleted medium was used to induce commitment. Total incorporation of [^{32}]UTP into RNA during nuclear run-off reactions was 60 x 10^6 and 31 x 10^6 cpm using 2.8 x 10^7 nuclei from proliferating and committed cells, respectively. A) Autoradiograph of filter discs hybridized with nuclear run-off transcription products from proliferative, "P", or committed, "C", nuclei: rows labeled "M13TK comp", "M13 col", and "M13" show hybridization to filters containing 0.5 pm of immobilized singlestranded phage DNA that is complementary to TK transcripts, colinear with TK transcripts, or parental mp19, respectively; columns labeled "9", "3", "1" and "0.3" designate the input (in millions of cpm) to each hybridization. B) radioactivity bound to each filter was quantitated by liquid scintillation counting and plotted as a linear regression function of input cpm to each hybridization: solid lines show the transcription signal from the TK-encoding strand in proliferating (open squares) or committed (open circles) cells; broken lines show the transcription signal from the opposite strand in proliferating (solid squares) or committed (solid circles) cells; each data point was corrected for nonspecific hybridization, calculated by linear regression analysis of mp19 hybridization plotted as a function of input cpm. C) at the same time that nuclei were prepared, total RNA was harvested from parallel cultures and 10 μg was assayed by RNase mapping to determine steady state TK mRNA level: arrows show the size of radioactive bands in nucleotides; lanes "Px" and "Py", and "Cx" and "Cy" contain RNA from duplicate proliferative and committed cultures, respectively; comparison of the protected signal in unknowns to the pseudo-mRNA standard curve indicates that proliferative and committed cells contained 150 and 43 TK mRNAs per cell, respectively.

Fig. 2.5



Photocopy. Best scan available



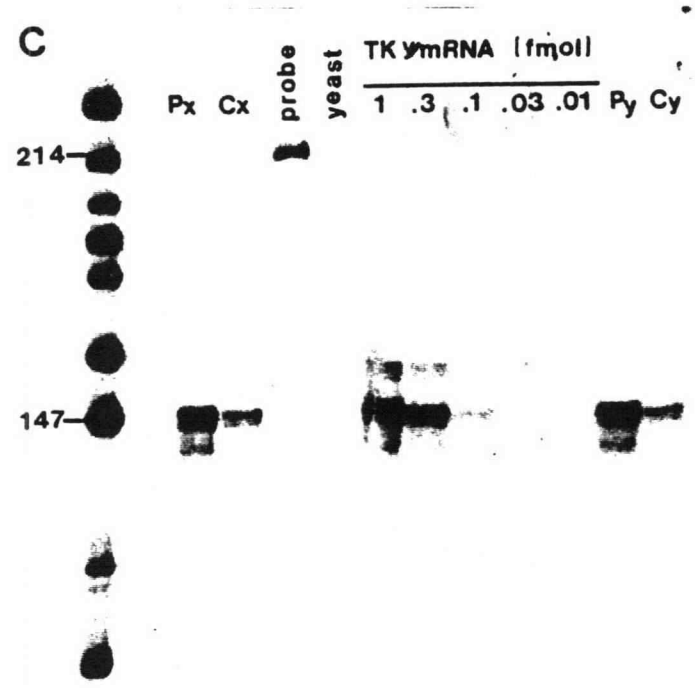


Table 2.1. Proliferative Rate, TK enzyme, and TK mRNA Levels in Liver, Heart, and Brain During Chicken Embryogenesis

Tissue type	Embryo age (days)	Proliferative rate[a]	TK enzyme/ mg DNA	TKmRNA[b]/ pg RNA	Total RNA[c]/ cell	TKmRNAs/ cell
		ΔDNA/48h	pm TMP/min	Molecules	Picograms	Molecules
Liver	8	215 (100)	65 (100)	0.83	6.2	5.15 (100)
	10	170 (79)	45 (69)	0.28	6.3*	1.76 (34)
	12	97 (45)	60 (92)	0.19	5.8	1.10 (21)
	14	58 (27)	50 (77)	0.12	5.5	0.66 (13)
	16	30 (14)	45 (69)	0.06	4.8*	0.29 (6)
	18	25 (12)	30 (46)	0.04	5.1	0.20 (4)
	20	45 (21)	22 (34)	0.37	5.0	1.85 (36)
	22	53 (25)	20 (31)	0.29	4.8	1.39 (27)
	24	NA	70 (108)	0.34	4.8*	1.63 (32)
Heart	8	175 (100)	155 (100)	1.36	8.7	11.83 (100)
	10	120 (69)	194 (125)	1.18	6.4*	7.55 (64)
	12	30 (17)	233 (150)	1.67	4.8	8.02 (68)
	14	65 (37)	237 (153)	2.19	3.7	8.10 (68)
	16	55 (31)	237 (153)	1.49	2.8*	4.17 (35)
	18	50 (29)	104 (67)	1.40	2.2	3.08 (26)
	20	35 (20)	34 (22)	1.27	1.7	2.16 (18)
	22	20 (11)	39 (25)	0.48	1.3	0.62 (5)
	24	NA	7 (5)	0.35	1.0*	0.35 (3)
Brain	8	65 (100)	135 (100)	0.97	3.9	3.78 (100)
	10	58 (89)	155 (115)	0.48	4.1*	1.97 (52)
	12	54 (83)	175 (130)	0.70	3.9	2.73 (72)
	14	50 (77)	160 (119)	0.70	3.9	2.73 (69)
	16	30 (46)	170 (126)	0.38	3.7*	1.41 (37)
	18	32 (49)	150 (111)	0.02	3.9	0.08 (2)
	20	25 (38)	140 (104)	0.03	3.9	0.12 (3)
	22	33 (51)	160 (119)	0.01	3.9	0.04 (1)
	24	NA	160 (119)	0.02	3.9*	0.02 (2)

Note. Values in parentheses are the data represented as a percentage of 8-day levels.

[a] Values are the percentage change in DNA content over a 48-hr interval beginning at the time indicated (3 organs per time point). Animals after 24 days were not assayed (NA).

[b] Absolute TK mRNA levels were determined essentially as described in Fig. 3.

[c] RNA per genome equivalent of DNA was determined as described under Materials and Methods. Each RNA value marked with an asterisk represents the average of four separate assays. Other values were obtained by interpolation.

CHAPTER 3:

Regulation of Thymidine Kinase Protein Levels during Myogenic Withdrawal from the Cell Cycle Is Independent of mRNA Regulation

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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 that 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 (Merrill *et al.*, 1984b; Gross *et al.*, 1987a; Liu *et al.*, 1985; Stuart *et al.*, 1985; Ayusawa *et al.*, 1986; Hofbauer *et al.*, 1987; Lewis and Matkovitch, 1986; Lewis *et al.*, 1983; Hendrickson *et al.*, 1980; Johnson *et al.*, 1978; Mariani *et al.*, 1981; Farnham and Shimke, 1985).

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 (Kaufman and Sharp, 1983) or TK (Hofbauer *et al.*, 1987) 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 (Linkhart *et al.*, 1981) 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

(Chamberlain *et al.*, 1985; Devlin and Emerson, 1978).

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 (Merrill *et al.*,1984b, 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 (Merrill *et al.*,1984b, 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.* (Merrill *et al.*,1984b) and Gross *et al.* (1987a) 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 (Hofbauer *et al.*, 1987; Lewis and Matkovich,1986; Stewart *et al.*, 1987; Travali *et al.*, 1988) 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 (Travali *et al.*, 1988; Kim *et al.*, 1988).

Quantitative measurements of TK mRNA levels in differentiating muscle cell transformants (Gross *et al.*, 1987a) 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 would 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 rate 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 (Merrill *et al.*, 1984b), 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 (Gross *et al.*, 1987b). A TK^- myoblast strain derived from the MM14D line (Merrill *et al.*, 1980) 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.* (1984b). Total RNA was isolated by extraction in guanidinium isothiocyanate and ultracentifugation through CsCl as described previously (Gross *et al.*, 1987a). Production of synthetic RNA probe and standards, and absolute TK mRNA quantitation *via* RNase mapping were described in detail elsewhere (Gross *et al.*, 1987a). Laser densitometry was used to compare signal intensities in standard and sample lanes of RNase quantitation gels. Capped synthetic RNAs were generated as described by Konarska *et al.* (1984). Northern analysis, done with MOPS (3-[N-morpholino]propanesulfonic acid)/formaldehyde agarose gels and nitrocellulose, was adapted from Lehrach *et al.* (1977).

Isolation of Nuclear and Cytoplasmic RNA

All subcellular fractionation procedures were carried out at 4° 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 (Labarca and Paigen, 1980). 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 (Fig. 3.1C). 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; the plasmid 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. Each of the bacterial expression vectors were transformed into DH5Δlac (a spontaneous derivative of DH1 (Hanahan, 1983) obtained from M.L. Berman).

Fig. 3.1A shows that 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-thiogalactopyranoside). Unlike the 116 kD native ß-gal band, the 139 kD TK/ß-gal band was smeared, probably reflecting poor solubility, a common problem with fusion proteins. 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 (Laemmli, 1970) 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 (1976).

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.

Because the TK epitope constituted only a fifth of the TK/ß-gal fusion protein, it was important to confirm that antibodies capable of binding chicken TK had been generated. Initially, antisera were screened using TK protein synthesized by *in vitro* translation of TK pseudo-mRNA. Optimal translation of SP6-generated TK pseudo-mRNA was obtained using rabbit reticulocyte lysate and capped mRNA. Wheat germ extracts and uncapped mRNA were fivefold and tenfold less effective, respectively. In reticulocyte lysates, TK activity was detected after translation of capped TK pseudo-mRNAs, suggesting that enzymatically active TK protein was produced. To screen sera for TK-antibodies, [^{35}S]Met-labeled *in vitro* translation products were mixed with immune or prebleed sera, precipitated using protein A agarose, and analysed on SDS polyacrylamide gels (Fig. 3.1B). Immune sera were able to precipitate a 25 kD protein only in reticulocyte lysates that had contained TK pseudo-mRNA, indicating that antibodies against the TK epitope of the fusion protein had been generated. Later, the presence of anti-TK antibodies was confirmed by immunoblotting of bacterial extracts containing ΔN15rTK (compare lanes u and i, Fig. 3.7).

The requirement for a cap structure and the optimal translation system were established using kits (Promega). For immunoprecipitation of labeled translation products, translation conditions were modified from Jackson and Hunt (1983); 30 µl translation mixtures contained: 22.1 µl micrococcal nuclease-treated rabbit reticulocyte lysate, 0.1 M KCl, 0.5 mM $MgCl_2$, 10 mM creatine phosphate, 0.15 mM Leu and Val, 0.1 mM each of 17 other amino acids, 5 µg/ml yeast tRNA, 16 µM hemin, 40 ng/µl creatine kinase , 1 U/µl RNasin (Promega), 10 µM [^{35}S]Met (1 Ci/µmol), and 33 ng/µl TK pseudo-mRNA. Translation reactions were done at 30°C for 60 minutes and stopped by freezing at -20°C. [^{35}S]Met-labeled *in vitro* translation products were analysed by

gel electrophoresis and fluorography.

Anti-TK antibody was purified from sera by double affinity chromatography. The methods of Carroll and Laughon (1988) 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 (Merrill *et al.*, 1984a) and sonicated twice for 5 seconds on ice. Sonicates were clarified by centrifugation, and 40 µl was electrophoresed on SDS polyacrylamide (15%) minigels (Laemmli, 1970). 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 (1985). 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

Although mouse myoblasts usually are maintained in Ham's F10-based medium (Linkhart *et al.*, 1981), 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. Withdrawal from the cell cycle in response to mitogen depletion was slightly slower in Ham's F12/DMEM-based medium; perhaps because the richer mixture of nutrients allowed coasting into S phase for a few hours. In labeling experiments, 10 cm cultures containing about 2 x 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.

Radiolabeled cells were dissociated with collagenase and collected by centrifugation. Clarified cell extracts were prepared as for TK activity measurements (Merrill *et al.*,1984a). Immunoprecipitations were carried out on ice. Cell extracts (50-100 µl) were incubated 1 hr with a titred amount of antibody (see below) 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 resuspended and heat-denatured in 40 µl loading buffer, and 28 µl was applied to SDS polyacrylamide (15%) minigels (Laemmli, 1970). Gels were soaked 1 hr in fix (10% MeOH, 10% TCA, 30% HOAc), 1 hr in water, and 30 min in Fluoro-Hance (Research Product International), and dried overnight between cellophane membranes (Bio-Rad). Dried gels were exposed to XAR-5 film at -70^o using an intensifying screen.

Pilot immunoprecipitation experiments, in which TK activity in Protein A agarose supernatants and precipitates were measured, established that 0.25 µl of the affinity-purified TK antibody stock effectively precipitated 10^5 cell equivalents of TK activity in transformants expressing high TK activity levels (1 pm TMP/min/µg DNA). Accordingly, to conserve antibody and minimize nonspecific binding in radioimmunoprecipitation experiments, 0.25 µl of antibody was used per 10^5 cells equivalents of extract. Pilot experiments also established that the antibody was chicken specific, as judged by its failure to precipitate mouse or human TK activity. Interestingly, enzyme activity was unimpaired by binding of chicken TK protein to antibody and immobilization on Protein A agarose.

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. 3.2 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 (Gross *et al.*, 1987a). 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 (data not shown).

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. 3.3). Transformants expressing low initial message levels, similar to levels found in proliferative tissues *in vivo* (1-20 copies/cell; Gross *et al.*, 1987a), 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 significantly different regulation was observed in each trial (asterisks in Fig. 3.3). 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 (Bowman, 1987) 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 (Bowman and Emerson, 1980). Therefore regulation of TK mRNA was likely mediated by a relatively 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. 3.4 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. 3.5). 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 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. 3.6 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. Taken together, 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, a rabbit antibody against chicken TK protein was generated using a bacterially-produced chicken TK/ß-galactosidase fusion protein as antigen (see Materials and Methods). The antibody was used to determine relative TK protein levels in proliferative and committed muscle cell transformants.

The number of TK protein molecules in proliferating vertebrate cells has not been determined. Presumably the protein is rare, as it has been difficult to isolate sufficient amounts to visualize on stained SDS polyacrylamide gels. 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-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. 3.7 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. 3.7B), 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. 3.7 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 yield information on 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. 3.8A 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 minutes (Fig. 3.8C). Therefore, the band intensity in cells pulsed 60 minutes in Fig. 3.8A and 90 minutes in Fig. 3.8B 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. 3.8A, 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. 3.8B, 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 3.8A 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. 3.8A were analyzed by SDS polyacrylamide gel electrophoresis. An autoradiogram of the gel (Fig. 3.9A) 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 (Chamberlain *et al.*,1985; Devlin and Emerson, 1978). 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. 3.9B, 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 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 (Liu *et al.*, 1985), thymidylate synthetase (Ayusawa *et al.*,1986), and TK (Stuart *et al.*, 1985) activity levels. Similarly, in several tissues of the developing chicken embryo, declines in TK mRNA levels can account for declines in activity levels (Gross *et al.*, 1987a). 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 (Merrill *et al.*, 1984b; Hofbauer *et al.*, 1987; Lewis and Matkovich, 1986; Kaufman and Sharp, 1983; Travali *et al.*, 1988; Goldsmith *et al.*, 1986). Most investigations indicate the regulatory information is not associated with the transcriptional promoter ((Merrill *et al.*, 1984b; Hofbauer *et al.*, 1987; Lewis and Matkovich, 1986; Kaufman and Sharp, 1983). Thus far, no specific genetic manipulation (including promoter replacement, intron removal, and 3' nontranslated region replacement) has reproducibly been shown to allow escape from S phase-dependent expression. However, a study on DHFR by Goldsmith *et al.* (1986) and recent work on TK by Travali *et al.* (1988) and Kim *et al.* (1988) 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 (Gross *et al.*, 1987a; Farnham and Shimke,1985; Santiago *et al.*, 1984), posttranscriptional (Kaufman and Sharp, 1983; Leys and Kellems, 1981; Groudine and Casimir, 1984), or both (Stewart etal., 1987) 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 (Gross *et al.*, 1987a). 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. 3.4 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* (1988) 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 (Groudine and Casimir, 1984), serum deprivation (Stuart etal.,1985; Thompsen etal.,1985), or drug inhibition (Hofbauer *et al.*, 1987), 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 largefold declines in the mRNAs encoding DHFR (Hendricksonet *al.*, 1980; Kaufman and Sharp, 1983) and thymidylate synthetase (Imam *et al.*,1987). 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 (Devlin and Emerson, 1978). Fibroblastic cells 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 (Geyer *et al.*, 1982); 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 (Gross *et al.*, 1987a; Stewart *et al.*, 1987). 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* (Gross *et al.*, 1987a) 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 (Thompson *et al.*, 1985), DHFR mRNA (Farnham and Shimke, 1986), or TS mRNA (Imam *et al.*, 1987) 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 Hunter,1987), and the activity of certain enzymes are known to be governed by protein kinases (Krebs *et al.*, 1959). Also, the *cdc2* and *cdc28* cell cycle control genes in yeast and the mammalian homolog of *cdc 2* encode enzymes involved in protein phosphorylation events (Lee *et al.*, 1988).

If the mRNA-independent decline in TK activity was due exclusively to posttranslational processes 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. Several observations indirectly bear on the question of whether TK activity is dependent on posttranslational modifications or ancillary factors. First, bacteria transformed with chicken TK sequences produced enzymatically active TK protein (see Materials and Methods). Since it is doubtful bacteria could supply ancillary factors or faithfully carry out putative posttranslational maturation events on a protein of vertebrate origin, production of enzymatically active chicken TK protein in bacteria argues against a requirement for posttranslational activation. Production of active TK protein in bacteria does not rule out the possibility that TK protein in vertebrate cells is subject to posttranslational inactivation. Second, the enzymatically active protein produced by *in vitro* translation of synthetic TK mRNA has the same electrophoretic mobility (Fig. 3.1) as the protein produced by cells (Figs. 3.7 and 3.8). Since cleavage or extensive glycosylation of nascent TK protein would likely result in a mobility shift, our finding of equivalent electrophoretic mobilities argues against major alteration of the protein in cells. Finally, in experiments in which cells were radiolabeled with [^{32}P]phosphate, we were unable to detect an immunoprecipitable TK-specific band (unpublished result). None of these observations eliminate the possibility that subtle alterations in TK protein occur, but were undetectable by our assays. 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 (1988) 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.

Acknowledgements

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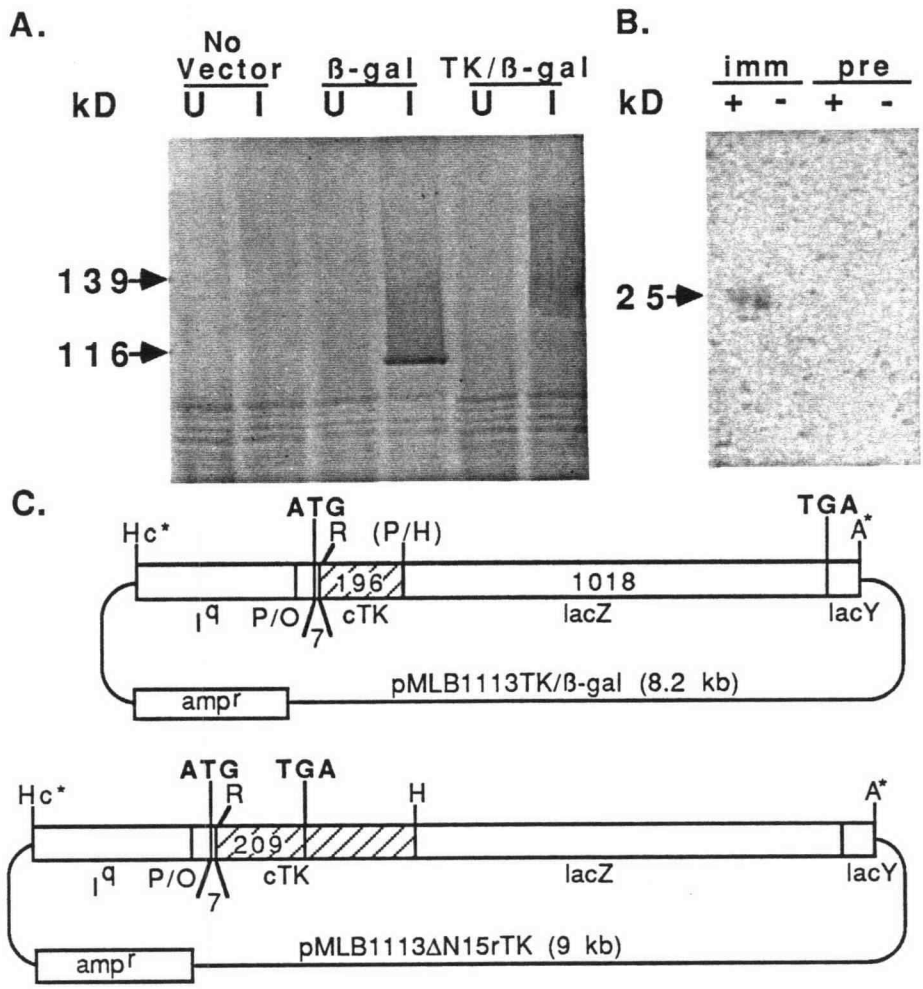
Fig. 3.1. Production of anti-chicken TK antibody. (A) SDS polyacrylamide gel (7%) showing total protein from bacterial cultures uninduced (U) or induced (I) with IPTG overnight. No vector indicates nontransformed cultures of the DH5Δlac host cell. Cultures of DH5Δlac transformed with the parental plasmid pMLB1113 (ß-gal) produced ß-gal (116 kD) and cultures transformed with pMLBTK/ß-gal (TK/ß-gal) produced a fusion protein (139 kD), when induced with IPTG. (B) Antiserum recognition of the TK epitope of the fusion protein. *In vitro* translation reactions were conducted in the presence (+) or absence (-) of capped TK pseudo-mRNA. [^{35}S]Met-labeled reaction products were mixed with either immune (imm) or pre-bleed (pre) serum overnight, and precipitated for 2 hours with protein A agarose. The precipitate was analysed on a SDS polyacrylamide (15%) gel. (C) Structure of plasmids which produce TK/ß-gal or ΔN15rTK. Hatched regions indicate TK coding sequences, open regions indicate sequences from the lac operon of *E. Coli*, and numbers indicate the number of amino acids used from each region in the gene product. Restriction sites are: R, EcoR1; P, Pvu2; H, Hind3; Hc, Hinc2; A, Ava1. Brackets indicate inactivated restriction sites and asterisks indicate non-unique restriction sites.

Fig. 3.1


A.
No Vector
ß-gal
TK/ß-gal
kD
U I U I U I
139
116
B.
imm pre
kD
+ - + -
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C.
ATG
R (P/H)
TGA
Hc*
A*
196
1018
I^q P/O 7 cTK
lacZ
lacY
pMLB1113TK/ß-gal (8.2 kb)
ampr
ATG TGA
Hc*
R
H
A*
209
I^q P/O 7 cTK
lacZ
lacY
pMLB1113ΔN15rTK (9 kb)
ampr

Fig. 3.2. 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, SP6-generated, 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 total RNA from either proliferative (P) or committed (C) populations of six independently derived transformant pools. The six left hand lanes contain serial dilutions of an SP6-generated RNA (TK pseudo-mRNA) which is colinear with native TK mRNA in the protected region. TK pseudo-mRNA signals were used to establish a standard curve from which absolute message levels in test samples were determined (numerals below each lane). Absolute levels in the right hand 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.

Fig. 3.2

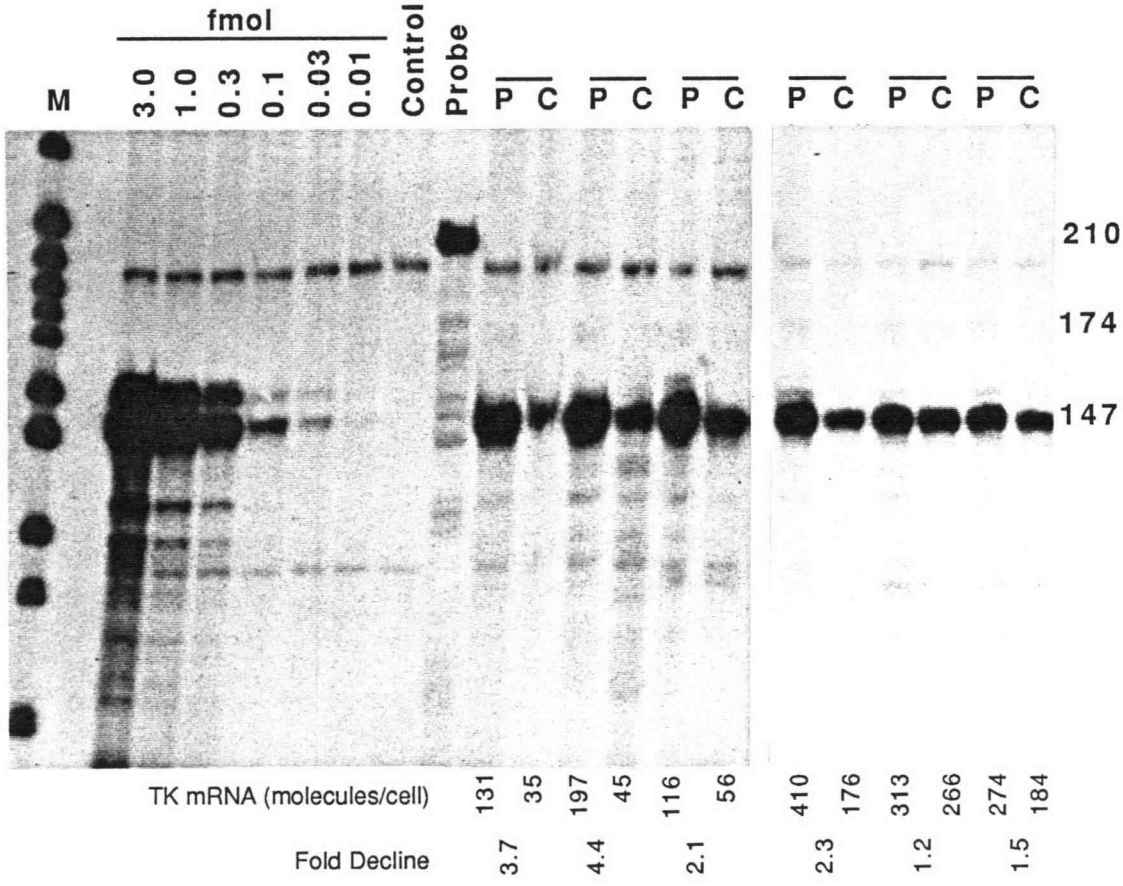


Fig. 3.3. Lack of effect of proliferative cell TK mRNA content on the efficiency of TK mRNA regulation. TK$^-$ myoblasts were transformed with pCHTKfl, and TK mRNA levels were measured in proliferative and committed populations as in Fig. 3.2. Data from 12 independent experiments were arranged in order 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.

Fig. 3.3

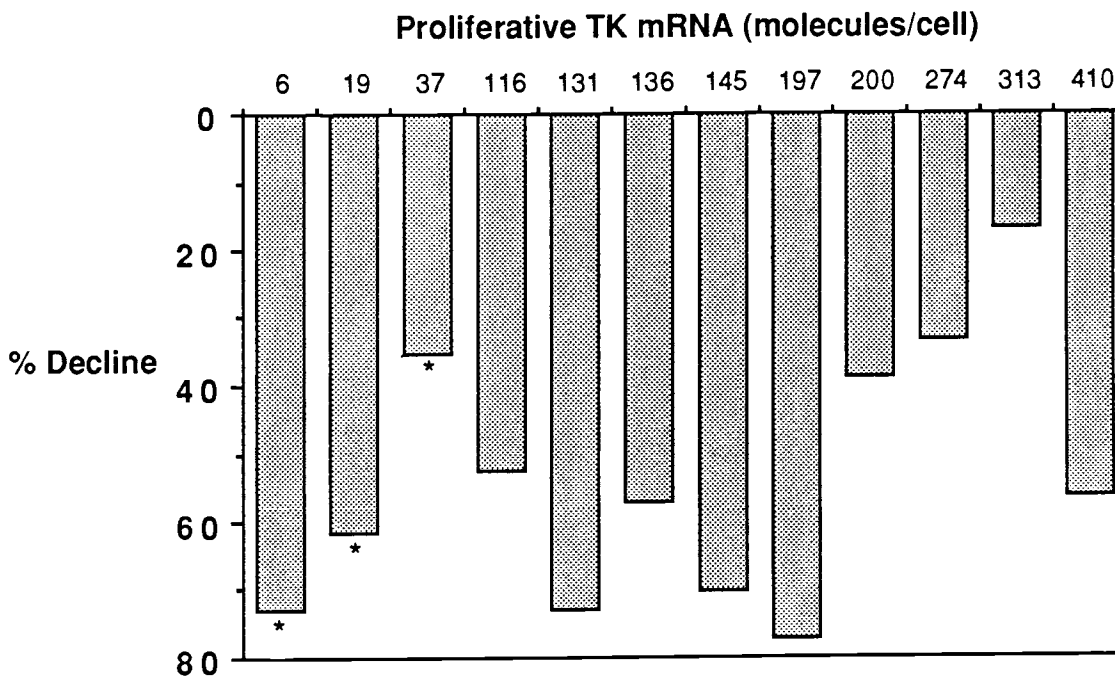

Proliferative TK mRNA (molecules/cell)
6
19
37
116
131
136
145
197
200
274
313
410
% Decline
0
20
40
60
80
*
*
*

Fig. 3.4. 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 thymidine phosphorylated/min/μg DNA.

Fig. 3.4

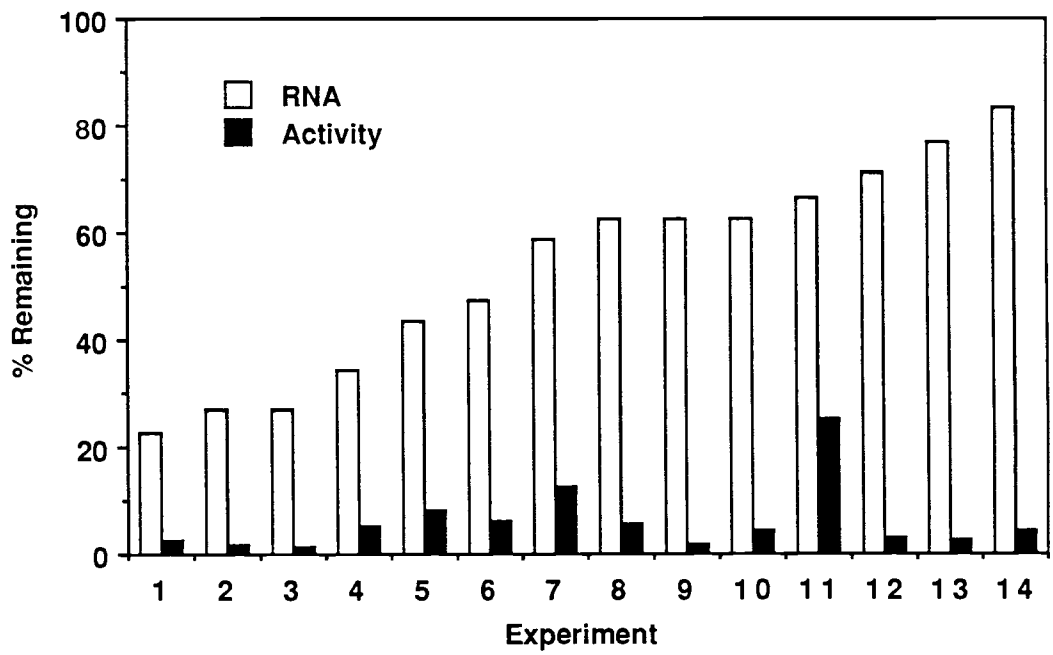

100
80
60
40
20
0
% Remaining
RNA
Activity
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Experiment

Fig. 3.5. Northern blot confirmation of TK mRNA size homogeneity during differentiation. Proliferative (P) and committed (C) cell RNA, isolated from three polyclonal muscle cell lines cotransformed with pCHTKfl and pKNeo, was fractionated on formaldehyde gels, blotted to nitrocellulose, and probed with a nick-translated 2.3 kb Kpn1/Hind3 fragment of pCHTKfl. The cell lines used and quantity of RNA analyzed 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).

Fig. 3.5

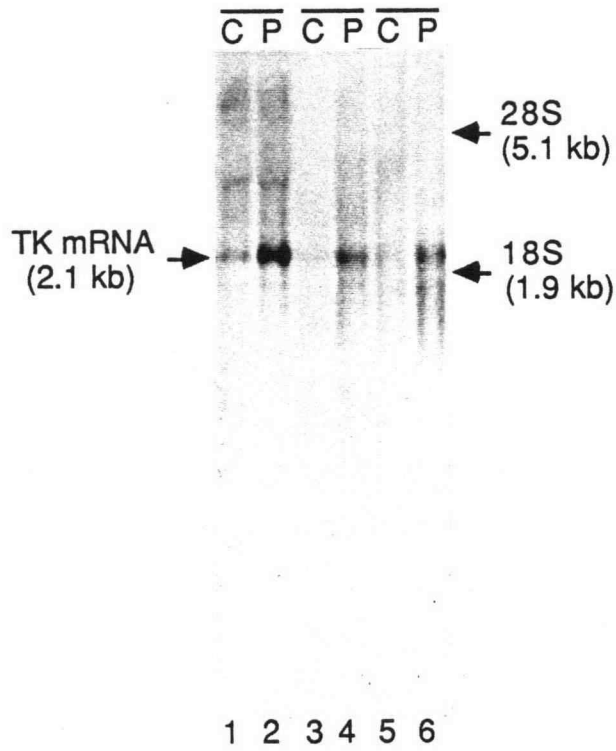


Fig. 3.6. TK mRNA regulation in nuclear and cytoplasmic compartments of muscle cells. Whole Cell (WC), nuclear (N), and cytoplasmic (Cy) RNA was isolated from proliferative (P) and committed (C) cultures of a polyclonal muscle cell transformant line carrying multiple copies of the chicken TK gene. The line, $H^{cfl:neo}1$pool, was derived by cotransformation of HGPRT$^-$ myoblasts with pCHTKfl and pKNeo. Standard curve, probe, and control lanes were as described in Fig. 3.2. TK precursor RNA protected a 174 base fragment and TK mRNA protected a 147 base fragment.

Fig. 3.6

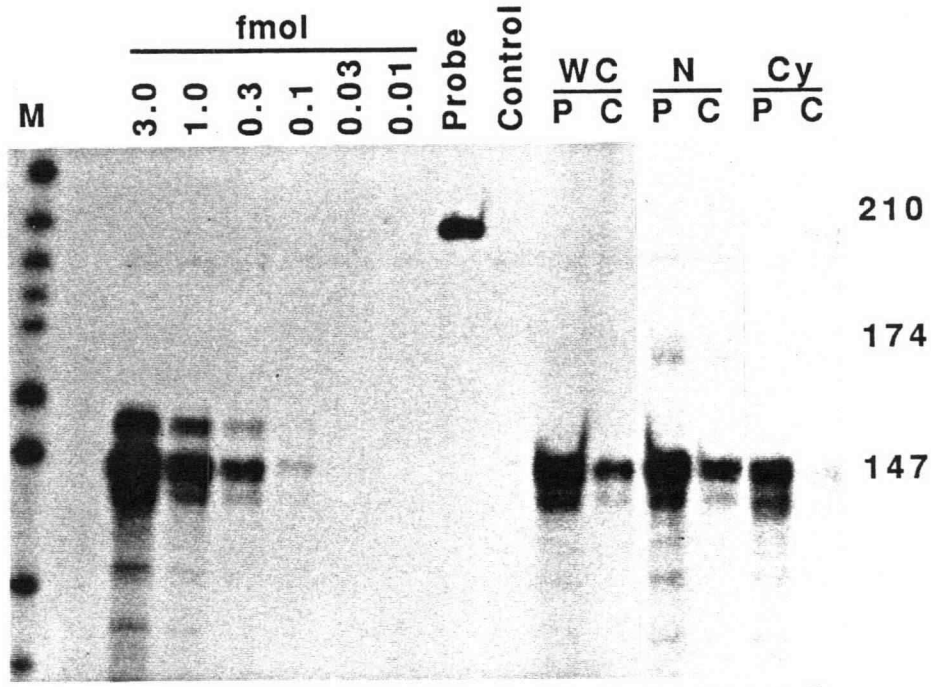
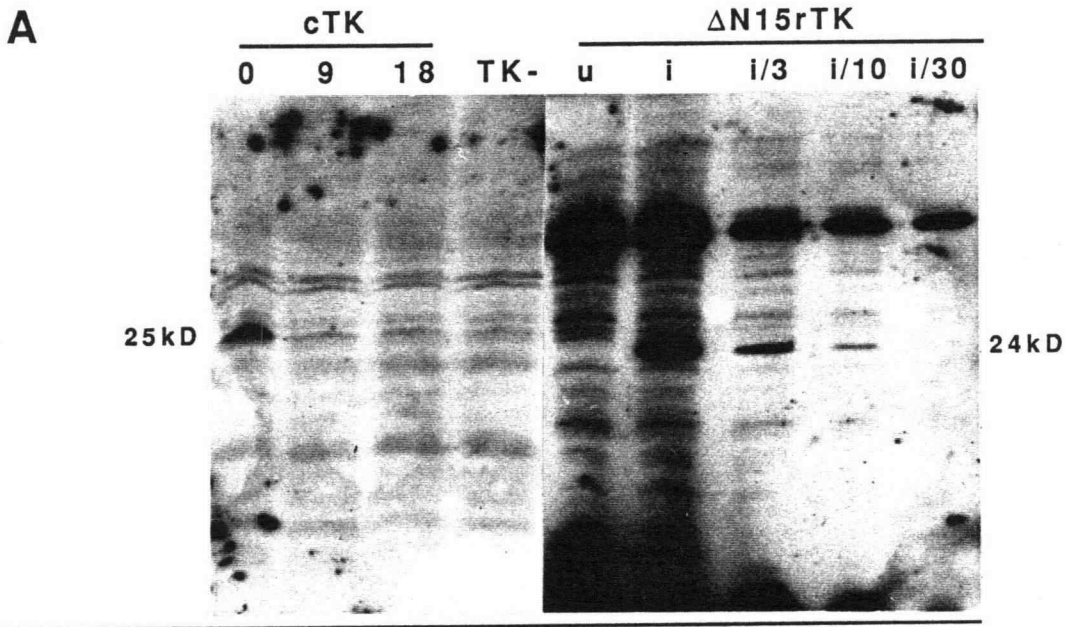
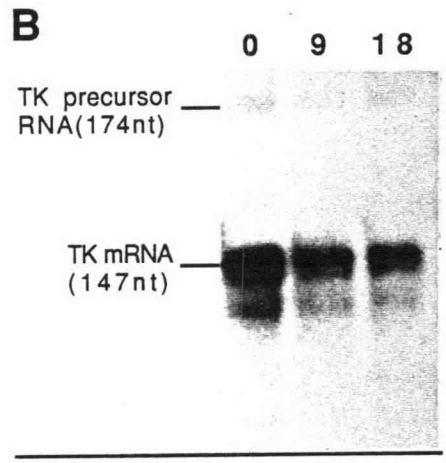


Fig. 3.7. Western blot of TK protein during muscle cell differentiation. (A) Protein from polyclonal muscle cell transformants carrying 50 copies of the chicken TK gene (cTK) was harvested at zero (0), nine (9), and eighteen (18) hours after inducing differentiation, and $2x10^6$ cell equivalents were 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 affinity purified anti-TK antibody and [^{125}I]protein A, and autoradiographed two months without an intensifying screen. 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).

Fig. 3.7



Band Intensity	320	77	66	55	829	180	36	3
Relative TK Protein	1.00	0.23	0.18					



TK mRNA/Cell	157	66	48
Relative TK mRNA	1.00	0.42	0.31

Fig. 3.8. 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.

Fig. 3.8

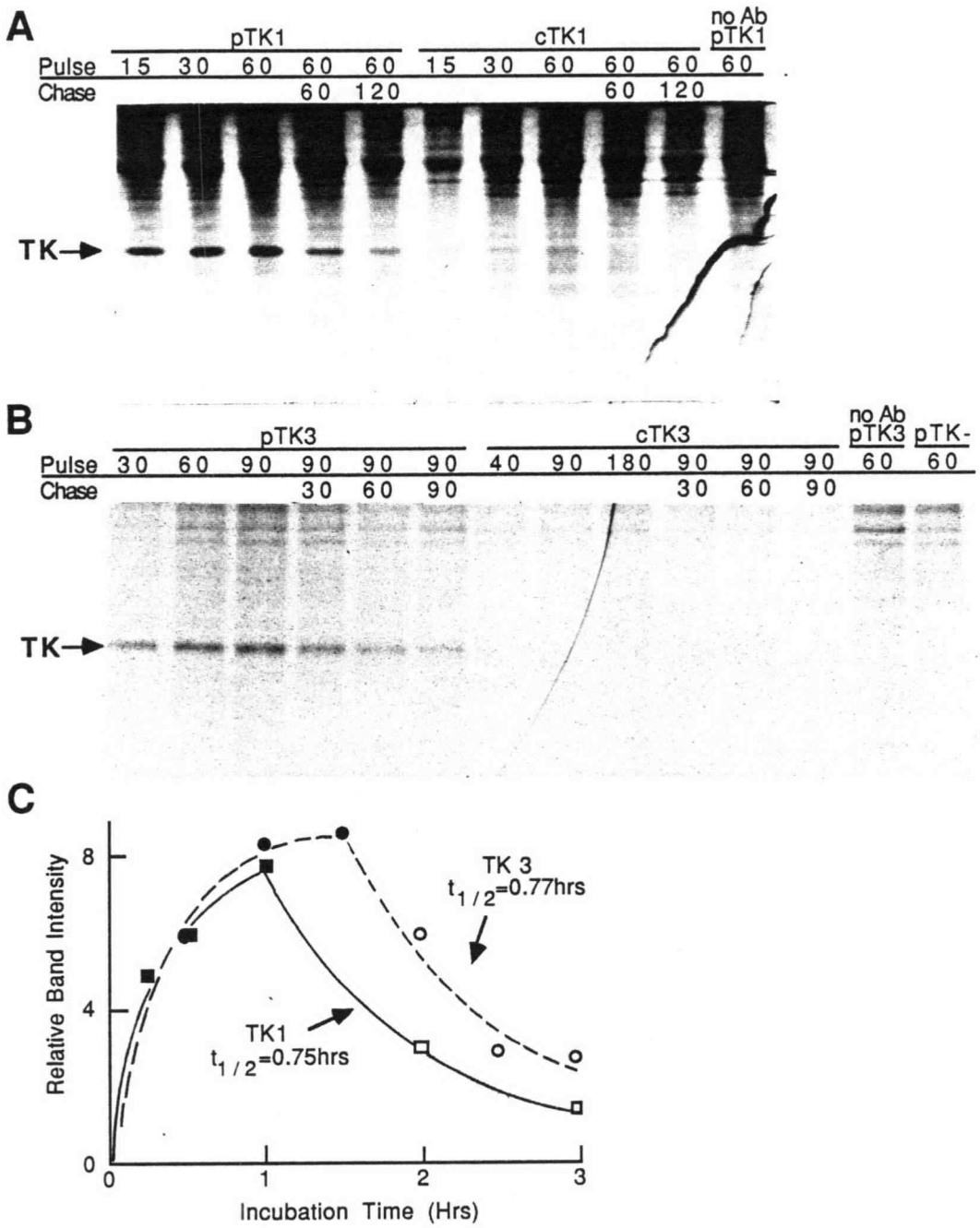
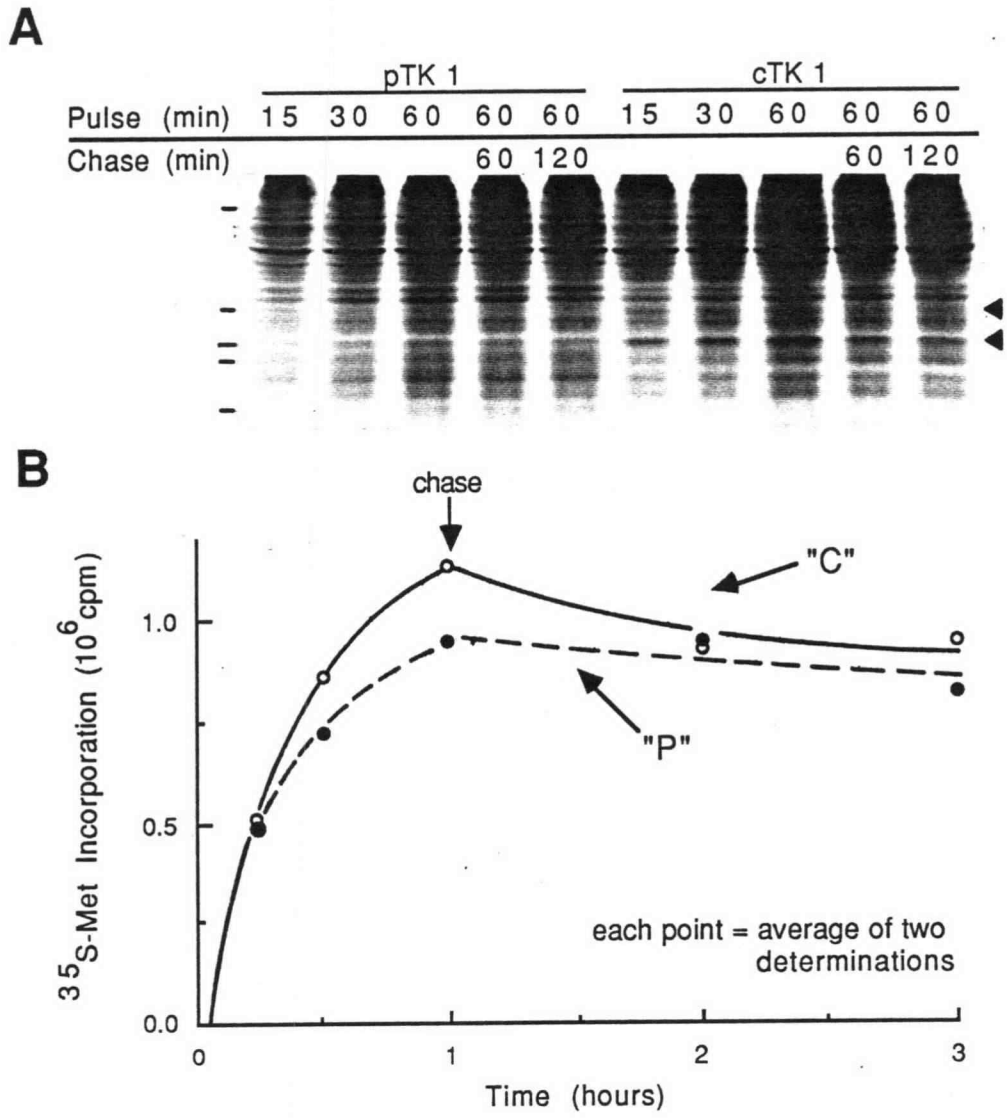


Fig. 3.9. Incorporation of [^{35}S]Met into total soluble protein in proliferating and committed muscle cells. Aliquots of the Protein A supernatant from the immunoprecipitation samples used in Fig. 3.8A were either run on denaturing SDS polyacrylamide gels and autoradiographed (A) or total protein was precipitated with 10% TCA and radioactivity in the precipitate determined by scintillation counting. 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.

Fig. 3.9


A
pTK 1
cTK 1
Pulse (min) 15 30 60 60 60 15 30 60 60 60
Chase (min) 60 120 60 120
B
chase
"C"
"P"
^{35}S-Met Incorporation (10^6cpm)
1.0
0.5
0.0
each point = average of two determinations
0
1
2
3
Time (hours)

CHAPTER 4:

Protein Coding Region Mediates the mRNA Independent Decline In TK activity During Myogenic Withdrawal From the Cell Cycle; General Mechanism for the Smallfold and Variable Decline in TK mRNA

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Abstract

The declines in thymidine kinase (TK) enzyme activity and mRNA during myogenic withdrawal from the cell cycle are regulated independently. Using a TK$^-$ myoblast cell line transformed with promoter switch, 3' region exchange, and exact intron deletion mutants, the *cis* acting regulatory information mediating regulation of either activity or mRNA levels was sought. Cis acting information mediating TK activity regulation was localized to the protein coding region, consistent with the observed translational or protein degradational level of regulation. Cis acting regulatory information mediating the decline in transcription of TK mRNA was also localized to the protein coding region of the gene. The latter result required equivocation because of the unusual location for transcriptional regulatory elements, the smallfold and variable decline in TK mRNA, the possibility of redundant control elements, and the controversy regarding possible S-phase dependence of the heterologous promoters used (HSV TK, metallothionein, SV40 early). Alternative, nonspecific models to explain TK mRNA regulation are discussed.

Introduction

Thymidine kinase (TK) is one of numerous replication associated enzymes that are expressed in a S-phase specific manner. S-phase specific expression of TK activity is accompanied by S-phase specific expression of TK mRNA. Recently several groups have used reverse genetics tosearch for the *cis* acting regulatory elements mediating S-phase specific expression of TK (Lewis and Matkovitch, 1986; Hofbauer *et al.*, 1987; Stewart *et al.*, 1987; Travali *et al.*, 1988; Kim *et al.*, 1988). We also have expended a great deal of effort on this problem.

During myogenic withdrawal from the cell cycle, TK activity was shown to be regulated in TK^- myoblasts transformed with the chicken TK gene (Merrill *et al.*,1984b). In contrast, myoblasts transformed with HSV TK gene showed constitutive expression of TK activity. To locate *cis* acting regulatory regions, Merrill *et al.* (1984b) have done a promoter switch experiment, in which the 5' flanking region of the chicken TK gene are replaced by that of the HSV TK gene and vice versa. Myoblasts transformed with the chicken TK gene under the control of the HSV TK promoter regulate TK activity whereas myoblasts transformed with the HSV TK gene under the control of the chicken TK promoter show constitutive expression of TK activity. Thus the promoter of chicken TK is inconsequential to regulation of TK activity. Similarly, a set of 3' region exchange mutant genes is regulated, indicating the 3' nontranslated region of chickenTK is not required for regulation of TK activity. Therefore, the *cis* acting regulatory information involved in regulating TK activity levels during myogenic withdrawal from the cell cycle has been localized to the internal region of the TK gene (i.e. the protein coding region and introns).

In the same report Merrill *et al.* have done a northern analysis indicating the decline in TK activity is accompanied by a qualitative decline in TK mRNA. A subsequent report (Gross *et al.*, 1987 or chapter 2 of this thesis) indicated the decline in TK mRNA is mediated at the level of transcription. Thus, the intragenic *cis* acting regulatory elements delineated by TK activity studies were initially thought to mediate their effect at the level of transcription.

However, by use of a quantitative assay for TK mRNA, Gross *et al.*(1987) also determined the decline in TK mRNA is insufficient to account fully for the decline in TK activity. More recently, Gross and Merrill (1988 or chapter 3 of this thesis) have exploited variability in TK mRNA regulation to show TK activity regulation is independent of TK mRNA regulation during myogenic withdrawal from the cell cycle. Because TK activity and TK mRNA are regulated by independent mechanisms, each

mode of regulation is expected to be mediated by a unique set of *cis* acting information.

This report describes an attempt to identify the *cis* acting regulatory information mediating either mode of regulation. To further define the location of the intragenic *cis* acting regulatory elements (Merrill *et al.*,1984) mediating TK activity regulation, a set of intron deletion mutants of the chickenmTK gene were transformed into myoblasts and tested for activity regulation. No intron deletion mutant consistently led to constitutive expression of TK activity. Hence, *cis* acting information mediating TK activity regulation must reside in the protein coding region.

To determine the location of *cis* acting regulatory information controlling TK mRNA levels, promoter switch, 3' region exchange, and intron deletion mutants of the chicken TK gene were tested for mRNA regulation during myogenic withdrawal from the cell cycle. Comparisons of mRNA regulatory phenotypes of mutant chicken TK genes was hampered by intrinsic variability of TK mRNA regulation (Gross and Merrill, 1988; Chapter 3). Numerous repetitions of experiments were required to observe a statistically significant difference in phenotype. Promoter switch mutants with either of two heterologous promoters and two 3' region exchange mutants failed to produce a completely constitutive phenotype. Numerous repetitions of the experiment with intron deletion mutants revealed partial alleviation of regulation. Formally, these results localize the *cis* acting regulatory information involved in TK mRNA regulation to the protein coding region, with a small contribution of intronic regulatory information. However, given the relatively smallfold decline in TK mRNA, the high degree of intrinsic variability in TK mRNA regulation, the controversial regulatory properties of the heterologous promoters used, and the unusual nature of transcriptional regulatory elements in the protein coding region, alternative non-TK-specific regulatory models are dicussed.

Materials and Methods

Construction of TK Mutants

The parental full length TK gene (fl in Fig. 4.1) extends from a synthetic Bam site located 775 bp upstream from the AUG translation start codon to a synthetic EcoR1 site located 2130 bp downstream from the UGA translation stop codon. The construction of Δ3'734Tx, Δ3'872Tx, VpΔ5' 2112,and VpΔ5'2177 were described by Merrill *et al.* (1984a). MpΔ5'2117 was constructed by attaching an EcoR1/Bgl2 fragment, containing the mouse metallotheionein-1 (MT-1) promoter and cap site (Mayo *et al.*,

1982), to the BamH1 linker on Δ5'2177. The construction of intron deletion mutants has been described previously (Gross *et al.*, 1988). All genes were cloned into pBR322 at appropriate sites.

Cell Culture and Transformation

Mouse muscle cells were grown as described elsewhere (Merrill *et al.*, 1984b), except that FGF isolated from bovine brains was used in place of chicken embryo extract as a source of mitogen. FGF enriched brain extracts were prepared by the method of Esch *et al.* (1985) and FGF was chromatographically purified by the method of Gospodarowicz *et al.* (1984). FGF preparations were used at concentrations which allowed maximal growth of myoblasts for 3 days after plating $5x10^5$ cells in 60mm dishes. To induce differentiation, cultures were rinsed twice with Ham's F10 and incubated in a defined mitogen-depleted medium consisting of Ham's F10 supplemented with 0.8 mM $CaCl_2$, 10^{-6} M insulin, 10^{-7} M dexamethasone. Muscle cells were transformed using the calcium phosphate precipitation method (Graham and van der Eb, 1973) with modifications (Corsaro and Pearson, 1981). Typically, $5x10^5$ cells in a 100 mm dish were exposed to calcium phosphate precipitates made with 10μg of TK plasmid(s) and 0.3 μg pKNeo. The internal control plasmid was cotransformed in either a 1:1 or 7:3 ratio with the test plasmid. All plasmids were linearized prior to transformation. HAT selection was performed as described previously (Merrill *et al.*, 1984b). Cotransformants with pKNeo were selected in 400 μg/ml G418 (GIBCO) and maintained in 100 μg/ml G418.

Isolation of RNA

One to five10 cm dishes (2-4 $x10^6$ cells/dish) were scraped in 1.6 ml of buffer Z (4 M Guanidinium isothiocyanate, 0.1 M ß-mercaptoethanol, 5 mM sodium citrate, 0.5% sarcosyl). The homogenate was sonicated 30 seconds at maximum with a small sonicator probe. Solid CsCl was added to the homogenate (1g per 2.5 ml final volume) and the volume adjusted to 2 ml with buffer Z. The homogenate was then layered on a 1ml CsCl pad (5.7 M CsCl, 0.1M EDTA) in a 3.0 ml heat seal tube and centrifuged at 49000 rpm in a 100.3 rotor (110000 x g) in a Beckman Tabletop Ultracentrifuge (TL-100) for 16-24 hours. After centrifugation the supernatant above the pad was removed with a syringe, the tube cut 1 cm from the bottom, and the remaining supernatant removed with a pasteur pipette. The clear, contact-lense like pellet was

resuspended in 400µl of1xTES (10 mM Tris-Cl pH 7.5, 5 mM EDTA, 1% SDS), mixed with 1/20 volume of 5 M NaCl, and immediately vortexed vigourously for 30 s with a half volume of redistilled phenol. The samples were worked up by adding a half volume of chloroform, extracting, removing the aqueous layer to a fresh tube, adding a full volume of chloroform, extracting, and precipitating the RNA in the aqueous layer in a fresh tube with 3 volumes of cold absolute ethanol.

Assays of TK activity and TK mRNA

TK activity was measured as described by Merrill *et al.* (1984b). Production of synthetic RNA probe and standards, and absolute TK mRNA quantitation *via* RNAse mapping are described in detail elsewhere (Gross *et al.*, 1987).

Results

Promoter Switch , 3' Region Replacement, and Intron Deletion Mutants

The full length chicken TK gene (fl, Fig. 4.1) is interrupted by six introns (thin lines). A seventh intron, in the 3' nontranslated region (stippled), is removed from rare TK mRNAs in some tissues (Merrill and Tufaro, 1986). Intron deletion constructs (Fig. 4.1 and Fig. 5.1) were made by combining cDNA and genomic fragments at shared restriction sites. The constructs were named for the introns that were deleted from the gene. For example, the mutant Δi1-6 lacks introns one through six. Fig. 4.1 also shows the structure of various promoter switch and 3' replacement mutants used.

Cis Acting Elements Regulating TK activity Levels

By examining the TK activity regulation of promoter switch and 3' region exchange mutants Merrill et al. (1984b) have demonstrated that *cis* acting regulatory elements are located in the intragenic region between 49 bp upstream of the start codon and 22 bp downstream of the stop codon, a region encoding introns as well as exons. In subsequent work (Gross and Merrill, 1988), we determined the mRNA-independent TK activity regulation was mediated at the level of translation or protein degradation. The translational and/or degradational models of TK activity regulation require *cis* acting information to be localized in the exons.

To test these models, TK^- mouse myoblasts were transformed with the intact gene

or the intron deletion mutants and TK enzyme activity was measured in proliferative and committed cultures (Fig. 4.2). In all mutant transformants, pooled and clonal, TK enzyme activity was regulated as tightly as in wild type transformants. Therefore, TK activity regulation is not mediated by intronic elements.

Myoblasts transformed with promoter switch (Vp5'2177) and 3' region exchange mutants (Δ3'-734Tx) also regulated TK activity as tightly as wild type transformants (Fig. 4.2), recapitulating results reported earlier by Merrill *et al.* (1984). A compilation of additional data collected for TK activity regulation in myoblasts transformed with the mutant and wild type genes is included as appendix 1.

Taken together, the results indicate the *cis* acting information involved in regulating TK activity during myogenic withdrawal from the cell cycle reside in or near the protein coding region. This conclusion is consistent with our previous report (Gross and Merrill, 1988) indicating TK activity is regulated mainly at the level of translation or protein degradation.

Cis acting Elements Regulating TK mRNA Levels

Fig. 4.1 shows the structure of various promoter switch, 3' region replacement, and intron deletion mutants that were cotransformed into muscle cells with pKNeo. Polyclonal transformant pools were tested for chicken TK mRNA regulation during myogenic withdrawal from the cell cycle by a sensitive, quantitative RNase protection method (Gross *et al.*, 1987). Fig. 4.3 shows a representative gel used to obtain absolute TKmRNA levels. The standards (0.3 to 0.01 fmol) consisted of a series of dilutions of an *in vitro* synthesized, spectrophotometrically quantitated TK pseudo-RNA. The intensity of the 147 base protected fragment in experimental samples was compared to a standard curve to give absolute mRNA levels.

Because TK mRNA is now known to be regulated independently of TK activity (Gross and Merrill, 1988), the intragenic *cis* acting information defined by activity measurements (Merrill *et al.*, 1984) can no longer be taken to represent elements involved in TK mRNA regulation. Consequently, a systematic examination of mRNA regulation in myoblasts transformed with promoter switch, 3' region replacement, and intron deletion mutants was undertaken. TK mRNA is regulated at the level of transcription during myogenic withdrawal from the cell cycle (Grosss *et al.*,1987). The most likely location for *cis* acting regulatory information controlling transcription is the 5' flanking region.

Therefore, regulation of chicken TK mRNA expressed from two heterologous

promoters was examined. Fig. 4.3 shows representative RNase protection gels used to determine TK mRNA levels in proliferative and committed populations of myoblasts transformed promoter switch genes. The full length gene (fl), containing the native promoter, typically showed a 2-3 fold decline in cTKmRNA levels between proliferative and committed muscle cells. If the HSV TK promoter was placed either 48 bp downstream (VpΔ5'2122) or 18 bp upstream (VpΔ5'2177) of the transcription start site (32 bp upstream of ATG; G. Merrill, unpublished observation) of the chickenTK gene, no significant change in chicken TK mRNA regulation was observed in repeated experiments with seperate transformant pools. The native promoter also was replaced with the metallothionein promoter (MTΔ5'2177) 18 bp upstream of the cap site. In a similar set of experiments no significant change in regulation was observed. Because regulation of TK mRNA is variable (Gross and Merrill, 1988), each experiment was repeated several times. The average fold decline in TK mRNA levels and the number of experiments performed with transformants of each construct are shown below. As expected, substantial variability was observed in TK mRNA regulation. Although poor regulation (less than 2-fold) was observed in some experiments, no example of constitutive expression (1-fold or no change) of TK mRNA was observed for any promoter switch mutants. Moreover, no promoter switch mutant consistently caused poor regulation of TK mRNA levels, suggesting the *cis* acting regulatory elements for mRNA regulation lie more than 32 bp downstream of the transcription start site. Alternatively, all three promoters tested mediate growth dependent mRNA expression.

A second likely location for *cis*-acting regulatory elements was in the 3' nontranslated region. To test this hypothesis, the chicken TK 3' nontranslated region was replaced by that of theHSV TK gene either 113 bp upstream of the stop codon (Δ3'872Tx) or 24 bp downstream of the stop codon (Δ3'734Tx). The chimeric genes showed no significant change in regulation in repeated experiments (Δ3'734Tx in Fig. 4.3; Δ3'872 is the internal control (Δ3'873Tx) in Fig 4.4). One construct (Δ3' 872Tx) which was used as an internal control in many experiments, was tested in 36 seperate experiments. The average fold regulation and standard deviation observed for this construct were very close to the values obtained for 24 experiments on the intact gene (2.8 ± 1.7 versus 2.7 ± 1.5, respectively, see Fig. 4.4). Therefore, the *cis* acting elements of mRNA regulation are more than 113 bp upstream of the chicken TK termination codon. Alternatively, the HSV TK termination sequences may contain regulatory elements that can substitute for the chicken TK elements. The sequences of these genes are not similar.

Intron deletion mutants with native promoters and 3' regions were tested for TK

mRNA regulation. Fig 4.4 shows all intronless genes can be regulated (left panel and Δi7). However, when the experiment was repeated several times, it became apparent regulation of TK mRNA was sometimes very poor in intronless genes. Occasional non-regulation was confirmed for intronless genes in experiments using an internal control gene (Δ3'872Tx). The right panels show two experiments in which the intronless test gene was not regulated but the control gene was. Hence non-regulation was not due to poor withdrawal from the cell cycle in a particular experiment.

Fig. 4.5 shows the fold decline in TK mRNA levels in numerous regulation experiments on different full length (fl) and intronless gene (Δi1-6) transformant pools. As expected, both genes showed a large spectrum of regulation when the experiments were ranked by the amount of regulation they exhibited. The regulation spectrum for the intronless gene had more cases of poor regulation and the degree of regulation was somewhat lower throughout the spectrum. Thus, removing introns from the chicken TK gene slightly alleviates regulation. However, removal of introns does not abolish TK mRNA regulation in all experiments. Moreover, TK mRNA is regulated 3 to 4-fold in some intronless transformants. Therefore the introns do not contain all of the essential *cis* acting information controlling TKmRNA regulation. Introns merely allow TK mRNA regulation to occur a little more efficiently, particularly in those experiments which show low regulation.

In summary, regulation of TK mRNA levels occurred when the native promoter or 3' nontranslated region of the chicken TK gene were replaced, or when the introns interrupting the protein coding region were deleted. Therefore, the *cis* acting information required for regulation of TK mRNA levels must reside in exonic sequence between 16 bases downstream of the start codon and 14 bases upstream of the stop codon. Based on the elongation of nascent transcripts in nuclear run-off assays, the decline in TK mRNA as cells withdraw from the cell cycle is at least partly, if not totally, due to repressed transcription of the TK gene (Gross *et al.* 1987; Conrad *et al.* 1987). Localization of the *cis* acting information to the exons suggests protein-encoding exonic sequences are capable of regulating the rate of transcription. Considering the unusual nature of this result, the variability of TK mRNA regulation, and the smallfold change in mRNA involved, the assumptions used to design the experiments are called into question. Alternative explanations which could explain the results are discussed below.

Discussion

Localization of *cis* acting information involved in TK activity regulation to within or near the protein coding region is supported by other observations. TK activity is known to be regulated at either the level of translation or protein degradation (Gross and Merrill, 1988/chapter 3). More recent experiments showed the distribution of TK mRNA in polysome profiles changed little during myogenic withdrawal from the cell cycle (unpublished observation). These results suggest TK activity is regulated at the level of protein degradation, a mode of regulation which would require *cis* acting elements to be in the protein coding region.

Localization of *cis* acting information involved in TK mRNA regulation to the protein coding region is less intuitive and requires equivocation. In designing experiments involving replacement of a chicken TK gene structure with an equivalent structure from a heterologous gene, one must be certain the structure from the heterologous gene lacks *cis* acting regulatory information involved in S-phase dependent expression. Promoter switch and 3' region replacement mutants were constructed with sequences from HSV TK and mouse metallothionein genes. Using a mouse fibroblast cell line and butyrate synchronization, Hofbauer *et al.* (1987) showed HSV TK mRNA to be constitutively expressed and MT mRNA to be declining as cells enter S-phase. These results suggest the heterologous structures we inserted into the chicken TK gene lack growth regulatory elements. Attempts have been made to recapitulate the results of Hofbauer *et al.* in our myoblast system. In several experiments, northern analysis and RNase protection assays showed HSV TK mRNA to decline during myogenic withdrawal from the cell cycle (unpublished observations). These result indicated the HSV TK sequences used in promoter switch and 3' region exchange mutants may have contained *cis* acting information suitable for growth dependent expression of mRNA.

Regulation of HSV TK mRNA during myogenic withdrawal from the cell cycle was suprising because HSV TK activity is constitutively expressed during this transition. However, the regulation of HSV TK activity may be independent of HSV TK mRNA regulation, as has been observed for chicken TK. Thus, for both chicken TK and HSV TK, there would be no obvious need for growth regulation of the mRNA.

In an effort to find a promoter which caused constitutive mRNA expression during myogenic withdrawal from the cell cycle, the SV40 promoter was attached to the bacterial gpt gene and the chimeric gene transformed into myoblasts. Northern blot analysis of RNA from proliferative and committed cells indicated the mRNA produced from the chimeric gene declined about the same extent as HSV TK mRNA and chicken TK mRNA during myogenic withdrawal from the cell cycle (unpublished observations). The bacterial gpt gene is not expected to include *cis* acting elements mediating growth

dependent expression of mRNA (this cannot be tested directly because a eukaryotic promoter is required to get expression in myoblasts). Therefore the SV40 promoter likely contains *cis* acting regulatory elements mediating growth dependent expression of mRNA.

Using a serum stimulation or viral infection protocol to stimulate entry of transformed CV1 cells into S-phase, Stewart *et al.* (1987) demonstrated the S-phase dependent increase of mRNA from a chimeric gene made of the SV40 early promotor driving the human TK gene. They concluded the body of the TK gene is sufficient to mediate cell cycle regulation. These investigators did not test the possibility of regulation of the SV40 early promoter themseves, but rather refer to a personal communication from N. Heintz, which indicates the SV40 promoter is constitutively expressed during the transition from G1 to S phase. Thus the SV 40 early promoter is also constitutive in the fibroblast system and regulated in the myoblast system.

The controversy regarding the growth regulation of HSV TK mRNA (our results; Kim *et al.*,1988; Hofbauer *et al.* 1987) and growth dependent expression from the SV40 promoter (our results; Heintz personal communication to Stewart *et al.*,1987), casts doubt upon the validity of the promoter switch experiments performed by us with the HSV TK promoter and by Stewart *et al.* with the SV40 promoter. In contrast, our promoter switch experiments with the metallothionein promoter find support in the report of Hofbauer *et al.* (1988). Considering we and Hofbauer *et al.* observe differences in HSV TK mRNA regulation in our respective systems, constitutive expression of metallothionein mRNA should also be confirmed independently during myogenic withdrawal from the cell cycle. This has not been done. Although promoter switch studies, which contend the body of the TK gene is sufficient to mediate mRNA regulation (this study; Stewart *et al.*,1987), are not neccesarily incorrect, their conclusions are not firm.

Two less controversial reports by Travali *et al.* (1988) and Kim *et al.* (1988) have implicated the TK promoter in cell cycle regulation of TK mRNA. Travali *et al.* used a chimeric gene, composed of the human TK promoter driving the bacterial CAT gene, to demonstrate S-phase specific expression of mRNA. They cited previous work to establish CAT as being constitutively expressed in the cell cycle. Thus, the human TK promoter controls S-phase specific expression of CAT activity. Kim *et al.* measured specific mRNA levels in fibroblasts transformed with chimeric genes composed of human TK promoter sequences and the bacterial neo gene. Their study implicates a 380 bp region of the human TK promoter in S-phase specific expression of mRNA. They also claim the HSV TK promoter, when fused to the neo gene, produces similar

mRNA levels in proliferative and quiescent cells. Therefore, Kim *et al.* (1988) use constitive expression of an HSV TK-neo construct to establish their result. If HSV TK promoter is indeed constitutively expressed, then our results are also valid and indicate the opposite conclusion, namely the *cis* acting regulatory elements are in the body of the TK gene.

If promoter switch experiments are incorrect because the heterologous promoters used, independently conferred cell cycle regulation, then the specificity of TK mRNA regulation would be in doubt. In order to negate the results of this report and the report of Stewart *et al.* (1987), the HSV TK, SV40, metallothionein, chicken TK, and human TK promoters would all need to confer S-phase dependent expression of mRNA. In addition, all five promoters might be expected to contain similar *cis* acting regulatory information.

Comparison of 5' flanking sequences for these genes revealed one similarity between all genes we studied. Sequences corresponding to the well known transcriptional elements such as TATA and CAAT are missing in the chicken TK and metallothionein genes, respectively. Regions of sequence similarity corresponding to the SP1 binding sites of the 1st and 2nd distal elements of HSV TK were found in the first 200 bp of chicken TK, metallothionein, and SV40 5' flanking regions (Fig. 4.6). The importance of SP1 binding sites for S-phase dependent expression of mRNA remains to be tested.

In view of the apparently contradictory evidence, two alternative hypotheses are discussed below. The first hypothesis suggests all promoters mentioned mediate cell cycle regulation of mRNA levels by a general mechanism that influences a wide variety of promoters. Consistent with this hypothesis is the smallfold decline and variability in the decline of TK mRNA, which may be expected from a relatively nonspecific mechanism. This hypothesis is intuitively appealing because transcription, the level at which TK mRNA expression is regulated, is normally mediated by 5' flanking sequences. In addition, a potential common element exists (Fig. 4.6). The promoter switch experiments, which suggest elements are located in the body of the TK gene, would be invalid because regulated promoters were substituted for a regulated promoter.

The second hypothesis suggests there are dispersed *cis* acting regulatory elements throughout the TK gene, each of which can mediate regulation of TK mRNA. This hypothesis would account for S-phase dependent expression of bacterial genes driven by TK promoters and for S-phase dependent expression of regulated genes driven by "nonregulated" promoters. In addition this model would be consistent with

the slight alleviation of mRNA regulation observed in intron deletion mutants. Interestingly, SP1 binding sites are observed in the promoter and introns of chickenTK. Smallfold declines and variability in chicken TK mRNA regulation are also consistent with dispersed elements, which would likely not mediate tight control but rather a preference for transcription of TK in proliferating cells.

The mechanism of TK mRNA regulation is likely to be a passive rather than active cellular event because it is not important in altering levels of a functional gene product.

Acknowledgements

We thank Steven McKnight, Bob Kingsbury, and Mark Kainz for constructing several parental plasmids, and Christine Davis for performing TK enzyme assays. This work was supported by NIH grant (GM34432) and RCDA (AG00334) to G.F.M.

Fig. 4.1. Mutants of the chicken TK gene. Hatched regions designate the protein coding domain, open wide bars are exons, solid bars are introns, horizontal striped regions indicate HSV TK sequences, checkered regions indicate metallothionein sequences, and the stippled region is an intron removed from the 3' nontranslated region of chickenTK during the biogenesis of rare messages in certain tissues (Merrill and Tufaro, 1986). Letters represent the following restriction enzyme recognition sites: B, BamH1; R, EcoR1; H, Hind3; P, Pst1; Bg, Bgl2; Sm, Sma1. Brackets indicate restriction sites that are dead and bars indicate synthetic oligonucleotide linkers. Cap indicates the expected transcription start site of the gene and the hexanucleotide AAUAAA designates the polyadenylation signal. The figure is drawn to scale; in the full length TK gene (FL) the distance between Bg and H is 772 base pairs. FL extends from a synthetic BamH1 linker 775 base pairs upstream from the start codon to a synthetic EcoR1 linker 2130 bp downstream from the stop codon. All constructs are aligned along the region used in RNase protection assay (Bg to Ps).

Fig. 4.1

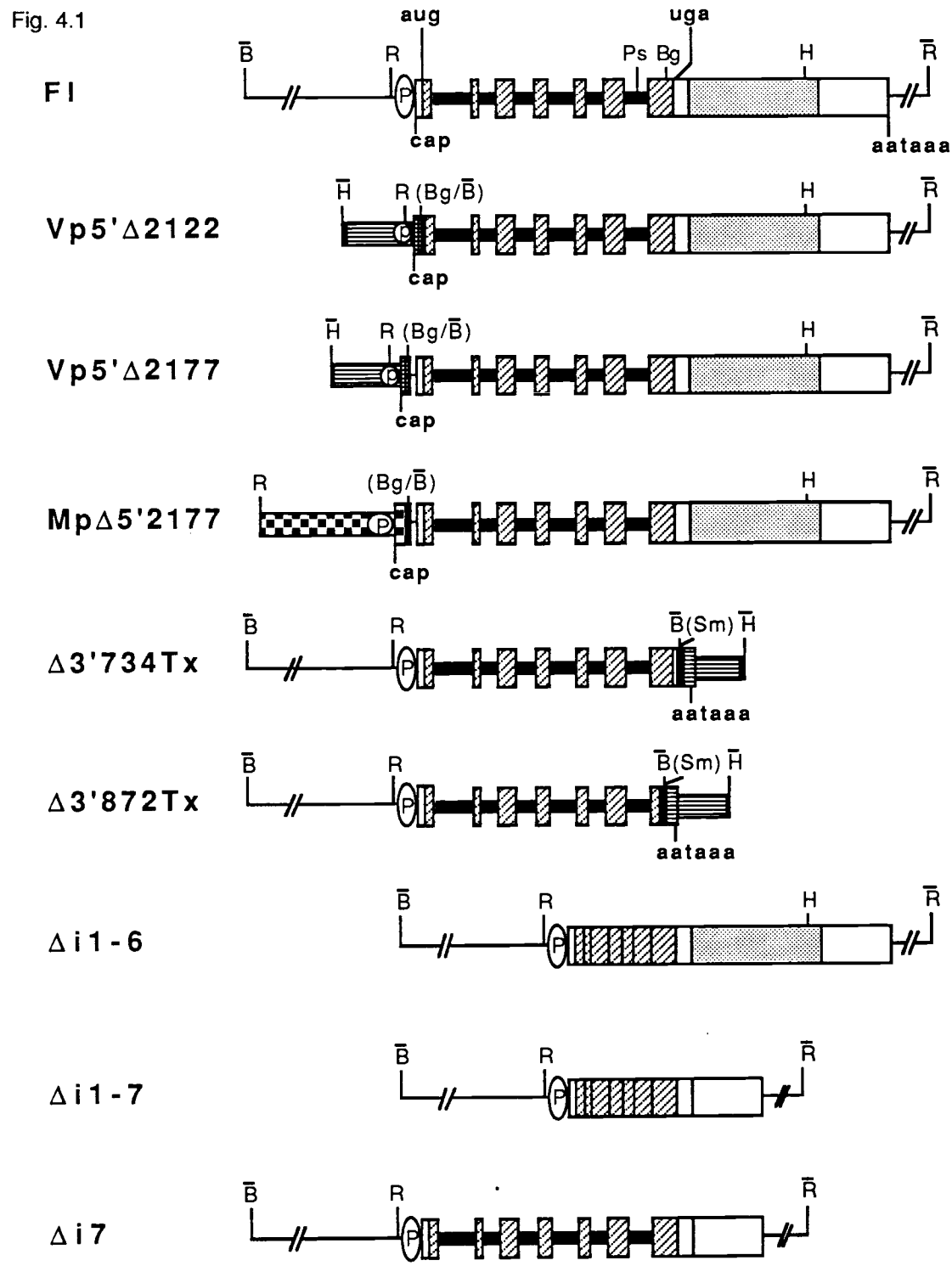

aug
uga
B̄
R
Ps
Bg
H
R̄
P
cap
aataaa
F I
H̄
R (Bg/B̄)
Vp5'Δ2122
Vp5'Δ2177
(Bg/B̄)
MpΔ5'2177
B̄(Sm) H̄
Δ3'734Tx
Δ3'872Tx
Δi1-6
Δi1-7
Δi7

Fig. 4.2. Regulation of TK enzyme activity in myoblasts transformed with mutant TK genes. For each cell line, TK enzyme activity was measured in proliferating myoblasts and in committed cells after 24 hours in mitogen depleted medium. Clonal (A, B, 1a, 1b, 1c) and pooled transformants were assayed.

Fig. 4.2

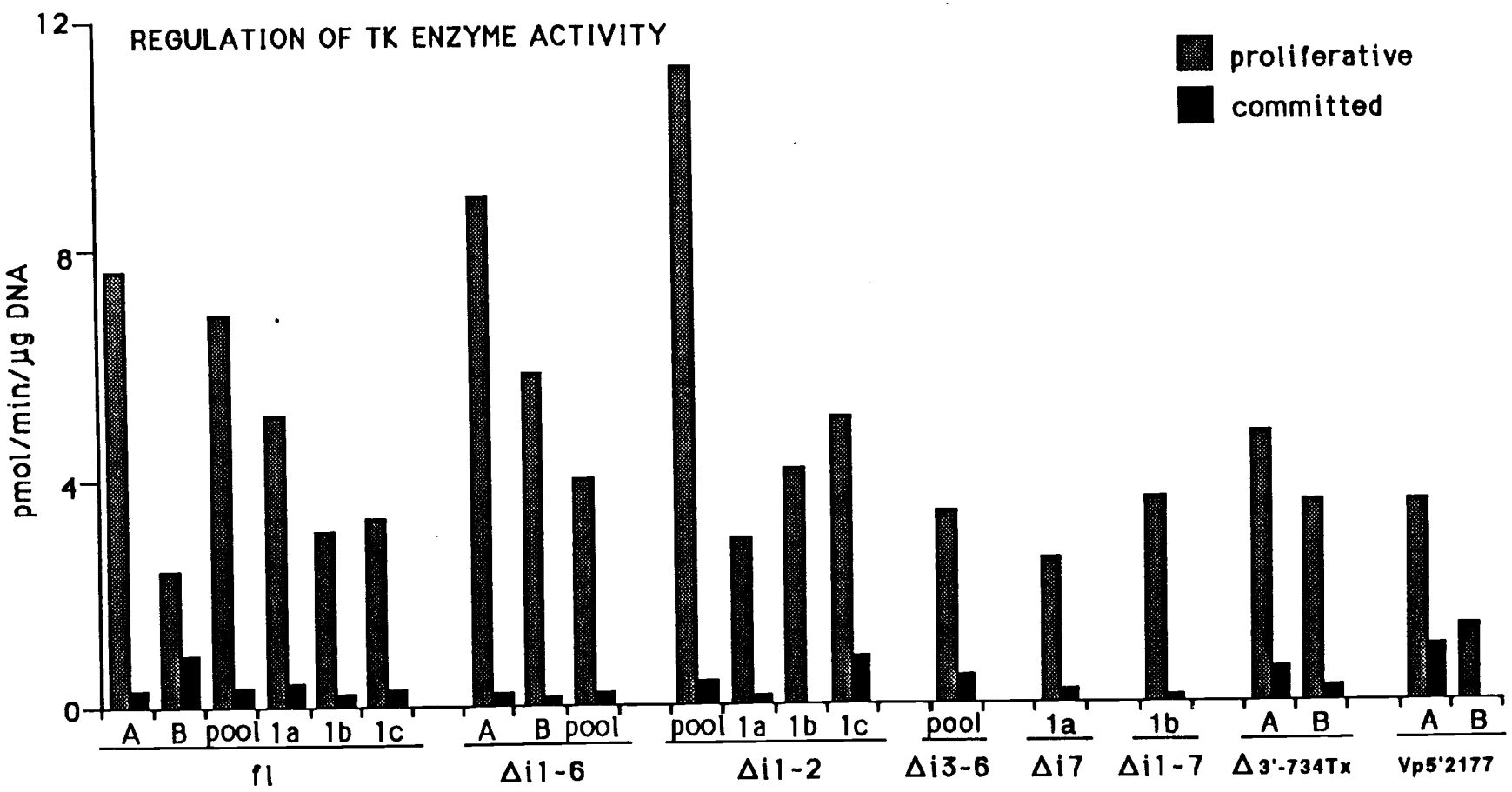
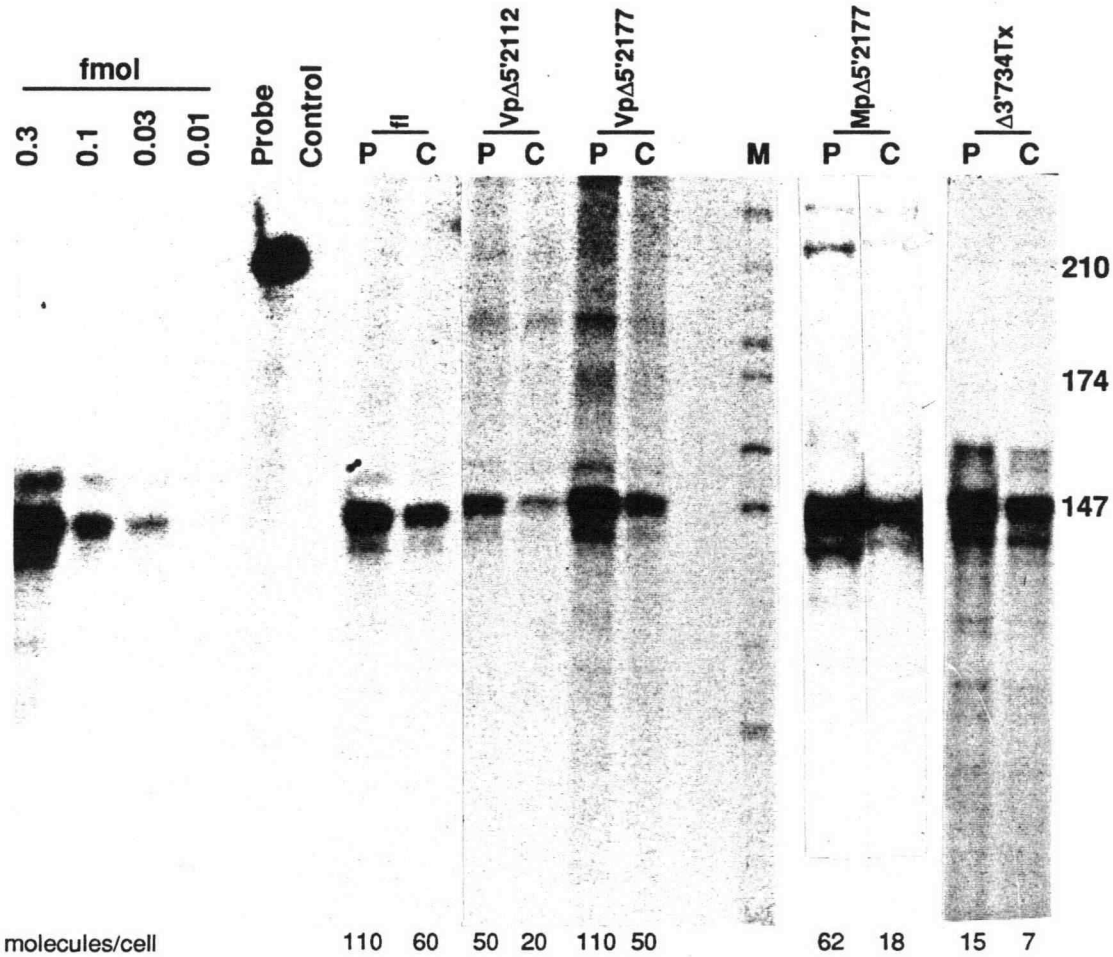

REGULATION OF TK ENZYME ACTIVITY
proliferative
committed
pmol/min/µg DNA
12
8
4
0
A
B
pool
1a
1b
1c
fl
Δi1-6
Δi1-2
Δi3-6
Δi7
Δi1-7
Δ3'-734Tx
Vp5'2177

Fig. 4.3. Regulation of mRNA in mouse myoblasts cotransformed with promoter switch or 3' region replacement mutants, and pKNeo. A representative RNase quantitation gel used to determine absolute levels of TK mRNA in muscle cell transformants is shown. TK mRNA and TK precursor RNA protect 147 and 174 base fragments, respectively. The four left lanes contain SP6-generated synthetic TK mRNA standards and 10μg of yeast RNA . The probe lane shows the nondigested 214 base probe. The control lane contains 10μg yeast RNA. Sample lanes contain 10 μg (left panel) or 40μg (right panels) of total RNA from proliferative (P) or committed (C) mouse myoblast transformant pools. Average fold declines, standard deviations, and number of experiments are given for each mutant tested. Repeated experiments include at least two different transformed cell lines.

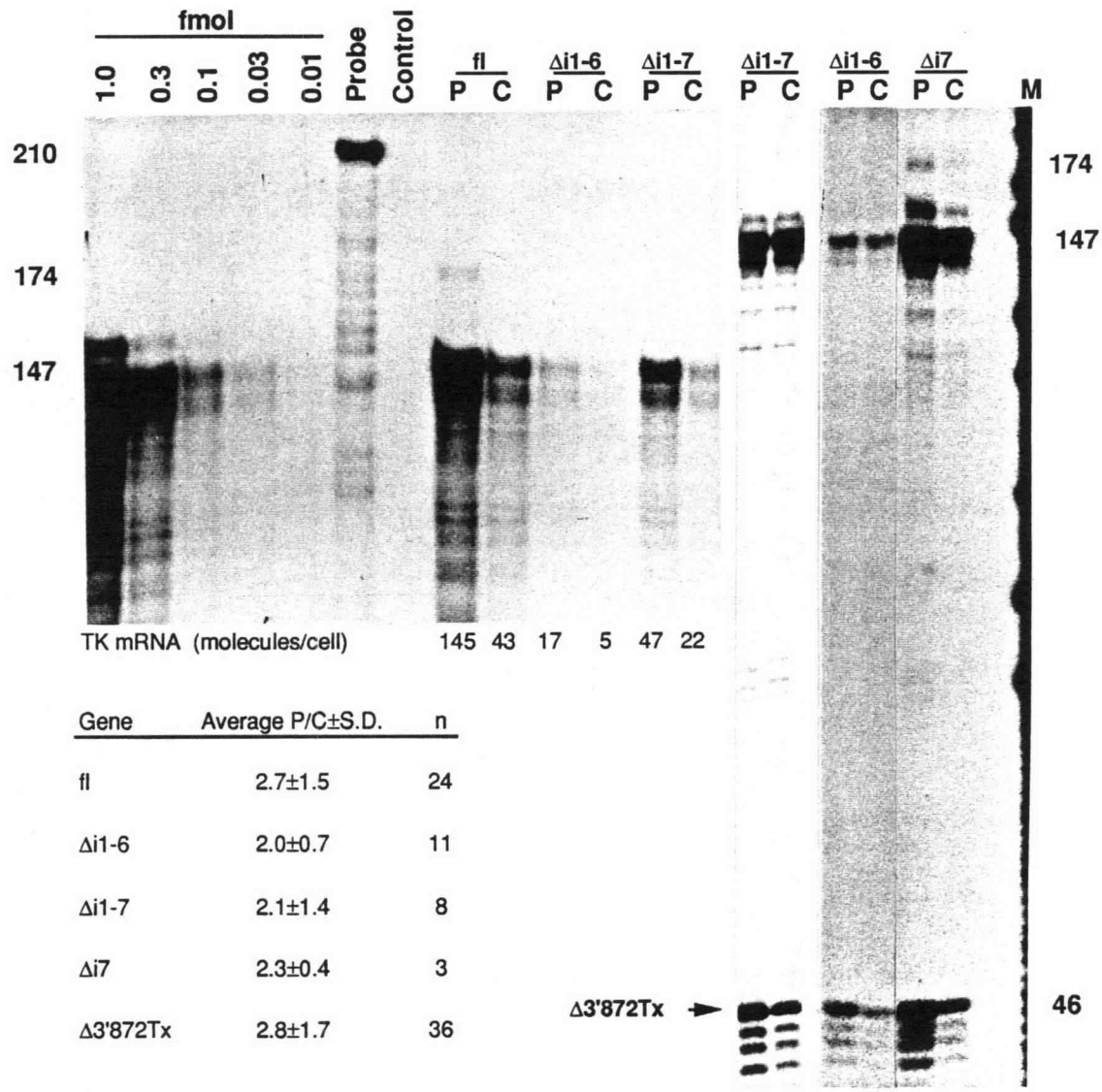
Fig. 4.3



molecules/cell	110	60	50	20	110	50		62	18	15	7
average P/C ± S.D.	2.7 ± 1.5		2.4 ± 0.8		2.4 ± 0.1			2.6 ± 1.3		2.3 ± 0.9	
number of experiments	24		5		3			4		2	

Fig. 4.4. Regulation of mRNA in mouse myoblasts cotransformed with intron deletion mutants and pKNeo. Sample lanes contain 20μg (left panel), 40μg (middle panel), and 20μg (right panel) of total RNA from polyclonal transformant pools. The right panels show a 46 base protected fragment expressed from a co-transformed internal control gene (Δ3'872Tx). Average fold decline, standard deviation, and number of experiments are indicated at lower left.

Fig. 4.4



Gene	Average P/C±S.D.	n
fl	2.7±1.5	24
Δi1-6	2.0±0.7	11
Δi1-7	2.1±1.4	8
Δi7	2.3±0.4	3
Δ3'872Tx	2.8±1.7	36

Fig. 4.5. Regulation spectra of full length and intronless genes. TK mRNA regulation data from TK- cells cotransformed with full length or intronless chicken TK genes and pKNeo were ranked from least to greatest regulation. Identical letters on the bar indicate repeated experiments with the same transformant pool. Brackets on the letters indicate monoclonal transformants. Bars with no letters are transformant pools that were tested only once.

Fig. 4.5

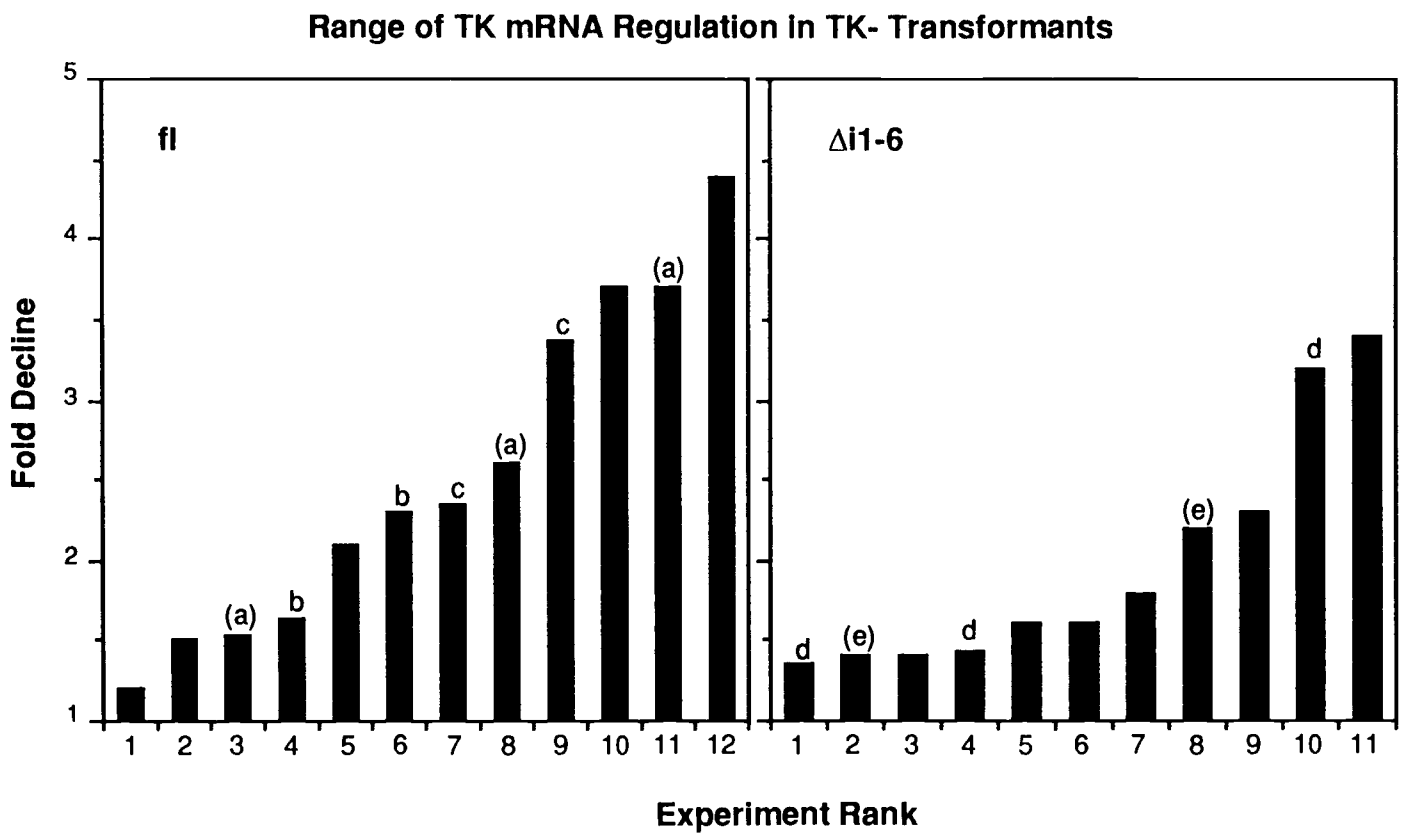

Range of TK mRNA Regulation in TK- Transformants
fl
Δi1-6
Fold Decline
Experiment Rank
1
2
3
4
5
(a)
b
c
d
(e)
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11

Fig. 4.6. Comparison of 5' flanking regions of genes displaying growth dependent mRNA expression. Gene sequences 200 bp upstream of the transcription start site were compared. First, searches for well defined transcription elements such as the TATA box, CCAAT box, 1st and 2nd distal elements of HSV TK, glucocorticoid response element, and SP1 site were conducted. The figure shows the most striking feature these genes all have in common, namely two SP1 consensi are arranged similarly to the SP1 consensi in the palindromic portions of the HSV TK 1st and 2nd distal elements. None of the other elements was common to all genes. Second, a search for novel elements was conducted. All possible pairs of the genes above were searched with a matrix homology program to identify sequences which particular pairs had in common. If a significant match (6 with no, 7 with 1, 8 with 2, 9 with 3, and 10 with 4 mismatches) between two genes was found, the other two genes were searched for that sequence. No significant, new sequence in common to all four genes was identified. Third, palindrome searches were conducted in each gene. If a large palindrome (>6) was found, the other three genes would be searched with the palindrome sequence. No palindromes, other than the one shown, were found in common in all four genes. Numbers in brackets indicate the position of the first nucleotide relative to the transcription start site. Pyrimidine (Y), purine (U), and any nucleotide (N) are used in describing the consensus sequence. Underlined bases are not identical in all 4 sequences.

HSV TK	(-105)	CCCCGCCCAG	-- 40 bp --	(-61)	CGGGGCGGCG
CHTK	(-97)	CTCCGCTCGG	-- 51 bp --	(-36)	CCGGGCGGCG
MT	(-141)	CTCCGCCCGA	-- 26 bp --	(-106)	CGGGGCGCGT
SV4OEarly	(-98)	CTCCGCCCAT	-- 75 bp --	(-14)	CGAGGCCGCC
	(-11)	CTCCGCCCAG	-- 54 bp --		
Consensus		CYCCGCCCUU	----------		CGPGGCGGGN
SP1 Consensi		CCGCCC			GGCGGG

CHAPTER 5:

Introns Are Inconsequential to the Efficient Formation of Cellular Thymidine Kinase Messenger RNA in Mouse L Cells

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Abstract

TK mRNA levels were determined in mouse L cells transformed with intron deletion mutations of the chicken TK gene. Whether normalized per cell, per integrated gene, or per internal control signal, intron deletion did not diminish the efficiency of TK mRNA formation in transformed L cells. The results demonstrated that introns are not required for efficient biogenesis of cellular mRNA in transformed mouse L cells.

Text

The general importance of introns for efficient gene expression in mammalian cells is an unresolved issue. Early work with recombinant SV40 virus showed convincingly that efficient formation of viral 16S mRNA requires the presence of an intron in the DNA template (Lai and Khoury,1979; Lee *et al.*, 1981; Hamer and Leder, 1979; Hamer *et al.*, 1979; Gross *et al.*, 1987); the intron requirement was manifested at a posttranscriptional level and could be satisified by substituting an intron from a heterologous gene. These results suggested that passage through a splicing pathway might be a general requirement for formation of stable cytoplasmic mRNA. Such a requirement could explain the poor transformation efficiency of various intronless minigenes (Lee *et al.*, 1981; Gasser *et al.*, 1982; Lewis, 1986). However, rigorous reaffirmation of the importance of introns to eucaryotic mRNA formation has not been reported. In fact, for certain viral, plant and yeast genes, evidence to the contrary has accumulated. Wildtype and intronless derivatives of the genes encoding adenovirus E1A protein (Svensson *et al.*, 1983; Carlock and Jones, 1981), polyoma virus T antigens (Treisman *et al.*, 1981; Zhu *et al.*,1984), and the Rous sarcoma virus envelope protein (Chang and Stoltzfus, 1985) were equally efficient in generating mRNA in infected cells. Similar results were obtained for bean phaseolin in transformed plants (Chee *et al.*, 1986) and yeast actin in transformed yeast (Ng *et al.*, 1985). Given these exceptions, a careful investigation of the importance of introns to expression of cellular genes in mammalian cells was warranted.

Direct comparison of mRNA levels in mammalian cells transformed with wildtype and intronless cellular genes has not been reported. Hofbauer *et al.* (1987) achieved expression of an intronless mouse thymidine kinase (TK) cDNA using a herpesvirus TK promoter, but did not compare expression levels to that of an intact gene. Evidence suggestive of an intron requirement for expression of mouse dihydrofolate reductase (DHFR) was reported by Lee *et al.* (1981) and Gasser *et al.* (1982), who showed that intron-free DHFR minigenes were less efficient than intron-containing minigenes in transforming $DHFR^-$ rodent cells to methotrexate resistance. Similarly, Lewis (1986) noted that an intron-free hamster TK minigene was ten-fold less efficient than intron-containing minigenes in transforming TK^- mouse L cells to HAT resistance. However, transformation efficiency is an indirect and potentially inaccurate measure of gene function. Furthermore, because of the large size of the mammalian DHFR and TK genes (due to the presence of large introns), direct comparison of intronless gene function to wildtype gene function was not feasible.

To investigate whether introns were required for efficient expression of cellular genes in animal cells, a series of intron deletion mutants of the chicken TK gene were constructed, transformed into L cells, and their level of expression quantitated. The full length chicken TK gene (fl) is interrupted by six introns. A seventh intron, in the 3' nontranslated region, is removed from rare TK mRNAs in some tissues (Merrill *et al.*,1984). Intron deletion mutants of the chicken TK gene were made by combining cDNA and genomic fragments at shared restriction sites (Fig. 5.1). The mutants were named for the introns that were deleted from the gene. For example, the mutant Δi1-2 lacks the first and second introns. Except for the removal of introns , all mutants were otherwise native, utilizing the normal TK promoter and polyadenylation signals.

As an initial test of the effect of intron deletion on gene expression, the mutants shown in Fig. 5.1 were used to transform TK$^-$ L cells to a HAT resistant phenotype. The transformation efficiency of the different mutants relative to the full length gene was determined in each of several independent transformation series, using the $CaPO_4$ method (Graham and van der Eb, 1973). The results gave no indication of a detrimental effect of intron deletion on gene function (data not shown). However, transformation assays could have obscured a significant effect of intron deletion on TK mRNA levels. For example, even if an intronless TK gene was ten-fold less effective in generating stable TK mRNA, enough mRNA may still be produced to allow growth in HAT medium.

As a more quantitative measure of mutant gene function, TK mRNA levels were measured in L cells cotransformed with intron deletion mutants and pKNeo, a plasmid conferring resistance to the drug G418. Resistance to 400 μg/ml G418 was used to select transformants because HAT might select cells transformed with greater numbers of weaker genes or smaller numbers of stronger genes, thereby obscuring any differences in observed TK mRNA levels. Furthermore, by transforming with a 20:1 ratio of TK-containing plasmid to pKNeo, cotransformants containing multiple copies of the TK gene were selected. Multicopy cotransformants facilitated direct determination of TK mRNA levels. Detection of TK mRNA was difficult in singlecopy HAT-selected transformants or transiently expressing transfectants. All G418 resistant colonies arising from a single transformation were pooled to minimize variability in TK expression from individual cotransformants.

TK mRNA levels in polyclonal cotransformant populations were determined by a quantitative RNase protection assay (Gross *et al.*, 1987) using total RNA isolated by the method of Glisin *et al.* (1974). Fig. 5.2 diagrams the RNase protection strategy and shows a representative quantitation gel. An aliquot of the undigested 214 base synthetic RNA probe is shown in lane 8. Hybridization of this probe to TK mRNA and

subsequent digestion with RNase, results in protection of a 147 base exonic fragment. Lanes 9-15 contain RNA isolated from cells cotransformed with fl, Δi1-6, Δi3-6, Δi1-2, Δi1-7, Δi7, and no TK (pKNeo only), respectively. To allow calculation of absolute TK mRNA levels in experimental samples, lanes 1-6 were loaded with known amounts of synthetic TK mRNA, generated using an SP6-based *in vitro* transcription system. In the transformation series shown in Fig. 5.2 (transformation series 9), Δi1-6 and Δi1-7 transformants (lanes 10 and 13) had less than half as much TK mRNA as fl transformants (lane 9). This result, seemingly suggestive of an intron requirement, was misleading. When TK mRNA molecules per cell were measured in several additional transformation series, as shown in the top set of entries in Table 5.1, no consistent detrimental effect of intron deletion was detected.

The number of TK mRNA molecules per cell varied considerably between different transformation series (Table 5.1). Similar variability was observed when TK enzyme activity levels were measured (data not shown). Variability in TK mRNA and enzyme levels could be due to random differences in gene copy number. This variability could obscure an effect of intron deletion on gene function. Furthermore, nonrandom, preferential integration or stability of intron deletion mutants could compensate for and mask negative effects of intron deletion on gene function.

To determine if intron deletion mutants were rearranged or preferentially integrated during transformation, TK gene copy number and integrity in cotransformants were analysed by Southern blotting (Southern, 1975). A representative blot of transformant DNA is shown in Fig. 5.3. In all samples, bands of the size expected for the input gene were evident. Thus, intron deletion neither enhanced nor inhibited rearrangement of the gene during the transformation process. To obtain gene copy number, the intensities of the sample bands in Fig. 5.3 (lanes 5-10) were compared to a standard curve generated with chicken liver DNA (lanes 1-3). Gene copy number data for five transformation series is summarized in the middle set of entries in Table 5.1. No consistent difference in integration efficiency was observed, although considerable random variation in gene copy number was evident. The random differences in gene copy number contributed to the variability in TK mRNA levels per cell. When TK mRNA levels were normalized to gene copy number levels (bottom set of entries in Table 5.1), much of the variability between transformation series was eliminated. However,even when normalized on a per gene basis, no apparent effect of intron deletion on TK mRNA expression was observed.

A final approach used to assess the effect of intron removal on gene expression was to include an internal control gene in each transformation. Individual transformants

are thought to integrate exogenous DNA as a single concatameric structure (Perucho *et al.* 1980). An internal control would allow mRNA expression to be normalized for differentially active chromosomal integration sites as well as for gene dosage. The internal control gene (2050tx) contains introns 1-6, but is truncated within exon 7 and joined to the HSV TK polyadenylation signals (Fig. 5.4, bottom). It produces a mRNA that protects only 46 bases of the probe used in the RNase protection assay. Southern blot analyses confirmed that the internal control gene and test gene were present in the transformants in the same 1:1 ratio as in the $CaPO_4$ transfection mixture (data not shown). A representative RNase protection gel of two transformation series using the internal control genes is shown in Fig. 5.4. The usefulness of the internal control gene was apparent for transformation series 12. If the TK mRNA produced from Δi7 (at 147 bases) was examined alone, one might conclude that deleting the seventh intron was detrimental to TK gene expression (compare Δi7 to fl). However, the level of mRNA produced from the internal control (at 46 bases) was also very low in Δi7. Normalized using the internal control, the efficiency of mRNA production was about the same for Δi7 and fl. Table 5.2 shows the relative efficiency of mRNA production in four transformation series using the internal control gene to normalize expression. The efficiency of mRNA production by the intron deletion constructs varied less than two fold from that of the full length gene. No detrimental effect of intron deletion was evident.

On the basis of quantitative TK mRNA measurements, normalized per cell, per gene, or per internal control, introns were inconsequential to the expression of chicken TK mRNA in mouse L cells. Our results indicate that the biogenesis of stable TK mRNA is not dependent on passage through a RNA splicing pathway, as has been suggested for SV40 16S mRNA (Lai and Khoury, 1979; Gruss and Khoury, 1980; Hamer and Leder, 1979; Hamer *et al.*, 1979). Our results also suggest that TK gene expression is not dependent on transcriptional regulatory elements located within introns, as has been demonstrated for certain eucaryotic genes (Queen and Baltimore, 1983; Gillies *et al.*, 1983; Banerji *et al.*, 1983). In this regard, it should be mentioned that a set of three SP1 binding site consensi are located within introns 1 and 2 of the chicken TK gene (Merrill *et al.*, 1984). These sites are missing in intron deletion mutants Δi1-6, Δi1-2, and Δi1-7; and yet TK mRNA is generated efficiently in mouse L cells tranformed with these templates.

Our results conflict with earlier transformation analyses, which suggested an intron requirement for mouse DHFR and hamster TK expression (Lee *et al.*, 1981; Gasser *et al.*, 1982; Lewis, 1986). Although chicken TK may differ from rodent TK and DHFR with

respect to a role for introns in efficient mRNA formation (perhaps due to the great difference in intron size), we suspect that parameters other than mRNA-generating capacity may have affected the transformation efficiency in these earlier studies.

The expression of three widely divergent eucaryotic genes have now been shown to be independent of RNA splicing or intronic information: bean phaseolin (Chee *et al.*, 1986); yeast actin (Ng *et al.*, 1985); chicken TK (the present study). These three cases represent the only studies in which intron deletion mutants containing native 5' and 3' flanking sequences were used and the efficiency of mRNA production by mutant and wildtype cellular genes was directly determined. The studies represent a consensus, suggesting that introns are not generally important for efficient production, transport or stabilization of eucaryotic mRNA.

For bean phaseolin and yeast actin, intron-independent expression was demonstrated in transformed organisms. For chicken TK, intron-independent expression was demonstrated in transformed mouse L cells, an established cell line that has been propagated in culture for over 20 years. It is possible that introns are required for efficient gene expression in euploid mammalian cells, but that L cells have overcome this requirement during the process of immortalization or during prolonged adaptation to *in vitro* conditions. To answer this question, the functionality of intron deletion mutants must be analyzed in transgenic organisms, in finite cell lines or in established cell lines displaying properties more characteristic of cells *in vivo*.

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Fig. 5.1. Intron deletion mutants of chicken thymidine kinase gene. Hatched regions designate the protein coding domain; open bars, exons; solid bars, introns; the stippled region, an intron removed from the 3' nontranslated region during the biogenesis of rare messages in certain tissues. Letters represent restriction enzyme recognition sites: B, BamH1; R, EcoR1; X, Xho1; K, Kpn1; S, Sst1; P, Pst1; Bg, Bgl2; H, Hind3. The hexanucleotide aauaaa designates the polyadenylation signal. The figure is drawn to scale; in fl the distance between Bgl2 and Hind 3 is 772 bp. Asterisks signify non-unique restriction enzyme sites. All genes extend from a synthetic BamH1 linker 775bp upstream from the start codon to a synthetic EcoR1 site 2130 bp downstream from the stop codon.

Fig. 5.1

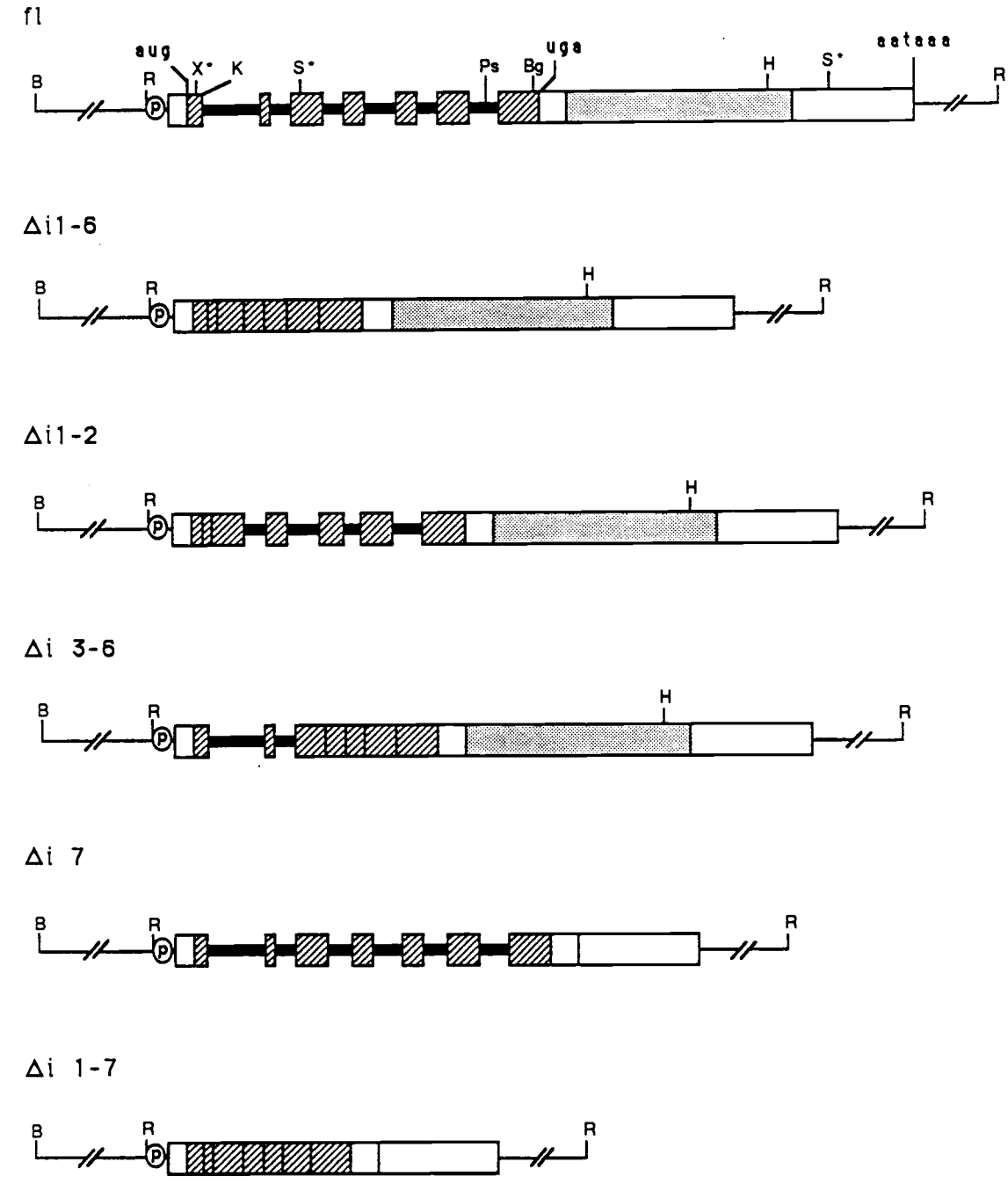
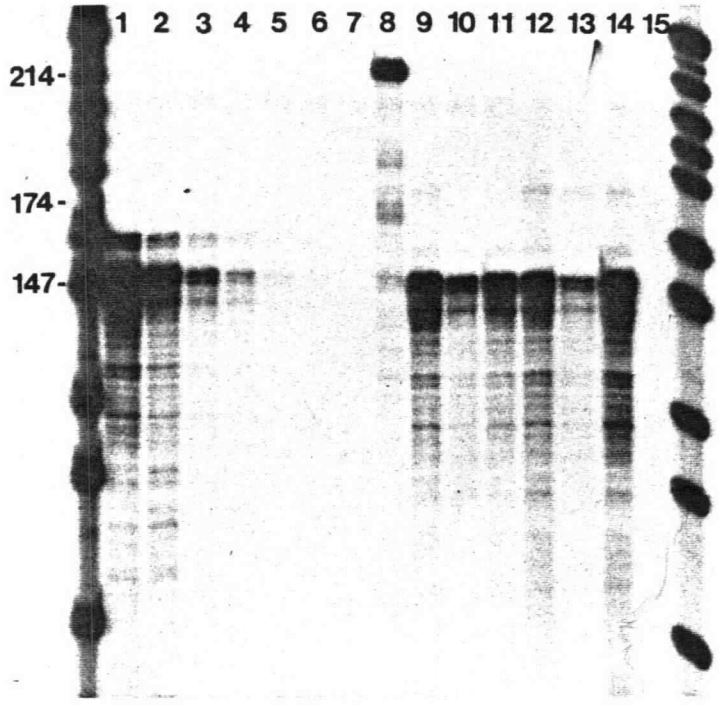


Fig. 5.2. Representative quantitation gel used to determine absolute levels of TK mRNA in mouse L cell cotransformants. Lanes 1-6, SP6-generated synthetic TK mRNA standards starting at 1.1 fmol and declining in half log intervals; Lane 7, control digestion using 10 μg yeast RNA; Lane 8, nondigested probe; Lanes 9-15, digestions using 10 μg of RNA from fl, Δi1-6, Δi3-6, Δi1-2, Δi1-7, Δi7, and pKNeo-only transformants, respectively. Markers are Msp1 digested pBR322. The diagram below illustrates the location and size of the probe and expected protected fragments: boxes indicate mRNA sequences; thick lines, intronic sequences; thin lines, plasmid sequences. The protein coding region is shaded.

Fig. 5.2



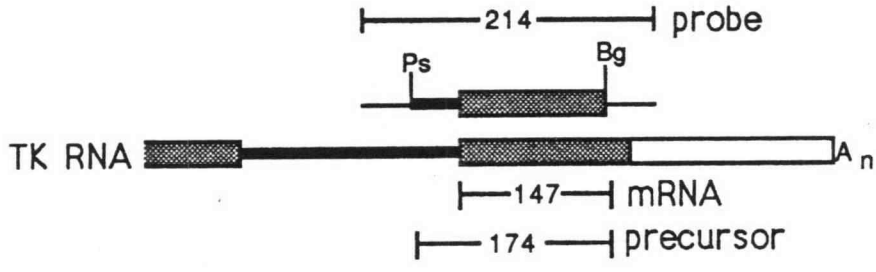


Fig. 5.3. Representative Southern blot used to determine TK gene structure and copy number in mouse L cell cotransformants. Lanes 1-3 contain 10, 3, and 1 μg of chicken liver DNA, respectively; Lanes 5-11 contain 1 μg of Hind3 and EcoR1 digested DNA from fl, Δi1-6, Δi1-2, Δi3-6, Δi7, Δi1-7 and pKNeo-only transformants, respectively. Markers are Hind3 digested lambda DNA. The blot was probed with a nick translated Kpn1/Bgl2 fragment of the chicken TK gene. Band intensities were compared by laser densitometry. Gene copy number per cell was calculated knowing the DNA content of mouse and chicken cells (10pg and 2.6pg, respectively), and assuming two TK genes per chicken cell.

Fig. 5.3

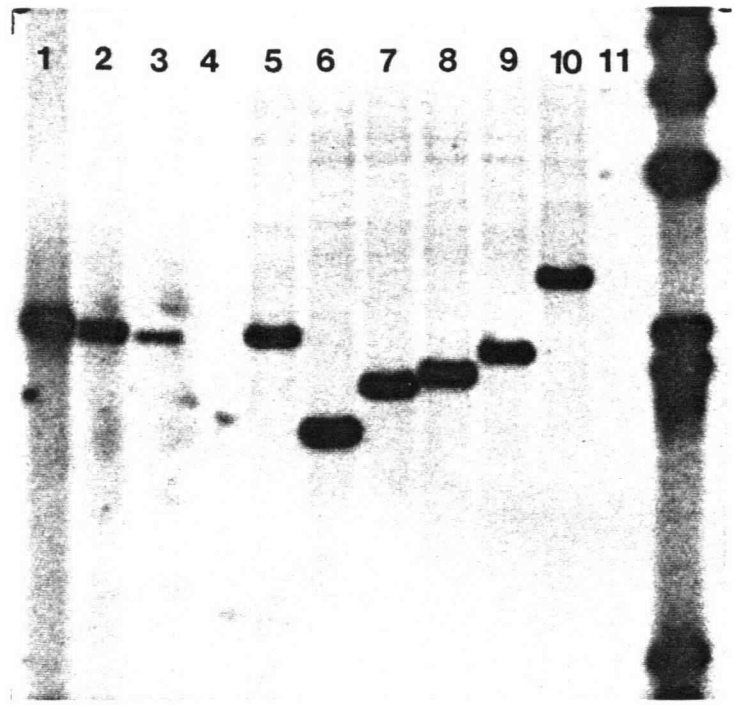


Fig. 5.4. Efficiency of TK mRNA production relative to a positive control gene in mouse L cell cotransformants. Using pKNeo as the selected gene, TK- L cells were cotransformed with equimolar amounts of each intron deletion mutant and an internal control gene, 2050Tx. The diagram below illustrates the RNase protection strategy for the internal control gene; boxes indicate mRNA sequences; thick lines, intronic sequences; thin lines, plasmid sequences. The protein coding region is shaded and Tx indicates the herpesvirus TK polyadenylation signals.

Fig. 5.4

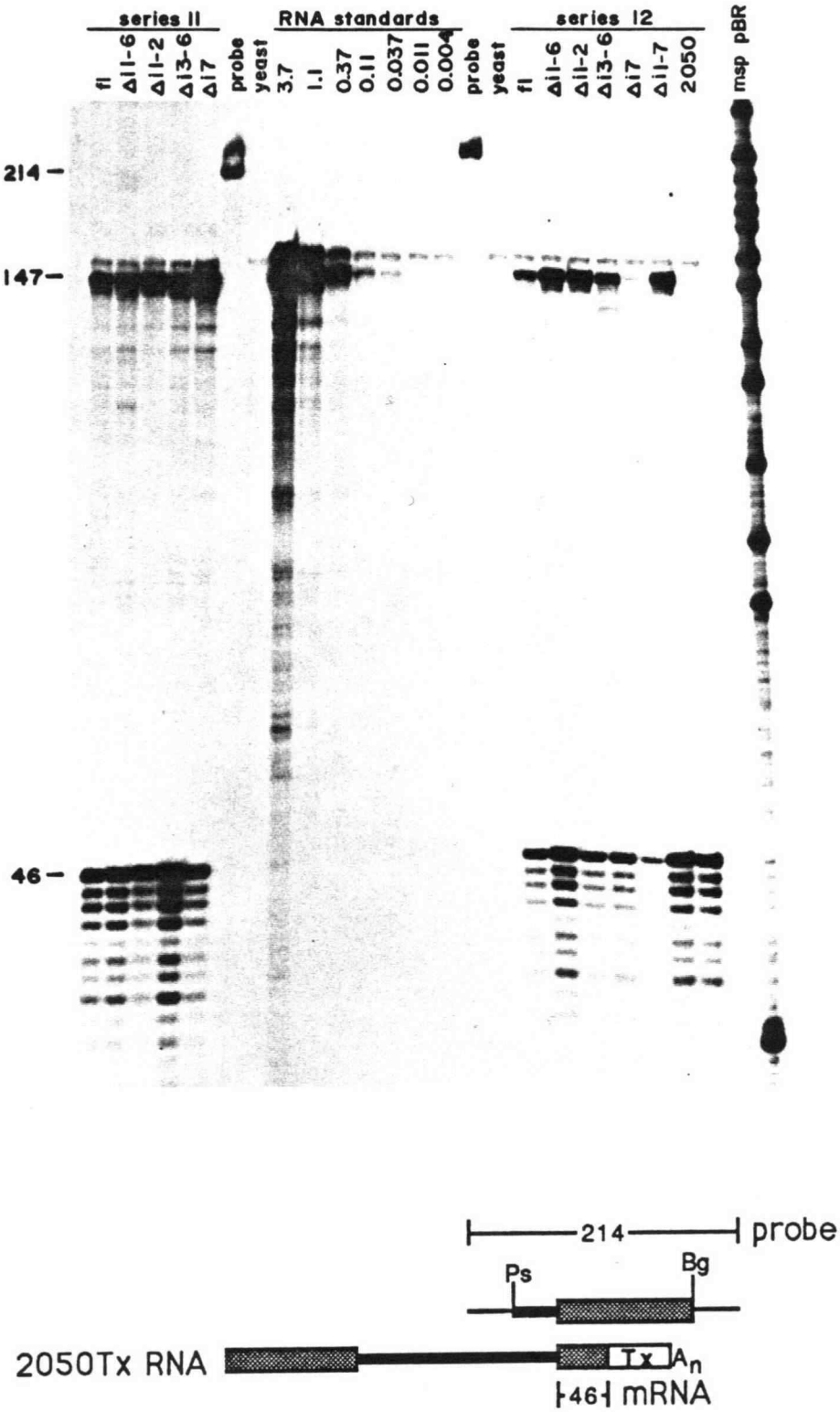


Table 5.1. Chicken TK mRNA and Gene Copy Number in Mouse L Cells Cotransformed With Intron Deletion Mutants[a]

Expression Parameter	Gene construct	Transformation Series						
		9	10	11	12a	12b	mean	± SD
TK mRNA/cell	fl	168	35	128	40	43	83	± 61
	Δi1-6	80	80	184	152	127	125	± 45
	Δi1-2	136	–	193	152	122	151	± 31
	Δi3-6	158	137	231	103	66	139	± 62
	Δi7	216	124	343	20	11	143	±140
	Δi1-7	59	134	–	205	103	125	± 61
TK genes/cell	fl	22	–	42	6	–	23	± 19
	Δi1-6	38	10	30	20	83	36	± 28
	Δi1-2	26	125	27	13	60	50	± 45
	Δi3-6	25	9	56	8	16	23	± 20
	Δi7	22	8	31	5	4	14	± 12
	Δi1-7	19	40	–	21	42	31	± 12
TK mRNA/gene	fl	7.6	–	3.0	6.7	–	5.8	± 2.4
	Δi1-6	2.1	8.0	6.1	7.6	1.5	5.1	± 3.1
	Δi1-2	5.2	–	7.1	11.7	2.0	6.5	± 4.0
	Δi3-6	6.3	15.2	4.1	12.9	4.1	8.5	± 5.2
	Δi7	9.8	15.5	11.0	4.0	2.8	8.6	± 5.2
	Δi1-7	3.1	3.4	–	9.8	2.5	4.7	± 3.4

[a] TK mRNA and gene copy levels were determined as described in Figs. 5.2 and 5.3, respectively.

Hyphens (--) indicate no data available.

Table 5.2. Efficiency of Chicken TK mRNA Production by Intron Deletion Mutants Relative To An Internal Control Gene

Gene Construct	Trans-formation Series	Expression of Mutant Gene[a]	Expression of Control Gene[b]	Efficiency of mRNA production by Mutant Gene[c]	
		(TKmRNA/cell)	(fl=1.00)	(fl=1.00)	(mean±SD)
fl	11	126.5			
	12a	40.6			
	12b	43.7			
	13	6.8			
Δi1-6	11	182.3	1.37	1.05	
	12a	152.1	9.67	0.39	
	12b	126.5	2.13	1.36	
	13	12.7	3.12	0.60	0.85±0.44
Δi1-2	11	194.2	1.08	1.42	
	12a	152.9	2.19	1.72	
	12b	121.9	1.00	2.79	
	13	72.3	11.05	0.97	1.73±0.77
Δi3-6	11	231.9	1.98	0.93	
	12a	105.2	4.45	0.58	
	12b	66.6	0.95	1.60	
	13	23.4	6.33	0.55	0.92±0.49
Δi7	11	343.4	0.84	3.23	
	12a	19.2	0.52	0.91	
	12b	11.9	0.32	0.85	
	13	55.6	5.01	1.64	1.66±1.10
Δi1-7	12a	207.3	10.48	0.49	
	12b	105.3	2.37	1.02	
	13	6.0	2.00	0.44	0.65±0.32
pKNeo	12a	ND	0.19	ND	
	12b	ND	2.06	ND	
	13	ND	1.70	ND	

[a] TK mRNA level per cell was determined by RNase protection as described in Fig.5.4.

[b] Efficiency of expression of 2050Tx internal control gene was determined by the intensity of the 46 base RNA signal (see Fig. 5.4); values are normalized to the 46 base signal in fl transformants.

[c] The efficiency of TK mRNA production by mutant genes was calculated by dividing the number of TK mRNAs/cell by the efficiency of expression of the positive control gene; values are normalized to the efficiency obtained with fl in each transformation series.

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APPENDICES

APPENDIX 1: Figures and Gels Illustrating Data in Table 2.1

Growth rate, TK activity, and TK mRNA levels in liver (Δ-Δ), heart (•--•), and ( ) during embryogenesis. (A) Growth rate of liver, heart, and brain were determined from smoothed DNA content plots as described in Fig. 2.3. (B) TK activity during development of liver, heart and brain were determined (see text, chapter 2). (C) TK mRNA levels in liver, heart, and brain were determined from the gels shown in D. (D) Quantitative RNase protection gels used to determine the TK mRNA levels in liver, heart, and brain plotted in C. Lane 1, end-labeled pBR322/Msp1 molecular weight markers; lanes 2-6, half-log increments of pseudo-mRNA between 0.01 and 1 fmol (liver and brain) and between 0.03 and 3 fmol (heart) mixed with 50 μg of yeast RNA; lane 7, an undigested control sample (containing probe, 3 fmol pseudo-mRNA, and 50 μg yeast RNA). Only a fraction of the control sample loaded on the gel (the whole sample was loaded in liver); lane 8, 50 μg of yeast RNA only; lanes 9-17, 50 μg of total RNA isolated from each organ at two day intervals from 8 days to 24 days postfertilization. The standards of each gel were used to determine the TK mRNA levels of samples on that gel.

Fig. A1.1

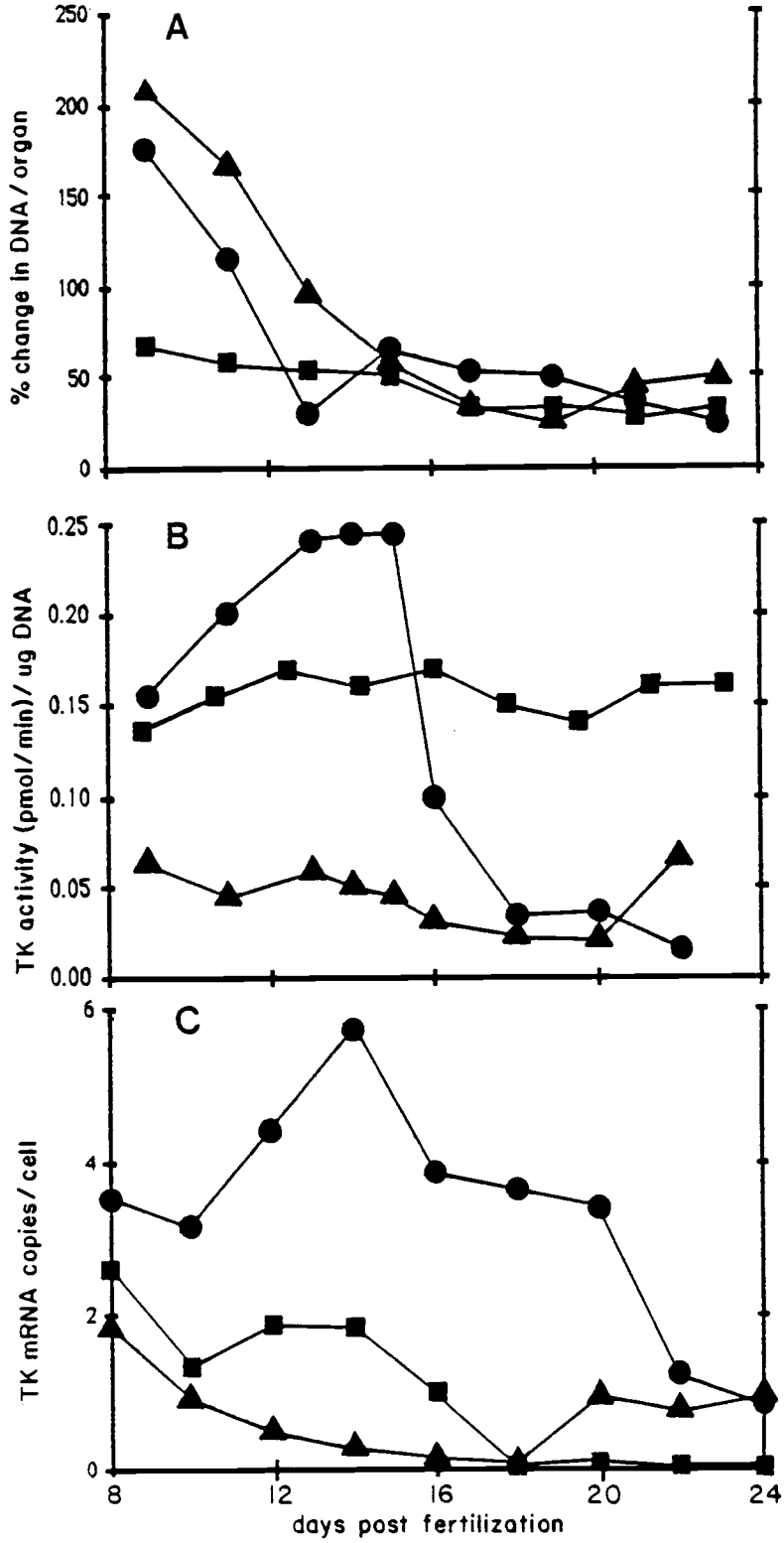
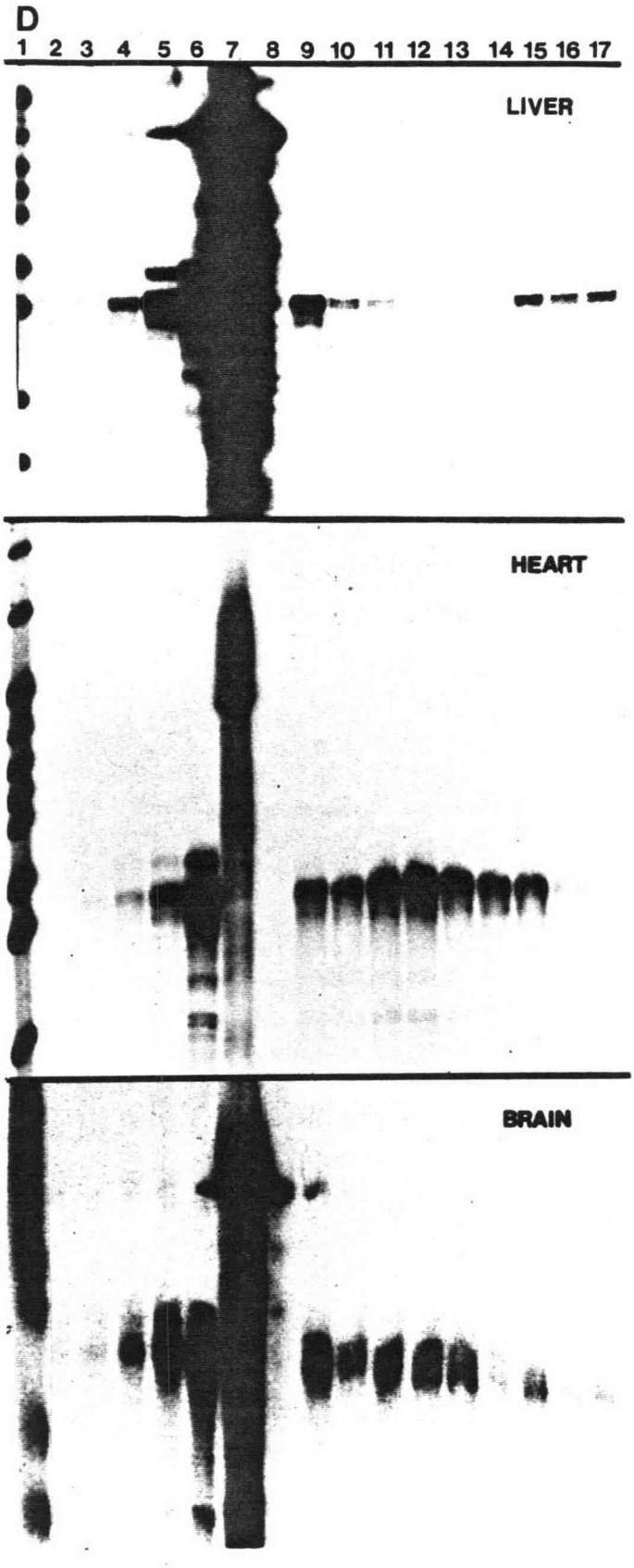

A
B
C
% change in DNA / organ
TK activity (pmol/min)/ ug DNA
TK mRNA copies / cell
250
200
150
100
50
0
0.25
0.20
0.15
0.10
0.05
0.00
6
4
2
0
8
12
16
20
24
days post fertilization

Fig. A1.1



APPENDIX 2: TK mRNA and Activity Regulation Measured in Parallel

This appendix is a compilation of data from all experiments on myoblast transformants in which TK mRNA regulation and TK activity regulation were measured in parallel. A subset of this data was used in chapter 3 to show TK activity regulation was independent of mRNA regulation (those experiments in which proliferative TK activities were 0.5 or greater). "Experiment" numbers correspond to my laboratory notebooks. Below MG experiment numbers are given the GJ experiment numbers (Dr. Merrill's notebooks) in which transformants were made. "Cell" indicates whether the experiment was done with H$^-$alpha (H$^-$) or TK$^-$ epsilon (TK$^-$) myoblast transformants. "Gene" indicates the gene(s) transformed into the myoblast line. Transformation ratio with pKNeo, pool number, sample number, or clone are indicated in brackets below the gene name to help colate information with notebooks. "P/C" indicates proliferative (P) or committed (C) myoblast samples. "Δ" indicates the fold change between P and C values.

Experiment	Host Cell	Gene(s)	P/C	TK mRNA	Δ	TK Activity	Δ
				molecules / cell	fold	pmol / min·μgDNA	fold
MG11-3-1	TK$^-$	cfl (11A)	P	5.6	3.7	0.51	85
			C	1.5		0.006	
		Δi1-6	P	15	2.2	0.59	~74
			C	6.8		~0.008	
		Vp5'Δ2177	P	5.9	-	0.24	3.4
			C	N.D.		0.07	
		Δ3'734Tx	P	29	1.7	3.2	7.8
			C	18		0.41	
MG11-3-2	TK$^-$	cfl (11A)	P	19	2.6	0.16	2.7
			C	7.5		0.06	
		Vp5'Δ2177	P	17	2.4	0.09	11
			C	7.1		~0.008	

		Δi1-6	P	27	1.4	0.39	49
			C	19		~0.008	
		Δ3'734Tx	P	243	2.9	2.4	20
			C	84		0.12	
MG11-3-5 GJ169	H-	cfI	P	339	8.1	7.8	13
			C	42		0.6	
		Δi1-7	P	22	1.1	2.2	110
			C	20		0.02	
MG11-4-2	TK-	cfI (11A)	P	37	1.5	0.35	14
			C	24		0.025	
MG14-1-2	H-	cfI : Neo (200:1)	P	409	2.6	1.8	75
			C	159		0.024	
MG15-1-1 GJ110 -17-11	H-	cfI : Neo (3:1)	P	128	1.6	3.0	48
			C	80		0.062	
		cfI : Neo (10:1)	P	145	1.6	3.8	42
			C	88		0.090	
		cfI : Neo (20:1)	P	262	2.0	3.4	25
			C	133		0.14	
		cfI : Neo (67:1)	P	199	1.4	5.0	27
			C	139		0.19	
		cfI (200:1)	P	466	2.8	17	55
			C	169		0.31	
MG15-2-1	H-	cfI (100:1)	P	152	3.3	4.7	70
			C	30		0.067	
MG16-18-1	H-	cfI : Neo (200:1)	P	157	3.3	1.0	18
			C	48		0.055	

MG16-18-3 GJ204	TK-	cfl (1)	P	200	1.6	0.89	23
			C	123		0.039	
MG25-1-3 GJ173	TK-	cfl Δ3'872Tx	P	136	2.4	1.3	1.2
			C	57		1.1	
		Δi1-6 Δ3'872Tx	P	5	1.3	0.47	39
			C	4		0.012	
		Δi1-2 Δ3'872Tx	P	105	2.2	0.91	1.3
			C	47		0.69	
		Δi3-6 Δ3'872Tx	P	37	1.1	1.1	1.4
			C	34		0.75	
		Δi7 Δ3'872Tx	P	179	1.8	1.5	1.8
			C	97		0.84	
	H-	Vp5'Δ2112	P	8	1.6	1.3	18
			C	5		0.075	
MG25-1-11 GJ210	TK-	cfl Δ3'872Tx (1)	P	131	3.7	1.4	56
			C	35		0.025	
		cfl Δ3'872Tx (3)	P	197	4.4	1.9	38
			C	45		0.050	
		cfl Δ3'872Tx (7)	P	116	2.1	1.4	16
			C	56		0.084	
		Δi1-6 Δ3'872Tx (2)	P	68	2.3	0.36	3.0
			C	29		0.12	
		Δi1-6 Δ3'872Tx (4)	P	44	1.6	0.38	127
			C	28		0.003	
		Δi1-6 Δ3'872Tx (8)	P	58	3.4	0.44	24
			C	17		0.018	

MG25-1-11 GJ204	TK-	cfl (1)	P	410	2.3	1.2	12
			C	176		0.096	
		cfl (3)	P	313	1.2	1.2	24
			C	266		0.051	
		cfl (5)	P	274	1.5	0.96	4.0
			C	184		0.24	
		Δi1-6 (2)	P	231	1.6	0.51	64
			C	146		~0.008	
		Δi1-6 (4)	P	86	1.8	0.39	2.8
			C	47		0.14	
		Δi1-6 (6)	P	171	1.4	0.90	33
			C	124		0.027	
MG25-1-13 GJ173	TK-	cfl Δ3'872Tx	P	145	3.4	0.30	38
			C	43		~0.008	
		Δi1-6 Δ3'872Tx	P	17	3.2	0.061	7.6
			C	5.4		~0.008	
		Δi1-7 Δ3'872Tx	P	47	2.1	0.10	13
			C	22		~0.008	
MG25-1-14 GJ169	TK-	Δi1-7	P	6.0	2.5	0.036	4.5
			C	2.4		~0.008	
	H-	Δi1-7	P	28	1.2	2.7	33
			C	23		0.083	

APPENDIX 3: MT-TK Regulation

Tabulatated below are regulation data from mouse myoblasts transformed with a metallothionein promoter/chicken TK structural gene fusion (MT-TK). Proliferative (P) or committed (C) myoblasts transformed with Mp5'Δ2177 were induced with 60 μM $ZnSO_4$ for indicated number of hours (suffix after P or C). The uninduced values were used in chapter 4 to demonstrate TK mRNA and activity regulation with a heterologous promoter. The first three experiments shown were done in a transformant line which also contained Δ3'872Tx as an internal control gene. Internal control (Control) mRNA levels were used to compute a "factor"by which test message levels were adjusted. TK activity levels were measured in parallel and were used to compute the efficiency of TK activity production on a per message basis. The first three experiments were done with an H^- alpha transformant pool and the last experiment was done with a TK - epsilon transformant pool.

Experiment	Time	TK mRNA	Control mRNA	Factor	Adjusted TK mRNA	TK Activity	Activity mRNA
		molecules cell			molecules cell	pmol/min μg DNA	
MG23-1-1							
	P0	62	26.5	1	62	6.5	104
	P2	252	37.0	0.72	181	5.8	32
	P4	158	6.4	4.1	648	2.3	4
	P6	163	14.1	1.9	310	3.3	11
	C0	18	5.8	1	18	0.04	2.2
	C2	170	12.2	0.48	82	0.16	2.0
	C4	273	12.8	0.45	123	0.84	6.8
	C6	336	18.7	0.31	104	0.57	5.5
MG23-2-1							
	P0	179	22.6	1	179	0.83	4.6
	P4	506	30.0	0.75	380	0.64	1.7
	P9	322	38.7	0.58	187	0.045	0.2

	P14	188	40.3	0.56	105	2.9	27.6
	P24	217	26.5	0.86	187	2.0	10.7
	C0	113	5.6	1	113	0.033	0.3
	C4	159	12.8	0.44	78	0.137	1.8
	C9	142	17.7	0.32	45	0.058	1.3
	C14	95	8.2	0.68	65	0.063	1.0
	C24	83	4.1	1.37	114	0.090	0.8
MG23-2-2							
	P0	77	73	1	77	10.1	130
	P3	118	154	0.47	72	10.7	149
	P6	-	124	0.59	-	11.8	-
	P12	83	90	0.81	67	13.1	196
	P21	53	100	0.73	39	7.5	192
	C0	20	40	1	20	0.50	25
	C4	181	113	0.35	63	0.39	25
	C6	369	132	0.30	111	0.65	6
	C13	675	374	0.11	74	8.65	117
	C21	713	278	0.14	100	16.70	167
MG23-3-1							
	P0	1000				0.094	0.1
	P4	3900				0.885	0.2
	C0	700				0.059	0.1
	C4	5100				0.708	0.1

APPENDIX 4: Supplementary Data For Chapter 5

TRANSFORMATION EFFICIENCY: Effect of intron removal on the transformation to HAT resistance. TK$^-$ mouse L cells were transformed with intron deletion mutants shown in Fig.5.1. The amount of linearized, TK-containing plasmid added to each transformation varied from 0.2 pmol (about 1 μg) in series 5 to 1.0 pmol (about 5 μg) in series 6 and 7. Cultures were placed in HAT medium at 48 hr after DNA. Cultures were fixed and stained at 14 days after DNA, and colonies greater than 16 cells scored with the aid of a dissecting microscope. ND means not determined.

TK ACTIVITY: TK activity in G418-resistant cell lines cotransformed with intron deletion mutants and pKNeo. TK activity was measured in five transformation series in exponentially growing tranformants. Series 12a and b are different passages of the same transformation. Asterisk indicates a sample omitted due to later Southern blot showing aberrant restriction fragment.

TK mRNA per CELL: Graphic representation of data in Table 5.1. TK mRNA levels in mouse L cells cotransformed with intron deletion mutants and pKNeo. Absolute TK mRNA levels in four independent transformation series. Series 12a and b are different passages of the same transformation series. ND indicates no data.

GENE COPY NUMBER: Graphic representation of data in Table 5.1. TK gene copy number in mouse L cells cotransformed with intron deletion mutants and pKNeo. Histogram showing gene copy numbers for the same four transformation series as in 'TK mRNAper Cell'. Series 12a and b indicate different passages of the same transformation. ND indicates no data.

TK mRNA PER GENE: Graphic representation of data in Table 5.1. TK mRNA per gene in mouse L cells cotransformed with intron deletion mutants and pKNeo. The histograms show the results of dividing the 'TK mRNA per Cell ' values in by the 'Gene Copy Number' values. Mean gives the average value for each construct in the other five sets of histograms.

Fig. A3.1

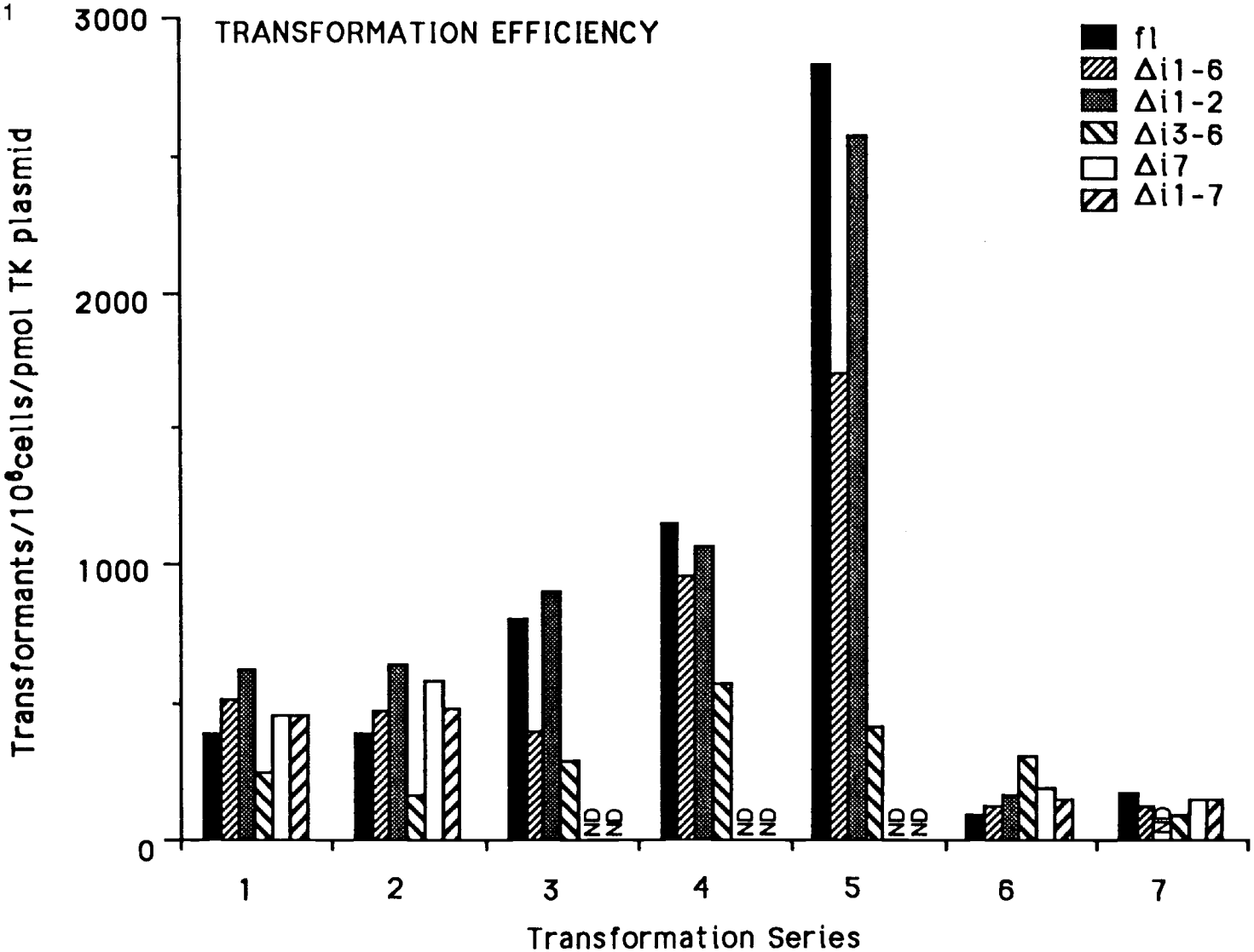

TRANSFORMATION EFFICIENCY
Transformants/10⁶cells/pmol TK plasmid
3000
2000
1000
0
1
2
3
4
5
6
7
Transformation Series
fl
Δi1-6
Δi1-2
Δi3-6
Δi7
Δi1-7

Fig. A3.2

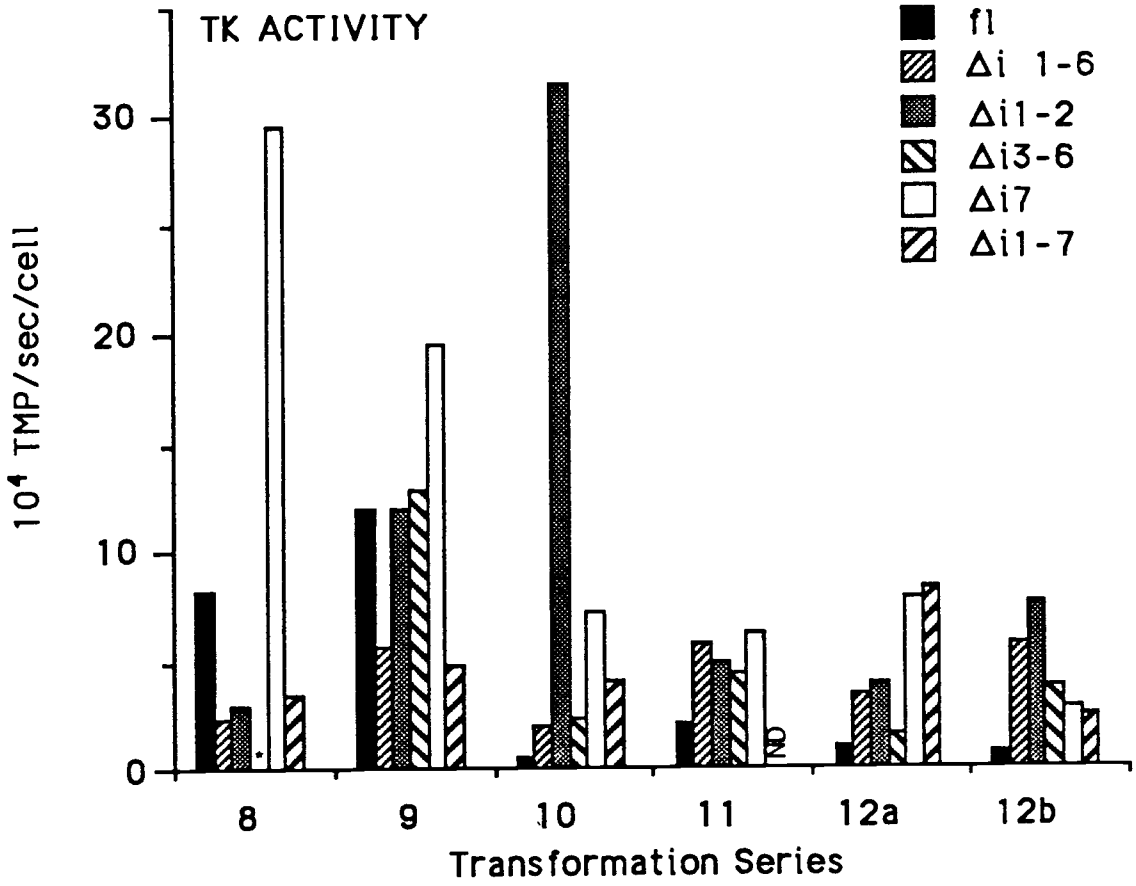

TK ACTIVITY
fl
Δi 1-6
Δi1-2
Δi3-6
Δi7
Δi1-7
10^4 TMP/sec/cell
30
20
10
0
ND
8
9
10
11
12a
12b
Transformation Series

Fig. A3.3

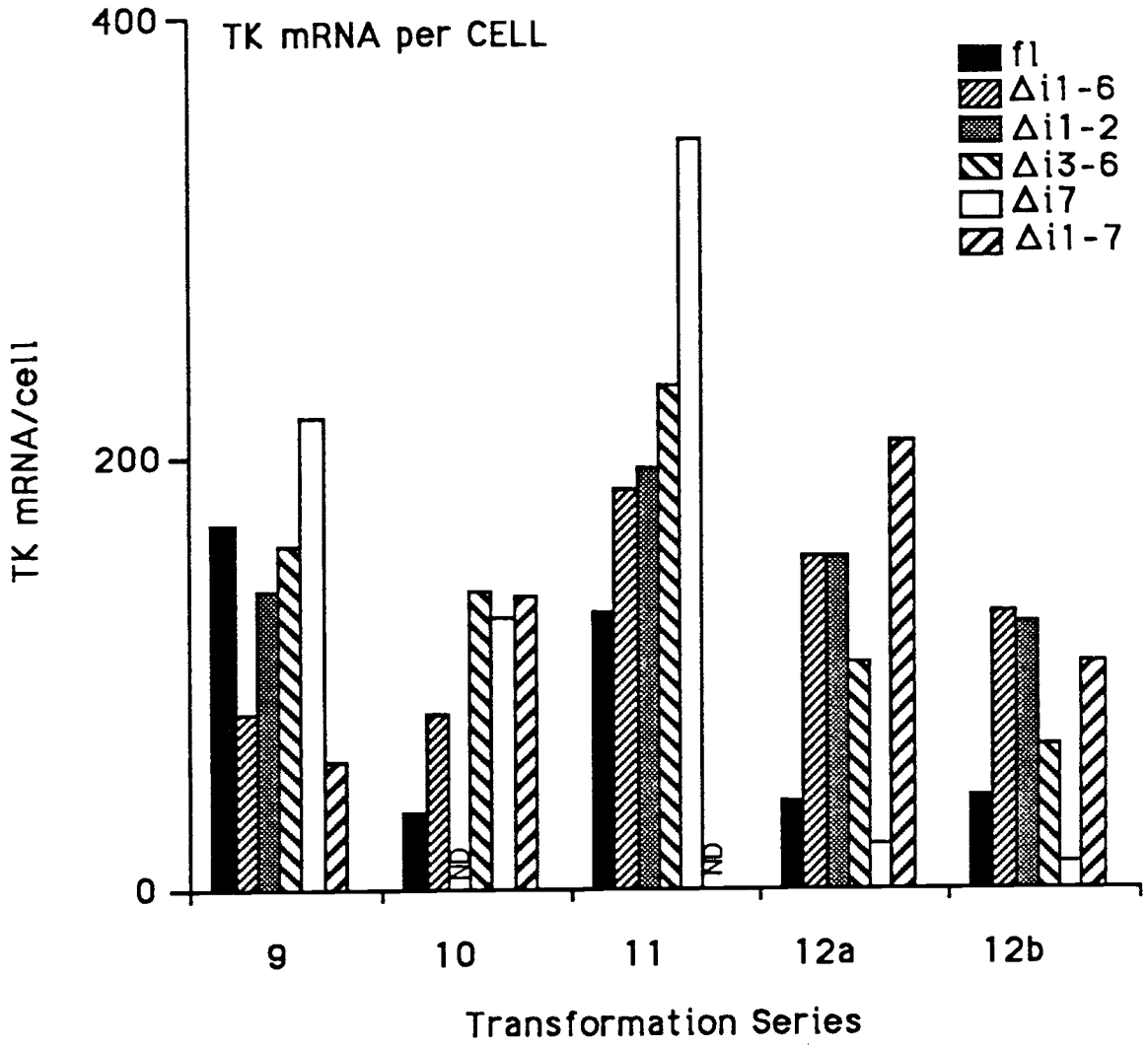

TK mRNA per CELL
fl
Δi1-6
Δi1-2
Δi3-6
Δi7
Δi1-7
TK mRNA/cell
400
200
0
ND
ND
9
10
11
12a
12b
Transformation Series

Fig. A3.4

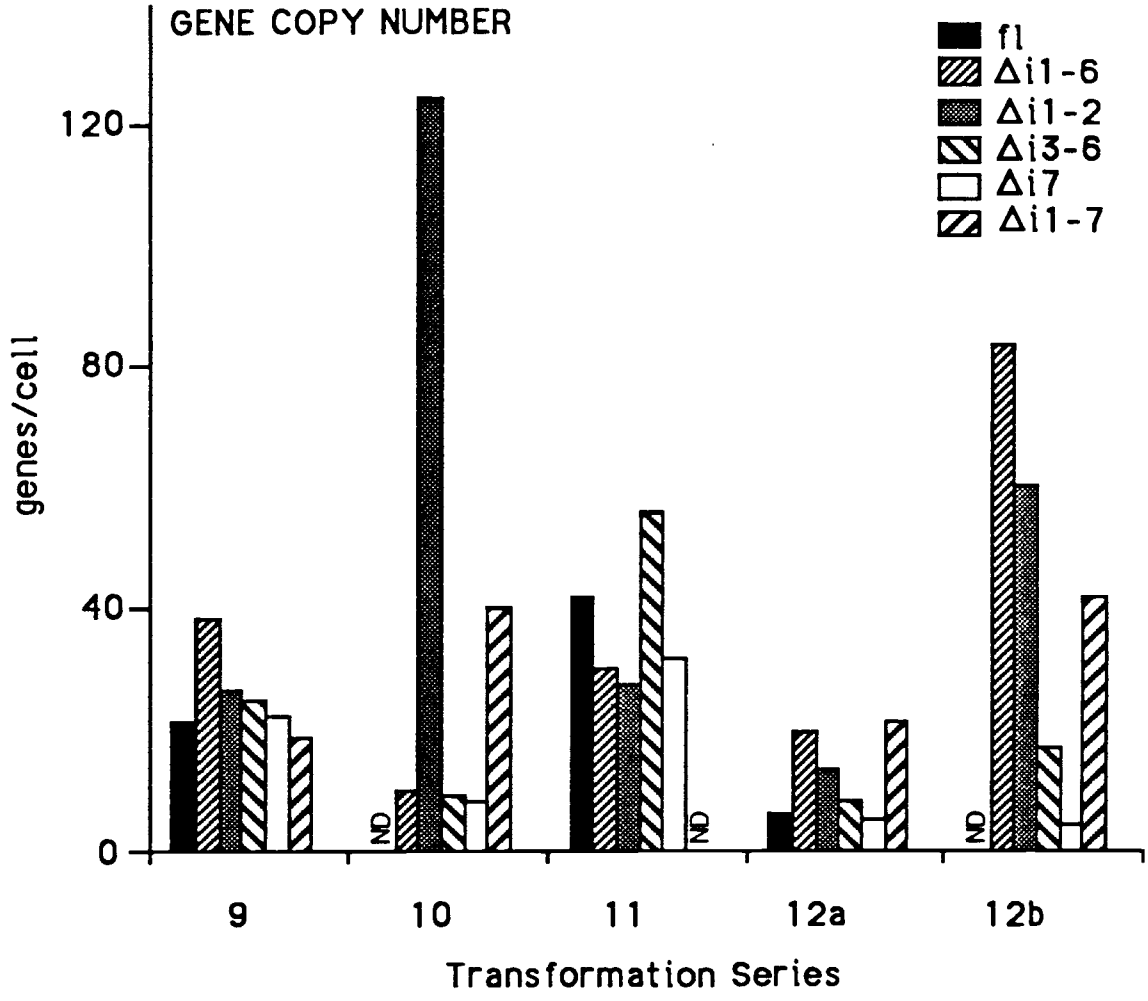
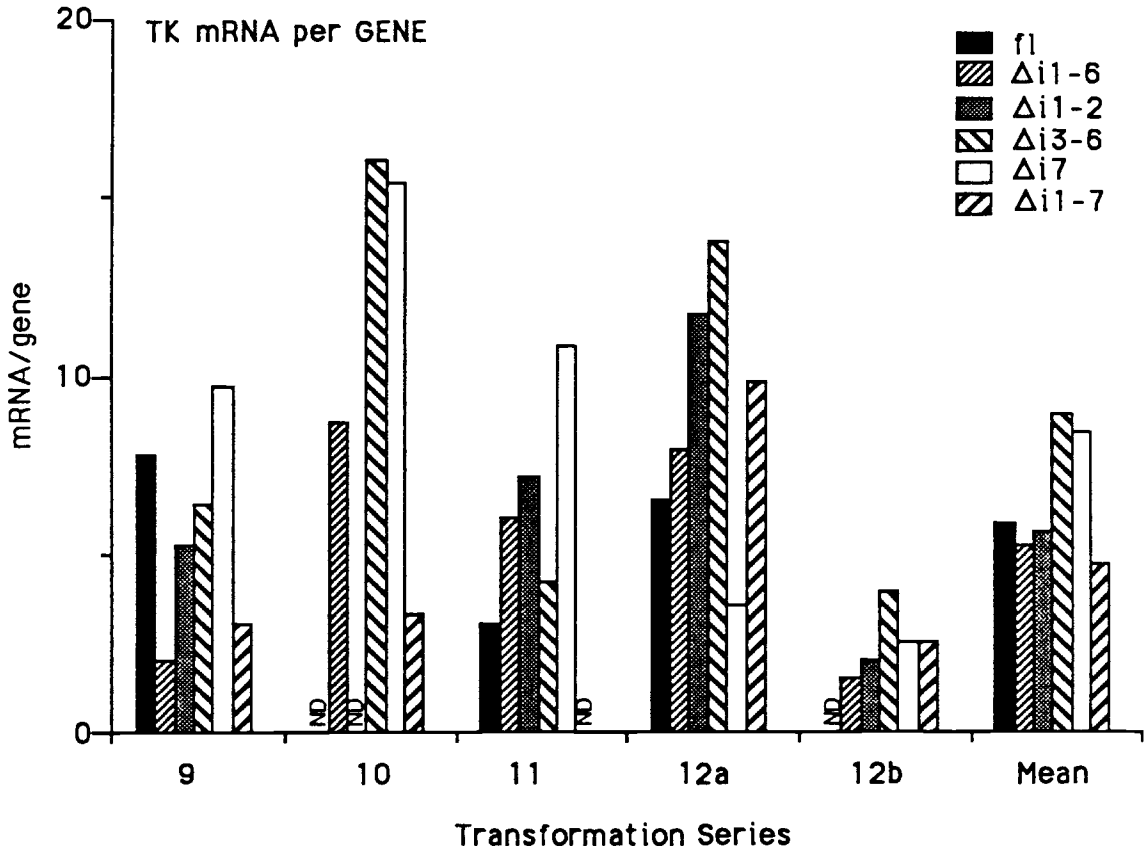

GENE COPY NUMBER
fl
Δi1-6
Δi1-2
Δi3-6
Δi7
Δi1-7
120
80
40
0
genes/cell
ND
ND
ND
9
10
11
12a
12b
Transformation Series

Fig. A3.5


TK mRNA per GENE
fl
Δi1-6
Δi1-2
Δi3-6
Δi7
Δi1-7
mRNA/gene
20
10
0
ND
ND
ND
9
10
11
12a
12b
Mean
Transformation Series

APPENDIX 5: *In Vitro* RNA Synthesis.

A. Template Preparation

1) Linearize 2-20 µg of transcription vector with appropriate enzyme for 2 hours.

- Certify complete digestion by minigel.
- 5' overhangs are best, blunts are marginal, and 3' overhangs are bad linearization sites. 3' overhangs allow polymerase to turn around and synthesize back on the non-coding strand.

2) Add GDW to 100 µl, 1/20 volume 5 M NaCl (RNA grade), 1/2 volume GDW-saturated phenol, vortex 1'; Add 1/2 volume chloroform (24:1, $CHCl_3$: Isoamyl alcohol), vortex 1', microfuge 3'.

3) Remove aqueous phase to fresh tube, extract with 1 volume of chloroform, and microfuge 1'.

4) Remove aqueous to fresh tube, add 3 volumes of 100% ethanol (stored at -20°C). Precipitate at -20°C overnight or at -70°C for 2 hours.

5) Do the following on ice. Microfuge 15' in cold room, remove supernatant with drawn out pasteur pipette, wash pellet with 200 µl of ice cold 70% ethanol, microfuge 5-15' in cold room, remove supernatant with drawn out pipette, and resuspend the pellet to 1 µg/µl in GDW (RNA grade).

B. Transcription Reactions

1) Assemble a transcription reaction in an eppendorf tube at room temperature.

a) Hot Reaction

- For generating high specific activity probes for use in southern or northern blots.
- This method limits the chemical concentration of the radioactive nucleotide, hence shortstops are frequent and full length transcripts are not made.

Conditions	Stock	Recipe
0.4-2 µg linearized vector	1 µg	1 µl
1x **Transcription Buffer**	**5x**	5µl
10 mM DTT	0.5 M DTT	0.5 µl
1.6 U/µl RNasin	40 U/µl	1 µl
0.5 mM rGTP, rCTP, rATP	2.5 mM each	5 µl
25-50 µCi [^{32}P]rUTP	10 µCi/µl	up to 11.5 µl
no cold rUTP	GDW	Q.S. to 24 µl
Polymerase (SP6 or T7)	20 U/µl	1µl

b) Warm Reaction

- Generates fairly high specific activity probes for use in RNase protection experiments.
- A moderate chemical concentration of the limiting nucleotide allows probes of several hundred bases to be synthesized to full length, but is low enough to allow a reasonable specific activity to be attained.
- The amount of nonradioactive rUTP in the recipe below can be lowered for probes shorter than 100 bases and raised for probes longer than 400 bases.
- The recipe shown has been used with successin numerous syntheses of a 210 base chicken TK probe.

Conditions	Stock	Recipe
2 µg linearized vector	1 µg/ µl	2 µl
1x **Transcription Buffer**	**5x**	4 µl
10 mM DTT	0.5 M DTT	0.4 µl
1.4 U/µl RNasin	40 U/µl	0.7 µl
0.5 mM rGTP, rCTP, rATP	2.5 mM each (-70°C)	4 µl
45 µM rUTP (cold)	300 µM	3 µl
50 µCi [^{32}P]rUTP	10 µCi/µl	5 µl
Polymerase (SP6 or T7)	20 U/µl	1µl

c) Cold reaction

- Used to generate up to 20 µg of a specific synthetic RNA that is not radioactive, therefore allowing spectrophotometric (A_{260}) quantitation as required for the standard curve of an RNase protection assay.
- RNA can be checked for size on a native 1x MOPS/1% agarose minigel (see MG 16-9-1 for example).
- RNA made by this method has been used successfully for in vitro translations (see MG 16-5-4).

Conditions	Stock	Recipe
2-4 µg linearized vector	1 µg/ µl	2-4 µl
1x **Transcription Buffer**	**5x**	10 µl
10 mM DTT	0.5 M DTT	1 µl
1.4 U/µl RNasin	40 U/µl	2 µl
0.5 mM rGTP, rCTP, rATP, rUTP	2.5 mM each (-70°C)	10 µl
	GDW	Q.S. to 48 µl
Polymerase (SP6 or T7)	20 U/µl	2 µl

2) Incubate transcription reactions at 40°C for 1 hour.

3) Add: 1 µl RNasin (promega 40 U/ ul); 2.5 µl tRNA (10 mg/ml) [omit for Cold transcription reaction]; 1 µl RNase free DNase (Promega 1U/µl).

4) Incubate 15' at 37°C. [Pour column for below]

5) Remove 1 or 2 µl to scintillation vial and save. [input counts]

6) Load the remaining sample on a 10 ml [use disposable pipette cut off at -2 ml and plugged with glass wool] G-50 (Fine)/**1xTES** column that has no buffer over the gel bed. Rinse the tube 2 times with 150 µl **1xTES** and load the washes on the column. Wait for washes to enter gel bed, then add 3 ml of **1xTES** to effect a seperation of two peaks (if radioactive, use minimonitor to follow them). The first peak (incorporated counts) is collected with an additional 1 ml of **1xTES** added to column. Collect sample in a 12.5 ml polypropylene tube resistant to chloroform. After the first peak has been collected, the second peak (unincorporated counts)should be near the 6 ml mark on the pipette. Dispose of column to radioactive waste.

7) Record volume of collected fraction (usually about 1-1.2 ml) and remove 5 µl to a scintillation vial.

[incorporated counts]

8) Add 1/20 volume 5M NaCl, add 1/2 volume GDW saturated phenol, vortex 1', add 1/2 volume chloroform, vortex 1', centrifuge 5' at maximum in tabletop centrifuge.

9) Remove aqueous layer (top) to fresh tube, add 1 volume chloroform, vortex 1', centrifuge 1' at maximum.

10) Remove aqueous layer (top) to 3 eppendorf tubes in 400 µl aliquots. To each tube add 1 ml of 100% ethanol, mix, and precipitate at -70°C at least 1 hour.

11) Centrifuge samples for 15' at 4°C in microfuge, remove supernatant with drawn out pasteur pipette, rinse each pellet with 200 µl ice cold 70% ethanol, centrifuge 5-15' at 4°C, remove supernatant as above.

12) Resuspend pellets in GDW. [All three pellets of warm probe are usually resuspended in a total of 200 µl] Store frozen.

C. Calculation of moles of RNA synthesized:

1) Compute % incorporation (% inc) from Cerenkoff counts taken in steps 5 and 7.

2) Compute the pmol of UTP (hot plus cold) added to the transcription reaction. [NEN [^{32}P]UTP usually comes at 13.2 pmol/µl]

3) pmol probe = (pmol input UTP(% inc/100)(4 NTP/UTP))/probe length

5xTranscription Buffer
0.2 M Tris pH 7.5
30 mM $MgCl_2$
10 mM spermidine

1xTES
10 mM Tris pH 7.5
5 mM EDTA
1 % SDS

APPENDIX 6: RNase Quantitation Assay

1) Mix test RNA (1-100 µg) with probe in 1.5 ml eppendorf tube to give roughly 1 fmol probe/µg total RNA (approximate molar ratio of 1 specific mRNA : 10 probe molecules).

- All pipetted volumes should be 5 µl or greater.
- Remember to include:

  a) Standards: Six standard lanes (3-0.01 fmol range is used typically for 10-50 µg of transformant total RNA). Standard dilutions are used so that one pipettes the same volume for each.

  b) No protecting fragment control: Same amount of total RNA as in test samples but the RNA used lacks the mRNA one is quantitating in test samples. Yeast RNA is often used. Control RNA is mixed with probe and treated identically to test samples.

  c) No digest control: Use mRNA containing total RNA or yeast RNA mixed with pseudo-mRNA. Control RNA is mixed with probe and treated identically to test samples except that RNAse is omitted in step 7.

2) Cover tubes with parafilm and use a dissecting needle heated in a bunsen burner to puncture approx. 6-8 small holes in the parafilm.

3) Place tubes in a "floater" foam rack in a lyophilization jar, cover the mouth of the jar with parafilm, make several small incisions in the parafilm with a razor blade, and freeze at -70°C (takes 15-30 minutes). [lyophilizer lid cracks if it freezes]

4) Quickly remove parafilm from mouth of jar, put lyophilizer cap on, and attach to lyophilizer. Keep under vacuum until samples are dry (50 millitorr/-150°C for 30 minutes).

-Do not allow samples to melt.

-If vacuum is poor, use more time (I have dried samples overnight by accident and the assay still worked).

-If volume of a sample is over 50 µl, use more time (I typically dry 1-3 hours in such a case).

-Steps 2-4 could be potentially replaced by a speed-vac drying step.

5) Remove samples from vacuum and immediately add 30 µl of Hybridization Master Mix (**HybMM**) through the holes in the parafilm. Remove parafilm with preflamed, cooled forceps, cap, vortex, briefly µfuge, and put in 55°C waterbath.

- Bring **HybMM** to lyophilizer

- If not done immediately the samples may rehydrate somewhat.

- **Hyb MM** (make fresh just before use)

Conditions	stock solution	recipe for 36 samples
80% deionized formamide	100%	864.0 µl
0.4 M NaCl	5 M	86.4 µl
1 mM EDTA	30 mM	36.0 µl
40 mM PIPES pH 7.0	1 M	43.2 µl
	GDW (DEP treated is OK)	50.4 µl

6) Hybridize at 55°C for 15-24 hours (optimal hybridization temperature should be determined for each probe).

7) Add 300 µl **RNase Mix** to each sample, cap tubes, flick several times to spread RNase to all surfaces inside the tube, place at 37°C for 1 hour.

- Add only **RDB** (no RNase) to no-digest control.

(Optimal digestion temperature/time should be determined for each probe)

-**RNase Mix** (make fresh just before use)

15 µl RNase T1 (1 mg/ml in GDW)

30 µl RNase A (10 mg/ml in 0.25 M Tris pH7.5)

7.5 ml RNase Digestion Buffer (**RDB**)

-**RDB** (100 ml of stock solution stored at room temperature)

10 mM Tris pH7.5

5 mM EDTA pH8.0

0.3 M NaCl

(Pour gel during RNase step because it needs to be pre-run overnight.)

8) Add 12.5 µl of Proteinase K/SDS (**PK/SDS**), flick tube hard 5 times to distribute PK/SDS to all surfaces, and incubate 15 min at 37°C.

-**PK/SDS** (make several minutes before use; becomes a turbid suspension which is mixed and quickly pipetted into the samples and controls)

stock	1 sample	30 samples
10% SDS	10 µl	300 µl
20 mg/ml PK (-20°C)	2.5 µl	75 µl

-During PK digestion, label two sets of tubes for extractions below and get aliquots of phenol, chloroform, and 10 mg/ml tRNA stock.

9) Microfuge samples 30" (to remove SDS from lip of tubes and prevent subsequent leakage). Add 200 µl phenol (no salt needed); Vortex 30"-1'; Quickspin samples; Add 2 µl 10 mg/ml tRNA; Add 200 µl $CHCl_3$; Vortex 30"-1'; spin 3-5' in µfuge.

10) Remove aqueous (top) phase carefully with a pipetman (2x200µl) to a fresh tube.

11) Add 400 µl $CHCl_3$; vortex 30"-1'; µfuge 1'.

12) Remove aqueous phase (top) to a fresh tube as in step 10.

13) Add 1 ml of 100% ethanol, cap, invert several times, and precipitate at -20°C until ready to run gel (typically overnight).

- Precipitation at -70°C for several hours is acceptable but may cause more salt to precipitate, potentially leading to salt effect on denaturing sequencing gels.

[Start pre-warming gel (see below) and then do the steps below.]

14) Spin down samples 15' in fixed angle µfuge at 4°C.

15) Immediately after run place in ice rack (crushed ice covered with aluminum foil, with holes punched in it for samples). This prevents pellets from sliding.

16) Remove supernatant (SN) with baked, drawn out pasteur pipette.

-For speed, I usually remove all SN except approx. 200 µl with a pasteur pipette that hasn't been drawn out(for all samples). I then go through the samples again and get the last 200 µl of each SN with a drawn out pipettte. This method is good because it allows tube walls to drain before the final SN removal, and allows use of a very small orifice on the drawn out pipette.

- The object is to keep the pellet compacted on the side of the tube.

17) Add 200 µl of ice cold 70% ethanol by running it down the sides of the tube. Flick the tube gently about 5 times to wash walls.

18) Spin 15' in fixed angle µfuge at 4°C; place immediately in ice rack; remove SN with drawn out pipette.

19) Dry pellet at 55-65°C for 3-5' (cap open); add 2 µl GDW and flick tube to get drop onto pellet; dissolve at 55-65°C for 3-5' (cap open).

20) Add 8 µl deionized formamide plus dyes (**DF+dyes**), close cap, incubate at 65°C for 15' briefly vortexing every 5'.

- **DF + dyes** (make less than 1 hour before use)
  - 980 µl deionized formamide (aliquot at -20°C)
  - 10 µl 1% bromopheol blue
  - 10 µl 1% xylene cylanol

21) Load 5 µl (half) of sample on a "hot to the touch" (from prewarming) **denaturing sequencing gel** in 37°C room.

- Save the remainder of samples at -20°C in case the gel runs badly.
- Markers: approx. 1-2 x 10^3 cpm pBR322/Msp1 per lane (2 µl DNA +8µl **DF+dyes**).

Denature 5' at 100°C, plunge into ice, then load.

- **Denaturing sequencing gel:** 0.3 mm/ 0.5xTBE/ 8M Urea/ 9% polyacrylamide (for 147 base TK fragment). Running buffer is 0.5xTBE (1.5 liter required for big gel rig).
- Pre-run overnight at 400 Volts.
- Pre-warm at 45 Watts constant power (big gel) or 20-30 Watts (narrow gel).

22) Run samples in at pre-warm wattage, then run at 20 W or 10W for big or narrow gel, respectively. Runs are usually 1.5-3 hours until Bromophenol blue is at the bottom.

23) Take down from gel rig, remove tape, remove spacers, and split plates apart with spatula or razor blade inserted in a bottom corner.

24) Lay 3MM paper on top of gel, then carefully peel back so that gel sticks smoothly to paper but comes off of glass plate.

25) Overlay gel on paper with Saran Wrap (not Handiwrap). Dry under vacuum at 80°C on gel dryer for 2-3 hours (it may take less time but I usually go longer to be sure).

26) Autoradiograph.

- Initially try an overnight exposure if fresh probe was used.

APPENDIX 7: Small Scale RNA Isolation

1) Rinse culture dishes twice with **1xPBS**. Tilt on edge to drain for 15 seconds. Aspirate remaining fluid.

2) Scrape 1-5 dishes (2-5 x 10^6 cells/dish) in 1.6 ml of **Guan-Thio**.

3) Sonicate homogenate 30" at maximum with small sonicator probe.

4) Add 0.8 g solid CsCl, invert to dissolve.

5) Adjust volume to 2 ml with **Guan-Thio**.

6) Layer homogenate on a 1 ml CsCl pad (5.7 M CsCl, 0.1 M EDTA) in a 3 ml heat seal tube.

7) Centrifuge at 49K rpm in a Beckman 100.3 rotor (110,000 x g) using a Beckman Tabletop Ultracentrifuge (TL-100) for 16-24 hours at 21°C.

[Work at room temperature for steps 8-14]

[Work quickly and process samples individually from step 8 to 11.]

8) Remove supernatant above the pad with a syringe.
   - Leave approx. 1 ml.
   - Need to poke two holes, one to let in air as you draw fluid from the other.

9) Cut the top of the tube off about 1 cm from the bottom.

10) Remove the remaining supernatant with a sterile pasteur pipette.

11) Take up the clear, contact-lens like pellet in 200 µl **1xTES**, followed by a rinse of 200 µl of **1xTES**. Transfer all 400 µl to a tube containing 200 µl phenol (GDW saturated) and 20 µl 5M NaCl. Vortex vigorously for 1'.

[Process samples together from step 12 onward]

12) Add 1/2 volume chloroform (24:1, $CHCl_3$: Isoamyl alcohol) to each sample, vortex 1', microfuge 3'.

13) Remove aqueous phase to fresh tube, add 1 volume of chloroform, vortex 1', and microfuge 1'.

14) Remove aqueous to fresh tube, add 3 volumes of 100% ethanol (stored at -20°C). Precipitate at -20°C overnight or at -70°C for 2 hours.

[Do the following on ice]

15) Microfuge 15' in cold room, remove supernatant with drawn out pipette, wash pellet with 200 µl of ice cold 70% ethanol, microfuge 5-15' in cold room, remove supernatant with drawn out pipette, and dissolve pellet in GDW (1 µg/µl).

- If there is lots of RNA, repeatedly heat sample at 55°C and vortex, until sample draws smoothly through a micropipette tip.

16) Quantitate by absorbance at 260/280 nm. [1 Unit at 260 = 50 µg/ml]

10xPBS

Conditions	stock	recipe
27 mM KCl	solid	2 g/l
15 mM KH_2PO_4	"	2 g/l
1.37 M NaCl	"	80 g/l
81 mM Na_2HPO_4	"	11.5 g/l

[To speed solvation, add salts to water, not vice versa]

Guan-Thio

Conditions	Stock	Recipe
4 M guanidinium isothiocyanate	solid	23.6 g
0.1 M ß-mercaptoethanol	12.8 M	391 µl
5 mM sodium citrate pH 7	1M	250 µl
0.5% Sarcosyl	solid	0.25 g
	GDW	Q.S to 50 ml

10xTES

Conditions	stock	recipe
10% SDS	solid	20 g
	GDW	160 ml
[Boil to sterilize, then add sterile ingredients below]		
0.1 M Tris pH 7.5	1 M	20 ml
50 mM EDTA	0.5 M	20 ml

APPENDIX 8: Nuclear/Cytoplasmic RNA Isolation

A. Harvesting Cells

1) Rinse culture dishes twice with **1xPBS**.

2) Scrape each dish in 1ml of 1xPBS. Option: Put a drop on a microscope slide, overlay with a cover slip, and examine under microscope; save slide for comparison to lysed cells below.

3) Centrifuge 10' at 500 rpm in tabletop centrifuge.

B. Nuclear/Cytoplasmic Fractionation; Use one of the two procedures below.

- Subcellular fractionation procedures are done with ice cold reagents in the cold room. [move vortexer, tabletop centrifuge to cold room in advance]
- These procedures were successful with myoblast cells (MG14-1-1; MG14-1-2).

Penman Procedure

1) Aspirate supernatant.

2) Vortex pellet on slow setting to loosen it. [It will spread around bottom of the tube]

3) Quickly add 2 ml **RSB(A)**, swirl briefly, and allow cells to swell 10'. [I used 12-30x10^6 cells]

4) Look at cells under microscope; if cells are lysed skip to step 6.

5) Break cells with 5 strokes (by hand) of a dounce homogenizer (I used the sanded pestle; Examine cells under microscope; if they are lysed, continue. If not repeat this step (perhaps with less strokes on the next try) until they are.

- Before douncing, all cells have a halo; 5 strokes usually reduces the size ofhalo.
- Excessive douncing to try to eliminate halo will break nuceii. The halo is effectively eliminated by a detergent step below [8 strokes was too much]
- Perhaps dounce step is not necessary at all considering the effectiveness of the following detergent step; However, I have not checked.

6) Centrifuge 10' at 500 rpm in tabletop centrifuge.

7) Remove the supernatant (Cyt #1) with a drawn out pipette to a 12.5 ml polypropylene tube. To Cyt#1 add 1/10 volume of **10xTES** and adjust to 0.2 µg/µl proteinase K (add 20 µl of 20 mg/ml PK) as quickly as possible. Mix and set at 55°C for 1-2 hours. [Have everything ready so the PK digestion can be set up quickly and pellet doesn't dry too long] Work up as below for cytoplasmic RNA.

8) Loosen pellet by slow vortexing. Quickly resuspend pellet in 2 ml **RSB (A)**.

9) Add 300 µl **Detergent Mix**, vortex (fast) for 3 seconds, centifuge 10' at 500 rpm in tabletop centrifuge.

10) Remove supernatant (cyt#2) and treat as in step 7.

11) Loosen pellet (nucleii)by vortexing (fast if needed); immediately add 1 ml of **1xTES** containing 400 µg Proteinase K (20 µl of 20mg/ml PK)[It should get very viscous]. Incubate at 55°C 1-2 hours. Prepare nuclear RNA as described below..

10xPBS

Conditions	stock	recipe
27 mM KCl	solid	2 g/l
15 mM KH_2PO_4	"	2 g/l
1.37 M NaCl	"	80 g/l
81 mM Na_2HPO_4	"	11.5 g/l

[Add salts to water, not vice versa]

RSB (A)

Conditions	stock	recipe
10 mM Tris pH 7.4	1 M	1 ml
10 mM NaCl	5 M	200 µl
1.5 mM $MgCl_2$	1 M	150 µl
	GDW	100 ml

10xTES

Conditions	stock	recipe
10% SDS	solid	20 g
	GDW	160 ml
[Boil to sterilize, then add sterile ingredients below]		
0.1 M Tris pH 7.5	1 M	20 ml
50 mM EDTA	0.5 M	20 ml

Detergent Mix

Conditions	stock	recipe
3.3 % deoxycholate	10% w/w deoxycholate	0.5 ml
6.6 % Tween 20	10% w/w Tween 20	1.0 ml

[Penman says to use Tween 40 but we do not have it]

Kainz Procedure

1) Aspirate supernatant; vortex pellet slowly till it is smeared all over the bottom of the tube; quickly add 2 ml **RSB Mix** and swirl briefly to resuspend cells.

2) Centrifuge 10' at 500 rpm in tabletop centrifuge.

3) Remove supernatant (cyt #1) carefully with a drawn out pipette and work up as in step 7 of method A.

4) Loosen pellet by slow vortexing; quickly add 2 ml of **RSB Mix** containing 0.5% NP40 (200 µl of 5% stock of NP40).

5) Vortex 15-20 seconds at maximum; let sit 5'; check appearance on scope, if OK continue, if cell halos are too large use the dounce homogenizer (5 strokes initially) until appearance of nucleii is correct.

6) Centrifuge 20' at 2000 rpm in tabletop centrifuge.

7) Remove supernatant (cyt #2) carefully with a drawn out pipette and work up as in step 7 of Procedure A.

8) Vortex pellet slowly to loosen it; quickly add 2 ml **RSB Mix** and swirl briefly to resuspend nucleii.

9) Centrifuge 10' at 2000 rpm in tabletop centrifuge.

10) Remove supernatant (cyt #3) carefully with a drawn out pipette and work up as in step 7 of Procedure A.

11) Work up pellet (nucleii) as in step 11 of Procedure A.

RSB Mix

Conditions	stock	recipe
10 mM Tris pH 7.5	1 M	20 µl
10 mM NaCl	5 M	4 µl
5 mM $MgCl_2$	1 M	10 µl
0.1 U/µl RNasin	40 U/µl	5 µl
10 mM DTT	0.5 M (frozen)	40 µl
	GDW	2 ml

C. Workup of PK/SDS Digestions to RNA [After 1-2 hours digestions at 55 °C]

[steps 1-3 are done at room temperature]

1) Add 1/20 volume 5M NaCl and 1/2 volume GDW saturated phenol, vortex 30-60" at maximum, add 1/2 volume Chloroform, vortex 30-60", centrifuge at maximum in tabletop centrifuge for 3'.

2) Remove aqueous (top) layer to fresh polypropylene tube, add 1 volume chloroform, vortex 1', and centrifuge 1' at maximum in tabletop centrifuge.

3) Remove aqueous (top) layer to fresh polypropylene tube, add 2.5-3 volumes ice cold 100% ethanol, precipitate at -70 °C for at least 1 hour. [If using a small amount of material, longer precipitation may be better]

[steps 4-6 are done at 4 °C]

4) Centrifuge at 10K rpm in SS34 rotor with adaptors for 15' [mark position of pellet so you canaspirate away from the pellet]; aspirate most of supernatant, leaving 0.5-1 ml; remove the remaining supernatant with a drawm out pipette.

5) Add 0.5ml of 70% ethanol, swirl and shake to wash tube walls, and centrifuge 5-15' at 10K rpm inm SS34 rotor with adaptors.

6) Remove supernatant with drawn out pipette.

7) Resuspend in water as appropriate. [200 µl is suggested; TNA is tough to dissolve. Use several cycles of vortexing and 65 °C incubation and then check by taking up the most concentrated samples with a P200 pipette tip, i.e. the sample should pipette smoothly]

8) Measure total nucleic acid (TNA) concentration by absorbance at 260 nm.

9) Measure DNA concentration by Hoechst Dye method. [allows determination of the degree of nuclear contamination in cytoplasmic samples and will be used as a check on the efficiency of oligo removal in steps 17-21]

10) Set up DNase digestions:

200 µl TNA sample
23 µl **10xDNase Buffer**
2 µl 1 U/µl DNase
4 µl 10 mg/ml Heparin (stored frozen)
1 µl RNasin

11) Incubate 1 hour at 37 °C.

12) Add 25 µl of 0.5 M EDTA, 13 µl of 5 M NaCl, and 150 µl GDW saturated phenol; vortex 30"; add 150 µl chloroform; vortex 30"; microfuge 3'.

13) Remove aqueous (top) phase to new tube; add 300 µl chloroform; vortex 30"; microfuge 1'.

14) Remove aqueous (top) phase to new tube; add 900 µl 100% ethanol; precipitate at -20 °C 3 hours at least.

15) Microfuge 15' at 4 °C; remove supernatant with drawn out pipette; add 200 µl of ice cold 70% ethanol; flick tube to rinse walls; microfuge 5-15' at 4 °C.

16) Remove supernatant with drawn out pipette; resuspend in GDW as appropriate. [40 µl is recommended; if nucleic acid concentration is high this step works better]; evaporate ethanol by incubating open tubes in 65 °C water bath for 5'.

17) Set up isopropanol precipitation to remove oligonucleotides by adding 1/4 volume of 10 M ammonium acetate and 3/4 volume isopropanol; allow to sit at room temperature 30'.

18) Microfuge 15' at room temperature [on a hot day microfuge in a cool place otherwise your samples may heat up to the point where they don't precipitate]; remove supernatant with drawn out pipette.

19) Add 40 µl ice cold 70% ethanol; flick to wash walls; microfuge 5-15' at 4 °C.

20) Remove supernatant with drawn out pipette; resuspend pellet in 100 µl GDW.

21) Quantitate RNA by absorbance at 260 nm. [check for agreement with predicted RNA in sample from measurements in steps 8 and 9, i.e. TNA-DNA]

10xDNase Buffer

Conditions	stock	recipe
200 mM HEPES pH 7.0	1M	2 ml
10 mM $CaCl_2$	0.1M	1 ml
50 mM $MgCl_2$	1 M	0.5 ml
10 mM $MnCl_2$	1 M	0.5 ml
	GDW	6 ml

APPENDIX 9: Polysome Profiles and RNA

1) Rinse culture dishes (4 ml/dish) with saline G plus 10 µg/ml cycloheximide (CH).

2) A. Myoblasts: Apply 4 ml Collagenase (C'ase) Master Mix (1 part C'ase, 3 parts Saline G,10 µg/ml CH), incubate 3-5' at 37°C until cells begin to slough off, rinse cells from dish by pipetting up and down 5 times, transfer 5 plates worth (20 ml) to a 50 ml tube containing 20 ml of F10C/15% horse serum(HS)/10 µg/ml CH, rinse the 5 plates successively with 10 ml of F10C/15% HS/ 10 µg/ml CH, and pool rinse with sample.
B. Fibroblasts: Apply 1.5 ml warm trypsin (containing 10 µg/ml CH), incubate 3-5' at 37°C until cells slough off the dish, add 2.5 ml cold DMEM/10% calf serum (CS)/10 µg/ml CH, pipette up and down 10 times to wash cells from dish, transfer 5 plates worth (20 ml) to a 50 ml tube containing 20 ml cold DMEM/10% CS/10 µg/ml CH, rinse the 5 plates with DMEM/10% CS/10 µg/ml CH, and pool rinse with sample.
[Dilution of c'ase or trypsin digestion with medium containing serum 'ties up' enzymes with serum proteins and keeps them from lysing cells.]

3) Centrifuge 5' at 500 rpm in tabletop centrifuge.

[Keep samples and all reagents ice cold from here on]

4) Aspirate supernatant.

5) Resuspend cells in 5 ml of 1xPBS containing 10 µg CH with 5 strokes of pipette. Transfer to a small Corex tube.

6) Centrifuge 5' at 500 rpm (use adaptors in tabletop centrifuge).

7) Aspirate all but approx. 5 ml of supernatant. Remove the remainder with a drawn out pipette.

8) Vortex pellet slowly to spread around on the base of the tube.

9) Quickly add 300 µl **Lysis Buffer** and let sit on ice 2'.

10) Centrifuge 10' at 13K rpm in pre-cooled SS34 rotor with adaptors.

11) Remove supernatant very carefully with a drawn out pipette and transfer to the top of a 15-50% sucrose gradient (see below).

- Use a fresh pipette for each sample.
- Have the gradients ready and balanced. Need to move quickly.

12) Centrifuge gradients for 130 minutes in precooled SW40 rotor at 32K rpm.

13) Analyse gradients at 260 nm, using the sucrose gradient analyser set at 0.5 sensitivity, 0.375 ml/min flow rate, and 30 cm/hour chart speed.

- Check flow cell to see if it is clean.
- Run sterile water containing 0.1% DEP [not autoclaved after DEP addition; not DEP-treated water.] through apparatus, rinse with sterile GDW.

- Use a 60% sucrose containing a xylene cylanol (any dye) as a pushing solution.
- Record the time difference between when the first sample enters the flow cell and when the first sample drops into a fraction tube. Put a mark on the chart as th first drop enters a fraction tube. This will allow allignment of profile with fractions.
- Collect 400 µl fractions in eppendorf tubes, i.e. 1.2 min/fraction at a flow rate of 0.375 ml/min (requires setting of 1x and 12 on our collector).
- Move fractions to ice as quickly as possible.

14) Freeze samples until they are pooled.

15) Align polysome profile with fractions, and decide which fractions to pool.

16) Thaw samples on ice and add 45 µl of the master mix below:

Conditions	stock	1xrecipe	70xMaster Mix
10 µg/sample	10 mg/ml	1 µl	70 µl
200 µg/ml proteinase K	20 mg/ml	4 µl	280 µl
1xTES	10x	40 µl	2.8 ml

17) Incubate at 55°C for 1 hour.

18) Pool fractions into samples representing various sizes of polysomes into polypropylene tubes.

- I have used fractions named 60S and 40S, and 1-2, 3-4, 5-7, 8-12, and >12 ribosomes (See MG30-3-1; MG30-3-2; MG30-4-1).

[Steps 19-21 are done at room temperature]

19) To each pool, add 1/20 volume 5M NaCl and 1/2 volume GDW saturated phenol, vortex 30-60" at maximum, add 1/2 volume Chloroform, vortex 30-60", centrifuge at maximum in tabletop centrifuge for 3'.

20) Remove aqueous (top) layer to fresh polypropylene tube, add 1 volume chloroform, vortex 1', and centrifuge 1' at maximum in tabletop centrifuge.

21) Remove aqueous (top) layer to fresh polypropylene tube, add 2.5-3 volumes ice cold 100% ethanol, precipitate at -70 °C for at least 1 hour. [If using a small amount of material, longer precipitation is better]

[steps 22-24 are done at 4 °C]

22) Centrifuge at 10K rpm in SS34 rotor with adaptors for 15'; aspirate most of supernatant, leaving 0.5-1 ml; remove the remainder with a drawn out pipette.

- Mark position of pellet so you can aspirate away from the pellet.

23) Add 0.5ml of 70% ethanol, swirl and shake to wash tube walls, and centrifuge 5-15' at 10K rpm inm SS34 rotor with adaptors.

24) Remove supernatant with drawn out pipette.

25) Resuspend in water as appropriate. [50 µl is suggested].

Lysis Buffer

Conditions	stock	recipe
250 mM NaCl	5 M	50 µl
25 mM $MgCl_2$	1 M	25 µl
50 mM Tris pH 7.5	1M	50 µl
0.5% Triton X-100	100%	5 µl
200 U/ml RNasin	40 U/µl	5 µl
10 µg/ml cycloheximide	1 mg/ml	10 µl
	GDW	Q.S. to 1 ml

10xPBS

Conditions	stock	recipe
27 mM KCl	solid	2 g/l
15 mM KH_2PO_4	"	2 g/l
1.37 M NaCl	"	80 g/l
81 mM Na_2HPO_4	"	11.5 g/l

[Add salts to water, not vice versa]

10xTES

Conditions	stock	recipe
10% SDS	solid	20 g
	GDW	160 ml
[Boil to sterilize, then add sterile ingredients below]		
0.1 M Tris pH 7.5	1 M	20 ml
50 mM EDTA	0.5 M	20 ml

Sucrose Gradients

- Practice making gradients because there is lots of fiddling about.
- For practice gradients, use blue dye (xylene cylanol) in one chamber. This will allow you to visualize the procedure and outcome better. Do not include dye in experiment because it absorbs at 260 nm, the wavelength used to analyse polsome profiles.

1) Use a 20 ml linear (cylindrical chambers, 10 ml each) gradient maker. Clamp into place over a stir plate. Rig an effluent tube of approximately 3 mm diameter flowing from the proximal chamber, through a peristaltic pump, to a 12.5 ml SW40 tube. Need to use proper peristaltic pump tubing, otherwise solutions will flow through while the pump is off. Insert a small stirring bar (0.5-1 cm long) into the proximal chamber.

2) Fill both chambers with sterile water containing 0.1% Diethylpyrocarbonate (DEP) [Not autoclaved after DEP addition; Not DEP-treated water.]. Check if water flows through pump while it is off; if it does, get the correct tubing to go through the pump. If it doesn't, turn the pump on fast and pump 0.1% DEP through the system. After

DEP-GDW is completely gone, flush the system with sterile GDW several times. Drain and turn pump off.

3) Add 6.3 ml of **15% Sucrose Buffer** to the distal chamber.

4) Briefly open the valve between the two chambers to allow approximately 0.3 ml to flow into proximal chamber. Remove the 0.3 ml from proximal chamber with a pipette.

- This step clears bubbles from the passage.

5) Add 5.5 ml of **50% Sucrose Buffer** to proximal chamber.

6) Set stirrer at optimum speed (2.5 on our small stainless steel stirrer). Adjust stir bar position so that it turns right next to the opening of the passage from the other chamber.

- If too slow, mixing is inefficient.
- If too fast, 15% sucrose can not enter proximal chamber.

7) Turn on peristaltic pump to a slow flow rate (20% at 10x setting on our Tris peristaltic pump) and allow level of solution in proximal chamber to drop just below level in distal chamber.

8) Open the switch between the chambers and observe schlearing as the two solutions mix. Adjust stir bar rate for optimal mixing and flow (the levels of fluid in each chamber drop similarly throughout the procedure). The stirring rate needs to gradually be slowed (to setting of 1) as the chambers get close to empty (not as much volume to mix).

9) Put the opening of tubing near the bottom of the SW40 tube and move it upward, just above the surface of the fluid, as it fills.

10) Store the gradients on ice until ready to load samples on them. [I have stored them for 1-2 hours with success.]

15% Sucrose Buffer

Conditions	stock	recipe
0.25 M NaCl	5 M	5 ml
25 mM $MgCl_2$	1 M	2.5 ml
50 mM Tris pH 7.5	1 M	5 ml
15% sucrose	solid, RNase free	15 g
1 µg/ml cycloheximide	1 mg/ml	100 µl
	GDW	Q.S. to 100 ml

50% Sucrose Buffer

Conditions	stock	recipe
0.25 M NaCl	5 M	5 ml
25 mM $MgCl_2$	1 M	2.5 ml
50 mM Tris pH 7.5	1 M	5 ml
50% sucrose	solid, RNase free	50 g
1 µg/ml cycloheximide	1 mg/ml	100 µl
	GDW	Q.S. to 100 ml

APPENDIX 10: *In Vitro* Translation

A. Promega Translations

- The relative effectiveness of rabbit reticulocyte lysates and wheat germ extracts in translating TK pseudo-mRNA was tested in MG16-5-4.

Rabbit Reticulocyte (RR) Lysates (nuclease treated and supplemented*)

1) Set up translation cocktails on ice.

2) Mix gently; incubate at 30°C for 1 hour; stop reaction by freezing.

3) To load on Laemmli minigel, dilute sample at least 1:6 with GDW.

Conditions	Stock	Recipe
70% Treated*RR lysate	100%	17.5 µl
20 µM amino acids (-Met)	1 mM	0.5 µl
	GDW	3.5 µl
40 ng/µl TK pseudo-mRNA	1 µg/µl	1.0 µl
1 µCi/µl [^{35}S]Met; 1 µM	10 µCi/µl; 10 µM	2.5 µl

* Promega analysis certificate states: Micrococcal nuclease treated RR lysates are optimized for mRNA translation by supplementing with hemin, calf liver tRNA, potassium acetate to 140 mM, magnesium acetate to 1 mM, and an energy generating system consisting of creatine phosphate and creatinephosphokinase.

Wheat Germ Translations

1) Set up translation cocktails on ice.

2) Mix gently; incubate for 2 hours at 25°C; freeze to stop reaction.

3) To load on a Laemmli minigel, dilute sample at least 1:6 with GDW.

Conditions	Stock	Recipe
50% Wheat Germ Extract*	100%	12.5 µl
80 µM amino acids (-Met)	1 mM	2.0 µl
100 mM potassium acetate	1 M	2.5 µl
	GDW	5.75 µl
40 ng/µl TK pseudo-mRNA	1 µg/µl	1.0 µl
0.5 µCi/µl [^{35}S]Met; 0.5 µM	10 µCi/µl; 10 µM	1.25 µl

* Promega analysis certificate states: Wheat germ extract is prepared by grinding wheat germ in an extraction buffer followed by centrifugation to remove the debris. Gel filtration is used to remove the endogenous amino acids and reduce the potassium ion concentration. The column buffer contains: 120 mM potassium acetate, 5 mM

magnesium acetate, 6 mM ß-ME, and 20 mM HEPES pH 7.6.

B. RR Translations from Scratch

-Uses treated (but unsupplemented) RR lysate obtained from John Lewis (stored in liquid nitrogen) and other components assembled from scratch (adapted from Jackson and Hunt,1983).

- This system works as well as the Promega RR kit in translating TK pseudo-mRNA (MG16-5-5).

- Lewis lysate does not work with Promega protocol given above; Similarly, Promega treated, supplemented RR lysate does not work with the procedure given below (MG16-5-5).

1) Assemble the translation cocktail shown below on ice.

-Do not allow RR lysate to heat up past 4°C.

2) Mix gently; incubate at 30°C for 1 hour; stop reaction by freezing.

3) To load on minigel, dilute sample at least 1:6 with GDW.

Conditions	Stock	Recipe
74% Treated RR lysate*	100%	22.1 µl
100 mM KCl; 0.5 mM $MgCl_2$	2M KCl;10 mM $MgCl_2$*(KM)	1.5 µl
10 mM creatine phosphate	0.2 M * (CP)	1.5 µl
no Met; 3 mMLeu, Val; 2 mM 17 others	**19 AA stock***(below)	1.5 µl
4.7 µg/ml yeast tRNA	1mg/ml (-20°C)	0.14 µl
16 µM Hemin	4 mM (-70°C)	0.12 µl
40 ng/µl Creatine Kinase	5 mg/ml (-20°C)**	0.24 µl
1.2U/µl RNasin (Promega)	40 U/µl (-20°C)	0.9 µl
0.5 µCi/µl [^{35}S]Met; 0.5 µM	10 µCi/µl; 10 µM	1.5 µl
17 ng/µl TK pseudo-mRNA	1 µg/µl	0.5 µl

* Indicates that disposable frozen (-20°C) aliquots of the stock are used.

** Made in 50% glycerol.

C. General Notes

- Capped RNA is translated better in any of th . systems described, but is not essential for a good signal.

-Potassium and magnesium concentrations are critical for good translation.

- In vitro translation experiments should include a negative control (no RNA) and a positive control (Bromo Mosaic Virus (BMV) RNA).

-BMV RNAs give proteins of the following sizes:

RNA	Protein
3234 bases	109K Daltons
2865	94K
2114	35K
876	20K

19 AA stock

Conditions	Molecular Weight	Recipe
3 mM L-Leucine	131.2 g/mol	39.4 mg
L-Valine	117.2	35.2
2 mM L-Glycine	75.1	15.0
L-Alanine	89.1	17.8
L-Isoleucine	131.2	26.2
L-Arginine	210.7	42.1
L-Asparagine	132.1	26.4
L-Aspartic acid	133.1	26.6
L-Cysteine (not cystine)	121.2	24.2
L-Glutamine	146.1	29.2
L-Glutamic acid	147.1	29.4
L-Histidine	191.7	38.3
L-Lysine	182.7	36.5
L-Phenylalanine	165.2	33.0
L-Proline	115.1	23.0
L-Serine	105.1	21.0
L-Threonine	119.1	23.8
L-Tryptophan	204.2	40.8
L-Tyrosine	181.2	36.2

- Dissolve in 100 ml GDW by warming on a heater/stirrer (not Hot).
- Bring to pH 7 with approximately 3 drops of 10 N KOH.
- Make 1 mM in DTT; add 200 µl of 0.5 M stock.

APPENDIX 11: Production and Isolation of Fusion Protein

A. Production of Fusion Protein

1) Inoculate a Fernbach flask (containing 1 liter of 2xYT, 50 µg/ml ampicillin and 0.2 mM IPTG) with a loop of glycerol stock of DH5 Δlac transformed with pMLB 1113 TK/ß-gal.

- Induction rangefinder for IPTG concentration is shown in MG 16-2-2.

2) Shake vigorously overnight at 37 °C.

3) Harvest culture by centrifugation at 4K rpm in GSA rotor (4 tubes at 250 ml).

- Harvest in mid-log phase has not been checked out and is perhaps preferable.
- Similarly, short IPTG induction times have not been tested.

4) Resuspend and pool pellets in 20 ml (2 x10 ml) of **Extraction Buffer** plus fresh PMSF at 1mM (add 100 µl of 0.2M PMSF in ethanol) at 4°C by pipetting.

- PMSF has a short half life in water, therefore is added fresh just before use.

5) Add lysozyme to 1 mg/ml; allow to sit 30' on ice.

6) Freeze at -70°C.

7) Thaw quickly by swirling in the 37°C bath.

- The object is to keep cells below 4°C at all times.

8) Sonicate on ice, five 20" bursts at maximum power with large probe.

-Do not allow sample to heat up.

- Optional to add more PMSF at this stage (to 1mM).

9) Add 1/10 volume of 5 M NaCl.

10) Clarify by centrifugation at 13000 x g for 10' at 4°C.

- Use SS34 rotor, yellow capped tubes, spin at 20K rpm.

11) Remove and save the supernatant, which does not contain the fusion protein (by Laemmli gel analysis).

- This supernatant would contain ß-galactosidase.
- Check an aliquot on a gel to be sure.

12) Solubilize and pool the pellets in 20 ml **Urea Buffer** by pipetting and stirring.

- The pellet should contain the TK/ß-gal fusion protein.

13) Clarify by centrifugation at 13000 x g for 10' at 4°C (see step 10). Discard pellet.

14) Dialyse supernatant 2-3 hours at room temperature against **Dialysis Buffer** (25-50 volumes).

15) Dialyse overnight at 4 °C against dialysis buffer (25-50 volumes).

16) Clarify by centrifugation at 13000 x g for 10' at 4°C (as in step 10).

- Supernatant is an enriched source of fusion protein which should be further purified by a preparative gel (see below).

Extraction Buffer

Conditions	stock	recipe
50 mM $NaPO_4$ pH 7.0	1 M	10 ml
10 mM ß-mercaptoethanol	12.8 M	156 µl
10 mM EDTA	0.5 M	4 ml

Urea Buffer

Conditions	stock	recipe
8 M Urea	solid	24 g
0.5 M Tris pH 7.9	1M	25 ml
0.5 M NaCl	5 M	5 ml
1 mM EDTA	0.5 M	100 µl
30 mM ß-mercaptoethanol	12.8 M	117 µl
1mM PMSF (add fresh)	0.2 M in ethanol	250 µl
	GDW	Q.S. to 50 ml

Dialysis Buffer

Conditions	stock	recipe
0.5 M Tris pH 7.9	solid	90.8 g
0.5 M NaCl	solid	43.5 g
10% glycerol	100%	150 ml
	GDW	Q.S. to 1.5 l

B. Preparative Gel Isolation of Fusion Protein

1) Prepare a 7% polyacrylamide Laemmli gel of size 0.2 x 10 x 23 cm.

2) Load enriched fusion protein supernatant or crude cell extract in a total volume of 6 ml in a well of 0.2 x 18 x 2.3 cm dimensions.

- Add 1.5 ml of fresh 4 x Laemmli sample buffer to 4.5 ml enriched fusion protein supernatant of step 16; boil 5-10', centrifuge at max for 5' in tabletop centrifuge and load supernatant (yield is 50 mg fusion protein).

- Crude cell extract: A 50 ml culture is grown and and induced as the the 1 l culture described above; harvest by centrifugation at maximum in table top centrifuge, resuspend pellet in 6 ml GDW, centrifuge again, resuspend cells in 6 ml GDW, add 2 ml 4 x Laemmli sample buffer, boil 5-10', centrifuge and load supernatant (yield is 1 mg of fusion protein).

3) Run gel at 100-200 V until bromophenol blue is at the bottom.

4) Surface stain (0.3% Coomasie G-250 in 100 mM NH_4acetate pH 4.5) for 15'.

5) Soak in destain (100 mM ammonium acetate pH 4.5) until fusion protein band is clearly discernable.

6) Excise band and elute with Elutrap in gel running buffer for 24 hours.

- May need to top of the trap with buffer after 12 hours.

- The sample well at positive pole should turn a deep blue color.

7) Check out size and purity of sample on a Laemmli minigel and quantitate protein concentration with a Bradford assay.

APPENDIX 12: Injection of Rabbits and Collection of Immuneserum

[Very important to check the number on the rabbits ear to make sure you are working with the your rabbit each time you remove a rabbit from a cage]

1) Collect prebleed serum from rabbits for several weeks before injection of antigen by **ear bleeds** (described below).

2)Emulsify antigen solution with an equal volume of Freunds Complete Adjuvant.

- Takes about 1 hour and should be done just prior to injection.
- Final volume injected into each rabbit is 1.6 ml.
- Good to inject 0.5-2 mg of antigen into each rabbit.
- If antigen solution is too dilute, concentrate it in an Amicon centrifugal ultrafiltration device (optimal to have 0.9 ml of antigen solution at 2 mg/ml for each rabbit).
- Emulsify by squirting the antigen/adjuvant mix back and forth through two 18 gauge needles (on 3 ml syringes) connected by tubing, until the emusion is too viscous to squirt through despite extreme effort.
- Test emulsion by allowing a small drop to fall on water in a beaker; if it remains beaded up for a short while then the emulsion is good; if it immediately spreads out then more emulsification is needed. Note: The samples I injected did not bead up but were too difficult to pass through a syringe again.
- An alternative method of emulsification is to use what looks like a miniature egg beater overnight [Harvey Holmes says this works well].

3) [check number] Shave the back of rabbit with electric shears at Lab Animal Resources (LAR).

- A swath about 2-3 inches on wide on each side of the backbone extending from the shoulders to the rump.

4) [check number] Inject 16 spots (8 on each side) with 0.1 ml of the emulsion using a 1 ml syringe and a 22.5 guage needle. To inject a spot:

a) Pull a fold of skin over your thumb.

b) Slip needle (bevel up) just under the skin.

c) Relax skin.

d) Inject 0.1 ml; pull back plunger to release pressure and withdraw needle.

- Be nice to the rabbit, pet him gently etc.
- Helps to have another person to firmly hold rabbit during injections, because he

sometimes jumps if you poke him too hard.

- Be prepared for the rabbit to jump and hold your needle so that you can quickly withdraw the needle when he jumps rather than jabbing it in further.

5) Return rabbit to cage and record injection on his card.

6) Wait 1 month.

7) Prepare booster solution by emulsifying (asdescribed above)an equal volume of antigen solution (2 mg/ml) with Freunds Incomplete Adjuvant.

- Incomplete adjuvant keeps rabbit from boosting immune response to certain antigenic components in the complete adjuvant.
- Prepare 2.2 ml of booster solution (expect to lose 0.2 ml in emulsification step).

8) Inject rabbit intramuscularly with booster solution, delivering 0.5 ml to each hind leg: [remember to check number on his ear]

a) Set rabbit on edge of table so that one hind leg hangs off the edge; get someone to hold rabbit firmly because they jump in inexperienced hands.

b) Find meaty part of leg behind the femur (hamstring) and hold it away from bone.

- You will cause extreme pain for the rabbit if you hit bone with your needle.

c) Quickly jab needle (22.5 gauge) in 0.5-1 cm deep into muscle, inject 0.5 ml, and withdraw; massage site gently to sooth rabbit and distribute injected material.

- To make sure your quick jab goes in the right amount, put your index finger on the side of the needle to serve as a stop at the right depth.

9) Pet rabbit, return to cage, record what you injected on rabbit card, and wait 10 days before collecting immune serum by **ear bleeds**.

Ear Bleeds

1) Remove rabbit from cage, check number, and bring to surgery table.

2) Harness rabbit firmly in a restrainer consisting of a nylon wrap with velcro fasteners.

- Pull hind legs back and flatten the rabbits hips, then tighten the restrainer; in this way the rabbit can not push up with his powerful hind legs and break his back against the restrainer.

3) Use a rounded scalpel blade (#10) to shave away hair covering the vein on the posterior edge of the ear. Shave a section about 2 inches long about halfway between

the tip and base points of the ear. Pick a section where the vein is straight.

3) Put a paper clip on proximal end of vein and wait for vein to swell up. [The main artery runs down the center of the ear, blood flows away from the body in it; however, blood flows toward the body in the vein on the posterior edge of the ear. Therefore the paper clip at the proximal end of the vein causes blood to pool in the vein, i.e. swelling]

4) Using a Q-tip, dab xylene on the tip of the ear (not the shaved area). [xylene helps dilate blood vessels]

5) Use a pointed sterile scalpel blade (#11) to puncture the vein on the posterior edge.

- Use a rolling motion.
- Hold a paper towel or gauze under ear so you don't cut yourself if you accidentally puncture through the ear.
- Have tube ready.

6) Allow blood to drip into tube.

- Use a gentle rapid stroking motion away from the body on the artery in the center of the ear to stimulate blood flow.
- If flow slows: dab more xylene on tip of ear, renew cut, or rub cut roughly with a paper towel to tear it open and remove the forming clot.
- Collect 10-15 ml in small rabbit and up to 40 ml in big rabbit (2x/week).

7) If enough blood is collected and the ear is still bleeding, remove clip, pinch the vein and bend ear just distal of the cut. After a while straighten ear; if it doesn't bleed, stop pinching.

8) Wash xylene from ear with soapy water or ethanol.

- Failure to do this will cause the ear to slough off cells.

9) Release rabbit from restrainer, check number, record bleed volume and date on his card and return him to cage.

- It is a good idea to cap full tubes of blood and move them to another table before releasing rabbit so that they don't get spilled if the rabbit acts up.

10) Allow blood to clot at room temperature for several hours (4 hrs is good).

- Longer clotting times lead to hemolysis (imparts a red tinge in serum), although this has no obvious detrimental effect.

11) Break up clot with a wooden stick; centrifuge 15' at maximum in tabletop centrifuge; hold clot in with the stick while decanting serum to a fresh tube; centrifuge again; use a pipette to cleanly transfer serum to a fresh tube; freeze the serum.

APPENDIX 13: Affinity Purification of Anti-TK antibodies

[all procedures done in cold room]
[Throughout this procedure, I used a fraction collector and monitored the absorbance of each fraction at 280 nm in a spectrophotometer. I used smaller fractions when peaks were eluting and larger fractions during washes.]
[Throughout the procedure a flow rate of 1 ml/ min was maintained by a peristaltic pump inserted below the column]

1) Pour a 30 ml column of ß-galactosidase-sepharose 4B in **1xPBS**.
   - Use a 1-3 cm diameter column with sealed adaptor at the top connected to a feeder tube.
   - Coupled column was made in MG16-15-1 by the method of Carroll and Laughon (1988).
   -ß-galactosidase enriched supernatant made in MG 16-3-1 (8.5 ml at 25 mg protein/ml) was dialysed 3 days against 3 changes of **1xPBS** (100 volumes each) and coupled to 30 ml of activated sepharose.
2) Allow 1-2 column volumes of 4 M guanidine HCl to pass thru column; monitor effluent to see how much protein is released. Use 1-2 ml fractions.
3) Rinse with 100 ml **1xPBS** and monitor effluent. Use 10 ml fractions.
4) If column is running clean, i.e. no absorbance released by 4M Guanidine HCl, then remove **1xPBS** from above the gel bed with a pipette.
5) Immediately apply the immune serum to the column. After 0.5- 1 column volume of seum has entered gel bed, begin to collect flow-through in a clean bottle.
   - If a large volume of immune serum is to be purified, pipette a small volume (5 ml) on the top of the gel bed and set up a siphon from the stock bottle to feed the column.
6) When serum has all entered the column bed, apply **1xPBS** and monitor effluent in 10 ml fractions until the absorbance drops and gets near background.
7) Pool all fractions with significant absorbance with the bottle of serum flow-through (step5).
   - The flow-through pool has reduced amounts of antibodies against ß-galactosidase or contaminant bacterial proteins coupled to the column.
   - The pool can be loaded on an an immunoaffinity column (TK/ß-gal-coupled sepharose) directly as described below, or it can be passed through the ß-gal-sepharose column again as in steps 2-7 to further reduce the

contaminating anti-ß-gal antibodies.

-I loaded the pool on the immunoexclusion column (steps 2-7) three times before continuing to the immunoaffinity column (steps 8-13). The third immunoexclusion passage still caught more absorbance units than were eluted from the first immunoaffinity column (step 13 below).

- There is a point of vanishing return. Pooling too many fractions of low absorbance will increase the volume that needs to be passed through each subsequent column at a flow rate of 1 ml/min. Too many successive columns will swell the volume considerably. For example, I started with 80 ml of serum and collected a pool of 300 ml after three successive passages.

- The material stuck to the immunoexclusion column is anti-ß-gal antibody, and needs to be removed by 4 M guanidine HCl before the column can be used again. [I have collected this anti-ß-gal antibody and coupled it to sepharose; perhaps this matrix can be used to isolate fusion protein in the future]

8) Pour a 10 ml column of TK/ß-gal-sepharose.

- Use a 1cm diameter column with sealed adaptor at the top connected to feeder tube.

- I coupled 30 mg of TK/ß-gal fusion protein (pooled from 5 preparations; see MG16-14-1 for details) to 10 ml of activated sepharose 4B as described by Carroll and Laughon (1988).

9) Wash column clean with 4 M guanidine HCL and **1xPBS** as in steps 2-4.

10) Apply pooled serum from first column (step 7). Collect flow through in a clean bottle. Flow through can later be reapplied to the column for a second round of isolation (repeat steps 10-13).

11) Wash the column with **BBS-Tween** until absorbance is low in effluent. Monitor 5-10 ml fractions.

12) Equilibrate the column with **1xPBS**. Use copious amounts to bring absorbance of effluent as low as possible (0.001 if possible).

13) Elute anti-TK antibodies from column with 1-2 column volumes of 4 M guanidine HCl. Monitor the eluate in 2 ml fractions. An elution peak of absorbance less than 0.2 can be expected on the first recovery. The size of the peak diminishes with each recovery.

- I pooled the peaks from three successive recoveries.

- Not all anti-TK antibodies are bound on the first passage of the immunoexcluded serum through the immunoaffinity column. Therefore, the flow through from step 10 is used to repeat steps 9-13 for a second (or

third, etc.) recovery of anti-TK antibodies.

14) Pool peak fractions from each recovery and dialyse 3 days (3 changes, 100 volumes each) against **1xPBS** to renature antibodies.

15) If neccesary, concentrate by ultrafiltration.

16) Determine titer of antibody with western blot strips containing ΔN15rTK (see MG 16-14-2 for example on the first bulk purification of anti-TK antibodies).

10xPBS

Conditions	stock	recipe
27 mM KCl	solid	2 g/l
15 mM KH_2PO_4	"	2 g/l
1.37 M NaCl	"	80 g/l
81 mM Na_2HPO_4	"	11.5 g/l

[Add salts to water, not vice versa]

BBS-Tween

Conditions	stock	recipe
0.125 M boric acid	solid	7.7 g
0.1% Tween 20 v/v	100%	1 ml
1 M NaCl	solid	58.4 g
Adjust pH to 8.3 with 10 M NaOH		
	GDW	Q.S. to 1 l

APPENDIX 14: Western Transfer

1) Remove SDS-polyacrylamide gel from plates; cut corner to designate orientation.

2) Soak 10-30 min in **Western Transfer Buffer** (200 ml reagent grade methanol, 100ml **10x Western Transfer Buffer,** 700 ml GDW)

-**10xWestern Transfer Buffer** (stored at room temperature)

25 mM Tris	30.3 g TrisOH
192 mM Glycine	144.0 g Glycine
	Q.S. to 1L

no pH adjustment needed; should be pH 8.3.

-Adding SDS to 0.1% (from 10% stock) helps transfer some proteins, for example, chicken TK.

3) Make a stack in the blotting tray as shown below:

top	a) top plastic
	b) bubble screen
	c) positive electrode plate
	d) bubble screen
	e) 2-3 scotrch brite pads
	f) 3 layers of 3MM paper
	g) nitrocellulose (NC)(prewet in GDW 4 hr for high capacity binding ofproteins)
	h) gel
	i) 3 layers of 3MM paper
	j) 2-3 scotch-brite pads.
	k) bubble screen
	l) negative electrode plate
	m) bubble screen
bottom	n) blotting tray

-Start adding layers from the bottom, keeping all layers wet with **Western Transfer Buffer** as you work toward the top.

- For layers i-f be very meticulous about removing bubbles between layers (use a clean glass test tube as a rolling pin).

4) Transfer overnight (15-24 hours) at 150 mA constant current in Genie plate electrode transfer apparatus.

5) Open apparatus, cut edges of NC to exact size of gel (also notch corner for orientation) and then remove NC from gel. Mark side of NC facing gel with pencil (expt#).

6) Place NC between two sheets of 3MM paper, hold together sandwich with paper clips, and bake in vacuum oven for 30 min at 80^{o}C.

7) Store blot dry until ready to use. See **Western Probing** protocol.

APPENDIX 15: Western Probing

1) Slide baked blot into **1xTBS** at 45° angle to wet it uniformly at room temperature (RT).

-**10xTBS**

conditions	recipe
0.2 M TrisOH	24.4 g/L
5.0 M NaCl	292.4 g/L
pH 7.5	adjust with HCl

2) Tranfer blot to **Blocking Solution** and shake for 1 hour at RT (use approx. 25 ml in 8 cm x11.5 cm pipette tip rack cover).

- **Blocking Solution** is 25 mg/ml BSA (Sigma, fraction V) in **1xTTBS.** This solution should be made fresh (i.e. within a wek of use) and filtered through whatman 1.

-**1xTTBS** is **1xTBS** with 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate).

3) Pick up blot with forceps, squirt in appropriate amount of first antibody, rock, and lower blot into solution again. Shake at RT overnight.

- Typically antibodies are used at 10^3 or 10^4 dilutions. Our stock of affinity purified anti-cTK antibody is used at 1:2000 for 3 hours (see MG16-14-2 for titer strips).
- If blot is large and/or antibody is expensive or at low titer, use a seal-a meal bag containing a small volume. Palpate occasionally or lay a heavy casserole on top of it and rock horizontally.

4) Pour off probing solution and wash 5 min in 1xTBS, 2x 5 min in 1xTTBS, and 5 min in 1xTBS.

- All washes are in approx. 50-100 ml/blot on horizontal shaker at room temperature.

5) Transfer to **Blocking Solution** containing either [^{125}I]Protein A ($2x10^5$ cpm/ml of10^6-10^7 cpm/μg Protein A specific activity) for 1hour, or a Horseradish peroxidase (HRP)-linked second antibody at the appropriate dilution (usually 1:2000) for 2 hours.

6) Repeat washes as in step 4.

7) a) For [^{125}I]Protein A blots, allow to air dry on 3MM paper, wrap in saran wrap, and expose to XAR-5 film.

- Screens will shorten exposure time required but will make bands fuzzy.

b) For HRP-linked second antibody blots, immerse blot in **Color Developer** for up to 30 minutes.

- **Color Developer** (made fresh just before use)

conditions	Stock	recipe
Phosphate Buffered Saline (PBS)		40 ml
4-Chloro-1-Naphthol (4CIN)		
in methanol (3mg/ml)	0.6 mg/ml	8.0 ml
30% solution (best if fresh)	0.1% H_2O_2	165 µl

Notes:

1) Color reactions are less sensitive than [^{125}I] protein A.

2) Alkaline phosphatase coupled second antibody from Promega is the most sensitive enzyme linked second antibody method I have tried.

3) The indicated concentration of [^{125}I] protein A ($2x10^5$cpm/ml) is very critical to reduce background. See MG16-17-8 (strips 6-10) for rangefinder experiment.

4) Various blocking conditions were compared in MG16-17-7.

5) Preabsorbing antiserum in 1ml of blocking solution containing TK$^-$ bacterial or eukaryotic extracts reduces background significantly.

APPENDIX 16: Coupling Proteins to Sepharose

[This procedure is almost identical to that in Carroll and Laughon, 1988]
[The procedures must be performed in a safe chemical hood, cyanogen bromide can be lethal. All vessels and instruments that contact CN Br should be decontaminated by wiping or soaking in dilute NaOH and left ovemight in the hood to allow the volatile gas to dissipate]

1) Wash the sepharose 4B (Pharmacia) with 5 column volumes of chilled, GDW on a coarse grained scintered glass funnel. Prepare at least 1-2 ml of sepharose 4B for every 10 mg protein to be coupled or every 1-2 mls of protein solution.
2) Suspend the washed sepharose in an equal volume of 2.5 M potassium phosphate buffer pH12.2 (353.4g/l K_3PO_4 and 145.4 g/l K_2HPO_4) in a beaker with gentle stirring and immersed in an ice bath.
3) In a seperate vessel and with the hood closed as much as possible, dissolve 1g CNBr in 1 ml of acetonitrile per 10 ml of gel to be activated.
4) Add the CNBrsolution dropwise to the gently stirring gel over a period of 2 minutes, continue stirring 8 more minutes.
5) Pour the activated sepharose onto the scintered glass funnel and wash the cake carefully with 10 volumes of cold GDW followede by 10 volumes of cold **1xPBS.**
- Break the vacuum before the cake dries and gets rifts.
6) Remove filter from hood and add activated sepharose cake to protein solution, agitate slowly overnight in cold room.
- The protein solution must not contain Tris or other free amino groups and should be dialysed to equilibrium with **1xPBS** (I dialysed samples with 3 changes of buffer (100 volumes) for 3 days).
7) The next day collect uncoupled filtrate on a scintered glass funnel and save it to measure the uncoupled protein concentration.
8) Suspend sepharose in an equal volume of 1 M ethanolamine /10 mM Tris, pH 8.5 for 2 hours at 4°C to block the remaining protein-reactive sites.
9) Wash and equilibrate the coupled sepharose in 1xPBS and store at 4°C.
- Addition of azide to inhibit bacterial growth is recommended by Carrol and Laughon; However, azide interferes with western blots, so I have not used it.