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Modulation of Sp1-dependent transcription by a *cis*-acting E2F element in *dhfr* promoter

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Abstract

The dihydrofolate reductase (*dhfr*) promoter contains *cis*-acting elements for Sp1 and E2F. Here we examined the cooperative regulation of *dhfr* gene transcription by Sp1 and E2F in human osteosarcoma cells, U2OS. Trichostatin A, an inhibitor of histone deacetylases, markedly stimulated *dhfr* promoter activity, a response that was enhanced by the deletion of an E2F element. In contrast, deletion of the *dhfr* Sp1 binding sites completely abolished promoter stimulation by trichostatin A. Cotransfection assays showed that activation of *dhfr* transcription by expression of E2F1/DP1 requires the reiterated Sp1 elements, whereas activation by Sp1 was enhanced by the deletion of the E2F element. Expression of HDAC1 with Sp1 suppressed promoter activity and suppression was not alleviated by coexpression of E2F1/DP1. These results suggest that HDAC1 acts through Sp1 to repress *dhfr* promoter activity, and that the E2F element modulates the activity of Sp1 at the *dhfr* promoter through a *cis*-acting mechanism.

Keywords: Dihydrofolate reductase; Sp1; E2F; HDAC; Transcriptional repression

Cellular transcription activity is at least in part regulated through the chromatin remodeling involving acetylation and methylation of histones [1,2]. Binding of transcriptional factors to DNA recruits histone acetyltransferases (HATs) that acetylate core histones, thereby enhancing nucleosomal relaxation and subsequently induction of transcription. On the other hand, several transcriptional factors such as CBF [3], hormone-dependent nuclear receptors [4], and Mad [5] can bind to histone deacetylases (HDACs) that remove acetyl groups from histones and stabilize nucleosomal structure, and thereby repress transcription. Thus, the balance between histone acetylation and deacetylation is known to modulate transcriptional activity in eukaryotic cells.

The transcription factor Sp1 associates with HDAC1 [6], and HDAC inhibitors have been shown to dramatically enhance the activities of promoters containing Sp1 binding sites [7–9]. One such promoter is that for dihydrofolate reductase (*dhfr*), which encodes an enzyme that catalyzes the synthesis of purines and thymidylate necessary for replication of DNA in the S phase of the cell-cycle [10]. Although transcription of the *dhfr* gene increases dramatically at the G1/S boundary after growth stimulation [11–13], *dhfr* mRNA is present throughout the cell-cycle [14,15]. Considerable effort has been made to identify the factors that control the

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constitutive expression of *dhfr* promoter and the factors that confer cell-cycle specific expression.

The *dhfr* promoter contains binding sites for the transcription factors Sp1 and E2F. It has been demonstrated that distinct GC boxes, responsible for Sp1 binding, direct transcription at two principal transcriptional initiation sites in rodents [16]. Only the most proximal GC box, at least in hamster cells, is needed to correctly initiate transcription at the major proximal site. Deletion of the first GC box abolishes transcription of the *dhfr* gene [17], despite the presence of the E2F-binding element downstream of the major transcriptional start. The reiterated Sp1 sites, which are required for selection of transcriptional start sites and regulate basal levels of expression, contribute to activation of *dhfr* during growth stimulation [18–20]. It has also been shown that the E2F site contributes to growth regulation [21,22]. Transcription of the *dhfr* is low in quiescent cells and is markedly activated by growth stimulation, with major increases occurring only after the phosphorylation of pRB family proteins, a process that activates E2F [23,24].

Previously, we demonstrated that repression of *dhfr* gene expression by E2F and Sp1 in the presence of low serum can be temporarily alleviated by the HDAC inhibitor trichostatin A (TSA) [25]. HDAC activity was recruited to the *dhfr* gene promoter during withdrawal from the cell-cycle, and complexes of Sp1/pRB and E2F4/p130 were observed to act together over time to mediate repression of *dhfr* gene expression in CHOC 400 cells. In this study, we investigated the potential function of HDAC as transcriptional repressor during growth arrest in a human osteosarcoma cell. Our results demonstrate that E2F element both negatively and positively regulates Sp1-dependent transcription through a *cis*-acting mechanism.

Materials and methods

Cell culture. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics in a humidified atmosphere of 5% CO_2.

Plasmids. A seris of DHFR reporter constructs were used in transient transfection assays as described previously [25]. Briefly, the pGL3-DHFR-WT luciferase reporter contains 230 bp of upstream sequence of the *dhfr* promoter that includes the transcription start site, the overlapping E2F elements, and the Sp1 binding sites (GC boxes I–IV). The pGL3-DHFR-Sp1 luciferase reporter includes nucleotides −230 to −85, which contain GC boxes I–IV, but not the E2F sites. The pGL3-DHFR-E2F luciferase reporter contains the two overlapping E2F sites alone.

RT-PCR analysis. Total RNA was extracted with RNAzolB (TEL-TEST, Inc.) and suspended in 50 μl of DEPC treated water. For first strand cDNA synthesis, 3 μg of total RNA was transcribed using an oligo(dT) primer and SuperScript RNase H-Reverse Transcriptase (Invitrogen) at 42 °C for 1 h. The mRNAs of genes of interest were amplified for 35 cycles from 10% total first strand cDNA [26]. The primer pairs for each gene spanned at least one intron to distinguish amplified products from cDNA from contaminating genomic DNA. The following primer pairs were used to amplify cDNA for cyclin A, cyclin E, DHFR, or GAPDH, respectively: Cyclin A (466 bp): 5′-TCC AAGAGGACCAGGAGAATATCA (sense), 5′-TCCTCATGGTAG TCTGGTACTTCA (antisense); Cyclin E (474 bp): 5′-AGTTCTCGG CTCGCTCCAGGAAGA (sense), 5′-TCTTGTGTCGCCATATACC GGTCA (antisense); DHFR (300 bp): 5′-CAGAGAACTCAAGGAA CCTCCAC (sense), 5′-TTAATGCCTTTCTCCTCCTGGAC (antisense); and GAPDH (983 bp): 5′-TCGGAGTCAACGGATTTGG (sense), 5′-AACTGTGAGGAGGGGAGATTCAG (antisense). Ten μl aliquots of each 50 μl PCR were resolved on a 2.0% agarose gel, the gel was stained with ethidium bromide, and DNA fragments were visualized with a UV-illuminator.

Immunoblotting. Cells (3×10^6) were washed with PBS twice and scraped from plates into Eppendorf tubes, suspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 100 mM PMSF, and proteinase inhibitors). The cell lysates were incubated at 4 °C for 30 min, and an equivalent amount of protein in the lysate (20–30 μg) was denatured with SDS sample buffer and subjected to electrophoresis on 6, 8, or 10% SDS–polyacrylamide gels. The proteins were electrotransferred to Immobilon-P membranes (Millipore) and specific proteins were detected with primary antibodies and visualized with secondary antibodies using ECL detection (Amersham) as described previously [25].

Transfection and reporter gene assays. Cells were plated at 3×10^5 cells per 60 mm-diameter culture dishes and transfection was performed by calcium phosphate coprecipitation method as described previously [25], or with Lipofectamine and Plus Reagent (Invitrogen) according to manufacturer's instruction. Each transfection contained a total of 7.2 μg DNA, including 2.4 μg of reporter plasmid and 4.8 μg of salmon sperm carrier DNA per dish, for calcium phosphate coprecipitation method, and 2 μg DNA per dish in the Lipofectamine and Plus Reagent method. Luciferase activity in cell lysates was determined using a luciferase assay kit (Promega) according to the manufacturer's instructions.

Antibodies and chemicals. Primary antibodies to cyclin A (C-19), cyclin E (HE111), cyclin B1 (GNS1), cdc25C (C-20), Sp1 (Pep-2), HDAC1 (H-51), and actin (C-11) were purchased from Santa Cruz Biotechnology. Trichostatin A (TSA) was purchased from Sigma.

Results

Effect of TSA on expression of cell-cycle regulators

To examine the role of HDAC in gene transcription regulated by E2F and Sp1, U2OS cells were treated with different concentrations of TSA for 24 h and the expression of protein products from several E2F-dependent genes was examined by Western blotting (Fig. 1A). Incubation with TSA for 24 h showed a dose-dependent increase in the expression of cyclin E and dose-dependent decreases in cyclin A, cyclin B_1, and cdc25C. No change was observed in the expression of Sp1 and HDAC1. We also examined the effects of TSA on mRNA levels 24 h after TSA treatment by RT-PCR assays (Fig. 1B). Consistent with the result of Western blotting, cyclin A expression was abolished by 100 nM TSA, whereas levels of cyclin E mRNA increased. Under these conditions TSA increased expression of *dhfr* mRNA markedly (Fig. 1B).

Activation of Sp1-mediated promoter activty by TSA

In contrast to the other E2F-dependent promoters studied in Fig. 1 (e.g., *cyclin E*, *cyclin A*, and *cdc25C*), *dhfr*

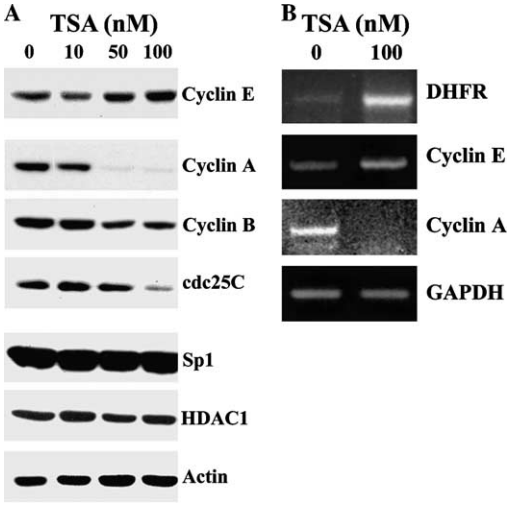

Fig. 1. Effect of trichostatin A on the expression of E2F targets. U2OS cells were treated TSA at the indicated concentrations for 24 h. Changes in the amounts of the endogenous cell-cycle proteins (A) and mRNA (B) were analyzed by Western blotting and RT-PCR, respectively.

is unique in that it contains four binding sites for Sp1. To analyze the roles of E2F and Sp1 in *dhfr* transcription, we used luciferase reporter genes linked to the wild-type promoter (DHFR-WT-luc), a mutated promoter containing the four upstream Sp1 sites but not the E2F sites (DHFR-Sp1-luc), or a promoter containing only the overlapping and inverted E2F sites at the wild-type position (DHFR-E2F-luc) (Fig. 2A). U2OS cells were transfected with the luciferase reporter plasmids and then treated with TSA at 100 nM for 24 h. Although these reporters expressed relatively low levels of luciferase activity when transiently expressed in log phase U2OS cells, TSA treatment for 24 h dramatically activated promoter activity; a 10-fold increase was observed for the DHFR-WT-luc and a greater than 14-fold increase was observed for the DHFR-Sp1-luc. The DHFR-E2F-luc plasmid construct showed low basal activity and only a 1.3-fold enhancement was observed in cells treated with TSA (Fig. 2B). These results suggest that the Sp1 sites are responsible for the majority of *dhfr* promoter activity, and that the *dhfr* promoter is negatively regulated by TSA-sensitive HDAC activity. These data also indicate that E2F acts to repress Sp1-dependent transcription. Transcription activity of pGL3-Basic was not affected by the treatment with TSA, suggesting that this regulation is promoter specific (data not shown).

HDAC activity is required for Sp1 to repress DHFR gene

The results shown in Fig. 2B were investigated further by examining the effects of expression of specific transcription factors, or combinations of transcription factors, with HDAC1 on reporter gene activity (Fig. 3). Ectopic expression of Sp1 increased the promoter activity

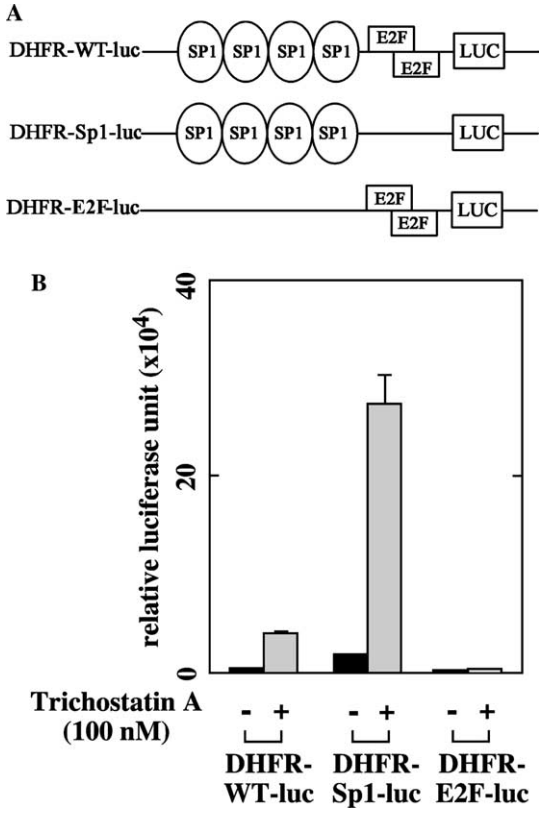

Fig. 2. Suppression of *dhfr* promoter through histone deacetylase activity and adjacent E2F elements. (A) Structure of reporter plasmids. (B) Induction of promoter activity by TSA. U2OS cells were transfected with reporters indicated and incubated with 100 nM TSA. Twenty-four hours after the transfection, activities of luciferase, and β-galactosidase in the cell lysate were determined. Bars represent standard deviations of duplicate independent experiments.

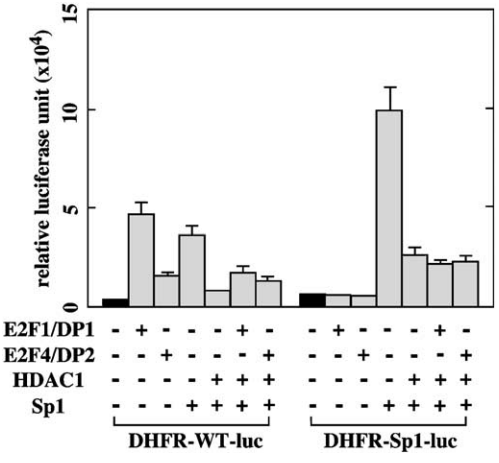

Fig. 3. Effect of ectopically expressed transcription factors on *dhfr* promoter activity. Cells were transfected with DHFR-WT-luc or DHFR-Sp1-luc together with expression vectors for the transcription factors indicated. Twenty-four hours after the transfection, activities of luciferase, and β-galactosidase in the cell lysate were determined. Bars represent standard deviations of duplicate independent experiments.

in DHFR-WT-luc, which was suppressed by coexpression of HDAC. Deletion of E2F element from the promoter (DHFR-Sp1-luc) markedly increased promoter

activity induced by Sp1. Cotransfection of E2F1 and DP1 with DHFR-WT-luc increased the promoter activity of DHFR-WT-luc as much as that of Sp1. E2F4 increased the activity in the presence of DP2, which translocates E2F4 into nucleus [27]. Since E2F1/DP1 or E2F4/DP2 did not increase the promoter activity of DHFR-E2F-luc (data not shown), *dhfr* promoter activation appears to require Sp1 elements. Neither E2F1 nor E2F4 was able to overcome HDAC1-mediated repression of promoter activity induced by Sp1, either in DHFR-WT-luc and in DHFR-Sp1-luc, suggesting that the repressive activity of the E2F element is not mediated through direct interaction of E2F protein with Sp1.

Discussion

The promoter of the dihydrofolate reductase gene, which has been well characterized in hamster, mouse, and human, contains Sp1 and E2F response elements in the vicinity of its transcription initiation region [19]. The position of the E2F site influences the transcription initiation position. The absence of functional Sp1 protein or Sp1-binding sites in the promoter almost totally abolishes *dhfr* transcription, suggesting that the *dhfr* E2F site is a very weak activator of transcription. The E2F site of the *dhfr* promoter is involved in downregulating the promoter during serum deprivation, whereas the majority of serum responsiveness relies on the Sp1 sites [20]. Although these studies suggest that Sp1 is primarily responsible for regulating levels of *dhfr* transcription, and that E2F acts primarily as a negative regulator, assigning specific roles for Sp1 and E2F in regulation of *dhfr* gene expression has generated considerable debate. We note, however, that screens for genes that are directly activated by E2F-1, E2F-2, or E2F-3 fail to identify *dhfr* as a direct target for transcriptional activation by E2F [28]. In hamster cells that express E2F-1 from a tetracycline-repressible promoter, increased levels of *dhfr* mRNA are only observed after passage through the G1 restriction point, suggesting that E2F is able to enhance activation of *dhfr* transcription only during specific phases of the cell-cycle (J. Magae and N.H. Heintz, unpublished data).

In the present study, we have shown that *dhfr* mRNA accumulates in TSA-treated cells and this effect appears to be linked to HDAC interactions with Sp1 bound to GC boxes I–IV. Hence, both Sp1/HDAC and E2F complexes regulate *dhfr* transcription in cycling cells. Moreover, transient transfection experiments suggest that binding of active E2F complex to the E2F element promotes *dhfr* transcription in a manner dependent on Sp1 sites, suggesting that E2F regulates the *dhfr* promoter negatively through the pRB family of tumor suppressor proteins, and once these proteins have been inactivated by phosphorylation, positively in cooperation with Sp1.

We previously showed the two overlapping E2F elements in *dhfr* may be occupied by different pairs of E2F family proteins in Chinese hamster cells [23,24]. The E2F2/DP1 complex appears to bind preferentially to the transcribed strand of the E2F element specifically at the G1/S boundary, whereas E2F4/DP1 complex binds to non-transcribed strand E2F element continuously during cell-cycle progression. E2F4/DP1 is predominant E2F family member proteins expressed in log phase cells, and majority of this complex is associated with a retinoblastoma family member, p107 [23,24,29]. Since p107 associates with Sp1 and represses its transcription activity [30], the repressor activity of E2F elements observed in the present study could be attributable to p107. On the other hand, the E2F2/DP1 complex, which appears specifically in G1/S boundary, may transactivate *dhfr* promoter in cooperation with Sp1 in a cell-cycle dependent manner. We also observed that p130/E2F4 complex accumulated in quiescent cells and generated a stably repressed state on the *dhfr* promoter in cooperation with Sp1/HDAC1 complex [25]. Taken together, we suggest that *dhfr* transcription is regulated during the cell-cycle progression through the recruitment and interactions of coactivators and repressors through both Sp1 and E2F.

E2F1 but not E2F4 directly binds to Sp1 and blocks the interaction of Sp1 [31,32]. As a result, E2F1 overrides HDAC1-mediated suppression of thymidine kinase promoter, which has E2F and Sp1 elements much like the *dhfr* promoter [6]. This is not the case in *dhfr* promoter, since the ectopic expression of E2F1 did not override HDAC1-mediated repression of Sp1-induced transcription activation. However, it is possible that E2F element differently regulates the transcription activity through the cooperation with Sp1 in the different manner in the different settings.

We also observed that expression of cyclin A was profoundly suppressed by TSA, despite the presence of Sp1 and E2F elements similar to *dhfr* promoter. Ectopic expression of E2F1 represses promoter activity of human telomerase reverse transcriptase in tumor cells, while it activates the promoter in normal human somatic cells [33]. Differences in the number and the arrangement of binding motifs might influence the factors recruited to the promoter, their mutual interactions, and thus entire promoter activity.

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