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Title: Repression of Dihydrofolate Reductase Synthesis During Myogenesis: Identification and Characterization of a Transcriptional Regulatory Mechanism

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Expression of DHFR enzyme and regulation of DHFR protein synthesis were investigated using a mouse muscle cell that could be induced to irreversibly withdraw from the cell cycle and differentiate. First, the relationship between DHFR enzyme levels and DHFR mRNA showed that although DHFR enzyme was expressed at the same level in all cells, it was synthesized only in proliferative cells. Persistence of DHFR protein in the absence of DHFR synthesis was due to protein stability. Based on the stability of DHFR protein, it is reasonable to postulate that regulation of DHFR synthesis acts not to compartmentalize DHFR activity to the replicative phase of the cell cycle, but rather, to match the rate of DHFR protein accumulation to the rate of cytokinesis.

Next, the mechanisms that restrict DHFR synthesis to growing cells were investigated. As cells withdrew from the cell cycle, levels of DHFR mRNA decreased 15- to 20-fold. The magnitude of the decrease was sufficient to account entirely for the decrease in DHFR protein synthesis rates. Furthermore, polysome analysis indicated that the residual DHFR mRNA in committed cells was engaged by the same number of ribosomes per message as proliferative cells (5 to 6). Thus, rates of DHFR synthesis were regulated solely by changes in the level of DHFR mRNA.

The mechanisms regulating DHFR mRNA levels were investigated. RNase protection assays indicated that levels of DHFR pre-mRNA decreased only 7-fold during commitment. Thus, DHFR mRNA regulation was bipartite: one mechanism reduced precursor levels 7-fold; a second

mechanism lowered DHFR mRNA levels an additional 2- to 3-fold. Nuclear run-on assays indicated that the rate of transcription of the DHFR gene decreased at least 7-fold during commitment, accounting entirely for the decrease in precursor levels. To further investigate the transcriptional mechanism, the DHFR promoter and contiguous sequences were fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene and the chimeric genes were transfected into muscle cells. Proliferative cell CAT mRNA levels indicated that DHFR sequences from -850 to +465 directed strong reporter gene expression. DHFR sequences from -850 to +60 directed only weak CAT expression (25-fold lower). Moreover, DHFR sequences from +61 to +465 augmented transcription 3-fold when fused between the heterologous Rous sarcoma virus promoter and CAT gene. Therefore a positive-acting element was positioned between +61 and +465.

To identify sequences required for regulation, chimeric gene expression was investigated during myogenesis. Sequences from -850 to +465 were sufficient for full (6-fold) down-regulation of reporter gene expression. In contrast, sequences from -850 to +60 did not confer regulation on reporter gene expression. Significantly, the +60 to +465 fragment was not able to confer regulation on the Rous sarcoma virus promoter. Thus, although sequences between +61 and +465 were sufficient for enhanced expression, neither sequences upstream nor downstream of +60 were sufficient for regulated expression. Therefore, regulation likely requires either multiple elements both upstream and downstream of +60, or a single element that spans +60.

Repression of Dihydrofolate Reductase Synthesis During Myogenesis: Identification and Characterization of a Transcriptional Regulatory Mechanism

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DEDICATION AND ACKNOWLEDGMENTS

"Education," wrote Albert Einstein, "is that which remains if one has forgotten all that he learned in school." My greatest debt is to he who gave me this education, a teacher who instructed me long before I began graduate school: my father, Arthur R. Schmidt. My education was given in practical mechanics. I learned a layman's version of the scientific method: identify a problem, predict the cause, formulate rational expectations based on this prediction, and test whether the expectations are correct. Although the object of my studies has shifted to the mechanisms of gene regulation, the approach is identical.

My father was a patient teacher and my best friend. On October 25, 1988, while we were camping together, he suffered a heart attack and died in my arms. In appreciation for all he gave me, I dedicate this thesis to my father.

In my opinion, the zenith of formal education, the degree of Doctor of Philosophy, embraces the value of integrating intellect, fact, and skill. When one is awarded a Ph.D., it indicates that they have attained sufficient facts to identify previously unanswered problems, sufficient intellect to reason beyond the facts, and sufficient skill to solve the problems. I am grateful to all who contributed to my formal education. Above all, I thank my thesis advisor, Gary Merrill. Gary provided outstanding direction in all facets of my graduate training. Numerous people additionally contributed to the work presented in this thesis. Technicians Becky Owen and Mark Kainz were the most generous and enjoyable contributors; previous graduate student Michael Gross was an effective role model and a good friend; undergraduates Andrew Pearson and Scott Bender were fast-learning, hard-working assistants; our departmental chairman, Chris Mathews, was a patient and attentive reviewer of my first two manuscripts and of this thesis; graduate student Christian Gross has been a good friend.

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Edward E. Schmidt
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CHAPTER 1

Introduction

THE CELL CYCLE

Proliferation is the concerted product of growth, replication, and division. Growth is the accumulation of cell mass; replication is duplication of the genetic material; division (cytokinesis) is fission of the parental cell into daughter cells. To ensure fidelity of transfer of the genetic material in eucaryotic cells, replication and cytokinesis occur in an orderly repetitive fashion termed the cell cycle. Thus, cells alternate between periods of replication, wherein a complete copy of the genome is synthesized, and division, wherein the genetic material is equally partitioned between the two daughter cells. In contrast, growth, as measured by rates of overall RNA and protein synthesis, occurs throughout the cell cycle.

The cell cycle is broken down into four periods: S, G2, M, and G1. Replication occurs in S phase, during which time the cell progresses from a diploid to a tetraploid state. After replication is completed, cells enter a tetraploid state termed G2. Segregation of the tetraploid genome into two diploid daughter cell equivalents occurs during the rapid and dramatic M phase. M phase culminates with cytokinesis. The mitotic machinery then rapidly decays as the diploid daughter cells enter G1 phase. The cycle is completed as G1 cells reenter S phase and begin another round of replication.

Progression through the cell cycle requires expression of numerous genes. By

isolating 35 mutant strains of the yeast *Saccharomyces cerivisae* that demonstrated growth-arrest when shifted to a nonpermissive temperature, Hartwell (1974, 1978) genetically characterized a series of *cell-division-cycle* (*cdc*) genes whose action is required for progression through the cell cycle. Individual *cdc* mutants arrest at different stages in the cell cycle. The greatest number of *cdc* mutants arrest during DNA synthesis (S phase). All *cdc* genes are required for progression through the cell cycle; however, that they are required is insufficient to implicate any of these genes in regulating cell cycle progression. For example, *cdc* 21, which is required for progression through S phase, encodes thymidylate synthase (Hartwell, 1978). Nevertheless, mammalian cells that overproduce thymidylate synthase by about 50-fold exhibit normal growth and cell cycle kinetics (Imam *et al.*, 1987; Navalgund *et al.*, 1980).

Embryonic cleavage: A simple cell cycle regulated by internal oscillators - Oogenesis, meiosis, and the early zygotic cleavages are convenient developmental events for investigations into cell cycle control. During maturation, the oocyte stockpiles replicative proteins and mRNAs encoding replicative proteins for the rapid burst of mitoses that follows. Despite the stockpile of replicative machinery, the mature oocyte arrests at the G2/M boundary in the cell cycle (reviewed by Whitaker and Steinhardt, 1985; Murray and Kirschner, 1989b). One can rationalize, then, that any components present in the cache of proteins, although possibly necessary, are either not limiting for entry into M phase or are not in an active conformation. Thus, any components present in an active conformation likely are not the cell cycle-regulatory factors. Included in this cache of proteins are DNA polymerase (Fansler and Loeb, 1969; Zierler *et al.*, 1985), ribonucleotide reductase small subunit (Standart *et al.*, 1985, 1986), histones (Woodland and Adamson, 1977), nucleoplasmin proteins (Earnshaw *et al.*, 1980), nuclear lamins (Lohka and Masui, 1983; Forbes *et al.*, 1983), components of the mitotic

spindle (Raff, 1975; Mohri *et al.*, 1976; Vallee and Bloom, 1983; Scholey *et al.*, 1985), actin (Mabuchi and Spudich, 1980), and myosin (Mabuchi and Okuno, 1975). In contrast to these stockpiled proteins, the synthesis or activity of the factor or factors limiting entry into M phase must be induced by fertilization. Consistent with this hypothesis, numerous studies have demonstrated that after fertilization, a short period of protein synthesis (35 min in *Stronglyocentrotus*) is required before the zygote can enter M phase (Hultin, 1961; Wilt *et al.*, 1967; Young *et al.*, 1969; Wagenaar and Mazia, 1978; Wagenaar, 1983; Newport and Kirschner, 1984; Gerhart *et al.*, 1984; Howlett, 1986). Presumably, during this period, the cell cycle-regulating factors accumulate sufficiently to induce entry into M phase. The replicative genes whose products are cached as mRNA in the oocyte could encode the proteins that limit entry into M phase. In this scenerio, however, it is not the limiting protein that regulates entry into M phase. Rather, the mechanism that prevents accumulation of the protein in the presence of the cognate mRNA regulates cell cycle progression. Indeed, in *Drosophila*, the meiotic cleavages and the first 13 zygotic cleavages occur before the onset of zygotic transcription (Gutzeit, 1980) and are not blocked by RNA polymerase inhibitors (Edgar *et al.*, 1986). However, inhibition of translation at any point during early development blocks entry into the subsequent M phase (Edgar and Schubiger, 1986; Newport and Kirschner, 1984), suggesting that release into M phase is regulated by a mechanism that relieves a translational block, allowing synthesis of a protein, which in turn, is limiting for cell cycle progression.

Models of cell cycle regulation allow predictions of properties of the regulatory factor. Although fertilization triggers the first nuclear divisions, once induced, cell cycle regulation is autonomous. Consequently, an intracellular "biological clock", or "timer" is required. Currently, the best models consistent with this requisite involve threshold-activation by an oscillator. By these models, the limiting factor is continuously synthesized; however, the cell cannot enter M phase until a threshold level

of the factor accumulates. Mitosis, then, must be accompanied by a rapid transient reduction in levels of the factor, such that mitosis will not occur again until threshold levels of the factor reaccumulate (Draetta *et al.*, 1989; Edgar and O'Farrell, 1989; Evans *et al.*, 1983; Gautier *et al.*, 1990; Labbe *et al.*, 1989b; O'Farrell *et al.*, 1989, 1990; Standart *et al.*, 1987; Swenson *et al.*, 1986; Whitfield *et al.*, 1989). Thus, levels of the limiting factor oscillate with the cell cycle.

Recent studies have implicated several cellular factors and genes in regulating progression through the early embryonic cell cycle (reviewed by Hartwell and Weinert, 1989; Murray and Kirschner, 1989b; O'Farrell *et al.*, 1989). Included are "maturation-promoting factor" (MPF, which is a chemically nondefined multicomponent cellular fraction that triggers entry of immature *Xenopus* oocytes into mitosis)(Masui and Markert, 1971; Gerhart *et al.*, 1984; Dunphy *et al.*, 1988; Gautier *et al.*, 1988), the "cyclin" genes (which are homologous to *Schizosaccharomyces pombe cdc*13, and like MPF, can trigger early cleavages)(Evans *et al.*, 1983; Goebl and Byers, 1988; Lehner and O'Farrell, 1989, 1990; Solomon *et al.*, 1988; Standart *et al.*, 1987; Swenson *et al.*, 1986), $p34^{cdc2}$ (a protein kinase encoded by the *cdc2* gene of *S. pombe*)(Draetta and Beach, 1988; Moreno *et al.*, 1989; Simanis and Nurse, 1986), and *string* (*stg*, which is homologous to *S. pombe cdc*25)(Edgar and O'Farrell, 1989). The evidence linking each of the above components to mitosis merits examination. Also, whether cell cycle progression in the early embryo is regulated by the same mechanism as somatic cell cycle progression merits consideration.

MPF will induce mitosis in immature oocytes and therefore likely contains all components necessary for regulating cell cycle progression (at least during early development). However, MPF is a complex cellular preparation. Identification of all of the components and clarification of the roles of each will probably be long in coming.

The cyclin genes (*cyclin A* and *cyclin B*) were first identified in several species of marine invertebrates (Evans *et al.*, 1983; Swenson *et al.*, 1986) as proteins that are

synthesized continuously after fertilization and are rapidly degraded near the end of M phase. Injection of synthetic cyclin A or B pseudo-mRNA into *Xenopus* oocytes induces meiosis 1 (Evans *et al.*, 1983; Murray *et al.*, 1989; Murray and Kirschner, 1989; Swenson *et al.*, 1986), implicating the cyclin genes in cell cycle control. It is noteworthy, however, that the oocyte contains a large stockpile of cyclin A and B mRNA (Swenson *et al.*, 1986). Thus, the endogenous cyclin mRNA in the oocyte probably is translationally inhibited, whereas the injected messages are translated.

Mitosis can be inhibited in *Xenopus* embryos by injecting RNase into the oocyte. However, when RNase treatment is followed by an injection with a large excess of RNase inhibitor and synthetic cyclin pseudo-mRNA, oocytes regain mitotic proficiency (Murray *et al.*, 1989). Therefore, the requisite of protein synthesis for entry into M phase is, more specifically, a requisite for cyclin synthesis.

All biologically-active preparations of MPF that have been characterized contain $p34^{cdc2}$ (Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Labbe *et al.*, 1989a,b). $p34^{cdc2}$ is necessary for mitosis in *S. pombe* (Simanis and Nurse, 1986). The sequence of $p34^{cdc2}$ shares similarity with known protein kinases (Lee and Nurse, 1988). Entry into M phase is accompanied by phosphorylation of histone H1 (Arion *et al.*, 1988; Labbe *et al.*, 1988; Langhan *et al.*, 1989). All preparations that demonstrate histone H1 kinase activity contain $p34^{cdc2}$, and mitosis will not occur in the absence of histone H1 kinase activity (Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Lee and Nurse, 1988; Murray and Kirschner, 1989a). Therefore, it is thought that $p34^{cdc2}$ is the enzyme responsible for histone H1 kinase activity, and, as a kinase, $p34^{cdc2}$ regulates mitosis. However, $p34^{cdc2}$ is constitutively present in cells (Draetta and Beach, 1988; Moreno *et al.*, 1989). Therefore, for $p34^{cdc2}$ to regulate the cell cycle, its activity, not its synthesis, must be regulated. This observation is inconsistent with $p34^{cdc2}$ determining the time of mitosis because it cannot explain the requisite of translation for entry into M phase. Also, histone H1 phosphorylation occurs in a rapid burst during mitosis, not as a

continuous process that achieves a threshold level at M phase (Arion *et al.,* 1988; Labbe *et al.,* 1988; Langhan *et al.,* 1989). Thus, $p34^{cdc2}$ does not fulfill the predictions of the threshold model for a cell cycle regulatory protein. $p34^{cdc2}$ likely does not determine the rate of cell cycle progression, but rather, is only a cog in the regulatory system.

An attractive model for regulation of the cell cycle has been proposed to explain the empirical activities of cyclin and $p34^{cdc2}$ (Murray and Kirschner, 1989a, b; Murray *et al.,* 1989). In this model, cyclin proteins are the threshold-acting regulatory molecules that determine the time of entry into M phase. Specifically, the activity of cyclins is to activate $p34^{cdc2}$. When cyclin levels reach the threshold, $p34^{cdc2}$ acts as the firing pin in the triggering mechanism by activating the mitotic enzymes through a series of rapid transient phosphorylations. During late mitosis, cyclin proteins are degraded, $p34^{cdc2}$ is inactivated, and G1 ensues. Consistent with this model, cyclin proteins are physically associated with $p34^{cdc2}$ (Booher *et al.,* 1989; Draetta *et al.,* 1989; Labbe *et al.,* 1989b; Meijer *et al.,* 1989; Pines and Hunter, 1989). Additionally, in the absence of cyclin proteins, neither histone H1 kinase activity nor MPF activity are detectable, and cells fail to enter mitosis (Booher and Beach, 1988; Minshull *et al.,* 1989; Murray *et al.,* 1989; Murray and Kirschner, 1989a).

A series of recent investigations into the roles of cyclin in regulating the early cleavages in *Drosophila* development, however, question the role of cyclins as the threshold regulators of cell cycle progression (Edgar and O'Farrell, 1989; Lehner and O'Farrell, 1989, 1990). A simple prediction from the threshold model is that a specific level of the regulatory factor is required for triggering mitosis. A corollary to this prediction, then, is that rapidly dividing cells must accumulate the regulatory factor (and thereby reach the threshold level) faster than slowly dividing cells. However, when the rate of cyclin accumulation was measured in the cells of *Drosophilia* embryos, Lehner and O'Farrell (1989) noted that after mitosis 13 (at which point the rapid synchronous cleavages end and cells assume distinct asynchronous rates of cell cycle progression) all

cells continue to accumulate cyclin proteins at similar rates. Interestingly, even cells that stop dividing after mitosis 14 accumulate levels of cyclin proteins higher than the levels achieved during each of the 14 early cleavages. The authors conclude that whereas cyclins are required at a threshold level for mitosis, rates of cyclin accumulation do not determine the time of entry into M phase in *Drosophila* embryos after the 13th cleavage.

Because the first 13 cleavages of *Drosophilia* development occur in the absence of transcription (using only maternally-derived proteins and proteins translated from maternally-derived messages)(Edgar and Schubiger, 1986), homozygous mutant embryos at the regulatory factor locus should complete the first 13 cleavages using maternally-derived messages, and then arrest in G2 of the 14th cell cycle. Established mutant strains of *Drosophila* that arrested division before cleavage 14 were screened to identify genes that regulate cell cycle progression (Edgar and O'Farrell, 1989). Eight P element insertion mutants in the *stg* allele (Jurgens *et al.*, 1984) demonstrated a homozygous recessive lethal phenotype that was preceded by a deficiency in the number of cuticular structures in the embryo. Histological analysis demonstrated that the *stg* mutants contained a reduced number of cells, which suggested the embryos were defective in cell proliferation (Edgar and O'Farrell, 1989). Subsequent analysis indicated that proliferation ceases after cleavage 13; however, curiously, mass accumulation and differentiation continue normally until a late embryo state (Edgar and O'Farrell, 1989). The *stg* gene was cloned and its expression was followed during development in wildtype flies. Similar to cyclin proteins, levels of *stg* protein fluctuate with the cell cycle, reaching a maximum at mitosis and then rapidly decaying. In contrast to cyclins, however, rates of accumulation of *stg* protein during the later cleavages match the rate of cell cycle progression (Edgar and O'Farrell, 1989).

Sequence analysis of the *stg* gene suggests it is a homolog of *S. pombe cdc*25 (Edgar and O'Farrell, 1989). Similar to *stg*, genetic analyses indicate that *cdc*25 is required for progression into M phase, and that overexpression of *cdc*25 causes premature

initiation of mitosis (reviewed by Edgar and O'Farrell, 1989). Subsequent experiments showed transfected *stg* genes can complement *cdc*25 mutations in *S. pombe*, indicating *stg* has been conserved as a *cdc*25 analog (i.e., exhibits similar function).

Both the cyclins and *stg* are required for cell cycle progression before and after cleavage 14; both proteins are encoded by maternal mRNAs for the first 13 cleavages and then are translated from zygotic messages; and both proteins are continously translated, but are rapidly degraded during mitosis, causing oscillations in the level of each protein. The studies by O'Farrell's group suggest that during the first 13 cleavages, *stg* is expressed above threshold; entry into M phase is limited by levels of cyclin. After cleavage 13, cyclin levels exceed threshold, and entry to M phase is limited by rates of accumulation of *stg* (O'Farrell *et al.*, 1989). The rationale for this is as follows. Cyclin levels are limited in the mature oocyte by a translational block; *stg* may not be subject to this block. Therefore, cyclin levels are a better regulator of zygotic induction. Levels of *stg* protein and *stg* mRNA show cell type-specific differences in rates of accumulation in the zygote. *In situ* labeling of nascent *stg* pre-mRNA shows differential rates of transcription of the *stg* gene in postblastula embryos (O'Farrell *et al.*, 1989). Thus, O'Farrell *et al.* (1989) postulate that rates of transcription of the *stg* gene are controlled by the "pattern forming" gene products, which are cell type-specific transcription factors. Thus, *stg*, by being transcriptionally regulated by tissue-specific factors, matches rates of proliferation to the embryonic tissue type in postblastula embryos.

Evidence in *S. pombe* suggests that there are several genes upstream of *cdc*25 (*stg*) in the regulatory cascade. Specifically, the gene *wee*1 acts as an inhibitor of *cdc*25. In strains of yeast deficient in both *wee*1 and *cdc*25, induced overexpression of *cdc*25 causes premature (and often lethal) entry into M phase; induced overexpression of *wee*1 causes a delay in entry into M phase resulting in a larger cell size (Russel and Nurse, 1986; 1987a). Expression of the *wee*1-dependent phenotype, in turn, is inhibited by overexpression of *nim*1 (Russel and Nurse, 1987b). The genes of both *wee*1 and *nim*1

have regions that show some sequence similarity with protein kinases (Russel and Nurse, 1987b), again suggesting phosphorylation is a key mechanism by which the proteins that control cell cycle progression are activated and inactivated.

Perhaps the most important implications that arise from Edgar and O'Farrell's (1989) results implicating *stg* as regulating cell cycle progression in the *Drosophila* embryo is that cell cycle progression is not regulated by a single mechanism in all cases. Indeed, other results from O'Farrell's group (Lehner and O'Farrell, 1990) suggest that maternal cyclin A, but not cyclin B, regulates the first 13 cleavages in *Drosophila* Thus, one is led to question what roles cyclin A, cyclin B, *stg*, or $p34^{cdc2}$ play in regulating postembryonic cell cycle progression.

Beyond the simple oscillator: Checkpoints and feedback in the somatic cell cycle - Unlike regulation of early embryonic cleavages, wherein only accumulation of an oscillating regulator is required for cell cycle progression, regulation of cell cycle progression in yeast and postembryonic metazoan cells is subject to feedback control by events throughout the cell cycle. Thus, as discussed above, *cdc* mutants have been isolated that arrest growth throughout the cell cycle (Hartwell, 1974, 1978). Each point at which passage through the cell cycle can be arrested is termed a "checkpoint" (reviewed by Murray and Kirschner, 1989b). For example, somatic cells have mechanisms for blocking cell cycle progression in S phase until DNA is completely replicated. Replication, in turn, is blocked by lesions in the DNA (reviewed by Varshavsky, 1983). Thus, each step is dependent on completion of the previous step.

Hartwell and Weinert (1989) liken the postembryonic cell cycle to a self-assembly process, much like self-assembly of T4 bacteriophage particles. T4 is assembled from preformed head, tail, and tail-fiber subunits by a defined pathway (reviewed in Mathews *et al.*, 1983). Although to form the phage particle, tail subunits must associate to form a tube, tail subunits do not self associate in solution. Rather, tail

subunits must assemble on the baseplate. Thus, tail assembly is dependent on accurate completion of the preceding step. Similarly, onset of any portion of the cell cycle is dependent on accurate completion of the previous step. In both T4 assembly and cell cycle progression, a series of checkpoints ensures fidelity.

Somatic cell generation times are determined by varying the length of G1 phase, not by delaying the G2/M transition. Thus, most somatic cells have S, G2, and M phases of similar duration; however, the length of G1 can vary from essentially zero to infinity (reviewed by Pardee, 1989). This alone indicates that progression through the somatic cell cycle is regulated differently than the mechanisms described for the embryonic cleavages.

When yeast or cultured somatic cells are treated with inhibitors of protein synthesis, a point in mid-G1 phase (termed "start") is found after which cells are committed to S phase and will synthesize DNA even in the absence of additional protein synthesis (Croy and Pardee, 1983; Campisi *et al.*, 1984; Hartwell, 1974, 1978; Pardee, 1989). Start is the putative restriction point at which cell cycle progression is regulated in postembryonic cells. Curiously, Nurse and Bissette (1981) reported that as well as being required for entry into M phase, the *S. pombe cdc2* gene was required for progression through mid-G1. Presumably both the substrates for the $p34^{cdc2}$ kinase and the mechanisms activating the $p34^{cdc2}$ kinase are distinct for passage through G1 and entry into M phase (Lee and Nurse, 1988).

Oncogenes and regulation of proliferation - Oncogenes can stimulate entry of postreplicative somatic cells into the cell cycle. Therefore, it is likely that oncogene products either act upon cell cycle-regulatory genes (directly or through second messengers) or are cell cycle-regulatory genes. It is curious that none of the putative cell cycle regulatory genes discussed above have been implicated as protooncogenes and none have been shown to interact with oncogene products. This observation suggests that

the mechanisms regulating somatic cell proliferation are, to a large extent, distinct from and more complex than the mechanisms regulating early embryonic cleavages.

Oncogenes are viral derivatives or mutated versions of normal cellular "protooncogenes" (reviewed by Bishop, 1987). Viruses probably salvaged oncogenes from cellular mRNA; cellular oncogenes generally are point-mutants of the endogenous protooncogene. Oncogenes disrupt regulatory mechanisms and allow aberrant cell proliferation. By characterizing oncogenes, one can infer the cellular functions of the cognate protooncogenes that are necessary for controlling proliferation. Such investigations reveal a complex network. The oncogene TGF-α is an EGF homolog; v-*sis* is a PDGF subchain homolog (Huberman *et al.*, 1987). v-*erb-B* is an EGF receptor; H-*ras* is a G-protein that probably acts in signal transduction; v-*src* is a protein kinase; and *fos* and *jun* encode transcription factors (reviewed in *Molecular Biology of Signal Transduction*, 1988). Thus, one is led to conclude that extracellular hormones and hormone receptors, second messengers and protein kinases, and transcriptional regulatory factors interact to modulate cell proliferation.

Nononcogenic regulators: Is half the story missing? - The embryonic cell cycle regulators induce entry into M phase; yeast and postembryonic metazoan cells are induced to traverse start and the various checkpoints; the oncogenes induce unchecked proliferation. Naturally, one is led to assume that proliferation and cell cycle progresssion are modulated solely by activators. However, it is not clear whether all regulatory factors are activators, or whether the repressors have simply escaped attention.

For an oncogene to be detected (and for an oncogene to fulfill its role in tumorigenesis), it must promote proliferation as a dominant trait. Thus, although mutations that inactivate protooncogenes are masked by heterozygous dominant effects, mutations that cause constitutive activation (i.e., conversion to an oncogene) induce

tumorigenic proliferation. In contrast, repressors would tend to inhibit proliferation as a dominant trait. Mutations that inactivate proliferation inhibitors would be masked by heterozygous effects; mutations that cause constitutive activity would inhibit proliferation, thereby preventing isolation of mutant clones. Therefore, only a subset of the regulatory genes (viz., the oncogenes) can be easily identified by their phenotype.

The absence of repressors from the library of known regulatory factors can lead one to assume that proliferation is controlled solely by positive effectors. However, such an assumption violates our understanding of metazoan evolution. In general, single cell organisms proliferate unchecked (although, as exemplified by yeast, individual steps in the cell cycle are feedback-regulated). Thus, a proliferative state can be considered the "ground state," upon which advancements were built. Genes that promote proliferation (the oncogenes) would be inconsequential to organisms that already proliferate unchecked. Genes that repress proliferation, on the other hand, allow the type of growth control that is required for progression to multicellular life.

Alternative approaches for studying regulation of proliferation - Despite great advances in our understanding of regulation of embryonic cleavages and yeast division, we are far from understanding the mechanisms of metazoan growth control. It is likely that the methods used to investigate regulation of the embryonic cleavages will be insufficient to resolve the mechanisms regulating proliferation in postembryonic metazoan cells. Although *Drosophila* provide a powerful genetic tool, most mutations affecting regulation of somatic cell proliferation will be lethal. Similarly, the mechanisms of cell cycle control are far too complex to allow interpretation of studies wherein total translation or transcription are inhibited.

Characterization of the activities of oncogenes has provided great inroads to the mechanisms controling somatic cell proliferation; however, we cannot yet describe a single branch of the regulatory network from signal to effect. A part of the problem is

that the chemical and biological activities of many of the identified regulators remain uncharacterized. For example, no enzymatic activity can be attributed to cyclin proteins. Cyclins probably act through direct protein-protein contacts (most likely with $p34^{cdc2}$); however, such hypotheses are difficult to verify. In contrast, some oncogenic proteins have been well characterized. For example, *jun* is a homolog of AP1, which is a cellular transcription factor. *jun* forms a heterodimer with *fos* via "leucine zipper" dimerization sites and binds to a specific DNA sequence, thereby activating transcription from adjacent promoters (reviewed by Johnson and McKnight, 1989; Perkins *et al.*, 1990). How *fos* and *jun* induce proliferation or tumorigenesis is unknown.

Because neither the studies on embryonic cleavages, yeast cell cycles, nor oncogene activities have unveiled the machinery that controls somatic cell proliferation, one is encouraged to attack the problem from other directions. One approach likely to yield information on regulation of proliferation is a series of rigorous molecular dissections of the mechanisms regulating expression of growth-dependent genes. The rationale for this prediction follows. At some point, genes that regulate proliferation must regulate expression of proliferation-dependent genes. Thus, when one investigates the mechanism regulating expression of a proliferation-specific gene, one is likely to identify a part of the mechanism regulating proliferation. By pursuing regulation in this "reverse direction," one may detect important regulatory factors, including both activators and repressors, that were missed in studies of cell cycle control and tumorigenesis.

GENE REGULATION

Every somatic cell in an organism is genetically identical; however, differences between cells in an organism are primarily determined genetically. The paradox is partially resolved when one notes that genes in different cells within an organism are differentially expressed. A large subset of genes are required for general cell function

and homeostasis. Although this subset will be ubiquitously expressed, the biochemical determinants that differentiate a hepatocyte, a myocyte, a chondrocyte, a thrombocyte, and an osteocyte can be traced to the differential expression of tissue-specific genes in each cell. Cells become committed to specific fates (i.e., committed to express only a specific subset of tissue-specific genes) during embryogenesis. How cell fate is determined is largely unknown.

Although a typical mammalian genome contains an estimated 4×10^4 genes, a cell will express only about 30% of these (Lewin, 1987), and at a given time, the cell likely expresses less than half again that number. Highly elaborate regulatory mechanisms are required to ensure that each gene is expressed (or repressed) appropriately.

Growth-dependent gene regulation *vs.* replication-specific gene regulation - Growth and replication are separable processes. Perhaps the best example of this has already been presented in characterization of the *Drosophila stg* mutants (Edgar and O'Farrell, 1989). The *stg*$^-$ mutants arrest cell cycle progression prior to the 14^{th} zygotic cleavage; however, growth and differentiation continue. The result is embryos that, macroscopically, are nearly normal, but histologically, are composed of only a fraction of the wildtype number of cells. The *stg*$^-$ embryo is not reduced in size. Rather, the cells of *stg*$^-$ embryos are megaloblastic.

Under most conditions, however, growth and replication are coordinated. When cells cease replication, accumulation of organic mass also must cease to maintain homeostasis. The importance of matching rates of accumulation of cellular constituents to the rate of cytokinesis varies directly with the stability of each molecule. The realized lifetime of a molecule in a cell will be determined both by the intrinsic half-life of the molecule and by the rate of cytokinesis (which partitions cellular material to the daughter cells). For a stable molecule in dividing cells, cytokinesis constitutes the major source of loss, whereas for an unstable molecule, cytokinesis contributes only

negligibly toward loss. Thus, if cell division ceases while synthesis and degradation rates remain constant, levels of the unstable molecule will increase only slightly, whereas levels of the stable molecule may increase by orders of magnitude.

To demonstrate the point, a comparison can be made between two hypothetical proteins, X and Y, which have intrinsic half-lives of 1hr and 100hr, respectively. To maintain constant levels in proliferative cells, both proteins are expressed such that on average, all cells in the population contain 10^5 molecules of each protein. If the cells undergo transition from a proliferative state (10-hr generation) to a postreplicative state (nondividing) without altering rates of synthesis or protein stability, how will cellular concentrations of each molecule be affected?

Both the intrinsic half-lives and cytokinesis cause exponential loss of the proteins. Protein X will have a realized half-life ($t_{1/2x}$) of 0.9091hr in proliferative cells (1hr intrinsic half-life, 10hr generation time); protein Y will have a realized half-life ($t_{1/2y}$) of 9.091hr (100hr intrinsic half-life, 10hr generation time). The loss constant (k) of each protein is defined by: $\ln 0.5 = kt_{1/2}$, and the instantaneous rate of loss equals kN_i, where N_i is the instantaneous concentration of protein. For molecule X in proliferative cells, $k_x = -0.763$. With 10^5 molecules per cell, the instantaneous rate of loss is: $r_{ix} = -7.63 \times 10^4$ molec cell^{-1}hr^{-1}. For Y, $k_y = -0.0763$; $r_{iy} = -7.63 \times 10^3$ molec cell^{-1}hr^{-1}. To maintain steady state levels of proteins X and Y, rates of synthesis in proliferative cells must equal the instantaneous rates of loss. Therefore, X is synthesized at rate: $s_x = 7.63 \times 10^4$ molec cell^{-1}hr^{-1}; Y is synthesized at: $s_y = 7.63 \times 10^3$ molec cell^{-1}hr^{-1} (note that s is given as a linear rate indicating that synthesis is independent of protein concentration).

When cell division ceases, protein is lost only to decay. Thus $t_{1/2x}$ = 1hr; $t_{1/2y}$ = 100hr. The postreplicative loss constants will be: $k_{ix} = -0.693$; $k_{iy} = -0.00693$. The instantaneous rates of loss ($N_i = 10^5$ molec) will be: $r_{ix} = -6.93 \times 10^4$ molec cell^{-1}hr^{-1}; $r_{iy} = -6.93 \times 10^2$ molec cell^{-1}hr^{-1}. However, because the synthesis rates

are unchanged (s_x = 7.63 x 10^4 molec $cell^{-1}hr^{-1}$; s_y = 7.63 x 10^3 molec $cell^{-1}hr^{-1}$), both proteins begin to accumulate. At any point in time, t, the number of molecules of either protein will be: $N_t = N_{t-1} + sdt + r_i dt$; $r_i = kN_{t-1}$. Because rates of decay (r_i) are concentration-dependent, they will increase as protein accumulates. Because rates of synthesis are concentration-independent, a new steady-state level of each protein will be approached in time. By solving for N_t as t goes from 0 to infinity in the equation $N_t = N_{t-1} + sdt + kN_{t-1}dt$, one finds that levels of protein X approach a steady state concentration of 1.5 x 10^5 molec $cell^{-1}$; levels of protein Y exceed 1.5 x 10^5 molec $cell^{-1}$ within 7 hours after ceasing replication, and approach a steady-state level of 1 x 10^6 molec $cell^{-1}$. Thus, in the absence of growth-dependent regulation, levels of Y are overexpressed by a factor of about 10-fold in postreplicative cells. To maintain levels of Y at 10^5 molec $cell^{-1}$, the rate of synthesis should decrease to match the instantaneous rate of decay of Y in postreplicative cells (s = 6.93 x 10^2 molec $cell^{-1}hr^{-1}$, an 11-fold decrease from the proliferative rate of 7.63 x 10^3 molec $cell^{-1}hr^{-1}$).

Matching gene expression to cellular conditions - In the previous sections of this dissertation, the mechanisms regulating of replication and growth were reviewed and regulation of gene expression was considered. This background serves to describe the various conditions or "growth states" to which cells must adapt during their life (including both transitions between phases in the cell cycle and the transition from a proliferative to a postreplicative state); it points out some key regulators active in determining the growth state of a cell; and it describes the changes in gene expression that will be necessary for adapting to the various states. At this point, one must consider how gene expression is coordinated with the growth state of the cell. Unfortunately, the mechanisms of coordination represent an important "missing link" in cell biology. To date, only speculative mechanisms can be proposed.

Genes that are expressed only at a particular period in the cell cycle are likely

regulated by activities that demonstrate either the same (activators) or the opposite (repressors) pattern of expression. In other words, the regulatory activity must oscillate with expression of the gene. Regulatory activities (or fractions of multicomponent activities) likely regulate numerous genes. For this reason, genes that demonstrate similar temporal patterns of expression are often considered as a "class."

A noteworthy requirement for cell cycle gene regulation is an internal oscillator. Thus, regulation of the cell cycle and regulation of cell cycle-dependent genes share a common requirement. Because they share a common requirement, and because the two events are tightly coordinated, they probably share the same oscillator. However, no experimental evidence directly links cyclin, *stg*, or $p34^{cdc2}$ to regulation of cell cycle-specific genes. Similarly, whereas cell cycle-regulated genes may be responsive to altered expression of various oncogenes, in no case has an oncogene been shown to determine the periodicity of expression of a gene. Rather, one may posit that there are intermediates through which the cell cycle-determining activity regulates gene expression.

To match rates of macromolecule synthesis to the rate of cell proliferation, two possible mechanisms can be postulated. First, some process in the synthetic pathway could be sensitive to the proliferative state of the cell. For example, a regulator that enhances rates of overall protein synthesis during periods of DNA synthesis (S-phase) could ensure accumulation of a second cell equivalent of all cell components prior to mitosis without causing overproduction of the components in postreplicative cells. By this model, a single regulator could control rates of synthesis of numerous macromolecules. Alternatively, macromolecule synthesis could be subject to feedback regulation. Thus, when levels of a macromolecule exceed a certain level, synthesis would abate regardless of the proliferative state of the cell. A modification of the latter model would be that rates of synthesis of numerous molecules (i.e., a "class" of proteins) are feedback inhibited by overexpression of a single molecule in the class. By this

modification, a single mechanism could control expression of multiple genes.

To date, the mechanisms of growth-dependent gene regulation have received little attention. Rather, most gene regulation studies have concentrated on replication-dependent, hormone-dependent, stress-dependent, tissue-specific, and developmental gene expression.

Levels of gene regulation - Gene expression is the net result of a complex pathway that allows a specific sequence of DNA in the genome to ultimately direct a specific chemical reaction at a precise location. Regulation determines when and at what rate the reaction will occur.

The first step in the gene expression pathway is transcriptional initiation (defined here as all events occuring before formation of the first phosphodiester bond in the nascent pre-mRNA). Included in initiation are conformational changes in the chromatin structure, binding of enhancer elements, assembly of the transcription complex at the promoter, melting of the DNA strands to make the template strand accessible, and incorporation of the first ribonucleotide. Initiation is followed by transcriptional elongation, which involves strand displacement, synthesis of a pre-mRNA molecule complementary to the sequence of the DNA template strand, and perhaps additional changes in chromatin structure. Transcripional termination results in pre-mRNA release and dissociation of the RNA polymerase complex. RNA processing follows, at which time the message is "capped" (addition of 7-methylguanosine-5'-triphosphate to the 5' end of the transcript), polyadenylylated (removal of 3' teminal residues and addition of adenylate moieties by a terminal transferase activity), and spliced (removal of introns) to yield mRNA. The mRNA is then transported to the cytoplasm. In the cytoplasm, mRNA is engaged by ribosomes during translational initiation. Translational elongation yields polypeptides composed of amino acids polymerized in a sequence determined by the nucleotide sequence of the mRNA. During translational termination the

polypeptide is released into the cytosol (or into a specific subcellular compartment). The protein may be posttranslationally modified, often including enzymatic removal of the N-terminal Met residue and covalent addition of various prosthetic groups (including phosphate moieties, various sugars, and nucleotide and fatty acid derivatives). The protein must fold into a specific conformation and associate with other proteins (often other subunits of a multimeric complex), coenzymes, or prosthetic groups (metal ions, hemes, allosteric effectors, etc.). Finally, for enzymatic activity, the protein must be provided substrates within specific concentration ranges. Formation of product from the substrates is the net effect of an actively expressed gene.

Theoretically, regulation of gene expression can occur at any point in the pathway. In addition, regulation can occur by altering the stability of any intermediate in the pathway or by sequestering an intermediate in an inaccessible compartment. In all cases, overall flux through the pathway will be altered. To identify which step in formation of a specific gene product is regulated, one generally proceeds backwards along the expression pathway, from enzyme activity to transcription, until a constitutively expressed intermediate is identified. When possible, to confirm the intervening reaction as the regulatory step, the rates of that reaction are compared under conditions of gene expression and repression. For example, if protein levels are regulated but translation rates are constitutive, one may hypothesize that protein stability is regulated. To confirm this, one could pulse-label cellular proteins, immunopurify the protein at various times during a chase period, and measure the rates of decay under conditions of expression and repression.

1. Transcriptional regulation - Because this thesis study involves identification and characterization of a transcriptional regulatory mechanism, mechanisms of transcriptional control will be elaborated upon. Because not all blocks of transcribed DNA are genes, the term "transcription unit" is established here to denote any continuous

region of transcribed DNA and the adjacent sequences required to direct that transcription (the term will be used extensively in CHAPTER 4).

Transcriptional regulation is any process that controls the rate of formation of pre-mRNA. By this definition, transcriptional regulation can occur by changing the rates of initiation or elongation. By far the most extensively studied form of regulation is at the level of initiation; the only examples of elongational regulation involve transcriptional pretermination.

a. Initiation - It has long been assumed that the first requirement for transcription is that the proximal chromatin must be in a conformation that is compatible with transcription. By this postulate, one predicts that some regions of chromatin are in an "active" conformation whereas others are in an "inactive" conformation. Although this model is teleologically pleasing in that it allows large numbers of genes to be irreversibly inactivated simply by sequestration, it has not been experimentally supported. Rather, indirect evidence *in vitro*, on transformed cells, and in transgenic animals suggests the model is incorrect. First, *in vitro* transcription of the albumin gene indicates: A) sequences sufficient for correct tissue-specific transcription lie within the 170 base-pair promoter; B) correct tissue-specific transcription does not require chromatin or supercoiling; and C) all cellular factors necessary for correct tissue-specific transcription are present in chromatin-depleted nuclear extracts (Gorski *et al.*, 1986; Maire *et al.*, 1989; Mueller *et al.*, 1990). Second, cultured cells transformed with genes fused to transcriptionally regulated promoters (e.g., albumin [Cereghini *et al.*, 1987; Maire *et al.*, 1989], creatine kinase [Buskin and Hauschka, 1989], and thymidine kinase [Gross *et al.*, 1987]) generally demonstrate correct tissue-specific and growth state-dependent transcription. Third, transgenic animals demonstrate correct tissue-specific and developmental state-dependent expression of genes containing transcriptionally regulated promoters

(e.g., αA-crystallin and transferrin [Klement *et al.*, 1989; Izerda *et al.*, 1989], pyruvate kinase and phosphoglycerate kinase 2 [Robinson *et al.*, 1989; Tremp *et al.*, 1989], and α-actin and myosin light chain [Petropolous *et al.*, 1989; Rosenthal *et al.*, 1989]). Finally, in transfected cells, inducible promoters (e.g., metallothionein and prolactin [Adler *et al.*, 1988; Imbra and Karin, 1987; Karin *et al.*, 1980]) rapidly transit from an inactive to an active state upon induction. Therefore, one must conclude either that sequences within the promoter are sufficient to determine the conformation of adjacent chromatin, or that chromatin conformation does not contribute to the transcriptional state of most promoters.

b. The basic promoter - Rates and loci of transcriptional initiation are determined largely by proteins ("transcription factors") bound to specific sequences of DNA (reviewed by Breathnach and Chambon, 1981). The highest density of binding occurs at and immediately upstream of the site of transcriptional initiation ("cap site"), in the region designated the promoter. In most eucaryotic RNA polymerase 2 (pol2)-transcribed genes, transcription initiates about 30 base pairs downstream of the "TATA element." The TATA element is the site bound by transcription factor 2D (TF2D)(Fire *et al.*, 1984; Hawley and Roeder, 1985, 1987; Hu and Manley, 1981; Matsui *et al.*, 1980; Nakajima *et al.*, 1988; Reinberg and Roeder, 1987a, b; Reinberg *et al.*, 1987; Sawadogo and Roeder, 1985a, b; Schmidt *et al.*, 1989; Wasylyk *et al.*, 1980). Additional "promoter factors" are necessary for transcription to initiate; however, not all factors are necessary in all cases. Rather, various combinations occur on different promoters. Transcription factors include Sp1 and CAAT-binding proteins, which bind DNA directly (Barberis *et al.*, 1987; Chodosh *et al.*,1988a, b; Connelly and Manley, 1989; Courey and Tjian, 1988; Gidoni *et al.*, 1985; Hatamochi *et al.*, 1987; Jackson and Tjian, 1988; Jones *et al.*, 1985, 1987; Kadonaga *et al.*, 1987), and TF2B and TF2E, which work via protein-protein interactions with TF2D and pol2 (Buratowski *et al.*, 1989; Burton *et*

al., 1986; Maity *et al.*, 1988; Reinberg *et al.*, 1987; Reinberg and Roeder, 1987a, b).

Basal transcription of a naked DNA template can be directed by a promoter containing only a TATA element and a single additional promoter element *in vitro*; however, all transcription units that are active *in vivo* probably require additional factor-binding sites. The additional sites may be located within the promoter or at a distance, and include binding sites for both general (e.g., the ubiquitous Sp1) and specific (e.g., the muscle-specific factor MyoD1) transcription factors. Elements located at a distance from the promoter are designated "enhancer elements." As well as being active at a distance, enhancer elements generally are active in either orientation.

c. The promoter as a conglomerate of discrete parts - An example of the complex composition of a transcription unit is demonstrated by the albumin gene. The albumin promoter contains at least seven different protein-binding sites (Gorski *et al.*, 1986; Herbomel *et al.*, 1989; Lichsteiner *et al.*, 1987; Tronche *et al.*, 1989). Binding sites include a TATA element and several distinct CAAT elements, including a binding site for nuclear factor 1 (NF1, a ubiquitous CAAT-binding protein) and a binding site for CAAT/enhancer binding protein (C/EBP, a transcription factor only expressed in tissues active in lipid metabolism [Birkenmeier *et al.*, 1989]). Also present are binding sites for hepatocyte nuclear factor 1 and D-binding protein (HNF1 and DBP, which are liver-specific transcription factors [Lichsteiner and Schibler, 1989; Mueller *et al.*, 1990]). Finally, a second HNF1-binding site located about 10 kilobase pairs upstream of the albumin promoter acts as an enhancer element (Pinkert *et al.*, 1987; Frain *et al.*, 1990).

The multicomponent assemblage of the albumin promoter gives the impression that promoters are conglomerates of discrete parts. As an extension of this observation, a recent report from Schaffner's group (Schatt *et al.*, 1990) is instructive. In this study, chimeric genes were constructed by fusing transcriptionally active elements.

Various combinations of promoter elements (viz., a TATA element, a GC element, and octamer element [OCT]), a general enhancer element (SV40 enhancer), and a specific promoter/enhancer element (glucocorticoid responsive element [GRE]) were fused to a reporter gene. The promoter elements were always assembled in the promoter region of the gene (zero or one copy of each); the SV40 enhancer was always fused as an enhancer at a site about 1.8 kb downstream of the reporter (zero or one copy per construct); GREs were fused either as promoter or enhancer elements (zero to six copies per construct)(all GRE data considered here are in the presence of glucocorticoid). Reporter gene message levels in transiently transfected cells demonstrated:

i. The TATA element is not required for transcription; however, it increases rates of transcription 5-fold. The TATA element determines the site of initiation; in its absence, initiation occurs at multiple sites.

ii. Promoters containing a TATA element and a single promoter element are transcribed weakly (GC or GRE) or not at all (OCT); additional elements augment activity exponentially (to a limit).

iii. Enhancer effects diminish with increasing distance from the site of initiation.

iv. Enhancers activate promoters poorly (GRE) or not at all (SV40) unless the construct contains a specific promoter element (distal GREs enhance transcription from GC/TATA, but not from OCT/TATA promoters; the SV40 enhancer works on both).

d. Repressors - All transcription elements considered above, and indeed, almost all characterized eucaryotic transcription elements, activate transcription. However, recent investigations indicate that repressors or "silencers" also contribute to the transcriptional activity of genes (reviewed by Levine and Manley, 1989). Theoretically, repressors can function through interactions with the DNA, the polymerase complex, other transcription factors, or a combination of the above. Three

models have been proposed. In the first, entitled "competition," the repressor competes with an activator for a specific binding site on the DNA. In the second, entitled "quenching" (Han *et al.,* 1989; Keleher *et al.,* 1988) the repressor interacts with either the polymerase complex or an activator via protein-protein contacts and inhibits the actvity of the polymerase or activator. The final model involves repression by an activator, and is termed "squelching" (Gill and Ptashne, 1988). Many activators function as heterodimers. In a scenario where subunit A dimerizes with either subunit B or subunit C, overexpression of B would titrate A as AB dimers, thus repressing expression of AC-dependent promoters.

By limiting gene expression, repressors increase the specificity of gene expression. For example, a hypothetical promoter consisting of a TATA element, a C/EBP-binding site (C/EBP is an activator expressed in liver and adipocytes), and an HNF1-binding site (HNF1 is an activator expressed only in liver) would be inactive in most tissues, mildly active in adipocytes, and highly active in liver. Addition of an adipocyte-specific repressor would prevent expression in adipocytes while maintaining both the C/EBP and HNF1 activational effects in liver. Addition of a binding site for a developmentally regulated repressor (expressed in all proliferative cells) would further limit expression to adult liver.

Repressors have been demonstrated on only a few genes. Repressors contribute to regulation of the yeast mating type determination gene *a-specific* (probably via quenching, Keheler *et al.,* 1988; Sauer *et al.,* 1988), the *Drosophila* homeobox genes *even-skipped* and *engrailed* (mechanism unclear, Han *et al.,* 1989; Jaynes and O'Farrell, 1988), the glucocorticoid-responsive prolactin gene (probably via squelching, Adler *et al.,* 1988), and the tissue-specific albumin, transthyretin, and collagen II genes (mechanisms unclear, Frain *et al.,* 1990; Maire *et al.,* 1987; Savagner *et al.,* 1989; Yan *et al.,* 1990).

The reason so many more transcriptional activators have been reported than

repressors likely is due to the different activities of the elements. Activators cause gene overexpression; overexpressed genes are more likely to be identified and studied. Repressors inhibit gene expression; repressed genes are less likely to be identified and studied. (E.g., one is more likely to propose an experiment asking, "what causes transcription of histone genes in S phase cells," than, "what causes histone genes to be repressed in non-S phase cells?") The argument is analogous to my rationale for overrepresentation of positive-acting proliferation regulators (i.e., the oncogenes, see section I,D).

e. TATA-less promoters - Some genes lack TATA elements. These "TATA-less" genes tend to be transcribed at low levels. TATA-less promoters include the SV40 major late promoter (Ayre and Dynan, 1988), the adenosine deaminase promoter (Chinsky *et al.*, 1989), the thymidylate synthase promoter (Deng *et al.*, 1989), the epidermal growth factor receptor gene promoter (Kageyama *et al.*, 1989), the cellular thymidine kinase promoter, the lymphocyte-specific terminal deoxynucleotidyl-transferase promoter (Smale and Baltimore, 1989), and the cellular dihydrofolate reductase promoter (Dynan *et al.*, 1986; Means and Farnham, 1990). The most thoroughly studied of these is the SV40 major late promoter.

Ayre and Dynan (1988) analyzed the SV40 major late promoter by measuring the promoter strength of deletion mutants and clustered point mutants across the 5' end of the major late gene in *in vitro* transcription assays and in transformed cells. Three sites were active in directing transcription: a region centered 31 base pairs upstream of the cap site, the cap site, and a region centered 29 base pairs downstream of the cap site. Although in TATA-containing genes transcription initiates 30 base pairs downstream of the TATA element (Schatt *et al.*, 1990), on the SV40 late promoter, mutations 30 base pairs upstream of the cap site only diminish the level of initiation; they do not affect the site of initiation. Rather, the site of initiation is determined by sequences at the

initiation site. Thus, in addition to diminishing transcription levels, mutations at the cap site result in ectopic initiation. Finally, mutations at a downstream site (located within protein coding sequences) diminish transcription. Identification of an intragenic transcription element was surprising because, based almost entirely on studies on TATA-containing genes, promoters had been thought to include only sequences at and immediately upstream of the cap site.

Surprisingly, Ayre and Dynan (1988) also demonstrated that neither the GC element-rich early promoter nor the SV40 enhancers (both located immediately upstream of the late promoter) affected rates of transcription from the late promoter *in vitro* or *in vivo.*

2. Premature termination - The only reports of eucaryotic gene regulation at the level of elongation have involved premature termination (reviewed by Yanofsky, 1988). Three eucaryotic genes known to be regulated by transcriptional pretermination are the SV40 gene encoding the 16S viral RNA (Hay and Aloni, 1985), the c-*myc* protooncogene (Bently and Groudine, 1986), and the c-*myb* protooncogene (Bender *et al.,* 1987). In the first two cases, pretermination is associated with RNA secondary structure. For c-*myc,* sequences immediately upstream of the pretermination site encode a complex stem loop that is structurally similar to the RNA stem loops associated with bacterial pretermination (attenuation)(Eick and Bornkamm, 1986; Nepveu and Marcu, 1986). However, the mechanism by which the c-*myc* stem loop causes pretermination is unclear. In the case of the SV40 16S gene, a viral protein, entitled "agnoprotein," binds an RNA secondary structure in the 5' leader sequence, resulting in pretermination (Hay and Aloni, 1985). The mechanism by which bound agnoprotein affects the activity of RNA polymerase is uncertain. (Interestingly, using an analogous mechanism, but to the opposite effect, HIV Tat protein activates viral transcription upon binding the "TAR" site of nascent pre-mRNA [Southgate *et al.,* 1990].) In the final example of elongational

pretermination, Bender *et al.* (1987) demonstrated that, whereas transcription is uniform across the entire c-*myb* gene in immature hematopoietic cells, only sequences upstream of the first intron are transcribed in mature hepatocytes. The authors propose that transcription aborts in the first intron during maturation; however, no mechanism has been identified. It is unknown whether c-*myb* pretermination is associated with RNA secondary structure. (Note: In late July, Kaufman's group reported that expression of the mouse adenosine deaminase gene is regulated by pretermination near the exon 1/intron 1 boundary [Maa *et al.*, 1990]. Thus, in placenta, transcriptional activity across the gene is homogeneous; in fibroblastic cells, most transcription aborts near the end of the first exon. Interestingly, evidence for a similar mechanism of regulation of the mouse dihydrofolate reductase gene is presented in CHAPTER 4 [Schmidt and Merrill, 1989b].)

3. Detecting transcriptional regulation - Unambiguous demonstration of transcriptional regulation can be difficult. The problems are exacerbated when attempting to detect transcription of a weakly-expressed single copy gene. The various methods that have been used are reviewed and evaluated here.

a. Drug inhibition - The simplest and most equivocal method of attempting to detect transcriptional regulation is the drug-inhibition study. Briefly, total cellular transcription is inhibited with actinomycin-D and the rate of message decay is measured during a subsequent chase period. A grossly simple-minded interpretation of such an experiment is that if rates of message decay are different, changes in RNA stability regulate expression; if rates of decay are similar, one infers that a transcriptional mechanism must regulate expression. The problem with this interpretation is that actinomycin-D poisons all cellular transcription. Treated cells are so far perturbed from their normal physiological state that it is impossible to distinguish whether

properties of the cells are innate or artifactual. Thus, if a message decays rapidly, it does not indicate that the message is labile in nontreated cells. It is just as likely, for example, that the transcriptional block indirectly induced decompartmentalization of ribonucleases.

b. In vivo-labeling - The second method occasionally used to detect transcriptional regulation is *in vivo* pulse/chase studies. As usually performed, *in vivo*-labeling studies measure rates of RNA decay. Therefore, when using *in vivo*-pulse/chase studies, as when using drug-inhibition studies, one can only infer transcriptional regulation based on whether or not RNA stability changes. In *in vivo*-labeling studies, newly synthesized RNA is radiolabeled by transiently incubating cells in radiolabeled RNA precursor. However, because cells are impermeant to nucleotides, either radiolabeled nucleosides or radiolabeled orthophosphate is used. Therefore, several intermediate steps (transport, conversion into triphosphonucleoside, translocation, transcription) are required prior to incorporation of radiolabel into RNA. As a result, there is a significant lag period between incubation and labeling. Generally, cells are pulsed for 10 min and RNA samples are harvested at 10 min intervals beginning 10 min after the end of the pulse.

Unlike drug-inhibition studies, *in vivo*-labeling is noninvasive. When correctly performed, results that indicate message stability changes are unambiguous. However, inference to whether or not transcription rates change may be ambiguous. For example, if, in response to a stimulus, message levels decrease and *in vivo*-labeling indicates message stability is unchanged, is it valid to conclude that message levels are transcriptionally regulated? No. It is possible that transcription rates and message stabilities are unchanged if a mechanism exists to destabilize pre-mRNA. As a nonabundant transient intermediate, levels of precursor are generally too low to detect. Furthermore, because *in vivo*-labeling cannot detect species that decay in less than about

20 min, *in vivo*-labeling lacks the resolution to measure decay rates of even relatively stable pre-mRNA species.

A modification of the *in vivo*-labeling method has been used in attempts to measure rates of transcription. In this technique, cells are transiently labeled for decreasing intervals of time and RNA is harvested immediately thereafter (Leys and Kellems, 1981; Leys *et al.*, 1984). Incorporation into a specific RNA species is then plotted as a function of time. During short intervals, labeling should approach a linear rate, which is representative of the rate of RNA synthesis (i.e., the transcription rate). However, the validity of the method is controversial because it is uncertain how rapidly intermediary metabolism allows ribonucleotide pools at the RNA polymerase to equilibrate. The linear rate of incorporation may represent the reaction rate of a kinase or the speed of a transporter, for example, rather than the rate of transcription.

c. Nuclear run-on - The nuclear run-on assay was developed to eliminate intermediary metabolism leading to equilibration of nuclear radionucleotide pools and thereby provide a more accurate measure of transcription rates (McKnight and Palmiter, 1979). In the nuclear run-on assay, cell metabolism is halted by rapidly shifting cells to 0^{o}C. Cells are then gently lysed to expose nuclei, and nuclei are incubated in the presence of radiolabeled ribonucleoside triphosphates and RNase inhibitors. After allowing intranuclear pools of radiolabeled nucleotide to equilibrate, cells are transiently shifted to a higher temperature. At the higher temperature, previously-engaged RNA polymerases resume elongation and radiolabel is incorporated into nascent transcripts. Assuming that the rate of transcriptional elongation is uniform on all genes, the amount of radiolabel incorporated into transcripts arising from a given gene is proportional to the number of RNA polymerase molecules engaged on the gene at the time of harvest (Appendix 1).

A curious property of nuclear run-on transcription is that total radionucleotide

incorporation rapidly reaches a maximum (3 to 10 min)(McKnight and Palmiter, 1979). Part of the reason is that, under standard conditions, RNA polymerases do not initiate in isolated nuclei. Additionally, in isolated nuclei, preengaged polymerases incorporate only several hundred nucleotides. As a result, initial (linear) rates of incorporation are rarely sought. Rather, as products are considered stable after formation in the presence of ribonuclease inhibitors, run-on reactions are allowed to go to completion (10 to 30 min), and total incorporation is measured.

One major drawback of the nuclear run-on assay is that it is insensitive. Generally, very high transcription signals are required for detection above background. High background results because all newly-synthesized transcripts are labeled and because the only method that has successfully been used for detecting specific run-on products is hybridization to filter-immobilized probes. First, because all transcripts are labeled, the average gene will only represent about 1/10,000 of the total incorporated radioactivty (based on an estimate of 10,000 active genes per cell). Weakly expressed genes may be expressed at less than 1% this level (representing 1 part per million of incorporated radiolabel). Second, as in any filter-bound probing assay, serendipitous association of radiolabeled RNA with the filter substrate will contribute to background.

An example of the problems of detecting specific run-on signals is exemplified by the cellular TK gene. Based on nuclear run-on assays, Groudine and Casimir (1984) reported that, despite relatively low levels of expression, the TK gene is transcribed at nearly the same rate as the globin gene in chick erythrocytes. However, when the experiments were repeated in developing chick tissues and in transformed cell cultures, Gross *et al.* (1987 and unpublished) found that nontransformed nuclei synthesized (non-*tk*) run-on products that hybridize to immobilized *tk* DNA, but not to pBR322 or M13 control DNA.

The second drawback of the nuclear run-on technique is that, unlike *in*

vivo-labeling, the nuclear run-on is invasive. Transcription rates are measured in nuclei devoid of their normal extranuclear milieu. Just as transcriptional initiation does not occur during run-on, it is likely that other properties of nuclear biochemistry are altered. For example, nuclei are exposed by treatment with mild detergent. Perhaps the detergent also alters DNA-protein or protein-protein interactions, thereby loosening nucleosome association or affecting transcription factor association. Also, DNA helix and RNA secondary structure stability may be different *in vivo* and *in vitro*. Whereas in the absence of transcriptional initiation, these perturbations cannot cause detection of artifactual transcription signals if genes are not engaged by RNA polymerase, they may affect pausing, elongation rates, or termination. Thus, actively transcribed genes may appear inactive if polymerase is stalled by secondary structure or caused to preterminate; genes engaged by blocked polymerases may appear active if the elongational block is disrupted.

In summary, whereas of the above assays, only the nuclear run-on measures transcription rates in the absence of RNA degradation, nuclear run-on results are not unequivocal. Nuclear run-on results are best experimentally substantiated by steady state mRNA and pre-mRNA measurements, *in vivo* RNA stability measurements, and promoter-switch studies (discussed below).

d. Promoter-switch - In most cases, transcriptional regulation likely is dependent on sequences at or near the promoter. As a result, a useful approach for distinguishing transcriptional and posttranscriptional regulation has been the promoter-switch. In promoter-switch studies, sequences adjacent to the 5' end of a gene are fused to a "reporter gene." The reporter gene is any transcribed sequence that is not posttranscriptionally regulated and that, when transcribed, imparts an easily detected phenotype on cells. The most popular reporter genes for studies in eucaryotic cells are the bacterial chloramphenicol acetyltransferase (CAT) and β-galactosidase (*lac z* or

"β-gal") genes. If fusion of the promoter region from a regulated gene imparts regulated expression on a reporter gene, one can conclude that intragenic sequences are not required for regulation. Therefore, regulation cannot result from posttranscriptional changes in message stability. Rather, regulation likely is transcriptional, occurs at initiation, and requires only proximal sequences. However, if the fusion gene is not regulated, one can only conclude that proximal sequences are insufficient for regulation. Nonregulated reporter gene expression does not indicate that regulation is posttranscriptional or even postinitiational.

By reversing the promoter switch, that is, replacing the promoter of a regulated gene with a well characterized nonregulated promoter, one can determine whether intragenic sequences are sufficient for regulation. If, when fused to a nonregulated promoter, the gene remains regulated, intragenic sequences are sufficient for regulation. These results favor a posttranscriptional model (such as RNA degradation) for regulation; however, they are also consistent with models wherein intragenic sequences regulate transcriptional initiation, pausing, or pretermination. If a gene becomes nonregulated when fused to a nonregulated promoter, regulation is almost certainly transcriptional (assuming all transcribed sequences are identical to the wild type gene).

e. Other - As each gene has its own deck of characteristics, specific genes occasionally can be assayed for transcriptional regulation by unusual methods. For example, in a landmark study, Gorski *et al.* (1986) demonstrated that *in vitro* transcription extracts could be prepared from liver and spleen that demonstrated correct tissue-specific transcription of the albumin gene. Although albumin expression was already known to be transcriptionally regulated (Tilghman and Belayew, 1982), the *in vitro* assay has been useful for assaying whether other tissue-specific genes are transcriptionally regulated. Various workers have used the Gorski *et al.* (1986) extracts to demonstrate transcriptional regulation of the liver-specifc α-fetoprotein

(Feuerman *et al.,* 1989), aldolase B (Tsutsumi *et al.,* 1989), D site-binding protein (Mueller *et al.,* 1990), pyruvate kinase (Vaulont *et al.,* 1989), and hepatitis B large surface protein (Chang *et al.,* 1989) genes, and for the pituitary-specific prolactin gene (Cao *et al.,* 1987; Schuster *et al.,* 1988).

Another novel method for detecting transcriptional regulation of a gene was used to demonstrate that *stg* expression is transcriptionally regulated in *Drosophila* embryos. Edgar and O'Farrell (1989; O'Farrell *et al.,* 1989) wanted to determine whether *stg* was transcriptionally regulated; however, no traditional approaches were amenable to their conditions. The problems were threefold: first, *stg* transcripts are expressed at very low levels; second, *stg* mRNA is only expressed in the proliferative tissues of the developing embryo (necessitating microdissection if transcription rates were to be determined by traditional methods); third, the authors wanted to compare transcription between individual expressing and nonexpressing cells within a single proliferative tissue as well as differences between tissues. To approach the problem, an *in situ* hybridization technique was used. The *stg* gene is expressed at such a low rate that pre-mRNA never accumulates. As a result, when radiolabeled *stg* cDNA probes were hybridized to whole embryo thin sections and exposed to photographic emulsion, in addition to cytosolic labeling, cells expressing *stg* mRNA exhibited two small "dots" in the nucleus; nonexpressing cells exhibited no cytosolic or nuclear labeling. The authors suspect that the nuclear dots represent nascent transcripts arising from each of the diploid copies of the *stg* gene. By this interpretation, the absence of nascent transcripts in nonexpressing cells indicates transcriptional repression (O'Farrell *et al.,* 1989). Although this is an interesting approach for detecting transcriptional activity on weak genes, it has yet to be rigorously tested, and has not yet been used on genes other than *stg.*

TISSUE CULTURE SYSTEMS FOR STUDYING GENE REGULATION

Regulation of replicative enzymes frequently is studied using growth-arrested fibroblastic cells. When fibroblast cultures reach confluency and are deprived of serum, the cells slowly withdraw from the cell cycle in G1 and enter a quiescent diploid state termed G0 (Johnson *et al.,* 1974; Pardee, 1989; Pardee *et al.,* 1978). When subsequently passaged into serum-containing medium, the G0 cells reenter the cell cycle, traverse G1, and initiate a new round of DNA synthesis (Johnson *et al.,* 1974; Pardee, 1989; Pardee *et al.,* 1978). Increases in the rate of synthesis or in the level of a particular replicative enzyme are often interpreted as representing a mechanism for S phase-specific synthesis of enzymes involved in DNA precursor synthesis (Johnson *et al.,* 1974; Pardee *et al.,* 1978). One caveat concerning this interpretation is that the conditions usually used to achieve quiescence result in a general depression of all macromolecular synthesis, as evidenced by a decrease in the overall rate of RNA and protein synthesis (Hendrickson *et al.,* 1980; Johnson *et al.,* 1974, 1976, 1978; Mauk and Green, 1973; Wiedemann and Johnson, 1979; Wu and Johnson, 1982). Therefore, following serum restoration, much of the observed increase in the levels of a particular gene product may simply reflect recovery from a metabolically depressed state. General metabolic recovery during serum stimulation may mask important changes in specific gene expression that normally occur during the G1/S transition of proliferative cells.

Skeletal muscle cell cultures are a useful system for studying the regulation of replicative and growth-dependent enzymes. Proliferating myoblasts, when placed in media lacking sufficient mitogenic activity to maintain exponential growth, irreversibly withdraw from the cell cycle in G1 and commit to terminal differentiation. Committed myocytes do not synthesize DNA, but are otherwise biosynthetically active as measured by rates of overall methionine incorporation, muscle-specific protein synthesis, and overall RNA synthesis (Devlin and Emerson, 1978; Jaynes et al., 1986).

DIHYDROFOLATE REDUCTASE

Attesting to the universality of basic metabolic pathways, organisms in all kingdoms contain DHFR genes. Additionally, DHFR genes have been detected in some viruses (Purohit *et al.*, 1981; Purohit and Mathews, 1984; Trimble *et al.*, 1988). Mammalian and bacterial DHFR genes are enzymatically and structurally similar; both the mammalian and bacterial DHFR enzymes have similar molecular weights. It is reasonable to speculate that the DHFR genes evolved from a common ancestor, thus representing one of life's very early and conserved enzymes.

DHFR enzyme - Tetrahydrofolate coenzymes are required for numerous one carbon transfer reactions throughout the life of the cell. In eucaryotes, dihydrofolate serves no known functions other than as an intermediate in the conversion of dietary folic acid to tetrahydrofolate. Conversion of folic acid to dihydrofolate and conversion of dihydrofolate to tetrahydrofolate are catalyzed by DHFR. Other than as an intermediate in folate assimilation, dihydrofolate is generated only by thymidylate synthase during *de novo* deoxythymidylate synthesis. As thymidylate synthase generates one mole of dihydrofolate for each mole of dTMP synthesized, demand on DHFR is greatest during DNA synthesis. A summary of the pathways by which *de novo* protein, RNA, and DNA synthesis are dependent on DHFR enzyme is presented in Appendix 2.

The DHFR gene - Mammalian DHFR genes are noted for several distinct characteristics. First, although DHFR is a relatively small protein (about 22 kD) it is encoded by a relatively large gene (30 kilobase pairs). The most extensively studied mammalian DHFR genes (hamster, human, and mouse) contain 5 introns. Introns range between 0.2 and almost 20 kilobase pairs (Crouse *et al.*, 1982; Fuji and Shimada, 1989; Looney and

Hamlin, 1987). Second, when cells (either in culture or *in situ*) are challenged over long periods of time with low doses of DHFR-inhibitors, clones occasionally arise in which the region of the chromosome containing the DHFR gene is duplicated by gene amplification. Finally, numerous partially or completely conflicting reports suggest that the DHFR gene is preferentially expressed under various conditions of growth vs. nongrowth, and at specifc periods during the cell cycle. Amplification is reviewed here because, as a part of this thesis study, cell lines containing amplified copies of the DHFR gene were generated. However, amplification *per se* was not studied. Rather, the amplified cells were only one of a number of tools used to study regulation of *dhfr* expression. DHFR expression is reviewed here because it is the subject of CHAPTER 2 of the thesis and because it provides a background for the investigations into regulation of DHFR expression presented in CHAPTERS 3-5. Literature on DHFR regulation is reviewed in the INTRODUCTION of each subsequent chapter.

1. Amplification - The drug methotrexate (MTX) is a folate analog that competitively inhibits DHFR activity. After long-term treatment of animals or animal cell cultures with low doses of MTX, resistant populations of cells can be isolated. Characterization of resistant tissue culture sublines indicates that cell survival is usually associated with overexpression of DHFR enzyme activity, overexpression of DHFR activity is due to overexpression of DHFR protein, and overexpression of DHFR protein is due to an increased rate of DHFR protein synthesis (Alt *et al.*, 1976). Molecular analysis of the mechanisms causing the increased rate of synthesis of DHFR protein indicates that DHFR mRNA is overexpressed in resistant cells, and further, that the number of DHFR genes per cell is increased proportionally (Alt *et al.*, 1978; reviewed by Schimke, 1982, 1984, 1988; Stark, 1986; Stark and Wahl, 1984).

The mechanisms of gene amplification have not been resolved. Studies into the source of the amplified genes indicate that the additional gene copies probably arise from

overreplication of specific chromosomal regions rather than from exogenous gene salvage (from dead cells or by cell-cell fusion) or from unequal sister chromatid exchange during mitosis (Alt *et al.,* 1978; Chasin *et al.,* 1982; Johnston *et al.,* 1986).

In attempts to understand the mechanism of amplification, various groups have either been studying single duplication events or have been characterizing the structure of amplified genes. Studies on individual duplication events have indicated: 1) gene amplification occurs in the absence of drug selection (Johnston *et al.,* 1983; Mariani and Schimke, 1984); 2) treatments that slow or interrupt DNA synthesis increase the frequency of amplification (Johnston *et al.,* 1986; Rice *et al.,*1986; Schuetz *et al.,* 1988; Sherwood *et al.,* 1988; Tlsty *et al.,* 1984); and 3) the DHFR gene is more likely to be amplified when in a transcriptionally active state (Johnston *et al.,* 1986). Based on the above evidence, one can conclude that amplification is a spontaneous event involving erroneous replication; the error is favored by treatments that transiently block replication. The association between transcriptional activity and amplification is not clear. Transcriptional alterations in DNA structure (nucleosomal arrangement, chromatin density, nuclear matrix association, etc.) may favor amplification, or the effect may be a secondary consequence of altered nucleotide metabolism arising from tetrahydrofolate depletion. Alternatively, inasmuch as amplification involves erroneous DNA synthesis, perhaps the mechanism that links transcription and DNA repair (Mellon *et al.,* 1986, 1987) is involved in initiating the amplification event.

Structural analyses of amplified DHFR genes indicate that the DHFR amplicon is generally large (from 100 to 500 kilobase pairs)(Looney and Hamlin, 1987; Looney *et al.,* 1988), that the amplified copies can be located immediately adjacent to the parental DHFR gene, on different chromosomes than the parental gene, or on minute chromosomes (Biedler *et al.,* 1980; Hamkalo *et al.,* 1985; Johnston *et al.,* 1983; Trask and Hamlin, 1985), that adjacent amplicons frequently, but not necessarily, are arranged in an head-to-head orientation (Looney and Hamlin, 1987) and that an origin of replication is

associated with the DHFR amplicon (Burhans *et al.,* 1986). Amplification probably initiates by erroneous reinitiation at an origin near the DHFR gene. Subsequently, the region of DNA present in triplicate (i.e., the amplicon) is inverted and inserted into the chromosome in the opposite orientation as the parental gene. It is noteworthy, however, that the junctions of recombinational insertion of the amplified copy, although occasionally occuring at regions of repetitive DNA (Anachkova and Hamlin, 1989) do not consist of a specific DNA sequence (Weith *et al.,* 1987). Moreover, of the 100 to 500 kilobases of DNA within DHFR amplicons, only the DHFR protein coding sequences (i.e., the *dhfr* exons) are necessary for amplification. Thus, several groups have successfully amplified DHFR cDNA minigenes fused to heterologous promoters (Gasser *et al.,* 1982; Gasser and Schimke, 1986; Kaufman and Sharp, 1982, 1983).

Because amplified gene copies often reside on independent minute chromosomes, and because minute chromosomes, like all chromosomes, require an origin of replication to multiply and persist, it had been suggested that a specific origin associated with the DHFR gene predisposes adjacent sequences to amplification (Burhans *et al.,* 1986; Handeli *et al.,* 1989). However, in a very recent report, Vaughn *et al.* (1990) demonstrate that amplification initiates at any of numerous origins throughout the *dhfr* amplicon, and suggest that all origins are equally capable of initiating amplification.

As the only DNA sequence requirement yet resolved for DHFR gene amplification is that a functional DHFR enzyme molecule be encoded and produced, it is reasonable to speculate that the apparent inherent propensity of *dhfr* (and several other genes, including adenosine deaminase [Kaufman *et al.,* 1986, 1987], thymidylate synthase [Rossana *et al.,* 1982], CAD [Giulotto *et al.,* 1986], multiple drug-resistance [Shen *et al.,* 1986], and *c-myc* [Wong *et al.,* 1986]) to amplify is a property of the enzyme, not the DNA. By this model, aberrant reinitiation of replication is a spontaneous error that occurs at random replication origins. Likewise, recombinational insertion (or circularization into minute chromosomes) of the amplified region of DNA is spontaneous

and sequence-independent. Preferential amplification of certain genes probably occurs as the combined result of the following properties of the cognate enzyme: 1) the enzyme represents an essential link in an essential metabolic pathway; 2) inhibitory conditions (usually competitive drugs) specifically inhibit that enzyme; and 3) the inhibitory stimulus works at an enzyme/inhibitor concentration-dependent threshold, such that small-fold overexpression of enzyme will allow cell survival (and thereby allow fixation of the genetic cause of overexpression in the population).

2. DHFR gene expression - An early study indicated that DHFR enzyme is synthesized in log-phase, but not in stationary-phase, fibroblastic cells (Alt *et al.* 1976). This report, although tame by today's standards, represented one of the first molecular studies on growth-dependent gene expression, and instigated a decade and a half of intense research. Since the Alt *et al.* (1976) report, the genes encoding numerous other replicative enzymes and proteins have joined the field. The list includes thymidine kinase, thymidylate synthase, ribonucleotide reductase, DNA polymerase, and the histones. Despite this attention, the mechanisms regulating the expression of any of the "replication-specific" genes remain obscure. The literature has been plagued by controversial and conflicting reports. Not surprisingly, the field of replicative gene expression has progressed much slower than, for example, the field of steroid hormone action.

Early studies on DHFR expression were based on comparisons of actively growing and growth-arrested amplified fibroblastic cell populations. Whether growth inhibition resulted from contact inhibition (Alt *et al.,* 1976, 1978; Kellems *et al.,* 1976; Leys and Kellems, 1981), serum deprivation (Gudewicz *et al.,* 1981; Johnson *et al.,* 1978; Wiedemann and Johnson, 1979; Wu *et al.,* 1982), drug-inhibition (Wu and Johnson, 1982), or viral infection (Yoder *et al.,* 1983), the studies generally agreed that DHFR is preferentially synthesized in proliferative populations.

Once investigators began asking whether DHFR is cell cycle regulated, reports from various labs became incongruous. Papers published within the past year contradict and challenge numerous studies published during the last decade. The various conflicting conclusions will be evaluated and interpreted here. Although it seems that most of the discrepancies finally have been scientifically resolved, it is uncertain whether the scientists involved have reached a consensus.

Somatic cells generally proliferate asynchronously. To study cellular properties at a given point in the cell cycle, it is necessary to obtain a population of cells which are all at the same point in the cell cycle (synchronized). For this reason, cell cycle studies are difficult. Two general approaches can be used to purify synchronous cell populations. By one approach (growth-arrest, see section III, above), cells are treated in such a way that cell cycle progression is blocked at a specific point. After all cells have reached that point, the population is synchronous. Upon removing the block, cells reenter the cell cycle and progress synchronously for one or two cell generations. By the second approach (cell sorting or mitotic selection), cells at a specific stage in the cell cycle are isolated from a growing asynchronous culture. The isolated cells are either studied immediately or allowed to grow synchronously until a specific stage in the cell cycle is reached.

Growth inhibition studies have suggested that DHFR enzyme levels, synthesis rates, and mRNA levels are low in arrested cells. Upon growth-restoration, mRNA levels, synthesis rates, and enzyme levels increase and reach a maximum as cells enter S phase (Johnson *et al.,* 1978; Kaufman and Sharp, 1983; Leys and Kellems, 1981; Weidemann and Johnson, 1979). However, when cells are synchronized by mitotic selection, although DHFR mRNA (Farnham and Schimke, 1986) and DHFR synthesis (Mariani *et al.,* 1981) remain S phase-dependent, DHFR enzyme levels increase less than two-fold as cells enter S phase (Mariani *et al.,* 1981). The cause of the contradiction is that, in the studies by Johnson's group, cell synchrony was achieved by serum-starving cells for

7 or more days. The low levels of DHFR enzyme detected in starved cells is not representative of DHFR enzyme levels at any point in the cell cycle. Rather, enzyme levels are artifactually reduced by long-term repression of all cellular metabolism.

A recent study from the Schimke group (Feder *et al.,* 1989) indicates that DHFR enzyme levels, synthesis rates, and mRNA levels do not change at all during the cell cycle. In the Feder *et al.* (1989) study, synchronous populations of cells in S, G1 and G2/M were isolated from actively proliferating populations by elutriation (a cell-sorting technique based on differences in the sedimentation velocity of cells at various cell cycle stages). The discrepancy between Feder *et al.* (1989) and Mariani *et al.* (1981) presumably arose because the cell population densities required to obtain large numbers of cells by the mitotic selection technique (used by Mariani *et al.,* 1981) caused partial artifactual repression of DHFR mRNA levels as a result of overcrowding. Feder *et al.* (1989) demonstrate that DHFR gene expression is cell cycle-independent; however, levels of DHFR mRNA, and consequently rates of DHFR synthesis, are affected by serum factors and cell-cell contact. The authors conclude that, although *dhfr* expression is growth state-dependent, it is not cell cycle regulated. Both the data and logic in the Feder *et al.,* (1989) paper appear sound. However, inasmuch as it contradicts numerous other apparently sound studies published by the Schimke group and others, the Feder report begs independent confirmation.

The report by Feder *et al.* (1989) is highly relevant to (and was published at the same time as) CHAPTER 2 of this thesis (Schmidt and Merrill, 1989a). In CHAPTER 2, I establish that levels of DHFR enzyme do not change as muscle cells withdraw from the cell cycle during myogenesis. The findings I present are consistent with Feder *et al.*'s (1989) model that DHFR enzyme is not cell cycle regulated. However, I demonstrate that levels of DHFR mRNA and rates of DHFR synthesis decrease during commitment. Thus, whereas DHFR mRNA levels and synthesis rates are not cell cycle regulated (Feder *et al.,* 1989), they are down-regulated during differentiation. Previous models had suggested

that DHFR was a replication-specific gene of the same "class" as TK, thymidylate synthase, deoxycytidine kinase, ribonucleotide reductase, ornithine decarboxylase, and DNA polymerase (Bello, 1974; Farnham and Schimke, 1985; Gross *et al.*, 1987; Gudas *et al.*, 1988; Johnson *et al.*, 1982; Merrill *et al.*, 1984; Navalgund *et al.*, 1980; Sherley and Kelly, 1988). Clearly this speculation was wrong in the case of *dhfr.* Interestingly, few of the other "replicative enzymes" listed above have been rigorously tested for cell cycle regulation. Feder *et al.* (1989) speculate that when careful analyses are performed, few genes will be truly cell cycle-regulated.

DHFR is not the first protein to have been stripped of a "cell cycle-regulated" title. Studies on the c-myc gene, again using growth-arrested cells, reported that c-*myc* was S phase-specific (Kelly *et al.*, 1983). However, when cell-cycle expression of c-*myc* mRNA and c-myc protein synthesis was investigated in elutriated cells, results indicated that c-*myc* was not cell cycle regulated (Hann *et al.*, 1985; Thompson *et al.*, 1985). c-*myc* expression, like DHFR expression, is sensitive to culture conditions (serum factors and population densities), and therefore, is a growth state-dependent gene, not a replication-dependent gene.

ORIENTATION TO FORMAT

The following four chapters constitute the results of my thesis research. Each chapter represents a separate facet of the project. Each of the following chapters has been prepared and submitted for publication, and thus, has been subject to peer review. Chapters are presented in publication format, including a brief SUMMARY, an INTRODUCTION to orient the reader to the rationale of each project and to provide background, a RESULTS section, FIGURES, and FIGURE LEGENDS to present data, and a DISCUSSION section wherein the implications of each study are considered. All references are compiled at the end of the thesis.

CHAPTER 2

Maintenance of Dihydrofolate Reductase Enzyme After Disappearance of DHFR mRNA During Muscle Cell Differentiation

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SUMMARY

Terminally differentiating mouse muscle cells were used to examine the relationship between myogenic withdrawal from the cell cycle and the levels of dihydrofolate reductase (DHFR) mRNA and DHFR activity. Differentiation was induced by removal of fibroblast growth factor activity from the medium. DHFR mRNA was measured by an RNase protection assay. DHFR activity was measured by a spectrophotometric assay and by a [^{3}H]methotrexate binding assay. Proliferative myoblasts contained four DHFR mRNA molecules and 1.8×10^5 DHFR enzyme molecules per cell. By 12.5 hours after induction, when [^{3}H]thymidine labeling indices showed that all cells had withdrawn from the cell cycle, DHFR mRNA levels had declined to 0.7 copies per cell. In contrast, myogenic withdrawal did not result in reduced DHFR activity. Qualitatively similar results, i.e., down-regulation of mRNA and constitutive expression of activity, were observed in a methotrexate-selected muscle cell line with > 50-fold amplification of the DHFR gene. Enzyme synthesis rate and stability measurements indicated that the persistence of DHFR activity in postreplicative cells was due to a long enzyme lifetime rather than to continued synthesis from residual normal DHFR mRNA or an alternative mRNA species not detected by the RNase protection assay. Unlike DHFR, thymidine kinase (TK) activity disappeared rapidly as muscle cells differentiated. Both DHFR mRNA and TK mRNA are expressed in a replication-dependent manner; however, the enzymes encoded by these messages are subject to different fates in postreplicative cells.

INTRODUCTION

Numerous proteins are preferentially synthesized during S phase. In some cases, S phase-specific synthesis leads to tight temporal compartmentalization of enzyme activity. For example, thymidine kinase (TK) is present in S phase cells, but decays rapidly after cells complete DNA replication. As a result, differentiated cells that have withdrawn from the cell cycle and synchronized cell populations that are traversing non-S phase portions of the cell cycle contain very little TK activity (Merrill *et al.*, 1984; Sherley and Kelly, 1988). In other cases, proteins synthesized preferentially during S phase persist after DNA replication is completed. For example, histone proteins are made during S phase (Borun *et al.*, 1967), but remain as structural components of chromatin in nonreplicating cells. In the latter case, S phase-specific synthesis may represent a homeostatic mechanism for balancing rates of protein accumulation with the growth state of the cell. For example, unabated synthesis of histones in cells that have stopped dividing would quickly lead to overaccumulation of histone proteins. To insure no more than a doubling in content prior to each mitosis, synthesis of many proteins, not just proteins involved in chromatin duplication, may be coupled to the DNA-synthetic phase of the cycle.

Many of the enzymes involved in DNA precursor biosynthesis are thought to be confined to S phase. This model is teleologically attractive because it posits that the enzymatic machinery for deoxynucleotide metabolism is expressed only when needed. In introducing studies on regulation of these enzymes, a common theme is to speculate the entire group is regulated similarly. DHFR and TK are frequently invoked as archetypal examples of S phase-specific enzymes. For both enzymes, mRNA levels and rates of protein synthesis are maximal during S phase (Farnham and Schimke, 1985; Johnson *et al.*, 1982; Mariani *et al.*, 1981; Sherley and Kelly, 1988; Thompson *et al.*, 1985). Rapid clearance of TK activity in nonreplicating cells is well established (Gross *et al.*, 1987; Merrill *et al.*, 1984; Sherley and Kelly, 1988). For DHFR, the situation is less

clear. Growth-arrested fibroblastic cells, blocked from replicating by serum deprivation, have several-fold lower DHFR activity levels than asynchronously growing cells or S phase cells synchronized by serum restoration (Johnson *et al.,* 1978; Wiedemann and Johnson, 1979). Such studies create the impression that DHFR levels are significantly depressed in nonreplicating cells. However, whether DHFR declines significantly during non-S phase portions of a normal cell cycle is uncertain. One study, in which DHFR levels were measured in cells synchronized by mitotic selection, revealed little decline in the per genome content of DHFR during the G2, M and G1 phases of the cell cycle (Mariani *et al.,* 1985). Furthermore, whether DHFR activity declines during differentiation is undetermined. The widely held assumption that DHFR is an S phase-specific enzyme merits reexamination.

Skeletal muscle cell cultures are a useful system for studying the regulation of replicative enzymes. When placed in media lacking sufficient mitogenic activity to maintain exponential growth, proliferating myoblasts irreversibly withdraw from the cell cycle in G1 and commit to terminal differentiation. Committed myocytes are incapable of synthesizing DNA but are otherwise biosynthetically active, accumulating large quantities of muscle-specific proteins (Devlin and Emerson, 1978; Jaynes *et al.,* 1986). Although differentiation of many species of primary and established myoblast lines is inducible by mitogen depletion (Doering and Fischman, 1974; Emerson, 1977; Konigsberg, 1971; Nadal-Ginard, 1978; Slater, 1976), clonally-derived mouse myoblast lines display a particularly sharp transition between the proliferative and postreplicative states (Linkhart *et al.,* 1981). In this paper, we used methotrexate-resistant and wildtype mouse myoblast strains to follow the fate of DHFR protein and mRNA during myogenic withdrawal from the cell cycle. We found that DHFR mRNA levels declined sharply, but DHFR protein persisted indefinitely. In contrast, TK activity was rapidly removed from postreplicative cells. These results indicate DHFR is not an S phase-specific enzyme in the same sense as TK. The stability of DHFR protein

suggests the reduction in DHFR mRNA levels after S phase functions to prevent overaccumulation of DHFR protein, not to reduce existing DHFR protein levels. DHFR mRNA regulation may represent a general regulatory mechanism designed to match the synthetic rates of many cellular proteins to the growth state of the cell.

EXPERIMENTAL PROCEDURES

Cell culture - Mouse skeletal muscle cells were grown as described by Merrill *et al.* (1984). Growth medium consisted of basal medium (Ham's F-10 supplemented with 0.8 mM $CaCl_2$, 10 U/ml penicillin G, and 0.5 mg/ml streptomycin sulfate) containing 15% horse serum and fibroblast growth factor extracted from bovine brain (Esch *et al.*, 1985). To induce differentiation, cells were rinsed twice with basal medium and incubated in basal medium supplemented with 10^{-6} M insulin as a maintenance factor. Withdrawal from the cell cycle was complete by 12.5 hours after induction, as determined by [^{3}H]thymidine incorporation (Fig. 2.1). Postreplicative myocytes were biosynthetically active, as evidenced by accumulation of muscle-specific proteins (Jaynes *et al.*, 1986).

All myoblast lines were derivatives of the MM14D cell line described by Linkhart et al. (1981). To establish DHFR mRNA and protein levels in nonamplified cells, two distantly related lines were used. One, TK$^-\varepsilon$, was deficient in thymidine kinase and has been described elsewhere (Merrill *et al.*, 1984). The other, H$^-\alpha$, was deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and has not been described previously. H$^-\alpha$ was selected in 6-thioguanine after mutagenesis with ethylmethylsulfonate. H$^-\alpha$ had a diploid number of chromosomes (n=40) and a doubling time of 13 hours (G1, S and G2 were 3, 7.5 and 2.5 hours, respectively). H$^-\alpha$ contained <5% the HGPRT activity of wildtype cells and was unable to survive in hypoxanthine, aminopterin, thymidine (HAT) medium.

The H$^-\alpha$R50A cell line containing amplified copies of the DHFR gene was generated from H$^-\alpha$ by serial selection in increasing concentrations of methotrexate (Sigma A-6770), initiating at 100 nM. The use of HGPRT$^-$ parental cells forced utilization of the *de novo* pathway for purine synthesis; therefore, use of hypoxanthine-free Ham's F10 and dialysis of purines from serum was unneccessary. H$^-\alpha$ cells were sensitive to 50 nM

methotrexate, as shown by failure to form colonies; H$^-$αR50A cells were resistant to 50 μM. Southern blots, that included standards generated from mouse liver DNA, showed that DHFR gene copy number was amplified 50- to100-fold.

Spectrophotometric DHFR enzyme determinations - Twenty 10-cm cultures were grown to a density of 5 X 10^5 cells per dish. Half of the cultures were induced to differentiate; the remainder received fresh growth medium. After 18 hours, cells were rinsed and scraped from dishes with saline, and a hemocytometer was used to determine the number of cells being assayed. Cells were centrifuged, resuspended in 600 μl 0.1 M KPO_4, pH 7.0, and sonicated for 10 sec on ice. Extracts were centrifuged for 15 min at 11,000 x g and supernatant assayed for DHFR activity. Each assay mixture contained 150 μl of clarified extract in 1.0 ml 0.1 M KPO_4, pH 7.0, 10 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol. DHFR enzyme activity was monitored by following changes in absorbance at 340 nm after supplementing extracts with 0.1 μM NADPH (Boeringer Mannheim 107-816) and 0.1 mM dihydrofolate (kindly prepared by Gerry Lasser as described, Mathews and Huennekens, 1963), or with only 0.1 μM NADPH (control) (Mathews and Huennekens, 1963; Osborn and Huennekens, 1958).

Methotrexate binding analysis - All manipulations were done on ice or at 4°C. Cultures were rinsed with saline and harvested by scraping in 200 μl of DHFR assay buffer (10 mM Pipes (piperazine-*N,N'-bis* (2-ethanesulphonic acid)), pH 6.4, 150 mM KCl, 0.6 mg/ml bovine serum albumin, 0.1 μM NADPH). Cells were sonicated for 10 seconds and 10 μl removed for fluorometric DNA analysis (Labarca and Paigen, 1980) to determine the number of cells per assay (Merrill *et al.*, 1984). The remaining lysate was clarified by centrifugation for 15 minutes in a microcentrifuge. Assay mixtures, containing 50 - 100 μl of clarified lysate and 100 nM [^{3}H]methotrexate (37.0 Ci/mmole, New England Nuclear, diluted with nonlabeled methotrexate to a final specific activity of about 1.6

mCi/mmole) in a 200-µl volume, were incubated for 10 minutes. Nonbound methotrexate was removed by adding 50 µl of a freshly prepared suspension of 33 mg/ml Norit (activated charcoal, Sigma C-5385) in 10 mM Pipes, pH 6.4, 150 mM KCl, 8.3 mg/ml bovine serum albumin. It was important to cut the pipet tips used for adding the Norit suspension to allow free passage of the larger particulates. Incubation was continued for 10 minutes with occasional mixing. Charcoal was removed by a 10-minute centrifugation (a horizontal-tube microcentrifuge was used to minimize pellet surface area). A 100-µl sample was carefully removed from the tube to prevent disturbing the charcoal pellet, added to 1 ml scintillation fluid (4 mg/ml Omnifluor in 1 part Triton X-100 to 2 parts toluene), and assayed by liquid scintillation. A parallel sample lacking cell extract was used as a control to establish the efficiency with which charcoal removed nonbound methotrexate; a sample lacking Norit was used as a control to empirically confirm the specific activity of the [^{3}H]methotrexate.

DHFR enzyme synthesis rate and enzyme stability determinations - The rate of DHFR synthesis in proliferative and postreplicative amplified cells was determined by blocking existing methotrexate binding sites and monitoring reemergence of methotrexate binding capability. Proliferative cells and cells induced to differentiate 20 hours previously were pulsed for 30 minutes with 100 µM nonlabeled methotrexate. Cells were then rinsed three times with serum-free medium and fed drug-free growth (proliferative cultures) or differentiation (postreplicative cultures) medium. At various times thereafter methotrexate binding was measured as described above.

The stability of DHFR was determined in proliferative wild type cells by a pulse-chase experiment. Cells were pulsed with 5.0 nM [^{3}H]methotrexate (37.0 Ci/mmole) for 20 minutes, rinsed, and fed growth medium containing 0.1 µM nonlabeled methotrexate. At various times thereafter, cells were rinsed extensively with saline, harvested by scraping, solubilized in scintillation fluid, and assayed by liquid

scintillation. Negative control values from samples containing only scintillation fluid and saline were subtracted from each determination.

TK activity assay - TK enzyme activity assays were performed as described by Merrill *et al.* (1984).

DHFR mRNA determinations - DHFR mRNA levels were determined by an RNase protection assay. Cellular RNA was purified as described previously (Gross *et al.,* 1987). The transcription template p4d735 was made by inserting the 735-base-pair Bgl2/Hind3 fragment of a mouse DHFR cDNA (from pSV2-dhfr, kindly supplied by P. Berg) into pGEM-4 (Promega Biotec) at Bam H1/Hind3. Transcription reactions were performed as described previously (Gross *et al.,* 1987), except the specific radioactivity of the probe was increased by raising the specific activity of $\alpha[^{32}P]$UTP in the reactions from 52 Ci/mmole to 190 Ci/mmole. Linearization at Dde1, followed by transcription from the SP6 promoter in the presence of radiolabeled nucleoside triphosphates, yielded a 175-base radiolabeled RNA probe complementary to DHFR mRNA. Linearization of the template at Eco R1, followed by transcription from the T7 promoter in the presence of only nonlabeled nucleotides, yielded a 795-base "pseudo-mRNA" (Fig. 2.2*A*). The quantity of pseudo-mRNA produced was determined by measuring the absorbance at 260 nm ($\varepsilon = 20$ ml mg^{-1} cm^{-1}). Known amounts of pseudo-mRNA were used to generate a standard curve for quantification of DHFR mRNA from cells. Pseudo-mRNA protected a larger fragment than cellular DHFR mRNA because it contained a short stretch of vector-derived sequence complementary to the radiolabeled probe. Values for cellular DHFR mRNA band intensities were multiplied by 1.14 to correct for the difference in size of the mRNA and pseudo-mRNA signal. Hybridization and RNase digestion conditions were as described previously for TK mRNA quantitation (Gross *et al.,* 1987).

RESULTS

DHFR enzyme activity is maintained as muscle cells differentiate - A spectrophotometric assay (Mathews and Huennekens, 1963; Osborn and Huennekens, 1958) was used to measure relative levels of DHFR enzyme activity in proliferative myoblasts and postreplicative myocytes. The myocyte population was prepared by incubating cells in mitogen-depleted medium for 18 hours, during which time all cells withdrew from the cell cycle and committed to terminal differentiation. Enzyme reaction rates were normalized to the number of cells per sample. Proliferative cells synthesized tetrahydrofolate at a rate of 0.48 fmole/min/cell; committed cells at 0.42 fmole/min/cell. Published turnover numbers for mammalian DHFR range from 1000-2200 $minute^{-1}molecule^{-1}$ (Domin *et al.*, 1982; Peterson *et al.*, 1975). Using a turnover value of 1500, proliferative myoblasts contained 1.9×10^5 DHFR molecules per cell; committed myocytes contained 1.7×10^5 molecules per cell. Based on spectrophotometrically-determined enzyme reaction rates, DHFR enzyme levels declined only slightly during myogenic withdrawal from the cell cycle.

Because of the large number of cells required for the spectrophotometric assay, a sensitive ligand-binding assay (Johnson *et al.*, 1978) was adapted to our system. In this assay, [^{3}H]-labeled methotrexate was incubated with cell lysates, nonbound ligand removed, and the portion bound measured by liquid scintillation. Under the conditions employed, binding was saturable and exhibited a dissociation constant of roughly 150 pM (Fig. 2.3). All subsequent methotrexate binding assays contained 100 nM methotrexate to insure binding site saturation. In most assays, the amount of radioactivity bound per µg DNA was determined and the number of DHFR molecules per cell was calculated, knowing the specific activity of [^{3}H]methotrexate in the assay buffer, the average DNA content of a mouse cell (10 pg), and the 1:1 stoichiometry of methotrexate binding to DHFR

(Werkheiser, 1961).

Methotrexate binding activity, TK activity, and [^{3}H]thymidine labeling index were assayed in parallel in muscle cultures induced to differentiate (Fig. 2.1). Viewed in context with the known cell cycle and commitment kinetics of MM14D cells (Linkhart *et al.*, 1981), the shape of the thymidine labeling index curve was consistent with the model that cells placed in mitogen-depleted medium completed the on-going round of replication and division and then withdrew from the cell cycle as they entered G1. All cells had exited the cell cycle by 12.5 hours, consistent with the 12 to 14-hour generation time of these cells. TK activity declined progressively as cells withdrew from the cell cycle, and was undetectable by 15 hours after induction. In contrast, no significant decline in the number of DHFR molecules per cell was observed; both proliferative and committed populations contained 1.8 X 10^5 DHFR molecules per cell.

In light of the well documented cell cycle regulation of DHFR enzyme synthesis (Johnson *et al.*, 1978; Liu *et al.*, 1985; Mariani *et al.*, 1981; Wiedemann and Johnson, 1979) and mRNA levels (Farnham and Schimke, 1985, 1986; Kaufman and Sharp, 1983; Leys *et al.*, 1984; Leys and Kellems, 1981; Liu *et al.*, 1984; Wu and Johnson, 1982) in fibroblastic cell lines, the maintenance of DHFR activity in postreplicative myocytes was surprising. We therefore asked whether DHFR mRNA also was constitutively expressed during myogenic withdrawal from the cell cycle.

DHFR mRNA rapidly declines as muscle cells differentiate - RNA prepared from cells at various times after inducing differentiation was assayed for DHFR-specific message using a quantitative RNase protection assay (Fig. 2.2*A*). Briefly, RNA was hybridized to a synthetic radiolabeled RNA probe. Nonhybridized single stranded RNA was removed with RNase, the products resolved on a denaturing polyacrylamide gel, and band intensities determined by laser densitometry. Known quantities of a synthetic DHFR "pseudo-mRNA" were assayed in parallel to generate a standard curve, thereby allowing determination of

absolute DHFR mRNA levels in experimental samples. DHFR mRNA protected 141 bases and pseudo-mRNA protected 161 bases of the RNA probe. The 13% greater length, and therefore radioactivity, of the fragment protected by pseudo-mRNA was considered in quantitation.

Figure 2.2*B* shows an autoradiogram of an RNase protection assay in which $5x10^6$ cell equivalents of H$^-\alpha$ cell RNA harvested 0, 12, and 25 hours after inducing commitment was assayed for DHFR mRNA content (*lanes 1-3*, respectively). Densitometrically-determined band intensities are shown below each lane. The strength of the 141-base DHFR mRNA signal in cellular RNA samples was compared to that of a range of DHFR pseudo-mRNA standards (*lanes 4-7*). The molar amount of DHFR message in cellular samples was interpolated from the standard curve. Proliferative cells (*lane 1*) exhibited 34 attomoles of DHFR mRNA per sample, or 4.1 message molecules per cell. DHFR mRNA levels declined precipitously during commitment (*lanes 2-3*). Although a DHFR mRNA signal was detectable in 12-hour cells, the band was too faint to quantitate accurately.

To better quantitate the decline in DHFR mRNA, particularly at later times after induction, the experiment described in Fig. 2.2 was repeated using H$^-\alpha$R50A cells, a methotrexate-selected derivative of the H$^-\alpha$ cell line that had amplified the DHFR gene 50 to 100-fold. Use of the amplified cell line, coupled with use of a higher specific activity RNA probe, allowed visualization of DHFR mRNA using fewer cell equivalents of RNA per assay. In *lanes 4-8* of Fig. 2.4, DHFR mRNA levels were assayed in 2 μg of amplified cell RNA harvested 0, 3, 7, 12, and 24 hours after induction. The absolute DHFR mRNA level listed below each lane was determined by comparison to pseudo mRNA standards (not shown). The zero-hour proliferative cultures (*lane 3*) contained 297 DHFR mRNA molecules/cell, a level 72-fold higher than that determined for the nonamplified H$^-\alpha$ parental cell line. Thus, the increase in DHFR mRNA levels was roughly proportional to the increase in DHFR gene copy number. As was observed in the parental cell line, DHFR mRNA levels declined significantly during commitment in the amplified cell line. By 12

hours *(lane 6)*, DHFR mRNA levels had declined 7.5-fold to a basal level of about 40 molecules/cell. Because the amplified cell line qualitatively recapitulated the pattern of expression observed in nonamplified cells, and yet contained greatly elevated levels of gene product, it should facilitate biochemical investigation of the regulatory mechanism governing DHFR mRNA and protein levels.

Figure 2.4 also includes data on DHFR mRNA levels in a second strain of nonamplified mouse muscle cells. The line used, $TK^{-}\varepsilon$ cells (Merrill *et al.*, 1984), although originally derived from the same mouse as $H^{-}\alpha$ cells, had had a separate cell culture history spanning several hundred cell generations. Lanes 10-14 show DHFR mRNA levels in 50 μg of $TK^{-}\varepsilon$ cell RNA at 0, 2.5, 5, 10, and 25 hours after inducing commitment. Relative band intensities, corresponding molar amounts of DHFR mRNA per sample, and DHFR mRNA molecules per cell are listed below each lane. Similar to results obtained with the amplified cell, DHFR mRNA levels in $TK^{-}\varepsilon$ cells declined to a basal level 6.1-fold lower than proliferative cells by 12 hours after induction (compare *lane 8* with *lanes 11* and *12*). Because the proliferative levels of DHFR mRNA in two distantly related mouse muscle cell lines were closely matched (compare Fig. 2.2, *lane 4* and Fig. 2.4, *lane 8*), we consider the calculated value of 4.1-4.3 copies per cell to be an accurate estimation of the number of DHFR mRNA molecules generally present in proliferating nonamplified muscle cells.

The maintenance of DHFR enzyme activity in differentiated muscle cells could be due to continued synthesis from residual normal DHFR mRNA or to synthesis from an alternative message; either a previously uncharacterized endogenous message, or an exogenous message from some cryptic microbial or viral contaminant. To distinguish these possibilities from continued DHFR activity as a result of a long-lived enzyme, we measured DHFR enzyme synthesis rates and DHFR enzyme stability *in vivo*.

Persistence of DHFR enzyme activity is the result of a long enzyme lifetime - The rate of

DHFR enzyme synthesis in proliferative and committed amplified muscle cells was assayed after blocking existing high-affinity binding sites with nonlabeled methotrexate. This method was valid because of the low dissociation rate and high affinity association of the DHFR-methotrexate complex (Fig. 2.3 and Goldman *et al.*, 1968; Johnson *et al.*, 1978; Werkheiser, 1961). Cells were assayed for reemergence of [^{3}H]methotrexate binding activity at specified times after blocking preexisting binding sites. Figure 2.5 shows results of a typical experiment. After a short lag, proliferative cells exhibited rapid exponential reemergence of methotrexate binding capability, doubling every 12-14 hours. The lag was attributed to dislodgement of some cells during the pulsing and rinsing manipulations, and to a delay in clearance of nonbound or loosely bound intracellular methotrexate. The 12 to14-hour doubling time for DHFR enzyme content per culture coincided with the 12 to 14-hour generation time for these cells.

As a second test of whether maintenance DHFR activity was the result of a long enzyme lifetime, the stability of DHFR *in vivo* was measured. Proliferative cells were pulsed with [^{3}H]methotrexate, rinsed 3 times, and maintained in medium containing excess nonlabeled methotrexate. Cells were rinsed, harvested, and assayed for radioactivity at various times thereafter. Again, because of the low dissociation rate of methotrexate from DHFR, loss of radioactivity after clearance of nonbound ligand was due primarily to DHFR degradation. Figure 2.6 shows results of an *in vivo* stability experiment. The relatively high signal immediately following the labeling period was attributed to nonbound and loosely bound intracellular [^{3}H]methotrexate not removed by rinsing (Goldman *et al.*, 1968). No further decline was observed during the first 8 hours, and less than a 40% decline was observed after 55 hours. By assuming all ligand diappearance at 55 hours was due to protein degradation, the minimum half-life of DHFR protein was calculated to be 75 hours. Thus, maintenance of DHFR activity in committed cells was not due to continued synthesis of DHFR, but rather, was due to continued existence of previously synthesized enzyme.

DISCUSSION

We have shown that DHFR enzyme activity was maintained after disappearance of DHFR mRNA during muscle cell commitment. Maintenance of activity resulted from a long *in vivo* lifetime of DHFR enzyme, not from continued synthesis using residual normal message or a cryptic message. The enzyme was so stable, in fact, that only a minimum half-life of 75 hours was calculable. To determine this value, the 55-hour time point in Figure 2.6, showing approximately a 40% decline in radioactivity, was used. However, after 55 hours in excess nonlabeled methotrexate, even a slow off rate would allow at least some dissociation of radiolabeled ligand. Furthermore, cell viability becomes questionable after such long incubations in methotrexate. Ligand dissociation and cell death would decrease the apparent enzyme lifetime. Our determination agrees with that of Alt *et al.* (1976), who reported a 50 to 60-hour minimum half-life for DHFR in growing fibroblasts, and that of Rogers and Rechsteiner (1988), who recently reported a 96 to 100-hour half-life for radiolabeled chicken DHFR microinjected into HeLa cells.

An enigma arises, then, as to how an enzyme can be cell cycle regulated if its *in vivo* half-life is several cell generations in length. Following its synthesis during S phase, the extreme stability of DHFR protein would preclude any significant decline in enzyme levels during the ensuing G2, M and G1 phases.

Our results, therefore, are incompatible with the commonly held idea that cells express significant levels of DHFR enzyme only during S phase. On close examination, results of previous studies on cell cycle regulation of DHFR are not in conflict with our findings. Numerous studies have established that DHFR enzyme levels increase when growth-arrested fibroblastic cells are released from inhibition and allowed to enter S phase (Johnson *et al.*, 1978; Wiedemann and Johnson, 1979); however, these studies did not address whether DHFR levels declined during subsequent phases of the cell cycle. In fact, treatments as severe as seven days of serum starvation were used to lower basal

DHFR activity prior to stimulation (Johnson *et al.,* 1978), and still a significant background was observed. When mitotic selection was used to synchronize cells and DHFR activity was normalized to cellular protein content (Mariani *et al.,* 1981), only a small decline in DHFR activity was observed as cells progressed beyond S phase. Furthermore, because protein accumulation continued unabated throughout the cell cycle, whereas DHFR synthesis was limited to S phase, the small decline in DHFR activity per unit protein was attributable entirely to increases in cellular protein, in effect diluting a constant amount of DHFR activity (Mariani *et al.,* 1981). Therefore, on the basis of our results and others, we conclude that only the rate of synthesis, not the level, of DHFR enzyme changes significantly during the cell cycle and during differentiation.

TK enzyme, in contrast to DHFR, is very labile in nonreplicating cells. As differentiating muscle cells withdraw from the cell cycle, TK activity declines rapidly (Fig. 2.1 and Merrill *et al.,* 1984), actually preceding the decline in TK mRNA levels (Gross *et al.,* 1987). A recent study using fibroblastic cells synchronized by centrifugal elutriation (Sherley and Kelly, 1988) suggested that the rapid decline in TK enzyme activity was due in part to cell cycle-dependent changes in TK enzyme stability. Although a change in protein stability may be important in controlling the levels of TK activity, DHFR enzyme apparently is not subject to a degradative mechanism of regulation.

By considering the roles of DHFR and TK in postreplicative cells, one can speculate why these enzymes are regulated differently. In postreplicative cells, tetrahydrofolate coenzymes are required for synthesis of purine ribonucleotides and certain amino acids. DHFR, being solely responsible for maintenance of tetrahydrofolate coenzyme levels, is required throughout the life of a cell. The rate of tetrahydrofolate oxidation will be greatest in replicating cells, when thymidylate synthetase generates one mole of dihydrofolate per mole of dTMP synthesized. However, in postreplicative cells, some DHFR activity will be required to maintain pools of reduced folates in the presence of tetrahydrofolate coenzyme turnover and non-enzymatic tetrahydrofolate oxidation (Baram

et al., 1988; Brown *et al.*, 1961; O'Dell *et al.*, 1947; Stockstad and Koch, 1967). Because cellular folates are limited (Goldman *et al.*, 1968), excess DHFR enzyme will not expand tetrahydrofolate pools in postreplicative cells. Rather, DHFR activity will simply become substrate-limited.

TK, on the other hand, uses nonlimited substrates. Because the TK reaction product, dTMP, is necessary only during DNA synthesis, TK has minimal function in postreplicative cells. To prevent overaccumulation of dTTP, it may be advantageous for TK activity to decline in postreplicative cells.

Whereas TK is regulated by a mechanism that actually confines TK activity to S phase, DHFR may be regulated by a more general mechanism that adjusts mRNA levels to the growth state of the cell. Because cellular proteins are partitioned at cytokinesis, proliferative cells must synthesize proteins at a rate sufficient to double protein content during each cell cycle, as well as replace protein lost to degradation. In postreplicative cells, the synthetic rate of each protein must decline until equal to its rate of degradation. For stable enzymes like DHFR, unabated synthesis in the absence of cytokinesis would rapidly overburden the cell with excess protein. In agreement with this, Figure 2.5 showed the amount of DHFR enzyme synthesized per proliferative culture increased exponentially, doubling every cell generation. Differentiated cultures showed no detectable rate of DHFR synthesis. We suggest that a general mechanism for reducing protein synthetic rates in postreplicative cells is a reduction in cognate mRNA. Muscle cells, particularly lines containing amplified copies of the DHFR gene, constitute a valuable system for studying this general control mechanism.

ACKNOWLEDGMENTS

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FIGURES AND LEGENDS

Fig. 2.1: **DHFR and TK enzyme levels during muscle cell differentiation.** At indicated times after inducing differentiation, [^{3}H]methotrexate binding activity (DHFR), TK activity, and thymidine labeling index were determined. A one hour exposure to 0.2 μCi/ml [^{3}H]thymidine followed by autoradiography was used in determining the latter (Merrill *et al.*, 1984).

Fig. 2.1

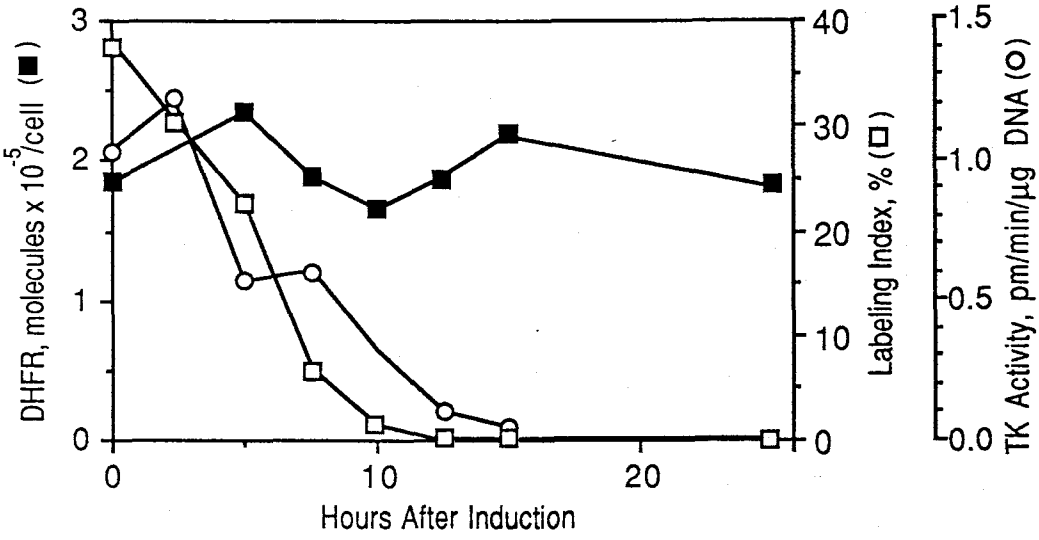
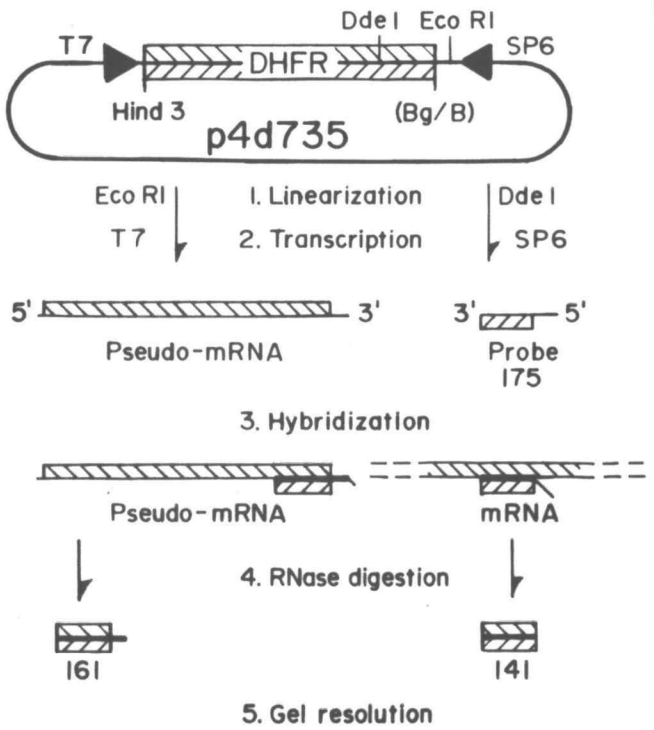
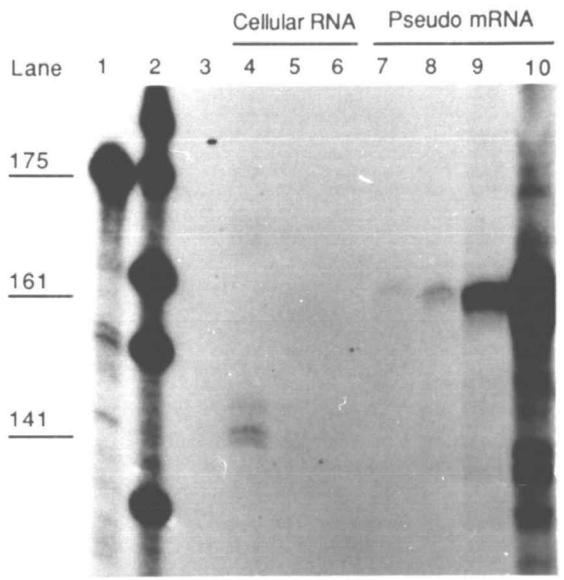

DHFR, molecules x 10^{-5}/cell (■)
Labeling Index, % (□)
TK Activity, pm/min/µg DNA (○)
Hours After Induction
0
10
20
30
40
1.5
1.0
0.5
0.0
1
2
3

Fig. 2.2: **Quantitation of DHFR mRNA using an RNAse protection assay.** *Panel A*, Schematic diagram of RNase protection protocol. To generate 175-base probe, plasmid p4d735 was linearized with Dde1 and transcribed with SP6 polymerase in the presence of [^{32}P]UTP. To generate "pseudo-mRNA", p4d735 was linearized at Eco R1, and transcribed with T7 polymerase in the presence of nonlabeled nucleotides. Probe was hybridized with either mRNA or pseudo-mRNA and RNase-digested, yielding 141-base and 161-base protected fragments, respectively. Fragments were resolved on 8 M urea, 9% polyacrylamide gels in 45 mM Tris-borate, pH 8.0, 1.0 mM EDTA. Abbreviations: B = Bam H1; Bg = Bgl2. *Panel B,* Effect of commitment on DHFR mRNA levels in nonamplified muscle cell line H$^-\alpha$. Lanes 3, 4, and 5 contain 50 µg of RNA harvested at 0, 12, and 25 hours after induction. As muscle cells contain 10 pg of RNA and RNA content remains constant during commitment (Gross *et al.,* 1987), 50 µg of RNA represents 5 x 10^6 cell equivalents. Lane 1 contains a 1:100 dilution of nondigested probe; lane 2 contains MspI-digested pBR322 markers (from top: 190, 180, 160, 147 and 122 bp); lane 3 is a control showing probe was not protected by 50 µg of yeast RNA. Lanes 6, 7, 8, and 9 contain 10, 30, 100, and 1000 attomoles (10^{-18} moles) of synthetic DHFR pseudo-mRNA. Band intensities, determined by laser densitometry, are listed below each lane in arbitrary units. ND = no detectable signal.

A



B



hours after induction	0	12	24				
band intensity	2.8	0.2	ND	1.2	3.1	16	120
attomol DHFR mRNA/sample	30	<10	<10	10	30	100	1000
molecules DHFR mRNA/cell	4.1	<1.4	<1.4				

Fig. 2.3: **Scatchard analysis of methotrexate binding activity in proliferative muscle cell extracts.** The standard methotrexate binding conditions described in Materials and Methods were used except each determination contained various concentrations of nonlabeled ligand in the presence of 37 μCi radiolabeled methotrexate. From the high affinity component of the curve, the kDa was calculated to be 150 pM ± 100 pM.

Fig. 2.3

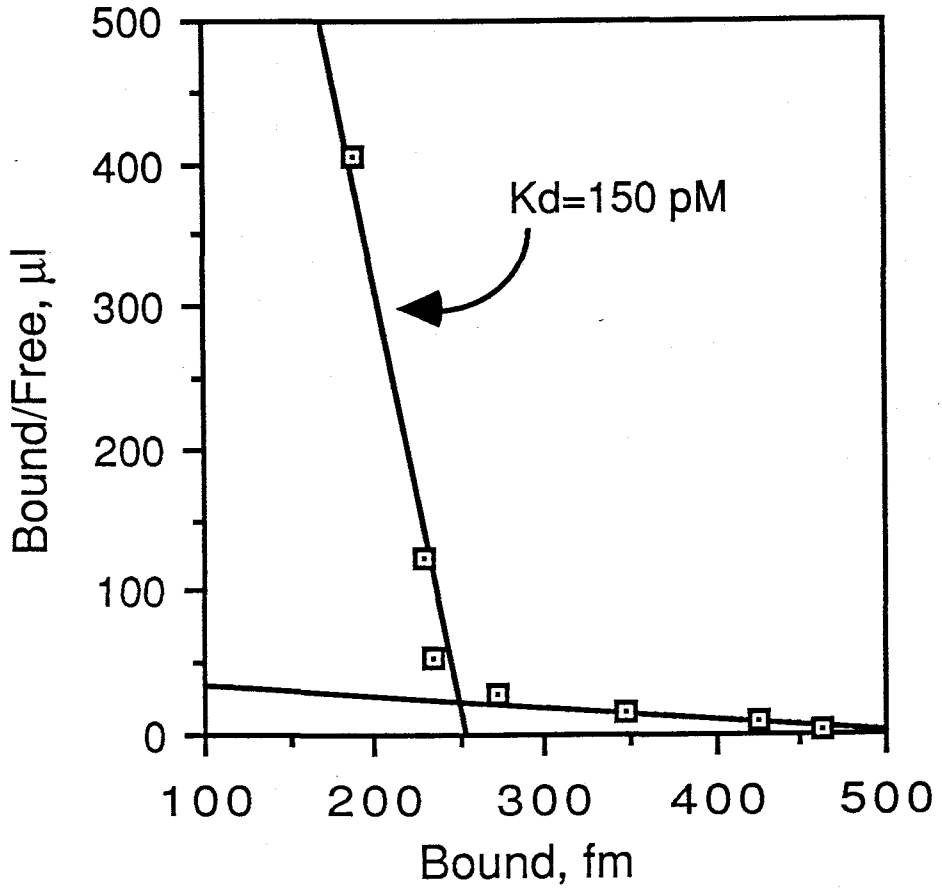
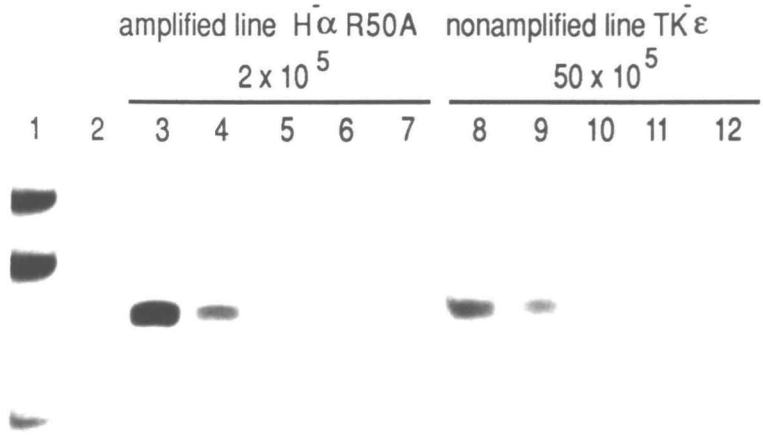

500
400
300
200
100
0
Bound/Free, µl
Kd=150 pM
100
200
300
400
500
Bound, fm

Fig. 2.4: **Effect of commitment on DHFR mRNA levels in amplified H$^-$αR50A cells and nonamplified TK$^-$ε cells.** Lane 1 contains Msp1-digested pBR322 markers (from top: 160, 147 and 122 bp). Lane 2 is a control showing probe was not protected by 50 μg of yeast RNA. Above the lanes, the cell line and the number of cell equivalents assayed are shown. Below, the hours of induction are listed, as well as the the relative band intensity, the number of attomoles of DHFR mRNA per sample, and the calculated number of DHFR molecules per cell. Lanes containing the pseudo-mRNA standards are not shown.

Fig. 2.4



hours of induction	0	3	7	12	24	0	2.5	5	10	25
band intensity	33	16	8.6	1.8	2.8	15	12	6.5	1.0	1.3
attomoles DHFR mRNA/sample	99	46	27	13	14	35	33	22	6	7
molecules DHFR mRNA/cell	297	138	81	39	42	4.3	4.0	2.6	0.7	0.8

Fig. 2.5: **DHFR synthesis in proliferative and postreplicative cells.**

Proliferative (closed squares) and committed (open circles) amplified muscle cell cultures were incubated for 30 minutes with 100 μM nonlabeled methotrexate to block existing DHFR molecules. Cultures were rinsed and incubation was continued in drug-free medium. At indicated times, [^{3}H]methotrexate binding activity was assayed as described in Materials and Methods. Symbols plus error bars represent the mean ± SD for determinations on 4 proliferative cultures or 3 committed cultures.

Fig. 2.5

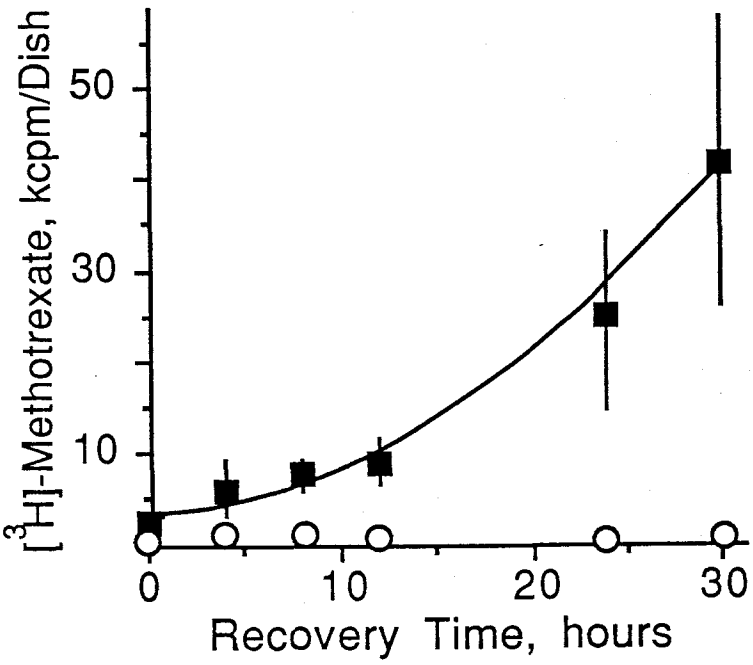
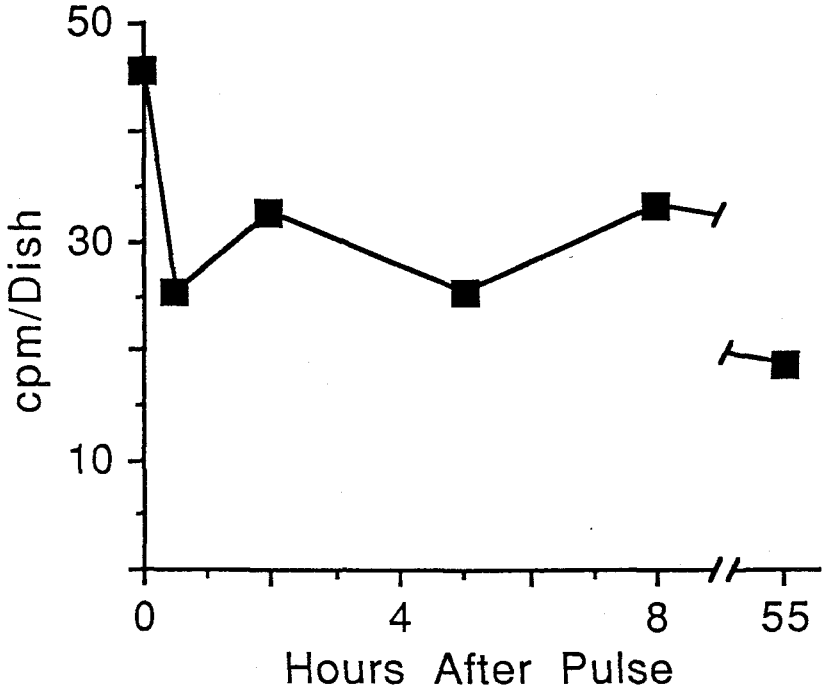

[3H]-Methotrexate, kcpm/Dish
50
30
10
0
10
20
30
Recovery Time, hours

Fig. 2.6: ***In vivo* DHFR stability**. Proliferating wild type cells were incubated 15 minutes with 5 nM [^{3}H]methotrexate (37 Ci/mmole). Cultures were rinsed and incubation was continued in medium containing 1.0 μM nonlabeled methotrexate. At indicated times, cells were rinsed and harvested for liquid scintillation counting. Background radioactivity of 17.5 cpm was subtracted from each determination.

Fig. 2.6



CHAPTER 3

Changes in Dihydrofolate Reductase mRNA Levels Can Account Fully for Changes in DHFR Synthesis Rates During Myogenic Withdrawal from the Cell Cycle

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Not yet published.

SUMMARY

A mouse muscle cell line resistant to 300 μM methotrexate was developed to determine whether the translational efficiency of dihydrofolate reductase (DHFR) mRNA is growth state-dependent. Cell line $H^{-}\alpha$R300T, containing an estimated 400-fold amplification of its DHFR gene, overexpressed DHFR mRNA and DHFR protein. As myoblasts withdrew from the cell cycle and committed to terminal differentiation, DHFR mRNA levels and DHFR synthesis rates decreased with quantitatively similar kinetics. After 15 to 24 hours, committed cells contained 5% the proliferative level of DHFR mRNA (80 molecules per committed cell) and synthesized DHFR protein at 6% the proliferative rate. At no point during commitment did the decrease in DHFR synthesis rate exceed the decrease in DHFR message. The decrease in DHFR mRNA levels during commitment was sufficient to account fully for the decrease in rates of DHFR synthesis. As a second means of investigating translational regulation, the polysomal distribution of DHFR mRNA was determined at various times after inducing commitment. At all times, DHFR mRNA remained polysomal and the average number of ribosomes per message remained constant (5 to 6 ribosomes per DHFR mRNA). The constancy of polysome size, along with the uniform rate of DHFR synthesis per message, indicated that DHFR mRNA was efficiently translated in postreplicative cells. The results support a model wherein replication-dependent changes in DHFR synthesis rates are determined exclusively by changes in DHFR mRNA levels.

INTRODUCTION

Synthesis of enzymes involved in DNA precursor biosynthesis preferentially occurs in replicating cells. For this class of enzymes, many studies have demonstrated that mRNA levels and protein synthesis rates are preferentially elevated in replicating cells (Alt *et al.,* 1976; Hendrickson *et al.,* 1980; Johnson *et al.,* 1976, 1978; Kellems *et al.,* 1976; Leys and Kellems, 1981; Leys *et al.,* 1984; Liu *et al.,* 1985; Mariani *et al.,* 1981; Merrill *et al.,* 1984; Santiago *et al.,* 1984; Schmidt and Merrill, 1989a; Sherley and Kelley, 1988; Wiedemann and Johnson, 1979; Wu and Johnson, 1982). However, in most cases, the possibility that translational control contributes to replication-specific enzyme synthesis has not been adequately tested.

Dihydrofolate reductase (DHFR) is an attractive enzyme to study because its expression can be increased two to three orders of magnitude above the wild type level by selecting strains of cells containing amplified copies of the DHFR gene. In highly amplified cell lines, DHFR is a major cell constituent, representing as much as 10% of soluble cellular protein (Alt *et al.,* 1976; Kellems *et al.,* 1976); however, by all measured criteria, DHFR regulation appears identical in amplified and wild type cells (Johnson *et al.,* 1978; Schmidt and Merrill, 1989a; Wiedemann and Johnson, 1979). Therefore, amplified cells are valuable for biochemical studies of the mechanisms regulating expression of the DHFR gene.

In the results that follow, quantitative measurements of DHFR mRNA levels, DHFR synthesis rates, and the polysomal distribution of DHFR mRNA were used to evaluate whether decreases in DHFR mRNA levels were sufficient to account for decreases in the rate of DHFR synthesis. The importance of this study is best illustrated by recent findings concerning S phase-dependent synthesis of thymidine kinase (TK, EC2.7.2.21). Numerous early studies, conducted using various growing and nongrowing cell

preparations, had established a positive correlation between TK mRNA levels, TK activity levels, and the replicative state of the cell population (Gross *et al.*, 1987; Groudine and Casimir, 1984; Hoffbauer *et al.*, 1987; Liu *et al.*, 1985; Merrill *et al.*, 1984; Schlosser *et al.*, 1981). However, more recent studies, in which TK mRNA levels and TK synthesis rates were measured quantitatively, revealed that TK synthesis rates are not primarily limited by levels of TK mRNA in non-S phase cells (Gross *et al.*, 1988; Gross and Merrill, 1989; Sherley and Kelly, 1988). Rather, the translational efficiency of TK mRNA is reduced (Gross and Merrill, 1989). If DHFR was subject to the same translational regulation as TK, amplified cells could be used to facilitate biochemical analysis of the translational control mechanism. To test this possibility, we specifically asked whether the translational efficiency of DHFR mRNA, like that of TK message, decreased as muscle cells withdrew from the cell cycle during commitment. We show that decreases in the rate of DHFR synthesis were matched temporally by decreases of equal magnitude in the level of DHFR mRNA. The results support a model wherein DHFR synthesis rates are limited solely by levels of DHFR message.

EXPERIMENTAL PROCEDURES

Parental muscle cell line $H^-\alpha$, a hypoxanthine-guanosine phosphoribosyltransferase-deficient substrain of the MM14D mouse skeletal myoblast line developed by Linkhart *et al.* (1981), was used to derive methotrexate-resistant cell line $H^-\alpha$R50T (Schmidt and Merrill,1989a). Cell line $H^-\alpha$R300T was derived from $H^-\alpha$R50T by further serial selection in 100, 200, and 300 μM methotrexate (Sigma, A-6770). Cells were grown for at least 10 cell generations at each selective step. $H^-\alpha$R300T was maintained in growth medium (basal medium [0.5X Ham's F-10, 0.5X DMEM, 0.4 mM additional $CaCl_2$, 15 mM Hepes, pH 7.2, 1% glucose, 2.45 mg/ml NaH_2CO_3, 10 U/ml penicillin G, 0.5 mg/ml streptomycin sulfate] supplemented with 15% horse serum, fibroblast growth factor activity from bovine brain [Esch *et al.,* 1985], and 300 μM methotrexate). Commitment was induced by rinsing cells twice with basal medium, followed by incubation in differentiation medium (basal medium supplemented with 300 μM methotrexate and 1 μM insulin).

DHFR synthesis rates were determined by *in vivo* labeling. Cultures were incubated 1 or 2 hr with 50 to 100 μCi/ml [^{35}S]Met (641 Ci/mmole, New England Nuclear) in methionine-free Ham's F12/DMEM (50%/50%, vol/vol) (GIBCO) supplemented with 1 μM insulin. Cultures were rinsed twice with ice cold phosphate-buffered saline and stored at -80°C. Cells were scraped from dishes and sonicated in either 10 mM Pipes, pH 7.0, 150 mM KCl; or 5 mM Tris, pH 7.8, 10 mM KCl, 10 mM $MgCl_2$ (both buffers gave identical results). DNA concentration was analyzed by the method of Labarca and Paigen (1980), and was used to calculate the number of cell equivalents in each lysate (10 pg DNA/cell). Extracts were clarified and proteins were separated by electrophoresis through 12% polyacrylamide/SDS gels as described by Laemmli (1970). Gels were fixed, stained with Coomassie, and destained as described previously (Gross and Merrill, 1988). Destained gels were photographed, washed 30 min in water, and incubated 1 hr in

1 M salicylic acid (Sigma, S-3007). Gels were rinsed with water, dried, and exposed to X-ray film with an intensifying screen. After exposure, Coomassie-stained DHFR bands were excised, incubated 12 to 24 hr in 0.2 ml 30% hydrogen peroxide at 65°C, mixed with 2.0 ml scintillation fluid (2 parts toluene, 1 part Triton X-100, 4.0 mg/ml Omnifluor), and assayed by liquid scintillation for 50 min.

DHFR mRNA levels were determined by RNase protection. The hybridization and digestion protocol, including a description of probes and DHFR pseudo-mRNA, has been published elsewhere (Schmidt and Merrill, 1989a,b). Protected fragments were analyzed by denaturing gel electrophoresis and autoradiography (Schmidt and Merrill, 1989a,b), or by liquid scintillation. In the latter case, RNase digestions were terminated by adjusting reactions to 0.5% (wt/vol) sodium pyrophosphate, 5% (wt/vol) trichloroacetic acid, 0°C. Samples were incubated on ice >30 min, and nucleic acids were collected by washing samples through pre-wet 1-cm diameter GFC filters (Whatman) with 5% trichloroacetic acid. Filters were rinsed with 70% ethanol, solubilized with 0.2 ml Soluene, mixed with 2.0 ml of 4 mg/ml Omnifluor in toluene, and assayed by liquid scintillation for 50 min.

Polysomal RNA preparations were isolated by velocity sedimentation using a modification of the methods of Gross and Merrill (1989). All manipulations were performed on ice or at 4°C unless otherwise noted. Cell cultures on 10-cm dishes were rinsed with phosphate-buffered saline containing 10 µg/ml cycloheximide and were either frozen at -80°C or harvested immediately (both procedures gave identical results). Cells were harvested by scraping in 300 µl 1X (fresh dishes) or 150 µl 2X (frozen dishes) lysis mix (1X = 50 mM Tris, pH 7.5 at 25°C, 250 mM NaCl, 25 mM $MgCl_2$, 0.5% Triton X-100, 0.1 U/µl RNasin [Promega], 20 µg/ml cycloheximide, 2 mM DTT). Lysates were transferred to 15 ml glass Corex tubes and clarified by centrifugation for 10 min at 13,000 rpm in a Sorvall SS-34 rotor. Clarified lysates were either stored frozen at -80°C, or used immediately (both procedures gave identical results). Clarified

lysates were carefully layered on 12-ml sucrose gradients (15-50% linear gradients in 50 mM Tris, pH 7.5 at 25°C, 250 mM NaCl, 25 mM $MgCl_2$, 20 µg/ml cycloheximide). Sedimentation was performed in a Beckman SW-40 rotor at 32,000 rpm for 130 min. Gradients were fractionated by injecting a 65% sucrose solution into the bottom of the centrifuge tubes (0.375 ml/min) and collecting the gradient at the top using an Isco model 185 density gradient fractionator. An Isco Type 6 optical unit (λ = 254 nm) and UA-5 recorder (calibrated immediately before each use) were used to monitor absorbance during fractionation. Aliquots of each gradient (0.5 ml) were collected and stored at -20°C. The time delay between a sample passing the optical unit and being recovered was determined, and used to align each aliquot with the scan. Aliquots were thawed and pooled to represent different polysomal and nonpolysomal fractions. Subsequent steps were performed at room temperature except as noted. Each pooled fraction was adjusted to 2.5 ml with water, and received 280 µl 10X TES (100 mM Tris, pH 7.5 at 25°C, 50 mM EDTA, 10% SDS). Yeast RNA was added to 25 µg/ml and proteinase K (Boehringer) was added to 20 µg/ml. Samples were incubated at 55°C for 45 min, adjusted to 250 mM NaCl, and were extracted with phenol/chloroform, extracted with chloroform, and precipitated with ethanol at -20°C. RNA was collected by centrifugation. RNA pellets were washed with 70% ethanol, resuspended in 20 µl water, 65°C, and assayed by RNase protection (10 µl/assay) (Schmidt and Merrill, 1989a,b).

RESULTS

Rates of DHFR synthesis during commitment - To determine the translational efficiency of a message, one must accurately measure both the cellular concentration of the mRNA and the rate of synthesis of the protein. We recently described a sensitive RNase protection assay for quantitating DHFR mRNA (Schmidt and Merrill, 1989a,b); an equally quantitative means of measuring DHFR synthesis rates was needed. Recovery of [^{3}H]methotrexate binding activity after blockage of existing sites with nonlabeled methotrexate (Johnson *et al.*, 1978; Schmidt and Merrill, 1989a; Wiedemann and Johnson, 1979) was judged to be too indirect, inaccurate, and insensitive a measure of DHFR synthesis. We therefore investigated whether DHFR was sufficiently abundant in amplified muscle cells to allow direct electrophoretic analysis. The previously described amplified muscle cell line H$^-$αR50T (Schmidt and Merrill, 1989b) did not produce an electrophoretically distinguishable DHFR protein band. Therefore, a more highly amplified substrain, H$^-$αR300T, resistant to 300 μM methotrexate, was isolated.

Proliferating H$^-$αR300T muscle cells were labeled briefly with [^{35}S]Met, and proteins in clarified whole-cell lysates were separated by SDS-gel electrophoresis. As shown in the autoradiogram in Figure 3.1, amplified H$^-$αR300T cells (*lane 2*) gave a strong band at 21 kDa, which corresponded to the molecular weight of DHFR protein. Nonamplified H$^-$α parental cells showed no band at 21 kDa (*lane 1*). The presence of a 21 kDa band in amplified cells, and its absence in nonamplified cells, was also evident when Coomassie-staining was used to visualize steady-state protein levels (not shown). In both the autoradiogram and the Coomassie-stained gel, all other protein bands were of similar intensity and mobility in amplified and nonamplified cells, with the exception of a band at 17 kDa. The band at 17 kDa was enriched in H$^-$αR300T extracts and may have been due to another protein encoded within the *dhfr*-amplicon.

Based on its mobility and its absence in control cells, we concluded that the

prominent 21 kDa band present in amplified cells represented DHFR protein. Published results have established that DHFR protein has a halflife of 50 to 100 hr in proliferative cells (Alt *et al.*, 1976; Rogers and Rechsteiner, 1988; Schmidt and Merrill, 1989a), and that there is no detectable change in the halflife of DHFR during muscle cell commitment (Schmidt and Merrill, 1989a). Because DHFR degradation is negligible during short labeling periods, DHFR-associated radioactivity after a 1- to 2-hr incubation with [^{35}S]Met accurately represents the rate of [^{35}S]Met incorporation into DHFR protein. Furthermore, the rate of [^{35}S]Met incorporation into total protein changes only slightly with commitment (Gross and Merrill, 1988), indicating that the specific activity of the [^{35}S]Met pool remains relatively constant. By normalizing DHFR-specific radioactivity to total [^{35}S]Met incorporation, the rate of DHFR protein synthesis can be accurately determined.

Having established that DHFR synthesis rates could be directly measured in H$^-$αR300T cells, we asked whether rates of DHFR synthesis changed during myogenic withdrawal from the cell cycle. Figure 3.2*A* shows a Coomassie-stained gel containing labeled H$^-$αR300T cell extracts harvested 1, 5, 10, and 15 hr after inducing commitment. (No cells withdraw from the cell cycle during the first two hours after induction, and virtually all cells withdraw by 15 hr [Schmidt and Merrill, 1989a]; thus, the 1-hr cells were fully proliferative and the 15-hr cells were fully committed.) Roughly equivalent amounts of the 21 kDa DHFR band were present at all time points, consistent with the previous finding that, because of the extreme stability of the protein, absolute levels of DHFR enzyme do not decrease significantly during commitment (Schmidt and Merrill, 1989a). An autoradiogram of the gel is shown in Figure 3.2*B*. The intensity of the 21 kDa DHFR signal decreased significantly during commitment. To relate the difference in intensity of the autoradiographic signal to the magnitude of change in DHFR synthesis rate, 0.1X and 0.3X dilutions of the proliferative (1-hr) cell extract were run in parallel lanes. Based on a comparison of autoradiographic signal intensities, DHFR synthesis

decreased to 10% that of proliferative cells by 15 hr after induction. A more quantitative measure of DHFR synthesis was obtained by liquid scintillation analysis of excised bands. Results are listed below each lane (cpm). Based on liquid scintillation results, the magnitude of the decrease in DHFR synthesis was 7.6-fold by 15 hr.

Translational efficiency of DHFR mRNA during commitment - To measure DHFR mRNA levels, an RNase protection assay was used (Schmidt and Merrill, 1989a,b). In this assay, cellular RNA or synthetic DHFR "pseudo-mRNA" standards are hybridized with radiolabeled RNA probes complementary to specific regions of the DHFR message. After RNase digestion, protected probe regions are separated from low molecular weight digestion products by gel electrophoresis or by precipitation with trichloroacetic acid. Comparison of cellular RNA signals to standards allows precise quantitation of the absolute number of DHFR molecules per cell. To ensure accuracy, two probes complementary to opposite ends of the DHFR protein coding region are used (Schmidt and Merrill, 1989b).

Figure 3.3 shows an RNase protection assay of DHFR mRNA levels in H$^-$αR300T cells. Proliferative H$^-$αR300T cells (*lane 7*) exhibited 1500 DHFR mRNA molecules per cell. (In contrast, proliferative nonamplified cells contain 4 DHFR messages [Schmidt and Merrill, 1989a].) Upon induction, levels of DHFR mRNA decreased, which indicated that the mechanisms regulating DHFR mRNA levels were not inactivated during selection of cell line H$^-$αR300T. Comparison of the 20-hr signal to the standard curve indicated that committed cells contained 156 DHFR mRNA molecules per cell (*lane 10*), a 9.6-fold decrease.

To test whether the translational efficiency of DHFR mRNA decreased during commitment, parallel measurements of DHFR synthesis rates and DHFR mRNA levels during muscle cell commitment were made. Determinations were done at 2-hr intervals to allow detection of possible transient changes in translational efficiency. Standards were included in both mRNA and synthesis rate measurements for accurate baseline calculation

and to maximize accuracy. Figure 3.4 shows the results from these experiments. At the end of each labeling period, parallel cultures were harvested for DHFR mRNA determinations. DHFR mRNA levels were measured in quadruplicate, using both the 5' and 3' probes; results represent average values. Decreases in the rate of DHFR synthesis were accompanied by decreases of similar or slightly greater magnitude in the level of DHFR mRNA. At no time during the commitment process did the decrease in synthesis rate exceed the decrease in mRNA level.

As an additional test for translational repression of DHFR synthesis, the polysomal distribution of DHFR mRNA was determined at various times during commitment. Usually, in reports of translational regulation, rates of translational initiation are reduced (Endo and Nadal-Ginard, 1987; Hershey *et al.,* 1986), often via sequestration of mRNA in nonpolysomal ribonucleoprotein (Aziz and Munro, 1987; Hentze *et al.,* 1987). Regulation of translational initiation would cause characteristic changes in the polysomal distribution of DHFR message. If the rate of translational initiation on a particular message was reduced, the mean number of ribosomes engaged on each mRNA would decrease, leading to a measurable shift in the message distribution to smaller polysomes. If initiation was blocked completely, such as when message is sequestered as RNP, the message would disappear from the polysomal fractions and appear in the more slowly sedimenting nonpolysomal fractions. Conversely, if translational elongation was inhibited, polysome size would be expected to increase (unless the elongational block was matched by a compensatory decrease in intiation). To determine the polysomal distribution of DHFR mRNA, $H^{-}\alpha$R300T lysates were sedimented through sucrose gradients, and RNA purified from pooled fractions was analyzed for DHFR message by RNase protection. Figure 3.5*A* shows a scan of absorbance (254 nm) of a representative gradient (1-hr sample), and demonstrates how polysomal numbers were assigned and how gradient fractions were pooled. *Panel B* shows the distribution of DHFR mRNA in the gradients prepared from 1-, 6-, 10-, and 20-hr cell lysates. At all times, only a small

proportion of the DHFR message was in the nonpolysomal material at the top of the gradient. Also, no significant shift in DHFR polysome size was observed during commitment. At all time points, an average of 5 to 6 ribosomes were engaged on each DHFR mRNA.

In conclusion, in comparing DHFR mRNA levels and DHFR synthesis rates, or in analyzing the polysomal distribution of DHFR mRNA, no evidence was found for translational repression of DHFR mRNA during commitment. Our results are consistent with a model whereby DHFR synthesis rates are limited solely by levels of DHFR mRNA.

DISCUSSION

Using various growing and nongrowing cell preparations, previous studies have established that rates of DHFR synthesis are replication-dependent (Alt *et al.,* 1976; Johnson *et al.,* 1978; Kaufman and Sharp, 1983; Kellems *et al.,* 1976; Leys and Kellems, 1981; Mariani *et al.,* 1981; Schmidt and Merrill, 1989a; Wiedemann and Johnsosn, 1979; Wu *et al.,* 1982). Likewise, previous studies have established that levels of DHFR mRNA are replication-dependent (Farnham and Schimke, 1986; Hendrickson *et al.,* 1980; Kaufman and Sharp, 1983; Kellems *et al.,* 1976; Leys and Kellems, 1981; Santiago *et al.,* 1984; Schmidt and Merrill, 1989a,b; Wu *et al.,* 1982). However, only two of the above studies measured DHFR mRNA levels and DHFR synthesis rates quantitatively and in parallel (Leys and Kellems, 1981; Wu *et al.,* 1982), and thereby could have detected changes in the translational efficiency of DHFR mRNA. Both studies investigated activation of the DHFR gene during induction of growth-arrested cells, and found no evidence for translational regulation of the DHFR gene. However, growth-arrested cells are metabolically depressed, as evidenced by greatly reduced rates of total RNA and protein synthesis (Hendrickson *et al.,* 1980; Johnson *et al.,* 1974, 1978; Mariani *et al.,* 1981; Mauk and Green, 1973; Wiedemann and Johnsosn, 1979). It was possible that in previous studies, general metabolic recovery following growth-induction masked significant changes in the translational efficiency of DHFR mRNA.

In the present study, to determine whether a translational mechanism regulated DHFR expression when cells ceased replication but remained otherwise metabolically active, DHFR mRNA levels and DHFR synthesis rates were quantitatively compared during muscle cell differentiation. Whereas rates of DHFR synthesis (and to a lesser extent the 17 kDa protein) decreased dramatically during commitment, rates of synthesis of all other detectable cellular proteins remained constant (Fig. 3.4*A*), demonstrating that the committed cells were metabolically active. At all times during the commitment process,

changes in the rates of DHFR synthesis were matched quantitatively by changes in the levels of DHFR mRNA. Furthermore, the polysomal distribution of DHFR message showed that shifts characteristic of translational regulation did not occur during commitment. It is possible to repress translation of an mRNA without causing a shift in the polysomal distribution of the message. For example, the rates of both translational initiation and elongation may change to the same degree. Although rare, there have been reports of translational repression without a corresponding shift in the polysomal mRNA distribution (Ballinger and Pardue, 1983; Berry *et al.*, 1988; Gross and Merrill, 1989; Skadsen and Scandolios, 1987; Thomas and Mathews, 1984). Interestingly, one of these is the translational regulation of TK (Gross and Merrill, 1989). Based on both the polysomal data and direct measurements of DHFR sythesis rates and mRNA levels, we concluded that, in contrast to TK mRNA, DHFR mRNA was translated with the same efficiency in committed cells as in proliferative cells.

Our investigations to date on DHFR regulation are consistent with the following model: As muscle cells complete replication, transcription of the DHFR gene is repressed. As a result, DHFR pre-mRNA levels decrease, leading to a decrease in DHFR mRNA levels. DHFR mRNA is translated at the same rate in proliferative and committed cells; however, because mRNA levels are much lower in committed cells, DHFR synthesis rates are greatly reduced. Because DHFR is a long-lived protein ($t_{1/2}$ = 50 to 100 hr), the decrease in DHFR synthesis does not lead to a reduction in cellular DHFR levels. Rather, it prevents overaccumulation of DHFR protein in nonreplicating cells. Because we have found that changes in DHFR synthesis rates in muscle cells are determined primarily by changes in DHFR mRNA levels, future investigations will be directed at resolving the mechanisms by which transcription of the DHFR gene is repressed during commitment.

FIGURES AND LEGENDS

Fig. 3.1. **Synthesis of DHFR in proliferative muscle cells.** Proliferative cultures of parental H$^-\alpha$ (*lane 1*) and amplified H$^-\alpha$R300T (*lane 2*) cells were incubated 1 hr in 50 μCi/ml [^{35}S]Met. Labeled proteins in clarified lysates (10^5 cell equivalents per lane) were separated by electrophoresis through a 12% polyacrylamide/SDS gel and visualized by autoradiography. The 21 kDa DHFR protein band visible in H$^-\alpha$R300T cells is indicated, as is a 17 kDa protein of unknown identity. Molecular weight markers (*lane M*) were (from top) 200, 97, 68, 43, 29, 18, and 14 kDa.

Fig. 3.1

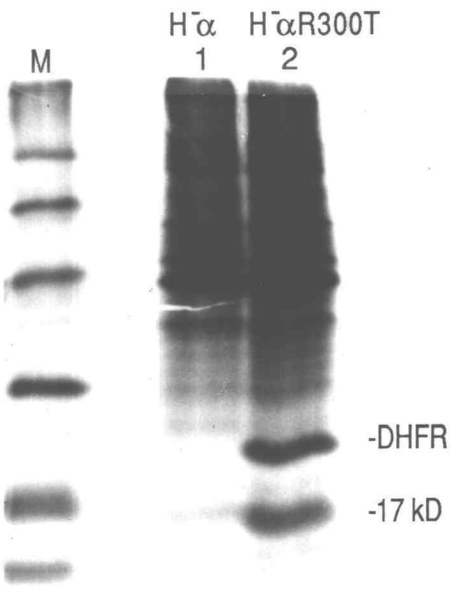


Fig. 3.2. **DHFR synthesis rates during commitment.** At indicated times after inducing commitment, H$^-$αR300T cells were incubated 2 hr in 100 μCi/ml [^{35}S]Met. Proteins in clarified lysates (10^5 cell equivalents per lane for undiluted sample) were separated by electrophoresis through 12% polyacrylamide/ SDS gels. Three gels were run; a typical gel is shown. *Panel A*, Coomassie-stained gel. *Panel B*, autoradiogram of the gel in *Panel A.* The DHFR bands were excised and analyzed by liquid scintillation. Radioactivity data (*cpm*) represent the average of all 3 gels. The DHFR band is indicated to the left of each panel.

Fig. 3.2

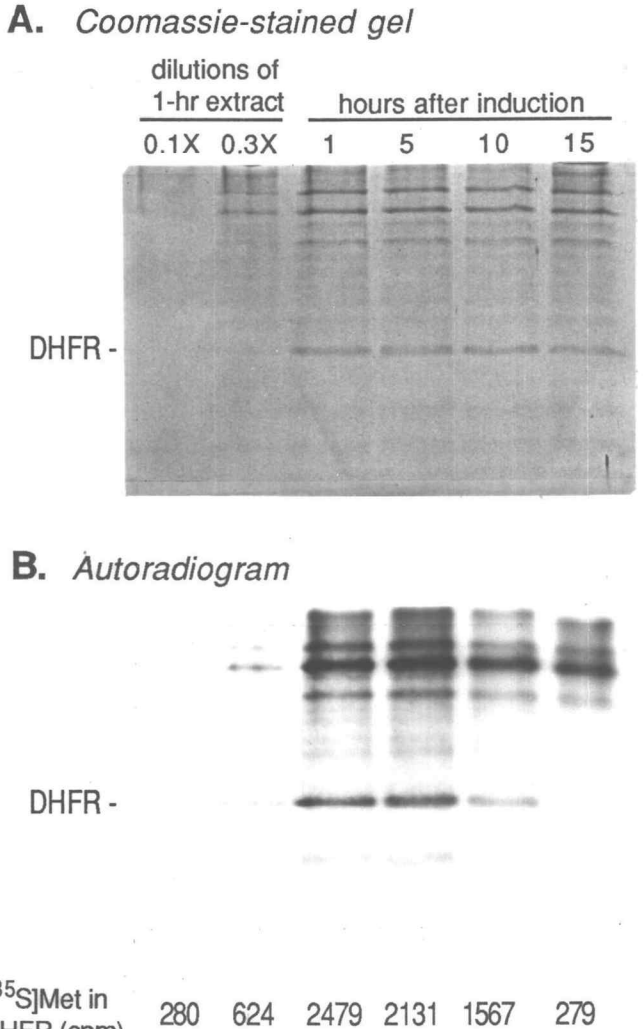
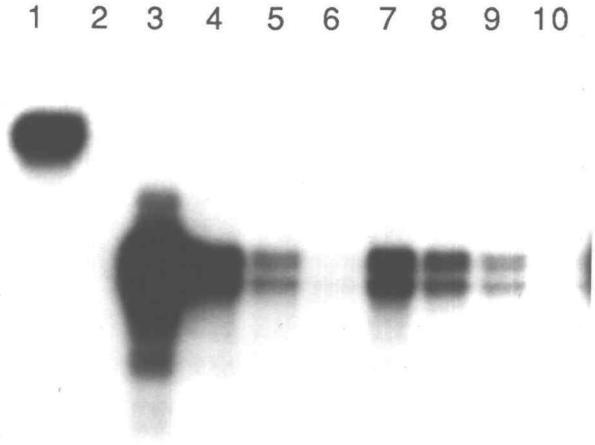

A. Coomassie-stained gel
dilutions of 1-hr extract
hours after induction
0.1X
0.3X
1
5
10
15
DHFR -
B. Autoradiogram
DHFR -
[35S]Met in DHFR (cpm)
280
624
2479
2131
1567
279

Fig. 3.3. **DHFR mRNA levels during muscle cell commitment.** RNA was harvested from H$^-$αR300T cells at specified times after inducing commitment, and DHFR mRNA levels were quantitated by RNase protection. Experimental samples contained 5 x 10^4 cell equivalents (0.5 μg) of RNA (*lanes 7-10*). Standard curve samples contained 640, 160, 40, and 10 attomoles of DHFR pseudo-mRNA (*lanes 3-6*). All hybridization mixtures contained 10 femtomoles of probe and 50 μg RNA (cellular or standard curve RNA was supplemented with yeast RNA to 50 μg). The lane designated *C* was a control that showed no signal arose from yeast RNA. The lane designated *P* contained 300 attomoles of nondigested probe. After RNase digestion, half of each sample was run on the gel shown. The remainder was precipitated with trichloroacetic acid and assayed by liquid scintillation. Standard curve signals were used to determine the number of attomoles of DHFR mRNA in each sample, which in turn were converted to DHFR mRNA molecules per cell based on the number of cell equivalents in each sample.

Fig. 3.3



P	C	standards		cellular RNA		
		hours after induction:		1	6	10	20
		attomoles DHFR mRNA/sample:		125	80	38	13
		molecules DHFR mRNA/cell:		1500	960	456	156

Fig. 3.4. **DHFR synthesis rates and DHFR mRNA levels during muscle cell commitment.** Parallel cultures of H⁻αR300T cells were harvested for DHFR synthesis rate and DHFR mRNA level determinations. *Panel A,* DHFR synthesis rates. Cultures were incubated 1 hr in 100 μCi/ml [^{35}S]Met. Proteins in clarified lysates (10^5 cell equivalents per lane) were separated by electrophoresis through 12% polyacrylamide/ SDS gels and visualized by autoradiography. DHFR bands were excised and assayed for associated radioactivity by liquid scintillation. The positions of the DHFR band and the 17-kD amplified band are indicated. All other bands represent rates of synthesis of other major cellular proteins, and serve as controls indicating that overall rates of protein synthesis were maintained during withdrawal from the cell cycle. The three leftmost lanes contain dilutions of the 1-hr H⁻αR300T sample (supplemented with proliferative cell extracts from identically-labeled nonamplified cells, such that 10^5 cell equivalents were loaded). Background radioactivity was determined by measuring the DHFR-associated radioactivity in dilutions of the 1-hr sample and extrapolating to zero amplified cell equivalents. DHFR mRNA levels were quantitated by RNase protection using 5×10^4 cell equivalents (0.5 μg) of RNA. After RNase digestion, samples were precipitated with trichloroacetic acid and assayed by liquid scintillation. Standard curve signals were used to determine the number of attomoles of DHFR mRNA in each sample, which in turn were converted to DHFR mRNA molecules per cell based on the number of cell equivalents in each sample. *Panel B* summarizes data from three polyacrylamide/SDS gel analyses of DHFR synthesis rates (*hatched bars*), and four RNase protection analyses of DHFR mRNA levels (*solid bars*). The inset shows total incorporation of [^{35}S]Met at each time point. To correct for possible variations in the specific activity of the [^{35}S]Met pool, DHFR-specific radioactivity was normalized to total incorporated radioactivity for each time point.

Fig. 3.4

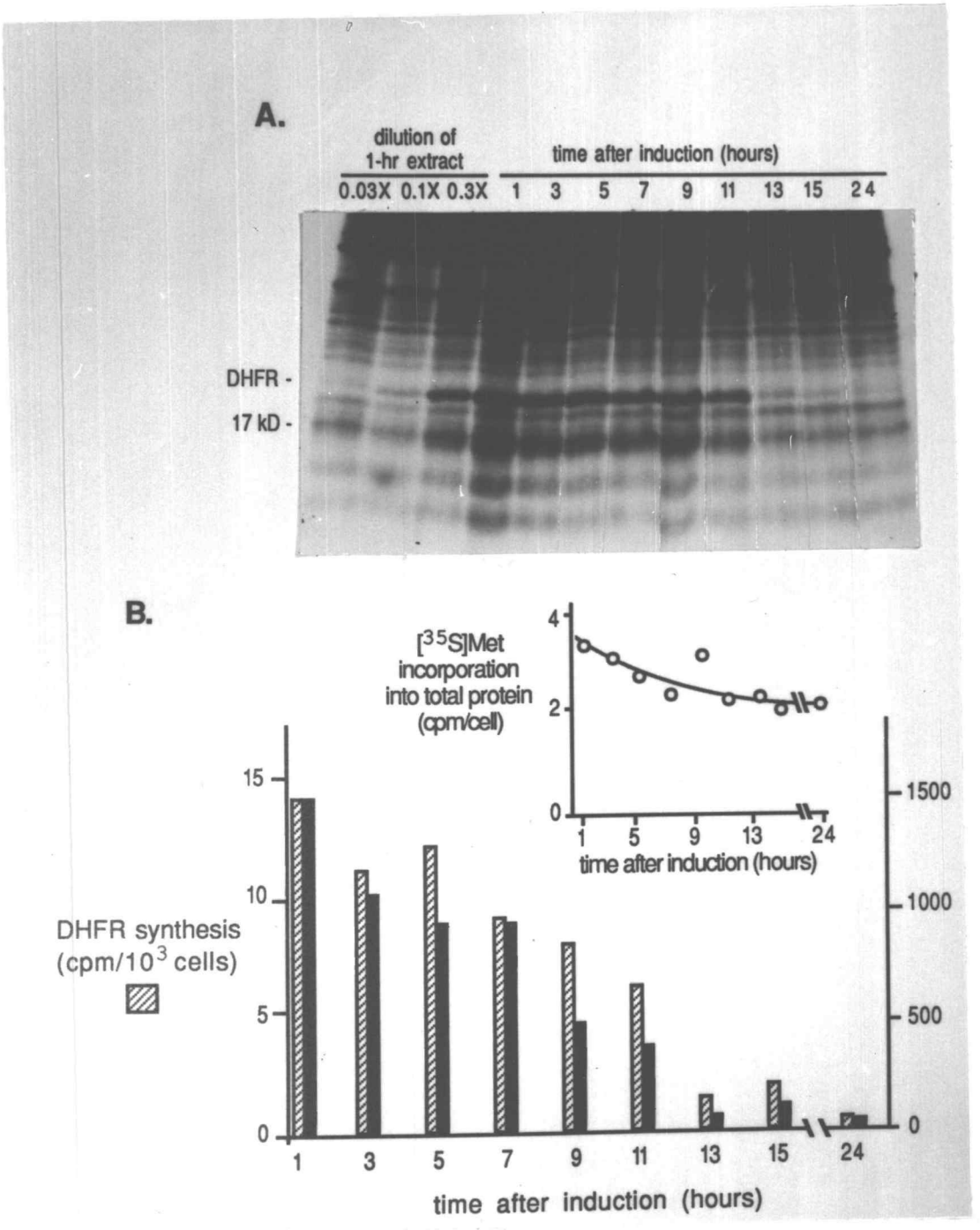
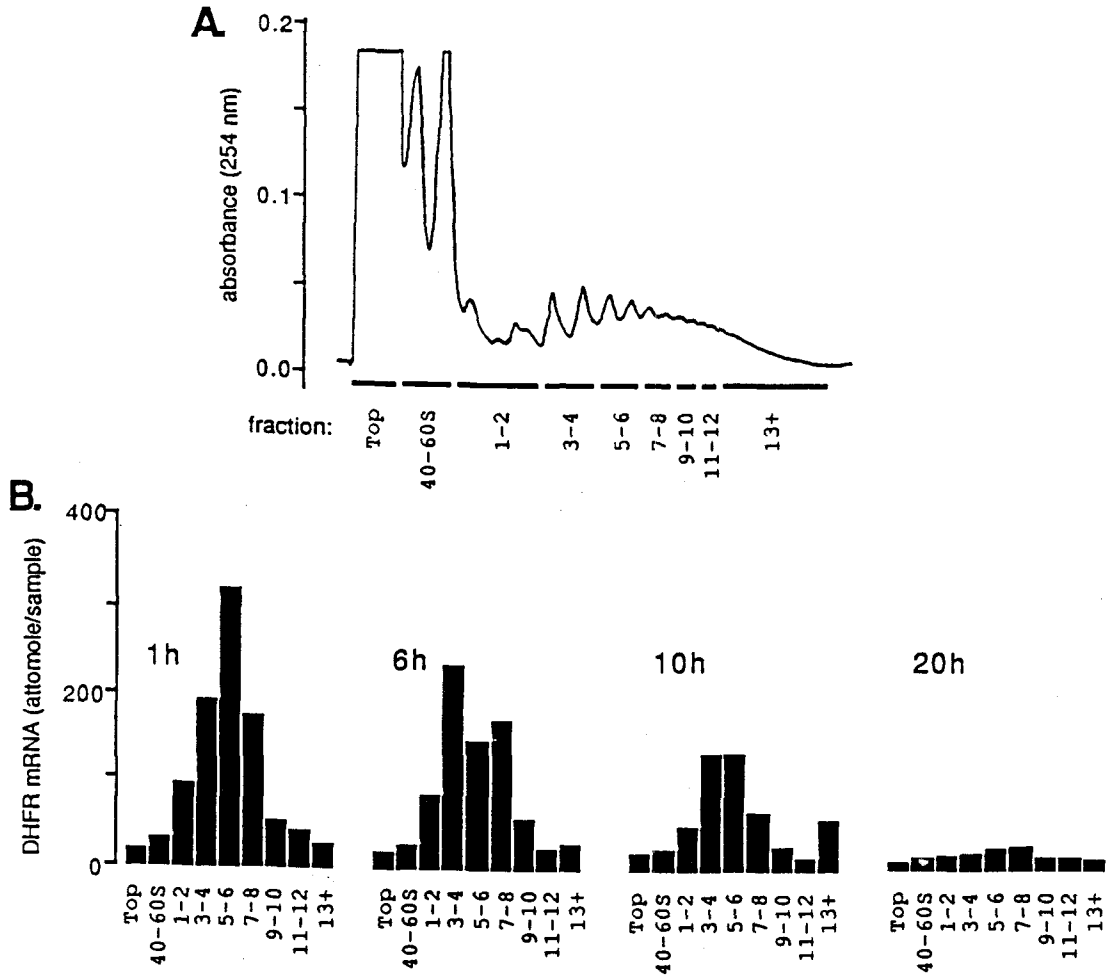

A.
dilution of 1-hr extract
0.03X 0.1X 0.3X
time after induction (hours)
1 3 5 7 9 11 13 15 24
DHFR -
17 kD -
B.
[35S]Met incorporation into total protein (cpm/cell)
4
2
0
1 5 9 13 24
time after induction (hours)
DHFR synthesis (cpm/10^3 cells)
15
10
5
0
1500
1000
500
0
1 3 5 7 9 11 13 15 24
time after induction (hours)

Fig. 3.5. **Polysomal distribution of DHFR mRNA during muscle cell commitment.** Lysates from 10^6 H$^-$αR300T cells were sedimented through sucrose gradients *Panel A,* absorbance profile of a representative gradient (1-hr sample) showing how fractions were pooled for DHFR mRNA analysis. Key: *Top,* slowly sedimenting absorptive material at top; *40-60S,* ribosomal subunits; *1-2, 3-4, 5-6, 7-8, 9-10, 11-12,* and *13+,* polysomes containing indicated number of ribosomes per message. *Panel B,* DHFR mRNA distribution at indicated times after inducing commitment. Half of the RNA from each fraction was analyzed by quantitative RNase protection using the 3' probe. RNase protection products were analyzed by liquid scintillation. Absolute quantities were determined by inclusion of DHFR pseudo-mRNA standards (468, 156, 47, and 16 attomoles) in each assay.

Fig. 3.5


A.
0.2
0.1
0.0
absorbance (254 nm)
fraction:
Top
40-60S
1-2
3-4
5-6
7-8
9-10
11-12
13+
B.
400
200
0
DHFR mRNA (attomole/sample)
1h
6h
10h
20h
Top
40-60S
1-2
3-4
5-6
7-8
9-10
11-12
13+

CHAPTER 4

Transcriptional Repression of the Mouse Dihydrofolate Reductase Gene During Muscle Cell Commitment

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SUMMARY

A differentiation-competent mouse muscle cell line containing 50- to 100-times the diploid number of DHFR genes was used to study regulation of DHFR mRNA levels during myogenic withdrawal from the cell cycle. Quantitative RNase protection assays showed that DHFR mRNA levels decreased 15-fold during commitment; DHFR pre-mRNA levels decreased 7-fold. Concomitantly, transcription of the DHFR gene, as measured by nuclear run-on assays, decreased between 7- and 30- fold. Nuclear run-on products were analyzed by hybridization to Southern blots of *dhfr*-containing plasmids. Control run-on assays performed on nonamplified parental cells indicated that run-on signals measured in amplified cells were *dhfr* amplicon-specific. Run-on signals were sensitive to α-amanitin, indicating RNA polymerase 2-specificity, and did not hybridize to pBR322 sequences, demonstrating hybridization stringency. Comparison of run-on signals hybridizing to DNA fragments representing either the 5' end of the gene or the entire gene showed that transcriptional repression occurred within the first 660 bases of the 30-kb gene, consistent with regulation at the level of either initiation or early pretermination. In contrast to the DHFR gene, DNA 5' to all but the first few bases of the DHFR coding region (between -1000 and +58 base pairs from the preferred cap site) showed strong run-on transcription in both proliferative and committed cells. Northern blot analysis using a probe complementary both to the *dhfr* coding region and the upstream region showed a uniform decrease in all detectable transcripts. No commitment-dependent changes in *dhfr* cap site usage, splicing, or polyadenylylation site usage were detected. Our results support a transcriptional model for regulation of DHFR mRNA levels.

INTRODUCTION

The enzyme dihydrofolate reductase (DHFR) catalyzes NADPH-dependent reductions of cellular folates, thereby maintaining pools of tetrahydrofolate coenzymes. Although required for numerous one carbon transfer reactions throughout the life of the cell, tetrahydrofolate coenzymes are enzymatically oxidized to the dihydrofolate state only by thymidylate synthase during *de novo* deoxythymidylate synthesis. Therefore, the demand on DHFR is greatest in cells synthesizing DNA. Coincident with changes in the demand on the enzyme, DHFR mRNA levels change during progression of growing cells through the cell cycle (Mariani *et al.*, 1981; Farnham and Schimke, 1985, 1986; Gasser and Schimke, 1986), and during release from serum starvation (Johnson *et al.*, 1978; Liu *et al.*, 1985; Mullner *et al.*, 1983; Wu and Johnson, 1982) or contact inhibition (Leys *et al.*, 1984; Leys and Kellems, 1981; Kellems *et al.*, 1976; Kaufman and Sharp, 1983). All previous studies generally agree that S phase cells exhibit high levels of DHFR mRNA, whereas G1 or "G0" cells exhibit relatively low levels. However, no consensus has been reached concerning the mechanisms regulating DHFR mRNA levels. Different studies have implicated changes in transcription rates (Farnham and Schimke, 1985, 1986; Gasser and Schimke, 1986; Wu and Johnson, 1982; Santiago *et al.*, 1984), changes in RNA stability (Kaufman and Sharp, 1983; Leys and Kellems, 1981), and changes in pre-mRNA stability (Leys *et al.*, 1984) in playing the primary regulatory role.

The DHFR gene is about 30 kilobases long, and contains 5 introns and 6 exons (Nunberg *et al.*, 1980; Crouse *et al.*, 1982). Transcription of *dhfr* initiates primarily at a site 55 bases upstream of the translation start site, and polyadenylylation occurs in a region about 1000 base pairs downstream of the translation stop codon (Farnham and Schimke, 1986; Sazer and Schimke, 1986; Hook and Kellems, 1988; McGrogan *et al.*, 1985). As many as 5 alternative cap sites and 11 alternative polyadenylylation sites are used at low frequencies (McGrogan *et al.*, 1985; Sazer and Schimke, 1986; Setzer *et al.*,

1980; Hook and Kellems, 1988). No growth state-dependent changes in initiation site or polyadenylylation site usage have been reported (Farnham and Schimke, 1986; Hook and Kellems, 1988). Exons account for only 1.6 kb of the primary transcript. Alternative splicing has not been observed. Initiating at about six sites in a region 200 base pairs upstream of the *dhfr* cap site, the opposite strand is transcribed (Farnham *et al.*, 1985; Crouse *et al.*, 1985). It is unclear whether the upstream region gives rise to a series of short (180- to 240-base), cell cycle-regulated, non-polyadenylylated, nuclear RNA species terminating at a single site 433 base pairs upstream of the *dhfr* cap site (Farnham and Schimke, 1986); or a series of long (3- to 5-kb), polyadenylylated, cytoplasmic RNA species (Crouse *et al.*, 1985; Fujii and Shimada, 1989). For our purposes, it was important to distinguish *dhfr* transcription from transcription of the upstream region. Therefore, experiments were designed to detect transcription of each region independently.

Differentiating muscle cells provide a convenient system to study DHFR mRNA regulation. When induced to differentiate, proliferative muscle cells irreversibly withdraw from the cell cycle in G1 phase. Committed myocytes are incapable of synthesizing DNA but are otherwise biosynthetically active (Devlin and Emerson, 1978; Jaynes *et al.*, 1986; Gross and Merrill, 1988). We selected a differentiation-competent mouse muscle cell line containing 100-fold amplification of its endogenous DHFR gene. Overexpression of the DHFR gene by the amplified cells facilitated biochemical analyses of transcription rates and transcript levels. Quantitative RNase protection assays were used to measure DHFR message and precursor levels; nuclear run-on assays were used to determine transcription rates (Gross *et al.*, 1987; McKnight and Palmiter, 1979).

Our findings indicated that the DHFR gene is transcriptionally regulated. DHFR mRNA and pre-mRNA levels decreased during muscle cell commitment, and nuclear run-on transcription rate determinations suggested that the decrease in precursor levels was due to repression of *dhfr* transcription. Quantitative comparison of the decreases in

mRNA and pre-mRNA suggested that a change in the stability of DHFR mRNA further lowered message levels. Our results are consistent with the hypothesis that the decrease in mRNA levels is due primarily to repressed transcription of the DHFR gene.

EXPERIMENTAL PROCEDURES

Cells lines and tissue culture - A diploid hypoxanthine-guanosine phosphoribosyltransferase-deficient subline of the MM14D mouse skeletal myoblast line developed by Linkhart *et al.* (1981), designated $H^-\alpha$, was used to derive the $H^-\alpha$R50T line. The parental $H^-\alpha$ cells were sensitive to 50 nM methotrexate (<1% colony formation). Parental cells were cultured in growth medium (basal medium [0.5X Ham's F-10, 0.5X DMEM, 0.4 mM additional $CaCl_2$, 15 mM Hepes, pH 7.2, 1% glucose, 2.45 mg/ml NaH_2CO_3, 10 U/ml penicillin G, 0.5 mg/ml streptomycin sulfate] supplemented with 15% horse serum and fibroblast growth factor activity from bovine brain (Esch *et al.*, 1985). To select the amplified cell line, parental cells were cultured for about 20 cell generations in growth medium containing 50 nM methotrexate. Surviving cells were serially selected in 0.1, 0.3, 0.5, 1, 3, 5, 10, 30, and 50 μM methotrexate, with at least 10 cell generations allowed at each selective step. Seven lines survived selection to 50 μM methotrexate. Each line was assayed for doubling time, DHFR gene copy (by Southern analysis using a standard curve generated with mouse liver DNA), DHFR enzyme levels, cell cycle withdrawal kinetics (by thymidine labeling index), thymidine kinase regulation during commitment, and regulation of DHFR mRNA levels during commitment. One of the methotrexate resistant cell lines, designated $H^-\alpha$R50T, was chosen for further study. Whereas proliferative $H^-\alpha$ cells have a 13-hr generation time, contain two copies of the DHFR gene and four DHFR mRNA molecules per cell, and synthesize DHFR at a rate of 1.5×10^5 molecules per cell per generation (Schmidt and Merrill, 1989a), proliferative $H^-\alpha$R50T cells had a 14-hr generation time, contained 100-200 copies of the DHFR gene and 500-700 DHFR mRNA molecules per cell, and synthesized about 1×10^7 DHFR molecules per cell per generation. By all other measured criteria the amplified cell line behaved identically to the parental cell line. Amplified cells were maintained in growth medium supplemented with 50 μM methotrexate. Commitment was induced by rinsing

cells twice with basal medium followed by incubation in differentation medium (basal medium supplemented with 50 μM methotrexate and 1 μM insulin as a maintenance factor).

Synthesis of RNA probes and standards - Plasmid p4d200 was made by inserting the 200-base pair Hind3/Rsa1 cDNA fragment from pSV2DHFR (Subramani *et al.*, 1981) into a pGEM-4 (Promega) vector that had been cut with Eco R1, blunted with the Klenow fragment of *E. coli* DNA polymerase 1, and cut with Hind3. By blunting the pGEM-4 vector at Eco R1 rather than ligating the Rsa1 of the insert to a blunt restriction site elsewhere in the polylinker region, the Taq1 site overlapping the Eco R1 site in pGEM-4 was inactivated, allowing subsequent digestion with Taq1 to cleave the DNA in the DHFR insert without cleaving between the SP6 promoter and the DHFR insert (Fig. 4.1*A*, see below). Plasmid p4d750 was generated by inserting the 750-bp Bgl2/Hind3 cDNA fragment from pSV2DHFR into pGEM-4 (Fig. 4.1*B*). Plasmid p3d3000 was constructed by inserting the 3.0-kb Eco R1/Hind3 fragment from pDR34 (Crouse *et al.*, 1982) into pGEM-3 (Fig. 4.2*A*). Schemes for transcription of probes and standards are depicted in Figures 4.1*A*, 4.1*B*, and 4.2*A*. Except as indicated, SP6 and T7 transcription reactions were performed as prescribed by Promega. Pseudo-mRNA standards were made by linearizing 1 μg plasmid p4d750 with Eco R1 followed by T7-catalyzed transcription in the presence of nonlabeled nucleotides. Pseudo-pre-mRNA standards were made by linearizing plasmid p3d3000 with Hind3, followed by T7-catalyzed transcription. Templates were degraded by adding three units RQ1 DNase (Promega) and incubating at 37^{o} C 1 hr. Standards were separated from nonincorporated nucleotides on 5 ml Sephadex G50-fine columns in 1X TES (10 mM Tris, pH 7.5, 1mM EDTA, 1 % SDS); quantities synthesized were determined by measuring the absorbance at 260 nm (ε = 20 ml mg^{-1} cm^{-1}). Ethidium bromide- stained agarose gels were used to confirm full-length synthesis.

All RNase protection probes were SP6-catalyzed transcripts synthesized in the presence of 13 μM α[^{32}P]UTP (190 Ci/mmole, New England Nuclear). The 5' probe template was made by digesting p4d200 with Taq1. The 3' probe template was generated by digesting p4d750 with Dde1. The intron probe template was made by linearizing p3d3000 with Bgl2. Transcription reactions were performed on 1 μg of template in 20 μl volumes. After transcription, samples were treated for 1 hr at 37^{o} C with three units of RQ1 DNase in the transcription reaction buffer. Deoxynucleotides and nonincorporated ribonucleotides were removed on 5 ml Sephadex G50-fine columns in 1X TES. The approximate concentrations of probes synthesized were deduced from the specific activity of the input [^{32}P]UTP and the amount of radioactivity incorporated into probe.

RNase quantitation protocol - RNA was harvested from cells by a modification of the guanidinium isothiocyanate/CsCl procedure (Gross, *et al.* 1987). RNA concentration was determined from the absorbance at 260 nm (ε = 20 ml mg^{-1} cm^{-1}). For detection of DHFR mRNA, 2-3 μg of cellular RNA was hybridized to 3-10 femtomoles of either 5' or 3' probe at 55^{o} C for 13-18 hr in 30 μl of hybridization solution (80% deionized formamide, 0.4 M NaCl, 1.0 mM EDTA, 1.0 M Pipes, pH 7.0). Parallel samples containing various quantities (from 10-1000 attomoles) of pseudo-mRNA were used as standards. All samples were adjusted to 50 μg total RNA with yeast RNA prior to hybridization. Control samples containing only probe and yeast RNA showed that all signal was mouse cell-specific and demonstrated completeness of RNase digestions (eg., Fig. 4.1*C*, lanes 2 and 14). Samples that were not RNased, containing only 200-2000 attomoles of probe and 10 μg yeast RNA, showed probe integrity (eg., Fig. 4.1*C*, lanes 1 and 13). For detection of pre-mRNA, hybridizations contained 20-50 μg of cellular RNA or 10-1000 attomoles of pseudo-pre-mRNA standards, and 3-10 femtomoles of the intron probe. Calibration controls (not shown) comparing equimolar amounts of pseudo-mRNA and pseudo-pre-mRNA using the 5' probe (which hybridizes to both

standards) corroborated absorbance measurements in showing both standards were at the same concentration, thereby validating direct comparison between results obtained with either set of standards.

After hybridization, samples were cooled to room temperature, 300 µl of RNase mix was added (10 µg/ml RNase A [Boehringer Mannheim], 0.35 U/µl RNase T1 [Boehringer Mannheim], 10 mM Tris, pH 7.5, 0.3 M NaCl, 5 mM EDTA), and samples were incubated at 37° C for 1 hr. RNase digestions were terminated by adding 20 µl PK mix (1.0 mg/ml proteinase K [Boehringer Mannheim], 0.5 mg/ml yeast RNA, 100 mM Tris, pH 7.5, 10 mM EDTA, 10% SDS), and incubated at 37° C for an additional 15 min. Samples were phenol/chloroform-extracted, chloroform-extracted, and precipitated 1 hr at -80° C in 70% ethanol. After centrifugation, pellets were washed with 70% ethanol, and resuspended in 2.0 µl water at 65° C. Samples received 8.0 µl loading mix (99% deionized formamide, 0.067% xylene cyanol, 0.033% bromphenol blue) and were incubated at 70-75° C for 10-20 min. Aliquots (2-5 µl) of each sample, while still at 70° C, were loaded onto 0.4-mm-thick, pre-run, 9% polyacrylamide, 8.0 M urea gels and run at 0.5-1.0 watts/cm-width in a 37° C ambient environment. After electrophoresis, gels were dried and exposed to X-ray film for 15-72 hr at -80° C.

After loading gels, a remaining aliquot of each sample was adjusted to a volume of 80 µl with water, and 20 µl of 10% sodium pyrophosphate and 100 µl 10% trichloroacetic acid (wt/vol) were added. Samples were incubated on ice >30 min. RNA was collected by centrifugation, and washed twice with cold 10% trichloroacetic acid. Pellets were resuspended at 65° C in 100 µl 0.25 M Tris, pH. 8.0, and added to glass scintillation vials containing 1.0 ml scintillation fluid (4.0 g Omnifluor, 333 ml Triton X-100, 667 ml toluene). Each sample was assayed for radioactivity by liquid scintillation for 50 min. Comparison of experimental samples to standards was used to obtain absolute values for *dhfr*-specific RNA in each sample. The standard curves showed reproducibly linear relationships between the amount of synthetic standards added and

associated radioactivity. Such precision was not obtained when measuring band intensities on autoradiograms using laser densitometry.

Nuclear run-on assays - Five 10-cm cultures at densities of 1-3 x 10^6 cells per dish were used for each nuclear run-on assay. All manipulations were performed on ice or at 4^o C unless otherwise noted. At specified times after inducing commitment, cells were rinsed twice with phosphate-buffered saline and harvested by scraping in the same. Cells were centrifuged at 500 rpm in an IEC HN-SII tissue culture centrifuge for 10 min and cell pellets were resuspended in 1.0 ml RSB (10 mM Tris, pH 8.0 at 4^o C, 10 mM NaCl, 5 mM $MgCl_2$). Cells were centrifuged at 500 RPM for 10 min and resuspended in 1.0 ml RSB. Cells were mixed by gentle vortexing and allowed to swell for 5 min. Samples were brought to 0.5% NP-40 and gently vortexed intermittently during the next 5 min. A 10 µl portion of each sample was removed, adjusted to 50 µl with 1X TES, boiled for five min, sonicated, and the absorbance was measured at 260 nm. Nuclei from lysed cells were collected by centrifugation at 2000 rpm for 20 min in the IEC centrifuge. Supernatant was removed and the pellet was dispersed by vortexing. Nuclei were resuspended in NFB (50 mM Tris, pH 8.7 at 4^o C, 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol) at a concentration of 3.0 mg nucleic acid/ml (ε = 20 ml mg^{-1} cm^{-1}).

Nuclear run-on reaction mixes were assembled on ice. Each reaction mix contained 100 µCi $\alpha[^{32}P]$UTP (800 Ci/mmole; the final concentration of UTP was 1.3 µM); 400 µM ATP, CTP, and GTP; 30% glycerol ; 70 mM KCl; 2.5 mM DTT; 2.6 mM $MgCl_2$; 0.5 U/µl RNasin (Promega); and 100 µg nuclei in a final volume of 100 µl. Nuclei were added last to all reaction mixes. When included, 0.1 µg/µl α-amanitin was added to the samples prior to nuclei. After adding nuclei, samples were mixed gently, incubated on ice for 10 min, and transferred to a 27^o C water bath for 20 min. Run-on reactions were terminated by adding 13 µl 10X TES, 10 µl 20 mg/ml proteinase K, and 5 µl 0.5 M EDTA. Samples were incubated at 37^o C for 1 hr, brought to 250 mM NaCl in a total volume of

400 µl, phenol/chloroform-extracted, chloroform-extracted, and precipitated overnight at -20° C in 70% ethanol. Precipitated nucleic acids were collected by centrifugation and washed twice with 70% ethanol. Samples were resuspended in 340 µl water at 65° C and cooled to room temperature. Samples received 40 µl 10X DNase buffer (20 mM Hepes, pH 6.6 at 25° C, 1mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM $MnCl_2$), 2.5 µl 1M DTT, 40 U RNasin, 5 U RQ1 DNase, and were incubated at 37° C for 80 min. DNase digestions were terminated by adding 40 µl 10X TES, 4 µl 20 mg/ml proteinase K, and 4 µl 0.5 M EDTA. Incubation at 37° C was continued an additional 15 min, NaCl was added to 250 mM, samples were phenol/chloroform-extracted, chloroform-extracted, and ethanol-precipitated at -80° C for 1 hr. Precipitated nucleic acids were collected by centrifugation, washed with 70% ethanol, and resuspended in 80 µl water at 65° C. Samples received 20 µl 10% sodium pyrophosphate and 100 µl 10% trichloroacetic acid, and were incubated on ice >30 min. RNA was collected by centrifugation. Pellets were washed three times with 10% trichloroacetic acid and resuspended in 400 µl 0.25 M Tris, pH 8.0 at 25° C. A portion of each sample was assayed for incorporated radioactivity by liquid scintillation. Samples were brought to 250 mM NaCl, ethanol-precipitated overnight at -20° C, centrifuged, washed with 70% ethanol, and resuspended in water at 3×10^7 cpm/ml.

Southern blots bearing *dhfr*-containing plasmids were used to probe nuclear run-on reaction products. Either 20 µg Taq1-digested pDR34 or 20 µg Pst1-digested pDHFR11 (Chang *et al.*, 1978) were loaded into 10-cm-wide troughs cast in 1% agarose/E buffer (40 mM Tris-acetate, pH 8.0, 2 mM EDTA) gels and run until the bromphenol blue dye marker had migrated 4-5 cm. Gels were stained with ethidium bromide for 15 min, destained in water for 1 hr, and photographed on a UV transilluminator. DNA in gels was denatured for 1.5 hr in 0.5 M NaOH, 1.5 M NaCl, rinsed in water, neutralized for 1.5 hr in 0.5 M Tris, pH 7.0, 3 M NaCl, and equilibrated for 1.0 hr in 20X SSC (3 M NaCl, 0.3 M sodium citrate). DNA was blotted onto

nitrocellulose membranes pre-equilibrated in 20X SSC. Nitrocellulose blots were washed gently for 5 min in 20X SSC, air dried face up on Whatman 3MM paper, and baked *in vacuus* 2 hr at 80° C. Blots were cut into 3.0 mm strips and stored in sealed tubes at 4° C. Strips were prehybridized in horizontal 4.0 ml polypropylene snap-cap Falcon tubes in 2.0 ml hybridization buffer (50 mM Pipes, pH 7.0 at 25° C, 0.5 M NaCl, 0.4 % SDS, 2 mM EDTA, 33% deionized formamide) submerged in a shaking water bath at 42° C for 3.5 hr. Nuclear run-on transcription products were denatured at 100° C for 10 min, and 3, 10, 30, or 100 x 10^4 cpm were added to 1.0 ml of hybridization buffer that had been prewarmed to 65° C in fresh 4 ml tubes. Nitrocellulose strips were removed from prehybridization tubes and transferred to hybridization tubes at 65° C. Tubes were sealed and incubated horizontally for 15 hr at 55° C, 9 hr at 50° C, and 12 hr at 45° C, submerged in a shaking water bath. Tubes were gradually cooled to 42° C, strips were removed and washed twice for 20 min at room temperature in ribowash (20 mM $NaPO_4$, pH 6.5, 50 mM NaCl, 1 mM EDTA, 0.1 % SDS). Strips were subsequently washed twice for 1 hr at 55° C in ribowash, twice for 5 min at room temperature in 2X SSC, once for 10 min at room temperature in 2X SSC, 0.1 μg/ml RNase A, and twice for 15 min at room temperature in ribowash. Strips were air dried face up on Whatman 3MM paper and exposed to film with an intensifying screen for 60 hr at -80° C. A second exposure for 6 days without an intensifying screen was also performed. Autoradiogram band intensities were determined using a Zeineh soft laser densitometer. Results from both exposures were qualitatively identical; however, because film background was lower and signal bands were more intense on the shorter exposure (with intensifying screen), data from the shorter exposure were used to calculate relative transcription rates. Band intensities were plotted as a function of input radioactivity (Fig. 4.3*C* and *D*). The slopes of best fit lines (assigned by linear regression) represented relative transcription rates.

Northern blot analyses - RNA was harvested from proliferative 3T6 R50 (Brown *et al.*,

1981) or from H$^-$αR50T cells at specified times after inducing commitment. For each sample, 10 μg of RNA was lyophilized and brought to 4.7 μl with water. Samples received 10 μl deionized formamide, 3.4 μl 37% formaldehyde, 2 μl of 10X Mops buffer (200 mM Mops acid, 50 mM sodium acetate, 10 mM EDTA), and 5 μl dye mix (20% Ficoll, 20 mM EDTA, 0.1% bromphenol blue) in order, mixing between each addition. Samples were incubated at 65^o C (15 min), plunged into an ice slush, and loaded onto 1% agarose, 2.5% formaldehyde, 1X Mops buffer gels. Gels were run overnight at 20-40 V and were washed gently for 1-2 hr in several changes of water. Gels were stained with ethidium bromide (3 min), destained in water (1 hr), and photographed to insure that lanes were equally loaded. RNA was fragmented by treating gels for 30 min at room temperature in 50 mM NaOH. Gels were rinsed twice with water and neutralized for 30 min in 0.25 M Tris, pH 7.0. After equilibrating gels in 20X SSC for 1 hr, RNA was blotted onto nitrocellulose sheets preequilibrated in 20X SSC. Nitrocellulose blots were subsequently washed gently at room temperature in 20X SSC and baked *in vaccus* at 80^o C for 2 hr. Baked blots were prehybridized for 4-24 hr at 42^o C in 10-15 ml 80% hybridization buffer (5X SSC, 25 mM $NaPO_4$, pH 6.5, 0.02% BSA [fraction V, Sigma A9647], 0.02% polyvinylpyrrolidone [Sigma PVP-40], 0.02% Ficoll 400, 0.25 mg/ml sheared salmon sperm DNA, 50% deionized formamide), 10% dextran sulfate. Blots were hybridized for 18-72 hr at 42^o C in 10-15 ml 80% hybridization buffer, 10% dextran sulfate, and 2 x 10^6 cpm of [^{32}P]-nick translation-labeled 3.4-kb Eco R1 fragment of pDR34 (see Fig. 4.3*A* for a restriction map of pDR34). After hybridization, blots were washed twice at room temperature for five min in 2X SSC, 0.1% SDS, and twice for 15-30 min at 50^o C in 0.1X SSC, 0.1% SDS. Blots were air- dried, covered with plastic wrap, and exposed to film for 18-72 hr at -80^o C with an intensifying screen.

RESULTS

DHFR mRNA regulation during muscle cell commitment - An RNase protection assay was used to measure levels of DHFR mRNA during myogenic withdrawal from the cell cycle. Briefly, cellular RNA or synthetic "pseudo-mRNA" standards were incubated with radiolabeled RNA probes complementary to specific regions of the DHFR message. After hybridization, single stranded RNA, including all nonhybridized probe, was removed by RNase digestion. Protected probe regions were separated from low molecular weight digestion products by gel electrophoresis or by precipitation with trichloroacetic acid. Two probes for measuring DHFR mRNA were used (Fig. 4.1*A* and 4.1*B*): one complementary to sequences at the 5' end of the DHFR message, near the translation start site; and one complementary to a region at the 3' end of the message, spanning the translation stop site. Use of two probes at opposite ends of the protein coding region provided assurance that the RNase protection assay was accurately measuring levels of intact mRNA rather than incomplete transcripts or mRNA degradation intermediates. Inclusion of pseudo-mRNA standards allowed absolute quantitation of DHFR mRNA in experimental samples. In addition, the standards controlled for interexperimental differences in hybridization efficiency and RNase digestion, and controlled for the nonlinear relationships between sample radioactivity and film response.

Muscle cell line $H^{-}\alpha R50T$, containing amplified copies of the DHFR gene, was used to measure levels of DHFR mRNA during myogenic withdrawal from the cell cycle. During the first two hours after mitogen depletion, all cells remain proliferative. Thereafter, as cells complete mitosis and enter G1, they withdraw from the cell cycle and commit to terminal differentiation (Linkhart *et al.*, 1981). By 15 hours, virtually all cells are committed (Schmidt and Merrill, 1989a; Gross *et al.*, 1987) and will not re-enter the cell cycle upon mitogen restoration (Linkhart *et al.*, 1981). Thus, cells harvested during the first 2 hr after induction are fully proliferative; cells harvested after 15 hr are fully

committed. At various times after inducing commitment, RNA was harvested from H$^-$αR50T cells and DHFR mRNA levels were measured by RNase protection (Fig. 4.1*C*). Using the 5' probe, we found that mRNA signals remained relatively constant during the first 7 hours after induction (*lanes 8-10*), but decreased sharply by 15 and 26 hours (*lanes 11* and *12*). A similar pattern of DHFR mRNA decline was observed when using the 3' probe *(lanes 20-24*). Standard curves were included for both the 5' (*lanes 3-7*) and 3' (*lanes 15-19*) probes. Due to overlapping vector sequences, pseudo-mRNA protected a larger region of the 3' probe than did mRNA (compare pseudo-mRNA signals in *lanes 15-19* to mRNA signals in *lanes 20-24*). In calculating absolute quantities of DHFR mRNA using the 3' probe, correction was made for the the difference in protected fragment size. The autoradiogram was scanned by laser densitometry; band intensities are listed below each lane in Figure 4.1*C*. Standard curves indicated that the decrease in DHFR mRNA was about ten-fold using either probe (compare *lanes 8* and *12*, and *lanes 20* and *24*).

Despite the inclusion of standards, quantitation of DHFR RNA levels by laser densitometry was imprecise. Variability between assays probably was due to problems in accurately assigning baselines to densitometry data. To more precisely measure mRNA levels, sample radioactivity was determined using a liquid scintillation-based assay. This assay was valid because autoradiographic results had established that all gel bands in experimental samples were *dhfr*-specific (compare sample *lanes 8-12* and *20-24* to yeast RNA control *lanes 2* and *14*). Briefly, low-molecular-weight molecules present after RNase digestion were removed by precipitating samples with trichloroacetic acid, and sample radioactivity was measured by liquid scintillation. When using liquid scintillation, quantitative results were highly reproducible. Autoradiograms of gels were still used as a visual representation of data to insure correct band sizes, *dhfr*-specificity, and completeness of RNase digestions. Figure 4.2*C* shows liquid scintillation data obtained by measuring the same samples shown in the autoradiogram in Figure 4.1*B*. Comparison

of sample radioactivity to standard curve values indicated that during commitment, DHFR mRNA levels measured using the 5' probe decreased 16-fold, from 670 messages to 42 messages per cell (*open squares*). DHFR mRNA measured using the 3' probe decreased 12-fold, from 500 messages to 42 messages per cell (*open circles*). Results of several experiments using both probes indicated that DHFR mRNA levels decreased 15-fold on average during commitment.

DHFR pre-mRNA levels during commitment - The reduction in mRNA levels as muscle cells withdrew from the cell cycle and committed to terminal differentiation could have resulted from increased degradation or decreased synthesis of DHFR mRNA. To distinguish between these possibilities, we developed a quantitative assay for DHFR pre-mRNA. If pre-mRNA levels persisted in postreplicative cells, it would indicate DHFR mRNA levels were regulated posttranscriptionally. If precursor levels decreased, it would indicate either DHFR pre-mRNA synthesis was repressed (transcriptional regulation) or DHFR pre-mRNA degradation was increased (posttranscriptional regulation).

Figure 4.2*B* shows an RNase protection assay of DHFR pre-mRNA levels using the same H$^-$αR50T cell RNA that was used for DHFR mRNA determinations in Figure 4.1*C*. Because cellular levels of pre-mRNAs were expected to be lower than levels of mature message, tenfold more cellular RNA was used in measuring pre-mRNA levels. The autoradiogram showed that DHFR pre-mRNA levels began to decrease after 3 hours, and continued to decrease for at least 26 hours (*lanes 8-12*). Liquid scintillation analyses (Fig. 4.2*C*) showed that DHFR pre-mRNA decreased 7-fold, from 18.2 copies per proliferative cell to 2.8 copies per committed cell. The decrease in DHFR precursor levels preceded the decrease in DHFR message, which was consistent with the hypothesis that decreased pre-mRNA levels were responsible, in part, for the reduction in mRNA levels. However, the decrease in precursor was of lesser magnitude than the decrease in mRNA (7-fold *versus* 15-fold, Fig. 4.2*C*). Thus, mRNA regulation involved both a small

(2.5-fold) precursor- independent component and a larger (7-fold) precursor-dependent component. When message and precursor levels were measured by laser densitometry of autoradiograms (Fig. 4.1*C* and 4.2*B*), decreases of slightly different magnitude were calculated for all DHFR RNA species (mRNA signal decreased about 10-fold, pre-mRNA signal decreased about 7-fold); however, again both precursor-dependent (7-fold) and precursor-independent (1.5-fold) mechanisms were required to explain the data.

In vitro nuclear run-on transcription rate assays - The decrease in DHFR pre-mRNA during muscle cell commitment was due either to reduced precursor synthesis (transcriptional regulation) or increased precursor degradation (posttranscriptional regulation). To distinguish between these possibilities, the relative rates of transcription of the DHFR gene in proliferative and committed cells were measured by nuclear run-on assays (McKnight and Palmiter, 1979). Nuclear run-on assays involve incubation of isolated nuclei with radiolabeled ribonucleoside triphosphates. Under these conditions, RNA polymerases that were already engaged on a template at the time of nuclei isolation resume transcription. Specific radiolabeled nuclear run-on transcription products are detected by hybridization to complementary DNA immobilized on nitrocellulose membranes. The extent of incorporation of radioactivity into a specific transcript is an indirect measure of the relative rate of transcription of the cognate gene (McKnight and Palmiter, 1979).

We used a Southern blot method to detect DHFR-specific transcription by isolated nuclei. Nitrocellulose strips containing electrophoretically-separated restriction fragments of DHFR-containing plasmids were incubated with nuclear run-on transcription products. Restriction maps of the plasmids used are shown in Figure 4.3*A*. The Southern blot method offered several advantages over the traditional method of using DNA that was simply dotted onto nitrocellulose filters. First, hybridization of

radioactivity to restriction fragments resulted in autoradiographic bands; thus, hybridization to immobilized DNA was distinguishable from background binding to the nitrocellulose. Second, the absence of hybridization to restriction fragments containing only vector sequences controlled for the stringency of hybridization and washing conditions. Third, the relative strength of hybridization to different DHFR-containing fragments revealed whether RNA polymerases were symmetrically or asymmetrically distributed on the DHFR gene. Fourth, hybridization to fragments flanking the *dhfr* transcription unit revealed whether significant transcription of adjacent extragenic sequences occurred during run-on reactions.

DHFR-specific transcription was detectable in run-on assays using nuclei from proliferative H$^-$αR50T cells. As shown in Figure 4.3*B*, nuclei from proliferative amplified cells synthesized [^{32}P]RNA that hybridized to the 2.6-kb and 0.6-kb *dhfr*-containing fragments of genomic clone pDR34 (*strips 5-8*), and to the 1.6-kb *dhfr*-containing fragment of cDNA clone pDHFR11 (*strips 21-24*). On strips 5-8, hybridization to a 1.4-kb fragment containing 60 base pairs of *dhfr* sequence and 1000 base pairs of upstream sequence was also detected (discussed below). For both sets of strips, specific signal intensities were proportional to input radioactivity. For example, the strengths of the 2.6-kb genomic signals on strips 5-8 increased linearly as the amount of [^{32}P]RNA added to the hybridization mixture was increased from 0.3 to 10.0 x 10^5 cpm. Thus, input RNA was not saturating the immobilized DNA. On the genomic strips, vector fragments were not resolved from the 1.4-kb mouse *dhfr* fragment and could not be used to assess the specificity of hybridization conditions. However, on the cDNA strips, the 4.4-kb vector fragment was clearly resolved from the 1.6-kb *dhfr* fragment. No hybridization to the 4.4-kb vector fragment was detected (*strips 21-24*), indicating that hybridization conditions were sufficiently stringent to prevent hybridization of run-on products to pBR322 sequences. Run-ons performed on amplified proliferative cell nuclei in the presence of α-amanitin showed no hybridization signal

(*strips 9-12* and *25-28*), indicating that the signals detected in the absence of drug were RNA polymerase 2-specific. Finally, strips incubated with products from parallel run-on reactions using nuclei from proliferative, non-amplified, parental cells showed little hybridization above background (*strips 1-4* and *17-20*), demonstrating that the signals detected using amplified cell nuclei arose specifically from the *dhfr* amplicon.

As muscle cells committed, *dhfr* transcription decreased precipitously. The run-on transcripts produced by committed cell nuclei showed little or no hybridization to *dhfr* sequences (Fig. 4.3*B*, *strips 13-16* and *strips 29-32*). Because bands arising from hybridization to the 0.6-kb genomic fragment were faint, only the 2.6-kb genomic and 1.6-kb cDNA fragments were used to quantitate commitment- dependent changes in *dhfr* transcription. Band intensities on 1- and 18-hour strips were measured by laser densitometry and plotted as a function of input radioactivity (Fig. 4.3*C* and 4.3*D*). The best fit line was determined for each data set by linear regression; the slope represents the relative transcription rate. Because bound radioactivity was determined by laser densitometry, whereas input radioactivity was measured by liquid scintillation, transcription rates are expressed as band intensity (units) per input radioactivity (cpm). Using the 2.6-kb genomic DNA hybridization signal, the calculated decrease in run-on transcription rate was 20-fold, from 156 units/input cpm to 8 units/input cpm (Fig. 4.3*C*). Using the 1.6-kb cDNA hybridization signal, the calculated decrease was 11-fold, from 78 units/input cpm to 7 units/input cpm (Fig. 4.3*D*). Given that the nuclear run-on signal in committed cells was faint, accurate quantitation of the decrease in transcription rate was difficult. Based on results of additional experiments and alternative algorithms for interpreting densitometric data, we estimate that *dhfr* run-on transcription decreased 7- to 30-fold during commitment. The magnitude of the change in transcription rate was sufficient to account for the 7-fold change in DHFR pre-mRNA levels.

As muscle cells committed to terminal differentiation, incorporation of radiolabeled

ribonucleotides into total RNA decreased about 1.6-fold. In other systems, the decrease in overall transcription was attributed primarily to growth-dependent differences in ribosomal gene transcription (Mauk and Green, 1973). Because we normalized run-on data per input cpm rather than per cell, committed cells were overrepresented by a factor of 1.6 in run-on hybridizations. Therefore, on a per-cell basis, *dhfr* transcription decreased 10- to 50-fold during commitment.

Surprisingly, a strong run-on signal was detected in proliferative cell nuclei using the 1.4-kb genomic restriction fragment, which contained only the first 58 base pairs of the *dhfr* coding region, but contained 1000 base pairs upstream of the DHFR initiation site. (Fig. 4.3*B*, *strips 5-8*). Because the immobilized DNA was not strand-specific, we could not resolve the orientation of the upstream signal. The upstream run-on signal was inhibited by α-amanitin (*strips 9-12*), indicating that RNA polymerase 2 was the enzyme transcribing upstream sequences. Unlike the DHFR gene, the nuclear run-on signal for the upstream region persisted as muscle cells committed to terminal differentiation (Fig. 4.3*B*, compare 1.4-kb bands in *strips 5-8* to *strips 13-16*), indicating that transcription of the upstream sequences continued after cells withdrew from the cell cycle. Laser densitometry showed that the transcription rate of the upstream region decreased only 1.8-fold, from 81 units/input cpm in proliferative cells to 46 units/input cpm in committed cells. Because the 0.6-kb *dhfr* probe (extending from +59 to +660 with respect to the major transcription initiation site) did not give a detectable signal in committed cells (Fig. 4.3*B*, compare 0.6-kb band in lanes 5-8 to lanes 13-16), the upstream transcription unit contained little or no sequence downstream of nucleotide +58. The upstream run-on signal may have arisen from transcription of a previously decribed upstream transcription unit that initiates at multiple sites in a region about 200 base pairs upstream of the major *dhfr* cap site, and extends in the opposite orientation of *dhfr* (Crouse *et al.*, 1985; Farnham *et al.*, 1985; Farnham and Schimke, 1986). Alternatively, the 1.4-kb run-on signal could have arisen

from hybridization of an as yet undefined transcript encoded within the DNA between -1000 and +58 base pairs from the *dhfr* cap site. Our data indicate that transcription of *dhfr* and of the region upstream of *dhfr* are independently regulated during muscle cell differentiation.

Northern blot analysis of steady state RNAs arising from the *dhfr* locus of amplified cells - Northern blots of amplified muscle cell RNA were used to determine whether the amplified DHFR gene in the H$^-$αR50T muscle cell line gave rise to transcripts of the expected sizes and to determine whether upstream transcription gave rise to any detectable RNA species. In addition, Northern blots were used to confirm and extend results of RNase protection assays. The 3.4-kb Eco R1 fragment of pDR34 (Fig. 4.3*A*) was used to probe Northern blots such that RNAs arising from all transcription signals detected in the nuclear run-on analysis (Fig. 4.3*B*) would hybridize to probe. Figure 4.4 shows that H$^-$αR50T muscle cells gave rise to the same DHFR RNA species as other established mouse cell lines. As a control, RNA from 3T6 R50 cells, a well characterized mouse fibroblast line containing amplified DHFR genes (Brown *et al.*, 1981; McGrogan *et al.*, 1985) was analyzed in parallel. All RNA species detected in proliferative muscle cell RNA (*lane 2*) were also present in 3T6 R50 cell RNA (*lane 1*). Likewise, Southern blot analysis (not shown) confirmed that the region of the amplicon immediately surrounding *dhfr* in H$^-$αR50T cells was qualitatively indistinguishable from that of parental cells. From the above evidence, we concluded that amplification did not alter the structure of the DHFR gene or its cognate mRNAs in our cell line.

No bands on the Northern blot were attributable to transcription of the upstream region. Using the mouse sarcoma cell line S180-500R, which contains amplified copies of the DHFR gene, Hook and Kellems (1988) have described all of the bands we detected and attributed each to *dhfr* transcripts with alternative initiation and termination sites (Fig. 4.4). Likewise, when we probed H$^-$αR50Tand 3T6 R50 Northern blots with the

1.6-kb Pst1 cDNA fragment of pDHFR11, which lacks upstream sequences (Fig. 4.3*A*), band patterns were identical to those depicted in Figure 4.4 (not shown). If the upstream transcripts were only 180-240 bases long as reported by Farnham and Schimke (1986), they may have been too small to detect on Northern blots. Alternatively, if upstream transcription gave rise to a longer mature transcript (Crouse *et al.*, 1985; Fujii and Shimada, 1989), it may have been too rapidly degraded to appear in steady state message pools, or may have co-migrated with a *dhfr* transcript and thereby been masked.

As muscle cells committed, levels of all RNA species detectable by Northern analysis decreased (Fig. 4.4, lanes 2-6). We found no evidence for growth-dependent differential cap site or polyadenylylation site usage, consistent with previous reports using synchronized fibroblastic cells (Hook and Kellems, 1988; Farnham and Schimke, 1986). The decrease in DHFR mRNA levels determined by Northern blot analysis corroborated findings based on RNase protection assays (Fig. 4.1*C*). It is noteworthy that no constitutive RNA species were detected despite strong continued transcription of the upstream region.

DISCUSSION

From previous studies, it has been unclear whether DHFR mRNA levels are transcriptionally or post-transcriptionally regulated. In studies on mitotically-synchronized cells, evidence for transcriptional regulation of *dhfr* expression is unchallenged (Farnham and Schimke, 1985, 1986). However, in studies using fibroblasts released from growth arrest, different groups report contradicting results. Wu and Johnson (1982) and Santiago *et al.* (1984) reported transcriptional activation of the DHFR gene during growth induction, whereas Leys and Kellems (1981) and Leys *et al.* (1984) reported no change in transcription rates, and attributed mRNA decreases to reduced transcript stability. Moreover, Kaufman and Sharp (1983) reported that, although a DHFR cDNA supplied with the adenovirus 2 major late promoter exhibited growth-dependent levels of DHFR mRNA in transfected cells, transcription rates were not growth-dependent, consistent with a posttranscriptional model for DHFR mRNA regulation. In contrast to previous studies on fibroblastic cells, we investigated the regulation of DHFR mRNA levels during the initial stages of myoblast differentiation. We have shown that DHFR pre-mRNA and mRNA levels decreased during myogenic withdrawal from the cell cycle and that the decrease was accompanied by a reduced rate of DHFR gene transcription. Although our data show transcriptional regulation of the DHFR gene during muscle cell commitment, it is uncertain whether the mechanism that regulates *dhfr* expression during myoblast commitment is identical to that regulating *dhfr* expression during progression through the cell cycle.

The decreases in DHFR gene transcript levels and transcription rates we report were not due to a general decrease in cellular biosynthetic activities. The overall rate of protein synthesis, as measured by [^{35}S]-methionine incorporation, is similar in proliferative and committed cells (Gross and Merrill, 1988). Further attesting to their anabolic activity, committed cells begin to accumulate muscle-specific proteins

(Chamberlain *et al.*, 1985; Jaynes *et al.*, 1986). In nuclear run-on reactions, we detected a small (1.6-fold) reduction in total nucleotide incorporation during commitment, which was attributed to growth-dependent changes in ribosomal gene transcription rates (Mauk and Green, 1973). However, as *dhfr*-specific radioactivity was measured as the percent of total incorporated radioactivity, the small decrease in total nucleotide incorporation does not affect our conclusion that the DHFR gene was transcriptionally repressed.

Theoretically, transcriptional repression could be due either to repression of initiation or to pre-termination in committed cells. However, a restriction fragment complementary only to bases 59 to 660 of the 30-kb primary *dhfr* transcript showed transcriptional repression of the DHFR gene in nuclear run-on assays (Fig. 4.3*B*, compare 0.6-kb band *on strips 5-8* to *strips 13-16*). Therefore, our results are consistent with a model wherein transcription of *dhfr* is regulated at the level of initiation or a model in which regulation occurs by transcriptional pretermination upstream of the region represented by the 0.6-kb restriction fragment. To determine which model is correct, one could test whether the *dhfr* promoter imparts growth-dependent regulation on an otherwise constitutive reporter gene. However, interpretation of such studies may be complicated if intragenic sequences participate in regulating *dhfr* transcription. Indeed, reports of promoter-switch experiments have not been forthcoming.

The region immediately upstream of the DHFR gene exhibited strong commitment-independent transcription. In proliferative cells, the run-on signal bound to the upstream genomic fragment was similar in intensity to the signal bound to the 2.6-kb genomic *dhfr* fragment (Fig. 4.3*B*, compare 1.4- and 2.6-kb signal intensities on *strips 5-8*). At 18 hours, strong hybridization to the 1.4-kb fragment persisted although binding to the 2.6- and 0.6-kb fragments was diminished. Because our immobilized DNA fragments were not strand-specific, we could not determine the orientation of the upstream run-on transcripts. Also, we were unable to detect upstream transcripts on

Northern blots, and have no data on transcript lengths or initiation sites. Therefore, either of two models could explain the signal hybridizing to the upstream fragment. First, the 1.4-kb signal could stem from RNA polymerase activity in the upstream region yielding transcripts distinct from the DHFR transcript. Included under this model would be generation of the previously described short (180- to 240-base) opposite strand transcripts (Crouse *et al.*, 1985; Farnham *et al.*, 1985; Farnham and Schimke, 1986). However, if upstream transcription was limited to only a 240-base-pair region (Farnham and Schimke, 1986) the strong signal we detected indicates that the number of RNA polymerase molecules per unit length (polymerase density) on the upstream region was about 10-fold greater than on the region of the DHFR gene represented by the 2.6-kb fragment. Alternatively, if opposite strand upstream transcription yields a long transcript, as suggested by Crouse *et al.* (1985) and Fuji and Shimada (1989), the polymerase density on the upstream region was similar to the polymerase density on the *dhfr*-coding region in proliferative cells. The second model consistent with our findings is that the upstream signal detected by the 1.4-kb fragment stems from transcription initiating at the DHFR promoter. Because the 1.4-kb fragment contains the DHFR promoter and 60 bases of the major DHFR transcript, it is possible that initiation from the DHFR promoter occurs at an extremely high and constitutive rate; however, most of the transcripts are rapidly preterminated. According to this model, even in proliferative cells, only about 1% of the transcripts extend into the *dhfr*-coding region represented by the 0.6- and 2.6-kb genomic fragments. In committed cells, fewer than 0.1% of the transcripts extend into *dhfr*-coding sequences. Until we can determine the orientation, length, and initiation site of the transcripts responsible for the 1.4-kb run-on signal, we cannot determine which model is correct.

Because the upstream region showed strong constitutive transcription, we investigated whether upstream transcription contributed to previous reports of growth-independent *dhfr* transcription (Leys and Kellems, 1981; Leys *et al.*, 1984; Kaufman and

Sharp, 1983). It did not. In the studies by Kellems' group, the cDNA probes used to detect *dhfr* transcription lacked upstream sequences (Leys and Kellems, 1981; Leys *et al.*, 1984). In the study by Kaufman and Sharp (1983), the chimeric constructs used lacked the *dhfr* promoter and upstream sequences. Therefore, transcription signals detected by both groups were not attributable to upstream transcription. The basis for the disparate findings regarding whether altered transcription rates are the major determinant of growth dependent changes in DHFR mRNA levels remains uncertain. Our results support a transcriptional model.

ACKNOWLEDGMENTS

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FIGURES AND LEGENDS

Fig. 4.1. **DHFR mRNA during muscle cell commitment.** *Panels A and B,* mRNA probe and pseudo-mRNA templates. Templates p4d200 and p4d750 were constructed as described in "Experimental Procedures." The pseudo-mRNA was an 800-base T7 transcript colinear with DHFR mRNA. The 5' probe was a 122-base SP6-generated RNA complementary to a 104-base region spanning the first intron junction of either DHFR mRNA or pseudo-mRNA. The 3' probe was a 175-base SP6 transcript complementary to a 141-base region within the sixth exon of DHFR mRNA, and a 161-base region of pseudo-mRNA. *Roman numerals* and *large open boxes* denote exons; *thick lines* denote intronic and 5' nontranscribed sequences; *thin lines* denote vector sequences; *arrowheads* denote viral transcription initiation sites and indicate direction; and *narrow open boxes* denote synthetic transcripts. THe abbreviations are: *B, Bam H1*; *Bg, Bgl2*; *D, Dde1*; *E, Eco R1*; *H, Hind3*; *R, Rsa1*; *T, Taq1*. *Parentheses* denote inactivated restriction sites. *Panel C,* DHFR mRNA RNase protection assay. RNA was harvested from H^{-}αR50T cells at specified times after inducing commitment. Experimental samples contained 3 x 10^5 cell-equivalents of RNA (3 μg). Standard curve samples contained 630, 250, 100, 40, and 16 attomoles of DHFR pseudo-mRNA (*lanes 3-7* and *15-19*). All hybridization mixtures contained 10 femtomoles of probe and 50 μg RNA (cellular or standard curve RNA supplemented with yeast RNA to 50 μg). *Lanes 2* and *14* (designated *C*) were controls showing no signal arose from yeast RNA. *Lanes 1* and *13* (designated *P*) contained 1 femtomole of nondigested probe. *Lane 25* contained Msp1-digested pBR322 markers (from bottom: 76, 90, 110, 122, 147, 160, 180, 190, 201, and 217 bases). Relative band intensities were determined by laser densitometry, and are listed below each lane.

Fig. 4.1

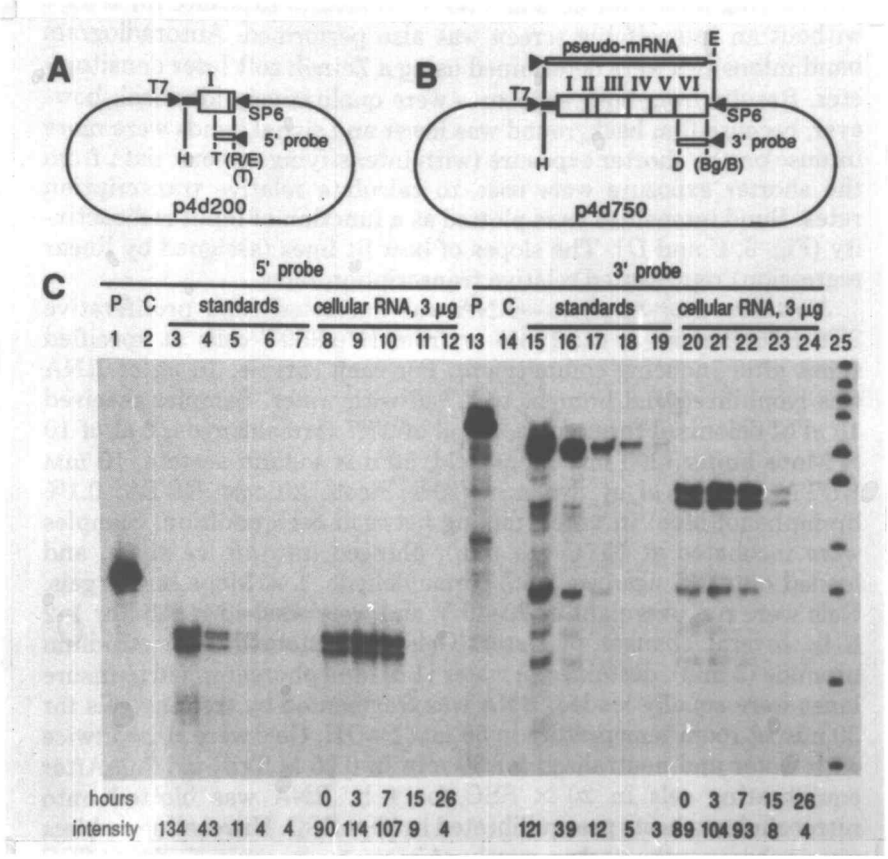
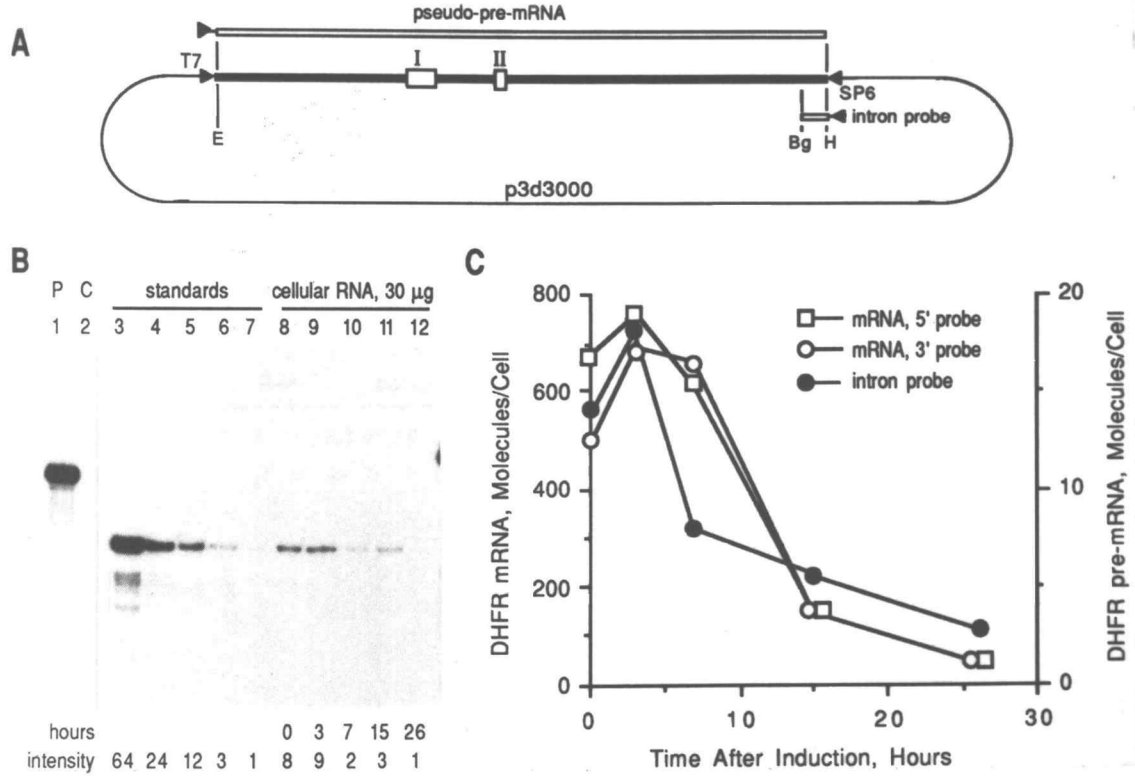

A
T7
I
SP6
5' probe
H
T (R/E)
(T)
p4d200
B
pseudo-mRNA
E
I II III IV V VI
T7
SP6
3' probe
H
D (Bg/B)
p4d750
C
5' probe
3' probe
P C standards cellular RNA, 3 µg
P C standards cellular RNA, 3 µg
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
hours 0 3 7 15 26 0 3 7 15 26
intensity 134 43 11 4 4 90 114 107 9 4 121 39 12 5 3 89 104 93 8 4

Fig. 4.2. **DHFR pre-mRNA levels during muscle cell commitment.** *Panel A,* intron probe and pseudo-pre-mRNA templates. Template p3d3000 was constructed as described in "Experimental Procedures". Pseudo-pre-mRNA was a 3.4-kb T7 transcript colinear with the *dhfr* transcript. The intron probe was a 161-base SP6-generated RNA complementary to a 136-base region entirely within the second intron of either DHFR pre-mRNA or pseudo-pre-mRNA. Designations as in Fig. 4.1. *Panel B,* DHFR pre-mRNA RNase protection assay. The same H⁻αR50T cell RNA that was assayed for DHFR mRNA content using exonic probes (see Fig. 4.1) was assayed for DHFR pre-mRNA content using the intron probe. Because pre-mRNA was expected to be rarer than mature message, tenfold more RNA (30×10^5 cell-equivalents, 30 μg) was used in measuring DHFR pre-mRNA. Hours of induction and band intensities are shown at bottom; lane designations are as described in Fig. 4.1. *Panel C,* quantitative comparison of DHFR pre-mRNA and mRNA during commitment. After electrophoretic analysis of RNase-treated samples, parallel aliquots were precipitated with trichloroacetic acid, washed, and assayed by liquid scintillation. Standard curves based on the amount of probe protected by DHFR pseudo-transcripts were used to determine the molar concentrations of DHFR transcripts in each sample. Molar values were converted to molecules/cell based on the number of cell-equivalents of RNA in each sample.

Fig. 4.2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T7
I
II
SP6
intron probe
E
Bg H
p3d3000
B
P C
standards
cellular RNA, 30 µg
1 2 3 4 5 6 7 8 9 10 11 12
hours
0 3 7 15 26
intensity 64 24 12 3 1 8 9 2 3 1
C
mRNA, 5' probe
mRNA, 3' probe
intron probe
DHFR mRNA, Molecules/Cell
DHFR pre-mRNA, Molecules/Cell
Time After Induction, Hours
800
600
400
200
0
20
10
0
10
20
30

Fig. 4.3. **Transcription of the DHFR gene and adjacent upstream sequences during muscle cell commitment.** Nuclear run-on assays were performed on nuclei harvested from H$^-$αR50T cells at 1 and 18 hours after inducing commitment and on nuclei harvested from proliferative (1-hr) H$^-$α cells. *Panel A*, restriction maps of the plasmids used to probe nuclear run-on reaction products. Genomic plasmid pDR34 was digested with Taq1; cDNA plasmid pDHFR11 was digested with Pst1. Intronic and 5' upstream sequences are depicted by *thick lines*; exonic sequences by *open boxes*; vector sequences by *thin lines*. DNA was separated on agarose gels and transferred to nitrocellulose blots. Blots were cut into strips. Taq1-digested pDR34 yielded three *dhfr*-containing fragments: a 1.4-kb fragment that included mouse sequences extending from 1000 base pairs upstream to 60 base pairs downstream of the major cap site (at -55 from AUG); a 0.6-kb fragment containing the remainder of exon 1, intron 1, exon 2, and the first 260 base pairs of intron 2; a 2.6-kb fragment containing 1.9 kilobases of intron 2. Pst1-digested pDHFR11 yielded a single 1.6-kb *dhfr*-containing fragment extending from 45 base pairs upstream of the major cap site to 950 base pairs downstream of the termination codon. Restriction fragment sizes are shown below each plasmid. Abbreviations as in Fig. 4.1*A*. *Panel B,* autoradiograms of strips after hybridization with nuclear run-on reaction products. Run-on reactions were performed using 10^7 nuclei harvested from wild type H$^-$α cells (denoted *wt*) or methotrexate-resistant H$^-$αR50T cells (denoted *R*) at specified times after induction (1 or 18 hours). Where indicated, α-amanitin (*α-am*) at 1.0 μg/ml was included during reactions. After run-on reactions were terminated, RNA was purified, incorporated radioactivity was determined by liquid scintillation, and the specified amounts of incorporated radioactivity (*input [^{32}P]RNA*) were incubated with strips containing either Taq1-digested pDR34 DNA (*top*) or Pst1-digested pDHFR11 DNA (*bottom*). Washed strips were exposed to X-ray film for 60 hours with a Dupont Cronex intensifying screen (*top*), or for 6 days without an intensifying screen (*bottom*). Background radioactivity on the strips represented

nonspecific binding of RNA to nitrocellulose. Lack of hybridization to vector fragments demonstrated binding specificity. At right are shown sections of the ethidium bromide-stained agarose gels prior to blotting; restriction fragment sizes are specified. *Panels C* and *D*, effect of commitment on DHFR transcription rates as determined by the hybridization signal present on the 2.6-kb genomic and the 1.6-kb cDNA probes. Densitometrically-determined band intensities were plotted as a function of the input radioactivity used in each hybridization. Best-fit lines were determined by linear regression; slopes represent relative transcription rates. Using the 2.6-kb genomic probe (*C*), we measured relative transcription rates of 156 and 8 units/input cpm for 1 and 18-hour nuclei (*closed* and *open circles*, respectively). Using the 1.6-kb cDNA probe (*D*), we measured relative transcription rates of 78 and 10 units/input cpm for 1 and 18-hour nuclei (*closed* and *open circles*, respectively).

Fig. 4.3

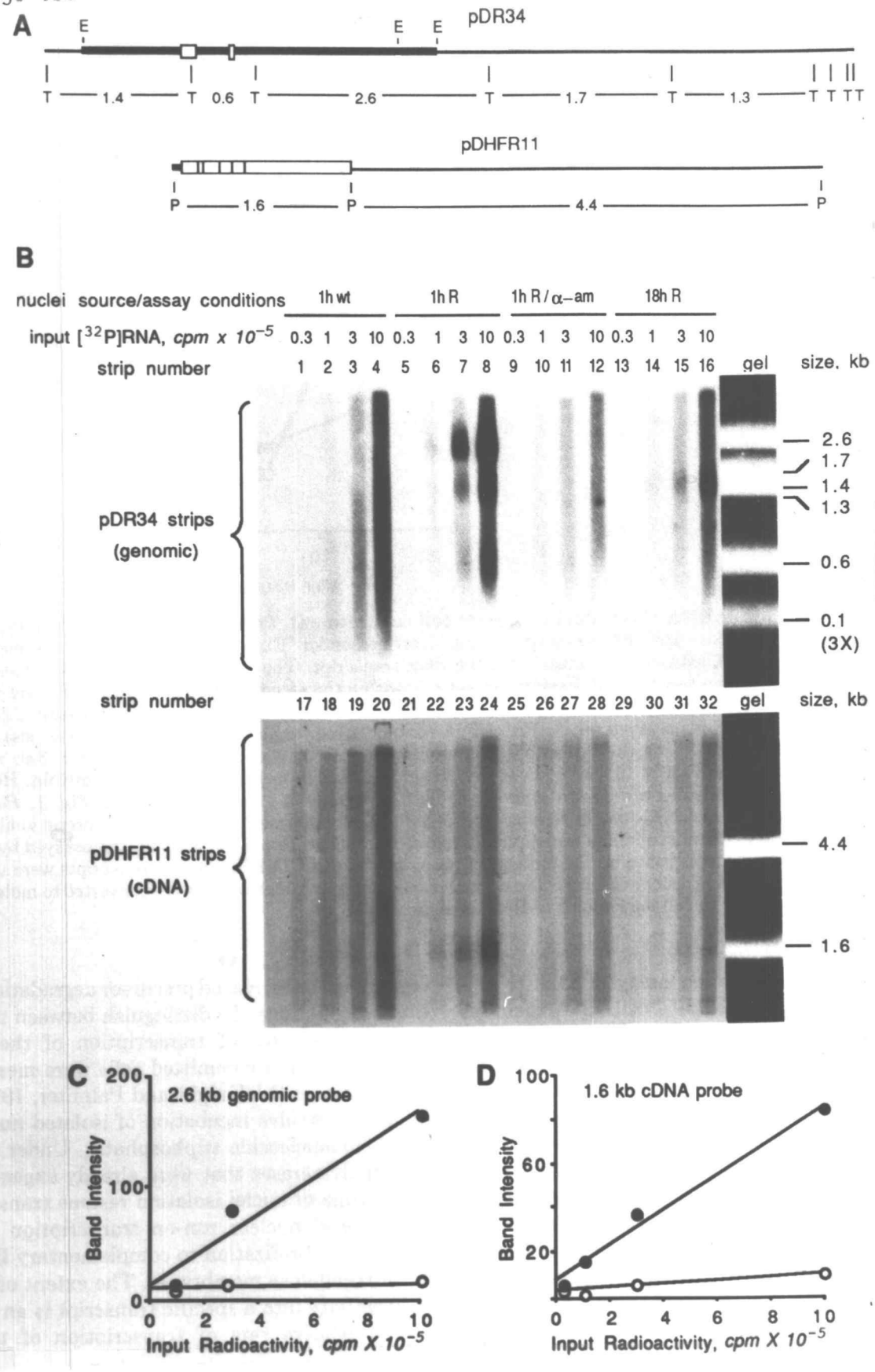
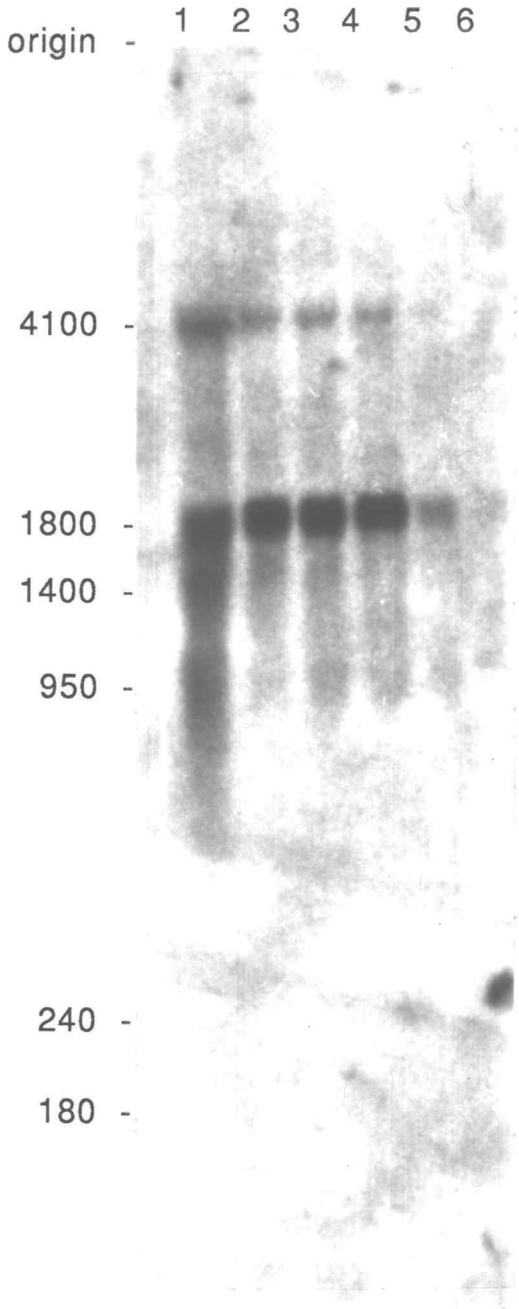

A
E
E
E
pDR34
T
1.4
T
0.6
T
2.6
T
1.7
T
1.3
T T TT
pDHFR11
P
1.6
P
4.4
P
B
nuclei source/assay conditions
1h wt
1h R
1h R / α–am
18h R
input [32P]RNA, cpm x 10−5
0.3 1 3 10 0.3 1 3 10 0.3 1 3 10 0.3 1 3 10
strip number
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
gel
size, kb
pDR34 strips
(genomic)
2.6
1.7
1.4
1.3
0.6
0.1
(3X)
strip number
17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
gel
size, kb
pDHFR11 strips
(cDNA)
4.4
1.6
C
2.6 kb genomic probe
Band Intensity
200
100
0
0 2 4 6 8 10
Input Radioactivity, cpm X 10−5
D
1.6 kb cDNA probe
Band Intensity
100
60
20
0 2 4 6 8 10
Input Radioactivity, cpm X 10−5

Fig. 4.4. **Northern analysis of *dhfr*-associated transcripts.** A Northern blot containing 10 μg RNA per lane from proliferative 3T6-R50 cells (*lane 1*) and from H^{-}αR50T cells harvested 0, 3, 7, 15, and 26 hours after inducing commitment (*lanes 2-6*) was probed with a nick-translated 3.4-kb Eco R1 DNA fragment isolated from pDR34 (see Fig. 4.3*A*). Sizes of detected DHFR mRNAs are indicated. The region where 180- to 240-base transcripts would appear, if detectable, is indicated.

Fig. 4.4



CHAPTER 5

An Intragenic Region Adjacent to the Dihydrofolate Reductase Promoter is Required for Replication-Dependent Expression

Edward E. Schmidt, Rebecca A. Owen, and Gary F. Merrill

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Coauthor contribution:

E.E.S.: Graduate student. Designed plasmids p4CAT1632, DHFR(-850/+60)pCAT, DHFR(-850/+465)pCAT, and RSVpDHFR(+61/+465)CAT. Constructed and cloned plasmids p4CAT1632, DHFR(-850/+60)pCAT, and DHFR(-850/+465)pCAT. Transformed, selected, subcloned and grew all eucaryotic cell lines used. Performed all tissue culture work. Harvested and prepared RNA samples with assistance from S.B and K.M. (listed in "Acknowledgments"). Synthesized all RNA probes and standards and performed all nuclease protection assays. Harvested DNA samples with assistance from R.A.O. and performed Southern blot analyses of transformed gene copy numbers. Performed all data quantitation with assistance from S.B. Performed all photography and Figure preparation. Wrote all drafts of manuscript. Proofread and corrected galley proofs.

R.A.O.: Laboratory technician. Prepared general laboratory and tissue culture reagents. Constructed and cloned plasmid RSVpDHFR(+61/+465)CAT. Prepared plasmid clean preps. Assisted with genomic DNA preparation. Proofread galley proofs.

G.F.M.: Research director. Designed, constructed, and cloned plasmid p4CAT148. Contributed to design and logic of experiments. Edited all drafts of manuscript and proofread galley proofs. Provided research space and materials. Paid salaries for E.E.S., R.A.O., and S.B., and paid tuition for E.E.S., from his N.I.H. research grant.

SUMMARY

The gene encoding dihydrofolate reductase (DHFR) is down-regulated as myoblasts withdraw from the cell cycle and commit to terminal differentiation. To localize *cis*-acting elements involved in regulating DHFR gene expression, the DHFR promoter and upstream region, together with differing amounts of contiguous intragenic sequence, were fused to the the bacterial chloramphenicol acetyltransferase (CAT) gene. The resulting fusion genes were stably transformed into muscle cells, and CAT mRNA levels were measured in proliferative myoblasts and committed myocytes. A gene consisting of the -850/+465 region of DHFR (numbers refer to distance in base pairs from transcription initiation site) fused to CAT was efficiently expressed in proliferating myoblasts and was appropriately down-regulated during commitment. A gene consisting of the -850/+60 region of DHFR fused to CAT was poorly expressed in proliferating myoblasts and was not down-regulated during commitment. When inserted between the Rous sarcoma virus promoter and CAT sequence of RSVpCAT, the +61/+465 region of the DHFR gene augmented CAT mRNA expression in muscle cell transformants but did not confer a regulated pattern of expression. Our data indicate that DHFR sequences between +60 and +465 are required but are not sufficient for replication-dependent expression. The DHFR sequences may be operating at either a transcriptional or posttranscriptional level.

INTRODUCTION

Dihydrofolate reductase (DHFR) is preferentially synthesized in growing cells (Feder *et al.*, 1989; Mariani *et al.*, 1981; Schmidt *et al.*, 1989a). The factors responsible for growth-specific synthesis likely are important for matching rates of synthesis of many proteins to the growth state of the cell. Regulatory factor identification depends on two prerequisites: establishment of the level of control and identification of the *cis*-acting regulatory elements. Replication-dependent expression of DHFR mRNA has been documented in several fibroblastic cell lines; however, depending on the cell culture system used, the relative contribution of transcriptional versus posttranscriptional processes has varied (Farnham and Schmike, 1985; Kaufman and Sharp, 1983; Leys *et al.*, 1984; Wu and Johnson, 1982). In no case has the *cis*-elements responsible for DHFR mRNA regulation been identified.

Using mouse muscle cells that irreversibly withdraw from the cell cycle when induced to differentiate we have demonstrated that DHFR pre-mRNA levels decrease during differentiation and that the decrease in pre-mRNA levels is associated with a decrease in DHFR gene transcription as measured by nuclear run-on assay (Schmidt and Merrill, 1989b). In the experiments that follow, we demonstrate that during myogenic withdrawal from the cell cycle, regulation of a reporter gene fused to the DHFR upstream region is dependent upon intragenic sequences within 465 base pairs of the transcription initiation site. Our results are discussed in context with recently published work by Farnham and Means (1990), who demonstrated that intragenic sequences are required for efficient transcription of DHFR templates *in vitro*.

EXPERIMENTAL PROCEDURES

Cell lines and transformations - All lines were derived from H-α, an hypoxanthine-guanine phosphoribosyltransferase-deficient subline of the MM14D mouse skeletal myoblast line developed by Linkhart *et al.* (1981). Culture conditions, commitment kinetics, and amplified cell line H-α R50T have been described elsewhere (Schmidt and Merrill, 1989a,b). Transformations were performed using 10 μg test gene plasmid DNA and 0.3 μg pKneo as described previously (Gross *et al.*, 1987); transformants were selected in 400 μg/ml G418. Transformations yielded 30 to 300 colonies per dish. Individual colonies were selected for clonal lines; pooled lines represented all colonies from a single dish.

RNase protection assays - DHFR mRNA levels were determined by quantitative RNase protection using the 3' probe described elsewhere (Schmidt and Merrill, 1989a,b) except that the specific activity of the probe was increased five-fold to 40 Ci/μmole. The probe hybridized to sequences entirely within the sixth exon of DHFR and did not overlap sequences in any of the test genes. Therefore, the probe detected only message arising from the endogenous DHFR gene.

To measure CAT message, a probe complementary to sequences at the 5' end of the CAT gene was designed. Plasmid p4CAT148 was constructed by inserting the 148-bp Hind3/Pvu2 fragment from RSVpCAT (Gorman *et al.*, 1982, provided by B. Howard) into Hind3/Hinc2-cleaved pGEM4 (Promega). Plasmid p4CAT1632 was constructed by inserting the 1632-bp Hind3/Bam H1 fragment from RSVpCAT into Hind3/Bam H1-cleaved pGEM 4 (Fig. 5.1). Probe was synthesized by linearizing p4CAT148 with Hind3 and transcribing with SP6 RNA polymerase in the presence of [^{32}P]UTP (190 Ci/mmole). Probe specific activity was 8 Ci/μmole. "Pseudo-mRNA" was synthesized by linearizing p4CAT1632 with Bam H1 and transcribing with T7 RNA polymerase in the

presence of nonlabeled nucleotides. Yield was determined spectrophotometrically (ε = 20 ml mg^{-1} cm^{-1}) and dilutions were used to generate standard curves. Transcription reactions, hybridizations, RNase digestions, and gel electrophoresis were as described (Schmidt and Merrill, 1989b). After autoradiography, gel bands were excised and solubilized overnight at 65° C in 300 µl 30% hydrogen peroxide. Cocktails received 3 ml scintillation fluid (4.0 g Omnifluor, 333 ml Triton X-100, 667 ml toluene) and were assayed for 50 min by liquid scintillation. Comparison to pseudo-mRNA standards allowed absolute quantitation.

Test gene construction - RSVpCAT was used as the source of the CAT/SV40 intron-polyadenyl- ylation site cassette. DHFR sequences were derived from pDR34 (Crouse *et al.*, 1982, provided by R. Schimke). To generate DHFR-promoter test genes, an intermediate cloning step was used. Fragments from pDR34 extending from the Eco R1 site at -850 to either the Taq1 site at +60 or the Rsa1 site at +465 were inserted into Eco R1/Acc1- or Eco R1/Hinc2-cleaved pGEM3 to yield p3DHFR(-850/+60)p and p3DHFR(-850/+465)p (numbers refer to distance in base pairs from the transcription initiation site). The DHFR fragments were subsequently excised by cleavage with Pvu2 and Hind3, which cut 46 base pairs upstream and 19 base pairs downstream from the DHFR insert, respectively. The Pvu2/Hind3 DHFR fragments were cloned upstream from a CAT reporter gene vector that was prepared by cleaving RSVpCAT with Nde1, filling with Klenow, and cutting with Hind3. Nde1/Hind3 cleavage completely excises RSV promoter sequences from RSVpCAT. To generate RSVpDHFR(+61/+465)CAT, the 2112-bp Taq1(filled)/Bam H1 fragment from DHFR(-850/+465)pCAT was inserted into Hind3(filled)/Bam H1-cleaved RSVpCAT.

RESULTS

Intragenic sequence requirements for expression and regulation of a reporter gene fused to the DHFR promoter - To identify *cis*-acting information involved in regulating DHFR gene expression, our approach was to analyze the expression pattern of chimeric genes containing portions of the DHFR gene fused to the bacterial cistron encoding chloramphenicol acetyltransferase (CAT). Although CAT enzyme activity is traditionally measured when CAT is used as a reporter gene, enzyme activity is removed from mRNA levels by numerous intermediate steps. We expected changes in CAT activity to lag far behind changes in CAT mRNA levels, and therefore be a poor indicator of mRNA regulation. Rather, chimeric gene expression was monitored by measuring CAT mRNA levels by the RNase protection assay described in Fig. 5.1.

Although DHFR mRNA was one of the first eucaryotic messages for which hybridization probes were developed (Alt *et al.,* 1978), the *cis*-acting information responsible for replication- dependent mRNA expression has thus far escaped identification. Based on the lack of progress in identifying *cis* regulatory elements, we speculated (Schmidt and Merrill, 1989b) that DHFR gene expression might be regulated by intragenic sequences. To investigate this possibility, the two chimeric genes shown at the top of Fig. 5.2 were constructed. Both plasmids contained DHFR sequences beginning at -850 (numbers refer to distance in base pairs from the transcription initiation site) and extending through the entire promoter, cap site, and translation start site. The DHFR portion of DHFR(-850/+60)pCAT ended at +60 (five bases downstream of AUG). The DHFR portion of DHFR(-850/+465)pCAT ended at +465 and contained all of exon 1, all of intron 1, and the first 14 bases of exon 2. If upstream signals were sufficient for regulation of DHFR mRNA, we expected both constructs to be regulated during commitment. If sequences between +60 and +465 were required, only DHFR(-850/+465)pCAT should be regulated. If sequences downstream of +465 were

required, none of the constructs should be regulated. As a control to check for changes in CAT mRNA stability during commitment, CAT message levels in muscle cells transformed with a gene consisting of the Rous sarcoma virus promoter fused to CAT (RSVpCAT) (Fig. 5.2) were determined.

Plasmids were cotransformed into muscle cells with pKneo and analyzed for replication- dependent expression of CAT mRNA. For each gene, several polyclonal and clonal transformant lines were established. Representative results from four DHFR(-850/+60)pCAT transformants (*2pB*, *2A*, *2B*, and *2C*), four DHFR(-850/+465)pCAT transformants (*3pA*, *3pB*, *3B*, and *3C*), and three RSVpCAT transformants (*5p*, *5A*, and *5B*) are shown in Fig. 5.3A. For each transformant, RNA was harvested before (*P*) or after (*C*) a 26-hr incubation in mitogen-depleted medium, an interval sufficient to induce all proliferative myoblasts in the population to withdraw from the cell cycle and commit to terminal differentiation (Schmidt and Merrill, 1989a; Linkhart *et al.*, 1981).

The proliferative cell data in Fig. 3A show that DHFR sequences between +60 and +465 were critical for efficient expression of the chimeric gene. Proliferative cell CAT mRNA levels were 25-fold higher in DHFR(-850/+465)pCAT transformants (lanes 13,15,17, and 19) than in DHFR(-850/+60)pCAT transformants (lanes 5,7,9, and 11). In fact, to visualize the CAT mRNA band in DHFR(-850/+60)pCAT transformants, 5-fold longer autoradiographic exposures were necessary (Fig. 5.3A, lower panel). Southern blots of selected transformants (lines *2pA*, *2A*, *3pA*, *3pB*, *3A*, and *3B*) indicated that differences in gene expression were not due to differences in gene copy number (not shown). We conclude that the +60/+465 region of the DHFR gene contains a positive-acting element that augments expression from the DHFR promoter.

Additional data in Fig. 5.3A address the issue of whether intragenic sequences are required for replication-dependent regulation of DHFR gene expression. CAT mRNA decreased between 3- and 10-fold during commitment in DHFR(-850/+465)pCAT

transformants (compare *P* and *C* samples in lanes 13-20). In contrast, CAT mRNA levels did not decrease during commitment in RSVpCAT transformants (compare *P* and *C* samples in lanes 21-26), indicating that the decrease in CAT mRNA in DHFR(-850/+465)pCAT transformants was not due to intrinsic instability of CAT mRNA in committed cells. The 6-fold average decrease in CAT mRNA expression from the DHFR(-850/+465)pCAT gene was similar to the 7-fold decrease in DHFR pre-mRNA expression from the native DHFR gene (Schmidt and Merrill, 1989b).

Significantly, CAT mRNA levels in DHFR(-850/+60)pCAT transformants did not decrease substantially during commitment (Fig. 5.3A, compare *P* and *C* samples in lanes 5-12). As a control to confirm that transformants appropriately regulated expression from the endogenous DHFR gene, RNA samples were analyzed for DHFR mRNA content (Fig. 5.3B). In all cases, expression of the endogenous DHFR gene was tightly regulated. Thus, poor regulation of CAT mRNA levels in DHFR(-850/+60)pCAT transformants was not an artifact of variation in host cell growth or regulatory properties. We therefore concluded that sequences between +61 and +465 were required in *cis* for regulation of reporter gene mRNA levels.

The +61/+465 region activates but fails to regulate expression from a heterolous promoter - Having established that sequences between +61 and +465 were required for regulated reporter gene expression from the DHFR promoter, we investigated whether these sequences were sufficient to confer regulation on an otherwise nonregulated heterologous promoter. The +61/+465 region of the DHFR gene was fused between the RSV promoter and CAT sequences of RSVpCAT to generate the plasmid RSVpDHFR(+61/+465)CAT (Fig. 5.2). CAT mRNA levels in muscle cells transformed with RSVpDHFR(+61/+465)CAT or RSVpCAT are shown in Fig. 5.4A. As a control to confirm that expression from the endogenous DHFR gene was appropriately regulated, samples also were analyzed for DHFR mRNA content (Fig. 5.4B). In proliferative cells,

CAT mRNA levels were 3-fold higher in RSVpDHFR(+61/+465)CAT transformants than in RSVpCAT transformants (Fig 5.4A, compare lanes 5 and 7). Thus, the presence of the +61/+465 DHFR sequences augmented expression from the RSV promoter. (We note that the enhancer region in the RSV long terminal repeat may have partially masked positive effects of the DHFR sequences on CAT mRNA levels.) Significantly, the +61/+465 DHFR sequences did not confer regulated expression from the RSV promoter. CAT mRNA levels did not decrease during commitment in RSVpDHFR(+61/+465)CAT transformants (Fig. 5.4A, compare lanes 5 and 6), even though DHFR mRNA levels were regulated normally (Fig. 5.4B, lanes 5 and 6). Thus, although the +61/+465 region augmented expression from a heterologous promoter, it was not sufficient to cause expression to be regulated. We conclude that the +61/+465 region was required but was not sufficient for regulation.

DISCUSSION

The -850/+465 region of the DHFR gene, when fused upstream of a CAT reporter gene, caused CAT mRNA levels to be regulated in a replication dependent manner. Both transcriptional and posttranscriptional mechanisms can be invoked to explain CAT mRNA level regulation. The chimeric transcript contained DHFR sequences at its 5' end that conceivably could have targeted the transcript for degradation in nonreplicating cells. We favor a transcriptional explanation for regulated CAT mRNA expression because the native DHFR gene in muscle cells is primarily regulated at the level of transcription (Schmidt and Merrill, 1989b). Furthermore, the 6-fold regulation of CAT mRNA expression in DHFR(-850/+465)pCAT transformants (Fig. 5.3A) was comparable in magnitude to the 7-fold regulation of DHFR pre-mRNA expression in amplified cells (Schmidt and Merrill, 1989b). Regardless of whether it works at the level of transcription or RNA stability, at least part of the *cis* regulatory information appears to reside within the transcribed region of the gene. CAT mRNA levels were not regulated when the CAT reporter gene was fused downstream of the -850/+60 region of the DHFR gene even though the DHFR region contained the entire DHFR promoter, cap site, and first 60 transcribed bases. The 400-bp fragment present in the regulated DHFR(-850/+465)pCAT gene but absent in the nonregulated DHFR(-850/+60)pCAT gene was not able to confer regulation on a heterologous promoter (Fig. 5.4A). Three explanations seem reasonable: 1) multiple elements located upstream and downstream of position +60 are required for DHFR regulation; 2) a single element located in the +61/+465 region is responsible for DHFR regulation but is incapable of influencing expression from the heterologous RSV promoter; 3) a single element centered near residue +60 is responsible for DHFR regulation. In the latter scenario, the regulatory element would have been bisected during construction of both DHFR(-850/+60)pCAT and RSVpDHFR(+61/+465)CAT and thus be nonfunctional.

In addition to being required for regulated expression, the +61/+465 region of the

DHFR gene was required for efficient expression. Unlike the regulatory function, the enhancement function of the +61/+465 region was operative on a heterologous promoter (Fig. 5.3A), suggesting that a positive-acting element resided entirely within this region.

With the exception of RSVpCAT, all of the chimeric genes used in this study (Fig. 5.2) contained early translation termination codons. A recent report by Urlaub *et al.* (1989) suggests that early nonsense codons can destabilize transcripts from the endogenous DHFR gene. In this study, several DHFR-deficient clones that arose after UV irradiation contained upstream stop codons and exhibited reduced levels of DHFR mRNA. Curiously, when DHFR cDNA or genomic genes containing the same nonsense mutations were transformed into cells, pretermination had no effect on message levels (Urlaub *et al.,* 1989). Several observations make it unlikely that the enhancer-like effect we detected in the +61 to +465 region was related to premature translation termination. First, as shown in the study by Urlaub *et al.* (1989), nonsense mutations only affect levels of mRNA arising from the endogenous DHFR gene; they are ineffectual on transformed-gene mRNA levels (Urlaub *et al.,* 1989). Second, all of our DHFR-containing constructs initiate translation out-of-frame with the CAT gene. DHFR(-850/+60)pCAT encodes a 64-amino acid polypeptide; DHFR(-850/+465)pCAT encodes a 33-amino acid polypeptide; RSVpDHFR(+61/+465)CAT encodes a 20-amino acid polypeptide. Third, although RSVpCAT encodes an intact CAT protein whereas RSVpDHFR(+61/+465)CAT contains an early termination codon, the later gene expressed higher levels of mRNA (Fig. 5.4). Thus, premature translational termination cannot explain the enhancer-like effect. Whereas it is possible that splicing of DHFR intron 1 (bases +141 to +450) is required for efficient mRNA expression, it is noteworthy that, inasmuch as all constructs we tested contained a downstream SV40-derived intron, the requirement would have to be relatively specific.

In considering the location and level of action of the positive-acting element, recently published work by Farnham and Means (1990) is highly relevant. These investigators

showed that sequences downstream from the DHFR cap site were important for transcription of DHFR templates *in vitro*. Specifically, a template truncated at position +275 was transcribed 2-fold more efficiently than a template truncated at position +60. Significantly, the +61/+275 region continued to augment *in vitro* transcription when placed upstream from the DHFR promoter in either orientation. Our studies provide *in vivo* support for their conclusion that an enhancer resides near the exon 1/inton 1 boundary.

The study by Farnham and Means (1990) also included DNase I footprinting analyses. Nuclear extracts gave two distinct footprints in the region thought to contain an enhancer. Interestingly, a third distinct footprint was evident that spanned +60, where we suspect regulatory information resides.

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FIGURES AND LEGENDS

Fig. 5.1. **CAT mRNA RNase protection asay.** CAT gene fragments (stippled boxes) were cloned into pGEM4 vectors (thin lines). In p4CAT148, the CAT gene Pvu2 site was destroyed by ligation to the vector Hinc2 site. Arrows denote size and orientation of synthetic RNAs; arrowheads denote site and orientation of phage promoters. Cleavage of p4CAT148 with Hind3 and transcription with SP6 polymerase yields a 193-base transcript that is complementary to 148 bases of CAT mRNA. Cleavage of p4CAT1632 with Bam H1 and transcription with T7 polymerase yields a 1647-base CAT pseudo-mRNA that is colinear with CAT mRNA.

Fig. 5.1

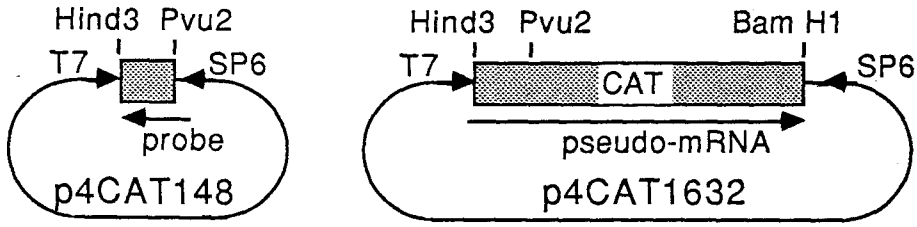

Hind3
Pvu2
T7
SP6
probe
p4CAT148
Hind3
Pvu2
Bam H1
T7
CAT
SP6
pseudo-mRNA
p4CAT1632

Fig. 5.2. **Plasmids used in cell transformations.** Numbering is in reference to the DHFR cap site (*+1, bent arrow*). *Open boxes* denote DHFR exonic sequences; *thick lines* denote DHFR intron 1; *thin lines* denote 5' nontranscribed sequences; *hatched boxes* represent the DHFR core promoter region (Dynan *et al.*, 1986); *thick arrows* denote the RSV promoter; other designations as in Fig. 5.1.

Fig. 5.2

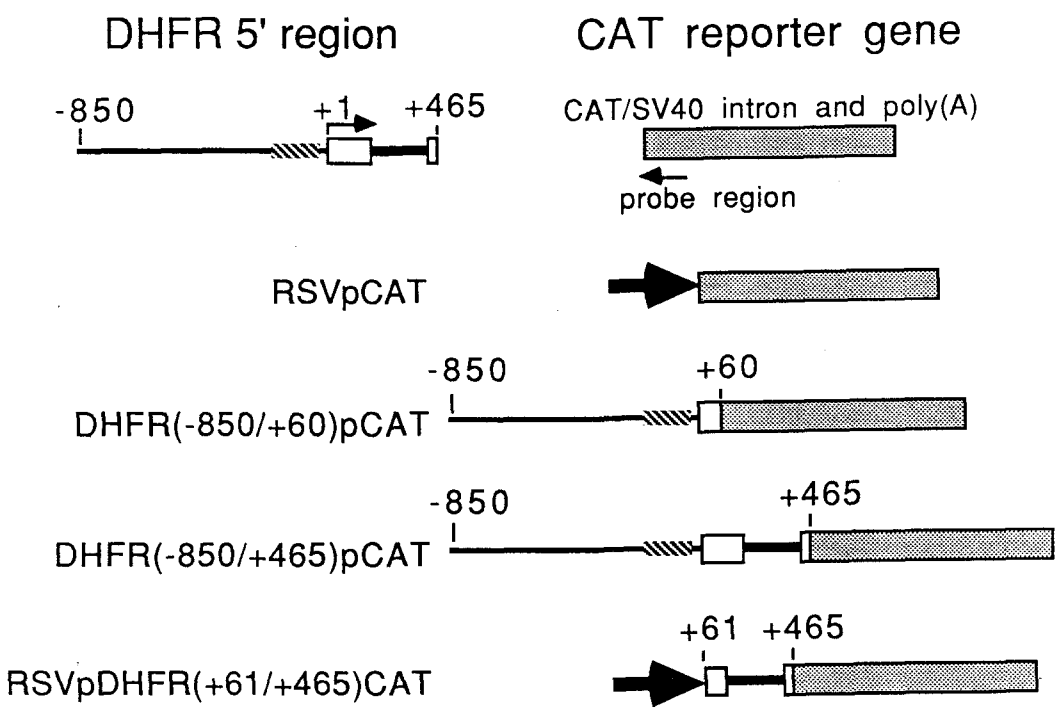

DHFR 5' region
CAT reporter gene
-850
+1
+465
CAT/SV40 intron and poly(A)
probe region
RSVpCAT
-850
+60
DHFR(-850/+60)pCAT
-850
+465
DHFR(-850/+465)pCAT
+61
+465
RSVpDHFR(+61/+465)CAT

Fig. 5.3. **Expression and regulation of DHFR/CAT fusion genes**. Myoblasts were transformed with DHFR(-850/+60)pCAT, DHFR(-850/+465)pCAT, or RSVpCAT. Both polyclonal (*2Pb, 3P, 3Pb,* and *5P*) and clonal (*2A, 2B, 2C, 3B, 3C, 5A,* and *5B*) lines were established. RNA was harvested from proliferative (*P*) and committed (*C*) transformants. A 26-hr incubation in mitogen-depleted medium was used to induce commitment. *Panel A,* CAT mRNA expression from test genes. Cellular RNA (10 μg) or synthetic pseudo-mRNA standards were hybridized with the CAT mRNA probe and digested with RNase. Half of each digest was separated by electrophoresis (*lanes 1-20* represent a single gel; *lanes 21-26* represent a separate gel). After a 36-hr exposure to X-ray film, the 148-base RNase-protected band derived from CAT mRNA (*CAT*) was excised and assayed by liquid scintillation. To better visualize signals arising from DHFR(-850/+60)pCAT, the remainder of each digest was run on an identical gel and exposed for 8 days (inset). Attomoles of CAT mRNA per sample are listed below each lane. *Panel B,* DHFR mRNA expression from the endogenous gene. A larger quantity (30 μg) of the same cellular RNA assayed for CAT mRNA above was analyzed for DHFR mRNA. Cell lines *2Pb* and *3B* were not analyzed because insufficient RNA remained. *Lanes 1-20* represent a single gel, *lanes 21-26* represent a separate gel. The standards give a larger RNase-protected fragment than cellular RNA because the DHFR probe hybridizes to 20 bases of vector sequence in DHFR pseudo-mRNA. Excised bands were assayed by liquid scintillation. Attomoles of DHFR mRNA per sample are listed below each lane.

Fig. 5.3

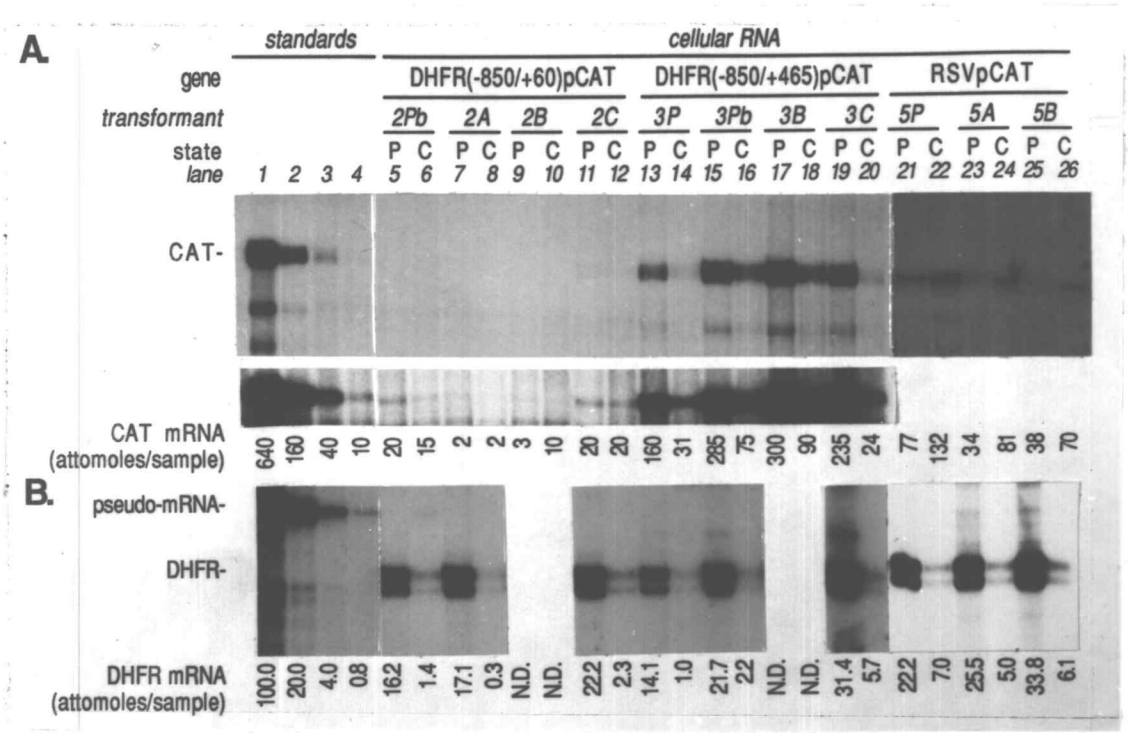
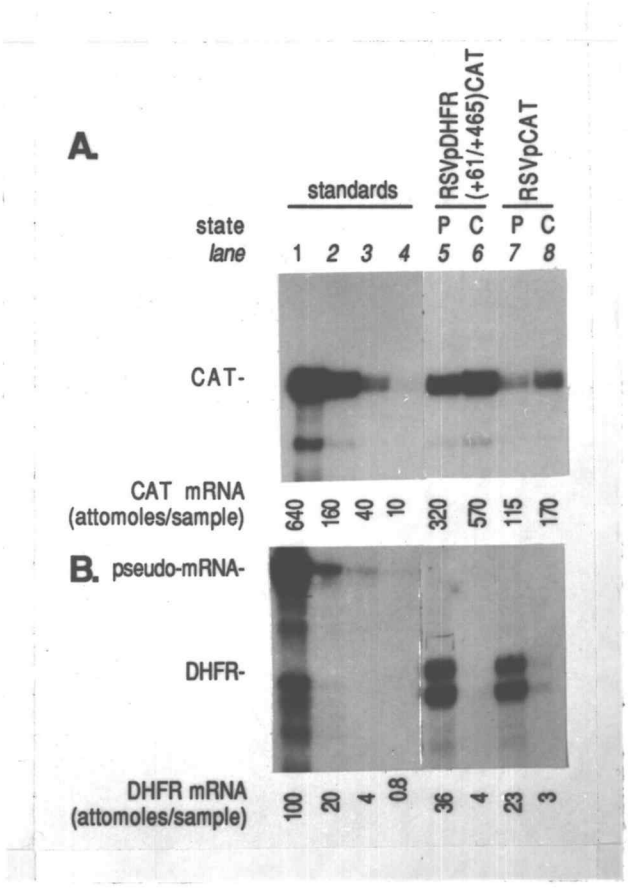

A.
standards
cellular RNA
gene
DHFR(-850/+60)pCAT
DHFR(-850/+465)pCAT
RSVpCAT
transformant
2Pb
2A
2B
2C
3P
3Pb
3B
3C
5P
5A
5B
state
P C P C P C P C P C P C P C P C P C P C P C
lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
CAT-
CAT mRNA
(attomoles/sample)
640 160 40 10 20 15 2 2 3 10 20 20 160 31 285 75 300 90 235 24 77 132 34 81 38 70
B.
pseudo-mRNA-
DHFR-
DHFR mRNA
(attomoles/sample)
100.0 20.0 4.0 0.8 16.2 1.4 17.1 0.3 N.D. N.D. 22.2 2.3 14.1 1.0 21.7 22 N.D. N.D. 31.4 5.7 22.2 7.0 25.5 5.0 33.8 6.1

Fig. 5.4. **Effect of intragenic DHFR sequences on RSVpCAT expression.** Proliferative (*P*) and committed (*C*) polyclonal myoblast lines transformed with RSVpDHFR(+61/+465)pCAT or RSVpCAT were analyzed for CAT mRNA or DHFR mRNA by RNase protection as described in Fig. 5.3. *Panel A,* CAT mRNA expression from test genes; ten micrograms of cellular RNA analyzed. *Panel B,* DHFR mRNA expression from endogenous gene; fifty micrograms of cellular RNA analyzed.

Fig. 5.4


A.
standards
RSVpDHFR (+61/+465)CAT
RSVpCAT
state
P C P C
lane
1 2 3 4 5 6 7 8
CAT-
CAT mRNA (attomoles/sample)
640 160 40 10 320 570 115 170
B. pseudo-mRNA-
DHFR-
DHFR mRNA (attomoles/sample)
100 20 4 0.8 36 4 23 3

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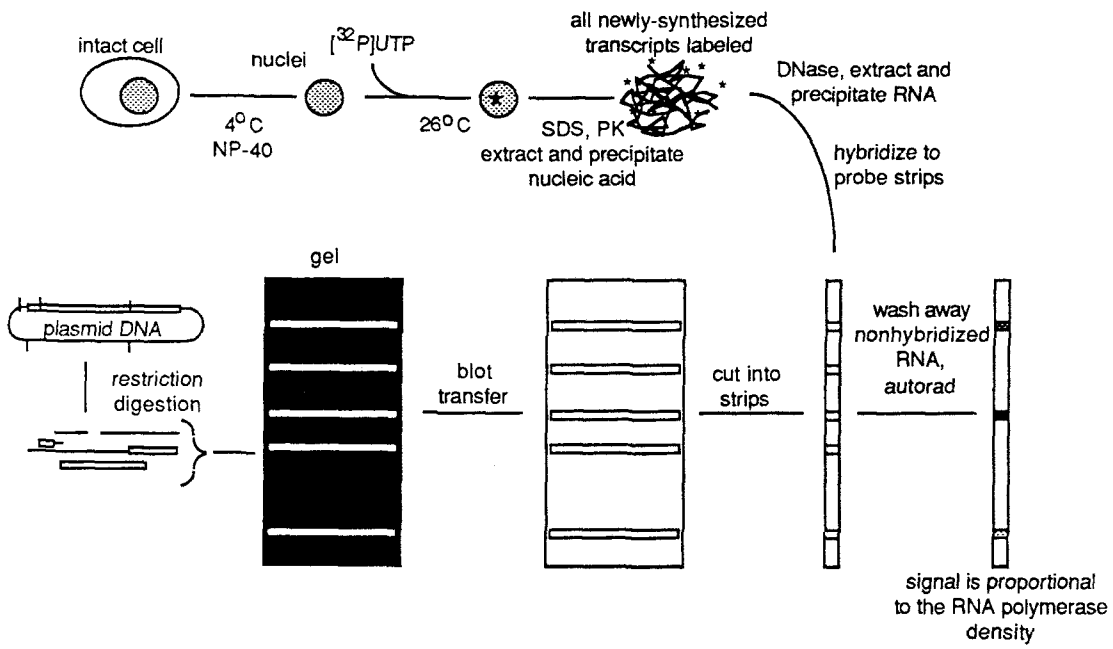
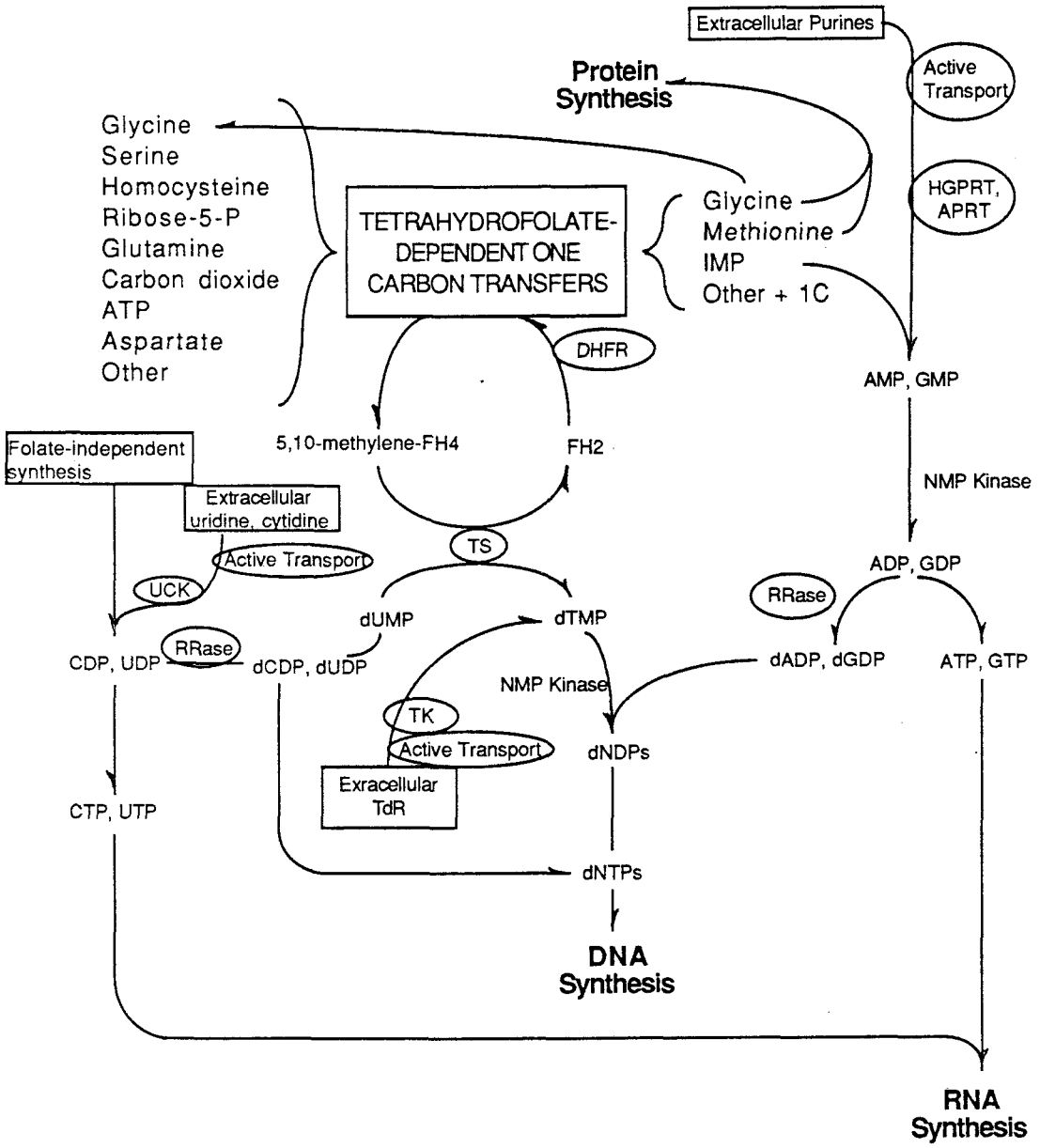
APPENDICIES

APPENDIX A. Nuclear run-on transcription rate determinations



Appendix B. Tetrahydrofolate requirements for DNA, RNA, and protein synthesis



APPENDIX C. Amplified muscle cell lines

Amplified muscle cell lines were generated by serial selection of parental cell line $H^-\alpha$ (Schmidt and Merrill, 1989a; CHAPTERS 2,3) in increasing concentrations of methotrexate (MTX). $H^-\alpha$ is an HGPRT-deficient subline of mouse myoblast line MM14D (Linkhart *et al.,* 1981). $H^-\alpha$ was raised in F10C-growth medium (Ham's F10 supplemented with 0.8 mM $CaCl_2$, 10 U/ml penicillin, 0.1 µg/ml streptomycin sulfate, 15% horse serum, and fibroblast growth factor extracted from bovine brain (Esch *et al.,* 1985, see APPENDIX D). Cells were fed F10C-growth medium every 1-3 days (frequency dependent on cell densities). For drug selection, MTX was added directly to F10C-growth medium. In 1988, after establishment of all amplified lines except $H^-\alpha$R300T (see below), general use of F10C-growth medium in our lab was replaced by F/D-growth medium (0.5X Ham's F10 [vol./vol.], 0.5X Dulbecco's modified Eagle's medium [vol./vol.], supplemented with 0.4 mM $CaCl_2$, 15 mM Hepes, pH 7.2, 1% glucose, 10 U/ml penicillin, 0.1 µg/ml streptomycin sulfate, 15% horse serum, and fibroblast growth factor extracted from bovine brain).

Ham's F10 and possibly serum contain hypoxanthine, thymidine, and glycine. Wildtype cells can use these substrates to make purines, pyrimidines, and proteins by the DHFR-independent salvage pathways. In contrast, because $H^-\alpha$ cells are HGPRT-deficient, they are absolutely dependent on DHFR activity for survival (see APPENDIX B, p. 185, for folate metabolism pathways). Therefore, amplified cells could be selected without first dialyzing medium components and without inhibiting auxiliary metabolic pathways.

Attempts to generate $H^-\alpha$ cells containing amplified copies of the DHFR gene were initiated in early 1986. My first approach was to simply add 100 nM MTX to the medium of growing cells (2-5 x 10^5 cells/10 cm culture dish). In four independent attempts, each including 2-10 culture dishes, no colonies resistant to 100 nM MTX arose.

As a second approach to obtain amplified muscle cells, $H^-\alpha$ cells were transformed with SV2DHFR (Subramani *et al.*, 1981) prior to selection in methotrexate. My rationale was that, by increasing the initial number of active DHFR genes in the starting cell line, one may increase the likelihood of an amplification event. Quadruplicate cultures of 2 x 10^5 cells were transformed by the $CaP0_4$/glycerol-shock method (APPENDIX E) with 10 µg total DNA containing ratios of 1:0, 1:1, 10:1, or 100:1 SV2DHFR:pKNeo. Two days after the glycerol shock, each culture was split into three dishes. One dish from each transformation was selected in 400 µg/ml G418; one dish was selected in 100 nM MTX; and one dish was selected in 300 nM MTX. No colonies arose on any dishes selected in MTX. Colonies arose on all G418-selected dishes that recieved pKNeo (1-20 colonies/dish). Twenty days after transformation, each dish was split into two cultures. One culture was selected in 300 nM MTX, one was maintained in 100 µg/ml G418. All cells died in MTX. G418-selected clones were expanded and frozen (labeled EES7-6-86), but were never used further.

In late 1986, the Schimke group published several reports indicating treatments that cause a transient block in DNA synthesis increase the frequency of amplification events (Johnston *et al.*, 1986; Rice *et al.*, 1986). Therefore, as a third approach, two cultures (2 x 10^5 cells/dish) were pulse-treated for 6 h with 300 nM MTX, rinsed, incubated for 48 h in nonselective medium, and selected in 100 or 500 nM MTX. All cells died in 500 nM MTX; however, several colonies arose in 100 nM MTX. When colonies became large (after about 15 days, 100-300 cells/colony), cultures were split into 1 frozen stock and 3 dishes. Dishes were selected in 100, 200, and 500 nM MTX. No colonies survived at any of the drug concentrations, which suggested that colonies on the parental dish (100 nM) were not stably surviving selection, but rather, overexpressed DHFR sufficiently to persist several extra generations in selective medium. The frozen stock was thawed into medium containing 50 nM MTX and was grown to about 2 x 10^5

cells/dish (about 2 weeks). Cells were split onto 2 dishes and were selected in 200 and 500 nM MTX. No stable colonies arose at 500 nM MTX; colonies surviving at 200 nM MTX were expanded to about 100 cells/colony and were passaged into 1 dish at the same drug concentration. When the cell density was about 3 x 10^5 cells/dish (about 10 days), 1/4 of the cells were passaged onto a dish containing 500 nM MTX and the remainder were frozen in 3 vials (labeled EES12-4-86 H$^-$αMTXR0.2μM). One week later, cells were split onto dishes containing 3, 5, 10, and 20 μM MTX. Nineteen days later, only the 3 and 5 μM dishes had colonies. The cells on the 5 μM dish, however, were very delicate and lysed upon harvesting with collagenase; the 3 μM cells (H$^-$αR3) cells survived the splitting procedure and were passaged onto plates containing 3, 5, 10, 20, and 50 μM MTX. Colonies appeared at 3, 5, and 10 μM. Two weeks after the passage, cells on the 10 μM dish (H$^-$αR10) were split onto dishes containing 10, 20, and 50 μM MTX; cells on other dishes were frozen (labeled E12287TC, H$^-$αR3 and H$^-$αR5). Within 16 days, colonies had grown on all dishes. The 10 and 20 μM cultures were frozen (E2587TC, H$^-$αR10 and H$^-$αR20). The dish at 50 μM was expanded and, after one passage, was split into the experiments in CHAPTER 2 (designated H$^-$αR50A, Schmidt and Merrill, 1989a). Cell lines H$^-$αR5 and H$^-$αR10 were subsequently selected directly in 50 μM MTX to yield lines H$^-$αR50B and H$^-$αR50C, respectively. However, only line A had an acceptable generation time (12 h); lines B and C had 20 and 40 h generation times, respectively.

The growing cultures of H$^-$αR50A were lost to contamination. When frozen vials were removed from liquid nitrogen (only two vials had been frozen) they exploded (as a result of bad silicone tube gaskets, which allowed liquid nitrogen to enter the frozen tubes; upon rapid thawing the gaskets warmed and sealed, trapping the liquid nitrogen, which evaporated and exploded the tubes). Thus, all H$^-$αR50A stocks were lost. Attempts to subclone rapidly growing lines of H$^-$αR50B or C failed to yield any cells with less than a 20 h generation (n = 30, range = 20.2 h to 95.4 h, average = 38 h). I reasoned that the

large selection steps used to obtain cell lines H⁻αR50A, B, and C forced the populations through numerous bottlenecks (at all steps, cells survival yielded only colonial growth), which allowed fixation of a slow-growth phenotype in lines B and C.

The time required to generate the first three amplified cell lines was about 7 months. Three additional months were spent trying to isolate a rapidly dividing subline after the extinction of H⁻αR50A. Due to the poor growth characteristics, all cell lines described in the preceding three paragraphs were discarded.

Rather than attempt further to isolate a rapidly-growing amplifiifed cell line from existing cultures, the amplification process was reinitiated with H⁻α cells. To reduce the likelihood that only slow-growing clones would arise during selection, I decreased the magnitude of selective steps, such that cell lines would not be forced through bottlenecks. Three parallel 10 cm cultures (10^5 cells/dish) were pulsed for 6 h with either 300 nM MTX, 3 mM hydroxyurea, or were left untreated. Pulsed cultures were rinsed twice with growth medium and were fed 10 ml growth medium. After 48 h in nonselective medium, 50 nM MTX was added. After 4 days in 50 nM MTX, cells were passaged into medium containing 100 nM MTX and 1 vial of each cell type was frozen. All three lineages (i.e., MTX-pulsed, hydroxyurea-pulsed, and mock-pulsed) exhibited similar cell survival in 100 nM MTX. After 2 weeks in 100 nM MTX, many cells still appeared viable; however, growth was very slow. To prevent selection of slow-growing clones, one vial of each lineage (frozen after selection in 50 nM MTX) was thawed into 50 nM MTX and grown in 50 nM MTX for 16 additional days (passaged once after 10 days). The selection pedigree of each cell line is exhibited in Fig. 1.

Seven lines survived selection to 50 μM MTX. Each line was assayed for general cell morphology, generation time, DHFR enzyme levels, DHFR Southern blot band intensity, and TK activity levels in proliferative and committed cells (as an indicator of commitment competence). Results are exhibited in Table 1. Cell line H⁻αR50T was the best cell line

in terms of DHFR overexpression, DHFR gene copy, generation time, and commitment competence, and therefore was chosen for the studies presented in CHAPTER 4 (Schmidt and Merrill, 1989b).

Although the cell line (T) chosen for the studies presented in this thesis originally arose from cells treated with a transient hydroxyurea block, it is noteworthy that the hydroxyurea treatment was unnecessary (Fig. 1, Table 1). In contrast to reports based on amplification of the DHFR gene in fibroblastic cells (Johnston *et al.*, 1986; Rice *et al.*, 1986), I observed no evidence that transient inhibition of DNA synthesis increased the frequency at which methotrexate-resistant myoblast clones arose. Rather, the most important features of my successful attempts to amplify the DHFR gene were that selection was initiated at low methotrexate concentrations and drug dosage was increased very gradually.

Cell line H$^-$αR300T was selected from H$^-$αR50T in three steps, using 100, 200, and 300 μM MTX (these selection steps were performed in F/D-growth medium, not F10C-growth medium). Line H$^-$αR300T contains about 800 copies of the DHFR gene (400-fold amplification), and has a generation time of about 14 h. H$^-$αR300T was used in the study presented in CHAPTER 3.

When H$^-$αR300T cells were grown in medium containing 500 μM MTX, cell densities increased very slowly and many floating cells were observed in the medium; however, the culture persisted and slowly expanded for several weeks at this drug concentration. After three passages, several vials of H$^-$αR500T cells were frozen. When H$^-$αR500T cells were thawed and grown at 300 μM MTX, populations expanded rapidly and few floating cells were observed.

A likely but untested explanation for the growth characteristics of H$^-$αR500T is that cells can amplify the DHFR gene only to a certain maximum level. At maximum amplification, DHFR gene copy is sufficent for full survival at 300 μM MTX, but

insufficient for survival at 500 μM MTX. At 500 μM MTX, only daughter cells that received more than 50% of the DHFR genes survive. The hypothesis has two inherent requisites: A) At least some of the amplicons must nonequally segregate (i.e., they must be episomal); and B) even cells that receive sufficient episomal copies of the amplicon to survive selection are incapable of reliably replicating all copies. As a result, only rarely will both daughter cells receive sufficient copies to survive selection.

In view of the above hypothesis, it is interesting to ask whether it is reasonable to propose that the amplified cells are unable to replicate the few extra copies of the DHFR gene required for survival of both daughter cells at each division. Based on the hamster DHFR amplicon (Looney and Hamlin, 1987; Looney *et al.,* 1988), DHFR amplicons contain an average of 300 kilobase pairs of DNA per repeat. In cells like H$^-$αR500T, that contain 800 copies of the DHFR gene, amplicon sequences represent 2.4 x 10^8 base pairs of DNA. Based on an estimate of 2-3 x 10^9 base pairs of DNA per mammalian genome (Lewin, 1987), DHFR amplicons in cell line H$^-$αR500T represent only about 10% of the cellular DNA content. Thus on a per mass basis, the increase in DNA content probably is not sufficient to hinder replication. It is possible, however, that local concentrations of the replicative equipment are insufficient to ensure initiation of replication at origins on all DHFR amplicons.

Fig. AC.1

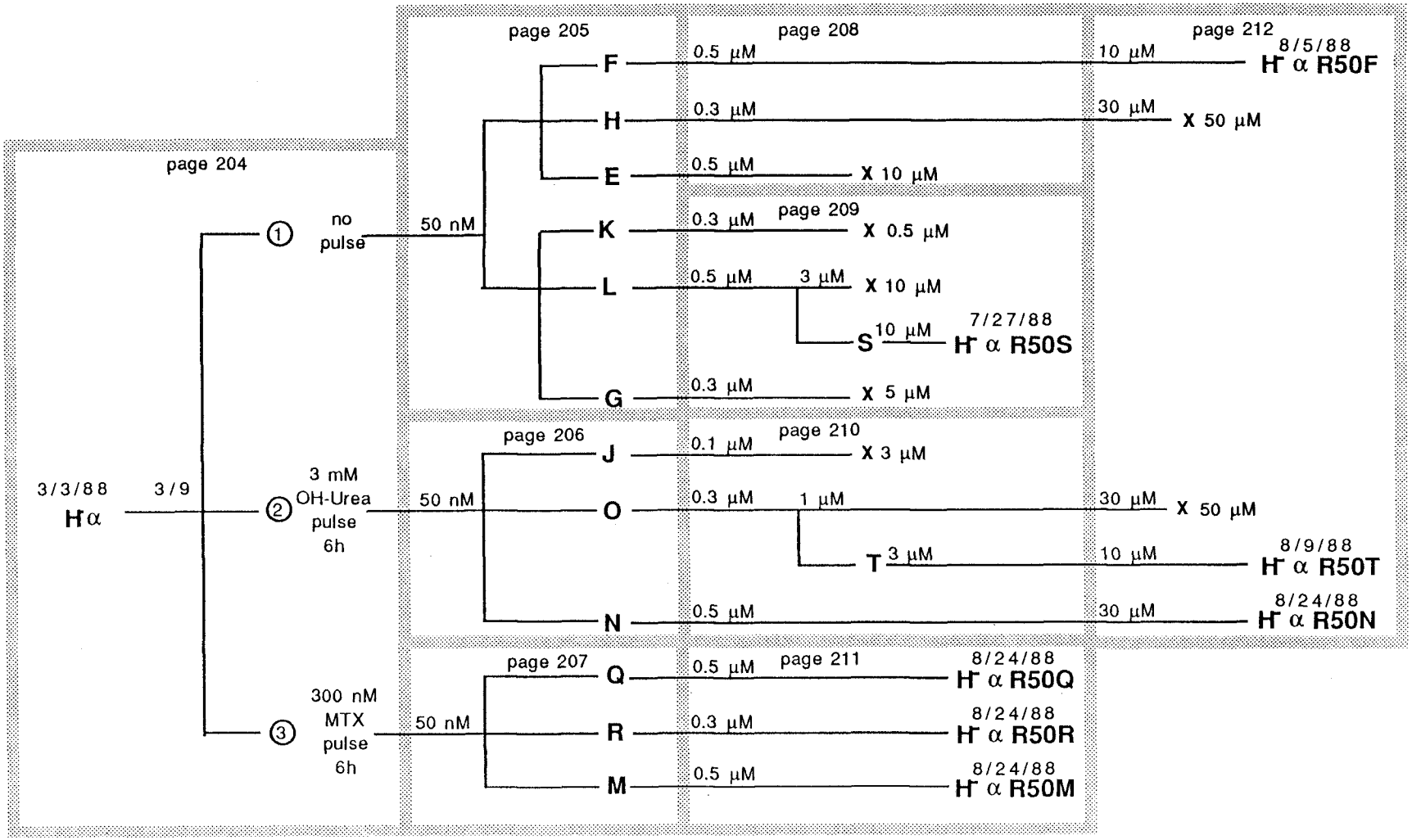

Derivation of amplified myoblast lines
overview
page 204
3/3/88
H-α
3/9
① no pulse
② 3 mM OH-Urea pulse 6h
③ 300 nM MTX pulse 6h
50 nM
page 205
page 206
page 207
page 208
page 209
page 210
page 211
page 212
F
H
E
K
L
S
G
J
O
T
N
Q
R
M
0.5 μM
0.3 μM
0.1 μM
1 μM
3 μM
10 μM
30 μM
X 10 μM
X 0.5 μM
X 5 μM
X 3 μM
X 50 μM
8/5/88
H- α R50F
7/27/88
H- α R50S
8/9/88
H- α R50T
8/24/88
H- α R50N
8/24/88
H- α R50Q
8/24/88
H- α R50R
8/24/88
H- α R50M

Fig. AC.1 (cont'd).

Derivation of amplified myoblast lines

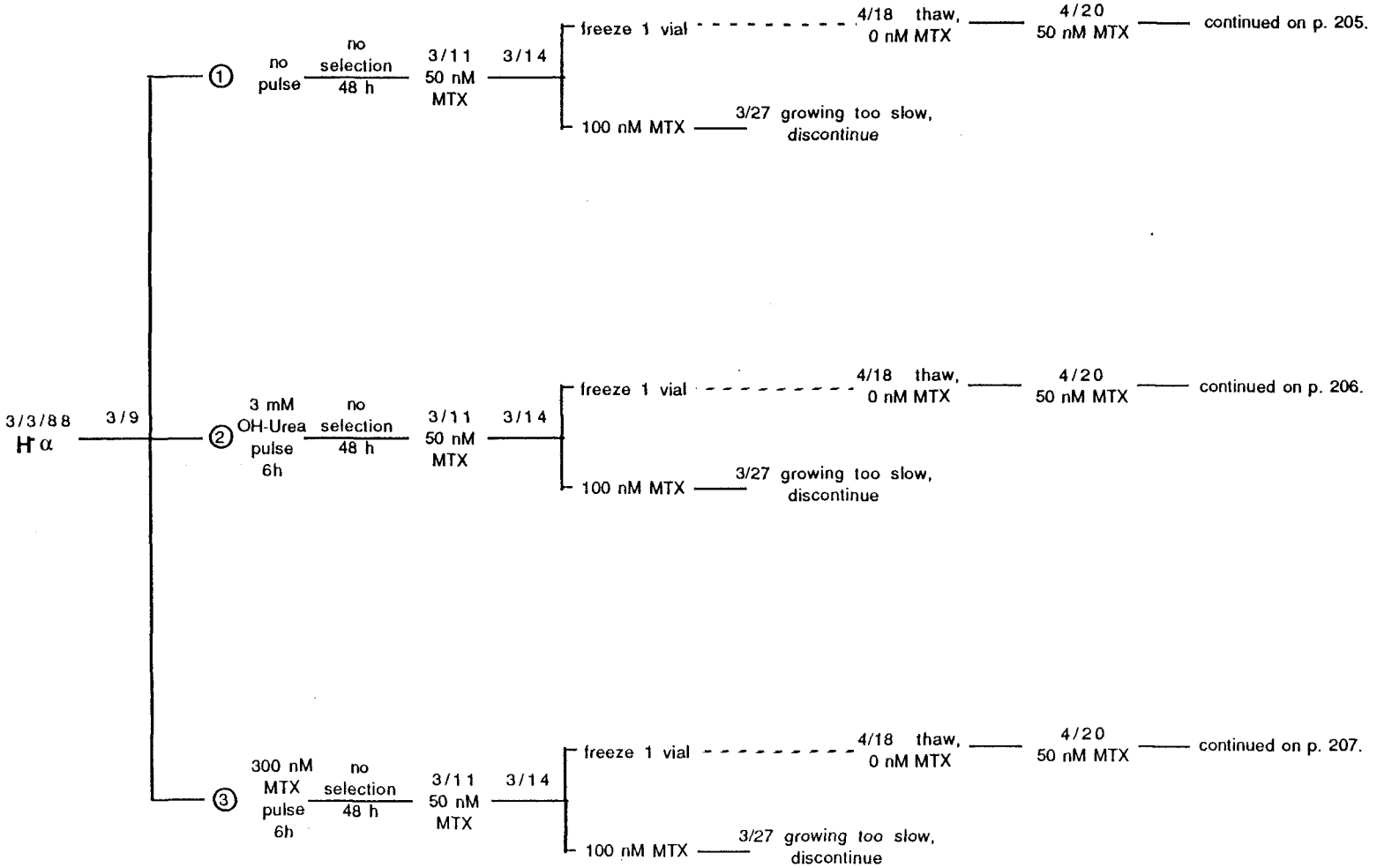
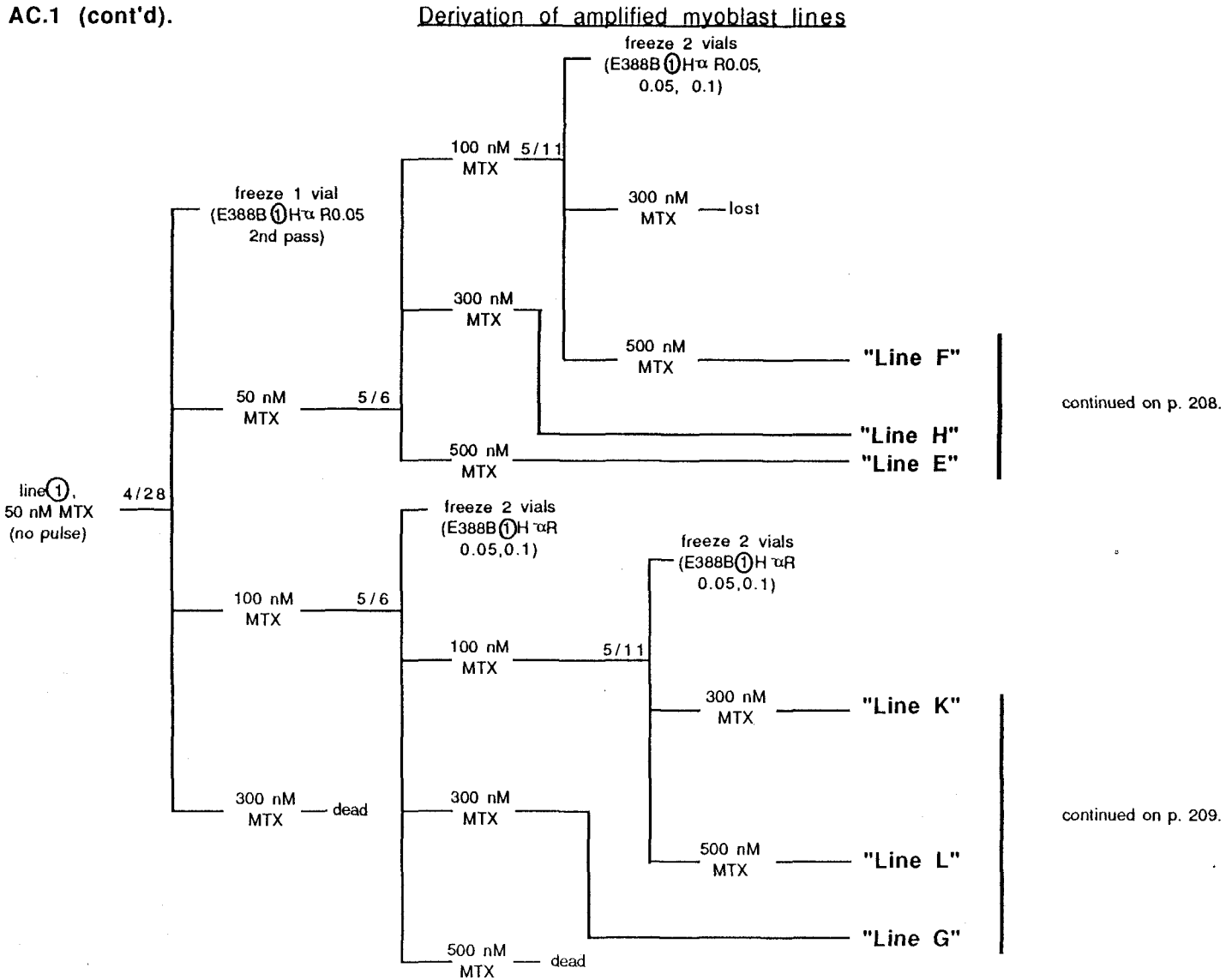


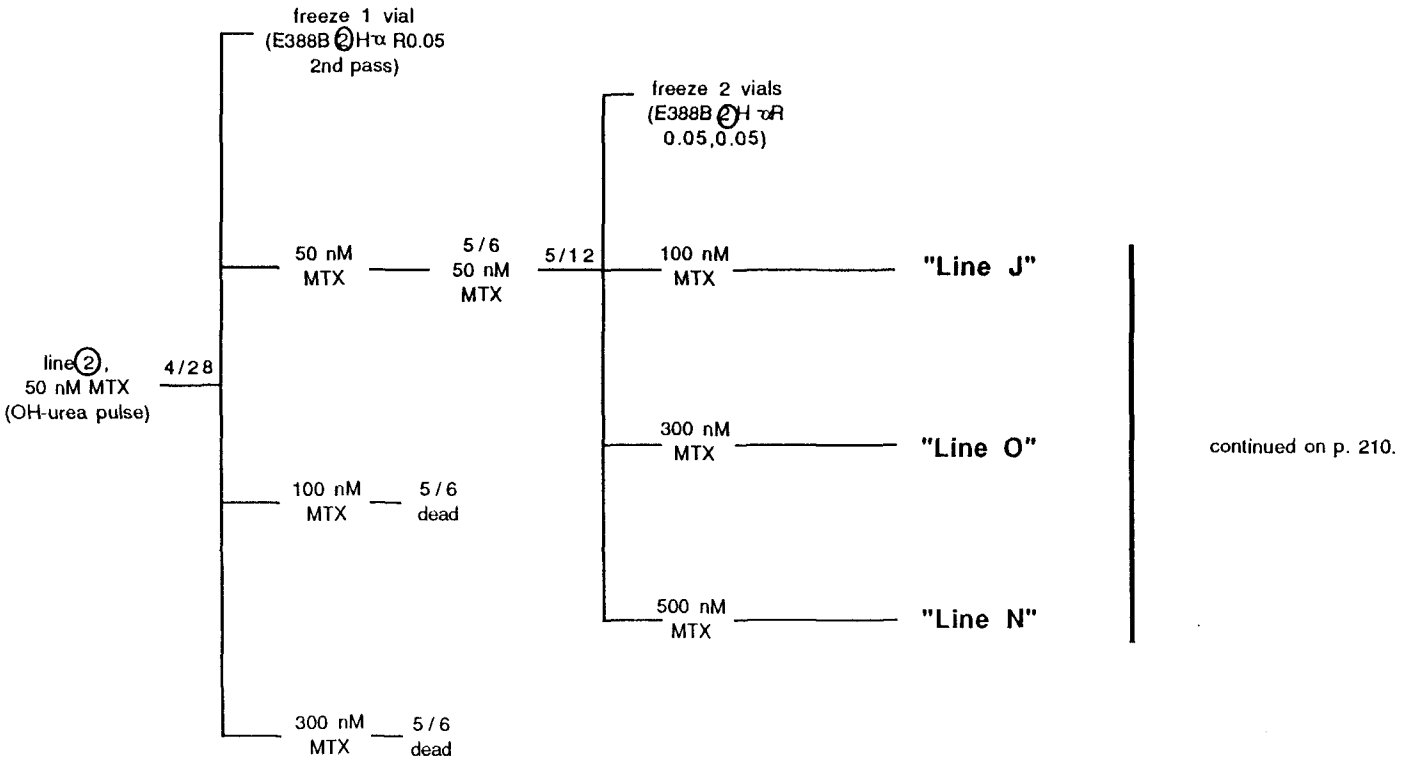
Fig. AC.1 (cont'd).



continued on p. 208.

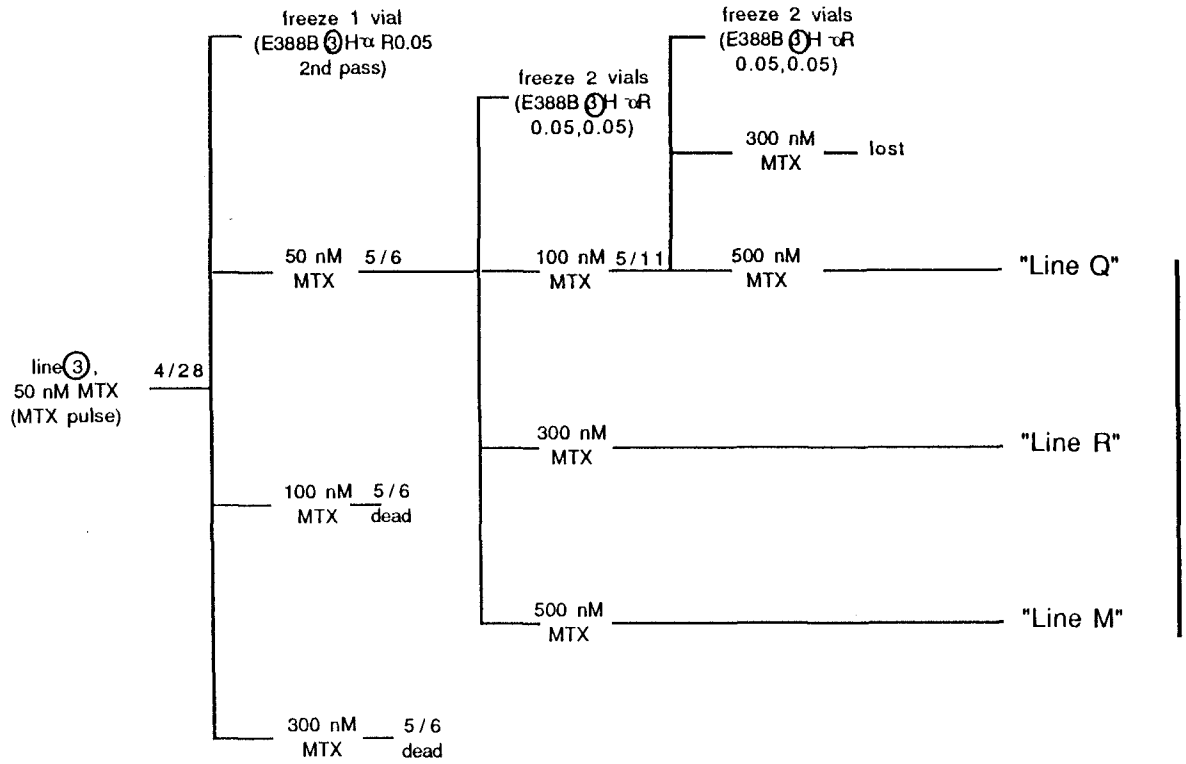
continued on p. 209.

Fig. AC.1 (cont'd). Derivation of amplified myoblast lines



continued on p. 210.

Fig. AC.1 (cont'd). Derivation of amplified myoblast lines



continued on p. 211.

Fig. AC.1 (cont'd). Derivation of amplified myoblast lines

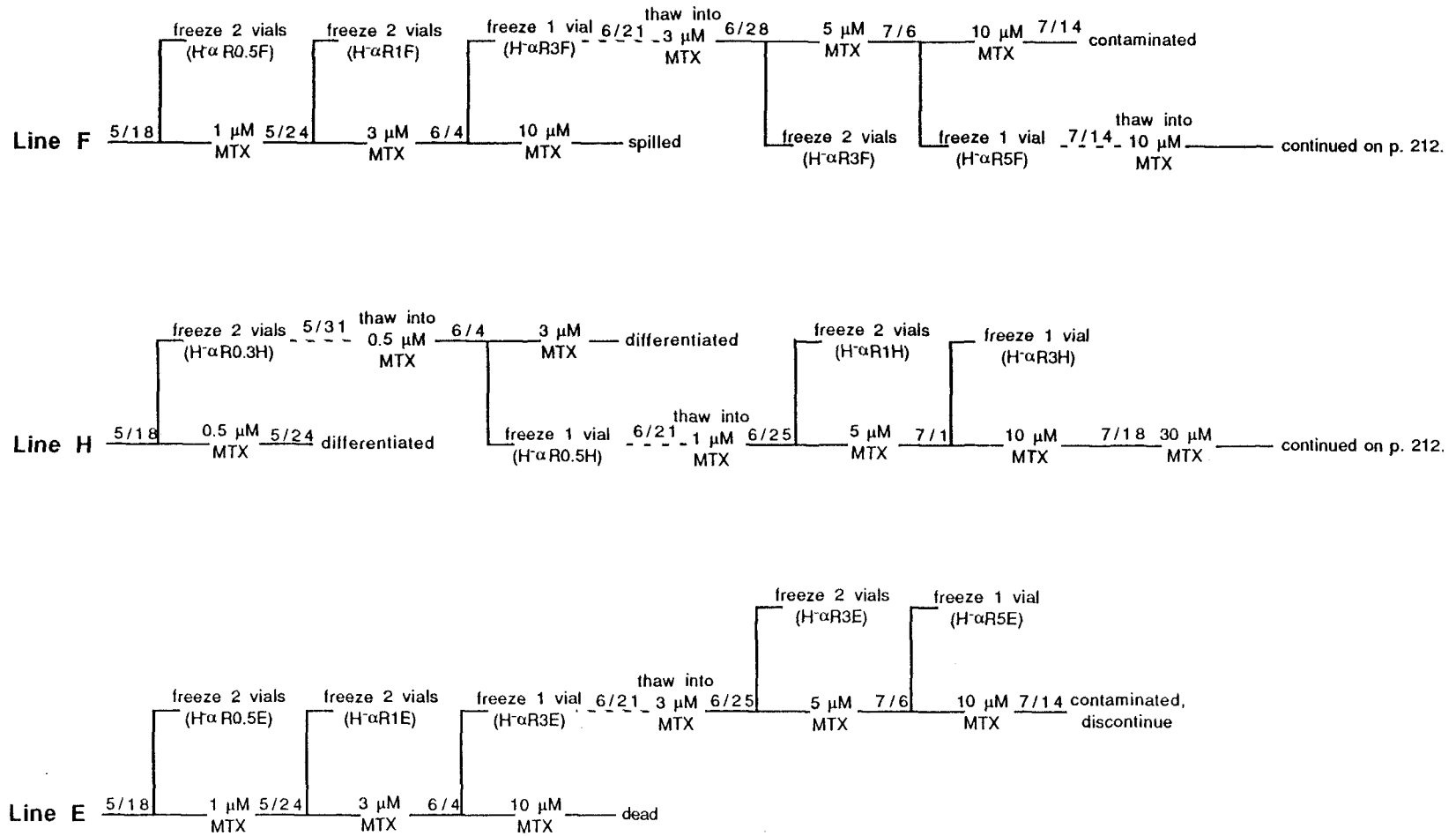


Fig. AC.1 (cont'd). Derivation of amplified myoblast lines

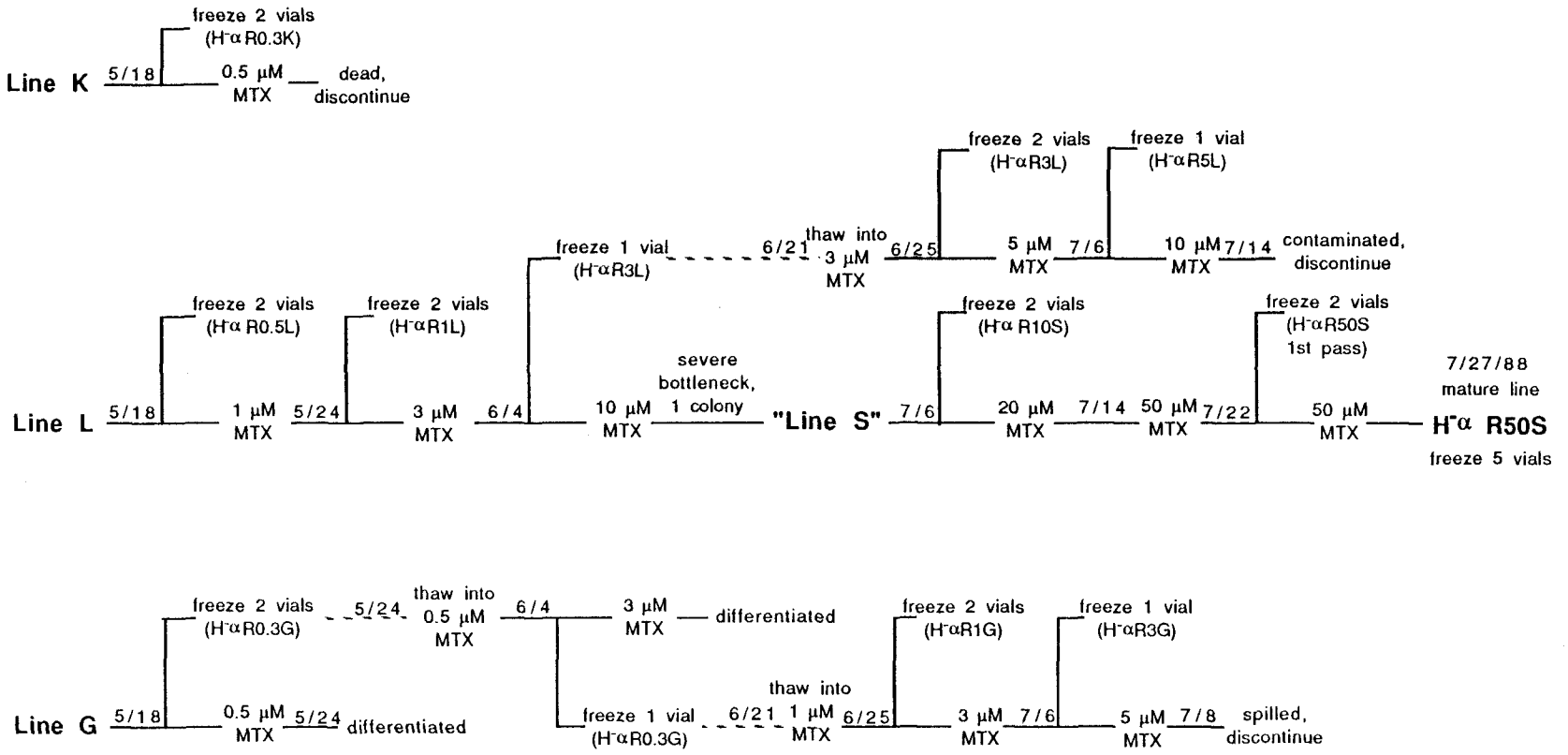


Fig. AC.1 (cont'd). Derivation of amplified myoblast lines

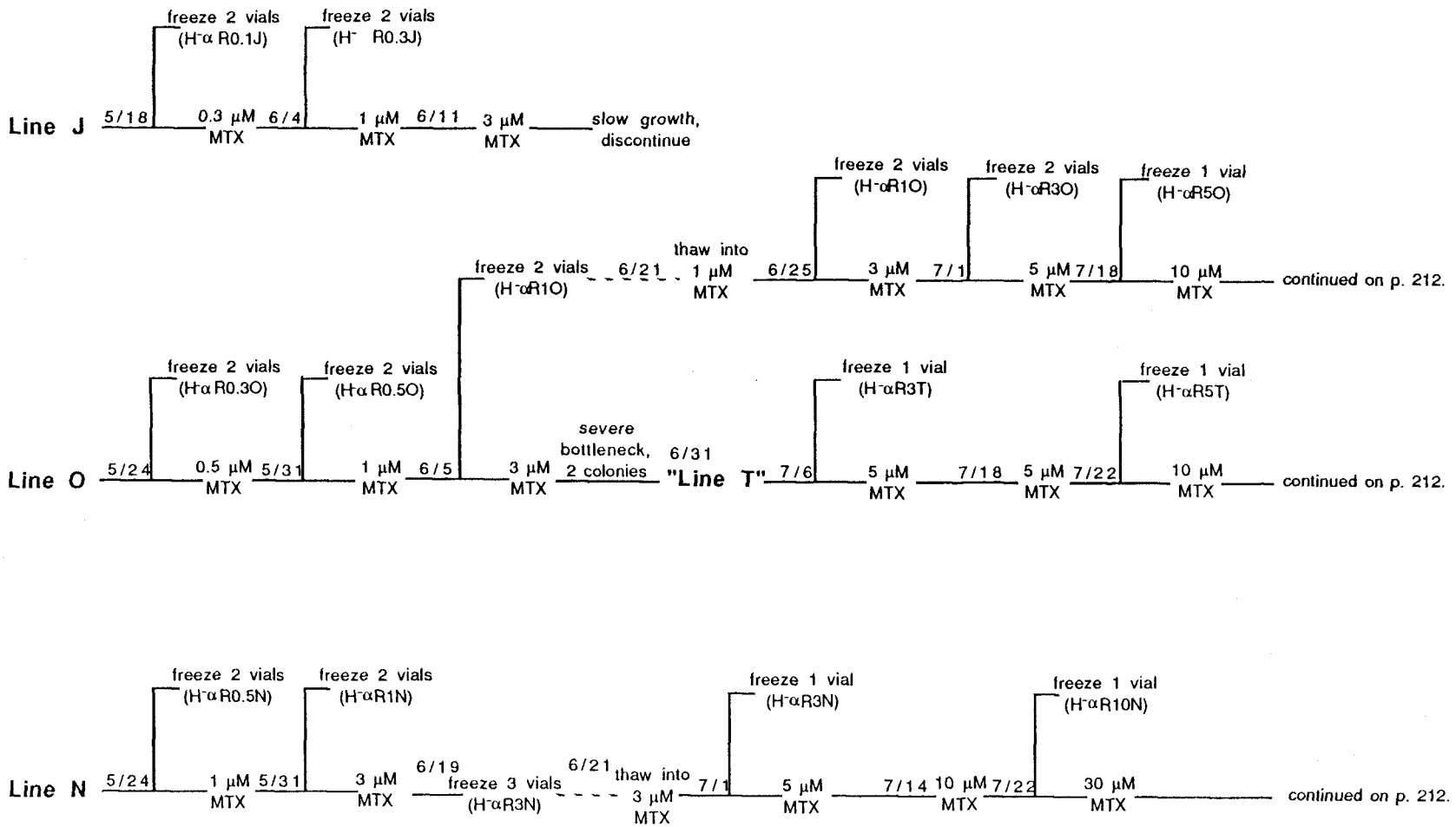


Fig. AC.1 (cont'd). Derivation of amplified myoblast lines

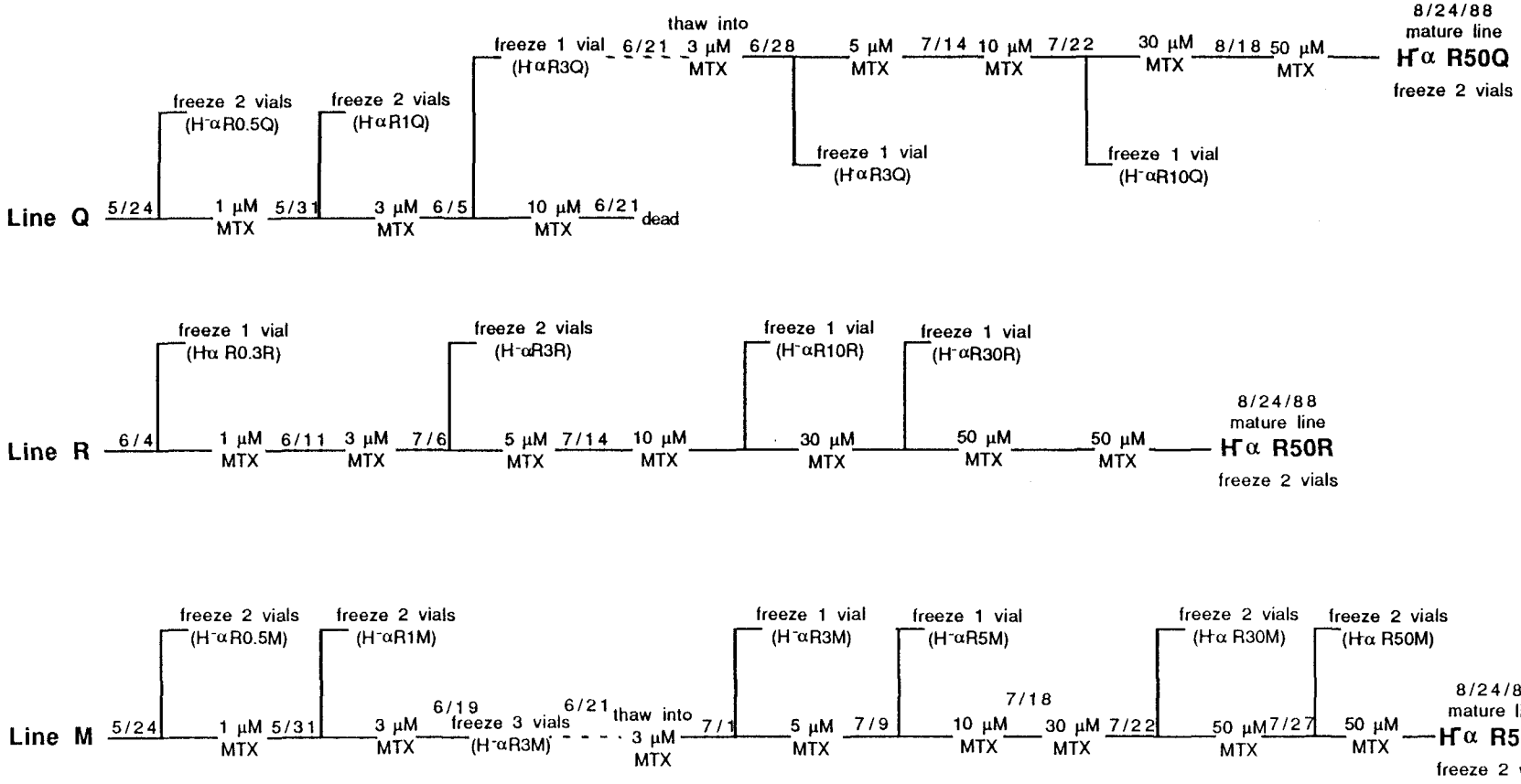


Fig. AC.1 (cont'd). Derivation of amplified myoblast lines

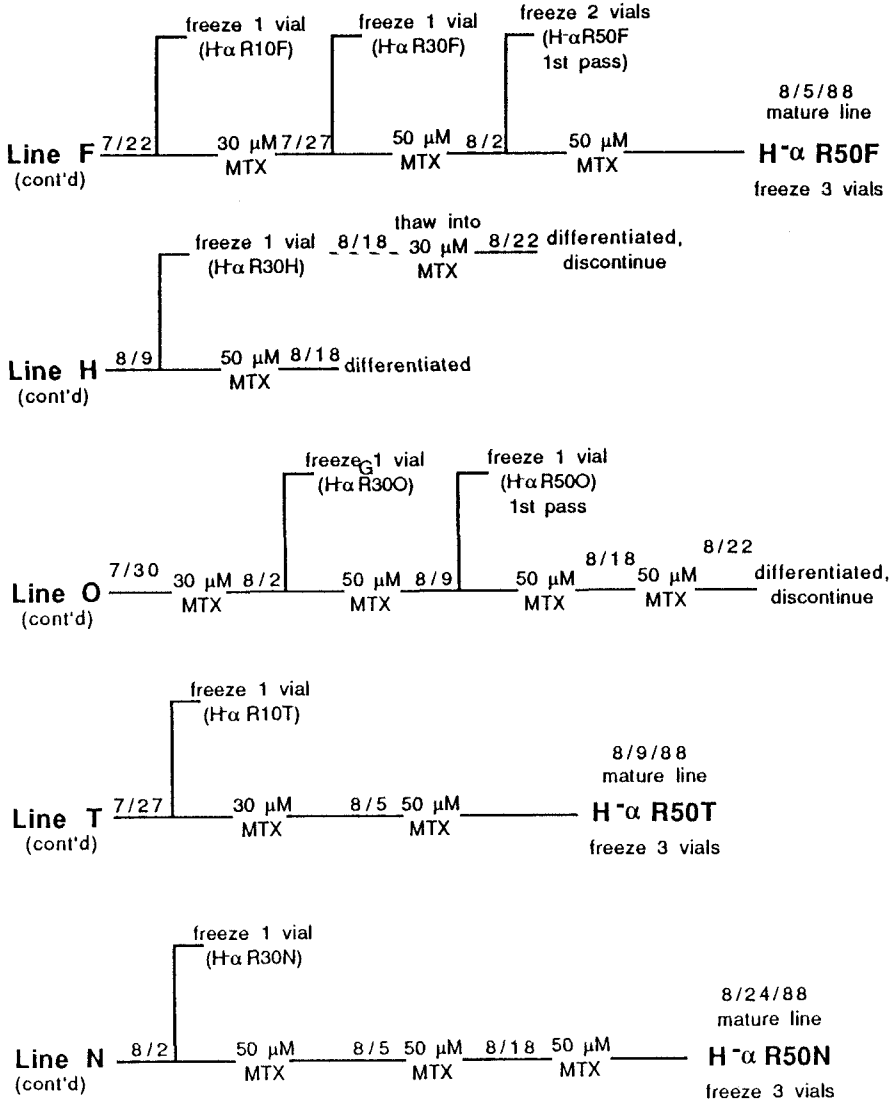


Table AC.1. Properties of amplified cell lines

cell line : HαR50-	F	M	N	Q	R	S	T
initial treatment	no pulse	MTX pulse	HO-urea pulse	MTX pulse	MTX pulse	no pulse	HO-urea pulse
cell morphology	myoblastic	myoblastic	fibroblastic[1]	myoblastic	myoblastic[2]	myoblastic	myoblastic
approx. generation	14 h	21 h	16 h	18 h	16 h	14 h	12 h
MTX binding[3] (molec/cell)	1.58×10^6	0.23×10^6	1.39×10^6	0.03×10^6	1.77×10^6	2.19×10^6	4.04×10^6
relative Southern band intensity	4[4]	2	1	1	3	4[4]	3
proliferative TK activity (pm/min/µg DNA)	0.41	0.09	0.88	0.42	0.35	0.38	1.26
committed TK activity (pm/min/µg DNA)	0.15	0.16	0.02	0.00	0.00	0.23	0.00

[1] Line N cells look like 3T6 cells with long dendrite-like outgrowths.

[2] Line R cells look myoblastic at low densities, at high densities colonies become three-dimentional "tumors."

[3] MTX-binding assayed after 21.5 h in drug-free medium.

[4] Lanes F and S on ethidium bromide-stained gel appeared slightly overloaded.

APPENDIX D. Crude bovine fibroblast growth factor preparation

Fibroblast growth factor activity was extracted from bovine brain by following the procedures of Esch *et al.* (1985) through the ammonium sulfate precipitations. The crude extract was affectionately named "brain smeg."

1. Perform all manipulations on ice or at 4°C; use glass-distilled water (GDW) for all solutions. Remove blood clots from 5 kg (final) fresh bovine brain (don't use frozen brains).
2. Grind brains 5 min in 4 liters 0.15 M $(NH_4)_2SO_4$/kg tissue. Transfer to plastic buckets; stir; adjust pH to 4.5 with 10 N HCl. Stir vigorously 2 h.
3. Centrifuge 30 min, JS4.2 rotors in Beckman J6B centrifuges, 4200 rpm.
4. Remove supernatant; measure volume; add 230 g $(NH_4)_2SO_4$/liter supernatant. Adjust pH to 6.0-6.5 with 10 N HCL; stir vigorously overnight (>15 h).
5. Centrifuge as above; remove supernatant; measure volume; add 300 g $(NH_4)_2SO_4$/liter supernatant. Stir vigorously 2 h.
6. Centrifuge as above; discard supernatant; resuspend pellets in a total of about 500 ml GDW. Transfer to dialysis bags leaving enough room so that after swelling, volume will be about 2 liters. Dialyze 3X (> 3 h each) against 20-30 liters GDW.
7. Pool dialysates and clarify by centrifugation in Sorvall GSA rotor, 7000 rpm, 30 min. Further clarify by centrifugation for 1 h at 26,000 rpm in Beckman type 30 ultracentrifuge rotor. Discard pellets.
8. Filter sterilize; determine FGF activity empirically by measuring thymidine incorporation by $H^-\alpha$ cells after 3 days growth. Brain smeg preparations are generally maximally active when added to medium at 0.5%-3.0%.

APPENDIX E. Myoblast transformation

The myoblast transformation procedure used for the studies in CHAPTER 5 was developed by C. Clegg in S. Hauschka's lab. The method was modified and passed as folklore by G. Merrill. Additional changes undoubtably arose during transfer of the information to me.

1. One day prior to transformation, split actively proliferating populations of $H^-\alpha$ myoblasts onto 6 cm dishes at densities of 2.5-5.0 x 10^5 cells/dish.
2. Replace F/D-growth medium three to five hours before transformation.
3. Mix test gene plasmid (3-10 µg, all give similar numbers of transformed colonies) with 0.1-0.3 µg pKNeo and adjust vol to 250 µl with GDW.
4. Add 250 µl 2X HBS.

2X HBS	for 250 ml
272 mM NaCl	4.0 g
10 mM KCl	0.185 g
1.4 mM NaH_2PO_4	0.050 g
11 mM glucose	0.50 g
21 mM Hepes,	2.50 g
pH 7.05 @ 25 ^oC.	Filter sterilize.

5. Use a baked/plugged Pasteur pipet in the pipet pump to continuously bubble (i.e., mix) the solution while slowly adding 31 µl 2M $CaPO_4$.
6. Incubate at room temp 45-60 min to allow precipitate to form.
7. Aspirate medium from dishes; however, take only what comes fast (about 95%). Add DNA precipitate and distribute by rocking.
8. Incubate at room temp 20 min; rock occasionally.
9. Add 3 ml F/D-growth medium and place in incubator 4 h.

10. Aspirate medium, rinse once with warm serum-free F/D, aspirate completely.
11. Add 1 ml of a mixture of 8.5 volumes 1X HBS to 1.5 volumes glycerol. Rock dishes to distribute.
12. Incubate at room temp 4 min (note: if numerous dishes are being transformed, glycerol-shock in sets of 5-10 to maintain 4 min incubation period).
13. Aspirate glycerol solution completely, rinse once with warm serum-free F/D, aspirate completely, add 3 ml F/D-growth medium, and return cultures to incubator.
14. One day later, replace F/D-growth medium.
15. One day later, passage culture onto one 10 cm dish in F/D-growth medium + 400 µg/ml G418 (selective medium).
16. Replace selective medium every day until the drug has substantially reduced cell densities (about 4 d), then switch to 3 d feeding schedule. Colonies begin to appear 5-7 days after initiating selection.
17. As individual colonies become large, feed more frequently or cells will differentiate. When colonies contain 100-300 cells, harvest a few clones in cloning cylinder and then pool the remaining colonies as a polyclonal population.
18. Expand clones and polyclonal populations at least once on 6 cm dishes before freezing or splitting into an experiment.

APPENDIX F. RNase protection assay

I. Hybridization:

A. Set up samples containing experimental or standard curve RNA, yeast RNA and probe[1]. Cap tubes loosely, leaving a small visible crack. Freeze >10 min -80°C in lyophilization jar. Cap jar and lyophilize >2 hr (1.5 hr if all samples <25 µl; 1 hr if all samples <15 µl).

B. Add 30 µl hybridization mix; close tubes; vortex to wash all surfaces of tubes; spin down 2 sec, 55°C 13-24 hr.

II. RNase digestion:

A. Remove tubes from 55°C water bath; centrifuge 10 sec; add 300 µl 0.3X RNase mix per tube[2]. Vortex, spin down 2 sec, 37°C 1 hr; proceed either to step IIIa or step IIIb.

IIIa. Gel work-up: (Always run at least one gel for each newly synthesized probe or when fresh batches of enzymes are employed to be sure probe is intact, template is degraded, RNase digestion conditions are correct, etc.).

A. Terminate RNase digestions by adding 20 µl PK mix, 37°C 15 min.

B. Extract with phenol/chloroform, extract with chloroform, precipitate with ethanol, 70% wash.

C. Resuspend in 2.0 µl GDW; add 8.0 µl DF dye mix (99% formamide, 0.0033% bromphenol blue, 0.0066% xylene cyanol), vortex, spin down, 75°C heating block 10-15 min, load aliquot on gel while still hot[3] (carry heating block into warm room to load gel). Remaining aliquot of samples can be stored at -20°C.

D. (Optional.) To remaining aliquot, add 80 µl GDW, 20 µl 10% NaPPi (wt/vol), 100 µl 10% TCA (trichloroacetic acid, wt/vol). Incubate on ice >30 min 0°C. Either:

1. Centrifuge tubes 15 min, 4°C; aspirate supernatant; add 100 µl 5% TCA; vortex well to wash walls and cap; centrifuge 5 min; aspirate; add 100 µl 5% TCA; vortex; centrifuge 5 min; aspirate to completion. Add 25 µl 1 M Tris, pH 8.0 @ 25°C, 75 µl GDW; vortex; spin down; 65°C 5-10 min; spin down; transfer to scintillation tubes containing 1.0 ml scintillation juice (333 ml Triton X-100, 667 ml toluene, 4 g Omnifluor); count 50 min [^{32}P] channel; or

2. Set a 1 cm GFC filter disc on the grate of a porcelain vacuum funnel. Pre-wet disc with about 1 ml 5% TCA, 1% NaPPi; load sample on GFC filter; wash tube with 200µl 5% TCA, 1% NaPPi; wash filter 3X with 3 ml ice cold 5% TCA, 1% NaPPi; count discs as above.

IIIb. Rapid liquid scintillation assay: (No gel).

A. Terminate RNase digestions by adding 60 µl 10% NaPPi, 400 µl 10% TCA, ice >30 min or 4°C overnight.

B. Set a 1 cm GFC filter disc on the grate of a porcelain vacuum funnel; pre-wet disc with about 1 ml 5% TCA, 1% NaPPi.

C. Load sample on GFC filter disc; wash tube contents with 200µl 5% TCA, 1% NaPPi; wash filter 3X with 3 ml ice cold 5% TCA, 1% NaPPi; count discs as above.

0.3X RNase mix

1X RD

10 µg/ml RNase A

0.35 U/µl RNase T1

1X RD (RNase digestion buffer)

10 mM Tris, pH 7.5 @ 25°C

5 mM EDTA

300 mM NaCl

PK mix

10X TES

1 µg/µl proteinase K

0.5 µg/µl Yeast RNA

10X TES

10 mM Tris, pH 7.5 @ 25°C

50 mM EDTA

10% SDS

ENDNOTES

[1]Probe control tube receives 0.5 fmole probe, 10 µg yeast RNA. Other tubes receive 5-25 fmole probe, appropriate RNA samples, and yeast RNA to 50 µg. Using more probe increases hybridization efficiency; however, certain probes (viz., the DHFR 5' probe, CHAPTERS 3,4) give substantial background when more than 5 fmole is used. Always include a control containing only probe and yeast RNA.

[2]For CAT and DHFR probes use 0.3X RNase mix; for TK "179" use 0.6-1.0X RNase mix. Probe control samples receive no RNase. Rather, 300µl 1X RD is added.

[3]Gel preparation protocol: To make a 9% polyacrylamide/urea gel, mix equal volumes of (18% polyacrylamide, 1% bis acrylamide, 0.5X TBE, 8 M Urea) and (0.5X TBE, 8 M urea [filtered solution]). Add 1 mg fresh ammonium persulfate (APS)/ml gel (20 well gel = 50 ml; 40 well gel = 100 ml). Mix until APS is dissolved, add 1µl TEMED/ml gel, mix rapidly, and pour gel. Remove tape from the bottom of the gel, remove comb, wash gel, put gel on rig, seal between tape and gel spacers with agarose, add 0.5X TBE to reservoirs, and hose out wells. For narrow (20-well) gels, pre-run overnight at 5 watts or 2-4 hr at 20 watts. Pre-warm gels 30 min at 20 watts, hose out wells, and load samples from heating block. Run gel at 15 watts (20 watts works, but gel smiles). For wide (40-well) gels, double wattage at all steps. Dry gel 4 hr, 80°C using house vacuum, or 1.5 hr, 80°C using water aspirator vacuum (preferred during the day when house vacuum is unreliable). Don't break vacuum until dryer has cooled completely. Expose gel to X-ray film.

APPENDIX G. Nuclear run-on assay

I. Preparation of nuclei.

1. Perform all manipulations on ice; make all solutions in glass-distilled water (GDW). Rinse 10 cm plates (1-10 x 10^6 cells/dish) with 5 ml phosphate-buffered saline (PBS). Scrape cells twice in 1 ml PBS/dish and collect in 15 ml conical tube.
2. Centrifuge cells in IEC tissue culture centrifuge, 500 rpm, 10 min. Draw off supernatant completely; disperse pellet by vortexing gently. Resuspend in a final volume of 5 ml RSB. Vortex gently 10 s; ice 5 min; vortex gently 10 s; check with phase contrast microscope to ensure lysis.
3. Centrifuge nuclei in IEC centrifuge at maximum speed (about 2200 rpm), 20 min. Draw off supernatant completely and disperse pellet by vortexing. Resuspend in 1 ml NFB per 20 plates of cells; vortex. Remove 10 µl and add to a tube containing 90 µl 1X TES; boil 5 min; sonicate 5 s; measure absorbance at 260 nm (1 OD = 50 µg/ml).
4. Adjust concentration to 3 mg/ml nucleic acid with NFB. Use fresh or freeze aliquots at -80°C.

RSB

10 mM Tris-HCl, pH. 7.5@25°C

10 mM NaCl

5 mM $MgCl_2$

10X TES

10 mM Tris, pH 7.5 @ 25°C

50 mM EDTA

10% SDS

NFB (nuclei freezing buffer)

50 mM tris-HCl, pH. 8.3 @25°C

5 mM $MgCl_2$

0.1 mM EDTA

40% glycerol

II. Nuclear run-on reaction

1. Assemble the following on ice:

   30% glycerol (include the contribution from NFB)

   2.5 mM DTT

   1-3 mM $MgCl_2$ (include the contribution from NFB)

   70 mM KCl

   0.25 U/µl RNasin

   400µM each of rATP, rCTP, rGTP

   100-250 µCi $\alpha[^{32}P]$UTP

   GDW to give final volume = 100 µl (include volume of nuclei).

2. Add 100 µg nuclei; triturate gently; ice 10 min; 26°C 13 min.
3. Stop reaction with 13 µl 10X TES, vortex. Add 10 µl 20 mg/ml proteinase K; vortex; 55°C 20 min.
4. Add NaCl to 250 mM, EDTA to 20 mM; extract with phenol/chloroform and chloroform; precipitate in ethanol.
5. Collect pellet by centrifugation; wash in 70 % ethanol; resuspend in 400 µl DNase mix; vortex well; incubate at 37°C 1 hour (vortex occasionally to break up remaining pellet).
6. Add 40 µl 10X TES, 100 µg proteinase K; 55°C 10 min.
7. Add NaCl to 250 mM, EDTA to 20 mM. Extract with phenol/chloroform and

chloroform; precipitate in ethanol.

8. Resuspend in 80 µl GDW; add 20 µl 10% NaPPi, 100 µl 10% TCA; ice 30 min.
9. Collect pellet by centrifugation. Wash with 5% TCA + 0.5% NaPPi until no extractable counts remain.
10. Resuspend in 400 µl GDW. Assay 2 µl by liquid scintillation. Add NaCl to 250 mM; precipitate in ethanol. Resuspend RNA and hybridize to immobilized probe (see CHAPTER 4).

DNase mix:

1 X DNase buffer

10 u/reaction RQ1 DNase

0.25 u/µl RNasin

5 mM DTT

50 µg/reaction yeast RNA.

10X DNase Buffer

200 mM Hepes, pH 7.0 @ 25^{o}C

10 mM $CaCl_2$

50 mM $MgCl_2$

10 mM $MnCl_2$

APPENDIX H: Table AH.1. Synthetic RNA probes and standards

transcript	description	template (reference)	linearization, transcription	comments
DHFR 5' probe (Taq1)	110-base complementary cDNA probe containing 3' 81 bases of exon 1 and 5' 15 bases of exon 2	p4D200 (T)[1] (EES5-22-88)	Taq1 SP6	0.3X RNase,[2] 3-5 fmole/rxn[3] (larger probe excess causes high background)
DHFR intron 2 probe	161-base complementary probe containing 136-base BamH1/ Hind3 fragment from intron 2	p3D136 (EES10-5-88 EES1-13-89)	EcoR1 SP6	0.3X RNase, 3-5 fmole/rxn pre-mRNA expressed at 1/50 level of mRNA
DHFR intron 2 probe (Alternate)	141-base complementary probe containing 136-base BamH1/ Hind3 fragment from intron 2	p3D3000 (EES10-5-88)	Bgl2 SP6	same as above
DHFR 3' probe (Dde1)	172-base complementary probe containing 141 bases of DHFR sequence entirely within exon 6 (spanning stop codon)	p4D750[1] (EES12-24-85, EES10-1-86)	Dde1 SP6	0.3X RNase, 3-100 fmole/rxn (no background even at large molar excess of probe); standard curve 161-bases, not 141
DHFR cap site probe	132-base probe complementary to sequences extending from -29 to +60 from cap site	p3D(-850/+465) (EES10-9-89, EES7-16-90)	Rsa1 SP6	With 0.3X RNase and 25 fmole/ rxn, background obscured signal; may work with 3 fmole/rxn (see EES7-17-90)

Table AH.1 (cont'd). Synthetic RNA probes and standards (cont'd)

transcript	description	template (reference)	linearization, transcription	comments
DHFR Ψ-mRNA	1600-base colinear cDNA transcript extending from about 50-bases upstream of cap to 78-bases downstream of UAA	p4D750[1] (EES12-24-85, EES8-10-87)	EcoR1 T7	20-bases of vector sequence at 3' end hybridize to identical sequences in DHFR 3' probe
DHFR Ψ-pre-mRNA	3000-base colinear genomic transcript extending from 850 bases upstream of cap site to an EcoR1 site near the 3' end of intron 2	p3D3000 (EES11-16-88, EES12-21-88)	EcoR1 T7	Hybridizes to the DHFR 5' probe, the DHFR intron 2 probe, and the DHFR cap site probe
CAT 5' probe	165-base probe complementary to 148 bases at the 5' end of the CAT cistron from Hind3 to Pvu2	p4CAT148[1] (EES9-18-89, GJ201)	Hind3 SP6	0.3X RNase, 3-100 fmole/ rxn; no background, very clean bands
CAT Ψ-mRNA	1652-base colinear transcript containing entire CAT cistron from SV2CAT	p3CAT1632 (EES10-13-89, EES12-23-89)	BamH1, SP6	

[1] p4D200⊘ = "p4D200;" p4CAT148 = "160-bp CAT H3/Pvu2 in pGEM" (GJ210) and "p4CAT160;" p4D750 = "p4D735."

[2] RNase concentrations refer to conditions given in APPENDIX F.

[3] Probe concentrations refer to the amount of probe to include in each hybridization reaction. Differences are based on the amount of intrinsic background arising from each probe. When background will permit, increased probe excess increases hybridization efficiency and signal strength.