Histone deacetylase plays an important role in chromatin remodeling and gene expression. The molecular mechanisms involved in cell-specific expression of CT: phosphocholine cytidylyltransferase $\alpha$ (CT$\alpha$) are not fully understood. In this study, we investigated whether or not histone deacetylation is involved in repression of CT$\alpha$ expression in quiescent C3H10T1/2 mouse embryo fibroblasts. We have examined the contributions of the Sp1 and E2F binding sites in the repression of CT$\alpha$ gene expression. Immunoprecipitation experiments showed that histone deacetylase 1 (HDAC1) and HDAC activity are associated with Sp1 in serum-starved cells or during serum stimulation. However, HDAC1 association with E2F was only detected in serum-starved cells. By chromatin immunoprecipitation assays, we detected both direct and indirect association of HDAC1 with the CT$\alpha$ promoter. Treatment with the HDAC inhibitor trichostatin A induced CT$\alpha$ expression. Our data suggest that HDAC1 plays a critical role in CT$\alpha$ repression and that Sp1 and E2F may serve as key targets for HDAC1-mediated CT$\alpha$ repression in fibroblasts.

Transition through the mammalian cell cycle is accompanied by the periodic expression of genes that participate in cell cycle-dependent processes. The transcription of several genes involved in DNA replication and mitosis, the DNA polymerase $\alpha$ (PC), cyclin-dependent kinase 2, and thymidine kinase genes (1, 2), increases as cells transit through G1 and enter S phase. Cell cycle progression is also sensitive to membrane lipids, which are required for CT activity (10–12). It was reported that the wave of PC synthesis that accompanies the G0–G1 transition is regulated by changes in CT activity, membrane affinity, and intracellular distribution (13). CT$\alpha$ expression increases when the cells reach S phase (14). We demonstrated that this activation is driven by the binding of Sp1 to the B site present in the CT$\alpha$ proximal promoter (15, 16). However, the molecular mechanism involved in G0 repression of CT$\alpha$ has not been fully elucidated. A recent study has demonstrated that Net acts as a repressor of CT$\alpha$ transcription (17).

The modification of core histones is important in alteration of chromatin structure and gene transcription. Acetylation of core histones unpacks the condensed chromatin and renders the target DNA accessible to transcriptional machinery, hence contributing to gene expression (18). Conversely, deacetylation of core histones by histone deacetylase (HDAC) increases chromatin condensation and precludes binding between DNA and transcription factors, leading to transcriptional silencing (19, 20). Previous studies have identified different mechanisms for transcriptional repression. One mechanism involves a direct interaction of retinoblastoma protein (Rb) with the E2F transactivation domain, resulting in masking of this domain and blocking its ability to stimulate transcription (21). Another mechanism is based on the ability of Rb to recruit chromatin remodeling proteins, such as HDACs, and assemble transcription repression complexes at E2F-regulated promoters (21–24). Other studies have demonstrated that Sp1 recruits HDAC to a promoter (21, 25).

We have examined the role of HDAC in repression of the CT$\alpha$ promoter. Our data support a dynamic model in which Sp1-HDAC and E2F-Rb-HDAC complexes cooperate to establish stable repression of CT$\alpha$ gene expression in quiescent cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The luciferase vector, pGL3-Basic, which contains the cDNA for *Photinus pyraris* luciferase, and the dual luciferase reporter assay system were obtained from Promega (Madison, WI). Lipofectamine Plus reagent, Dulbecco’s modified Eagle’s medium (DMEM), and fetal bovine serum were from Invitrogen. Anti-Rb, anti-HDAC1, anti-Sp1, and anti-E2F (Santa Cruz) are commercially available. ECL immunoblotting reagents were purchased from Amersham Biosciences, and trichostatin (TSA) was from Sigma.

**Cell Culture**—C3H10T1/2 mouse embryo fibroblasts were cultured in DMEM supplemented with penicillin G (100 units/ml), streptomycin (100 $\mu$g/ml), and 10% fetal bovine serum (FBS) in a 5% CO2 humidified incubator at 37°C. Cells were arrested in G0 by incubation in culture medium with low serum (0.5%) for 48 h and released by the addition of fresh medium containing 10% FBS. Transient transfections with CT$\alpha$ promoter-luciferase reporter plasmids containing deletions at the 5’ end of the murine
promoter, LUC.C7 (−1268/+38) and LUC.C8 (−201/+38) (1 μg), were performed using a cationic liposome method. LUC.C7 (−1268/+38) and LUC.C8 (−201/+38), inserted into the promoterless luciferase vector pGL3-basic (Promega), were prepared as described previously (26). All dishes received 1 μg of pSV-β-galactosidase (Promega) as a control for transfection efficiency. Luciferase assays were performed using the Promega assay system, as recommended by the manufacturer, and lumino-
metric measurements were made using a Fluskan Ascent FL Type 374 luminometer (Thermolabsystems). Luciferase activity was normalized to the protein content or β-galactosidase activity. LUC.C7ΔE2F was constructed with the QuickChange kit (Stratagene) using LUC.C7 as a template and the following set of primers: E2Fup (gACCggCggCAggAgATgCCA-
GACCTCGAGGCTGGGATCCATCCgtgGCTTTg/TTgCCCTCgCCTCTACTCCTgCTC)/ChIPCT1 (5′-CCACCAgCCgCCCGCTTgTGg) and CB001 (5′-CCACA-
CATCCggAAATgTGg) and CB002 (5′-CACCggATgTA ggCAGATgTC). PCRs were performed using 2.5 μl of template DNA, 1.5 μM MgCl₂, and 20 pmol of each primer for 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C 1 min. For the assay performed on plasmids LUC.C7ΔE2F and LUC.C7delSp1, we used a set of primers, ChIPCT1 and GLP2, chosen specifically to detect the episcopal CTα promoter but not the chromosomal one.

Nuclear Extract Preparations—Total nuclear extracts of C3H10T1/2 cells grown to G₀ or S phase of the cell cycle were prepared as described by Andrews and Faller (27).

Immunoblot Analysis—Nuclear proteins (10 μg) from C3H10T1/2 fibroblasts were heated for 3 min at 90°C in 62.5 mM Tris-HCl (pH 8.3), 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 1% SDS, and 0.004% bromophenol blue. The samples were electrophoresed on a 10% SDS-polyacrylamide gel in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 0.1% SDS buffer. The proteins were then transferred to nitrocellulose by electroblotting in transfer buffer (25 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% (v/v) methanol). Following transfer, the membrane was incubated for 1 h at room temperature or overnight at 4°C with 5% skim milk in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 (T-TBS) and incubated for 1 h with antibody raised against the protein as indicated. Immunoreactive proteins were detected using the enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer’s instructions.

Immunoprecipitation—Nuclear extracts were prepared as described above from cells collected after 20 h of cell cycle induction. 200 μg of nuclear extract protein were incubated with 5 μg of polyclonal anti-Sp1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-E2F (Santa Cruz Biotechnology) in 1 ml final volume of immunoprecipitation buffer (1% (v/v) Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% (v/v) Nonidet P-40). The reaction was incubated for 1 h at 4°C and then incubated for 30 min with 50 μl of 10% Protein A-Sepharose (Staphylococcus aureus, Cowan strain), and the complex was washed three times with immunoprecipitation buffer. The pellet was resuspended in 30 μl of concentrated electrophoresis sample buffer and boiled, and the supernatant was loaded onto a SDS-polyacrylamide gel and electrophoresed. Proteins were transferred to polyvinylidene difluoride membranes and probed with appropriate antibodies.

FIGURE 1. Induction of CTα expression during G₀ by the HDAC inhibitor trichostatin A. A LUC.C7 stable cell line was synchronized for 48 h in DMEM plus 0.5% FBS, followed by incubation in the same medium with the indicated concentration of TSA or Me₂SO as a control. After 20 h, luciferase activity and protein concentration were measured. The data represent the average ± S.D. of three independent experiments where luciferase activity and protein concentrations were measured in duplicate.

Treatment with Trichostatin—Stably transfected LUC.C8 (−201/+38), LUC.C7ΔE2F, and LUC.C7ΔSp1C. C3H10T1/2 fibroblasts were grown under normal conditions for 24 h and synchronized in G₀ phase with DMEM containing 0.5% FBS for 72 h. The cell cycle was induced by adding DMEM containing 10% FBS, and TSA was added at the concentrations indicated. Samples were collected at 20 h (5 phase) after induction of the cell cycle, and luciferase activity was measured.

HDAC Activity—Nuclear extracts were immunoprecipitated using anti-Sp1 or anti-E2F antibodies, and HDAC activity was measured following the manufacturer’s instructions.

RESULTS

Is the G₀ Repression of CTα Regulated by HDAC?—We have previously shown that in C3H10T1/2 mouse embryo fibroblasts, the CTα gene is transcriptionally regulated by Sp1. Binding of Sp1 to the consensus Sp1 binding site B is essential for CTα activation during the S phase of the cell cycle (15). The interaction of the DNA-binding proteins was direct and shown to be strongly enhanced during the S phase due to Sp1 phosphorylation by cyclin-dependent kinase-2 (16). Since CTα transcription is low during the G₀ phase of the cell cycle, we investigated whether HDAC is involved in such repression. To address this question, we used a LUC.C7 (−1268/+38) (26) stable line of C3H10T1/2 cells. Cells were synchronized for 48 h by serum deprivation (G₀ phase), and after this time, duplicate samples were incubated for 20 h with the indicated concentrations of TSA or dimethyl sulfoxide as a control. TSA is a nonreversible inhibitor of HDAC (28). We analyzed the luciferase/protein ratio as a measure of CTα expression. When cells were treated with TSA, a 2.8-fold increase in CTα expression was observed, consistent with a mechanism whereby HDAC causes repression through chromatin remodeling (Fig. 1).

HDAC1 Binds the CTα Promoter—The association between HDAC1 and the CTα promoter was monitored by ChIP assay using chromatin prepared from C3H10T1/2 fibroblasts that were grown to different phases of the cell cycle (determined by [3H]thymidine incorporation into DNA; data not shown). Target sequences were detected by PCR using two sets of primers as described under “Experimental Procedures” and in Fig. 2A. A portion of the CTα promoter was found in association with anti-HDAC1 immunoprecipitate in chromatin extracted from G₀- and S-phase cells (Fig. 2B). As a control, chromatin was immunoprecipitated in the absence of specific antibodies, and in these samples, no significant quantity of CTα promoter was detected. Although the PCR is not quantitative, clearly, the amount of CTα promoter associated with HDAC1 decreased as cells progressed through the G₀-S transition.

Interactions among HDAC1, Sp1, and E2F—HDAC1-Sp1 and HDAC1-E2F interactions were observed by immunoprecipitation experiments.
HDAC1 Represses CTa Expression

Nuclear extracts obtained from resting and S-phase C3H10T1/2 mouse embryo fibroblasts were immunoprecipitated with anti-Sp1 or anti-E2F antibodies and the co-immunoprecipitated HDAC1 was detected by immunoblot with anti-HDAC1 antibody. As Fig. 3 shows, Sp1 directly or indirectly binds HDAC1 in both phases of the cell cycle. Whereas HDAC1 was expressed at a higher level in replicating cells, the affinity to Sp1 seemed to be the same. In the case of immunoprecipitation of E2F, HDAC1 was only immunoprecipitated during the G0 phase as expected from previous models where the progression of the cell cycle induced a release of HDAC from E2F (29).

HDAC activity associated with Sp1 and E2F was also analyzed in the same nuclear extracts as described above. In agreement with the immunoblot experiments, we observed that Sp1 is associated with significant HDAC activity in both G0 and S phases, whereas E2F is associated with HDAC activity in G0 phase (Fig. 4), with much less activity found in the S phase. HDAC activity was also measured in nuclear extracts treated with TSA to assess the effectiveness of the assay, and HDAC activity was inhibited by 50% (data not shown).

CTa Expression Is Regulated by E2F and Sp1—E2F regulates several families of genes whose products are required for cell progression. In G0 or G1 cells, E2F sites in the promoters of these genes are generally occupied by complexes that include E2F proteins and Rb or other members of the pocket family. Experimental data indicate that Rb-E2F repressive complexes function in association with HDAC (24, 30, 31) and that E2F appears to play a role in both repression and activation (32).

Investigation of the DNA sequence of the proximal promoter for the CTa gene revealed a putative E2F binding site at the -212 position upstream of the transcriptional start sites (see Fig. 2A). Using the luciferase reporter construct LUC.C7 (−1268/+38), we constructed a plasmid (LUC.C7ΔE2F) harboring a point mutation in the putative E2F binding site (see “Experimental Procedures”). C3H10T1/2 fibroblasts were transfected with the wild type LUC.C7 or mutated LUC.C7ΔE2F plus pSV-β-galactosidase as a control for transfection efficiency. Samples were collected at G0 phase and S phase. We also analyzed the activity of a reporter construct LUC.C7Δ Sp1(C) harboring a mutation in the previously identified Sp1 binding site C that was shown to be a negative regulator of CTa expression (15). The activity of the LUC.D3 (−52/+38) construct, which excludes both the E2F and the Sp1 binding sites (see Fig. 2A), was also analyzed. As Fig. 5 shows, mutation of the E2F binding site abruptly decreased CTa expression in both phases analyzed. Like LUC.D3, the mutation in E2F not only decreased the basal activity in G0 but also affected S-phase activation. In contrast, mutation of the Sp1 binding site C resulted in enhanced expression of CTa in the G0 phase as expected from previous models where the progression of the cell cycle induced a release of HDAC activity in both situations (29, 33).

FIGURE 2. In vivo association of HDAC1 with the CTα promoter. A, a schematic representation of the CTα promoter−15 flanking region, location of the Sp1 and E2F binding sites, the luciferase reporter plasmids with the indicated mutations, and the five primers used for PCR amplification in the ChIP assays. The lines between arrows represent the PCR products. B, a ChIP assay was performed with anti-HDAC1 antibody or a nonrelated antibody anti-IgG as a control. The pairs of primers (ChIPCT1/−201 and CB001/CB002) were used to amplify the DNA isolated from the ChIP assay. The data are representative of three separate experiments. MW, molecular weight markers. G0 and S, phase of the cell cycle.

FIGURE 3. HDAC1 co-immunoprecipitates with Sp1 and E2F. Nuclear extracts obtained at G0 or S phase were immunoprecipitated with the indicated antibodies. Total nuclear extract obtained from nonsynchronized cells (7) was used as a control. HDAC1 was detected by immunoblot analysis with anti-HDAC1 antibody.

FIGURE 4. Sp1 and E2F interact with HDAC. Nuclear extracts from C3H10T1/2 fibroblasts at the G0 or S phase of the cell cycle were immunoprecipitated with anti-Sp1 antibody or anti-E2F antibody. Associated HDAC activity (ratio of fluorescence to mg of protein) was measured as described under “Experimental Procedures.” The data represent the means ± S.D. of three independent experiments.

FIGURE 6. Association of E2F, Rb, and HDAC with the CTα promoter in the G0 phase of the cell cycle. ChIP assays were performed with anti-E2F, -Rb, or -HDAC antibodies. A nonrelated antibody, anti-IgG, was used as a control. The pairs of primers (ChIPCT1/−201 and CB001/CB002) as shown in Fig. 2A were used to amplify the DNA isolated from the ChIP assay. The data are representative of two separate experiments. MW, molecular weight markers.
**HDAC1 Represses CTα Expression**

We provide evidence for the first time that HDAC1 is involved in the repression of murine CTα expression in quiescent cells. The repression of CTα expression in C3H10T1/2 mouse embryo fibroblasts arrested in G0 phase could be partially mitigated by inhibition of HDAC activity. TSA treatment of the LUC.C7 cell line (Fig. 1), but not in cells transiently transfected with the same construct (data not shown), restores whereas no significant amount was detected with a nonspecific antibody, anti-IgG. Although the PCR used was not quantitative, there were differences in the intensities of the bands that may reflect a direct or indirect interaction between each protein with DNA (33).

**Is HDAC Recruited to the CTα Promoter by either Sp1 or an E2F-Rb Complex?**—Luciferase reporter constructs LUC.C7ΔE2F and LUC.C7ΔSp1(1C), with mutations in the E2F and Sp1(1C) binding sites, respectively, were transfected into C3H10T1/2 fibroblasts. After synchronization in G0 phase, the cells were analyzed by ChIP assay using antibodies against Sp1, Rb, HDAC, and E2F (Fig. 7). The fragment of the CTα promoter harboring the corresponding mutation in each case was analyzed by designing primers ChIPCT1 and GLP2 (see Fig. 2A), which specifically recognize the plasmids. As a negative control, we used a sample incubated with a nonrelated antibody anti-IgG. Mutation in the CTα binding site does not affect the association between the CTα promoter with Sp1, HDAC1, or E2F-Rb. In contrast, a mutation in Sp1(1C) decreases the association with HDAC1 but has no effect on E2F and Rb association. Because the primers amplified a fragment of the CTα promoter that includes three Sp1 binding sites, we did not evaluate the effect of a mutation in the Sp1 binding site C on Sp1 association. Thus, the interaction of HDAC1 with the CTα promoter depends at least in part on the Sp1(1C) binding site.

We also analyzed whether or not inhibition of HDAC activity by TSA would alter luciferase expression in LUC.C8 (–201/+38), LUC.C7ΔE2F, and LUC.C7ΔSp1(1C) stable cell lines. Cells were synchronized for 48 h by serum deprivation, after which duplicate samples were incubated with the indicated concentration of TSA or dimethyl sulfoxide as a control. We analyzed the luciferase/protein ratio as a measure of CTα expression. Mutation in the Sp1(1C) binding site did not abolish the induction of luciferase activity caused by TSA (Fig. 8A). However, mutation of E2F decreased the induction by TSA, but we still observed a 1.7-fold induction of luciferase activity after treatment. In agreement with our previous results, analysis of LUC.C8, which excludes the E2F binding site, revealed an increase in CTα expression in both phases of the cell cycle after TSA treatment (Fig. 8B).

**DISCUSSION**

We provide evidence for the first time that HDAC1 is involved in the repression of murine CTα expression in quiescent cells. The repression of CTα expression in C3H10T1/2 mouse embryo fibroblasts arrested in G0 phase could be partially mitigated by inhibition of HDAC activity. TSA treatment of the LUC.C7 cell line (Fig. 1), but not in cells transiently transfected with the same construct (data not shown), restores
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CTα expression. After the episomal CTα promoter, which is methylation-free, is transfected into fibroblasts, it will form minichromatin but not in a repressive chromatin structure as does the endogenous CTα promoter (34). We also demonstrated by ChIP assays that HDAC associates in vivo with the CTα proximal promoter. Based on this result, it would be logical to ask how CTα is repressed in quiescent C3H10T1/2 fibroblasts by HDAC1.

Sp1 Recruits HDAC to the CTα Promoter and Regulates Its Expression in Quiescent Cells—CTα expression during cell cycle progression is mainly regulated by Sp1. Three Sp1 binding sites have been identified in the CTα proximal promoter (see Fig. 2A); Sp1 site B drives CTα induction in G1-S phase, whereas site A acts as a transcriptional enhancer. Conversely, the Sp1 binding site C appears to be a repressor of CTα expression (15). HDAC1 was readily recovered in immunoprecipitates of Sp1, and this interaction persisted in serum-starved cells, supporting the notion that Sp1 plays a role in repression of CTα. Doetzlhofer et al. (25) suggest that Sp1 can serve as a target for HDAC1-mediated transcriptional repression, although Sp1 is usually known as a positive transcriptional factor. Moreover, in different cell lines, gene repression is regulated by the association of HDAC1 or -2 and Sp1 or Sp3 (35, 36). Given that Sp1 associates with HDAC1 and Sp1 binds and regulates CTα expression, it is likely that Sp1 may serve as a target for HDAC1-involved CTα suppression. To address this hypothesis, we analyzed by ChIP assay the ability of a mutated Sp1 binding site to recruit HDAC1 (Fig. 7). A limited quantity of CTα promoter harboring a mutation in the Sp1 binding site C was immunoprecipitated with anti-HDAC antibody. However, this mutation did not abolish the effect of the HDAC-specific inhibitor TSA on CTα expression; the LUC.C7ΔSp1(C) stable cell line showed a 3.5-fold increase in luciferase reporter activity after treatment (Fig. 8A). Taken together, these results suggest that HDAC-mediated regulation of CTα expression in quiescent cells is in part due to a Sp1-HDAC complex. However, because we still observed regulation in the absence of Sp1 binding site C, we propose that additional protein(s) could be involved in HDAC recruitment to the promoter.

E2F Cooperates with Sp1 in Recruiting HDAC—Binding sites for E2F are found in the promoters of genes whose expression occurs at G1/S transition, and repression of promoters containing E2F-binding sites has been shown to involve binding of E2F/Rb pocket protein complexes to these sites (37). One of the mechanisms of transcriptional repression is based on the ability of Rb to recruit chromatin remodeling proteins, such as HDACs, and assemble transcriptional repression complexes at E2F-regulated promoters (22–24). With this information in mind, we evaluated the presence of E2F-HDAC complexes by immunoblotting, using nuclear extracts obtained during G0 and S phases (Fig. 3). We only detected the complex in extracts obtained in G0 phase, in agreement with a previous report that Rb is released after phosphorylation by cyclin D-cyclin-dependent kinase 4 during G1-S phase (38). To further investigate the role of E2F in CTα regulation, we analyzed the activity of a reporter construct having a point mutation in the putative E2F binding site found in the −210 position with respect to the transcriptional start point (Fig. 5). Surprisingly, this mutation decreased CTα expression to a similar extent as LUC.D3 (−54/+38), in which the promoter is almost entirely deleted. This result indicates that, as with other cell cycle-dependent genes, CTα expression is regulated by E2F.

E2F, Rb, and HDAC1 were detected on the CTα promoter by ChIP assay (Fig. 6). The different intensities of the PCR band may reflect the type of interaction between the factors and the DNA, with E2F acting as the anchor protein to recruit Rb+HDAC. The role of E2F in HDAC-dependent repression of CTα was examined by measuring the ability of a mutant E2F binding site to allow for the CTα promoter to be immuno-precipitated by various antibodies (Fig. 7). Because mutation of the E2F binding site did not affect the binding of E2F, HDAC, and Rb to the promoter, we can speculate that these and probably other proteins such as Sp1 form a multimolecular complex that regulates CTα expression (Fig. 9). The cooperation between E2F and Sp1 is supported by the observation that members of these two families interact with one another (39–41). In concurrence with this result, a stable cell line with a mutated E2F binding site is still sensitive to TSA, showing a 1.7-fold increase in luciferase activity (Fig. 8A). On the basis of these results, we propose that an interaction between E2F and Sp1 or other proteins not yet identified as part of the complex cooperates in recruiting HDAC to the CTα promoter.

In conclusion, this work describes a model for the control of the murine CTα promoter in quiescent cells (Fig. 9). During growth arrest, E2F binds Rb+HDAC1, and the nearby Sp1 recruits HDAC1. Thus, both E2F-Rb and Sp1 bind HDAC1, thereby causing repression of the expression of the CTα promoter and, thereafter, a decrease in PC biosynthesis, which should impact on membrane biogenesis. The model is in agreement with reports on regulation of the dihydrofolate reductase promoter (42, 43) and thymidine kinase promoter (25), which have shown that Sp1 in addition to E2F plays an active role in the growth control of transcription. Further experiments will be necessary to clarify the type of interaction between each of these proteins.

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