THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 38, Issue of September 19, pp. 36496–36504, 2003
Printed in U.S.A.

Che-1 Arrests Human Colon Carcinoma Cell Proliferation by Displacing HDAC1 from the *p21*$^{WAF1/CIP1}$ Promoter*

Received for publication, June 24, 2003, and in revised form, July 2, 2003
Published, JBC Papers in Press, July 7, 2003, DOI 10.1074/jbc.M306694200

Monica Di Padova‡§, Tiziana Bruno‡, Francesca De Nicola‡¶, Simona Iezzi‡, Carmen D'Angelo‖, Rita Gallo¶, Daniela Nicosia¶, Nicoletta Corbi§, Annamaria Biroccio‖, Aristide Floridi‡¶, Claudio Passananti**, and Maurizio Fanciulli‡ ‡‡**

*From ‡Laboratory B and the ‖Experimental Chemotherapy Laboratory, Experimental Research Center, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, the ¶Department of Experimental Medicine, Via Vetoio, Coppito 2, University of L'Aquila, 67100 L'Aquila, and the **Istituto di Biologia e Patologia Molecolari, Consiglio Nazionale delle Ricerche, Viale Marx 43, 00137 Rome, Italy*

Che-1 is a recently identified human RNA polymerase II binding protein involved in the regulation of gene transcription and cell proliferation. We previously demonstrated that Che-1 inhibits the Rb growth-suppressing function by interfering with Rb-mediated HDAC1 recruitment on E2F target gene promoters. By hybridization of cancer profile arrays, we found that Che-1 expression is strongly down-regulated in several tumors, including colon and kidney carcinomas, compared with the relative normal tissues. Consistent with these data, Che-1 overexpression inhibits proliferation of HCT116 and LoVo human colon carcinoma cell lines by activation of the cyclin-dependent kinase inhibitor p21$^{WAF1/Cip1}$ in a p53-independent manner and by promoting growth arrest at the G_1 phase of the cell cycle. Che-1 activates p21$^{WAF1/Cip1}$ by displacing histone deacetylase (HDAC)1 from the Sp1 binding sites of the *p21*$^{WAF1/Cip1}$ gene promoter and accumulating acetylated histone H3 on these sites. Accordingly, Che-1-specific RNA interference negatively affects *p21*$^{WAF1/Cip1}$ transactivation and increases cell proliferation in HCT116 cells. Taken together, our results indicate that Che-1 can be considered a general HDAC1 competitor and its down-regulation is involved in colon carcinoma cell proliferation.

Histone acetyl transferases and histone deacetylases (HDACs)[1] affect gene expression by regulating the acetylation of histone and non-histone proteins (1–3), and HDAC inhibitors, such as sodium butyrate, trichostatin A, or suberoylanilide hydroxamic acid, inhibit proliferation of several tumor cell lines and tumor growth *in vivo* (4–10). These compounds act selectively on genes, altering the transcription of only ~2% of expressed genes in cultured tumor cells (11), and several structurally different histone deacetylase inhibitors are in phase I or II clinical trials for cancer treatment (12, 13). Although the mechanism/s of HDAC inhibitor-induced growth arrest has not been fully established, several studies have shown that these compounds strongly activate the expression of the cyclin-dependent kinase inhibitor p21$^{WAF/CIP1}$ (p21) (8, 14, 15) through six Sp1 sites on the *p21* gene promoter (16–21). Consistent with these observations, it has been demonstrated that p21 is absolutely required for butyrate-induced growth arrest (14).

Che-1, initially identified by its ability to interact with the subunit 11 of human RNA polymerase II in a yeast two-hybrid screen, is a human nuclear protein (22, 23). The studies performed on this protein and its homologues in rat and mice strongly support the idea that Che-1 is involved in the regulation of gene transcription and cell proliferation (22–26). Recently, we have demonstrated that Che-1 contacts the Rb pocket region and competes with HDAC1 for Rb binding site, removing HDAC1 from the Rb-E2F complex *in vitro* and from the E2F target promoters *in vivo* (27). Furthermore, we found that Che-1 expression can thus regulate E2F-dependent transcription and cell proliferation (27), supporting a novel mechanism of Rb inactivation. Although Rb mutations are not frequent, disruption of Rb activity occurs in the majority of human tumors through several mechanisms, including viral oncoprotein binding or deregulated Rb phosphorylation by mutations of cyclins or cyclin-dependent kinase inhibitors (28). Therefore, it is possible that Che-1 expression could also be altered during neoplastic transformation. Indeed, as the E2F pathway is activated in highly proliferative cancer cells, we might expect Che-1 levels to be altered similarly.

This study was designed to analyze Che-1 expression in multiple matched tumor/normal clinical samples. We found that Che-1 is down-regulated in several human cancers, including colon carcinomas, and that its overexpression in colon carcinoma cell lines induces a growth arrest. Notably, Che-1 exerted its functions displacing HDAC1 from Sp1 binding sites of the *p21* gene promoter activating p21 expression. These results show that Che-1 down-regulation is involved in colon carcinoma proliferation and together with our previous observations strongly support Che-1 as a general HDAC1 competitor.

MATERIALS AND METHODS

Cancer Profiling Array—A radioactive probe was generated using a Che-1 cDNA fragment. The probe was hybridized against a cancer profiling array (BD; Clontech), following the manufacturer's instructions.

Antibodies—The following rabbit polyclonal antibodies were used: anti-Che-1 (22), anti-green fluorescent protein (GFP) (BD; Clontech), anti-human Sp1 (sc 59, Santa Cruz Biotechnology), anti-HDAC1 and

* This work was supported by the Italian Association for Cancer Research (A.I.R.C.), Italian Ministry of Health (Ministero della Sanità), Telethon project A160, and Ministero Istruzione Università e Ricerca. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of an Italian Federation for Cancer Research fellowship.
‡‡ To whom correspondence should be addressed. Tel.: 3906-52662566; Fax: 3906-52662566; E-mail: fanciulli@.ifo.it.

[1] The abbreviations used are: HDAC, histone deacetylase; GFP, green fluorescent protein; GST, glutathione *S*-transferase; BrdUrd, bromodeoxyuridine; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; RFP, red fluorescent protein; si, small interfering; ChIP, chromatin immunoprecipitation.

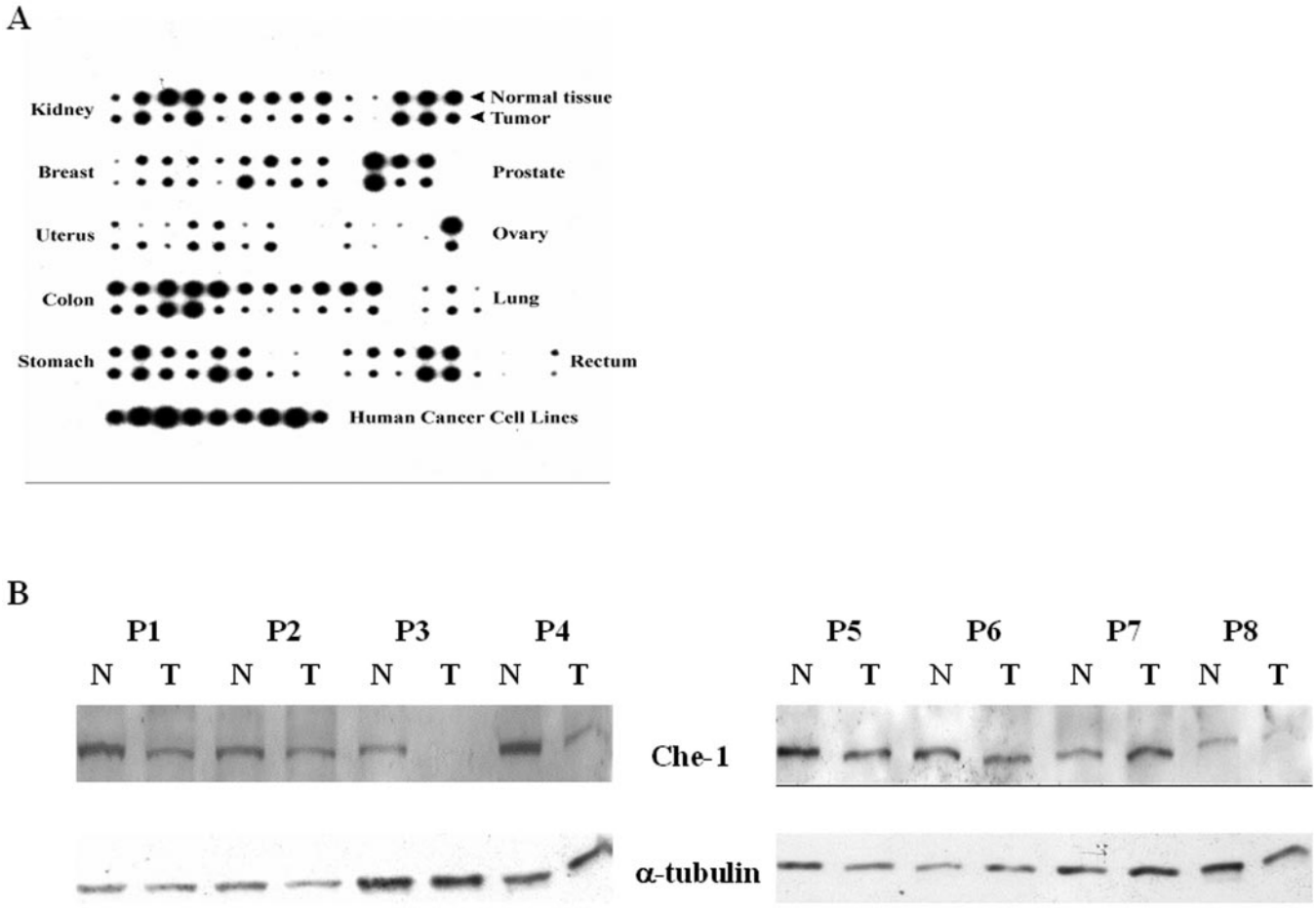

FIG. 1. **Che-1 is down-regulated in various human colon carcinomas.** *A*, a radioactive probe was generated from Che-1 cDNA for Southern blotting of an array of matched normal and tumor tissues. *B*, lysates from frozen tumor (*T*) and normal tissues (*N*) of eight colon carcinoma patients (*P*) were subjected to Western blot analysis with anti-Che-1 and anti-α-tubulin antibodies.

acetylated histone H3 (Upstate Biotechnology), anti-Myc (Chemicon International). Mouse monoclonal antibodies anti-Myc 9e10 (Invitrogen), α-tubulin and human p21 (sc-8035 and sc-6246; Santa Cruz Biotechnology), and bromodeoxyuridine (BrdUrd) (Roche Applied Science) were also used.

Plasmids—The Myc-tagged Che-1 mammalian expression vector and its deletions have been previously described (22). The *p21* promoter fused to a luciferase reporter was a gift from Dr. P. L. Puri (Salk Institute, San Diego, CA). The complete open reading frame of human Sp1 was generated by PCR and cloned into the pGEX4T1 vector (Amersham Biosciences) to produce the GST-Sp1 fusion protein.

Cell Culture, Transfections, and Analysis—LoVo and HCT116 human colon carcinoma cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HCT116 p21−/− and p53−/− were kindly provided by B. Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD) (29). Transfections were carried out by BES-calcium phosphate precipitation as previously described (22). Cell proliferation was evaluated by BrdUrd labeling and detection assay (Roche Applied Science) 24 h after transfection with Myc-tagged Che-1 or with GFP vectors as negative control. The immunofluorescence staining for BrdUrd was performed as previously described (30). Cell cycle analysis of GFP-transfected cells was performed as previously described (31). The data are presented as bivariate distribution dot plots, showing DNA content (red fluorescence) along the *x*-axis *versus* GFP (green fluorescence) along the *y*-axis. Cell cycle distribution of GFP-positive cells is shown as linear propidium iodide. Annexin apoptosis assay of red fluorescent protein (RFP)-transfected cells was analyzed as previously described (32). Adherent cells were stained with vibrant apoptosis assay (Molecular Probes, Eugene, OR). The data are presented as bi-parametric dot plots showing the fluorescein isothiocyanate-annexin V (green fluorescence) *versus* RFP. Flow cytometric analyses were performed using FACScalibur (BD PharMingen). For each analysis 20,000 gated events of GFP- or RFP-positive were collected. Data analyses were performed using Cell Quest (BDIS) and ModFit LT (Verity Software House, Topsham, ME). Colony-forming efficiency assays were performed following transfections with the same amounts of plasmids carrying G418 or puromycin resistance. Drug selection was initiated 2 days following transfection, and dishes were stained with methylene blue after 7 or 14 days. Luciferase assays were performed as previously described (22), and pCMVβ-galactosidase expression plasmid was used as an internal standard.

RT-PCR Analysis—For semi-quantitative RT-PCR analysis, cells were harvested 36 h after transfection and total RNA isolated using TRIZOL reagent (Invitrogen). RT-PCR was performed using a Platinum quantitative RT-PCR kit (Invitrogen) following the manufacturer's instructions. PCR products were separated onto 2% agarose gel. PCR conditions were: 1 cycle at 95 °C 5 min, followed by 25 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. The following primers were employed: *p21* forward, 5′-ATGTCAGAACCGGCTGGGGAT-3′; *p21* reverse, 5′-CCTCTTGGAGAAGATCAGCCG-3′; *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* forward, 5′-CCAAGGTCATCCATGACAAC-3′; *Gapdh* reverse, 5′-TTACTCCTTGGAGGCCATGT-3′.

Immunoprecipitations and Western Blot Analysis—For immunoprecipitation experiments cells were lysed by incubation at 4 °C for 30 min in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5 mM EDTA, 0.5 mM EGTA, 100 mM NaF, 3 mM $NaOV_4$, 10 nM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin). After high speed centrifugation, the lysates were precleared with 20 μl of protein A/protein G beads (Santa Cruz Biotechnology) and immunoprecipitated by standard procedures. Frozen tissue specimens were mechanically disaggregated, and cell suspensions were incubated at 4 °C for 30 min in lysis buffer. Lysates were centrifuged at 16,000 × *g* for 20 min at 4 °C and supernatants assayed for protein concentration. For each sample, equal amounts of total protein were loaded. Western blots were prepared by standard procedures using antibodies described above. Immunoreactivity was detected by ECL chemoluminescence reaction (Amersham Biosciences).

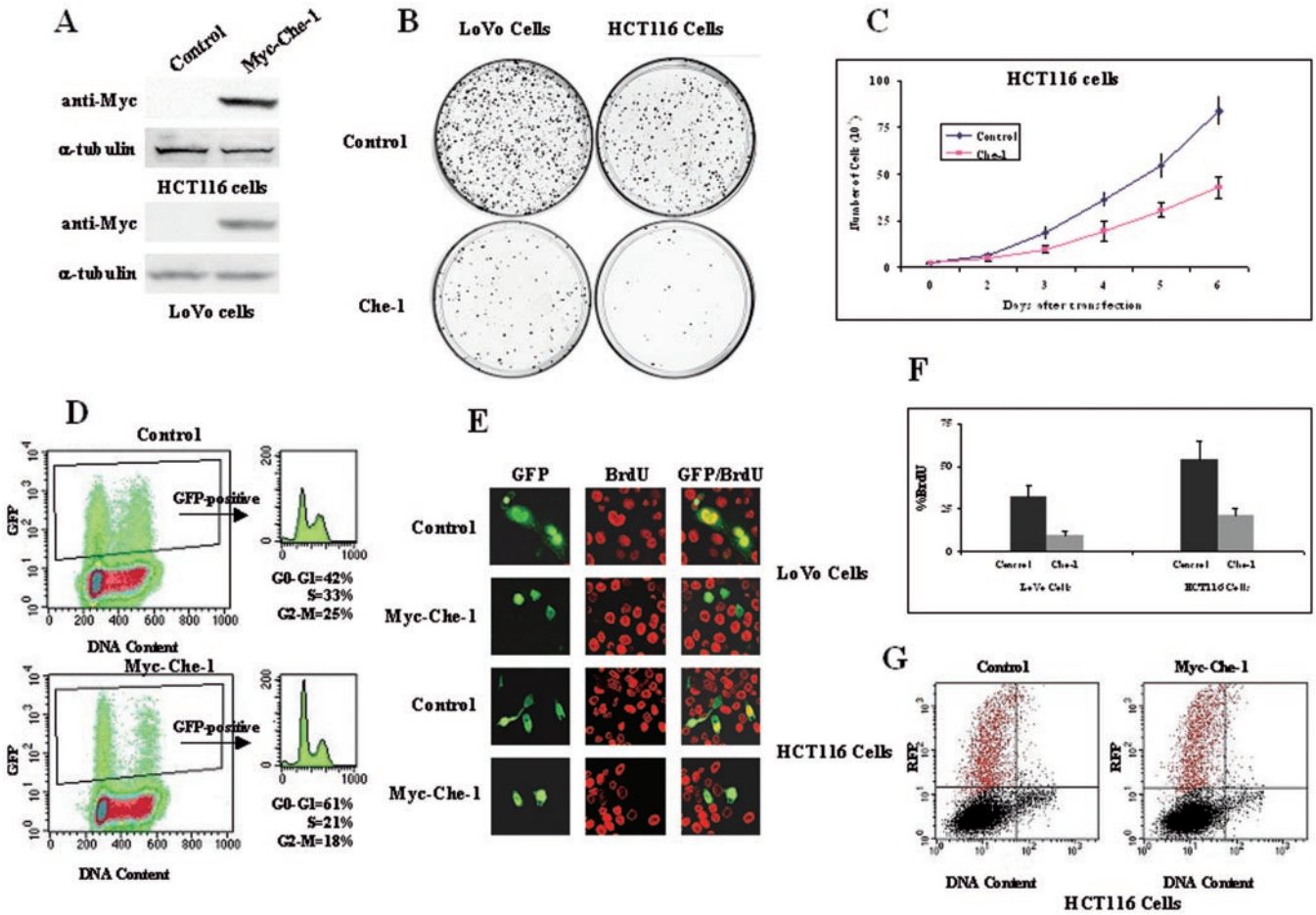

FIG. 2. **Che-1 induces G_1 block in human colon carcinoma cells.** *A*, lysates from HCT116 and LoVo cells transiently transfected with empty vector (*Control*) and Myc-Che-1, respectively, were subjected to Western blot analysis with anti-Myc and anti α-tubulin antibodies to verify protein expression. *B*, HCT116 and LoVo cells were transiently transfected with empty pCMV expression vector (*Control*) or Myc-Che-1 and a vector carrying neomycin-resistance gene (10:1 ratio) and selected for 14 days in G418. Parallel plates were stained with methylene blue and scored. Representative HCT116 and LoVo cells plates are shown. *C*, cell proliferation analyses of HCT116 cells transiently transfected with empty vector (*control*) and Myc-Che-1, respectively. Data are presented as the mean ± S.D. from three independent experiments performed in duplicate. *D*, Myc-Che-1 or empty vector (*control*) were cotransfected with RFP (10:1 ratio) into HCT116 cells. Cells were fixed and analyzed for annexin V fluorescein isothiocyanate-green fluorescence of RFP-positive cells. *E*, Myc-Che or empty vector (*Control*) were cotransfected with GFP (10:1 ratio) into HCT116 cells. Cells were fixed and stained for propidium iodide to analyze the DNA content of GFP-positive cells. *F*, HCT116 and LoVo cells transiently transfected as in *panel E* and visualized by a double fluorescence analysis with anti Che-1 or anti-GFP-specific antibodies and anti-BrdUrd monoclonal antibody. *G*, quantification of BrdUrd-positive HCT116 and LoVo cells. Data are presented as the mean ± S.D. from three independent experiments.

Pull down Analysis—Pull down experiments were performed as previously described (22). *In vitro* transcription and translation were carried out with TNT-coupled reticulocyte lysate systems (Promega) and L-[^{35}S]methionine (>1,000 Ci/mmol; Amersham Biosciences), following the manufacturer's instructions.

Chromatin Immunoprecipitation Assay (ChIP)—ChIP assays were performed as previously described (27). In each experiment, signal linearity was tested by amplifying increasing amounts of the DNA template. Generally, DNA representing 0.005–0.01% of the total chromatin sample (input) or 1–10% of the immunoprecipitated was amplified using promoter-specific primers. Immunoprecipitation with no specific immunoglobulins (Santa Cruz Biotechnology) was performed as negative control. PCR conditions were: 1 cycle at 95 °C for 2 min, followed by 30 cycles at 95 °C for 1 min, 68 °C for 1 min, and 72 °C for 2 min. The following p21 promoter-specific primers were employed for PCR amplifications: Sp1 1–2 forward, 5′-TCCGGGACCGGCTGGCCT-3′; Sp1 1–2 reverse, 5′-GCTCGGCCCACCGCGCCG-3′; Sp1 3–6 forward, 5′-GCGGGTCCCGCCTCCTTG-3′; Sp1 3–6 reverse, 5′-TCTGG-GCCGCCGGCCCGG-3′.

RNA Interference in Human Cells—The 22-nucleotide small interfering RNA (siRNA) duplexes corresponding to nucleotides 191–212 of the human Che-1 sequence and to nucleotides 122–143 of the negative control GFP sequence were synthesized by Xeragon. RNA interference was performed as described by Elbashir *et al.* (33).

RESULTS

Che-1 Is Down-regulated in Human Colon Carcinomas—To evaluate Che-1 expression in different tumor samples, the Che-1 cDNA was used as probe on a filter containing 68 cDNA pairs from tumor and the corresponding normal tissues of individual patients, enabling the study of Che-1 expression in normal and tumor samples side by side. Surprisingly, Che-1 transcript levels showed a substantial decrease in several tumors of kidney, prostate, and colon relative to matched normal tissue (Fig. 1*A*). In particular, 9 of 11 colon cancers present on the filter showed a significant decrease of Che-1 expression. To confirm this result, we analyzed Che-1 expression in normal and tumor tissues from patients affected by colon cancer. Densitometric analysis of Western blots revealed that Che-1 protein levels were decreased in five of eight tumor extracts (P1, -3, -4, -6, -8) when compared with colon normal tissue (Fig. 1*B*), whereas in the other patients Che-1 levels did not change (P2, -5) or slightly increased in tumor cells (P7) (Fig. 1*B*). Taken together, these results indicate that Che-1 is down-regulated in various colon carcinomas.

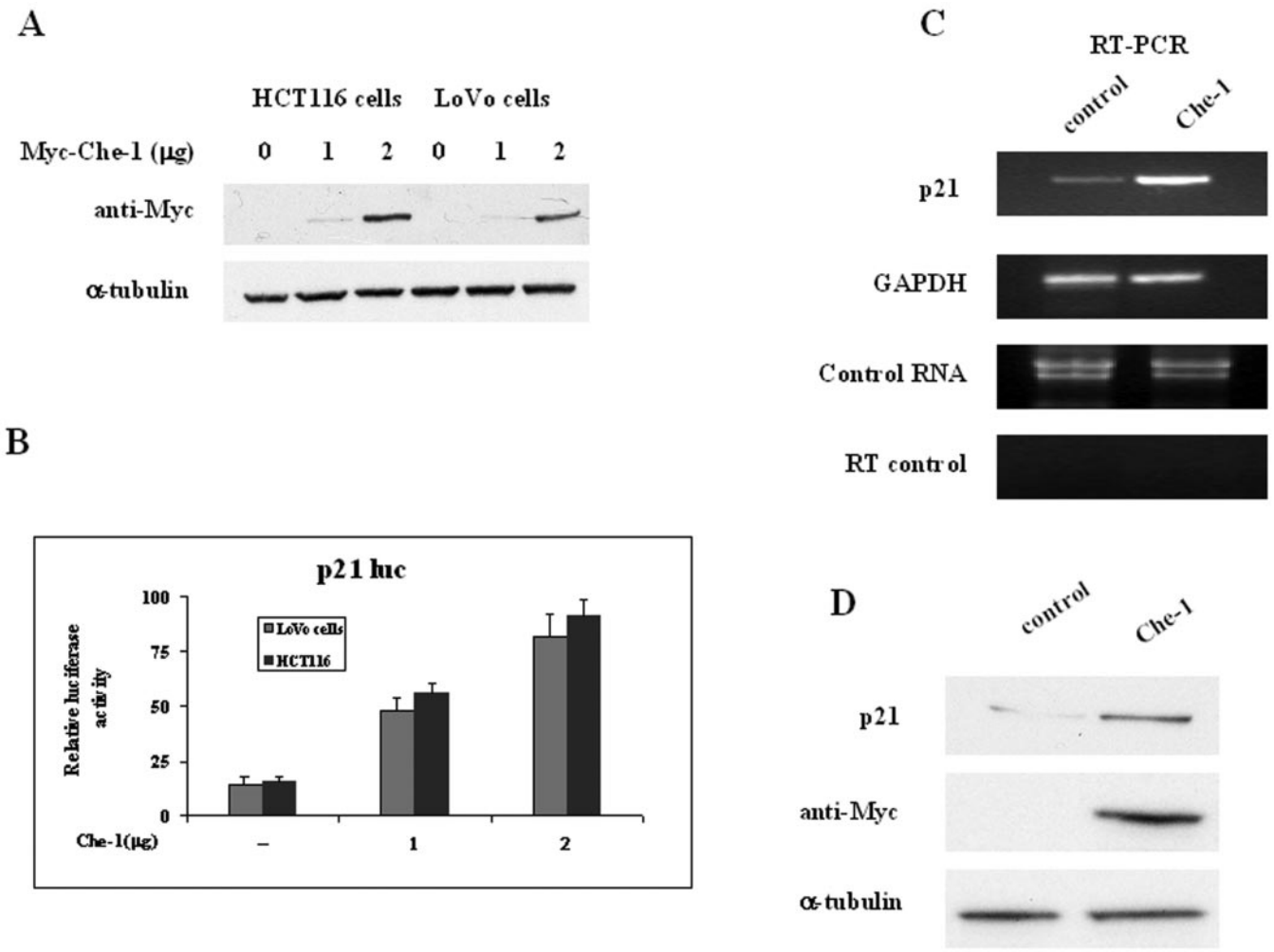

FIG. 3. **Che-1 increases p21 expression.** *A*, lysates from HCT116 and LoVo cells transiently transfected with the indicated amounts of Myc-Che-1 were subjected to Western blot analysis with anti-Myc and anti-α-tubulin antibodies to verify protein expression. *B*, HCT116 and LoVo cells were transiently transfected with 1 μg of the *p21* promoter luciferase reporter and the indicated amounts of Myc-Che-1. The total amount of transfected DNA was normalized with pCMV empty vector (−). Data are presented as the mean ± S.D. from three independent experiments performed in duplicate. *C*, RNA from HCT116 cells transiently transfected as described in *panel A* was isolated. Equal amounts of RNA (RNA input) were analyzed by RT-PCR (25 cycles) for expression of the *p21* gene. The RT control lanes represent RT-PCR in the absence of reverse transcription. *D*, lysates from HCT116 cells transiently transfected with empty vector (*Control*) or Myc-Che-1, respectively, were subjected to Western blot analysis with anti-p21, anti-Myc, and anti-α-tubulin antibodies.

Che-1 Induces Growth Arrest of Human Carcinoma Cell Lines—To determine whether the down-regulation of Che-1 expression had relevance to the growth of this type of tumor, Che-1 was overexpressed in two human colon carcinoma cell lines, HCT116 and LoVo (Fig. 2*A*), and colony-forming efficiency assays were performed. Parallel plates containing the same number of cells were transfected with control or Che-1 expression constructs, selected with G418, and the number of colonies/plate screened 14 days later. In both cell lines, Che-1-transfected cells showed more than 70% reduction in colony-forming efficiency compared with cells transfected with the empty vector (Fig. 2*B*). Consistently, HCT116 cells expressing Che-1 protein exhibited a decreased rate of proliferation (Fig. 2*C*). To evaluate whether the growth reduction produced by Che-1 overexpression could be because of apoptosis, an annexin V assay was performed. As is shown in Fig. 2*D*, no differences were observed between cells transfected with RFP and Che-1 or empty vector.

These findings, underscoring an involvement of Che-1 in the regulation of colon carcinoma cell proliferation, prompted us to analyze the growth properties of HCT116 cells overexpressing Che-1. Cotransfection of GFP enabled the flow cytometric analysis of the transfected (+GFP) cell population. Fig. 2*E* shows that transient transfection of Che-1 into HCT116 cells induced a strong accumulation in G_1 phase as compared with cells transfected with empty vector (61 *versus* 42%), parallel to a decrease of cells in S phase (21 *versus* 33%). These results were confirmed evaluating BrdUrd incorporation in HCT116 and LoVo cells transfected with Che-1. BrdUrd was added to the media, and cells were incubated for an additional 6 h before fixation and immunofluorescence staining with anti-BrdUrd antibody (Fig. 2*F*). Che-1 overexpression substantially decreased the fraction of BrdUrd-positive cells (Fig. 2*G*), indicating that Che-1 can inhibit DNA synthesis in these cells. Altogether, these results clearly indicate that Che-1 can inhibit colon carcinoma cell proliferation inducing growth arrest.

Che-1 Stimulates p21 Expression—It has been extensively demonstrated that one of the most important proteins involved in the G_1 arrest of the cell cycle is the cyclin-dependent kinases inhibitor p21 (34–35). Moreover, several HDAC inhibitors are able to activate p21 expression and in such way they arrest cell growth of several tumors (8, 14–21). For these reasons, we analyzed whether Che-1 was able to stimulate p21 expression. Che-1 was co-transfected with the *p21* gene promoter fused to luciferase into HCT116 and LoVo cells (Fig. 3*A*). These experiments showed that in both cell lines Che-1 stimulated lucifer-

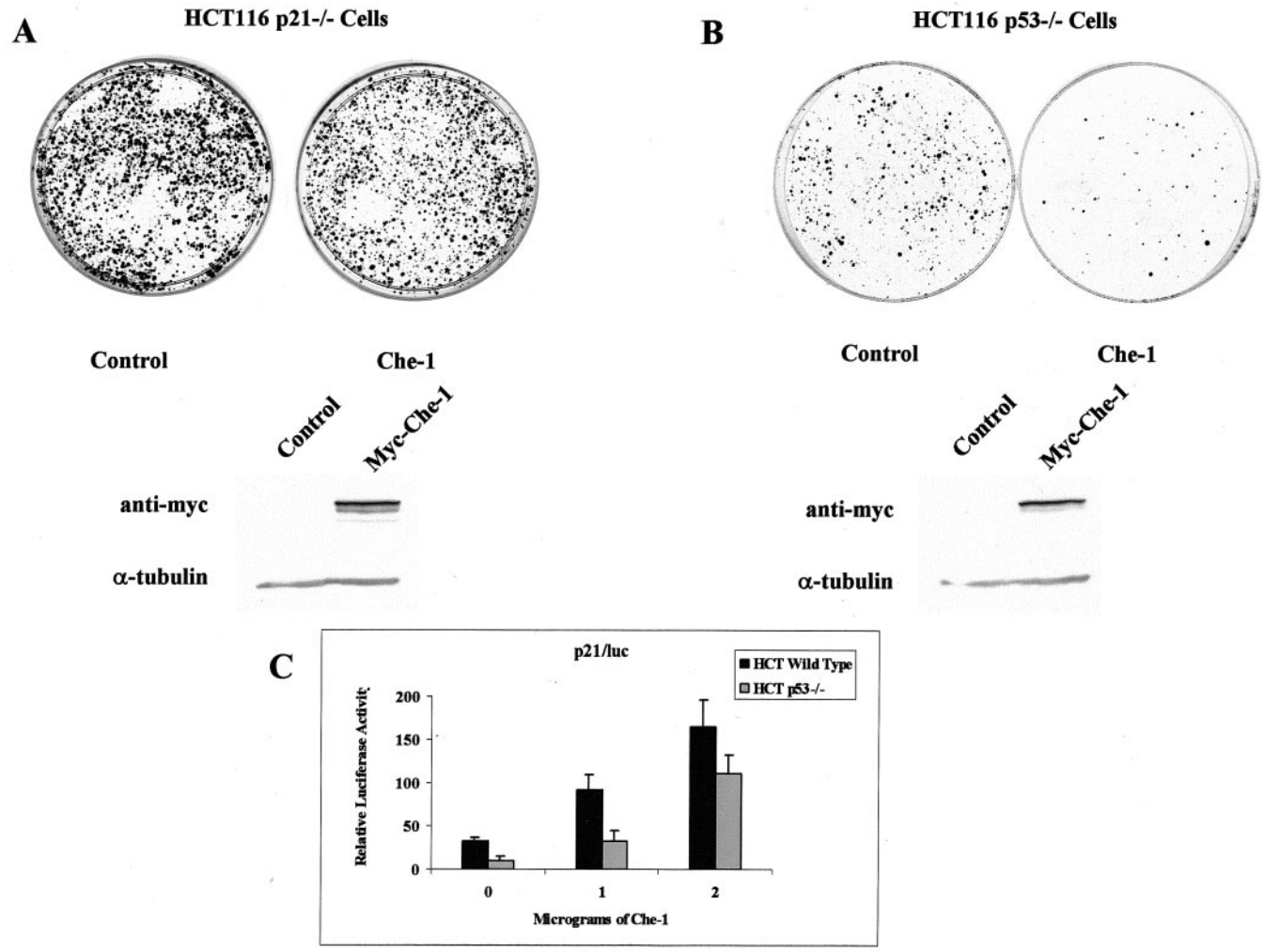

FIG. 4. **p21, but not p53, is required for Che-1-mediated growth inhibition.** HCT116 p21−/− (*A*) and HCT116 p53−/− (*B*) cells were transiently transfected with empty pCMV expression vector (*Control*) or Myc-Che-1 and a vector carrying puromycin resistance gene (10:1 ratio) and selected for 7 days in purumycin. Parallel plates were stained with methylene blue and scored. In the *lower panels*, the expression levels of Myc-Che-1 and α-tubulin were assayed by Western blotting. *C*, HCT116 and HCT116 p53−/− cells were transiently transfected with 1 μg of the *p21* promoter luciferase reporter and the indicated amounts of Myc-Che-1. The total amount of transfected DNA was normalized with pCMV empty vector (−). Data are presented as means ± S.D. from three independent experiments performed in duplicate.

ase activity in a dose-dependent manner (Fig. 3*B*). To evaluate whether the results observed performing transcriptional analysis correspond to a real regulation of the expression of *p21 in vivo*, a semi-quantitative reverse transcriptase-PCR was carried out. The expression levels of *p21* mRNA were specifically up-regulated in HCT116 cells following Che-1 overexpression (Fig. 3*C*). Finally, consistent with the previous analysis, the amounts of p21 protein in HCT116 cells transfected with Che-1 were found significantly increased (Fig. 3*D*). Taken together, these results strongly indicate that Che-1 stimulates p21 expression in colon carcinoma cells.

p21, but Not p53, Is Required for Che-1-mediated Growth Inhibition—To determine whether p21 activation is required for Che-1-mediated growth inhibition, the effect of Che-1 overexpression was analyzed in *p21*-deleted HCT116 cells (29). Che-1 did not inhibit cell growth in *p21*−/− cells (Fig. 4*A*), indicating that p21 is critically important in the growth arrest induced by Che-1. Because the transcription of the *p21* gene is directly activated by p53 oncosuppressor (36–38), we evaluated whether p53 could mediate Che-1 growth inhibition and p21 activation by overexpressing Che-1 in *p53*-deleted HCT116 cells. Che-1 overexpression in p53−/− cells produced a growth inhibition (Fig. 4*B*) comparable with wild-type HCT116 cells (Fig. 2*B*). Consistently, *p21* activation by Che-1 in p53−/− and wild-type HCT116 cells had similar results (Fig. 4*C*), demonstrating that Che-1 stimulates the *p21* gene independently from p53. Therefore, taken together these results indicate that p21, but not p53, is required for growth inhibition by Che-1.

Che-1 Can Abolish the HDAC1 Binding to Sp1—It has recently been reported that HDAC inhibitors can induce p21 expression in a p53-independent fashion (8, 14–21, 39). The transcriptional activation of the *p21* gene by these inhibitors is promoted by chromatin remodeling following acetylation of histones H3 and H4 in the *p21* promoter region (39, 40). Moreover, HDAC recruitment on the *p21* promoter was shown to be mediated by six conserved GC boxes that are binding sites for the transcription factor, Sp1 (16–21). Because we recently demonstrated that Che-1 can compete with HDAC1 for Rb binding (27), we evaluated whether Che-1 could also displace HDAC1 from Sp1. To test this hypothesis ^{35}S-labeled Che-1 protein was incubated with bacterial recombinant GST and GST-Sp1 fusion proteins. Fig. 5*A* shows that although GST control protein did not bind Che-1, GST-Sp1 was able to bind Che-1. These results were confirmed when extracts from HCT116 cells were immunoprecipitated with anti-Sp1 antibody and the precipitated proteins assayed by Western blot for the presence of Che-1. The results show that cellular Che-1 is indeed complexed with Sp1 protein (Fig. 5*B*), indicating a phys-

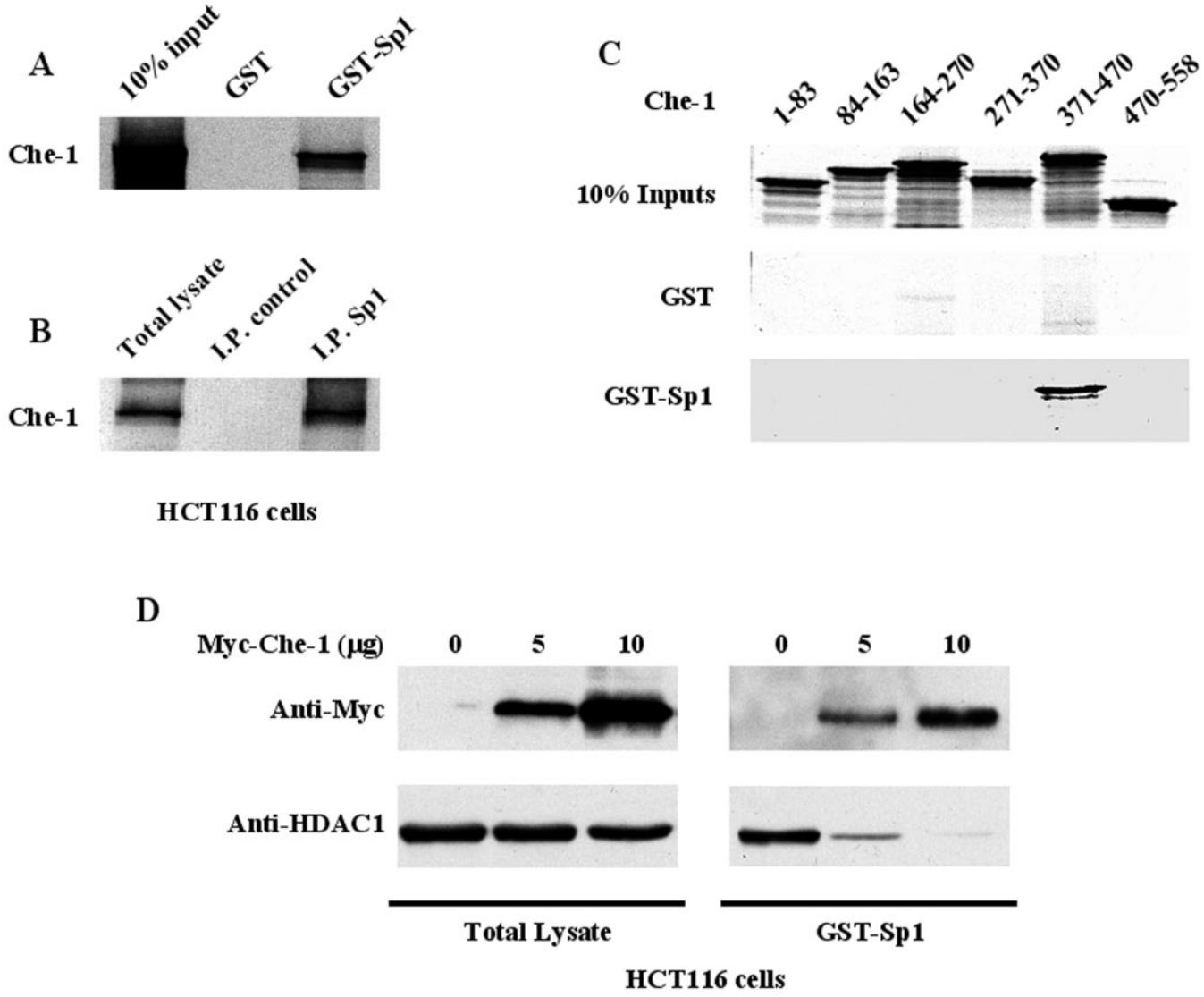

FIG. 5. **Che-1 competes with HDAC1 for Sp1 binding.** *A*, labeled Che-1 was subjected to GST pull down analysis using GST or GST-Sp1 beads. *B*, whole cell extracts of HCT116 cells were immunoprecipitated with preimmune serum as a negative control or anti-Sp1 and analyzed by Western blot using anti-Che-1 polyclonal antibody. *C*, GST pull down assay of ^{35}S-labeled Che-1 fragments using GST or GST-Sp1-Sepharose beads. Amino acid end-points for each Che-1 construct are indicated *above* lanes. *D*, *in vitro* binding analysis of GST-Sp1 fusion protein with lysates from HCT116 cells transiently transfected with 10 μg of empty vector or 5 or 10 μg of Myc-Che-1. The membrane was analyzed by Western blot using specific anti-Myc and anti-HDAC1 antibodies.

iological interaction between Che-1 and Sp1. To determine the regions of Che-1 involved in this interaction, six polypeptides covering the whole Che-1 protein (22) were used to perform *in vitro* transcription/translation and GST pull down assay. Peptide fragment-(371–470) showed the ability to bind GST-Sp1 fusion protein (Fig. 5*C*), indicating that Che-1 does not contact Sp1 with the region involved in HDAC1 displacing from Rb (27). Nevertheless, Che-1 transfection in HCT116 cells led to a marked decrease in the ability of GST-Sp1 to retain HDAC1 protein from cell extracts (Fig. 5*D*), indicating that the physical interaction between Che-1 and Sp1 can interfere with HDAC1 recruitment by Sp1.

Che-1 Displaces the HDAC1 Associated to Sp1 Binding Sites of the p21 Promoter in Vivo—To determine whether Che-1 can decrease the level of HDAC1 at the Sp1 binding sites of the *p21* gene promoter *in vivo*, HCT116 cells were transfected with Myc-Che-1 (Fig. 6*B*), and ChIP assays were performed by immunoprecipitating cross-linked genomic DNA with specific antibodies. Immunoprecipitated DNA was analyzed by PCR, utilizing promoter-specific primers that encompass the six Sp1 binding sites (Fig. 6*A*) under conditions of linear amplification (Fig. 6*C*). The expression of exogenous Che-1 resulted in an increase of Che-1 levels at the Sp1 binding sites of the *p21* promoter with a parallel decrease of HDAC1 amount, as measured by ChIP (Fig. 6*D*), thus confirming the ability of Che-1 to displace HDAC1 from Sp1. Accordingly, Che-1 overexpression did not change Sp1 presence on the chromatin, but its presence on the chromatin produced an increase in the level of acetylated histone H3 in association with the Sp1 binding sites of the *p21* promoter (Fig. 6*D*). Taken together, these results are consistent with the hypothesis that Che-1 efficiently competes with HDAC1 for Sp1 binding and in such a way activates *p21* transcription.

siRNA of Che-1 Inhibits p21 Transcription and Increases Cell Proliferation—The results described above show that Che-1 was down-regulated in several human colon carcinomas and that exogenous Che-1 expression inhibits cell proliferation by p21 induction. Conversely, inhibition of Che-1 should lead to decreased *p21* transcription and to an increase of cell proliferation. To test this hypothesis, the siRNA technique (34) was

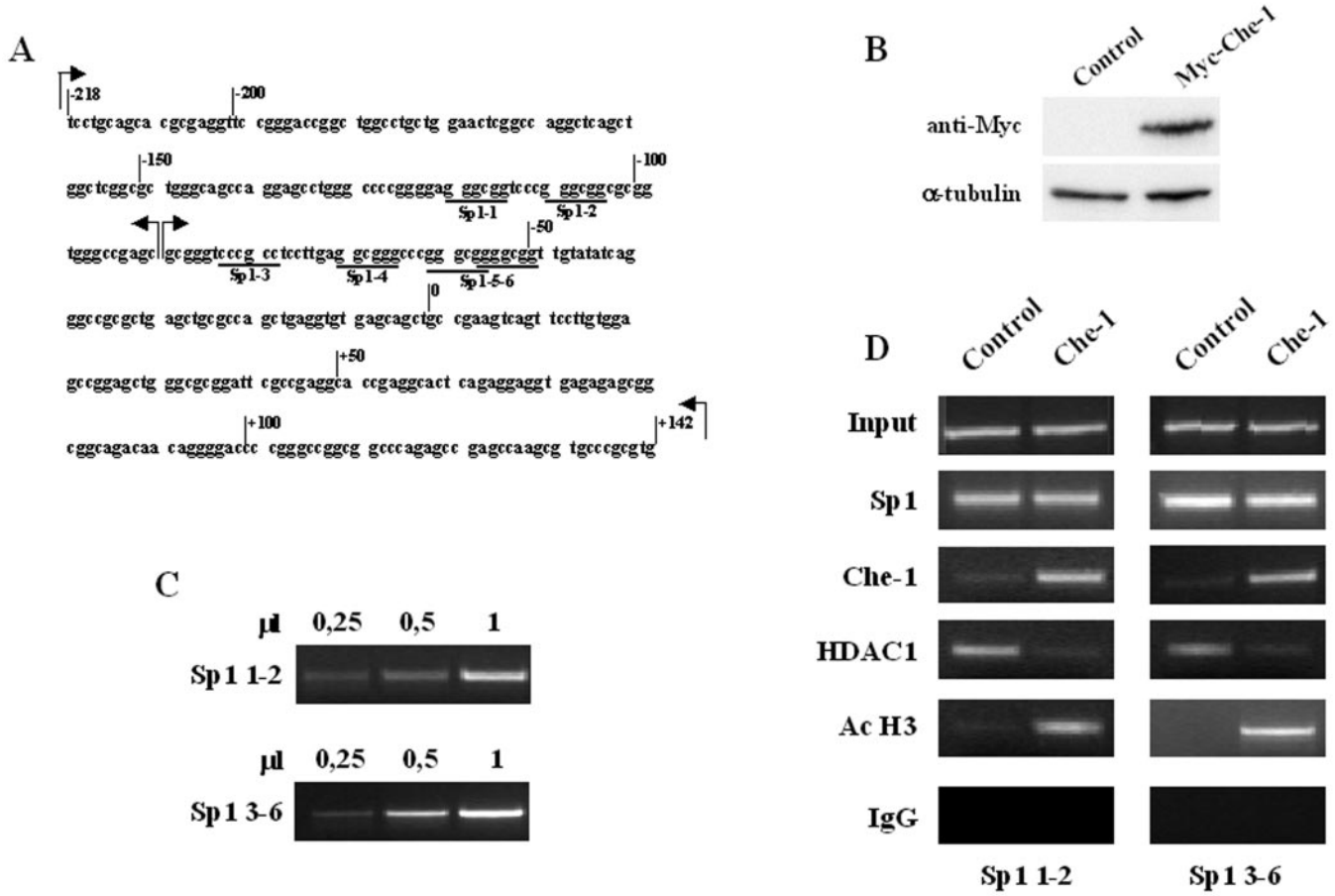

FIG. 6. **Che-1 displaces the HDAC1 associated to Sp1 binding sites of the *p21* promoter *in vivo*.** *A*, DNA sequence of the 5′-end region of the *p21* promoter; nucleotides are *numbered* from the transcription initiation site. Consensus sites for Sp1 binding are indicated. *Arrows* indicate starting points of primers used for ChiP analysis. *B*, lysates from HCT116 cells transiently transfected with empty vector (*Control*) or Myc-Che-1, respectively, were subjected to Western blot analysis with anti-Myc and anti-α-tubulin antibodies to verify protein expression. *C* and *D*, lysates from HCT116 cells transfected as described in *panel B* were subjected to ChiP using specific polyclonal antibodies, anti-HDAC1, anti-Che-1, anti-Sp1, or anti-acetylated histone H3. *C*, increasing amounts of input samples (0.25, 0.5, 1 µl) were used as template in PCR amplifications performed using primers specific for the different regions of the *p21* promoter that include the Sp1 binding sites. *D*, immunoprecipitates from each sample were analyzed by PCR, and a sample representing linear amplification (0.5–1 µl) of the total input chromatin (*Input*) was included in the PCRs as control. Additional control included a precipitation performed with no specific IgGs.

used to reduce Che-1 expression in HCT116 cells. Cells transduced with double-stranded (dsRNA) oligonucleotides to Che-1 (siChe-1) showed a specific reduction of Che-1 expression compared with control, GFP dsRNA (siGFP)-transduced cells (Figs. 7, *A* and *B*). Che-1 reduction resulted in a dramatic decrease of the *p21* transcription (Fig. 7*C*). Furthermore, as expected, cells with a reduced Che-1 expression exhibited an increased rate of proliferation (Fig. 7*D*). Taken together, these results strongly indicate that in colon carcinoma cells, Che-1 can affect cell proliferation by stimulating p21 expression.

DISCUSSION

The original purpose of this work was to evaluate Che-1 expression in human tumors. Indeed, because Che-1 was shown to activate E2F1 by overcoming Rb block, an induction of its expression in tumor cells was a rational hypothesis. Surprisingly, we found that Che-1 is down-regulated in several tumors, especially in almost all of the colon carcinomas examined. Furthermore, the data presented here show a specific involvement of Che-1 expression in the growth arrest of human colon carcinoma cell lines, exerted by p21 induction. Moreover, we showed that this activation is p53-independent and that it is achieved by displacing HDAC1 from Sp1 sites on the *p21* promoter.

HDAC1 is one of the major regulators of chromatin structure and gene expression. This protein is recruited by a variety of transcriptional regulators to specific genomic regions, such as Rb, Mad, or Sp1, thereby mediating the repression of the corresponding target genes (41, 42). Tight control of HDAC1 expression is essential for normal cell cycle progression of mammalian cells. Indeed, overexpression of HDAC1 in mouse fibroblasts or disruption of the *HDAC1* gene in mouse embryonic stem cells led to impaired cell proliferation (43, 44). Significantly, HDAC1-null cells displayed reduced proliferation rates, increased levels of p21, and specific hyperacetylation at the Sp1-binding sites of the *p21* promoter (44). Moreover, several studies have pointed to the possible involvement of aberrant acetylation in human cancer, and a number of HDAC inhibitors have been characterized that inhibit tumor growth *in vitro* and *in vivo* (12, 13, 41). These compounds cause cell cycle arrest, apoptosis, and/or differentiation in cultured transformed cells essentially inducing expression of the *p21* gene, and the Sp1 sites within the *p21* promoter are essential for the activation of p21 by HDAC inhibitors (14–21).

Recently, we have produced evidence that Che-1 is involved in the activation of E2F-dependent promoters and cell proliferation affecting Rb growth suppression (22). Che-1 exerts this inhibition, at least in part, by competing with HDAC1 for the Rb binding site and displacing HDAC1 from E2F target promoters during G_1/S transition (27). In this report, we show that Che-1 also contacts Sp1, and more importantly Che-1 is able to

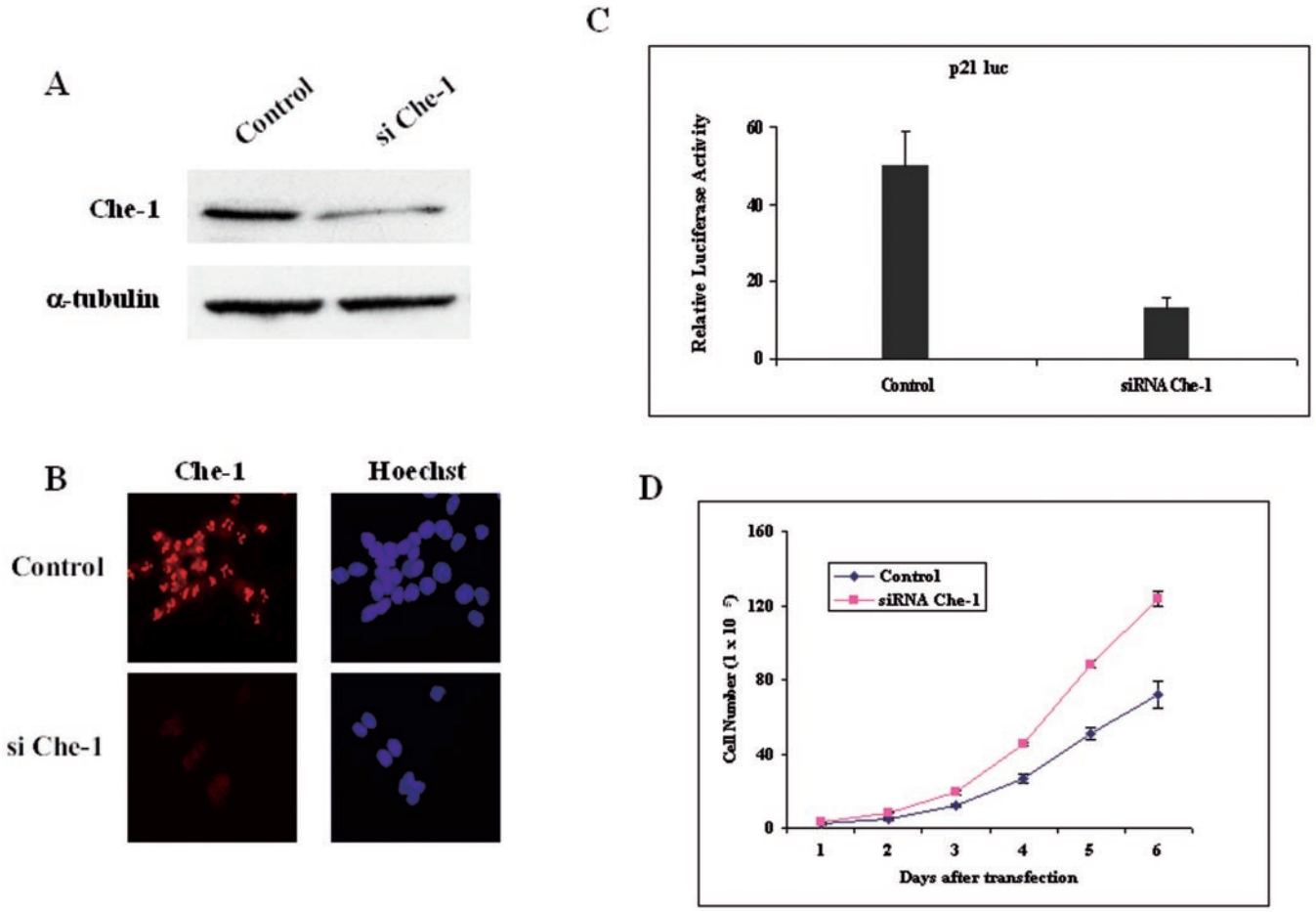

FIG. 7. **Che-1 silencing inhibits p21 transcription and increases cell proliferation.** *A*, silencing of Che-1 in HCT116 cells. Western blots of cells transfected with GFP siRNA (*Control*) or Che-1 siRNA, probed with anti-Che-1 antibody. The blot was stripped and reprobed with anti-α-tubulin antibody. *B*, double fluorescence analysis with anti Che-1-specific antibody and Hoechst of HCT116 cells transfected with GFP siRNA (*Control*) or Che-1 siRNA. *C*, HCT116 cells were transiently transfected with 1 μg of the *p21* promoter luciferase reporter and GFP siRNA (*Control*) or Che-1 siRNA. Data are presented as the mean ± S.D. from three independent experiments performed in duplicate. *D*, cell proliferation analyses of HCT116 cells transiently transfected with GFP siRNA (*Control*) or Che-1 siRNA. Data are presented as means ± S.D. from three independent experiments performed in duplicate.

displace HDAC1 from it. This action should not be mediated by the same mechanism previously described for Rb (27), because distinct regions of Che-1 are responsible for the bindings to polymerase II, Rb, and Sp1 (Ref. 22 and Fig. 5*C*). Further studies will be necessary to elucidate the mechanism by which Che-1 abolishes HDAC1/Sp1 binding. Nevertheless, our findings demonstrated that Che-1 reduced the presence of HDAC1 on the *p21* promoter, increasing the levels of acetylated histone H3 in association with the Sp1 binding sites (Fig. 6*D*). On the basis of these observations, we could assume that Che-1 exerts its function by recruiting polymerase II and displacing HDAC1 from Sp1 on the *p21* promoter. Consistent with these results, the importance of Che-1 on p21 induction was further confirmed by siRNA analysis. Indeed, gene silencing of Che-1 resulted in a strong inhibition of the *p21* promoter transactivation (Fig. 7*C*) and in an increase of cell growth rate (Fig. 7*D*). Therefore, these results support a model where Che-1 acts as a general HDAC1 competitor, recruiting polymerase II and activating the transcription. Additional experimental data will provide new evidence to confirm or invalidate this hypothesis and to better characterize the role of Che-1 in the regulation of transcription.

Che-1 was characterized as a protein that can activate E2F1 and cell proliferation by overcoming Rb block (22, 27), and therefore its effects on colon carcinoma cells would seem paradoxical observations. However, a way to reconcile these observations is to point out that one of the key functions of HDAC1, besides acting as a growth inhibitor (45), is to prevent the expression of cycline-dependent kinase inhibitors like p21 and p27 in cycling cells (44). Therefore, it is possible that the effects of Che-1 on cell proliferation could be influenced by which function of HDAC1 is predominant in a determinate cell contest.

The down-regulation of Che-1 was observed in most of the human colon carcinomas tested. However, further analysis will be necessary to generalize this finding and consider Che-1 as a possible target in tumor treatment. The Che-1 inhibition seems to be carried out at the transcription level, and isolation and characterization of the *Che-1* gene promoter[2] will provide the possibility to shed light on the factors that regulate Che-1 transcription. Nevertheless, we cannot rule out that other mechanism/s, such as the regulation of protein degradation, could affect the presence of Che-1 protein in colon carcinoma cells.

Acknowledgments—We acknowledge Dr. S. Soddu for fruitful discussion. We thank Annalisa Onori for technical assistance.

REFERENCES

1. Grunstein, M. (1997) *Nature* **389,** 349–352
2. Kouzarides, T. (2000) *EMBO J.* **19,** 1176–1179

[2] Monaco, L., Passananti, C., and Fanciulli, M. (2003) *Gene*, in press.

3. Strahl, B. D., and Allis, C. D. (2000) *Nature* **403,** 41–45
4. Bernhard, D., Ausserlechner, M. J., Tonko, M., Loffler, M., Hartmann, B. L., Csordas, A., and Kofler, R. (1999) *FASEB J.* **13,** 1991–2001
5. Butler, L. M., Agus, D. B., Sher, H. I., Higgins, B., Rose, A., Cordon-Cardo, C., Thaler, H. T., Rifkind, R. A., Marks, P. A., and Richon, V. M. (2000) *Cancer Res.* **60,** 5165–5170
6. Finzer, P., Kuntzen, C., Soto, U., zur Hausen, H., and Rosl, F. (2001) *Oncogene* **20,** 4768–4776
7. Yamashita, Y., Shimada, M., Harimoto, N., Rikimaru, T., Shirabe, K., Tanaka, S., and Sugimachi, K. (2003) *Int. J. Cancer* **103,** 572–576
8. Han, J-W., Ahn, S. H., Park, S. H., Wang, S. Y., Bae, G-U., Seo, D-W., Kwon, H-K., Hong, S., Lee, H. Y., Lee, Y-W., and Lee, H-W. (2000) *Cancer Res.* **60,** 6068–6074
9. Butler, L. M., Zhou, X., Xu, W-S., Scher, H. I., Rifkind, R. A., Marks, P. A., and Richon, V. M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 11700–11705
10. Fournel, M., Trachy-Bourget, M-C., Yan, P. T., Kalita, A., Bonfils, C., Beaulieu, C., Frechette, S., Leit, S., Abou-Khalil, E., Woo, S-H., Delorme, D., MacLeod, A. R., Besterman, J. M., and Li, Z. (2002) *Cancer Res.* **62,** 4325–4330
11. Van Lint, C., Emiliani, S., and Verdin, E. (1996) *Gene Expr.* **5,** 245–253
12. Marks, P. A., Rifkind, R. A., Richon, V. M., Breslow, R., Miller, T., and Kelly, W. K. (2001) *Nat. Rev. Cancer* **1,** 194–202
13. Marks, P. A., Richon, V. M., Breslow, R., and Rifkind, R. A. (2001) *Curr. Opin. Oncol.* **13,** 477–483
14. Archer, S. Y., Meng, S., Shei, A., and Hodin R. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6791–6796
15. Richon, V. M., Sandhoff, T. W., Rifkind, R. A., and Marks, P. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10014–10019
16. Sowa, Y., Orita, T., Minamikawa, S., Nakano, K., Mizuno, T., Nomura, H., and Sakai, T. (1997) *Biochem. Biophys. Res. Commun.* **241,** 142–150
17. Han, J-W., Ahn, S. H., Kim, Y. K., Bae, G-U., Yoon, J. W., Hong, S., Lee, H. Y., Lee, Y-W., and Lee H-W. (2001) *J. Biol. Chem.* **276,** 42084–42090
18. Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani-Fujita, N., Matsukawa, Y., Tokino, T., Yamagishi, H., Oka, T., Nomura, H., and Sakai, T. (1997) *J. Biol. Chem.* **272,** 22199–22206
19. Huang, L., Sowa, Y., Sakai, T., and Pardee, A. B. (2000) *Oncogene* **19,** 5712–5719
20. Xiao, H., Hasegawa, T., and Isobe, K. (1999) *J. Cell. Biochem.* **73,** 291–302
21. Sowa, Y., Orita, T., Minamikawa-Hiranabe, S., Nakano, K., Mizuno, T., Nomura, H., and Sakai, T. (1999) *Cancer Res.* **59,** 4266–4270
22. Fanciulli, M., Bruno, T., Di Padova, M., De Angelis, R., Iezzi, S., Iacobini, C., Floridi, A. and Passananti, C. (2000) *FASEB J.* **14,** 904–912
23. Thomas, T., Voss, A. K., Petrou, P., and Gruss, P. (2000) *Dev. Biol.* **227,** 324–342
24. Lindfors, K., Halttunen, T., Huotari, P., Nupponen, N., Vihinen, M., Visakorpi, T., Maki, M., and Kainulainen, H. (2000) *Biochem. Biophys. Res. Commun.* **276,** 660–666
25. Page, G., Lodige, I., Kogel, D., and Scheidtmann, K. H. (1999) *FEBS Lett.* **462,** 187–191
26. Kogel, D., Plottner, O., Landsberg, G., Christian, S., and Scheidtmann, K. H. (1998) *Oncogene* **17,** 2645–2654
27. Bruno, T., De Angelis, R., De Nicola, F., Barbato, C., Di Padova, M., Corbi, N., Libri, V., Benassi, B., Mattei, E., Ch'ersi, A., Soddu, S., Floridi, A., Passananti, C., and Fanciulli, M. (2002) *Cancer Cell* **2,** 387–399
28. Harbour, J. W., and Dean, D. C. (2000) *Genes Dev.* **14,** 2393–2409
29. Waldman, T., Kinzler, K. W., and Vogelstein, B. (1995) *Cancer Res.* **55,** 5187–5190
30. Gallo, R., Zazzeroni, F., Alesse, E., Mincione, C., Borello, U., Buanne, P., D'Eugenio, R., Mackay, A. R., Argenti, B., Gradini, R., Russo, M. A., Maroder, M., Cossu, G., Frati, L., Screpanti, I., and Gulino, A. *J. Cell Biol.* **158,** 731–740
31. Del Bufalo, D., Biroccio, A., Soddu, S., Laudonio, N., D'Angelo, C., Sacchi, A., and Zupi, G. (1996) *J. Clin. Invest.* **98,** 1165–1173
32. Biroccio, A., Benassi, B., Filomeni, G., Amodei, S., Marchini, S., Chiorino, G., Rotilio, G., Zupi, G., and Circolo, M. R. *J. Biol. Chem.* **277,** 43763–43770
33. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) *Nature* **411,** 494–498
34. Harper, J. W., Adami, G. R., Wei, N., Keiomarsi, K., and Elledge, S. J. (1993) *Cell* **75,** 805–816
35. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993) *Nature* **366,** 701–704
36. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parson, R., Trent, J. M., Lin, D., Mercer, W. E., Klinzer, K. W., and Vogelstein, B. (1993) *Cell* **75,** 817–825
37. Gu, Y., Turck, C. W., and Morgan, D. O. (1993) *Nature* **366,** 707–710
38. Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J., and Reed, S. I. (1994) *Cell* **76,** 1013–1023
39. Sambucetti, L. C., Fischer, D. D., Zabludoff, S., Kwon, P. O., Chamberlin, H., Trogani, N., Xu, H., and Cohen, D. (1999) *J. Biol. Chem.* **274,** 34940–34947
40. Kim, Y. B., Lee, K. H., Sugita, K., Yoshida, M., and Horinouchi, S. (1999) *Oncogene* **18,** 2461–2470
41. Cress, W. D., and Seto, E. (2000) *J. Cell. Physiol.* **184,** 1–16
42. Ng, H. H., and Bird, A. (2000) *Trends Biochem. Sci.* **25,** 121–126
43. Bartl, S., Taplik, J., Lagger, G., Khier, H., Kuchler, K., and Seiser, C. (1997) *Mol. Cell. Biol.* **17,** 5033–5043
44. Lagger, G., O'Carroll, D., Rembold, M., Khier, H., Tischler, J., Weitzer, G., Schuettengruber, B., Hauser, C., Brunmeir, R., Jenuwein, T., and Seiser, C. (2002) *EMBO J.* **21,** 2672–2681
45. Zhang, H. S., and Dean, D. C. (2001) *Oncogene* **20,** 3134–3138

Che-1 Arrests Human Colon Carcinoma Cell Proliferation by Displacing HDAC1 from the *p21*$^{WAF1/CIP1}$ Promoter

Monica Di Padova, Tiziana Bruno, Francesca De Nicola, Simona Iezzi, Carmen D'Angelo, Rita Gallo, Daniela Nicosia, Nicoletta Corbi, Annamaria Biroccio, Aristide Floridi, Claudio Passananti and Maurizio Fanciulli

J. Biol. Chem. 2003, 278:36496-36504.
doi: 10.1074/jbc.M306694200 originally published online July 7, 2003
