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The E2F consensus site is found within the promoters of several genes that are preferentially expressed in replicating cells. An E2F site is located at the transcription start of the dihydrofolate reductase (DHFR) gene promoter and is required for growth-dependent DHFR promoter regulation in serum-stimulated quiescent fibroblasts. In this dissertation, the importance of the E2F site in repressing DHFR promoter activity in postreplicative muscle cells was investigated.

During myogenic withdrawal from the cell cycle, we showed that the five-fold decrease in DHFR promoter activity was dependent on an intact E2F site. In transient expression assays, the E2F site-binding transcription factors DP1 and E2F1 derepressed DHFR promoter activity in differentiated muscle cells. DP1 and E2F1 mRNA levels did not change significantly during myogenic withdrawal from the cell cycle, suggesting that DP1 and E2F1 activity was not regulated transcriptionally. To investigate possible posttranslational modifications of DP1 and E2F1 activity, an *in vitro* band shift assay was used. Neither stimulatory E2F1/DP1-like dimeric nor inhibitory E2F1/DP1/Rb-like multimeric E2F site binding activity changed significantly during myoblast cell cycle withdrawal. If repression of DHFR promoter activity is due to changes in the relative abundance

of stimulatory and inhibitory E2F site binding activity, the changes were either too subtle to detect or were not preserved using *in vitro* band shift assays.

E1a proteins transactivate E2F site-containing promoters by displacing inhibitory Rb-like proteins from activating E2F1/DP1 dimeric transcription factors. To investigate whether Rb-like proteins are involved in the repression of DHFR promoter activity during myogenesis, a DHFR promoter/reporter gene was cotransformed with a plasmid encoding the adenovirus E1a oncoprotein. In contrast to control transformants, reporter gene expression did not decrease in E1a transformants induced to withdraw from the cell cycle. E1a induced derepression of DHFR promoter activity was dependent on an intact E2F site and was not a consequence of a failure of E1a transformants to withdraw from the cell cycle. The absolute and relative abundance of dimeric and multimeric E2F site-binding activity was unaltered in E1a transformants. Surprisingly, the E2F site -containing endogenous DHFR, TK and TS genes continued to show strong regulation in E1a transformants, suggesting that additional factors, that act outside of the DHFR promoter region, serve a redundant regulatory role in repressing DHFR gene expression in postreplicative muscle cells.

# E2F Site-Dependent Regulation of *Dihydrofolate Reductase* Promoter Activity During Myogenesis

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Kurt Schesser, Author

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## Chapter 1

## Introduction

The elucidation of the mechanisms controlling the relative activities of gene expression during growth and development has been one of the primary aims of biological research in the latter half of this century. Recombinant DNA technology developed in the last twenty years has made possible the identification of gene sequences that are critical for regulated gene expression. The paradigm that has emerged is that specific DNA sequences are recognized by transcription factors that determine whether or not a particular gene is expressed. Major effort is currently being made to understand the molecular mechanisms by which regulatory transcription factors activate or repress gene expression and how the transcription factors themselves are regulated.

The research described herein is focused on the regulation of a gene that is preferentially expressed in growing cells. The enzyme dihydrofolate reductase (DHFR) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. Tetrahydrofolate serves as an intermediate in a number of one-carbon transfer reactions that are required for the *de novo* synthesis of glycine, purines, and thymidine monophosphate. The demand for DHFR enzymatic activity is greatest in replicating cells and indeed this enzyme was one of the first to be demonstrated to be preferentially synthesized in mitotically-growing cells (Alt et al., 1976). The regulation of *DHFR* gene expression has likewise been shown to occur in a number of cell lines in which growth status can be experimentally manipulated. Regulation of the *DHFR* gene has

emerged as a model system for studies aimed at elucidating the mechanisms involved in controlling the expression of growth-specific genes.

The importance of controlling the expression of growth-specific genes can best be understood by considering that such control is largely lost in tumor cells and perhaps be overactive in dystrophic cells. To remain proliferating, normal cells require the association of extracellular polypeptide growth factors with specific receptors located at the outer face of the cellular membrane. These interactions initiate a signal transduction pathway that activates a number of physiological and genetic processes in order to prepare the cell to undergo a round of division. One event that is believed to occur relatively late in the mitogen response process is the activation of genes, such as *DHFR,* that are required for DNA replication. Therefore it is expected that deciphering the cellular events that bridge *DHFR* gene activation with the mitogenic signal transduction pathway in normal cells will result in a better understanding of what exactly goes awry in tumorgenesis.

In the remaining sections of this chapter, I will first describe the molecular anatomy of the mouse *DHFR* gene then discuss the promoter elements thought to be important for basal transcription. Next, I will discuss systems that have been used to study regulated *DHFR* expression and introduce the muscle cell we used. Finally I will review what was known about *DHFR* gene regulation when I commenced my graduate studies in 1990. The following chapters of the thesis will describe the experimental results of my graduate research, including discussion sections that integrate my observations with observations made by others using other experimental systems.

## The *DHFR* Locus

The DHFR enzyme is a small protein encoded by a large gene. The 187-residue DHFR protein is encoded by a gene encompassing approximately 31 kilobases (Crouse et al., 1982). The mouse *DHFR* gene is organized into six exons and five introns and is

very similar, in both organization and sequence homology of the protein coding regions, to both the hamster and human *DHFR* genes (Mitchell et al., 1986; Yang et al., 1984). There is a considerable amount of size heterogeneity among DHFR transcripts due to multiple initiation and polyadenylation sites (Sazer and Schimke, 1986; Setzer et al., 1980; Setzer et al., 1982). Although transcription initiates at multiple sites, most transcripts initiate at 55 nucleotides upstream of the ATG (henceforth the major transcription start site will be used as a reference point and designated +1) (Farnham and Schimke, 1986b). Another transcription start site, located at -60, is utilized by approximately 15% of DHFR transcripts (Sazer and Schimke, 1986). Still other start sites, located in the -400 to -500 region of the *DHFR* promoter, are used by a small percentage of transcripts (McGrogan et al., 1985). The multiple upstream transcription start sites may be a consequence of bidirectional activity of the *rep* gene promoter that is located partially within the *DHFR* promoter and is oriented in the opposite direction (discussed below) (McGrogan et al., 1985). For other genes, data suggests the utilization of a particular transcription start site *in vivo* may be determined by the developmental pathway of the cell (Linton et al., 1989). Furthermore, start site usage of cloned *DHFR* minigenes can be manipulated experimentally by mutating promoter elements known to bind transcription factors (Blake et al., 1990). The relationship, if any, between where transcription initiates and the mechanisms controlling *DHFR* expression is unknown at present. The existence of alternative start sites may well be more a consequence of the architecture of the *DHFR* promoter and not be, at least in terms of *DHFR* expression and/or regulation, functionally significant. In the remaining sections of this study only the major *DHFR* transcription initiation site will be considered.

## The *DHFR* Promoter Region and Basal Expression

The mouse *DHFR* gene promoter was the first bidirectional promoter described in higher eukaryotes (Crouse et al., 1985). *Rep-3a*, the gene that shares its promoter region with *DHFR*, encodes a protein that bears significant sequence homology to bacterial genes involved in mismatch DNA repair (Linton et al., 1989). The function of Rep-3a in eukaryotic cells is unknown. The *DHFR* and *rep-3a* transcripts initiate approximately 200 base pairs apart and elongate in opposite directions. The sharing of the same promoter region by the *DHFR* and *rep-3a* genes is probably functionally significant since in a number of different experimental systems the two genes appear to be regulated in a similar fashion (Farnham and Schimke, 1986a; Schmidt and Merrill, 1989b). In the remaining sections of this thesis only promoter activity giving rise to DHFR transcripts will be considered.

The *DHFR* promoter region contains a relatively high density of unmethylated CpG dinucleotides in what has been termed a CpG island (Gardiner-Garden and Frommer, 1987). It is thought that methylation of CG-rich promoter regions has an inhibitory affect on gene expression and indeed there exist cellular mechanisms that insure that such regions remain unmethylated (Shimada et al., 1987). Another distinctive feature of the *DHFR* promoter is a lack of the TATA box sequence motif commonly found in pol2 promoters. In promoters that contain one, the TATA box is thought to be the nucleation site for assembly of the transcription preinitiation complex. I will begin my review about what is known about basal expression of the *DHFR* gene by a discussion of the promoter element, the GC box, that is thought to be functionally equivalent to the TATA box.

The GC box sequence motif, GGGCGG, is found singly and multiply in numerous cellular and viral promoters. Within the mouse *DHFR* promoter there are four GC boxes, each of which is contained within a 29-bp repeated element. The four 29-bp elements are tandemly repeated, with 18-bp spacers between them, and span the *DHFR*

promoter from -218 to -29. Mutations within the GC boxes have been shown to decrease the activity of the mouse and similarly organized hamster *DHFR* promoters in both *in vitro* transcription and transient transfection assays (Farnham and Schimke, 1986b; Ciudad et al., 1992). Mutation of the GC box proximal to the transcription start site has the most deleterious effect on promoter activity; the effects on promoter activity become successively less severe as the more distal GC boxes are mutated.

There have been a number of reports indicating that proper transcription initiation from the *DHFR* promoter is dependent on a correctly spaced and intact proximal GC box (Means and Farnham, 1990; Smith et al., 1990; Blake et al., 1990). The relationship between the proximal GC box and initiation suggests that the transcription factors binding at the GC box are involved in the assembly of the transcription preinitiation complex. In promoters containing a TATA box (usually located 25-30 base pairs upstream of the transcription start site), the transcription factor binding at the TATA box, the TATA-binding protein (TBP), is believed to be the necessary initial event in the formation of the preinitiation complex (reviewed in Rigby, 1993). Bound TBP is thought to serve as both the foundation and to some degree the scaffolding for several general transcription factors and pol2 that are required for proper initiation.

The way in which the preinitiation complexes are constructed in non-TATA box-containing promoters is beginning to emerge. In pol2 and pol3 promoters, neither of which contain a TATA box, TBP has been demonstrated to play an essential role in initiation (reviewed in Sharp, 1992). In non-TATA pol2 promoters, TBP is thought to function in a similar fashion as in TATA-containing promoters in the formation of the transcription preinitiation complex. In the absence of a TATA box, TBP is thought to functionally interact with the promoter region by making contacts, either directly or indirectly, with DNA-bound transcription factors (Wiley et al., 1992; Hoey et al.,

1993). In the *DHFR* promoter, the GC box-binding transcription factor Sp1 is believed to link the transcription machinery, at least initially, to the DNA (see below).

The transcription factor Sp1 is thought to play a major role in basal *DHFR* gene expression. Using *in vitro* footprinting, cloned Sp1 has been demonstrated to bind specifically at the GC boxes located in the hamster and mouse *DHFR* promoters (Blake et al., 1990; Means and Farnham, 1990). In *in vitro* transcription assays, Sp1 has a large influence on the transcriptional activity of *DHFR* promoter-containing templates (Schmidt et al., 1989; Farnham and Cornwell, 1991). Additionally, in *Drosophila* cells, which do not contain Sp1, a transfected *DHFR* promoter/reporter gene is inactive unless a Sp1-encoding plasmid is included in the transfection cocktail (Swick et al., 1989). To date, Sp1 is the only well-characterized transcription factor that binds at the *DHFR* GC boxes and affects transcriptional activity of the gene. It is likely, however, that a number of different transcription factors bind GC boxes (Kageyama and Pastan, 1989).

Like other DNA-binding transcription factors, Sp1 can be separated into discrete functional domains. The DNA-binding domain is comprised of three zinc fingers that specifically bind the GC box (Kadonaga et al., 1987). Additionally, mutagenesis has revealed four separable transcriptional activation domains (Courey and Tjian, 1988). Studies of transcriptional activation have shown that Sp1 interacts, most likely via one of its glutamine-rich activation domains, with the TBP-associated factors (TAFs) 110 and 250 (Hoey et al., 1993; Kokubo et al., 1993; Ruppert et al., 1993). If Sp1 does in fact tether the components of the basic transcription complex to the DNA, it would appear that the proximal GC box in the *DHFR* promoter serves as the functional equivalent of a TATA box. In this scenario, Sp1 binding at the GC box is the necessary first, and perhaps limiting, step in the construction of the transcriptional machinery.

It is worth noting that GC boxes are found in a large number of viral and cellular promoters in contexts that vary both in terms of distance from the start site and in

positioning relative to other promoter elements. In fact the mechanism of Sp1 transcriptional activation has been most thoroughly studied using synthetic promoters containing GC boxes located upstream of a TATA box (Pascal and Tjian, 1991). Possibly, multiple mechanisms are involved in Sp1 transcriptional activation. Any particular promoter may use only a subset. It will be interesting to see if there are any structural or functional differences in the interactions between Sp1 and TBP when they are both bound to the DNA, as is the case for TATA box-containing promoters, or when only Sp1 is bound to the DNA, as is the situation for non-TATA promoters.

The mouse, hamster and human *DHFR* genes have a perfectly conserved 17-bp sequence flanking their transcription start site (-11 to +6 in the mouse promoter). Within the sequence element are two copies of the consensus E2F site, TTT(C/G)(C/G)CG(C/G), originally described as a promoter element located in the adenovirus E2 gene. (The role the E2F site plays in regulated *DHFR* expression will be covered in the remaining chapters of the thesis.) The two E2F sites partially overlap one another and are oriented in opposite directions. Even though the spacing between the E2F site and the proximal GC box is conserved among the three *DHFR* genes, other promoter elements are thought to determine where transcription initiation occurs, as location of the start site differs in the three genes. The mouse gene initiates within the 3' end of the E2F site, and the hamster and human genes initiate either one or four base pairs, respectively, upstream of the 5' end of the E2F site.

Cell extracts contain proteins that specifically bind the E2F site of the *DHFR* promoter (see Chapter 2 for a review of E2F site-binding proteins) (Shimada et al., 1986; Means and Farnham, 1990; Blake and Azizkhan, 1989). There have been contradictory reports, including that presented in Chapter 2, of the effects of E2F-site mutations on *DHFR* promoter/reporter gene activity in asynchronously growing cells. A hamster *DHFR* promoter/CAT gene with a mutated E2F site gives five-fold less activity than a wildtype reporter gene when introduced into HeLa cells (Blake and

Azizkhan, 1989). Surprisingly, the same mutated E2F site gene gives two-fold more activity than a wildtype reporter gene when introduced into LMTK and Vero cells (Hiebert et al., 1991). Mutation of the E2F site in a mouse *DHFR* promoter/luciferase gene also leads to an increase in asynchronous cell expression levels in transfected HeLa cells (Means et al., 1992). No simple explanation reconciles these seemingly contradictory observations. In fact, a number of factors could contribute to the different relative activities of reporter genes containing wildtype or mutated E2F sites. One possible explanation for the apparent cell type-specific differences observed for the hamster gene is that the relative levels of stimulatory and inhibitory E2F site-binding transcription factors may vary in different cell lines. The opposite effects of mutating the E2F site in DHFR/CAT and DHFR/luc reporter genes in transfected HeLa cells may be due to the reporter gene. It has been reported that the activity of bacterial promoters can be greatly influenced by the fused downstream reporter gene (Forsberg et al., 1994). It was proposed that reporter genes differ in their topology and that this may determine the accessibility of the upstream promoter region to transcription factors. Although the effect of mutating the E2F site on asynchronous cell expression levels is uncertain, it is clear that the site and its associated transcription factors play a dominant role in linking *DHFR* gene expression to the growth state of the cell.

## Regulated *DHFR* Expression

When one moves beyond the question of "how" a gene is expressed to inquiries about "when" a gene is expressed, what is required for analytical purposes is a method to enrich for cells that are in a common growth state. Due to the variability in the length of G1, even a perfectly synchronized cell population (i.e. all cells in the exact same point of the cell cycle) will, after two or three doubling times, return to asynchronous growth. A variety of cell culture systems have been developed to study gene expression as a function of cell growth state. Most systems may be classified

roughly into two types; those that exploit the morphological changes of cells that occur during the cell cycle, and those that make use of cell lines whose growth states may be experimentally manipulated. Still other systems utilize drugs that influence the growth status of the cell by inhibiting a process, such as replication, transcription or mitosis. Different systems that have proved useful for studying *DHFR* regulation will be described below.

A useful property of the *DHFR* gene that has greatly facilitated the analysis is that the *DHFR* locus can be selectively amplified in response to sublethal doses of the folate analog methotrexate (MTX) (reviewed in Stark and Wahl, 1984). *DHFR* amplification has attracted a great deal of interest due to the fact that tumor cells isolated from MTX-treated patients and laboratory animals often have either amplified the *DHFR* locus or carry extra copies of the *DHFR* gene on small extrachromosomal elements referred to as double minutes. The mechanisms involved in selective gene amplification are currently unknown, but amplication most likely occurs during, and indeed may be dependent on, replication. The size of the amplified unit is highly variable, from a minimum that is just sufficient to encompass the *DHFR* structural gene and core promoter region to as large as 2000 kilobases. Importantly, there have been no reported instances in which amplified *DHFR* genes are regulated any differently than nonamplified, single-copy versions.

In several experimental systems, using a variety of different types of analysis, *DHFR* gene expression has been shown to be strongly influenced by the growth status of the cell. One of the first and still widely used experimental system to demonstrate regulated *DHFR* expression involves inducing non-growing cells to reenter the cell cycle. Regulation is inferred from differences in either mRNA and protein synthesis rates or mRNA and protein levels between non-growing and growing cells. These systems employ fibroblastic cell lines that can be experimentally manipulated to withdraw from the cell cycle and enter a state, termed $G_0$, that is characterized by a

general depression of the rates of all macromolecular synthesis (Johnson et al., 1974). Withdrawal from the cell cycle occurs early in G1 during a period of the cell cycle when fibroblasts, and probably most other normal and immortalized cells, are sensitive to serum, and, for some cell lines, growth factor concentrations (henceforth all media constituents affecting growth will be termed mitogens) (reviewed in Norbury and Nurse, 1992). If mitogen levels are sufficient for another round of cell division, cells transverse what is termed the restriction point (Pardee et al., 1978), which occurs approximately two hours before the onset of S phase in many fibroblastic cells. After passage of the restriction point, cells do not require mitogens to complete the remainder of the cell division cycle.

If mitogen levels fall below that required for restriction point transversal, cells collect in $G_0$. Over a span of a few days an entire population of cells will arrest in $G_0$. Upon restoration of mitogens, and replating to a lower cell density if the culture was confluent, quiescent $G_0$ cells reenter the cell cycle and enter S phase in approximately 10-16 hours, depending on cell type. Mitogen-stimulated cultures often exhibit enough synchrony to allow enrichment for cells that are in particular phases of the cell cycle.

The resumption of growth, and more specifically for our purposes here, preparation for DNA synthesis, is associated with the increased expression and activity of several genes and their products. Historically, an increase in the expression of a gene following mitogen restoration was sufficient for the gene to be labeled as "cell cycle" regulated (Pardee et al., 1978). It was thought that entry into S phase following $G_0$ was equivalent to S phase entry during a normal cell cycle. There are two major problems with this interpretation. First of all, as was mentioned above, quiescent cells have an overall lower rate of metabolism and therefore any increase in gene activity that occurs following mitogen restoration may be due simply to an overall increase in the cellular metabolism rate. The increase in gene activity therefore will

tend to be overestimated due to the fact that gene activity in quiescent cells is lower than at any point of the cell cycle and therefore such increases in gene activity are not reflective of what occurs during a normal cell cycle. Secondly, there is some evidence that at least some genes are regulated differently during the first cell cycle following serum stimulation and subsequent cell cycles (Blanchard et al., 1985; Thompson et al., 1985). Although an increase in gene activity immediately prior to or during S phase following mitogen stimulation does not, by itself, entitle a gene to be referred to as "cell cycle" regulated, it is indicative of at least what can be called "growth-specific" regulation.

Since amplification of the *DHFR* gene was relatively easy to achieve (see above) and an increase in reduced folate pools likely would have to occur before a quiescent cell could begin DNA synthesis, *DHFR* expression studies became a widely used model for studying the mechanisms involved in controlling growth-specific gene expression. Using metabolic pulse/chase [$^{35}$S]-methionine labeling, early serum restoration studies clearly demonstrated that increased DHFR enzyme activity in late G1/early S phase is due to an increase in the DHFR protein synthesis rate (Johnson et al., 1978; Wiedemann and Johnson, 1979). It was additionally shown that there is a corresponding increase in DHFR mRNA levels after serum stimulation (Wiedemann and Johnson, 1979). The stability of DHFR mRNA was reported to be the same in quiescent and growing cells, which suggested that changes in DHFR mRNA levels during cell cycle reentry result from an increase in the transcription rate (Hendrickson et al., 1980). This hypothesis was confirmed by measuring the transcription rate in quiescent and growing cells using a nuclear run-on assay (Wu and Johnson, 1982). The nuclear run-on assay involves labeling isolated nuclei with radioactive ribonucleoside triphosphates and allowing engaged RNA polymerases to continue elongation (McKnight and Palmiter, 1979). By measuring the radioactivity incorporated into specific transcripts, polymerase density on a particular gene can be determined. Proliferating

fibroblasts have a greater polymerase density on *DHFR*, and hence a higher transcription rate, compared to the polymerase density on *DHFR* genes in quiescent cells (Wu and Johnson, 1982). Taken as a whole, these studies indicate that the increase DHFR enzyme activity observed when quiescent cells are induced to reenter the cell cycle is primarily due to an increase in the transcription rate of the *DHFR* gene.

Although it has been clearly demonstrated that a transcriptional mechanism operates to increase DHFR mRNA and enzyme levels during the transition from nongrowth to growth, it has been much more difficult to determine if the *DHFR* gene is preferentially transcribed at any point within a normal cell cycle. Analogous to the reasoning used above for growth-specific regulation, the expectation would be that *DHFR* would be preferentially expressed sometime after the restriction point (early G1) and possibly well into S phase.

In order to study cell cycle specific-gene expression, two different methods have been developed for the enrichment of cells at specific points during a normal cell cycle. One takes advantage of the fact that cultured cells become less adherent to the substratum during mitosis and can be selectively removed by mechanical means. In practice, a culture dish containing a growing population of cells is shaken and the medium, containing the M-phase cells, is replated on a fresh culture dish. The replated cells constitute a synchronized population that can be analyzed for cell cycle-specific gene expression at various times after replating. The technique, commonly known as a mitotic shake off, has been used in amplified cell lines to show that both the *DHFR* gene transcription and *DHFR* protein synthesis rates increase at the G1/S boundary (Farnham and Schimke, 1985; Mariani et al., 1981). These data suggest that *DHFR* expression is under the control of a regulatory mechanism that prepares cells for S phase.

An alternative method to enrich for cells in specific cell cycle compartments is centrifugal elutriation which exploits the fact that cells at different phases of the cell cycle differ in size and therefore can be sorted by differential sedimentation. Using this method of cell-separating, no differences were observed in DHFR mRNA levels between G1, S, and G2/M cell populations (Felder et al., 1989). These data would appear to support a model in which *DHFR* is expressed constitutively throughout the cell cycle, and is at odds with conclusions drawn from mitotic shake off and serum restoration studies. These seemingly contradictory observations have not been reconciled, although other experimental systems (see below) strongly suggest that *DHFR* expression is regulated during the cell cycle.

The strong correlation between increased *DHFR* expression and the growth state of the cell has manifested itself, albeit with a slight twist, in studies concerning the genetic alterations that occur in cells during viral infection. During a viral infection reduced folates are required for the synthesis of viral DNA. Therefore it would be expected that virally-infected cells would exhibit at least some of the properties characteristic of late G1 and/or S phase. Indeed it has long been established that following infection with either polyomavirus or adenovirus, quiescent cells enter a S-like phase (defined as an increase in the incorporation rate of labeled nucleotide analogs) (Dulbecco et al., 1965; Shimojo and Yamashita, 1968). Concomitant with this S phase induction, DHFR mRNA levels have been demonstrated to increase due primarily to an increase in the transcription rate (Gudewicz et al., 1981; Kellems et al., 1979; Yoder et al., 1983; Yoder and Berget, 1985). Supporting a model that virus-induced *DHFR* activation occurs at the level of transcription, *in vitro* transcription from a *DHFR* promoter is more efficient using extracts prepared from adenovirus-infected HeLa cells than with extracts made from uninfected cells (Farnham and Schimke, 1986b). Elucidation of the gene activation pathways utilized during viral infection have had a major influence on the thinking of how cellular genes

that are required for mitotic growth are regulated. Descriptions of recent studies will be presented in Chapter 3, where it will be shown that an adenovirus gene is sufficient to derepress *DHFR* promoter activity in muscle cells that have withdrawn from the cell cycle.

Differentiation-competent cell lines have further strengthened the association between *DHFR* expression and mitotic growth. In the best characterized system, that involving a skeletal muscle cell line able to be experimentally induced to undergo myogenesis, DHFR protein synthesis rate, mRNA levels, and transcription rate have all been shown to decrease during the differentiation process (Schmidt and Merrill, 1989b; Schmidt and Merrill, 1991). DHFR protein has been demonstrated to be extremely stable *in vivo* with the consequence that DHFR protein levels do not decrease significantly during differentiation, despite the decrease in the DHFR protein synthesis rate (Schmidt and Merrill, 1989a).

In some respects myogenic withdrawal from the cell cycle, one of the initial events during the differentiation process, is similar to the withdrawal from the cell cycle that occurs when fibroblastic cells become quiescence. In both cases, cell cycle withdrawal occurs prior to the restriction point in G1. In both cases, withdrawal is a consequence of an assessment of culture conditions made during early G1. Fibroblastic cells, such as those of the human HeLa and mouse NIH 3T3 lines, withdraw from the cell cycle when mitogens contained in the serum become limiting. To remain proliferative, muscle cells require both serum and fibroblast growth factor (FGF) (reviewed in Florini et al., 1991). If FGF levels fall below a threshold required for restriction point traversal, muscle cells withdraw from the cell cycle and undergo numerous genetic, physiological, and morphological changes characteristic of myogenic differentiation.

This latter point raises important differences between the processes that give rise to quiescent and differentiated cells. Quiescent cells are in a reversible, non-

replicative state that, upon serum addition, can reenter the proliferative cell cycle. In contrast, differentiated cells are in a postreplicative state that, even upon reexposure to FGF, cannot reenter the cell cycle. These two non-growth states are also characterized by different patterns of gene expression. As the name implies, quiescent cells have a lower overall rate of metabolism than proliferating cells, as measured by total protein synthesis rate. Although there are likely a few genes that are preferentially expressed during the quiescent state, quiescence is best characterized by a general repression of all gene activity. In contrast, there is only an approximate 1.6-fold decrease in the rate of precursor incorporation into RNA and protein as muscle cells withdraw from the cell cycle and differentiate (Schmidt and Merrill, 1989b). Myogenic induction is associated with the activation of a large array of genes encoding muscle-specific transcription factors and structural proteins (reviewed in Olson, 1990). Although characterized by an absence of mitotic growth, myogenic differentiation is associated with a substantial amount of cellular growth as can be observed an obvious increase in the cytoplasmic/nuclear volume ratio. Therefore, although *DHFR* expression is repressed in both quiescent and differentiated cells, in the former the repression may well be a consequence of a general repression of all gene activity. In contrast, myogenic repression of *DHFR* expression occurs against a background of active cellular metabolism and therefore is most certainly a specific mechanism responsible for repressing the expression of genes required for mitotic growth.

Differentiation studies complement growth restoration studies by providing a means to compare and contrast the elements responsible for the repression and activation, respectively, of gene expression. In addition, studies of the repression of genes such as *DHFR* during the differentiation process may yield important insights into the pathology of cancer. Carcinogenesis is thought to be a multistage process involving sequential activation or inactivation of a number of genes that either directly

or indirectly play a role in controlling cell proliferation and differentiation. Since proliferation and differentiation are usually mutually exclusive processes, a cancer cell must be immune to the signals that operate in early G1 that instruct normal cells to cease proliferation and initiate a differentiation program. Therefore elucidating the mechanisms controlling the differentiation-specific repression of *DHFR* expression is an important first step in understanding the molecular circuitry linking the regulation of gene expression with events occurring in early G1.

The work described in this thesis concerns the role played by the E2F site and its associated transcription factors in the repression of *DHFR* expression during myogenesis. The results reported herein complement a growing body of literature dealing with the regulation of E2F site- containing genes. The results confirm certain aspects of current models for E2F site-containing gene regulation and extends the models to an experimental system capable of differentiation. Additionally, evidence will be presented suggesting that the current model is not adequate to completely describe the regulation of growth-specific genes in postreplicative cells.

# CHAPTER 2

## Regulation of *DHFR* Promoter Activity in Postreplicative Muscle Cells Requires an E2F Site


### Abstact

The E2F consensus site is found within the promoters of several DNA synthesis genes. Protein interactions at the E2F site in the dihydrofolate reductase (*DHFR*) promoter are required for growth-dependent promoter regulation in serum-stimulated quiescent fibroblasts. The importance of protein interactions at the DHFR E2F site for promoter regulation in differentiating muscle cells was investigated. In muscle cells transformed with DHFR promoter/reporter genes, correct regulation of reporter gene expression during myogenic withdrawal from the cell cycle was dependent on an intact E2F site. In transient expression assays, plasmids encoding the E2F site-binding transcription factors DP1 and E2F1 derepressed DHFR promoter activity in differentiated muscle cells, suggesting that the activities of DP1- or E2F1-like proteins might be limiting in differentiated cells. DP1 and E2F1 mRNA levels did not change significantly during myoblast withdrawal from the cell cycle, suggesting that DP1 or E2F1 activity was not regulated transcriptionally. *In vitro* band shift assays showed that muscle cells possessed E2F site binding activity with electrophoretic mobilities characteristic of E2F1/DP1 dimers and E2F1/DP1/Rb-like multimers. Neither dimeric nor multimeric E2F site binding activity changed significantly during myoblast cell cycle withdrawal. If changes in DHFR promoter activity are due to changes in the relative abundance of stimulatory dimeric binding activity and inhibitory multimeric binding activity, the changes were either too subtle to detect against the background of bulk binding activity or were not preserved under the conditions of *in vitro* analysis.

## Introduction

Most eukaryotic genes characterized to date have discrete sequence elements located in their upstream noncoding regions that bind transcription factors. Transcription factors can be classified broadly into two categories: basal factors, that bind at all or most promoters and are necessary for the construction of the transcription complex, and regulatory factors that bind only a subset of promoters and control when and how much the gene will be expressed. Transcription factors that play a regulatory role are thought to account in large part for the cell type-specific gene expression that gives rise to specialized tissues. Therefore, a necessary first step in understanding development is the identification of promoter elements that bind regulatory transcription factors. In this Chapter an element is identified in a cellular promoter that appears to be necessary for the proper expression of the corresponding gene during growth and differentiation.

Two interdependent processes occur during terminal skeletal muscle differentiation: irreversible withdrawal from the cell cycle and induction of muscle specific genes. To remain proliferative, the MM14D muscle cell line requires fibroblast growth factor (FGF). If FGF levels fall below a threshold level, proliferating myoblasts withdraw from the cell cycle and initiate the myogenic differentiation program. Therefore, at least in permanent muscle cell lines, the interaction between FGF and its receptor is the ultimate determinant as to whether a cell will complete another round of replication or differentiate.

The analysis of the factors that control the expression of developmentally regulated genes has been useful in deciphering the intracellular flow of information that operates during growth and differentiation. For example, phenotypic differentiation in muscle cells is controlled by a family of muscle-specific transcription factors that regulate the expression of muscle-specific genes during myogenesis. The activity of the muscle creatine kinase (MCK) promoter and enhancer is totally dependent on the direct

binding of muscle-specific transcription factors to a conserved enhancer sequence known as the E box (Lassar et al., 1989). The muscle-specific transcription factors MyoD, myogenin, myf5, and MRF4 share homology within a basic-helix-loop-helix (bHLH) motif that has been demonstrated to mediate DNA binding and dimerization (reviewed in Weintraub, 1993). The transcriptional activity of at least one of the members of this family, myogenin, is inhibited by the peptide growth factor FGF by a mechanism involving the reversible phosphorylation of the DNA-binding region of myogenin by protein kinase C (Li et al., 1992a). Removal of FGF results in the dephosphorylation of myogenin thereby restoring its ability to transcriptionally activate the MCK promoter. Although there are other seemingly redundant cellular processes that control the activity of the muscle bHLH transcription factors (for example see Gu et al., 1993; Li et al., 1992b; Benezra et al., 1990), the mechanism described by Li et al. (1992a) neatly links an upstream effector molecule, FGF, with a downstream process, MCK promoter activity, that is intimately involved in muscle cell differentiation.

During myogenic cell cycle withdrawal, muscle-specific gene activation is preceded by a reduction in the level of transcripts that encode proteins required for DNA synthesis. The elucidation of the elements that mediate the differentiation-specific repression of the DNA synthesis genes has awaited the identification of promoter sequences that are required for such regulation to occur. With such a promoter sequence identified, it may soon be possible, as in the case of the MCK promoter, to ascertain the molecular circuitry that links events occurring at the cell surface with the control of DNA synthesis gene expression.

Based on studies using serum-synchronized fibroblasts, regulation of growth-specific gene expression is thought to occur primarily by a mechanism mediated by the E2F site promoter element. E2F binding sites are found in the promoters of several cellular genes that encode products that primarily function in the G1 and S phases of

the cell cycle (Merrill et al., 1992). E2F site-containing genes encode proteins, such as DHFR, thymidylate synthase, DNA polymerase alpha and ribonucleotide reductase, that are directly involved in DNA synthesis, or transcription factors, such as c-myc, N-myc and B-myb, that are thought to control the expression of other genes. It has become quite clear that the E2F site is required for the efficient expression of genes required by replicating cells.

E2F was originally defined as a cellular transcription factor, induced early during adenovirus infection, that bound the viral E2 gene promoter in band shift assays (Kovesdi et al., 1986). The E2F binding site consensus, TTT(C/G)(C/G)CG(C/G), is required for both efficient basal transcription and increased expression induced by serum, viral oncogenes, or viral infection (Blake and Azizkhan, 1989; Lam and Watson, 1993; Means et al., 1992; Mudryj et al., 1990; Ogris et al., 1993; Thalmeier et al., 1989; Wade et al., 1992). The genes encoding a family of proteins, termed E2F1, 2 and 3, and DP1, that bind the E2F site and stimulate E2F site-containing promoters have recently been cloned (Girling et al., 1993; Helin et al., 1992; Ivey-Hoyle et al., 1993; Johnson et al., 1993; Kaelin et al., 1992; Krek et al., 1993; Lees et al., 1993). Extracts from uninfected cells contain large multimeric complexes that bind the E2F site and are believed to be tethered to the DNA by the E2F site-binding proteins (Bagchi et al., 1990; Chittenden et al., 1993; Mudryj et al., 1991). Antisera recognizing Rb, p107, cyclin A, cyclin E and $p33^{cdk2}$ have been used to identify components of the multimeric complexes (Bandara et al., 1991; Chittenden et al., 1993; Devoto et al., 1992; Lees et al., 1992; Mudryj et al., 1991; Shirodkar et al., 1992). The presence of multimeric E2F site-binding complexes *in vitro* correlates with the inhibition of E2F site-containing promoters *in vivo* (Hiebert et al., 1992; Ogris et al., 1993). The E2F site appears to link the expression of replication-specific genes with the cell cycle control machinery.

The gene encoding dihydrofolate reductase (DHFR) is one example of a DNA synthesis gene that is preferentially expressed in growing cells. During myogenic withdrawal from the cell cycle, DHFR mRNA levels decrease approximately 15-fold, which is matched by a similar decrease in the DHFR protein synthesis rate (Schmidt and Merrill, 1989b; Schmidt and Merrill, 1991). An E2F binding site is located at the transcription start site of the DHFR gene promoter and is required for efficient DHFR promoter/reporter gene expression in asynchronously growing cells (Blake and Azizkhan, 1989). In addition, the E2F-binding site is required for increased DHFR promoter activity in quiescent cells induced with serum (Means et al., 1992). To investigate the role the E2F binding site plays in the regulation of DHFR promoter activity during terminal differentiation, we determined the effect that mutating the E2F site consensus has on DHFR promoter regulation during mouse muscle cell differentiation. We also measured DP1 and E2F1 mRNA levels, E2F site binding activity, and the effect of ectopic expression of DP1 and E2F1 during muscle differentiation.

Our results showed that an intact E2F site was critical for regulation of DHFR promoter activity during myogenic cell cycle withdrawal and muscle cell differentiation. Also, DP1 and E2F1 overexpression derepressed the DHFR promoter in transfected cells. However, repression of DHFR promoter activity was not associated with a decrease in DP1 or E2F1 mRNA levels or a decrease in the amount or mobility of E2F site binding activity measured in band shift assays. If changes in DHFR promoter activity are due to alterations in the level or activity of E2F/DP dimer bound at the DHFR promoter E2F site, the alterations were not detectable when bulk E2F site binding activity was measured.

## Materials and Methods

### Cell cultures and transformations

A hypoxanthine guanine phosphoribosyltransferase-deficient derivative of the diploid mouse skeletal myoblast line MM14D (Linkhart et al., 1981) was grown on gelatin-coated culture dishes in basal medium (DMEM/F12 [Gibco] containing 2.45 g/L $NaHCO_3$, 15 mM Hepes [pH 7.2], 10 units/ml penicillin G, and 0.5 mg/ml streptomycin sulfate) supplemented with 15% horse serum and either pure basic fibroblast growth factor (FGF) (a gift from B. Olwin, U. Wisconsin), or FGF-rich, ammonium sulfate-fractionated bovine brain extract (Esch et al., 1985).

Myoblasts grown in pure FGF required frequent FGF replenishment and were maintained as follows. Cells ($10^5$ per 10-cm dish) were inoculated into serum-supplemented medium containing 0.5 ng/ml FGF. Medium was supplemented with 0.5 ng/ml FGF at 12 h, replaced with medium containing 1 ng/ml FGF at 24 h, supplemented with 1 ng/ml FGF at 36 h, replaced with medium containing 2 ng/ml FGF at 48 h, and supplemented with 2 ng/ml FGF at 48 h. RNA was prepared either 12 hours after the final FGF supplementation, when cells were still fully proliferative (as measured by morphology, BUdR-staining and cloning efficiency) or at 72 hours after the final FGF supplementation, when cells had completely withdrawn from the cell cycle and differentiated.

Myoblasts grown in brain extract required less frequent medium replenishment because mitogen levels were high. Brain extract-grown cells were induced to withdraw from the cell cycle by rinsing cultures twice with basal medium, followed by incubation in mitogen free medium (basal medium supplemented with 1 $\mu$m insulin [Schmidt and Merrill, 1989b]).

A modified calcium phosphate precipitation procedure (Gross et al., 1987) was used to transform cells with 5 $\mu$g of a DHFR promoter/reporter gene (either DHFR promoter/CAT or DHFR promoter/luciferase) and 0.3 $\mu$g pKneo. Stable transformants

were selected in 1 mg/ml G418 (Gibco). Transformants were pooled (20-50 colonies/dish) and expanded in 100 μg/ml G418. For transient transfection assays, 2 μg of a DHFR promoter/luciferase gene and 8 μg of an effector gene were cotransfected two days after muscle cells were induced to differentiate by FGF depletion (i.e. 60 hours after the final FGF supplementation). Following the glycerol shock, conditioned media was fed back to cultures and cells were harvested 20 hours later.

**Plasmids**

Plasmids pWTluc and pNWluc (Means et al., 1992) (provided by P. Farnham, U. Wisconsin) contain DHFR promoter sequences (-270 to +20) fused to the luciferase reporter gene. In pNWluc, the wild-type DHFR sequence from -11 to +11 (ATTTCGCGCCAAACTTGACGGC) was mutated to gccctatatCAAAtccagtaat. The plasmids wtDpCAT and mutDpCAT were prepared by inserting the *Hin*dIII DHFR promoter fragments from either pWTluc or pNWluc into the *Hin*dIII site of a CAT vector plasmid prepared by removing the RSV promoter from RSVpCAT (Schmidt et al., 1990) by digesting with *Nde*I and *Hin*dIII, blunting with Klenow DNA polymerase, and religating, thereby regenerating a *Hin*dIII site immediately upstream of the CAT coding region. The plasmid
(-850/+16)DHFRp/CAT was derived by digesting p3Dp(+300)CAT, a plasmid containing the (-850/+300) DHFR promoter fused via a *Hin*dIII linker to CAT, with *Hin*dIII, resecting the DNA using exonuclease III and SI nuclease, ligating *Hin*dIII linkers to the resected termini, digesting with *Sma*I to cleave the DNA at position -256 in the DHFR promoter, and ligating the resulting -256 to +16 DHFR promoter fragment to a gel-purified vector fragment prepared by digesting p3Dp(+300)CAT with *Sma*I and *Hin*dIII.

MTpDP1 was constructed by inserting the 1.5 kb blunted *Eco*RI/*Hin*dIII pGC fragment, containing the entire DP1 cDNA coding sequence (Girling et al., 1993)

(provided by N. B. La Thangue, NIMR, London), into a vector prepared by digesting mMT-1, containing the mouse metallothionein (MT) promoter (Mayo et al., 1982) (provided by R. Palmiter, U. Washington), with *Bgl*II, blunting with Klenow polymerase, and digesting with *Hin*dIII.

MCKpDP1 was constructed by first inserting the 1.5 kb blunted *Eco*RI/*Hin*dIII fragment of pGC into Bluescribe to generate BSDP1. BSDP1 was digested with *Not*I, blunted with Klenow polymerase, and digested with *Cla*I. The resulting DP1-encoding fragment was inserted into a vector containing the mouse muscle creatine kinase (MCK) promoter prepared as follows: the 3300-bp blunted *Eco*RI/*Hin*dIII fragment from p3300MCK (provided by J. Buskin, U. Washington) was inserted into a vector prepared by digesting pAAlucA (Means et al., 1992) with *Pst*I, blunting with Klenow polymerase, and digesting with *Hin*dIII. The resulting plasmid, MCKpluc, was subsequently digested with *Pst*I, blunted with Klenow polymerase, and digested with *Cla*I which removed the luciferase coding region and generated termini compatible with insertion of the DP1 fragment.

The plasmid pCDNAIIImE2F1 (Li et al., 1994) (provided by P. Farnham, U. Wisconsin) contains the mouse E2F1 coding region under the control of the CMV early promoter. In this study this plasmid is denoted as pCMVpE2F1.

Descriptions of the CAT and DHFR 3' riboprobe template plasmids and riboprobe synthesis have been published elsewhere (Schmidt and Merrill, 1989b; Schmidt et al., 1990). A riboprobe template containing E2F1 was constructed by removing the 700-bp *Xho*I fragment from pCMVpE2F1, that contains the terminal 40 base pairs of the coding region and 610 base pairs of 3' untranslated sequence, followed by religation of the vector. The resulting plasmid was digested with *Bam*HI and used in a SP6 polymerase-catalyzed transcription reaction to generate a riboprobe complementary to a 216-nt region near the 3' end of mouse mE2F1 mRNA. A DP1 riboprobe template was constructed by cloning the 212-bp *Pst*I/*Bam*HI fragment from pGC (Girling et al.,

1993) into Bluescribe. The resulting plasmid was digested with *Hin*dIII and used in a T7 polymerase-catalyzed transcription reaction to generate a riboprobe complementary to a 212-nt region near the 3' end of mouse DP1 mRNA. Riboprobe specific activity ranged from 2-5 $x10^8$ cpm/$\mu$g.

**RNA isolation and analysis**

Total cellular RNA was isolated by a modified guanidinium isothiocyanate/CsCl procedure (Gross et al.,1987). RNA concentration was determined by $A_{260}$ (1 OD=40 $\mu$g/mL), and integrity and concentration confirmed by electrophoresis through MOPS/formaldehyde 1% agarose gels and ethidium bromide-staining (Sambrook et al., 1989). RNA was analyzed by a RNase protection assay described previously (Schmidt and Merrill, 1989b) with the following modifications. Cellular RNA (25 $\mu$g) was incubated with 10 fmol riboprobe for 18 hours at 57° C. Samples were analyzed by electrophoresis through pre-run, 8% polyacrylamide, 8 M urea sequencing gels, using 1X TBE (90 mM Tris-borate, 2 mM EDTA) as gel buffer and 0.5X TBE as running buffer.

**Luciferase assay**

For luciferase assays on stable transformants, cells (2.5-4.0 x $10^5$ per 6-cm dish) were inoculated into growth medium containing FGF-rich brain extract, fed fresh growth medium at 24 hours, and switched to insulin-supplemented basal medium to induce myogenic withdrawal from the cell cycle at 48 hours. Cells were harvested for luciferase assays either at the time of the switch (when cells were proliferating), or at specified intervals thereafter. For luciferase assay on transiently-transfected myocytes, cells were grown in pure FGF medium as described above (see Cell cultures and transformations). Cells were harvested by washing cultures once with cold PBS and scraping up cells in 1 ml cold PBS. Cell pellets were frozen at -20° C. Extracts

were prepared and luciferase assays performed using the Enhanced Luciferase Assay Kit (Analytical Luminescence Laboratory). Substrate A and B (25 μl each) were added to 2.5 μl extract and counted for 30 s using a liquid scintillation counter (Beckman, model LS8000) modified for photon detection. Luciferase activity was calculated by dividing the number of photons by the extract protein concentration as determined by Bradford assay.

**Electrophoretic mobility shift assays**

Cells were harvested by washing cultures with cold PBS followed by scraping up cells in 1 mL PBS. Whole cell extracts were prepared by resuspending cell pellets in 100 μL buffer A (10 mM Hepes [pH 7.9], 15 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) followed by three consecutive freeze/thaws. Extracts were clarified and stored at -80° C in small aliquots. Protein concentration of extracts was determined by a Bradford assay. Band shifts were performed by incubating 5-10 μg cell extract with 20 fm end-labeled oligonucleotide probe and 0.1 mg/mL salmon sperm DNA in 50 mM Tris [pH 8], 122 mM NaCl, 0.2 mM EDTA, 1 mM DTT and 10% glycerol (Bandara et al., 1991) for 15 min at room temperature. In detergent-treated reactions, DOC was included at a final concentration of 1.25% and NP40 (1.25% final concentration) was added after 10 min incubation at room temperature. Reactions were loaded on pre-run (45 min at 200V) 6% native polyacrylamide gels in 0.25X TBE. Electrophoresis was done at room temperature for 2-3 hours at 120V.

Oligonucleotides used in binding reactions contained either a wildtype E2F site (5'-AGCTGTTTCGCGCCAAACAGCT-3', 5'-AGCTGTTTGGCGCGAAACAGCT-3') or a mutated E2F site (5'-AGCTGTTTCTCGCCAAAC-3', 5'-AGCTGTTTGGCGAGAAAC-3'). Oligonucleotides (100 pm of each) were annealed in a 10 μl volume of EST (1 mM EDTA, 50 mM NaCl, 10 mM Tris [pH 7]). Duplexed oligonucleotides (10 pm) were end-labeled in 50 μl reactions containing 30 pm [∂-$^{32}$P]ATP (3000 Ci/mmole), 45 U T4 polynucleotide

kinase, 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT and 0.1 mM Spermidine. Nucleotides were removed on 5-ml P6 columns (BioRad) in EST. Specific activities of end-labeled oligonucleotides were 2-5 $x10^8$ cpm/$\mu$g.

## Results

### DHFR promoter regulation requires an intact E2F site

Muscle cell withdrawal from the cell cycle and differentiation is accompanied by reduced expression of genes involved in DNA synthesis such as *DHFR* and *TK* (Schmidt and Merrill, 1989b; Gross et al., 1987). Mouse muscle cells remain proliferative if medium FGF levels are maintained above a threshold level. If cells are not regularly given fresh FGF and are allowed to deplenish residual FGF in the medium, proliferating myoblasts irreversibly withdraw from the cell cycle to form committed myocytes that then differentiate to form multinucleated myotubes (Linkhart et al., 1981). Under our standard culture conditions (see Materials and Methods), FGF deplenishment resulted in complete myogenic commitment and extensive morphological differentiation within 60 hours.

To identify *cis*-acting information required for *DHFR* regulation during myogenic withdrawal from the cell cycle, we analyzed DHFR promoter activity in cells stably transformed with genes consisting of DHFR promoter fragments fused upstream of the bacterial CAT gene. As shown in Figure II.1a, a DHFR promoter fragment from −270 to +20 (relative to the transcription start site) was sufficient to cause CAT mRNA levels to decrease four-fold as proliferative myoblasts irreversibly withdrew from the cell cycle and differentiated (compare lanes 1 and 2). Endogenous DHFR mRNA levels decreased in parallel with the reporter gene (Fig. II.1b, lanes 1 and 2).

Figure II.1. E2F site requirement for CAT mRNA regulation in DHFR promoter/CAT muscle cell transformants induced to differentiate by FGF depletion. Total RNA (25 μg) from proliferating myoblasts (mb) or differentiated myocytes (mc) was analyzed by RNase protection for CAT mRNA (A) or DHFR mRNA (B). Myoblasts transformed with DHFR promoter/CAT reporter genes containing a wildtype (lane 1 and 2) or mutated (lane 3 and 4) E2F site were induced to differentiate by allowing FGF depletion. A 72-h induction period was used. To determine absolute CAT mRNA levels in experimental samples, dilutions of T3 RNA polymerase-generated CAT pseudo-mRNA were assayed in parallel (pseudo-mRNA lanes). Band intensities were quantitated by laser densitometry. Absolute mRNA levels (mRNA/cell) calculated by extrapolation from pseudo-mRNA standards, are shown, as is the change relative to myoblast levels (percent remaining). Yeast RNA (Y) was probed to insure that signals arising in experimental lanes were specific. Arrows designate expected mobilities of fragments protected by CAT and DHFR message.

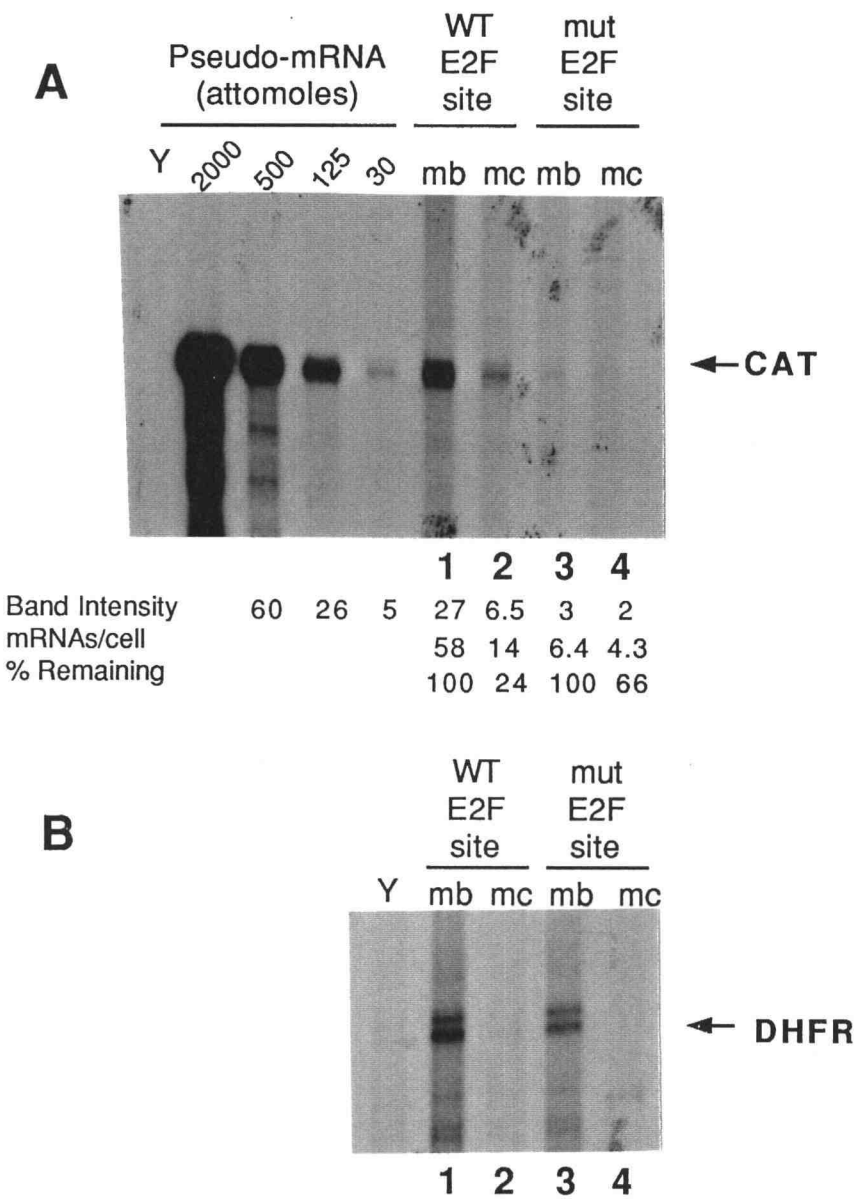
A
Pseudo-mRNA (attomoles)
WT E2F site
mut E2F site
Y
2000
500
125
30
mb
mc
mb
mc
CAT
1 2 3 4
Band Intensity 60 26 5 27 6.5 3 2
mRNAs/cell 58 14 6.4 4.3
% Remaining 100 24 100 66
B
WT E2F site
mut E2F site
Y
mb
mc
mb
mc
DHFR
1 2 3 4

Figure II.1

A similar regulated pattern of expression was obtained when muscle cells transformed with a (−850/+16)DHFR promoter/CAT gene were analyzed (described later, see Fig. II.5).

Previously Schmidt et al. (1990) reported that a (−850/+60)DHFR promoter fused to CAT was constitutively expressed in postreplicative muscle cells and concluded that intragenic information was required for myogenic DHFR promoter regulation. On further examination, the (−850/+60)DHFR promoter/CAT gene showed a variable pattern of regulation. In different experiments, the gene showed low, constitutive expression or moderate, regulated expression (data not shown). Although the (-850/+60)DHFR promoter/CAT gene gave variable results, the (−270/+20)DHFR/CAT and (−850/+16)DHFR/CAT genes always showed a regulated pattern of expression. Thus, the earlier conclusion by Schmidt et al. (1990) that intragenic sequences downstream from +60 were required for regulation of DHFR promoter activity was incorrect.

Means and coworkers (1992) have demonstrated that the E2F site located immediately upstream of the mouse DHFR gene transcription start site is required for late G1 induction of a DHFR promoter/luciferase reporter gene in serum-synchronized fibroblasts. To investigate whether the site was required for DHFR promoter regulation during muscle cell differentiation, we analyzed CAT mRNA levels in proliferative and postreplicative muscle cells stably transformed with a (−270/+20)DHFR promoter/CAT gene containing a mutated E2F site. As shown in Figure II.1a, mutation of the E2F site resulted in the CAT reporter gene being expressed at a low and constitutive level in both proliferating and postreplicative cells (compare lanes 3 and 4). Parallel measurements indicated that endogenous DHFR mRNA levels were regulated normally in the transformant population (Fig. II.1b, compare lanes 3 and 4).

In a second series of experiments, myoblast withdrawal from the cell cycle was induced by switching cells from brain extract medium to mitogen free basal medium supplemented with insulin (see Materials and Methods). Mitogen-free medium induced rapid withdrawal from the cell cycle, but did not allow full morphological differentiation. As earlier shown in FGF deplenishment experiments, withdrawal from the cell cycle in mitogen-free medium was associated with a four-fold decrease in DHFR/CAT gene expression when the E2F site was intact (Fig. II.2a, compare lanes 1 and 2) but not when the E2F site was disrupted (compare lanes 3 and 4). Parallel measurements showed that endogenous DHFR mRNA levels were regulated normally in both transformant populations (Fig. II.2b, compare lanes 1 and 2, and lanes 3 and 4).

The DHFR promoter/CAT genes used in the above experiments were expressed at barely detectable levels in postreplicative muscle cells, as measured by RNase protection assays. In order to better quantitate the decrease in DHFR promoter activity during myogenic cell cycle withdrawal, the firefly luciferase gene was used as a reporter gene. Studies of fibroblastic cells (Means et al., 1992) suggest luciferase enzyme has an intracellular half-life sufficiently short, relative to the time scale of cell cycle withdrawal, to make it possible to determine DHFR promoter activity from luciferase enzyme activity. (CAT enzyme has a relatively long half-life making it necessary, as was done above, to measure mRNA levels.)

Muscle cells were stably transformed with a (–270/+20)DHFR promoter/luciferase gene containing either a wildtype or mutated E2F site. As measured by luciferase activity, wildtype DHFR promoter activity decreased five-fold by 16 hours after inducing cell cycle withdrawal by mitogen removal (Fig. II.3). In contrast, mutated DHFR promoter activity remained essentially unchanged throughout the course of the experiment. Interestingly, the wildtype and mutated E2F site-containing DHFR promoters, when fused to the luciferase reporter gene, had essentially the same absolute activities in proliferating cells. This is in stark contrast to the

Figure II.2. E2F site requirement for CAT mRNA regulation in DHFR promoter/CAT muscle cell transformants induced to withdraw from the cell cycle. Transformants described in Fig. II.1 were grown in brain extract medium and induced to withdraw from the cell cycle by switching cells to mitogen-free medium. An 18-h induction period was used. Total RNA (25 $\mu$g) from either proliferating myoblasts (mb) or postreplicative myocytes (mc) was analyzed for CAT mRNA (A) or DHFR mRNA (B) as in Fig. II.1. Band intensities were quantitated by laser densitometry and were used to calculate absolute mRNA levels (based on pseudo-mRNA standards). Arrows designate expected mobilities of fragments protected by CAT and DHFR message.

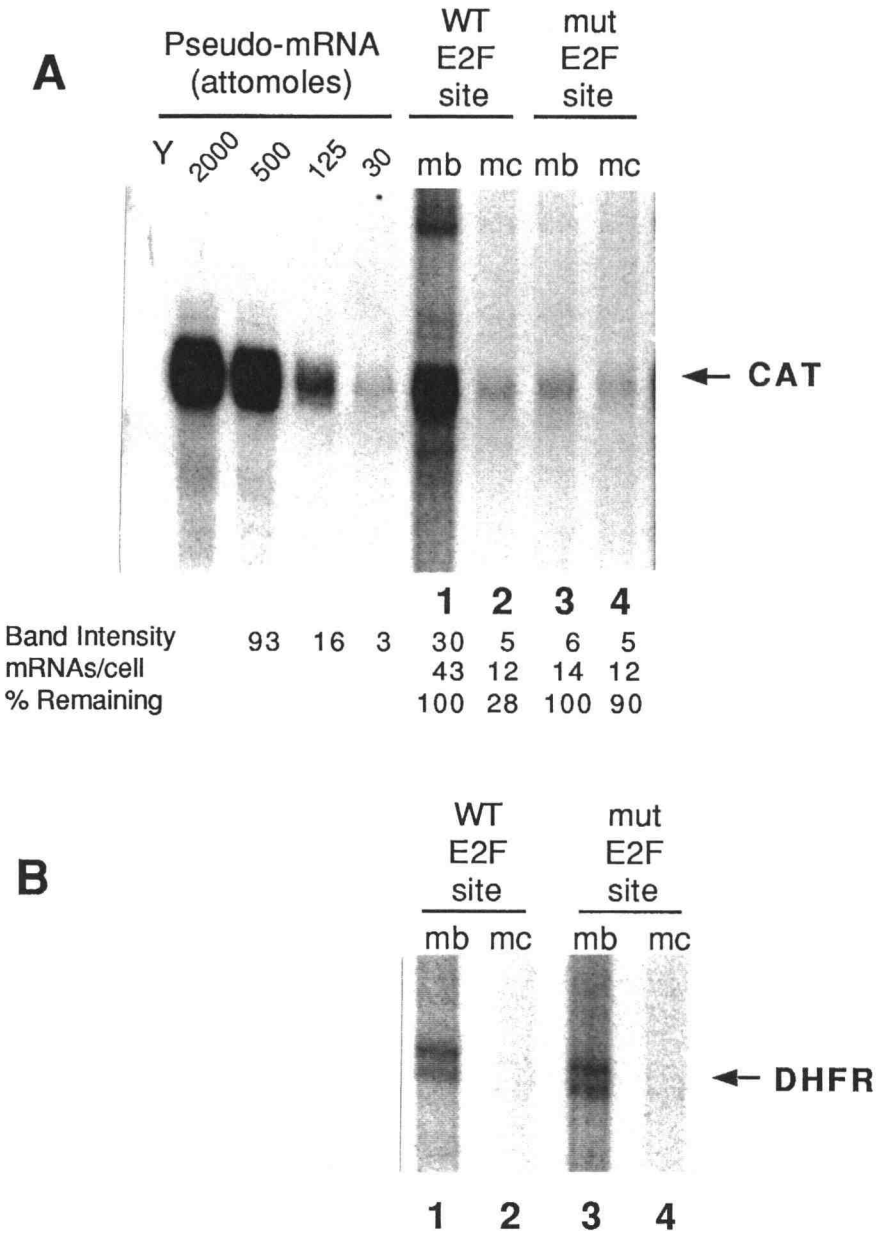
A
Pseudo-mRNA (attomoles)
WT E2F site
mut E2F site
Y
2000
500
125
30
mb
mc
mb
mc
CAT
1
2
3
4
Band Intensity
93
16
3
30
5
6
5
mRNAs/cell
43
12
14
12
% Remaining
100
28
100
90
B
WT E2F site
mut E2F site
mb
mc
mb
mc
DHFR
1
2
3
4

Figure II.2

Figure II.3. E2F site requirement for luciferase activity regulation in DHFR promoter/luciferase muscle cell transformants induced to withdraw from the cell cycle by FGF removal. Cells transformed with DHFR promoter/luciferase reporter genes containing either a wildtype or mutated E2F site were grown in brain extract medium and induced to withdraw from the cell cycle by switching to mitogen-free medium. At indicated times thereafter, cultures were harvested and assayed for luciferase activity. Activity was normalized to protein content and represented as a percentage of initial levels.

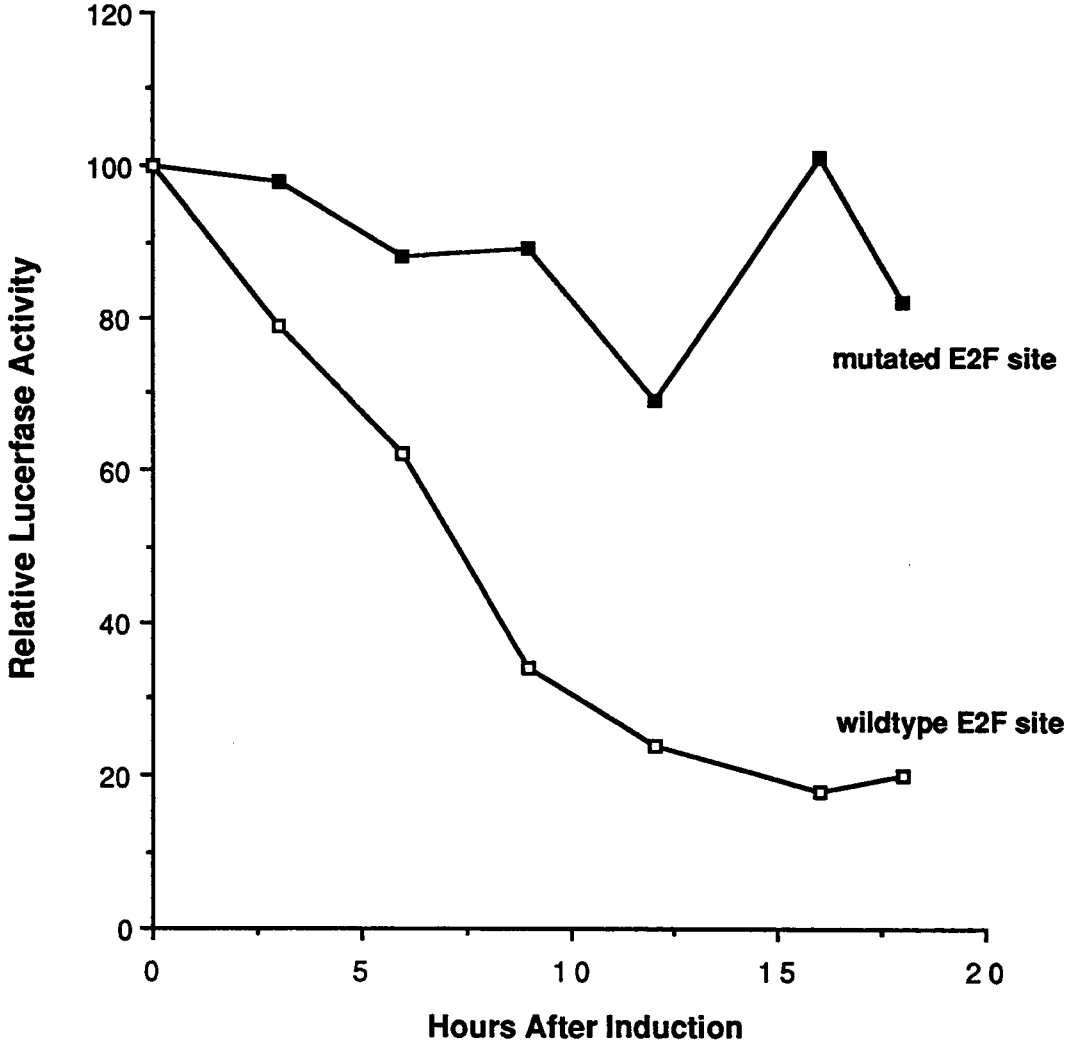
120
100
80
60
40
20
0
Relative Lucerfase Activity
0
5
10
15
20
Hours After Induction
mutated E2F site
wildtype E2F site

Figure II.3

DHFR promoter/CAT reporter gene, where mutation of the E2F site resulted in significantly reduced expression in proliferating cells (Figures II.1a and II.2a). This seeming incongruity has been observed in other experimental systems (Blake and Azizkhan, 1989; Means et al., 1992) and may reflect properties of the reporter genes (Forsberg et al., 1994; also see Chapter 1).

On the basis of CAT mRNA and luciferase activity measurements, using two methods to induce withdrawal from the cell cycle, we conclude that DHFR promoter activity was regulated as muscle cells withdrew from the cell cycle and that regulation was dependent on an intact E2F site. The above reporter gene experiments indicated that the E2F site was necessary for linking DHFR promoter activity with the growth state of the cell.

## DP1 and E2F1 transactivation of DHFR promoter activity

Recently, a number of transcription factors have been cloned that specifically bind E2F sites and appear to play a role in regulating E2F site-containing promoters. Two of the first E2F site-binding proteins identified, DP1 and E2F1, can activate E2F site-containing promoters in transient transfection assays (Bandara et al., 1993; Krek et al., 1993). To test whether DP1 and E2F1 affect DHFR promoter activity in muscle cells, a DHFR promoter/reporter plasmid was cotransfected with either DP1 or E2F1 expression plasmids into differentiated muscle cells in a transient transfection assay.

As shown in Fig. II.4, the expression level of a (−270/+20)DHFR promoter/luciferase gene was 5.4-fold higher in proliferative myoblasts as compared to differentiated myocytes. Transfection of differentiated myocytes with the plasmid MCKpDP1, that encodes the mouse DP1 cDNA under the control of the mouse muscle creatine kinase promoter, induced DHFR promoter activity 11.5-fold relative to control myocytes transfected with a plasmid containing only the MCK promoter. Ectopic DP1 expression also induced a DHFR promoter/luciferase gene containing a

Figure II.4. Transactivation of DHFR promoter activity by DP1 and E2F1 in differentiated muscle cells. Myoblasts were induced to differentiate by allowing FGF depletion. Two days after induction, myocytes were cotransfected with 2 mg of a plasmid encoding a (−270/+20)DHFR promoter/luciferase gene containing either a wildtype or mutated E2F site and 8 mg of either a control plasmid or a DP1 or E2F1 expression plasmid. Cells were harvested 20 hours after transfection and assayed for luciferase activity. To compare DHFR promoter activity in proliferative and differentiated muscle cells, myoblasts were transiently cotransfected with a (−270/+20)DHFR promoter/luciferase gene and the plasmid RSVpCAT encoding the bacterial CAT gene under the control of the Rous Sarcoma Virus promoter (Schmidt et al., 1990). Following transfection, myoblasts were either refed FGF (proliferative) or conditioned media (differentiated) and harvested two days later. Luciferase activity was normalized to protein content. The values shown represent the mean ± one standard deviation for four independent transfections and are normalized to control cell levels, arbitrarily set at 1.

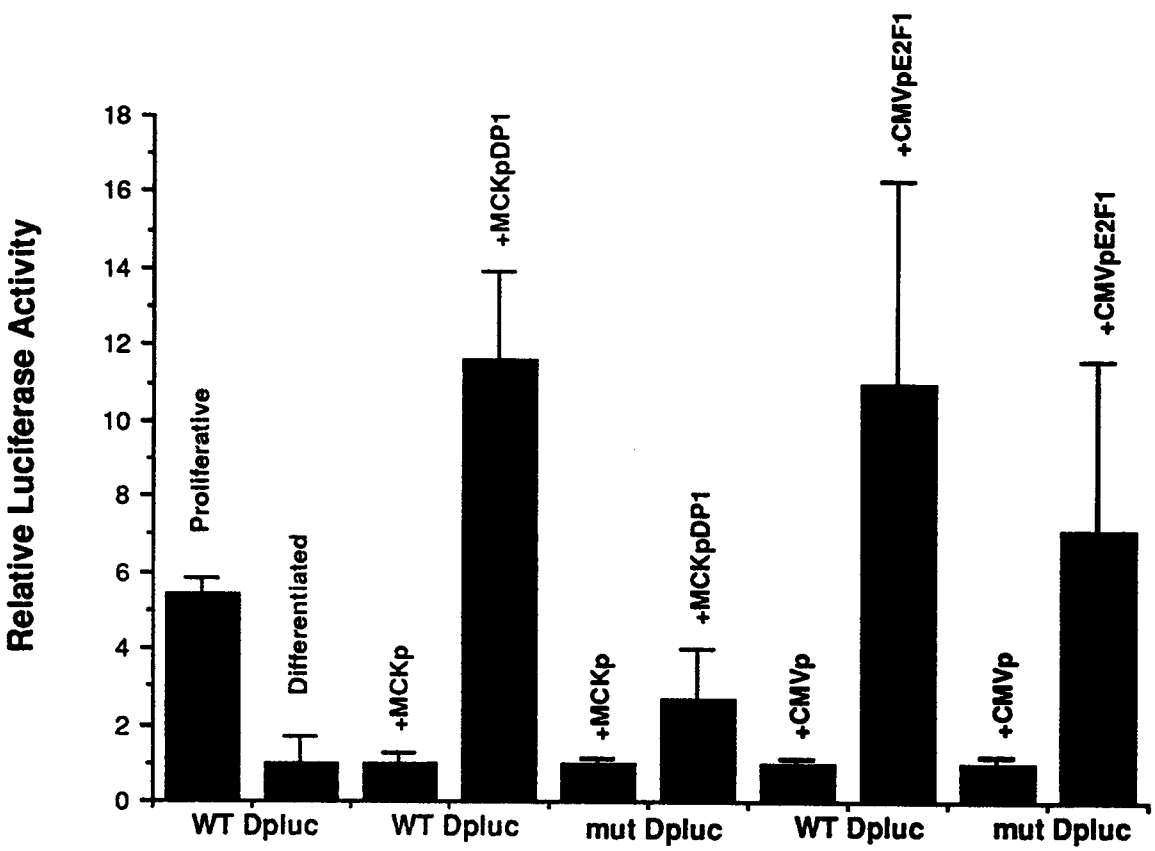
Relative Luciferase Activity
18
16
14
12
10
8
6
4
2
0
Proliferative
Differentiated
+MCKp
+MCKpDP1
+MCKp
+MCKpDP1
+CMVp
+CMVpE2F1
+CMVp
+CMVpE2F1
WT Dpluc
WT Dpluc
mut Dpluc
WT Dpluc
mut Dpluc

Figure II.4

mutated E2F site, but to a much lower extent (2-fold) then the wildtype promoter. Similar to DP1, the plasmid CMVpE2F1, encoding the mouse E2F1 cDNA under the control of the cytomegalovirus promoter, induced DHFR promoter activity 11-fold relative to a control plasmid containing only the CMV promoter. Unlike DP1, ectopic E2F1 expression also significantly induced a DHFR promoter/luciferase gene containing a mutated E2F site (7-fold induction relative to a control plasmid containing only the CMV promoter). Induction of a mutated E2F site-containing DHFR promoter may indicate that ectopic E2F1 expression in differentiated muscle cells can increase DHFR promoter activity in a non-E2F site-dependent fashion. Alternatively, transient transfections may result in such high intracellular levels of E2F1 that sufficient E2F1 binding still occurs on a mutated E2F site. We conclude that ectopic expression of the E2F site binding proteins DP1 and E2F1 can override the regulatory processes that repress DHFR promoter activity in differentiated muscle cells.

To test further whether DP1 overexpression would affect DHFR promoter activity during myogenic withdrawal from the cell cycle, muscle cells were stably cotransformed with a (−850/+16)DHFR promoter/CAT gene and a plasmid, MTpDP1, that encodes the mouse DP1 cDNA under the control of the mouse metallothionein promoter. In control transformants, CAT mRNA levels decreased 6.5-fold by 16 hours after inducing cell cycle withdrawal by mitogen removal (Fig. II.5). In contrast, in MTpDP1 transformants, CAT mRNA levels did not decrease significantly by 16 hours after inducing cell cycle withdrawal.

The metallothionein promoter is zinc-inducible (Mayo et al., 1981), the effect of zinc on CAT mRNA levels in MTpDP1 and control transformants was therefore investigated. As shown in Fig. II.5, addition of zinc at 16 hours after induction had no effect on CAT mRNA levels at 3 and 6 hours after zinc addition in either transformant population. Therefore, we conclude that ectopic DP1 expression renders the DHFR

Figure II.5. DP1 deregulation of a DHFR promoter/CAT gene in early passage stable muscle cell transformants. Muscle cells were cotransformed with the (–850/+16)DpCAT reporter gene, and either a Bluescribe control plasmid (non-DP1) or the DP1-encoding plasmid MTpDP1 (DP1). Cells were grown in brain extract medium, and total RNA was isolated at indicated times after cultures were switched to mitogen-free medium. At 16 hours after induction, cultures were exposed to 60 $\mu$M $ZnCl_2$. CAT mRNA levels were measured by RNase protection as described in Fig. II.1. Band intensities were quantitated by laser densitometry. Arrow designates expected 148-residue fragment protected by CAT mRNA. *Msp*1-digested SP64 molecular weight markers are shown in lane M, a dilution of undigested probe in lane P, and a negative control digestion with yeast RNA in lane Y.

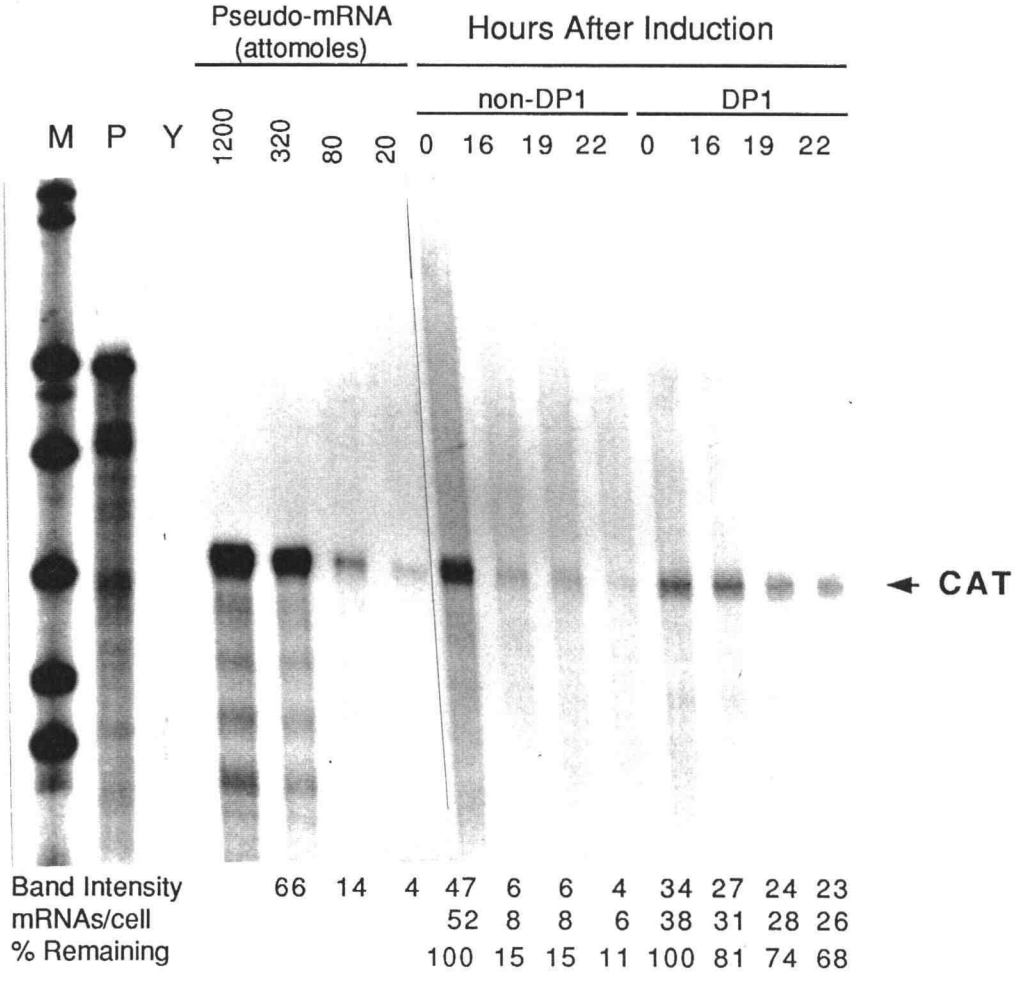
Pseudo-mRNA (attomoles)
Hours After Induction
non-DP1
DP1
M P Y
1200 320 80 20
0 16 19 22 0 16 19 22
CAT
Band Intensity 66 14 4 47 6 6 4 34 27 24 23
mRNAs/cell 52 8 8 6 38 31 28 26
% Remaining 100 15 15 11 100 81 74 68

Figure II.5

promoter insensitive to the cellular mechanisms responsible for repressing promoter activity during myogenesis.

Similar results were observed in myoblasts stably cotransformed with MTpDP1 and a (−270/+20)DHFR promoter/luciferase gene. In the experiment shown in Fig. II.6a, DHFR promoter activity, as measured by luciferase activity, decreased four-fold in control transformants by 12 hours following myogenic induction. In contrast, DHFR promoter activity did not decrease in MTpDP1 transformants throughout the course of the experiment. (The initial increase in luciferase activity of the MTpDP1 transformants in the experiment shown was not reproducible.)

Although the data in Fig. II.6a show that ectopic DP1 expression deregulated DHFR promoter activity at early passages after DNA transformation, with subsequent passages, a regulated pattern of expression was restored. For example, as shown in Fig. II.6b, by four-passages after DNA transformation (24 cell generations), DHFR promoter activity was as tightly regulated in MTpDP1 transformants as in controls. The phenomenon of initial deregulation of DHFR promoter activity followed by wildtype-like regulation was observed in three separate polyclonal MTpDP1 transformant populations. The most likely explanation of evolution towards the regulated phenotype is that those transformants expressing low levels of DP1 have a growth advantage over those expressing higher levels, and with extended passaging, cells with subactivating levels of DP1 predominate. This suggests that ectopic expression of DP1 not only is sufficient to deregulate DHFR promoter activity during myogenic withdrawal from the cell cycle but also has an effect, albeit a negative one, on the physiology of replicating cells.

Figure II.6. Restoration of DHFR promoter activity regulation after serial passaging of MTpDP1 transformants. Muscle cells cotransformed with the (−270/+20)DHFR promoter/luciferase reporter gene, and either a Bluescribe control plasmid (non-DP1) or the DP1-encoding plasmid MTpDP1 (DP1) were grown in FGF-rich medium and analyzed for luciferase activity at indicated times after cultures were switched to FGF-free medium. (A) DHFR promoter activity 16 cell generations after transformation. (B) DHFR promoter activity 24 cell generations after transformation.

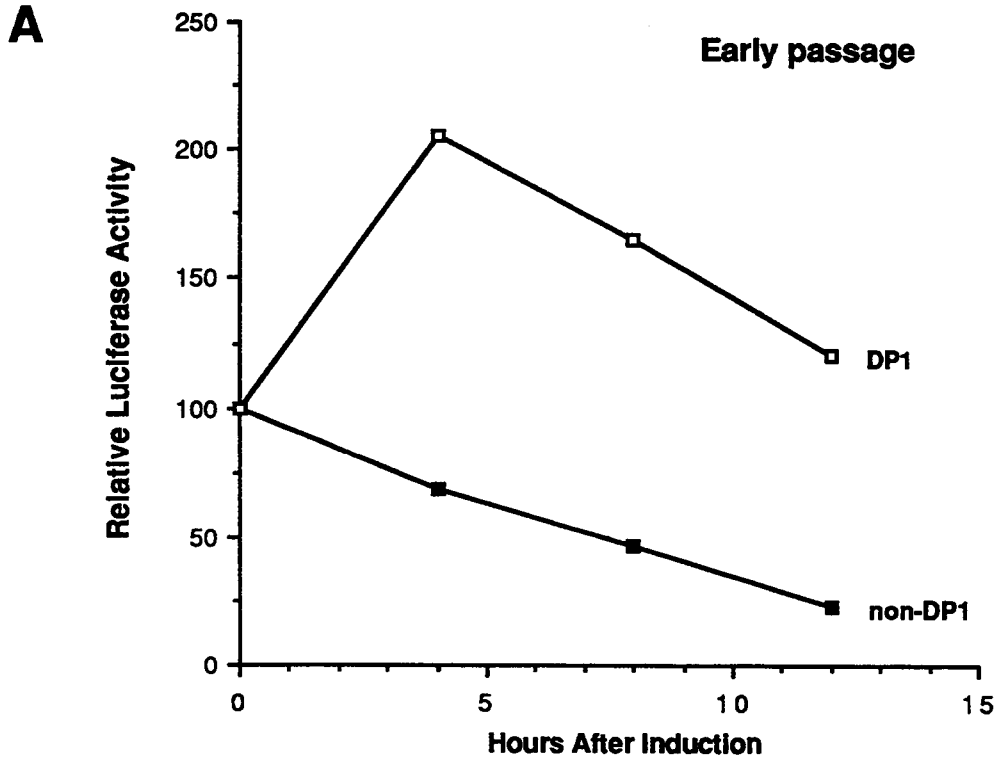
A
Early passage
Relative Luciferase Activity
250
200
150
100
50
0
DP1
non-DP1
0
5
10
15
Hours After Induction

Figure II.6A

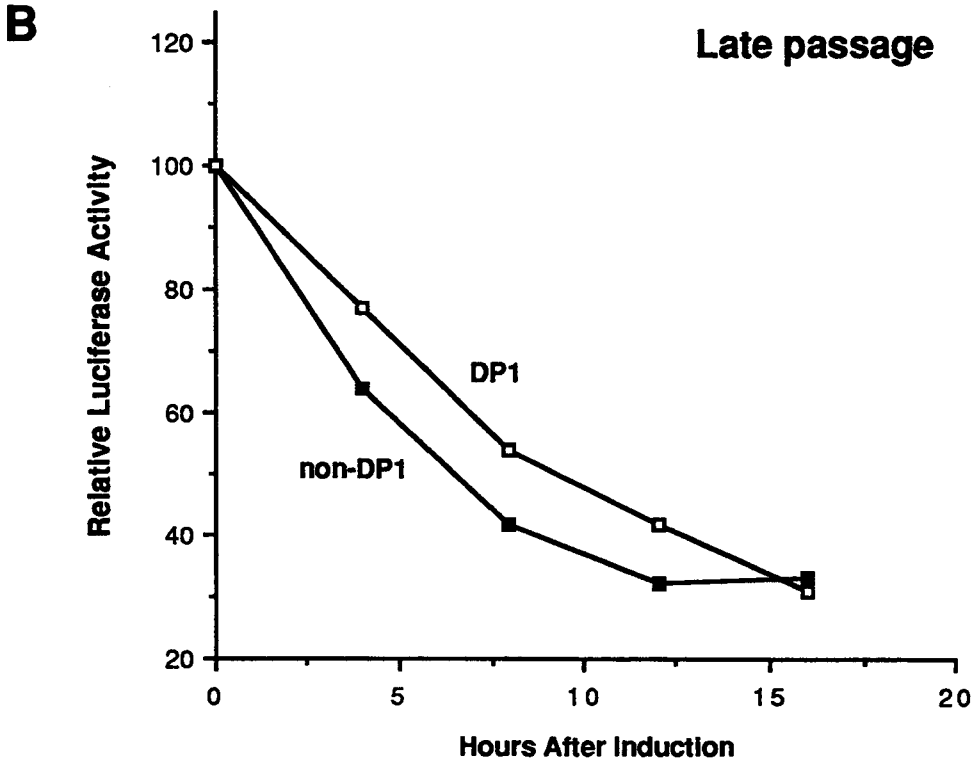
B
Late passage
120
100
80
60
40
20
Relative Luciferase Activity
DP1
non-DP1
0
5
10
15
20
Hours After Induction

Figure II.6B

## DP1 and E2F1 mRNA levels during myogenesis

DP1 and E2F1 mRNA levels have been reported to increase 5- and 15-fold, respectively, near the G1/S boundary in serum-stimulated quiescent fibroblasts (Slansky et al., 1993; Li et al., 1994). Induction of DP1 and E2F1 mRNA levels during reentry into the cell cycle coincides with increased DHFR promoter activity (Li et al., 1994). Therefore, it has been proposed that DP1 and E2F1 activity is regulated at the transcriptional level and that the decrease in DP1 and E2F1 mRNA levels in quiescent fibroblasts is the basis for reduced DHFR promoter activity.

To investigate whether reduced DHFR promoter activity in postreplicative muscle cells is accompanied by reduced DP1 and E2F1 mRNA levels, an RNase protection assay was developed to measure DP1 and E2F1 mRNA. Fig. II.7a shows CAT mRNA levels from proliferating and postreplicative muscle cells stably transformed with a DHFR promoter/CAT gene. The same RNA was analyzed for endogenous DP1 and E2F1 mRNA levels (Figs. II.7b and II.7c, respectively). In contrast to the almost five-fold decrease in CAT mRNA levels by sixteen hours after induction, DP1 and E2F1 message levels remained relatively constant. Thus, reduced reporter gene expression was not accompanied by a reduction in DP1 or E2F1 mRNA levels. The lack of a correlation in the regulation of reporter gene mRNA levels and DP1 and E2F1 mRNA levels is consistent with a model in which the activity of these transcription factors was not governed by transcriptionally mediated changes in the levels of DP1 and E2F1 protein.

## E2F binding activity during myogenesis

In contrast to the model proposing that the activities of DP1 and E2F1 are regulated at the transcriptional level (described above), an alternative model proposes that E2F site-containing promoters are regulated by inhibitory proteins that associate with E2F site-bound transcription factors. Zhu et al. (1993) and Cobrinik et al. (1993) have reported an inverse relationship between the

Figure II.7. Levels of CAT, DP1 and E2F1 transcripts in DHFR promoter/CAT muscle cell transformants during myogenesis. Myoblasts were grown in brain extract medium and induced to withdraw from the cell cycle by switching cells to mitogen-free medium. Total RNA was harvested at 0, 8, and 16 hours after induction and analyzed by RNase protection. (A) CAT mRNA levels were measured as described in Fig. II.1. (B) DP1 mRNA levels were measured using a riboprobe complementary to a 212-nucleotide region near the 3' end of the mouse DP1 mRNA. (C) E2F1 mRNA levels were measured using a riboprobe complementary to a 216-nucleotide region near the 3' end of mouse E2F1 mRNA. The expected protected fragments are denoted by an arrow. CAT, DP1 and E2F1 band intensities were quantitated by laser densitometry and are reported as a percentage of proliferative levels.

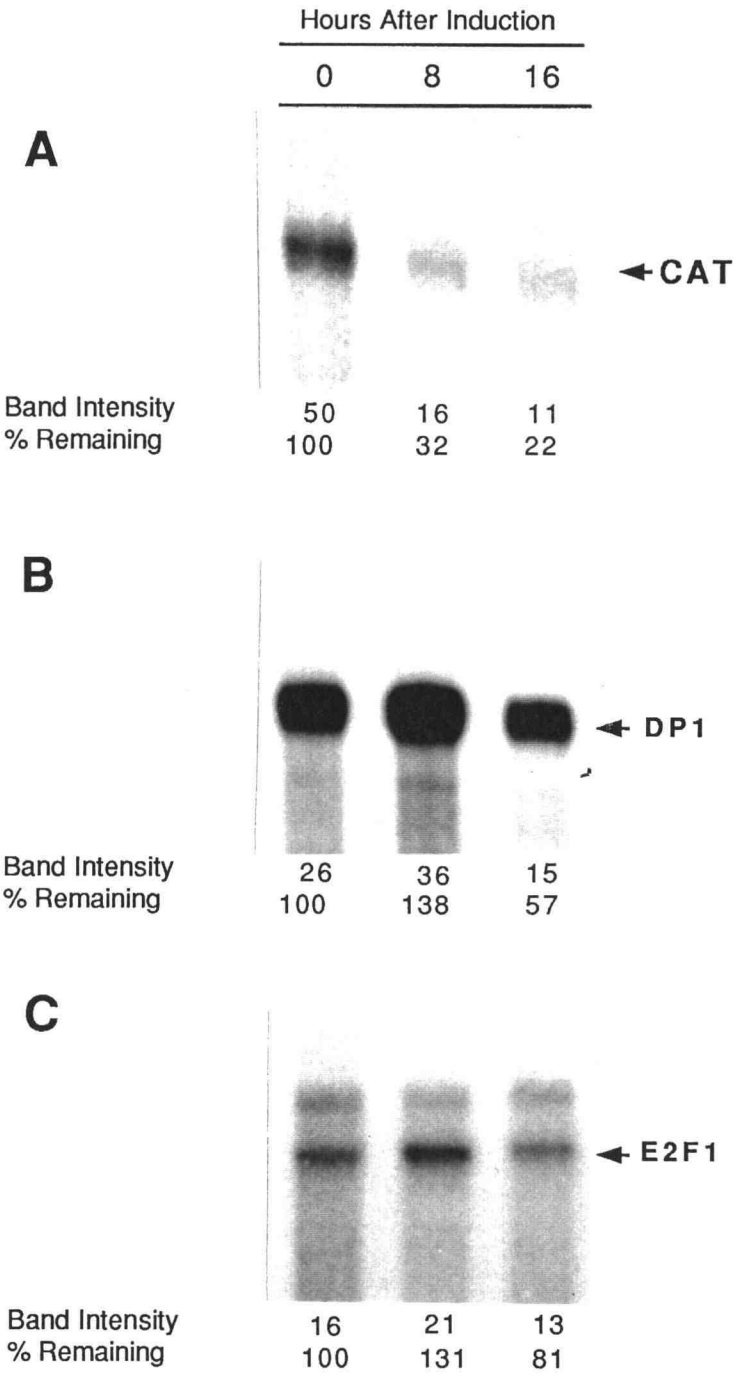
Hours After Induction
0
8
16
A
CAT
Band Intensity
% Remaining
50
100
16
32
11
22
B
DP1
Band Intensity
% Remaining
26
100
36
138
15
57
C
E2F1
Band Intensity
% Remaining
16
100
21
131
13
81

Figure II.7

presence of inhibitory proteins in multimeric E2F site complexes and the activity of E2F site-containing promoters. Cell extracts contain two types of E2F binding activity as observed in electrophoretic mobility shift assays (or band shift assays as the technique is commonly called). Faster migrating complexes contain dimerized E2F1/DP1-like transcription factors and slower migrating multimeric complexes contain, in addition to E2F1/DP1-like proteins, Rb-like proteins, cyclins and cyclin-dependent kinases (Bandara et al., 1991; Devoto et al., 1992; Mudryj et al., 1991; Hiebert et al., 1992). In serum-stimulated quiescent fibroblasts, the appearance of dimeric E2F1/DP1-like E2F site binding activity *in vitro* has been correlated with the *in vivo* activation of E2F site-containing promoters (Mudryj et al., 1991; Hiebert et al., 1992; Dou et al., 1994).

To monitor E2F activity in muscle cells, we used assay conditions identical to those of Bandara et al. (1991), who have carried out extensive characterization of the dimeric and multimeric E2F site binding complexes in extracts from mouse F9 cells and other cell types. In the experiment shown (Fig. II.8), a radiolabeled E2F site-containing oligonucleotide was incubated with whole cell extracts prepared from myoblasts and, as a positive control, F9 embryo carcinoma cells. Binding activity with mobilities characteristic of both dimeric and multimeric E2F complexes was observed. The specificity of both binding activities was demonstrated by including an excess of unlabeled competitor oligonucleotide in the binding reactions. An oligonucleotide containing a wildtype E2F site competed for binding (lanes 4 and 8), an oligonucleotide containing a mutated E2F site did not (lanes 5 and 9). Bandara and La Thangue (1991) have shown that the detergent desoxycholate (DOC) disrupts the protein-protein interactions of multimeric E2F complexes but not the protein-DNA interaction of E2F1/DP1 dimeric E2F complexes. The majority of E2F binding activity in muscle and F9 cell extracts was resistant to the DOC, suggesting that it represents dimeric E2F1/DP1-like

Fig. II.8. E2F site binding activity in mouse muscle cell and F9 embryo carcinoma cell extracts. Whole cell extracts were prepared under conditions that preserve the association of E2F1/DP1 with other proteins such as Rb, p107 and cyclin/CDK complexes (Bandara et al., 1991). Equal amounts of extract protein (8 mg) were assayed in each sample. A 40-fold molar excess of unlabeled oligonucleotide containing either a wildtype (WT) or mutated (mut) E2F site was included as a competitor as indicated. The detergent desoxycholate (DOC), which disrupts the association of E2F1/DP1-like proteins with other proteins such as Rb, p107 and cyclin/CDK complexes (Bandara et al., 1991), was included as indicated.

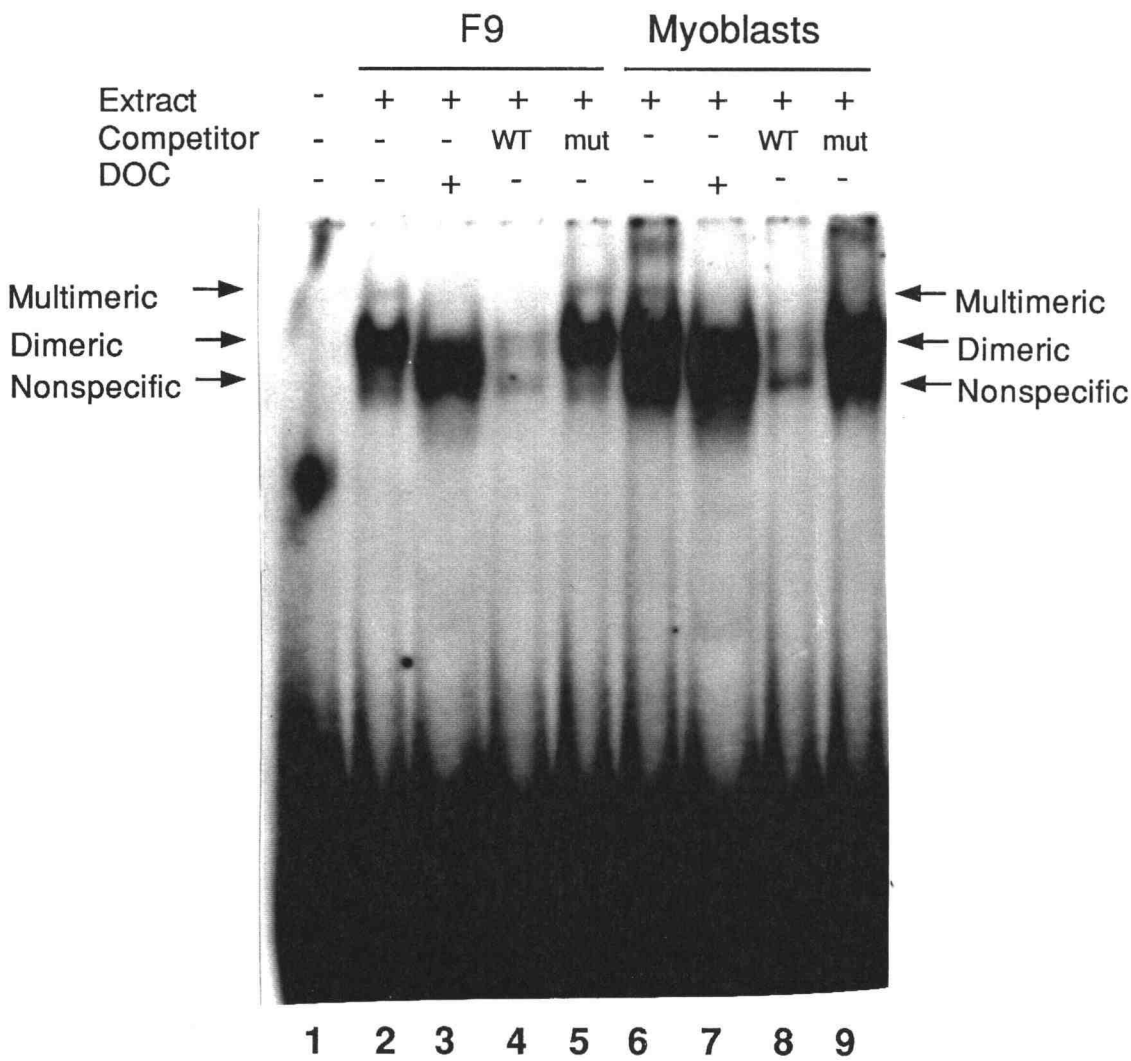
F9
Myoblasts
Extract - + + + + + + + +
Competitor - - - WT mut - - WT mut
DOC - - + - - - + - -
Multimeric
Dimeric
Nonspecific
Multimeric
Dimeric
Nonspecific
1 2 3 4 5 6 7 8 9

Figure II.8

proteins. The faint slower-migrating band observed in muscle cell and F9 extracts was DOC-sensitive, suggesting that it represents the Rb-containing multimeric E2F complex. Therefore, we conclude that muscle cell extracts contain E2F site binding activity characteristic of E2F1/DP1-like dimers and E2F1/DP1/Rb-like multimers.

In order to test whether E2F site binding activity changes in a growth-dependent fashion and can be correlated with reduced DHFR promoter activity in postreplicative muscle cells, whole cell extracts were prepared from (-270/+20)DHFR promoter/luciferase transformants at various times after inducing cell cycle withdrawal. Parallel cultures were assayed for luciferase activity in order to compare E2F binding activity with DHFR promoter activity.

As shown in Fig. II.9a, the absolute and relative levels of dimeric E2F binding activity remained constant as muscle cells withdrew from the cell cycle. The levels of multimeric E2F complexes did not increase. In extracts prepared from parallel cultures, luciferase activity decreased five-fold 15 hours after induction (Fig. II.9b).

The lack of a correlation between multimeric E2F complexes and low DHFR promoter activity suggests that the formation of such complexes on E2F sites is not required for the growth-specific repression of E2F site-containing promoters. Alternatively, only a subset of E2F activity may be involved in DHFR promoter regulation and be subject to regulation. Binding of inhibitory proteins to the regulatory subset of E2F activity may not be detectable against a background of nonregulatory cellular E2F binding activity.

Figure II.9. E2F site binding activity and luciferase reporter gene activity in muscle cells induced to withdraw from the cell cycle. Myoblasts transformed with the DHFR promoter/luciferase reporter gene were grown in brain extract medium and harvested at indicated times after switching cells to mitogen-free medium. (A) Band shift assay. Extracts were prepared and assayed as in Fig. II.8. Equal amounts of extract protein (8 mg) were assayed in each sample. A 40-fold molar excess of unlabeled oligonucleotide containing either a wildtype (WT) or mutated (mut) E2F site was included as a competitor as indicated. Band intensities were determined using a PhosphorImager and were normalized relative to the proliferative signal. (B) Comparison of dimeric E2F site binding activity and luciferase reporter gene activity. Luciferase activity, assayed in parallel cultures, and represented as a percentage of initial levels, was plotted alongside the dimeric E2F binding activity measured in panel A. Vertical bars represent one standard deviation for four determinations for luciferase activity and the range of two determinations for dimeric E2F binding activity.

**A**

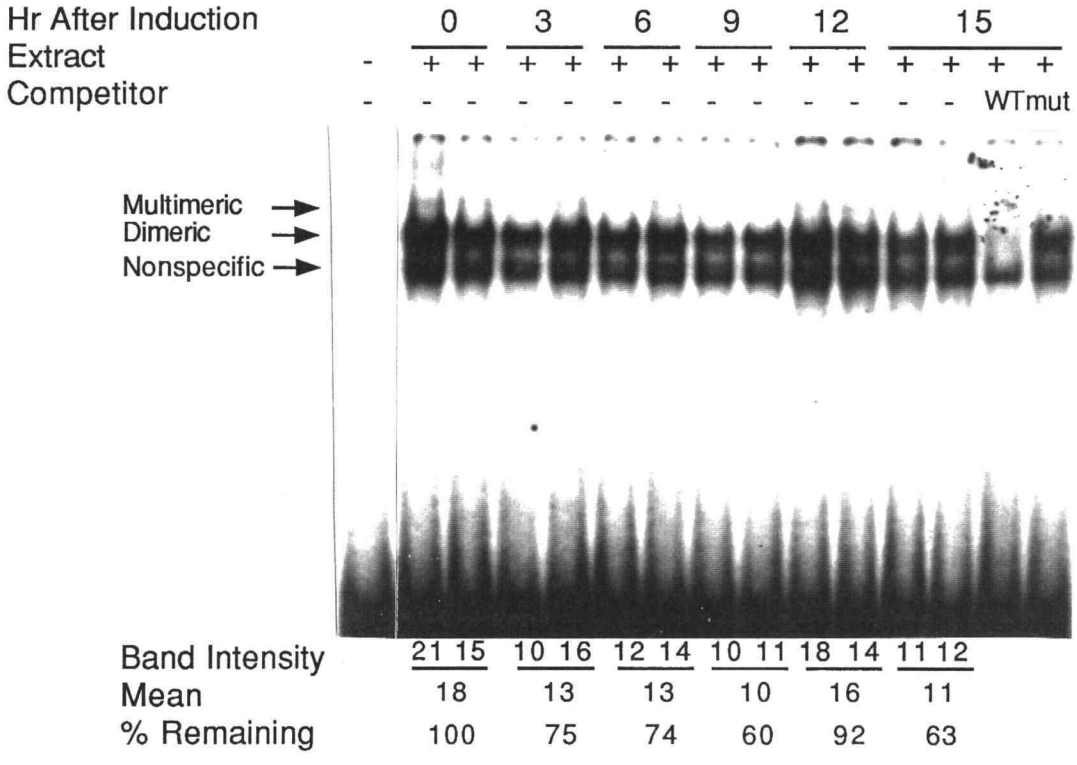


Figure II.9A

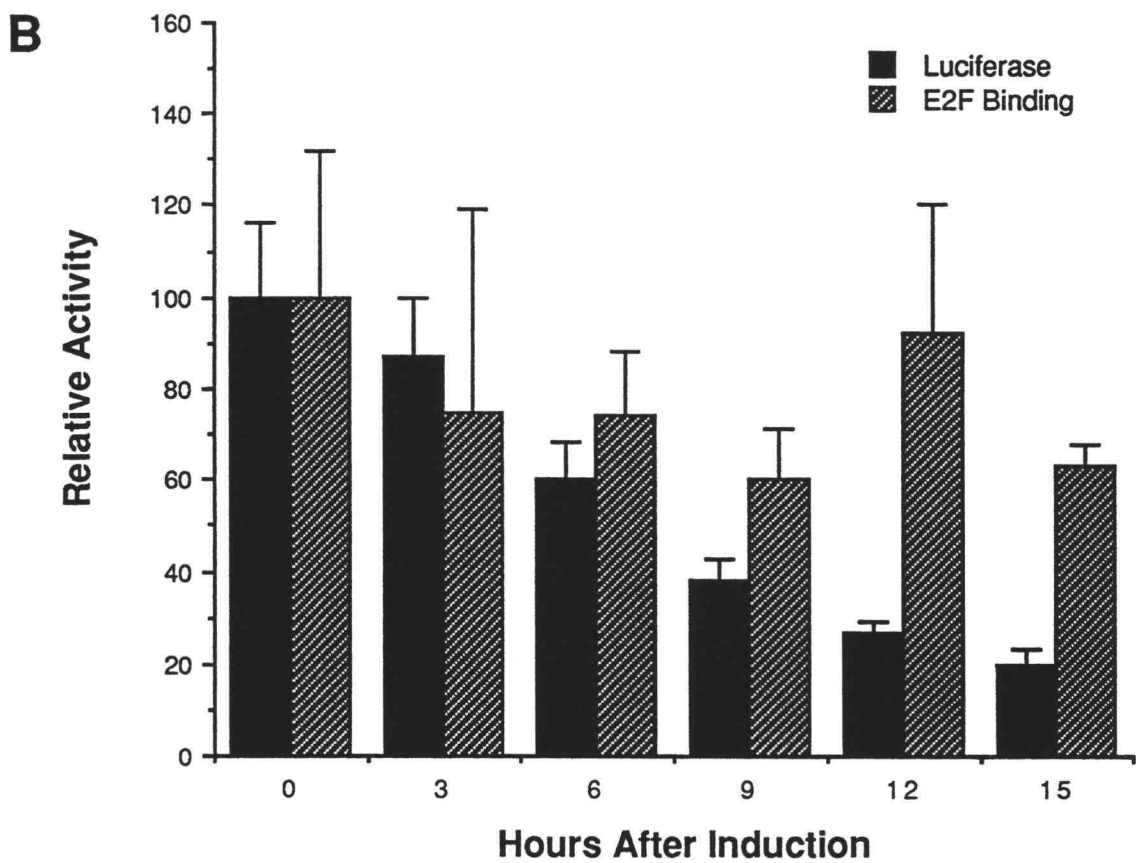
B
160
140
120
100
80
60
40
20
0
Relative Activity
Luciferase
E2F Binding
0
3
6
9
12
15
Hours After Induction

Figure II.9B

## Discussion

In serum-stimulated quiescent fibroblasts, an intact E2F site is required for increased DHFR promoter activity near the G1/S boundary (Means et al., 1992). We have demonstrated that an intact E2F site was also required for the repression of DHFR promoter activity in muscle cells induced to withdraw from the cell cycle. Therefore, the same element mediates promoter repression in both metabolically-depressed quiescent fibroblasts and metabolically-active postreplicative muscle cells.

A number of transcription factors have recently been cloned that specifically bind the E2F site (reviewed in La Thangue, 1994). The E2F site-binding transcription factors so far characterized can be classified into two separate families based on their similarities to either E2F1 or DP1 (Helin et al., 1992; Kaelin et al., 1992; Girling et al., 1993). We showed that both DP1 and E2F1 transactivated a DHFR promoter/reporter gene in differentiated muscle cells and that full transactivation was dependent on an intact E2F site.

Although the mechanism of how these transcription factors regulate promoter activity is currently not completely understood, recent reports indicate that DP1 and E2F1 activity may be controlled by both transcriptional and posttranslational processes. Li et al. (1994) showed that E2F1 mRNA levels increased 15-fold near the G1/S boundary in serum-stimulated quiescent fibroblasts and DP1 transcripts increased 5-fold. Since the activation kinetics of a DHFR promoter/luciferase gene during cell cycle reentry more closely matched increased E2F1 mRNA levels, the authors proposed that E2F1 protein levels may be rate limiting for DHFR promoter activity during late G1/early S. In further support of the model, the same authors showed that constitutive expression of an E2F1 gene results in a greater than 20-fold increase in DHFR promoter activity in quiescent fibroblast, thereby masking the increase in DHFR promoter activity normally observed upon serum stimulation (Slansky et al., 1993).

Unlike the mRNA levels of a DHFR promoter/CAT gene, E2F1 and DP1 mRNA levels did not significantly decrease during myogenic cell cycle withdrawal. The difference between our results and those mentioned above (Slansky et al., 1993), in which E2F1 and DP1 mRNA levels were low in noncycling cells and 5 to 15-fold higher in cycling cells, may be due to the fact that the mRNA measurements of noncycling fibroblasts were done approximately two days after cell cycle withdrawal (Slansky et al., 1993; Li et al., 1994). In contrast, we measured E2F1 and DP1 transcript levels at 8 and 16 hours after inducing cell cycle withdrawal. A slow decrease in E2F1 and DP1 mRNA levels may eventually occur in differentiated muscle cells, but cannot account for the rapid decrease in DHFR promoter activity. Another possible explanation for the differing results between the muscle cell differentiation and serum-starved fibroblast systems, is that the context in which cell cycle withdrawal occurs is vastly different in the two experimental systems. In differentiating muscle cells, the large many-fold decrease in DHFR mRNA levels occurs against a background of great metabolic activity. In contrast, fibroblasts induced to withdraw from the cell cycle by serum starvation undergo a general repression of all metabolic activity and a reduction in nearly all gene expression. Therefore it is not surprising that DHFR, E2F1, and DP1 mRNA levels are all lower in quiescent fibroblasts compared to metabolically active replicating cells. In any case, based on mRNA measurements, we conclude that a transcriptionally-mediated decrease in E2F1 and DP1 protein levels cannot account for the decrease in DHFR promoter activity in postreplicative muscle cells. If E2F1 and DP1 activity are regulated during myogenesis, regulation must occur posttranscriptionally.

Other mechanisms regulating E2F activity have been proposed based on the identification of a number of proteins that have been shown to form multimeric complexes on the E2F site *in vitro* in a growth-dependent fashion. Recently Li et al. (1994) demonstrated that the majority of the E2F binding activity in serum-starved quiescent fibroblasts exists as multimeric complexes that have been shown by others to

contain Rb-like proteins and E2F site-binding transcription factors. Upon reentering the cell cycle from the quiescent state, the majority of the E2F binding activity appears as faster migrating complexes that are thought to represent dimeric E2F site-bound transcription factors (Hiebert et al., 1992; Li et al., 1994). Rb-like proteins are thought to play an inhibitory role in controlling E2F site-containing promoters (described more fully in Chapter 3), as activation of E2F site-containing promoters is correlated with the appearance of dimeric E2F site binding activity. We expected to observe an increase in multimeric E2F complexes in extracts prepared from muscle cells that had been induced to withdraw from the cell cycle. Contrary to expectations, no increase in multimeric E2F site binding activity was observed, even though in parallel assays DHFR promoter activity showed the usual 5-fold decrease. As a control to insure that the extraction and/or binding conditions used did not disrupt multimeric E2F complexes, whole cell extracts prepared from F9 embryonic carcinoma cells were assayed for E2F binding activity. The F9 band shifts matched the published observations made by others (Bandara et al., 1991). The F9 extracts contained a faint, slower-migrating E2F complex that was detergent sensitive and comigrated with the slower-migrating muscle cell E2F complex. Therefore we conclude that the conditions used in this study were compatible with the formation of stable E2F complexes.

However, it should be noted that E2F binding activity varies greatly among primary and permanent cell lines (Bandara and La Thangue, 1991; Chittenden et al., 1993). In Chapter 3, *in vivo* biological evidence will be presented that strongly suggests that Rb-like proteins do in fact play a role in the repression of DHFR promoter activity during myogenesis. Perhaps only a subset of E2F complexes are relevant with respect to DHFR promoter regulation and changes in the ratio of multimeric to dimeric complexes in the physiologically relevant subset occur, but are present in such low amounts, either within the cell or following the extraction procedure, as to be beyond the levels of detection in band shift assays.

In summary, our results establish that myogenic repression of DHFR promoter activity is dependent on an intact E2F binding site. Furthermore, our data are consistent with a model in which the activities of DP1- and E2F1-like transcription factors are repressed in postreplicative cells, but such repression is not associated with a decrease in DP1 or E2F1 mRNA, a decrease in dimeric E2F1/DP1-like DNA binding activity, or an increase in multimeric Rb/E2F1/DP1-like DNA binding activity.

# CHAPTER 3

## The Adenovirus E1a Oncoprotein Antagonizes E2F Site-Dependent Regulation of *DHFR* Promoter Activity During Myogenesis


### Abstract

Several genes preferentially expressed in proliferating cells are controlled by activating and inhibitory factors that associate with a specific promoter element called an E2F site. For example, the adenovirus E1a oncoprotein transactivates E2F site-containing promoters by displacing inhibitory retinoblastoma (Rb)-like proteins from the activating transcription factor E2F. To test whether Rb-like proteins repressed E2F site-containing promoters during myogenesis, myoblasts were stably cotransformed with a DHFR promoter/CAT gene and an E1a-encoding plasmid. Unlike reports using other muscle cell lines, E1a did not alter the competency of MM14D mouse myoblasts to withdraw from the cell cycle and form myotubes. In contrast to control transformants, CAT mRNA levels did not decrease during differentiation of E1a transformants. Additionally, E1a activated a DHFR promoter/luciferase gene when transiently transfected into differentiated muscle cells. Although the DHFR promoter/CAT gene was completely deregulated in the E1a transformants, the endogenous genes encoding DHFR, thymidine kinase (TK) and thymidylate synthase (TS) continued to show strong regulation. Band shift assays were used to monitor dimeric E2F1/DP1-like binding activity and multimeric Rb/E2F1/DP1-like binding activity in muscle cell transformants. The absolute and relative abundance of dimeric and multimeric E2F site-binding activity was unaltered in E1a transformants. The data demonstrate that E1a levels sufficient to deregulate DHFR promoter/reporter genes in differentiating muscle cells were not sufficient to deregulate endogenous E2F

site-containing genes, dissaggregate multimeric E2F site binding activity, or inhibit differentiation.

## Introduction

An intact E2F site is necessary for the repression of the mouse *DHFR* gene during myogenic withdrawal from the cell cycle (see Chapter 2). A number of cellular and viral gene products have been shown to participate in the E2F site-mediated control of gene expression. These E2F site-associated proteins include cyclins, cyclin-dependent kinases, E2F site-binding proteins, and tumor suppresser proteins such as the product of the retinoblastoma gene (*rb*).

Individuals with a germ-line mutation in one allele of the *rb* gene have a 95% chance of developing retinoblastoma tumors (reviewed in Gallie et al., 1991). Cells from retinoblastoma tumors show loss of heterozygosity at the *rb* locus, resulting in loss of the remaining functional allele. Since the absence of the gene product is associated with tumorgenesis, the *rb* gene product is believed to act in normal cells to constrain growth.

Recapitulating its clinical characteristics, the *rb* gene blocks cell cycle progression when introduced into cultured $rb^-$ tumor cells by either microinjection, viral infection, or transfection (Hinds et al., 1992; Huang, et al., 1988). Accumulating evidence suggests the *rb* gene product is involved in regulating the cell cycle during differentiation. For example, in transgenic $rb^{-/-}$ mice, which survive to about 12 days post-gestation, death appears to be at least partially due to a lack of differentiation of the hemopoietic and neuronal cell lineages (Jacks et al., 1992; Clarke et al., 1992). Fetal $rb^{-/-}$ mice develop histologically normal skeletal musculature, and furthermore, muscle cell lines established from such animals can be induced to form morphologically normal multinucleated myotubes (Schneider et al., 1994 and references therein). However, in contrasts to myocytes containing a

functional *rb* gene, *rb* $^{-/-}$ myonuclei could be induced with serum to reenter S phase (Schneider et al., 1994). S phase reentry suggests that Rb protein contributes to the maintenance of the terminally differentiated state.

E2F site-containing promoters are repressed in cells overexpressing Rb and the Rb-related protein p107 (Hiebert et al., 1992; Weintraub et al., 1992b; Zhu et al., 1993). Repression appears to require physical binding of Rb protein to E2F site-binding transcription factors (Flemington et al., 1993; Helin et al., 1993; Zamanian and La Thangue, 1992). Furthermore, the E2F1 binding domain and the growth suppression activity of Rb have been mapped to a region of Rb known as the pocket domain, which has been shown to be the site of almost all naturally occurring Rb loss-of-function mutations (Qin et al., 1992). The pocket domain of Rb and p107 is also the binding site of viral oncoproteins (DeCaprio et al., 1988; Dyson et al., 1989; Shirodkar et al., 1992). Adenovirus E1a proteins and SV40 large T antigen are able to overcome the growth suppression activity of Rb-like proteins, at least in part, by a mechanism mediated through the E2F-binding sites contained in the promoters of growth-specific genes (Hiebert et al., 1991; Zamanian and La Thangue, 1992). In electrophoretic mobility shift assays, E1a protein dissociates the multimeric complexes that bind E2F sites, leaving the E2F1/DP1 dimer as the only bound species (Bagchi et al., 1990; Bandara and La Thangue, 1991; Krek et al., 1993). The appearance of dimeric E2F1/DP1-like binding activity also correlates with the activation of E2F site-containing promoters in serum-stimulated quiescent fibroblasts (Dou et al., 1994; Hiebert et al., 1992; Mudryj et al., 1991). During early adenovirus infection, the release of what is believed to be transcriptionally-active E2F results in the induction of both cellular and viral genes that contain E2F sites in their promoters. The data to date suggest that E1a displaces Rb-like proteins from E2F1/DP1, thereby removing negative-acting factors from E2F-driven promoters.

Although Rb, p107 and p130, are closely related to one another structurally, they differ in their interactions with other regulatory proteins. For example, a complex containing p107, cyclin A, p33$^{cdk2}$, and E2F site-binding transcription factors forms in mid-S phase and the appearance of the complex is correlated with the repression of E2F site-containing promoters (Devoto et al., 1992). Originally it was thought that the sole substrate of the cyclin A-p33$^{cdk2}$ kinase was p107, since p107 became hyperphosphorylated in late S-phase when cyclin A levels are at their maximum and cyclin A-p33$^{cdk2}$ phosphorylates p107 *in vitro* (Peeper et al., 1993). Recent evidence suggest that the primary *in vivo* substrate of cyclin A-p33$^{cdk2}$ may be the E2F site-transcription factor DP1 (Krek et al., 1994). Phosphorylation of DP1 results in loss of DNA-binding activity and thus would neatly explain the reduction of E2F site promoter activity observed in mid-S phase of the cell cycle.

Repression of E2F site promoter activity during early G1 and $G_0$ (the term used for the cell cycle state of noncycling cells) is thought to be mediated by Rb and p130. Although p130 is believed to be the predominant pocket protein present in extracts prepared from G1/$G_0$ cells (Cobrinik et al., 1993), the following discussion will be limited to the much better characterized Rb protein. A substantial fraction of the E1a-dissociable multimeric E2F complexes detectable in G1/$G_0$ cell extracts contain underphosphorylated Rb (Chellappan et al., 1991). As the G1/S boundary is approached, Rb becomes progressively phosphorylated, which results in a loss of affinity for E2F1 and presumably other members of the E2F1 family of transcription factors. The *in vitro* appearance of dimeric E2F/DP1 correlates with the *in vivo* activation of E2F site promoters that occurs during late G1 (Hiebert et al., 1992), suggesting that phosphorylation is involved in regulating the activity of Rb during the cell cycle.

The phosphorylation of Rb is believed to be catalyzed by cyclin-dependent kinases (Cdk). Many of the phosphorylation sites of Rb fit the Cdk consensus site and

overexpression of cyclins can overcome Rb-dependent growth arrest in certain cell lines (Dowdy et al., 1993). The physiologically relevant Cdk responsible for Rb phosphorylation has not been determined, although *in vitro* studies have demonstrated that $p33^{cdk2}$ forms stable complexes with G1 cyclins E and D (reviewed in Sherr, 1993). A variety of evidence suggests that cyclin E regulates the entry into S phase. Cyclin E mRNA and protein levels start to rise in late G1 and reach a maximum just after the G1/S boundary. Overexpression of cyclin E in fibroblasts shortens the length of time cells spend in G1 and reduces the serum requirement for traversing the G1/S boundary (Ohtsubo and Roberts, 1993). Two cellular factors that interfere with the G1/S transition, TGFß1 and rapamycin, specifically reduce the stability of cyclin E/$p33^{cdk2}$ complexes (Koff et al., 1993; Sherr, 1993). These studies suggest that cyclin E must reach a threshold level and stably interact with Cdks in order for a cell to enter S phase.

Based on the timing of their appearance, the D cyclins (D1, D2 and D3) are thought to regulate the cell cycle progression during mid/late G1 phase. Specifically, a number of studies have suggested that the D cyclins may control whether the restriction point is traversed. Cyclin D mRNA and protein levels start to rise in early G1 and reach a maximum in late G1. Unlike the expression pattern of other cyclins that are linked to intrinsic determinants of cell cycle progression, expression of D-type cyclins are regulated by extrinsic determinants such as mitogen levels (Matsushime et al., 1991). The idea that D-type cyclins serve as growth factor sensors has been borne out by the fact that they are overexpressed in a number of mouse and human tumors (for example see Wang et al., 1994). Furthermore in cultured cells, high cyclin D levels antagonize granulocyte, muscle cell and lymphocyte differentiation (Kato and Sherr, 1993; Rao et al., 1994; Bodrug et al., 1994). Although the signaling pathway linking growth factor receptors and cyclin D

expression is presently unknown, recent progress has been made in deciphering downstream cyclin D-mediated events.

Unlike other cyclins, the D cyclins contain in their amino termini the sequence Leu-X-Cys-X-Glu that is also found in the E1a and large T oncoproteins, where the sequence is required for Rb and p107 binding. The sequence motif is required for the *in vitro* association of D cyclins with Rb and is competed by oncoprotein-derived peptides that include the motif (Dowdy et al., 1993). In insect cells coinfected with appropriate baculovirus vectors, D cyclins form stable complexes with Rb, and the complexes become destabilized upon superinfection with wild-type Cdk4 but not a kinase-defective Cdk4 (Kato et al., 1993). In *rb*-deficient SAOS-2 cells, cotransfecting plasmids encoding cyclin D2 and Rb resulted in hyperphosphorylated Rb. When Rb was expressed alone or with other cyclins it remained unphosphorylated (Ewen et al., 1993). These results suggest a model in which the D cyclins target Rb for phosphorylation during mid/late G1. As mentioned above, hyperphosphorylation of Rb prevents it from interacting with E2F complexes and has been correlated with increased E2F site promoter activity during late G1/S. Therefore, when mitogens decrease below a threshold level required to support another round of replication, D-type cyclin levels may become rate limiting for Rb phosphorylation, with the result that Rb remains associated with and continues to repress E2F site promoters. Although these studies indicate a role for D cyclins in late G1, it is still unknown how the D cyclins trigger restriction point traversal earlier in G1.

The E2F site in the dihydrofolate reductase (DHFR) promoter is required for repressing DHFR promoter activity during myogenic cell cycle withdrawal and muscle cell differentiation (see Chapter 2). In order to test whether Rb-like proteins are involved in repressing DHFR promoter activity in postreplicative muscle cells, we cotransformed myoblasts with DHFR promoter/reporter genes and E1a-encoding plasmids. E1a completely deregulated reporter gene expression during myogenic cell

cycle withdrawal through a mechanism dependent on an intact E2F site. Surprisingly, E1a transformants continued to regulate endogenous E2F site-containing genes and exhibited unaltered *in vitro* E2F site binding activity. Our results suggest that Rb-like proteins play a significant role in the repression of DHFR promoter activity in postreplicative muscle cells. Furthermore, other factors, acting outside of the DHFR promoter region, serve a redundant role in limiting DHFR gene expression in postreplicative muscle cells.

## Materials and Methods

### Cell cultures and transformations

A modified calcium phosphate precipitation procedure (Gross et al., 1987) was used to transform cells with 7 μg E1a effector plasmid (either pJN20 or MTpE1a), 3 μg reporter plasmid (either DHFR promoter/CAT or DHFR promoter/luciferase) and 0.3 μg pKneo. Stable transformants were selected in 1 mg/ml G418 (Gibco). Transformants were pooled (20-50 colonies/dish) and expanded in 100 μg/ml G418.

### Plasmids

The reporter genes, (–850/+16)DHFR promoter/CAT and (–270/+20)DHFR promoter/luc were described in Chapter 2. The plasmid pJN20 (Carlock and Jones, 1981) (provided by N. Jones, ICRF, London) contains a 2.8-kb *Eco*RI/*Hin*dIII Adenovirus 5 genomic fragment (map units 0-7.8) cloned into pBR327.

MTpE1a was constructed by first inserting the 961-bp *Hin*dIII/*Hin*cII 13S E1a cDNA fragment from pSVN20 (Velcich and Ziff, 1988) (provided by D. Barnes, Oregon State U.) into Bluescript (KS+) to generate pBlueE1a. A blunted 1-kb *Bam*HI/*Xho*I fragment encompassing the 13S E1a coding region was isolated from pBlueE1a and inserted downstream of the mouse metallothionein (MT) promoter in a 5.1-kb vector

prepared by digesting mMT-1 (Mayo et al., 1982) (provided by R. Palmiter, U. Washington) with *Hin*dIII, blunting with Klenow polymerase, and digesting with *Bgl*II.

Descriptions of the CAT and DHFR 3' riboprobe template plasmids and riboprobe synthesis have been published elsewhere (Schmidt and Merrill, 1989b; Schmidt et al., 1990). The mouse TK riboprobe template was constructed by inserting the 348-bp *Eco*RI/*Cla*I fragment from the 5' region of a mouse TK cDNA clone (Hofbauer et al., 1987) (provided by E. Wintersberger, U. Vienna) into Bluescript (KS+) to generate pBSmTK330. TK riboprobe synthesis was performed by digesting pBSmTK330 with *Eco*R1 followed by T3 RNA polymerase-catalyzed transcription (Schmidt and Merrill, 1989b). The mouse TS riboprobe template, pMTS-687 (provided by L. Johnson, Ohio State U.) contains a 687-bp *Pst*I fragment from the middle of the TS cDNA coding region (Deng et al., 1986). TS riboprobe synthesis was performed by digesting pMTS-687 with *Bgl*II followed by T3 polymerase-catalyzed transcription. Riboprobe specific activity ranged from 2 to 5 x$10^8$ cpm/$\mu$g.

**DNA isolation and analysis**

Cells were washed with cold PBS (100 mM NaCl, 81 mM $Na_2HPO_4$, 19 mM $NaH_2PO_4$) and collected by scraping in 400 $\mu$l 1X TES (10 mM Tris, pH 7.5, 5 mM EDTA, 1% SDS) containing 200 $\mu$g/ml proteinase K (Boehringer). Lysates were incubated 30 m at 55° C, adjusted to 250 mM NaCl, extracted with phenol-chloroform, and precipitated with ethanol. Pellets were resuspended in 400 $\mu$l TE (10 mM Tris, pH 7.5, 1 mM EDTA) containing 100 $\mu$g/ml RNase A and incubated 15 m at 37° C. Proteinase K was added (200 $\mu$g/ml) and samples incubated 30 m at 55° C. NaCl addition, phenol-chloroform extraction and ethanol precipitation was done as before. Pellets were resuspended in 50 $\mu$l TE and DNA concentrations determined by $A_{260}$. DNA was digested with *Eco*RI and *Hin*dIII, which generates a 2.8-kb fragment containing the entire E1a coding region. Digested DNA (15 $\mu$g) was fractionated on 1% agarose

gels and blotted onto nitrocellulose as described (Schmidt and Merrill, 1991). Blots were prehybridized 5 h at 42° C in Stark's buffer (50% formamide, 0.75 M NaCl, 0.075 M sodium citrate, 25 mM sodium phosphate, pH 6.5, 1X Denhardt's [Sambrook et al., 1989], 250 μg/ml denatured salmon sperm DNA). Blots were hybridized 16 h at 42° C in 80% Stark's buffer, 20% dextran sulfate using a $^{32}$P-labeled 2.8-kb pJN20 E1a fragment as probe (2 x $10^7$ cpm/μg). Probe was prepared using a Prime-it kit (Stratagene). Blots were washed and exposed to film for 24 hours as described (Schmidt and Merrill, 1989b).

**Immunocytochemistry**

Cells were immunostained for myosin using an antibody (MF20) raised against native chicken myosin (Bader et al., 1982). Cells were fixed with AFA (75% ethanol, 10% formaldehyde, 5% acetic acid), preincubated 1 h at 37° C with TBS (25 mM Tris, pH 7.5, 137 mM NaCl, 2.7 mM KCl) containing 1% filtered horse serum, and incubated 45 m at 37° C with a $10^{-1}$ dilution of primary antibody (MF20) in TBS/1% serum. Cells were washed twice with TBS/1% serum, incubated 45 m at 4° C with biotinylated mouse anti-IgG secondary antibody (Vector Laboratories), washed twice with TBS, incubated 1.5 h at 4° C with an avidin:biotinylated horseradish peroxidase complex, and stained using a peroxidase substrate kit (Vector Laboratories).

Cells were pulsed and stained for BUdR incorporation using components from a cell proliferation kit (Amersham). Briefly, cultures were incubated 1 h at 37° C with BUdR in thymidine-free basal medium containing 15% horse serum, washed twice with PBS, and fixed with 95% ethanol:5% acetic acid. Fixed cells were rehydrated with PBS, incubated 1 h at 20° C with mouse anti-BUdR antibody, washed with PBS, incubated 30 m at 20° C with peroxidase-conjugated goat anti-mouse IgG, washed with PBS, and treated with peroxidase substrate solution for 30 m.

**Miscellaneous**

Cell culture, RNA isolation and analysis, luciferase assays, transient transfection assays and band shift assays were described in Chapter 2.

## Results

**E1a derepression of DHFR promoter activity**

To determine whether E1a proteins affect the regulation of E2F site-containing genes during myogenesis, MM14D muscle cells were cotransformed with pKneo, a (-850/+16)DHFR promoter/CAT gene, and either the E1a-encoding plasmid pJN20 or a control plasmid. To confirm that G418-selected transformants contained E1a sequences, DNA from non-E1a and E1a transformant populations was analyzed by Southern blot hybridization (Fig. III.1). Using plasmid DNA as a standard (lane 1), the E1a line (lane 3) contained about five copies of the E1a-encoding plasmid per cell.

Total RNA from proliferating and differentiated muscle cells was isolated from non-E1a and E1a transformants, and analyzed for CAT mRNA. In non-E1a transformants the expected pattern of DHFR promoter activity was observed. CAT mRNA levels decreased to barely detectable levels in differentiated myocytes (Fig. III.2, compare lanes 1 and 2 with lanes 3 and 4). In contrast, in E1a transformants, a deregulated pattern of DHFR promoter activity was observed. CAT mRNA levels did not decrease in differentiated myocytes (Fig. III.2, compare lanes 5 and 6 with lanes 7 and 8). Proliferative myoblast levels of CAT reporter gene mRNA were about two-fold higher in E1a transformants than non-E1a transformants (Fig. III.2, compare lanes 1 and 2 with lanes 5 and 6).

E1a derepression of DHFR promoter activity was even more striking when cells were grown in FGF-rich brain extract and induced to withdraw from the cell cycle by switching cells to mitogen-free medium. In the experiment shown in Fig. III.3, cell cycle withdrawal of non-E1a and E1a transformants was confirmed by FACS. As shown

Figure III.1. Southern blot analysis of E1a sequences in muscle cell transformants. DNA (15 μg) from cells cotransformed with the (−850/+16)DpCAT reporter gene, and either a control plasmid (lane 2) or the E1a-encoding plasmid pJN20 (lane 3) was digested with *Eco*R1 and *Hin*dIII, and analyzed by blot hybridization using a radiolabeled 2.8-kb *Eco*R1/*Hin*dIII E1a fragment from pJN20 as probe. An aliquot of *Eco*R1/*Hin*dIII-digested pJN20 was used as a standard (lane 1). M designates *Hin*dIII/*Eco*R1-digested lambda DNA molecular weight markers.

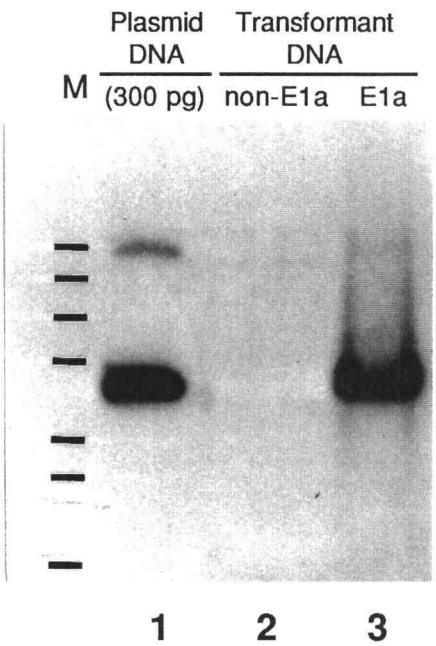


Figure III.1

Figure III.2. Effect of E1a on CAT mRNA regulation in DHFR promoter/CAT muscle cell transformants induced to differentiate by allowing FGF depletion. Muscle cells cotransformed with the (-850/+16)DpCAT reporter gene, and either a control plasmid (non-E1a) or the E1a-encoding plasmid pJN20 (E1a) were grown in purified FGF and induced to differentiate by allowing FGF depletion (see Materials and Methods) (a 72-h induction period was used). Total RNA (25 $\mu$g) from proliferating myoblasts (mb) or differentiated myocytes (mc) was analyzed by RNase protection for CAT mRNA. Arrow designates expected mobility of riboprobe fragment protected by CAT mRNA. Duplicate samples represent independent RNA isolations.

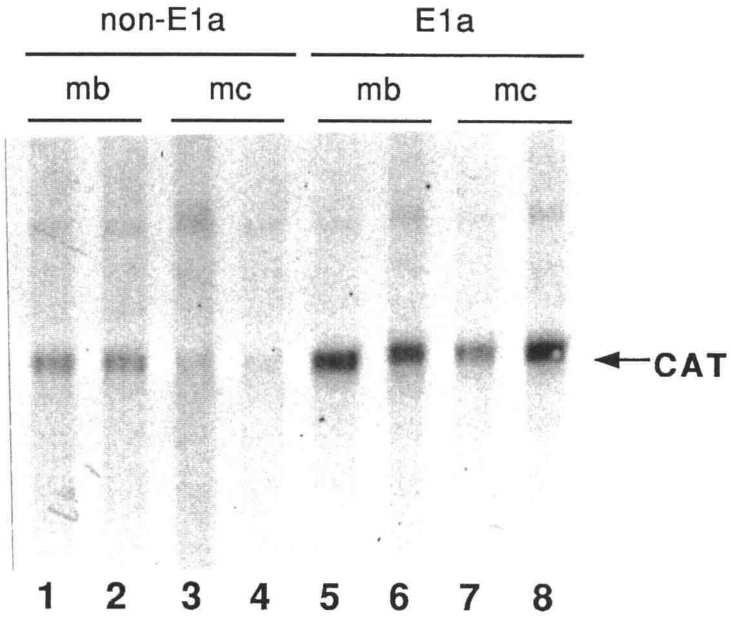


Figure III.2

Figure III.3. Effect of E1a on CAT mRNA regulation in DHFR promoter/CAT muscle cell transformants induced to withdraw from the cell cycle by incubation in mitogen-free medium. E1a or control transformants (described in Fig. III.2) were grown in brain extract medium. Total RNA was isolated at indicated times after cultures were switched to mitogen-free medium. CAT mRNA (in 25 $\mu$g cellular RNA) was measured by RNase protection. Arrow designates expected mobility of riboprobe fragment protected by CAT mRNA. Band intensities were quantitated by laser densitometry. Absolute mRNA levels (mRNAs/cell) calculated by extrapolation from pseudo-mRNA standards, are shown, as is the change relative to myoblast levels (% remaining).

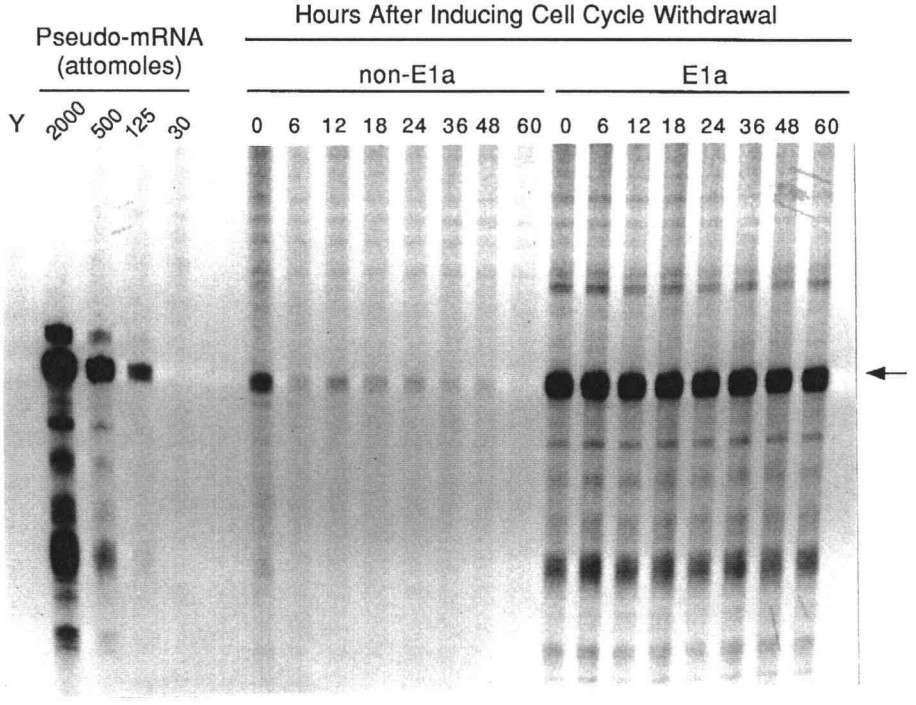
Hours After Inducing Cell Cycle Withdrawal
Pseudo-mRNA (attomoles)
non-E1a
E1a
Y 2000 500 125 30
0 6 12 18 24 36 48 60 0 6 12 18 24 36 48 60
Band Intensity 143 43 13 ND 14 3 5 4 3 2 ND ND 43 50 43 44 42 48 36 38
mRNAs (x10)/cell 18 4 6 4 4 2.5 - - 55 64 55 56 54 61 46 49
% Remaining 100 22 36 24 22 14 - - 100 116 100 102 98 112 84 89

Figure III.3

in Fig. III.3, in non-E1a control cells, DHFR promoter/CAT gene mRNA levels decreased five-fold by 36 hours after induction. In contrast, in E1a-transformed cells, CAT mRNA remained constant throughout the course of the experiment.

Similar effects of E1a on reporter gene activity were obtained when myoblasts were cotransformed with a DHFR promoter/luciferase gene and a plasmid encoding the 13S form of E1a cDNA under the control of the metal-inducible mouse metallothionein promoter. Stable transformants were assayed for luciferase activity at various times after inducing cell cycle withdrawal by incubation in mitogen-free medium. In the experiment shown in Fig. III.4, luciferase activity in non-E1a transformants decreased six-fold by 12 hours after induction. In contrast, luciferase activity in E1a-transformants remained constant for at least 16 hours after induction. The addition of 60 $\mu$M zinc 16 hours after induction had no effect on luciferase activity in either the non-E1a or E1a transformants. Although we did not confirm that zinc elevated E1a expression from the MTpE1a fusion gene, zinc strongly increases expression of metallothionein promoter/TK fusion genes in myocytes (Mayo et al., 1981; Gross and Merrill, unpublished). Thus, the failure of zinc to further induce luciferase activity in MTpE1a transformants suggested that E1a protein was already at levels sufficient to maximally derepress reporter gene expression.

Thus, using two different DHFR promoter/reporter genes, two different conditions to induce cell cycle withdrawal, and two different E1a effector plasmids, our data indicates that E1a overcame the cellular mechanism(s) that inhibited DHFR promoter activity in postreplicative muscle cells. The results are consistent with those of Slansky et al. (1993), who showed that constitutively-expressed E1a abolishes the induction of DHFR promoter activity in serum-stimulated quiescent cells. In summary we conclude that an E1a-antagonizeable process inhibited DHFR promoter activity during myogenic withdrawal from the cell cycle.

Figure III.4. Effect of E1a on luciferase activity in DHFR promoter/luc muscle cell transformants induced to withdraw from the cell cycle by incubation in mitogen-free medium. Muscle cells cotransformed with the (-270/+20)DHFR promoter/luc reporter gene, and either a control plasmid (non-E1a) or a plasmid encoding the 13S form of E1a under the control of the mouse metallothionein promoter (MTpE1a). Myoblasts were grown in brain extract medium. At indicated times thereafter, cultures were harvested and assayed for luciferase activity. At 16 hours after induction, cultures were exposed to 60 $\mu$M $ZnCl_2$ (arrow). Activity was normalized to protein content and represented as a percentage of initial levels.

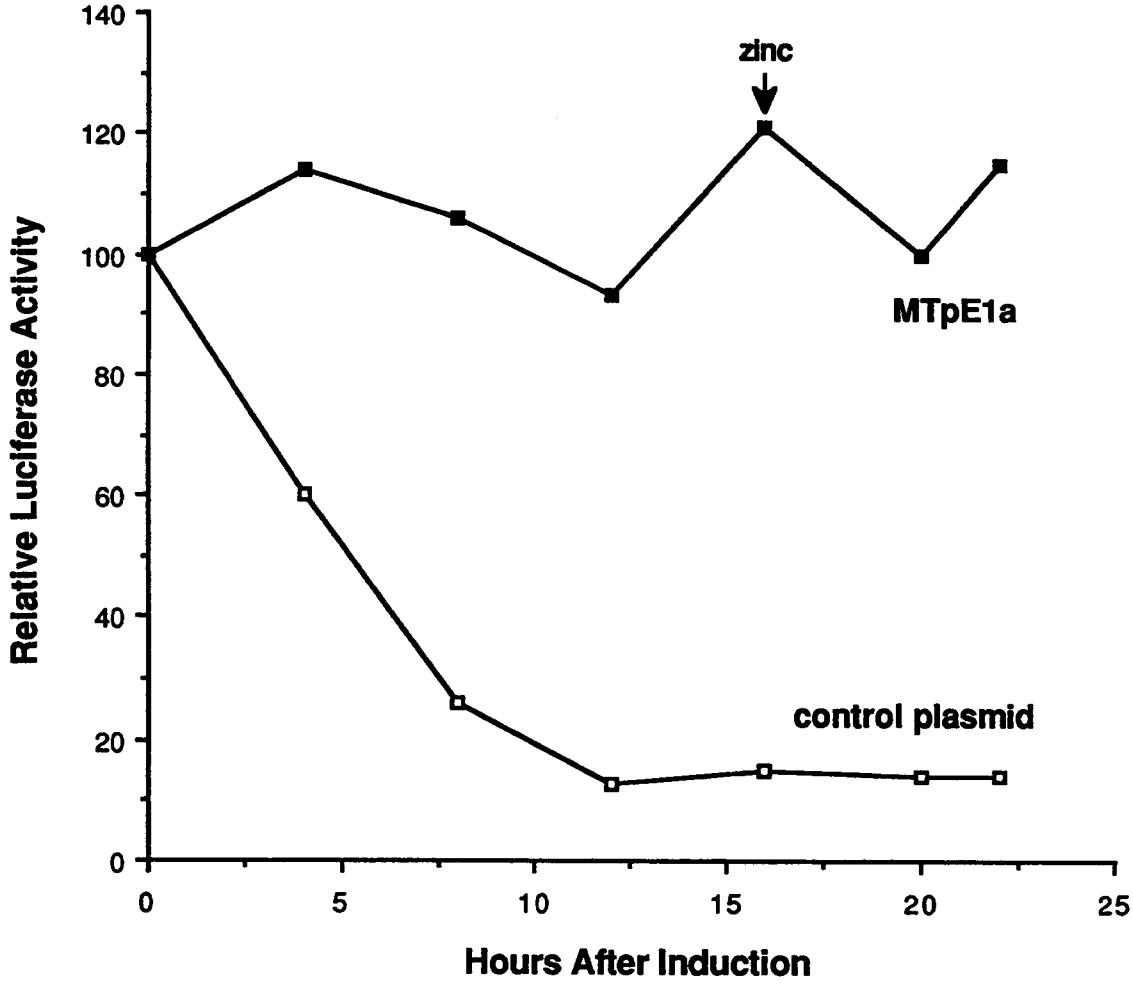
140
120
100
80
60
40
20
0
Relative Luciferase Activity
zinc
MTpE1a
control plasmid
0
5
10
15
20
25
Hours After Induction

Figure III.4

**E2F site requirement for E1a-mediated transactivation**

To test whether E1a induced derepression of DHFR promoter activity is dependent on an intact E2F site, differentiated muscle cells were cotransformed in a transient transfection assay with a DHFR promoter/luciferase gene containing either a wildtype or mutated E2F site and either a control plasmid or MTpE1a. In the experiment shown in Fig. III.5, a DHFR promoter/luciferase gene containing a wildtype E2F site was induced 22-fold when cotransfected with MTpE1a into differentiated muscle cells. In contrast, a DHFR promoter/luciferase gene containing a mutated E2F site was induced only 2-fold by MTpE1a. These data suggest that E1a induced deregulation of DHFR promoter activity in postreplicative muscle cells is mediated primarily through the E2F site.

**The effect of E1a on the differentiation program**

The myogenic differentiation program of rat L6 and L8 muscle cell lines has been reported to be blocked by E1a, and recently, SV40 large T antigen has been reported to drive myonuclei back into S phase (Braun et al., 1992; Gu et al., 1993; Webster et al., 1988). One explanation for constitutive DHFR promoter activity in E1a-transformed muscle cells incubated under differentiation-inducing conditions is that E1a is inhibiting cell cycle withdrawal and differentiation.

To determine whether E1a inhibited MM14D mouse myoblast differentiation, myocytes were fixed 60 hours after FGF deplenishment and immunostained with an antibody to muscle-specific myosin heavy chain. Myosin is only expressed in muscle cells that have withdrawn from the cell cycle and have achieved a fully differentiated state. As shown in Fig. III.6, panels a and b, E1a and non-E1a transformants were equally competent in forming myosin-positive myotubes. Therefore, at the levels achieved in our transformants, E1a did not inhibit MM14D mouse muscle cell differentiation.

Figure III.5. Transactivation of DHFR promoter activity by E1a in differentiated muscle cells. Myoblasts were induced to differentiate by allowing FGF depletion. Two days after induction, myocytes were cotransfected with 2 mg of a plasmid encoding a (-270/+20)DHFR promoter/luciferase gene containing either a wildtype (WT) or mutated (mut) E2F site and 8 mg of either a control plasmid (non-E1a) or the E1a expression plasmid MTpE1a (E1a). Cells were harvested 20 hours after transfection and assayed for luciferase activity. Values shown represent the mean $\pm$ one standard deviation for four independent transfections and are normalized to control cell levels, arbitrarily set at 1.

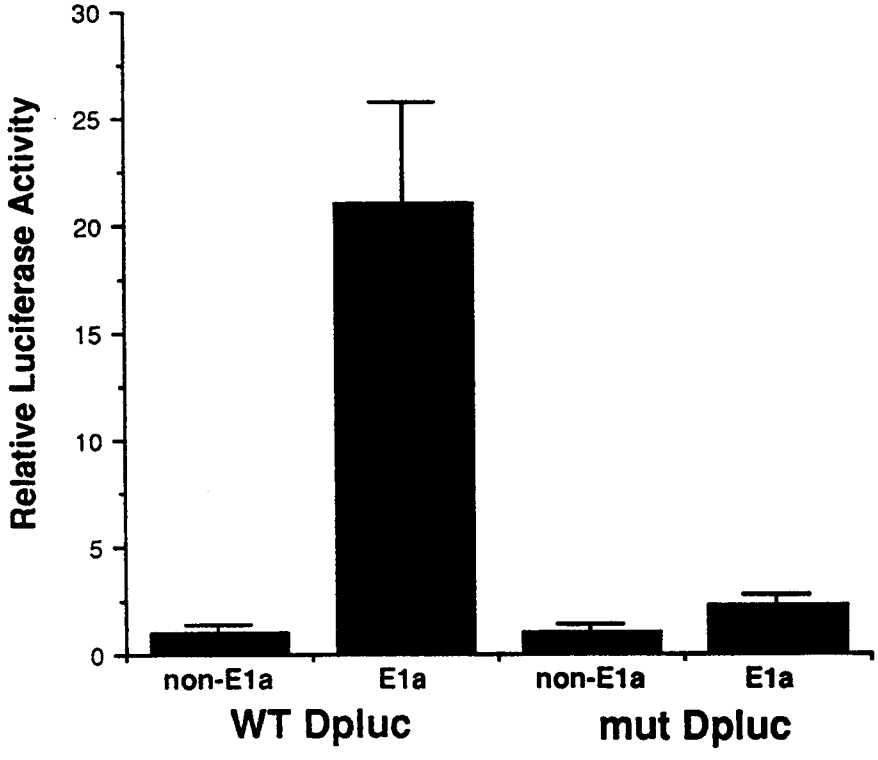
Relative Luciferase Activity
30
25
20
15
10
5
0
non-E1a
E1a
non-E1a
E1a
WT Dpluc
mut Dpluc

Figure III.5

Figure III.6. Effect of E1a on muscle cell differentiation and withdrawal from the cell cycle. E1a (panels b, d, and e) or control (a and c) transformants were grown in purified FGF and induced to differentiate by FGF depletion (Materials and Methods). At 72 hours after induction, cultures were fixed and immunostained for myosin (a and b) or pulsed with BUdR and immunostained for BUdR-containing DNA (c-e). Because withdrawal from the cell cycle was nearly complete in both populations, nonrepresentative fields showing rare BUdR-positive cells (arrows) are shown in panels c-e. Scale bar equals 100 $\mu$ in a-d and 160 $\mu$ in e.

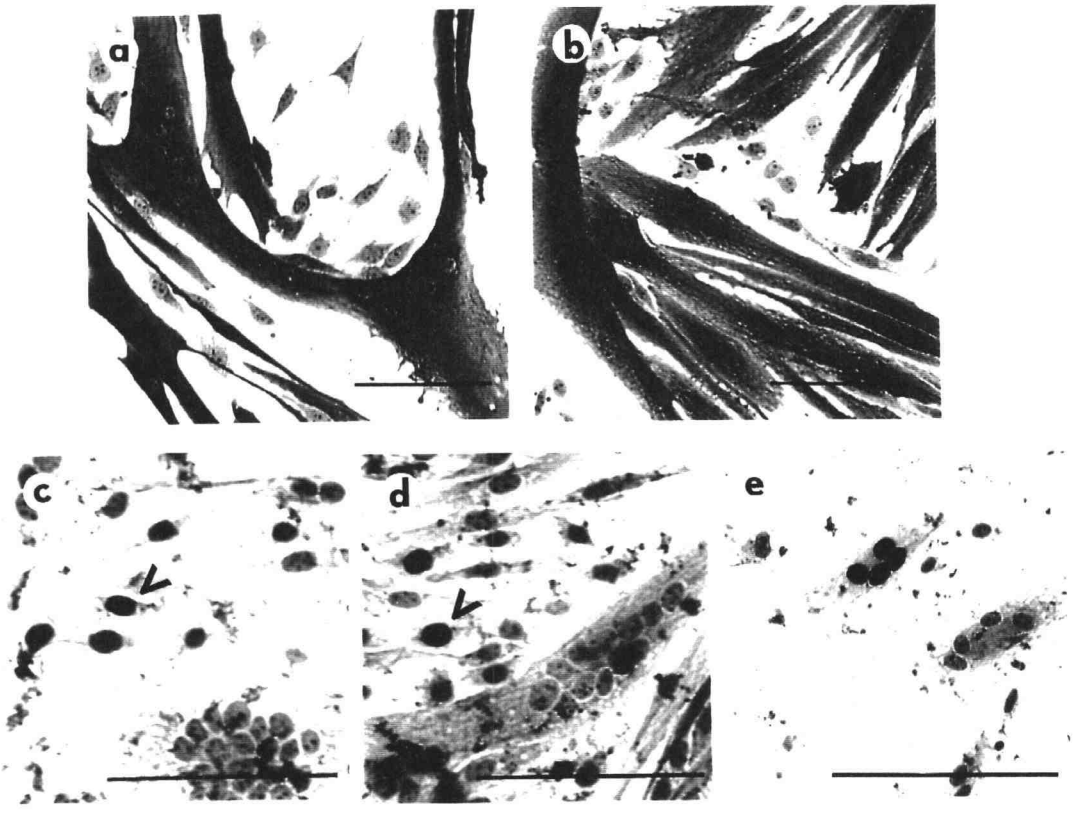


Figure III.6

To determine whether E1a affected MM14D myoblast withdrawal from the cell cycle, FGF-grown muscle cell transformants were pulsed briefly with the thymidine analog bromodeoxyuridine (BUdR) at various times after inducing differentiation by allowing FGF depletion and immunostained with anti-BUdR antibody. As shown in Fig. III.6, panel c, very few (< 0.5%) BUdR-positive nuclei were present 60 hours after induction in control non-E1a transformants. As shown in Fig. III.6, panel d, BUdR-positive nuclei were equally rare after induction in E1a transformants. Nonrepresentativefields containing rare labeled cells had to be used in Fig. III.6 (panels c and d), in order to demonstrate the difference between labeled and unlabeled cells. The only detectable difference between the non-E1a and E1a transformants were extremely rare fields in the latter in which a myotube containing several BUdR-positive nuclei was observed (Fig. III.6, panel e). This phenomenon was never observed in non-E1a myotubes and may represent a situation in which the level of E1a was sufficient to cause the myonuclei to initiate DNA synthesis.

**E1a and endogenous gene expression**

We investigated whether E1a affected the regulation of endogenous genes containing E2F binding sites. The expectation was that E1a would derepress endogenous gene expression in a fashion similar to that observed for the DHFR promoter/CAT gene. The RNA previously analyzed for CAT mRNA (Fig. III.2) was probed for endogenous DHFR, thymidine kinase (TK) and thymidylate synthase (TS) mRNA by RNase protection. Like *DHFR*, the genes encoding TK and TS have E2F consensi in their promoter regions (Merrill et al., 1992). As expected, endogenous DHFR mRNA levels decreased to barely detectable levels as non-E1a transformants differentiated (Fig. III.7a, compare lanes 1-2 with 3-4). Contrary to expectations, DHFR mRNA levels also decreased to barely detectable levels as E1a transformants differentiated (Fig. III.7a, compare lanes 5-6 with 7-8). The failure of E1a to derepress endogenous E2F site-containing genes

Figure III.7. Effect of E1a on endogenous DHFR, TK and TS mRNA levels in muscle cells induced to differentiate by allowing FGF depletion. E1a or control transformants (described in Fig. III.2) were grown in purified FGF and induced to differentiate by FGF depletion (see Materials and Methods). A 72-h induction period was used. Total RNA (25 $\mu$g) from proliferating myoblasts (mb) or differentiated myocytes (mc) was analyzed by RNase protection for DHFR mRNA (A) or TK and TS mRNA (B). Arrows designate bands with mobilities expected for fragments protected by DHFR, TK and TS mRNA. Yeast RNA (Y) was probed to insure that signals arising in experimental lanes were specific.

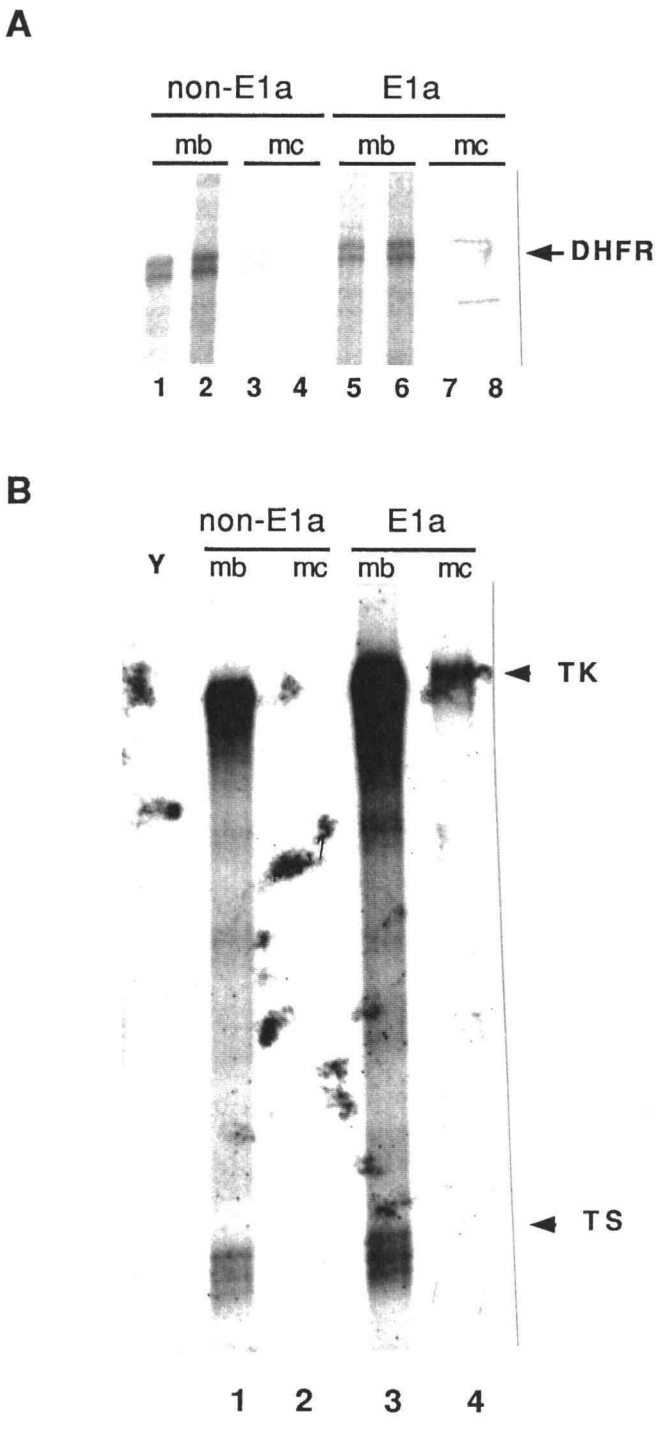
A
non-E1a
E1a
mb
mc
mb
mc
DHFR
1 2 3 4 5 6 7 8
B
non-E1a
E1a
Y
mb
mc
mb
mc
TK
TS
1 2 3 4

Figure III.7

was also evident when TK and TS mRNA levels were determined. As shown in Fig. III.7b, TK and TS mRNA decreased severalfold as non-E1a transformants differentiated (compare lanes 2 and 3),and a similar decrease in TK and TS mRNA occurred as E1a transformants differentiated (compare lanes 4 and 5).

In contrast to the complete deregulation of reporter gene expression, E1a did not deregulate endogenous DNA synthesis gene expression. The continued regulation of endogenous gene mRNA levels suggested that factors acting outside of the -850/+16 DHFR promoter region and not abrogated by E1a played a redundant role in DNA synthesis gene regulation during myogenesis.

**E2F site binding activity in E1a transformants**

Two types of E2F binding activity are observed in electro-phoretic mobility shift assays. The faster migrating complex contains dimerized E2F1/DP1-like transcription factors and is correlated with increased activity of E2F site-containing promoters (Girling et al., 1993; Ogris et al., 1993; Li et al., 1994). The slower migrating complex contains, multimerized E2F1/DP1-like proteins, Rb-like proteins, cyclins and cyclin-dependent kinases and is correlated with inhibition of E2F site-containing promoters (Bandara et al., 1991; Devoto et al., 1992; Mudryj et al., 1991; Hiebert et al., 1992). Adenovirus E1a proteins, either expressed *in vivo* or added to cell extracts *in vitro*, dissociate multimeric E2F complexes, leaving dimeric E2F1/DP1-like proteins as the only bound species (Bagchi et al., 1990; Bandara and La Thangue, 1991).

To test whether E1a affected the formation of multimeric complexes on the E2F site in muscle cell transformants, an electrophoretic mobility shift assay was used. In the experiment shown (Fig. III.8), a radiolabeled E2F site-containing oligonucleotide was incubated with whole cell extracts prepared from non-E1a and E1a transformants (lanes 6-13). Parallel band shift assays were performed using extracts prepared

Figure III.8. E2F site binding activity in whole cell extracts prepared from F9 embryo carcinoma cells and either control or E1a muscle cell transformants. Extracts were prepared under conditions that preserve the association of E2F1/DP1 with other proteins such as Rb, p107 and cyclin/CDK complexes (Bandara et al., 1991). Equal amounts of extract protein (8 mg) were assayed in each sample. A 40-fold molar excess of unlabeled oligonucleotide containing either a wildtype (WT) or mutated (mut) E2F site was included as a competitor as indicated. The detergent desoxycholate (DOC), which disrupts the association of E2F1/DP1-like proteins with other proteins such as Rb, p107 and cyclin/CDK complexes (Bandara et al., 1991), was included as indicated.

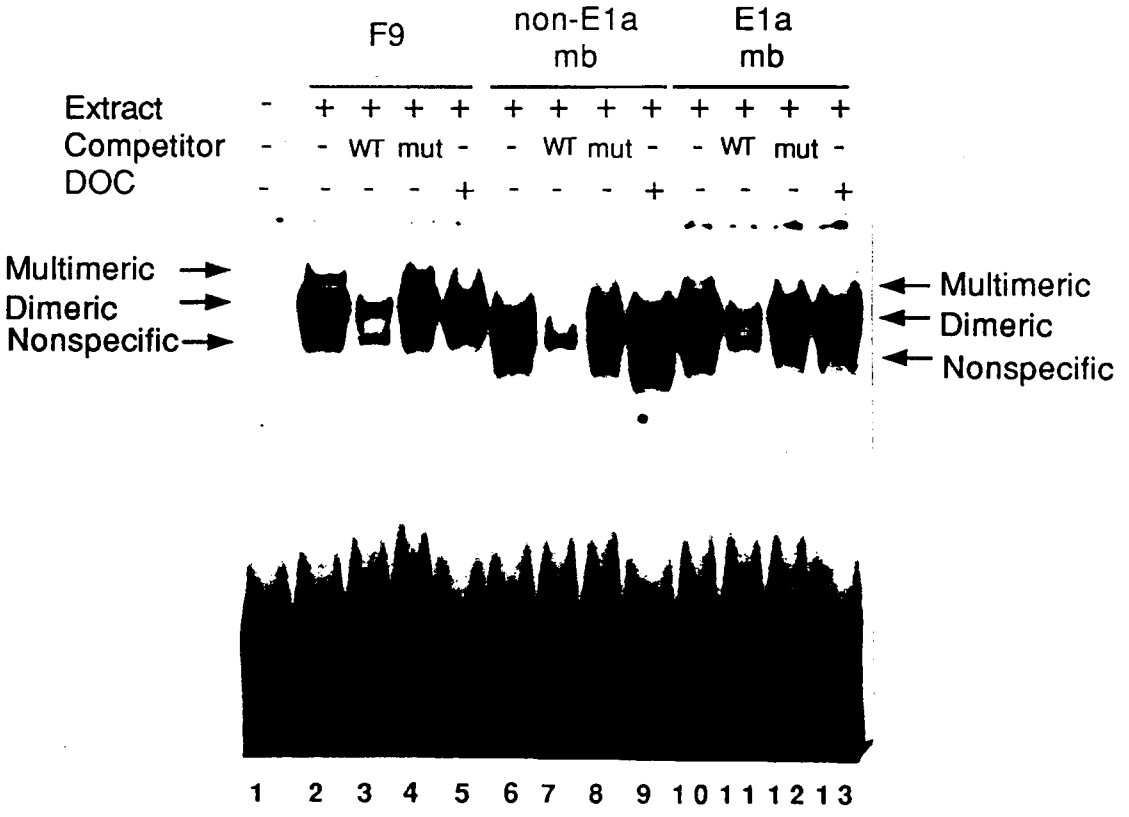
F9
non-E1a mb
E1a mb
Extract - + + + + + + + + + + +
Competitor - - WT mut - - WT mut - - WT mut -
DOC - - - - + - - - + - - - +
Multimeric
Dimeric
Nonspecific
Multimeric
Dimeric
Nonspecific
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure III.8

from F9 embryo carcinoma cells (lanes 2-5). The majority of the E2F binding activity in non-E1a and E1a cell extracts, as well as in the F9 cell extracts, was resistant to the detergent DOC, suggesting that it represents dimeric E2F1/DP1-like proteins. A faint, detergent-sensitive, slower-migrating band was observed in extracts prepared from both muscle cell transformants, and comigrated with the previously characterized Rb-containing multimeric E2F complexes present in F9 extracts (Bandara et al., 1991; see also Chapter 2).

To test whether E1a affected E2F site binding activity in postreplicative muscle cells, whole cell extracts were prepared from non-E1a and E1a transformants at various times after inducing cell cycle withdrawal by incubating cultures in mitogen-free medium. In both populations the absolute and relative levels of dimeric and multimeric E2F binding activity remained constant as muscle cells withdrew from the cell cycle (Figs. III.9a and III.9b). Therefore, we conclude that E1a levels sufficient to deregulate a DHFR promoter/reporter gene in postreplicative muscle cells *in vivo* did not inhibit the formation of multimeric E2F complexes, as detected *in vitro*. The results do not rule out the possibility that E1a antagonizes multimeric E2F complexes that form *in vivo* but are not detected under our *in vitro* conditions.

Figure III.9 E2F site binding activity in control (A) and E1a (B) muscle cell transformants during withdrawal from the cell cycle. Myoblasts were grown in brain extract medium and harvested at indicated times after switching cells to mitogen-free medium. Equal amounts of extract protein were assayed in each sample. A 40-fold molar excess of unlabeled oligonucleotide containing either a wildtype (WT) or mutated (mut) E2F site was included as a competitor as indicated. Band intensities were determined using a PhosphorImager and were normalized relative to the proliferative signal. Binding activity of dilutions of the proliferative cell extracts are shown in the indicated lanes and were done to insure that both the binding reactions and the detection process were linear with respect to input of extract. Band intensities were determined using a PhosphorImager and are normalized relative to the proliferative signal.

A) Non-E1a Transformants

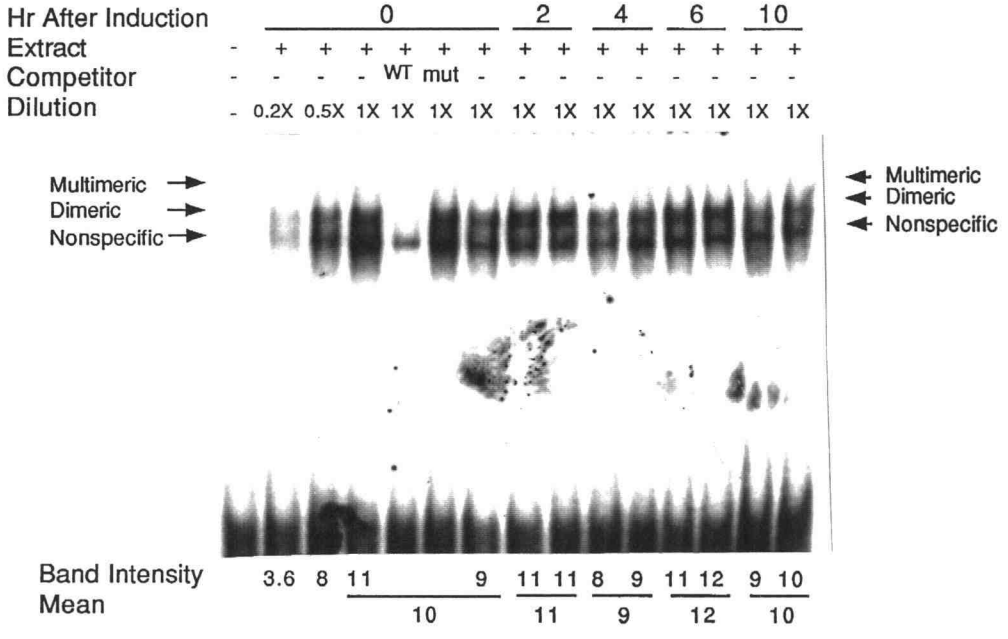


Figure III.9A

B) E1a transformants

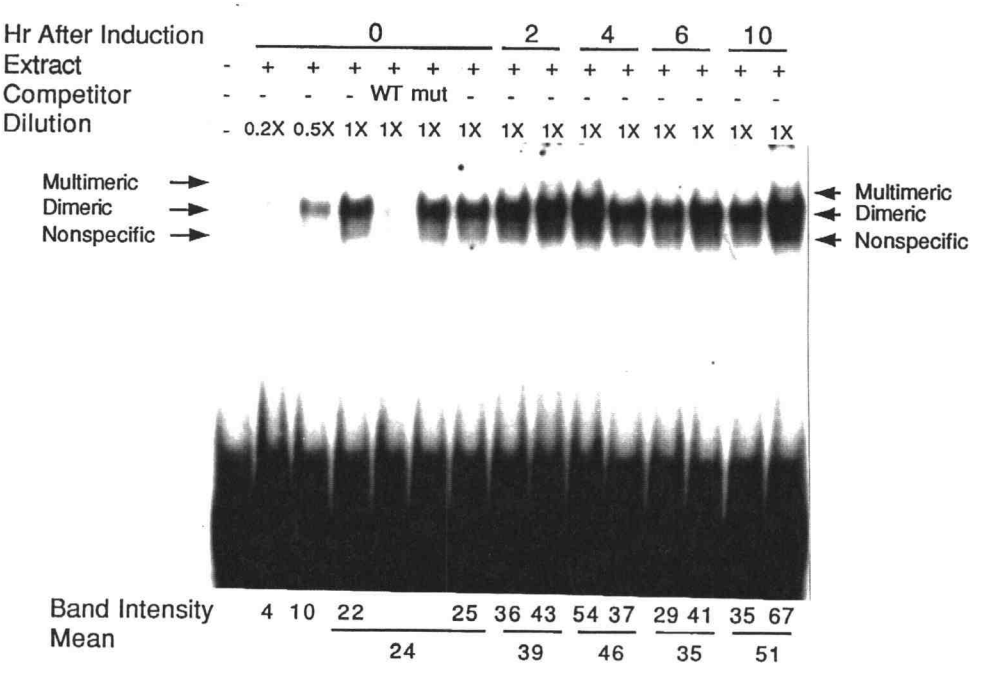


Figure III.9B

## Discussion

In the past, E1a effects on gene expression have been difficult to dissociate from E1a effects on cell proliferation. Our results on muscle cells thus have added significance in that they establish that E1a deregulation of DHFR promoter activity was not due to an E1a effect on muscle cell withdrawal from the cell cycle or differentiation. Immunological BUdR incorporation and myosin staining assays showed that E1a-transformed myoblasts withdrew from the cell cycle and formed myosin-positive myotubes with roughly the same kinetics as non-E1a transformants. The absence of an E1a effect on cell cycle withdrawal or differentiation in muscle cells contrasts with results of earlier studies that showed that microinjection of either E1a-encoding plasmids or purified E1a proteins into quiescent fibroblasts induced DNA synthesis (Kaczmarek et al., 1986; Stabel et al., 1985). Furthermore, E1a has been shown to block differentiation in other muscle cell lines (Braun et al., 1992; Webster et al., 1988). The discrepancy between our results and those of others may be due to differences between cell lines. Unlike aneuploid L6 and L8 myoblasts, MM14D myoblasts are diploid and may be less predisposed to oncogenic effects of E1a. Alternatively, effects on cell proliferation may be achieved only at high E1a levels, and high E1a levels may be toxic to diploid MM14D myoblasts. In the absence of ameliorating proteins, E1a has been shown to trigger apoptosis in euploid primary cells (Rao et al., 1992). Therefore, in introducing E1a by cotransformation with pKNeo, we may be selecting for transformants that express lower, non-oncogenic levels of E1a. Rare BUdR-labeled myonuclei may reflect regions where sufficient E1a has accumulated to trigger a terminal G1/S-like phase.

In the mouse BC3H1 muscle cell line, low-level E1a expression only partially blocked differentiation (Mymryk et al., 1992). Although E1a inhibited expression of creatine kinase and beta-actin, it did not affect the expression pattern of tropomyosins,

myosin light chain 1 and alpha-actin. In another study, immortalization of primary cultured embryonic rat brain cells by transformation with E1a did not inhibit the ability of the cells to differentiate in low serum, unless cells were cotransformed with other oncogenes (Galiana, et al., 1993). Therefore E1a is not necessarily incompatible with differentiation.

In contrast to the complete deregulation of DHFR promoter/reporter genes, the endogenous DHFR, TK and TS genes were regulated normally in differentiating E1a transformants. Regulation of the endogenous genes in E1a transformants was surprising since others have shown that DHFR mRNA levels increase during early adenovirus infection of cycling HeLa cells (Yoder et al., 1983), and that TK and DHFR mRNA levels increase when SV40 large T antigen is conditionally expressed in quiescent fibroblasts (Mudrak et al., 1994; Ogris et al., 1993). Again, the discrepancy between our results and others may be due to differences in the cell lines used or in the levels of oncoprotein achieved. E1a and T-antigen are polyfunctional oncoproteins, and contain two distinct regions that are required, in conjunction with other viral or cellular oncoproteins, to induce a transformed phenotype in primary cells. One region of E1a, designated conserved domain 2, binds Rb and the Rb-related proteins p107 and p130 (Cobrinik et al., 1993; Dyson et al., 1989; Whyte et al., 1988), and is required for increased activity of E2F site-containing promoters. A second domain, located near the N-terminus, binds a 300-kD cellular protein and is required for induction of DNA synthesis in primary Baby Rat Kidney cells (Stein et al, 1990). Deregulation of endogenous DNA synthesis genes may require more than the E1a domain responsible for E2F site derepression. These additional domains may have a higher threshold concentration for effectiveness than that required for E2F site derepression.

The fact that DHFR promoter/CAT genes were sensitive to E1a deregulation, whereas endogenous genes were not, suggested that additional systems were controlling

endogenous DNA synthesis gene mRNA levels. The additional regulatory systems most likely operate at the transcriptional level. In *DHFR*-amplified muscle cells, nuclear run-on assays indicate that myogenic withdrawal from the cell cycle is associated with a greater than fivefold reduction in RNA polymerase density downstream from position +60 on the DHFR coding region (Schmidt and Merrill, 1989b). A similar increase in polymerase density occurs in serum-induced fibroblasts (Santiago et al., 1984; Wu and Johnson, 1982).

Where might additional regulatory information reside? Farnham and Means (1990) showed that efficient *in vitro* transcription of a template containing the DHFR promoter requires intragenic sequences. In the same study, several protein-binding sites were detected in the intragenic region. Possibly transcription factors that bind within the DHFR coding region may play a role in growth-specific DHFR regulation. Alternatively, additional *cis*-acting information may lie far upstream or downstream from the DHFR coding region.

Although the additional regulatory information has not been localized, the differential regulation of DHFR promoter/reporter genes and the endogenous DHFR gene in E1a-transformed muscle cells indicate that two separate cellular mechanisms control the level of DHFR transcripts during myogenesis; one E1a-sensitive and the other E1a-resistant. Factors acting outside of the -850 to +20 DHFR promoter region serve a redundant regulatory role in limiting DNA synthesis gene expression in postreplicative cells.

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Appendex A: Transient Transfection Protocol for Differentiated Myocytes

The results of transiently introducing DHFR promoter/reporter genes along with plasmids encoding DP1, E2F1 and E1a into differentiated muscle cells are shown in Figs. II.4 and III.5.

1. Grow myoblasts on 6-cm dishes in purified FGF following the feeding schedule described in Materials and Methods, Chapter 2.

2. Perform transfection 48 hours after final FGF addition when cells are well fused.

3. Prepare DNA as follows: Bring DNA (typically 2 $\mu$g DHFR promoter/luciferase and 8 $\mu$g of a transactivator or control plasmid) to a volume of 250 $\mu$l with Millique water. Add 250 $\mu$l 2X HBS (see below) and mix by pipetting. Bubble in 31 $\mu$l 2 M $CaCl_2$. Incubate at room temperature for 1 hour.

4. Remove and save culture medium from dishes and store at $4^{O}$ C.

5. Add DNA cocktail to cells and incubate at room temperature for 30 min with occasionial rocking to insure that DNA solution bathes entire surface of dish.

6. Add fresh prewarmed medium containing 15% horse serum and incubate for 3.5-4 hrs at $37^{O}$.

7. Prewarm conditioned medium saved in step 4.

8. Remove medium/DNA from dishes, add 2 ml of fresh medium supplemented with 15% sterile glycerol, and incubate 4 min at room temperature.

9. Remove medium/glycerol from dishes and wash once with fresh prewarmed medium.

10. Add prewarmed conditioned medium saved from step 4 to dishes.

11. Harvest cells 16-24 hrs following transfection (see Appendex B).

<u>2X HBS</u>
272 mM NaCl
10 mM KCl
1.4 mM $NaH_2PO_4$
11 mM glucose
21 mM Hepes
pH 7 @ $25^{O}$

Appendex B: Reporter Gene Assays

Extracts prepared as described below can be assayed for luciferase, CAT and ß-galactosidase activity.

Extractions (Using components from the Enhanced Luciferase Assay Kit sold by Analytical Luminescence Laboratory, San Diego, Ca)

1. Wash cells once with cold PBS.

2. Scrape cells in 1 ml cold PBS and place in a 1.5 ml eppindorf.

3. Microfuge 5 sec and remove supernatent. Cell pellets can be either processed immediately or frozen at $-20^{0}$.

4. Resuspend cell pellets in 150 µl extraction buffer (dilute extraction buffer in kit 1:3 with distilled water).

5. Incubate at $4^{0}$ for 10-15 min.

6. Vortex 5 sec, and allow tubes to warm to room temperature (10-15 min).

7. Vortex 5 sec, and microfuge for 2 min.

8. Extracts can either be assayed immediately (see below) or stored at $-20^{0}$.

Luciferase Assay

1. In a 0.5 ml eppindorf, add 25 µl substrate A (from luciferase assay kit, see above) to 2.5 µl cell extract and mix by pipetting 3-4 times.

2. Add 25 µl substrate B, mix by pipetting, and <u>immediately</u> place in a scinillation counter that is set-up to count photons (having the machine count for 30 sec works well).

3. Normalize luciferase activity by protein content as measured by Bradford assay.

<u>Note</u>
Rapid decay of luciferase enzyme activity makes it absolutely required that Step 3, in which the enzyme substrate luciferin is added, be performed consistently between samples in terms of the amount of time taken from the addition of substrate B to the beginning of the counting interval.

ß-galactosidase Assay

1. Add 90 µl cell extract (prepared above) to 910 µl ZB buffer (see below). Include a negative control containing 90 µl extraction buffer.

2. Add 180 µl ONPG (Φ-nitrophenyl-ß-D-galactoside: 13 µM in ZB buffer) and incubate at $28^{O}$ until the solution turns noticeably yellowish as compared to the negative control (1-2 hrs).

3. Terminate reaction by adding 450 µl 1 M $Na_2CO_3$.

4. Determine $A_{420}$ using negative control as blank.

5. nmoles ONP in samples = $A_{420}/0.0045$ X 1.5 ml.

6. Normalize to protein content of extract as determined by Bradford assay.

ZB buffer

60 mM $Na_2HPO_4 \cdot 7\ H_2O$
40 mM $NaH_2PO_4 \cdot H_2O$
10 mM KCl
1 mM $MgSO_4 \cdot 7\ H_2O$
pH 7
33 mM ß-mercaptoethanol (added just before use)

CAT Assay

1. In a scinillation vial, place 50 µl extract and 200 µl of a master mix containing 1.25 mM Chloroacetylphenicol (CAP), 100 mM Tris (pH 7.8), 8 µM acetyl-CoA, and 2 µM acetyl-[$^{14}$C]CoA.

2. For standard: In place of extract add 0.1-1 units of CAT enzyme (in a final volume of 50 µl with extraction buffer [see above]) to master mix and process in parallel with experimental samples.

3. Gently overlay extract/master mix with 2 ml econofluor and count 10-15 times, 2 min each, using scinillation counter.

4. Calculations: Plot slopes for each standard and determine slope units per unit CAT. Then from slopes of experimental samples CAT activity can be determined. Activities should be normalized to protein content to compare relative CAT activities between samples.

## Appendex C: Electrophoretic Mobility Shift Assays

### Preparation of Cell Extracts

1. Wash cells once with cold PBS and collect cells by scraping in 1 ml cold PBS into a 1.5 ml eppindorf.

2. Microfuge for 5 sec and carefully remove supernatent. Cell pellets can either be stored at -20$^{0}$ or processed immediately.

3. Resuspend pellets in 100 $\mu$l buffer A (10 mM Hepes [pH 7.9], 15 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT).

4. Freeze cells in liquid nitrogen for 15-30 sec followed by thawing at 37$^{0}$ for 1-1.5 min.

5. Repeat freeze/thaw two times, vortexing 5 sec after every thaw. (Although following freeze/thaws cells may look intact when viewed with a microscrope, soluble proteins are released into the supernatent.)

6. Clarify extracts by microfuging for 2 min.

7. Aliquot extracts into 10-15 $\mu$l volumes and store at -80$^{0}$.

8. Determine protein content by Bradford assay.

### Preparation of End-Labeled Oligonucleotides

1. To hybridize single-stranded oligos, combine 200 pm of each oligo (resuspended in EST [TE/50 mM NaCl]) in a final volume of10 $\mu$l EST.

2. Place in boiling water for 1 min and allow water to cool to room temperature overnight.

3. Label 10 pm duplexed oligo in a 50-$\mu$l volume reaction containing 30 pm [$\partial$-$^{32}$P]ATP (3000 Ci/mmole), 45 U T4 polynucleotide kinase, 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT and 0.1 mM Spermidine.

4. Incubate at 37$^{0}$ for 30 min.

5. Add 50 $\mu$l EST and load on a P6 column equilibrated with EST in a 5-ml disposable pipette.

6. Using a hand-held monitor to follow radioactivity through the column, collect the first peak which represents labeled oligos. No separation of peaks indicates that the labeling reaction failed to occur.

7. Calculate specific activity by dividing total cpms collected off column by $\mu$g oligo (assuming 100% recovery, 10 pm of a 22-bp oligo weighs 0.0726 $\mu$g). Typical specific activities are 2-5 x 10$^{8}$ cpm/$\mu$g.

Binding Reactions and Electrophoresis Conditions

1. Combine 5-10 μg cell extract (as prepared above) with 20 fm probe, 2 μg salmon sperm DNA, and 0-1 pm unlabeled oligonucleotide competitor in a 20 μl reaction containing 50 mM Tris [pH 8], 122 mM NaCl, 0.2 mM EDTA, 1 mM DTT and 10% glycerol. In detergent-treated reactions, add DOC to a final concentration of 1.25%.

2. Mix gently by pipetting followed by a flash spin and incubate at room temperature for 15 min. (In DOC reactions, after 10 min at room temperature add NP40 to a final concentration of 1.25% and continue to process in parallel with other samples.)

3. Add 3 μl tracking dye (25% Ficoll and 0.002% dye), mix gently by pipetting and load samples on a pre-run (1 hr at 200V) 6% polyacrylamide gel at room temperature prepared by combining 6 ml 30:1 acrylamide/bisacrylamide (deionized overnight with Amberlite MB-1 ion exchange resin), 2 ml 0.5X TBE, 400 μl 20% APS and 40 μl TEMED.

4. Run samples at 120 V for 2-3 hrs.

5. Following electrophoresis, adhere gel to Whatmann paper, dry for 1-2 hrs, and expose to X-ray film for 1-2 days or Phospholmager screen for 12-24 hrs.