Journal of Cellular Biochemistry 67:24–31 (1997)

Distinct Roles for Sp1 and E2F Sites in the Growth/Cell Cycle Regulation of the DHFR Promoter

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Abstract Dihydrofolate reductase activity is required for many biosynthetic pathways including nucleotide synthesis. Its expression is therefore central to cellular growth, and it has become a key target for cancer chemotherapy. Transcription of the dihydrofolate reductase gene is regulated with growth, being expressed maximally in late G_1/early S phase following serum stimulation of quiescent cells. This regulation is directed by a promoter which contains binding sites for only the transcription factors Sp1 and E2F. In this study, the role of these promoter elements in growth/cell cycle regulation of dihydrofolate transcription was addressed directly by transient transfection of Balb/c 3T3 cells with mutant promoter-reporter gene constructs. The E2F sites were found to repress transcription in G_0 and early G_1 but did not contribute to the level of transcription in late G_1/S phase. In contrast, Sp1 sites were able to mediate induction of transcription from the dihydrofolate reductase promoter, as well as a heterologous promoter, following serum stimulation of quiescent cells. These findings add dihydrofolate reductase to a growing list of genes at which E2F sites are primarily repressive elements and delineate a role for Sp1 sites in the growth/cell cycle regulation of transcription. J. Cell. Biochem. 67: 24–31, 1997.

Key words: growth control; transcription; repression; dihydrofolate reductase; retinoblastoma; Balb/c 3T3 cells; TATAA-less promoter; gene expression

Dihydrofolate reductase (DHFR) catalyzes the regeneration of tetrahydrofolates which are required cofactors in several metabolic pathways, including those for nucleotide precursor synthesis [Hakala and Taylor, 1959]. Since tetrahydrofolates are rapidly depleted in the cell, DHFR activity is required for DNA synthesis. As such, it is important in cancer chemotherapy, being the major target for methotrexate. The central position of DHFR in cellular proliferation and cancer chemotherapy has led to much interest in the control of its expression. As with many genes required for DNA synthesis, DHFR expression is transcriptionally regulated during the growth/cell cycle, with peak expression occurring in late G_1 to early S phase following stimulation of quiescent (G_0) cells [Johnson et al., 1978; Farnham and Schimke, 1985].

Comparison of the DHFR promoter from human, hamster, and mouse reveals that it is highly conserved; the core promoter lacks both TATAA and CAAT elements but contains multiple binding sites for Sp1 upstream of an inverted repeat sequence containing two overlapping binding sites for the transcription factor E2F [Azizkhan et al., 1986; Blake and Azizkhan, 1989]. The presence of Sp1 and E2F sites is a common feature of many promoters that are induced in mid–late G_1 [for review see Azizkhan et al., 1993], indicating that their presence may be important for this induction.

Previously, we have shown that the Sp1 sites in the DHFR promoter are required for efficient transcription from this promoter [Blake et al., 1990]. Furthermore, we have also shown that the Sp1 sites mediate the induction of DHFR

Abbreviations: CAT, chloamphenicol acetyl transferase; DHFR, dihydrofolate reductase; GH, human growth hormone.

Contract grant sponsor: American Cancer Society, contract grant number CB317, CB-82794.

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Received 26 June 1997; accepted 27 June 1997

transcription in response to methotrexate and other antimetabolic drugs [Eastman et al., 1991]. This modulatory role of Sp1 is in keeping with subsequent reports of a direct role of Sp1 sites in the modulation of transcription from a number of promoters [for review see Black and Azizkhan, 1996]. However, the role of Sp1 sites in the growth/cell cycle regulation of transcription has not been evaluated directly.

The hamster DHFR promoter was the first nonviral promoter shown to bind E2F [Blake and Azizkhan, 1989]. E2F has subsequently been shown to be involved in growth/cell cycle regulation of many genes including DHFR [for review see Azizkhan et al., 1993; Slansky and Farnham, 1996; Nevins, 1992]. E2F DNA binding is due to heterodimers comprised of a member of the E2F family (E2F 1-5) and a member of the DP1 family (DP1-2) of proteins. E2F activity is regulated by binding to the retinoblastoma gene product (pRb) and the related pocket proteins, p107 and p130. This binding is regulated, in turn, by phosphorylation of pocket proteins which occurs in mid–late G_1 and leads to the release of "free" E2F and the induction of E2F-dependent transcription. It was initially thought that pocket proteins modulated E2F-dependent transcription simply by blocking its transactivation activity. However, recent studies have determined that pocket proteins contain transcription repression domains that can actively repress transcription when brought to promoters by E2F [Weintraub et al., 1995]. Indeed, at a number of promoters it appears that E2F sites act solely as repressive elements in G_0/early G_1 and have no stimulatory role in late G_1/S [for review see Black and Azizkhan, 1996; Zwicker and Muller, 1997]. Although the E2F sites in the mouse DHFR promoter have been shown to be involved in its growth/cell cycle regulation [Means et al., 1992], these studies did not differentiate between repressive and activating functions of the E2F sites.

In order to clarify the roles of the E2F and Sp1 sites in the growth/cell cycle regulation of DHFR transcription, the present study directly addressed their roles through the use of various promoter-chloramphenicol acetyl transferase gene (CAT) constructs. This approach revealed that each of these elements has significant and distinct roles: whereas the E2F sites act primarily as repressive elements in G_0 and early G_1, the Sp1 sites contribute positively to induction of transcription in late G_1.

METHODS

Cell Culture

Low passage Balb/c 3T3 mouse fibroblast cells were maintained at 37°C and 10% CO_2 in Dulbecco's modified Eagle medium with 4.5 g/l glucose (DMEM-H) (Gibco/BRL, Grand Island, NY) plus 10% calf serum (Colorado Serum Company, Denver, CO) and penicillin/streptomycin (Gibco/BRL).

Plasmid Constructs

The hamster DHFR-CAT construct is the pDHF-210/CAT promoter construct described previously [Blake et al., 1990]; it contains hamster DHFR sequence from nucleotide positions −210 to −23 (relative to the translation start site at +1) cloned 5′ to the bacterial chloramphenicol acetyl transferase (CAT) gene and poly adenylation signal from the SV40 small t antigen in a pUC18 vector. The DHFRΔSp1-CAT is described as pDHF-113/CAT in Swick et al. [1989]. It is like DHFR-CAT except that it contains only the region of the DHFR promoter from −113 to −23 upstream of the CAT gene; thus, it lacks the binding sites for Sp1. The DHFRΔE2F-CAT construct was made by site-directed mutagenesis of DHFR-CAT which replaced the central CG pair of the dyad E2F site (positions −57/−56) with a TA dinucleotide [Blake and Azizkhan, 1989]. The Δ53MLP-CAT construct (provided by Dr. Albert Baldwin) contains the TATAA box and transcriptional initiation region of the adenovirus major late promoter (nucleotides −53 to +33 relative to the major transcription start site at +1) upstream of the CAT gene and poly adenylation signal from the SV40 small t antigen. The 5XSp1Δ53MLP-CAT construct was made by annealing two single-stranded Sp1 binding site–containing oligonucleotides (5′-GATCGGGGCGGGC-3′ and 5′GATCGCCCCGCCCC3′) and ligating them into an engineered BamH1 site 30 bp 5′ to the TATAA box of Δ53MLP-CAT. The resulting construct contains five tandem copies of the Sp1 consensus binding site upstream of the adenovirus sequences in Δ53MLP-CAT.

Transient Expression Analysis

Cells were transfected by calcium phosphate precipitation essentially as described [Swick et al., 1989]. Cells (3×10^5 per 10 cm dish) were seeded 24 h prior to transfection. A precipitate containing 10–18 μg of reporter DNA and 2–5

μg of a transfection control plasmid, pXGH5 (mouse metallothionein I–human growth hormone expression construct; Nichols Institute Diagnostics, San Juan Capistrano, CA), was left on the cells for 15–18 h in a 5% CO_2 incubator. The cells were then glycerol-shocked (15% glycerol), rinsed with isotonic buffer, and placed in medium with 0.5% calf serum at 10% CO_2 for 48 h. Alternatively, 24 h after transfer to 0.5% serum, calf serum was added to a 20% final concentration, and the cells were incubated in 10% CO_2 for an additional 24 h. Cells were harvested, and cell extracts were prepared as previously described [Swick et al., 1989]. In time course experiments, multiple dishes were transfected with the same precipitate, and cells were harvested at the indicated times after serum addition. Equal volumes of extracts from serum-starved or serum-stimulated cells, representing an equivalent number of initially transfected cells, were assayed for CAT activity using [^{3}H]acetyl CoA in a liquid scintillation assay as described [Lin et al., 1996]. Levels of human growth hormone (GH) in the medium were determined using the HGH-TGES system of Nichols Institute Diagnostics.

CAT activity values were normalized on the basis of levels of growth hormone (GH) expressed from the pXGH5 plasmid, which was not responsive to serum growth factors [Jensen and Azizkhan, unpublished observations]. The GH levels increased linearly with time; therefore, a linear regression analysis of GH levels yielded a Y-intercept value which reflected the overall efficiency of DNA transfection; this value was used to normalize CAT activities for each precipitate in time course experiments.

[^{3}H]thymidine Incorporation

Following serum starvation as above, medium was replaced with medium containing 0.5–20% fetal bovine serum and 2 μCi [^{3}H]thymidine/ml. At various times thereafter, cells were rinsed twice with ice-cold phosphate buffered saline and incubated for 1 min in ice-cold 5% trichloroacitic acid containing 2 mM thymidine. Cells were then rinsed with phosphate buffered saline, dried, and solubilized in 1 M NaOH for 4 h at room temperature. The resultant solution was neutralized with 1 M HCl, and its ^{3}H content was determined by scintillation counting.

RESULTS

Previously, transient transfection analysis has revealed that the region of the hamster DHFR promoter from −210 to −23 bp (relative to the translation initiation site) is able to confer full promoter activity [Swick et al., 1989]. Since this region contains only sites for E2F and Sp1 as determined by DNA footprinting [Swick et al., 1989; Blake and Azizkhan, 1989], the role of these sites in growth/cell cycle regulation of DHFR transcription could be determined directly using promoters which were mutated to eliminate either Sp1 or E2F binding. Transient transfection assays were used since these had been used in previous studies of the hamster DHFR promoter and in growth analysis of the mouse DHFR promoter [Blake and Azizkhan, 1989; Swick et al., 1989; Eastman et al., 1991; Means et al., 1992]. The structures of the promoter-CAT constructs studied are listed in Figure 1, and the experimental procedure is outlined in Figure 2A. Briefly, the various promoter constructs were transfected into Balb/c 3T3 cells with a cotransfection control (a construct with the mouse metallothionein I promoter driving expression of human growth hormone). Following transfection, cells were serum-starved for 24 h and induced to reenter the cell cycle by addition of serum to 20% or left in low serum medium. After another 24 h, cells were harvested, and the expression of CAT was determined and normalized against expression of the cotransfection control. [^{3}H]thymidine uptake and flow cytometric analysis revealed that ≥85% of cells were in G_0/G_1 following serum starvation and began to enter S phase between 10 and 12 h after serum addition (Fig. 2B and data not shown).

Consistent with regulation of the endogenous DHFR gene [Farnham and Schimke, 1985; Johnson et al., 1978], the DHFR-CAT construct showed pronounced growth/cell cycle regulation using this protocol; CAT activity in cell lysates from cells transfected with the DHFR promoter was induced approximately thirtyfold (29.3 ± 4.2, $n = 5$) by 24 h after addition of serum to serum starved cells (Fig. 3A, DHFR-CAT). In keeping with the dependence of DHFR promoter activity on its Sp1 binding sites in cycling cells [Swick et al., 1989], the DHFRΔSp1-CAT construct showed very low activity in both serum-starved and serum-stimulated cells (data not shown). In contrast, the

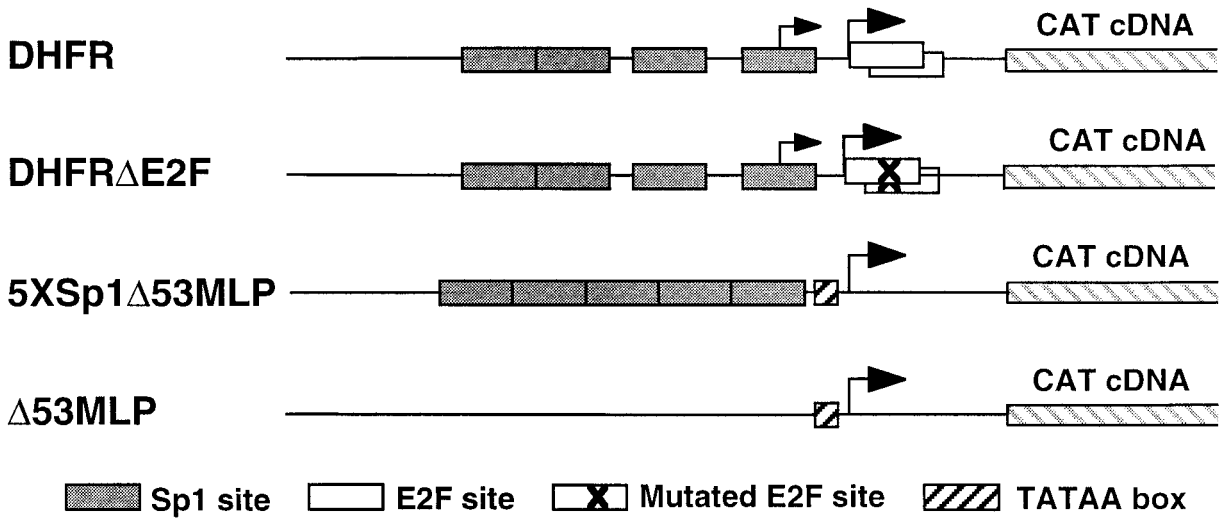

Fig. 1. Schematic representation of promoter-reporter constructs. The promoter regions of the various plasmid constructs used for transfection are diagrammed to show the relative positioning of Sp1 sites, E2F sites, TATAA boxes, and transcriptional start sites.

construct with the E2F sites mutated (DHFRΔE2F-CAT) showed significant activity and was induced by serum addition (Fig. 3A). The level of CAT activity from this construct in serum-stimulated cells was not significantly different ($P < 0.05$, n = 5) from that of the wild-type DHFR promoter (Fig. 3A). However, a significant difference between these two promoters could be seen in serum-starved G_0 cells, where the E2F mutated promoter had approximately threefold (2.8 ± 0.30, n = 4) higher activity than the wild-type promoter (Fig. 3A,B). Although the activity of the DHFR promoter and the DHFRΔE2F promoters were essentially the same in serum-stimulated cells, the elevated level of transcription from the DHFRΔE2F promoter in serum-starved cells resulted in a reduction in the relative serum induction of these two promoters; the DHFRΔE2F promoter was only induced approximately thirteenfold (12.8 ± 2.8, n = 4) by serum stimulation compared to the approximately thirtyfold induction of wild-type DHFR promoter (Fig. 3C). Together, these data indicate that the E2F sites in the DHFR promoter act to repress transcription in G_0 rather than to enhance transcription in late G_1/S phase. Moreover, since the DHFR promoter contains binding sites for only E2F and Sp1 [Blake and Azizkhan, 1989; Swick et al., 1989], the serum inducibility of the DHFR promoter with the E2F sites mutated indicates that Sp1 sites can mediate serum/growth induction of this promoter.

The ability of Sp1 sites to confer serum inducibility to an heterologous promoter was determined by placing five Sp1 sites upstream of the adenovirus major late promoter TATAA box and initiator (5XSp1Δ53MLP-CAT). The adenovirus major late promoter construct (Δ53MLP-CAT) showed low but detectable activity in serum-starved cells and was induced approximately threefold (3.1 ± 0.18, n = 4) by serum addition (Fig. 3A,C). In contrast, the construct containing the five Sp1 sites (5XSp1Δ53MLP-CAT) showed a much greater degree of serum inducibility (11.0 ± 1.1-fold, n = 4), indicating that Sp1 sites can also confer serum responsiveness to an heterologous promoter (Fig. 3A,C).

To determine if the serum induction of the DHFR promoter correlated temporally with that of Sp1 site-dependent transcription, we undertook a time course of the induction of various promoters. In cells transfected with the DHFR-CAT construct, CAT activity was low in G_0 and early G_1 cells but was seen to accumulate between 12 and 15 h following serum addition (Fig. 4), consistent with the previously observed induction of DHFR promoter activity in late G_1 [Means et al., 1992]. Very similar kinetics of induction were seen for the DHFR promoter with the E2F sites mutated (Fig. 4); although the activity DHFRΔE2F-CAT con-

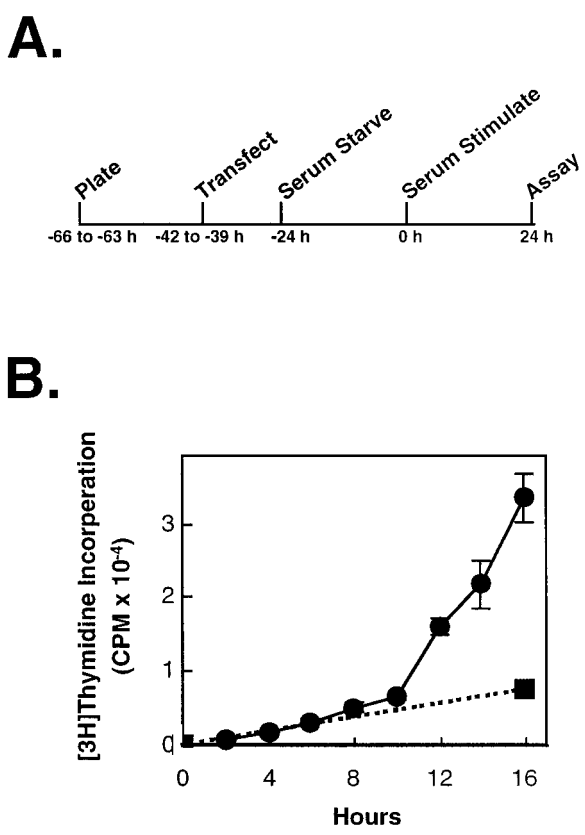

Fig. 2. Protocol for serum starvation and transfection of Balb/c 3T3 cells and kinetics of the $G_0 \rightarrow S$ phase transition. **A:** The timing of the various treatments that cells received. **B:** Levels of DNA synthesis ([^{3}H]thymidine incorporation) in cells at various times. Following starvation for 24 h, cells were placed in medium containing [^{3}H]thymidine and either 20% serum (●) or 0.5% serum (■). At the indicated times, incorporation of ^{3}H into trichloracetic acid perceptible material was determined. Data are averages of duplicates ± standard error and are representative of three experiments.

struct in G_0 and early G_1 cells was higher than that of the DHFR-CAT construct, the activity of both the wild-type and DHFRΔE2F promoters remained essentially constant for 12 h and was induced thereafter. These data indicate that the kinetics of DHFR induction can be mediated by the Sp1 sites and do not require the E2F sites. However, other parameters involved in this regulation remain to be defined since the kinetics of induction of the 5XSp1Δ53MLP-CAT construct differed from that of the DHFR promoter; it showed increased activity within 2–4 h of serum addition, and its activity increased steadily over 24 h.

DISCUSSION

The data presented herein indicate that the Sp1 and E2F sites play significant and distinct roles in the growth/cell cycle regulation of the hamster DHFR promoter. The similarity between the promoter activity of the E2F site mutant DHFR promoter (DHFRΔE2F) and the wild-type DHFR promoter in late G_1 and S phase indicates that the E2F sites play, at most, a minor positive role in the induction of the hamster DHFR promoter in Balb/c 3T3 cells. However, the significantly higher activity of the DHFRΔE2F promoter in G_0 and early G_1 indicates that the E2F sites play a role in repressing the activity of the DHFR promoter at these stages of the growth/cell cycle. Comparison of the fold induction by serum of the wild-type DHFR promoter (approximately thirtyfold) with that for the E2F site mutated promoter (approximately thirteenfold) indicates that this repression contributes significantly to the differential in the expression from the DHFR promoter in G_0 and late G_1/S phase cells.

E2F sites appear to act as repressive rather than activating elements at a number of other promoters, including those for B-myb, E2F1, CDC2, and HsORC1 [Lam et al., 1995; Cress and Nevins, 1996; Tommasi and Pfeifer, 1995; Ohtani et al., 1996], indicating that a predominantly repressive role may be a common function of E2F sites. In a previous study, we detected a positive role for the E2F sites in the hamster DHFR promoter in cycling HeLa cells [Blake and Azizkhan, 1989]. However, HeLa cells constitutively express the papilloma virus E7 protein which binds to and inactivates pRb and related pocket proteins [Pagano et al., 1992]. Since levels of pRb have been found to determine whether E2F sites act as positive or negative elements [Weintraub et al., 1992] and p130 has been shown to mediate repression in G_0 cells [Smith et al., 1996], a negative role for E2F sites would be expected to be limited to cells with functional pocket proteins (such as Balb/c 3T3 cells), and a significant positive role of the E2F sites may reflect disruption of pocket protein function.

The DHFRΔE2F promoter contains binding sites for only Sp1 [Swick et al., 1989; Blake and Azizkhan, 1989]; induction of its activity 12–16 h after serum stimulation of serum-starved cells indicates that Sp1 sites can contribute directly to the upregulation of the DHFR promoter in late G_1. The ability of Sp1 sites to confer serum inducibility was confirmed by the enhanced serum induction of the 5XSp1Δ53MLP-CAT construct compared with the Δ53MLP-CAT con-

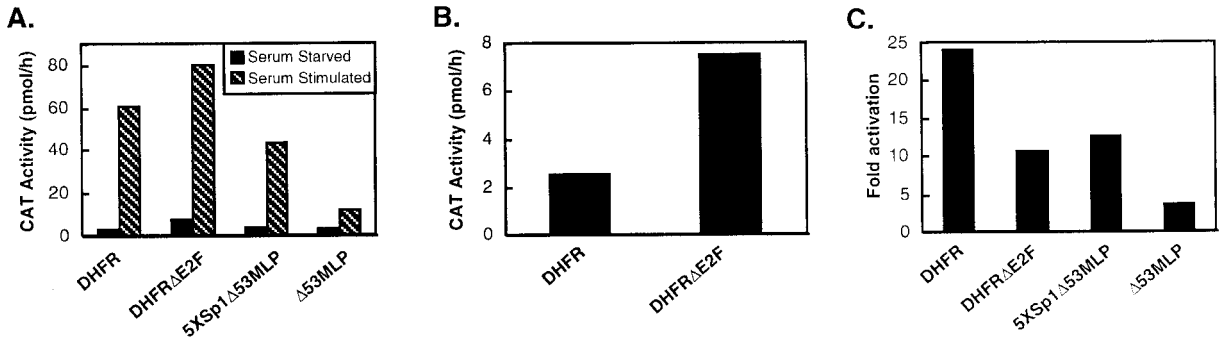

Fig. 3. Growth regulation of Sp1 site–containing promoters. **A:** Balb/c 3T3 cells were transfected with the indicated promoter-CAT constructs prior to being serum-starved for 24 h. Cells were then either left in low serum medium (serum-starved) or stimulated by addition of serum to 20%. After 24 h, cells were harvested, and CAT activity was measured and normalized against the expression of the human growth hormone cotransfection control. Data are from a single experiment which was typical of at least four experiments. **B:** Data from A for serum-starved cells transfected with DHFR-CAT or DHFRΔE2F-CAT plotted on an expanded axis to more clearly show the difference in transcription from these two promoters. **C:** For each promoter-CAT construct, normalized CAT expression in serum-stimulated cells was divided by that in serum-starved cells to give the fold activation upon serum stimulation.

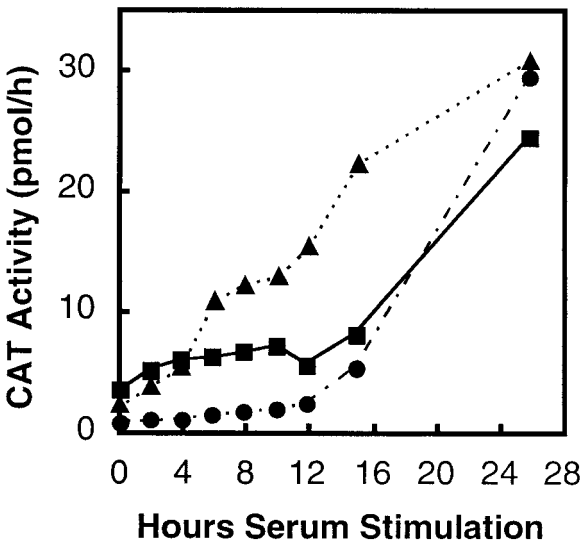

Fig. 4. Time course for serum stimulation of promoter activity. Cells were transfected with DHFR-CAT (●), DHFRΔE2F-CAT (■), or 5XSp1MLPΔ53-CAT (▲), serum-starved as described in Fig. 3, and CAT expression was determined at the indicated times following serum stimulation. Data are representative of at least three experiments.

struct. Although these results demonstrate that Sp1 sites can confer serum responsiveness to a heterologous promoter, a significant difference between the kinetics of induction of the DHFRΔE2F and 5XSp1Δ53MLP promoters was seen. This may be due to either 1) the Δ53MLP sequences in the 5XSp1Δ53MLP which showed a low level of serum inducibility or 2) the spacing of the Sp1 sites in the two promoters [Segal and Berk, 1991]. Regardless of these differences, the data clearly demonstrate that Sp1 sites can confer serum inducibility on different promoters and, at least with respect to the DHFR promoter, this induction occurs in late G_1 following reentry of cells into the cell cycle.

It has been reported previously that growth control of the mouse DHFR promoter in NIH 3T3 cells was mediated solely through E2F sites [Means et al., 1992; Fry et al., 1997]. Since the mouse and hamster promoters are highly homologous and contain only Sp1 and E2F sites, this study initially appeared to be in contradiction to our findings. However, careful analysis of the data indicates that no contradiction exists. As observed here with the hamster promoter, mutation of the E2F sites caused an approximately threefold reduction in the fold activation of the mouse DHFR promoter seen upon serum stimulation of quiescent NIH 3T3 cells [Fry et al., 1997]. Moreover, the E2F site mutant mouse promoter was induced approximately fourfold by serum stimulation [Means et al., 1992; Fry et al., 1997], indicating that Sp1 sites contribute positively to the growth regulation of the mouse as well as the hamster DHFR promoter. Our study has confirmed a role for the E2F sites in the growth regulation of the DHFR promoter and has further characterized it by determining that these sites mediate repression of transcription in G_0 and early G_1. The fact that mutation of the E2F sites in the mouse DHFR promoter led to higher activity in cycling and G_0 NIH 3T3 cells [Means et al., 1992; Fry et al., 1997] indicates that they also act as a repressive element in this pro-

moter. Thus, the roles of E2F and Sp1 sites delineated here for the hamster DHFR promoter seem to hold for the mouse promoter and are therefore a conserved feature of the regulation of this gene.

Although Sp1 sites have been considered constitutive promoter elements, a growing body of evidence indicates that they are involved in the regulation of transcription. For example, Sp1 sites mediate effects of some viral oncogenes and are involved in the monocycte-specific expression of CD14, the interferon induction of IL6 expression, the glucose induction of acetyl-CoA carboxylase, and TGFβ induction of $p15^{INK4B}$ [for references see Black and Azizkhan, 1996]. Our findings indicate that a direct role for Sp1 sites in modulation of promoter activity may be more general since they represent the first time that an ability to mediate growth/cell cycle–related signals has been attributed to Sp1.

At present the mechanism underlying the observed growth/cell cycle regulation of Sp1-dependent transcription is unclear. Since neither the levels nor synthesis of Sp1 [Lin et al., 1996; Jensen and Azizkhan, unpublished data] nor Sp1 site occupancy on the DHFR promoter (Wells et al., 1996) change during the $G_0 \rightarrow S$ phase transition, these mechanisms would not appear to be involved. However, differential binding of Sp1 family members may play a role since Sp3 can repress transcription selectively at promoters (including that of DHFR) which contain multiple Sp1 sites [Birnbaum et al., 1995; Majello et al., 1997]. Sp1 phosphorylation has been implicated in the regulation of Sp1-dependent transcription [Leggett et al., 1995; Daniel et al., 1996] and may play a role here since the phosphorylation of Sp1 is induced in late G_1 at a time consistent with the induction of transcriptional activity [Black, Jensen, Lin, and Azizkhan, manuscript in preparation). Although the data demonstrate that binding sites for E2F are not required for upregulation of DHFR transcription in late G_1, E2F may be involved in regulation of Sp1-dependent transcription. Previously, we and others have found that Sp1 can physically and functionally interact with E2F [Lin et al., 1996; Karlseder et al., 1996]. Since this interaction is induced in mid–late G_1 [Lin et al., 1996], it represents a mechanism by which E2F may contribute to the upregulation of Sp1 site-dependent transcription. Sp1 activity is also regulated by pocket proteins: it is repressed by p107 and activated by pRb [Datta et al., 1995; Kim et al., 1992; Udvadia et al., 1993]. Therefore, differential expression and/or interaction of these proteins through the growth/cell cycle could also account for the regulation of Sp1 site-dependent transcription. These possibilities are being actively pursued in this laboratory.

In conclusion, the studies presented here have helped to clarify the role of DHFR promoter elements in the growth/cell cycle regulation of its transcription. They have placed DHFR into a growing class of genes for which the E2F sites act primarily as repressive elements in G_0 and early G_1. Furthermore, they have delineated a direct role for the Sp1 sites in the activation of DHFR transcription in late G_1; this represents the first occasion where Sp1 sites have been ascribed a direct role in the growth regulation of transcription. Since E2F and Sp1 sites are common features of many growth/cell cycle–regulated promoters, these findings are likely to have wider implication for the growth regulation of gene expression and the control of cellular proliferation.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the following for many helpful discussions: Dr. Al Baldwin, Dr. Jenny Ting, Dr. Nancy Zeleznik, and members of the Azizkhan lab, past and present.

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