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Human Fibroblast Commitment to a Senescence-Like State in Response to Histone Deacetylase Inhibitors Is Cell Cycle Dependent

VASILY V. OGRYZKO, TAZUKO H. HIRAI, VALYA R. RUSSANOVA, DAVID A. BARBIE, AND BRUCE H. HOWARD*

Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development, Bethesda, Maryland 20892-2753

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Human diploid fibroblasts (HDF) complete a limited number of cell divisions before entering a growth arrest state that is termed replicative senescence. Two histone deacetylase inhibitors, sodium butyrate and trichostatin A, dramatically reduce the HDF proliferative life span in a manner that is dependent on one or more cell doublings in the presence of these agents. Cells arrested and subsequently released from histone deacetylase inhibitors display markers of senescence and exhibit a persistent G_1 block but remain competent to initiate a round of DNA synthesis in response to simian virus 40 T antigen. Average telomere length in prematurely arrested cells is greater than in senescent cells, reflecting a lower number of population doublings completed by the former. Taken together, these results support the view that one component of HDF senescence mimics a cell cycle-dependent drift in differentiation state and that propagation of HDF in histone deacetylase inhibitors accentuates this component.

Cellular senescence in human diploid fibroblasts (HDF) and other human cell types has been extensively studied (17, 26, 35, 53, 73, 85) yet remains incompletely understood. On the basis of current knowledge, it is not unreasonable to suppose that multiple mechanisms contribute to the senescence phenotype and that the relative contributions of such mechanisms may vary for different cell types and conditions of cell propagation. At least two senescence-associated changes, increased oxidative stress (74, 75) and telomere shortening (33, 34, 55, 87), have been intensively studied and are widely viewed as important components of in vitro aging. Less well studied is a third aspect of senescence, namely, its apparent relatedness to differentiation (4, 16, 26, 48, 57). Although these two processes are not necessarily synonymous (60, 88), in many respects senescence resembles a partial or aberrant form of terminal differentiation, with cells appearing to acquire a phenotype that is suboptimal for tissue function and maintenance. HDF proliferative potential is dependent primarily on the number of rounds of DNA synthesis completed rather than the cumulative time in culture (17, 19, 27); thus, to the extent that senescence is akin to terminal differentiation, it most closely resembles model systems (e.g., murine erythroleukemia or promyelocytic leukemia cells) where a requirement for passage through the cell cycle has been demonstrated (7, 45, 46).

Our attention to the differentiative aspects of senescence was first prompted by experiments designed to compare cell cycle arrest mechanisms in senescent and quiescent cells. Transient expression of simian virus 40 (SV40) T antigen is strongly mitogenic, i.e., is dominant over cell cycle arrest mechanisms, in both senescent cells and serum-deprived quiescent fibroblasts (29, 39, 80). We and others observed that in senescent HDF, but not quiescent cells, the domain in T antigen which mediates binding to retinoblastoma (Rb) family proteins is required for efficient stimulation of DNA synthesis (8a, 65). This requirement is likely to reflect the failure in senescent cells to downregulate $p21^{Waf1/Cdi1/Sdi1}$ (52), which in turn leads to accumulation of the *Rb* gene product (pRb) in its antiproliferative hypophosphorylated form (76). In the course of these and related experiments, we noted that HDF deprived of serum in the presence of sodium butyrate resemble senescent HDF by the above criteria; that is, both senescent and butyrate-treated cells exhibit a strong response to the mitogenic action of wild-type T antigen (wt T) but are only weakly stimulated when the pRb-binding domain of T antigen is inactivated (54, 65). Butyrate-treated cells resemble senescent HDF additionally in that they exit the cell cycle with a G_1 phase DNA content and exhibit pRb hypophosphorylation despite being maintained in high-serum-concentration medium (54). These results led us to consider the possibility that the antiproliferative pathways activated by sodium butyrate might be similar to those operative in senescent HDF. If such treatment could be shown to hasten entry of HDF into a senescence-like state, we surmised, it would reinforce the view that a major component of HDF senescence is mechanistically related to differentiation.

MATERIALS AND METHODS

Plasmids. Cell surface marker vector pCMV-IL2R (25) and expression vectors pCMV-TAg and pCMV-TAg(Rb^-) (54) were described previously.

Cell culture and transfections. WI-38 HDF and HeLa S3 cells were obtained from the American Type Culture Collection. Responsiveness of butyrate-propagated WI-38 cells to T antigen was assessed by an assay which combines transient transfection with affinity cell sorting (65). In this assay HDF were electroporated with pCMV-IL2R, with or without an appropriate T-antigen expression vector, and maintained at subconfluence in medium containing 0.2 to 0.5% fetal calf serum for ~48 h, after which the culture was briefly labeled with 10 μM bromodeoxyuridine (BrdUrd) (Sigma) or 10 μCi of [^{3}H]thymidine (Dupont/NEN). Immediately after being labeled, the successfully transfected subpopulation was purified by affinity sorting.

Flow cytometry. Nuclei prepared from affinity-sorted cells were fixed with ethanol, denatured with 2 N HCl, and consecutively stained with monoclonal antibody against BrdUrd (Becton-Dickinson) and propidium iodide (Becton-Dickinson). Analysis was performed by using a FACScan flow cytometer with a doublet discrimination module and LYSYS II software (Becton-Dickinson).

Retinoblastoma protein analysis. Extracts were prepared from WI-38 HDF for Western analysis (immunoblotting) with G3-245 monoclonal anti-pRb antibody (PharMingen) as described elsewhere (79).

* Corresponding author. Phone: (301) 496-9038. Fax: (301) 480-9354. Electronic mail address: howard@helix.nih.gov.

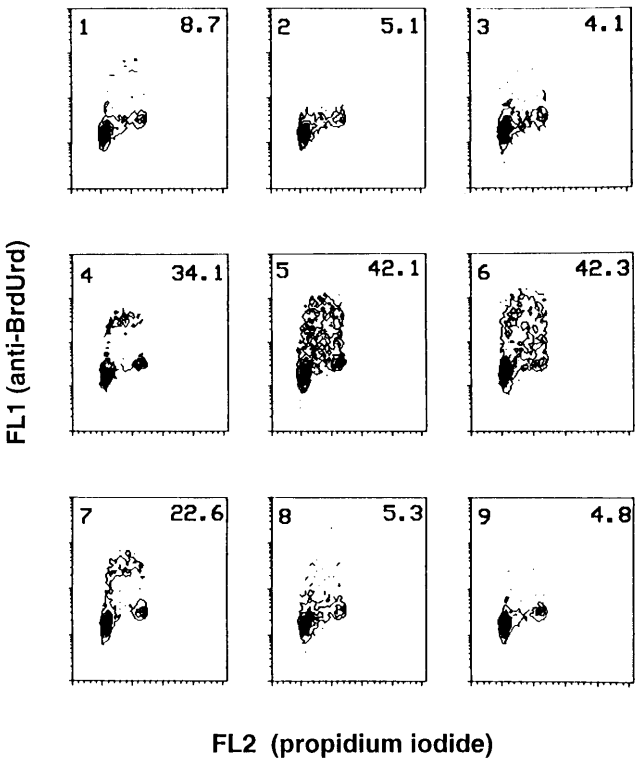

FIG. 1. T antigen versus Rb$^-$ T antigen. WI-38 HDF were transfected with pCMV-IL2R (2 μg), with or without pCMV-TAg (5 μg) or pCMV-TAg(Rb$^-$) (5 μg), and maintained for 48 h in medium containing 0.3% fetal bovine serum (FBS) plus 5 mM sodium butyrate or 100 μM trichostatin A as indicated below. Prior to being harvested, cultures were incubated for 30 min in 10 μM BrdUrd and then subjected to affinity sorting to isolate Tac-antigen-positive cells (65). Nuclei prepared from sorted cells were stained with fluorescein-coupled anti-BrdUrd and propidium iodide. Abscissa and ordinate values represent fluorescence intensity due to propidium iodide (FL2) and anti-BrdUrd (FL1), respectively, in all panels. Transfections: panels 1 to 3, control (pCMV-IL2R alone); panels 4 to 6, pCMV-IL2R plus pCMV-TAg; panels 7 to 9, pCMV-IL2R plus pCMV-TAg(Rb$^-$). Posttransfection conditions: panels 1, 4, and 7, 0.3% FBS alone; panels 2, 5, and 8, 0.3% FBS plus 5 mM sodium butyrate; panels 3, 6, and 9, 0.3% FBS plus 100 ng of trichostatin A per ml. The percentage of cells in S phase is indicated by the numeric value in the upper right corner of each panel.

RESULTS

Inhibition of histone deacetylase is a very well-described action of butyrate (9, 67, 82); however, there existed no corroborative evidence that this activity could account for the above-described effects on HDF cell cycle control. The drug trichostatin A has been reported to be a highly specific inhibitor of histone deacetylase, as evidenced by the fact that extracts from trichostatin-resistant cell lines contain histone deacetylase that is refractory to inhibition by this agent (90). To determine whether trichostatin A could induce a profile of T-antigen responsiveness similar to that induced by sodium butyrate, WI-38 HDF were transfected with expression plasmids encoding either wt T or a pRb-binding-deficient form of T antigen (Rb$^-$ T). After maintenance for 48 h in low-serum-concentration medium with or without trichostatin A (100 ng/ml) or sodium butyrate (5 mM), transfected cells were briefly labeled with BrdUrd and harvested for flow cytometry. As shown in Fig. 1, HDF expressing wt T displayed a strong mitogenic response in either the presence or the absence of these agents, whereas cells expressing Rb$^-$ T were stimulated strongly only in their absence. Thus, treatment with either trichostatin A or butyrate dramatically attenuated the response to Rb$^-$ T, supporting the notion that sodium butyrate activates Rb family antiproliferative activity in HDF by a mechanism related to inhibition of histone deacetylase.

Persistent growth arrest. Having shown that transient exposure of serum-deprived HDF to either of two histone deacetylase inhibitors can induce a growth arrest state in which the pattern of T-antigen responsiveness is reminiscent of senescence, we were encouraged to determine whether cell cycle transit in the presence of these agents could alter HDF proliferative potential. As a first step to test this possibility, WI-38 HDF and HeLa cells were passaged in either the presence or the absence of butyrate, at relatively low concentrations, i.e., ranging from 0.01 to 0.5 mM. WI-38 cells were found to be very sensitive to this agent, exhibiting a measurably reduced proliferative life span even at the lowest concentration tested (Fig. 2A and B). HeLa cells, in contrast, doubled at a nearly normal rate through 30 population doublings at the highest concentration tested (Fig. 2C). The lack of HeLa growth inhibition argues against nonspecific toxicity as an explanation for the reduced WI-38 proliferative potential, particularly since the responses of HeLa and WI-38 cells were similar with respect to butyrate-induced histone hyperacetylation (unpublished data). The proliferation of NIH 3T3 mouse fibroblasts was likewise found to be unaffected by propagation in 0.5 mM butyrate, i.e., growth curves were superimposable for cultures maintained for at least 3 weeks with and without the agent (data not shown). Human colon carcinoma cells have been reported to exhibit morphological changes when placed in 0.5 mM butyrate; however, even when cells are propagated for 30 days under such conditions, phenotypic changes are fully reversible in the great majority of them (38). Overall, these results are in keeping with previous reports that butyrate affects cell proliferation in a highly differentiation-specific manner (7, 24, 43).

To examine whether WI-38 HDF that had completed several rounds of DNA synthesis in the presence of sodium butyrate were committed to a persistent growth arrest state, these cells were propagated in 0.5 mM butyrate until they failed to undergo at least one population doubling within a 2-week period. After butyrate removal, the cells were replated into multiple flasks at a density low enough to permit several cell doublings and then sampled at various time points. Control cultures that remained in butyrate or had not been treated with this agent were included. As expected, WI-38 cells that had not been exposed to butyrate rapidly reached confluence and thereafter increased in number only gradually (Fig. 2D). Conversely, cells that had been propagated in butyrate underwent less than one doubling over an 18-day period. No difference was observed in growth rate between cells that had and cells that had not been released from 0.5 mM sodium butyrate, indicating that the effect of this agent was essentially irreversible over the time interval tested. For simplicity, HDF that were propagated in butyrate until growth arrest and then released will hereafter be referred to as post-butyrate-arrested (PBA) cultures.

Senescence phenotype. Parameters of HDF senescence include increased cell size and reduced saturation density, as well as altered expression of a number of gene products that have been proposed to serve as biomarkers of aging. With respect to biomarkers, pH 6 β-galactosidase is particularly useful, since this activity has been found to distinguish senescent from quiescent cells (20). In human keratinocytes, senescence can also be distinguished from the normal pathway of terminal differentiation (20). In experiments to examine these parameters, we found that senescent and PBA cultures exhibit similarly reduced saturation densities and altered cell morphology; moreover, pH 6 β-galactosidase activity is prominent in both senescent and PBA cultures but not in proliferating HDF (Fig. 3).

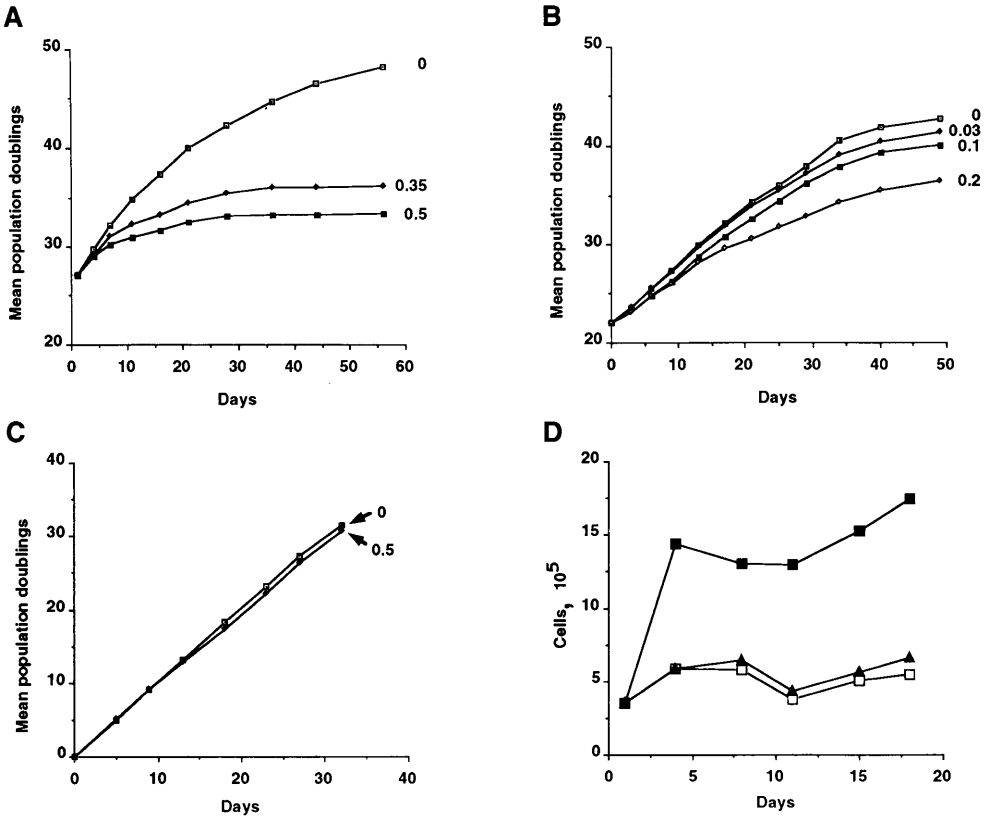

FIG. 2. Growth curves. (A and B) At intervals of 3 to 4 days, WI-38 HDF were split to a density of 10^4 cells per cm^2 in medium with 10% fetal bovine serum (FBS) until cells reached a state of near-senescence; thereafter, intervals were increased until the population failed to double after 2 weeks. Numbers associated with growth curves indicate sodium butyrate concentrations (millimolar). (C) HeLa S3 cells were split at intervals of 3 to 4 days to a density of 10^4 cells per cm^2 in the same growth medium as that used for WI-38 HDF. Numbers associated with growth curves indicate sodium butyrate concentrations (millimolar). (D) WI-38 cells were propagated in medium containing 10% FBS and 0.5 mM sodium butyrate until the growth rate decreased to less than one doubling per 2 weeks (the mean population-doubling [MPD] level was 34). These cells and control, untreated WI-38 HDF (MPD level, 25) were split into replicate flasks at a density of 10^4 cells per cm^2 for maintenance in medium containing 10% FBS with or without butyrate. Individual flasks were harvested for cell counts at the indicated times. Symbols: ■, control, untreated cells; □, cells in 0.5 mM sodium butyrate; ▲, cells incubated without sodium butyrate.

pRb hypophosphorylation. It has been reported previously that senescent HDF, like their quiescent counterparts, exhibit pRb hypophosphorylation (76). To further characterize the state of PBA HDF, these cells were examined with respect to pRb phosphorylation levels. Controls comprised HDF that had not been exposed to butyrate, as well as cells rendered senescent by a conventional protocol. Western analysis of pRb demonstrated that PBA cells, like senescent cells, exhibit pRb hypophosphorylation despite the presence of high-serum-concentration medium (Fig. 4, lanes 1 and 2). Young cells under identical conditions, by comparison, exhibited rapid proliferation (data not shown) and concomitant pRb hyperphosphorylation (Fig. 4, lane 3). The reason for the markedly reduced pRb levels in both senescent and PBA cells relative to levels in young cells is not known, although differences between immunoreactive pRb levels in young and old cells are evident in results of at least one earlier study (76).

T-antigen responsiveness. Normal senescent cells are blocked primarily in a G_1-like phase (30, 89) but can be induced to enter a single round of replicative DNA synthesis in response to SV40 T antigen (29, 39, 80). Flow-cytometric analysis was employed to determine the cell cycle distribution of PBA HDF, as well as to examine whether they are responsive to mitogenic stimulation by SV40 T antigen. Senescent cells served as a control in these experiments. As shown in Fig. 5A and C, PBA cells closely resemble senescent cells in being predominantly arrested with a 2*N* DNA content. Furthermore, both cell types could be stimulated to undergo DNA synthesis by T antigen (Fig. 5B and D).

Quantification of these responses revealed an eightfold increase in the percentage of senescent cells in S phase (from 2.5 to 20% of the total population), compared with a fivefold increase for PBA cells (from 5 to 25%). We conclude that propagation of WI-38 HDF in butyrate does not cause nonspecific irreversible cell damage but rather induces cells to enter an arrest state that, by the above-described parameters, resembles normal senescence.

Cell cycle dependence. The onset of growth arrest in old HDF is determined primarily by a counting mechanism that measures cumulative population doublings rather than calendar time, i.e., the time that cells are maintained in culture (17, 19, 27, 64). If butyrate acts on this putative counting mechanism to induce a senescence-like state, then its effect on maximum proliferative potential might be manifested only when the cells undergo replicative DNA synthesis in the presence of this agent. To determine whether butyrate treatment was selective in this regard, WI-38 HDF were rendered quiescent by confluence and serum deprivation and then maintained in the presence or absence of 0.5 mM butyrate for 36 days, i.e., roughly the time required for such cells to reach a senescence-like growth arrest state when propagated in the presence of this concentration of butyrate (Fig. 1 and unpublished results).

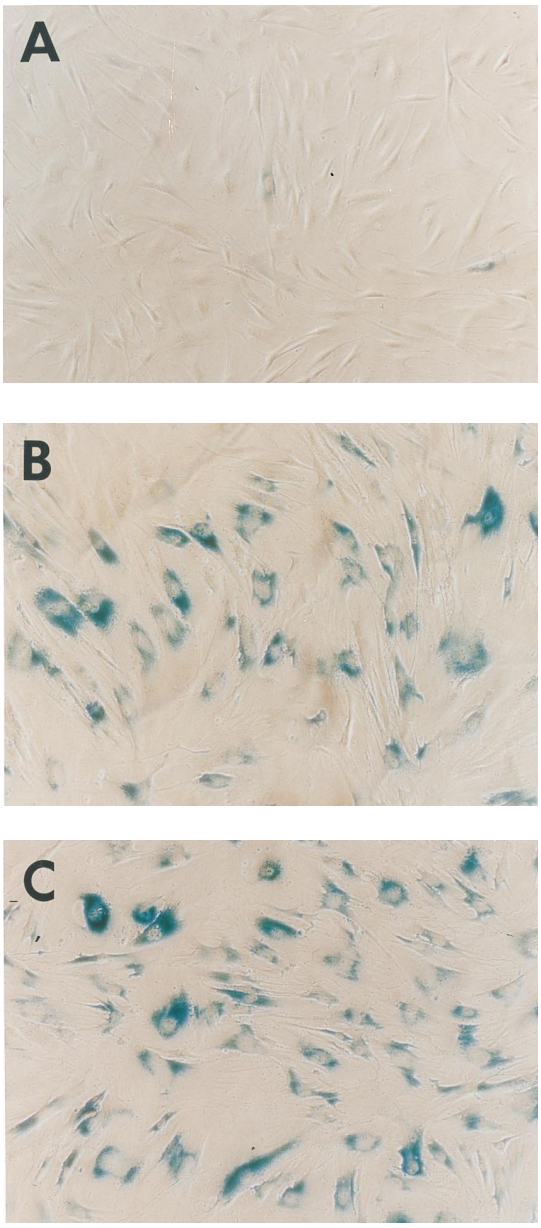

FIG. 3. Comparison of PBA and senescence phenotypes. With the exception of cells used in the experiment represented in panel A, HDF were propagated until cultures failed to undergo at least one population doubling over an interval of 2 weeks. Cells were prepared for detection of pH 6 β-galactosidase as described elsewhere (20). (A) Young HDF (mean population-doubling [MPD] level, 22); (B) PBA HDF (MPD level, 33); (C) senescent HDF (MPD level, 56).

Alternatively, quiescent cells were incubated in high concentrations of butyrate (5 or 20 mM) for 24 h. No cell toxicity was evident, even at the latter, very high butyrate concentration (data not shown). After restoration to normal growth medium, these cells, in parallel with control cells that had been treated identically except for exposure to butyrate, were assayed for growth rate and/or maximum proliferative potential. As seen in Fig. 6A and B, such treatments had no detectable effect on either of these growth parameters for the duration of the experiments.

In parallel studies, the antiproliferative effects of trichostatin A in HDF were further investigated. These studies involved a

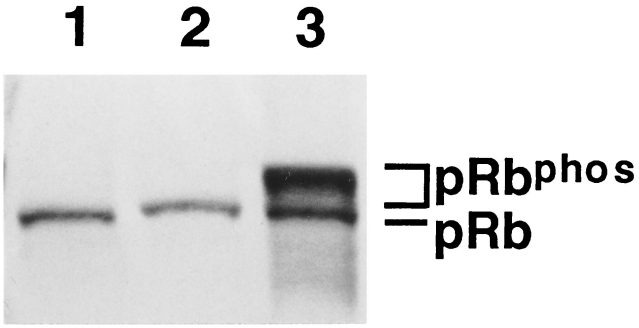

FIG. 4. pRb phosphorylation levels. Western blot of pRb extracted from WI-38 cells maintained in medium containing 10% fetal bovine serum. Lanes: 1, senescent HDF (mean population-doubling [MPD] level, 52); 2, HDF arrested in 0.5 mM butyrate (MPD level, 33); 3, young, proliferative HDF (MPD level, 22). Threefold less extract was loaded in lane 3 than in the other lanes. pRb^{phos}, phosphorylated pRb.

dose-response experiment similar to that performed initially with butyrate, as well as treatment of quiescent cells. In the latter case, confluent, serum-deprived cells were either maintained for 30 days in appropriate levels of trichostatin A (50 ng/ml) or treated for 24 h with a higher concentration of this agent (200 ng/ml). In all respects, trichostatin A mimicked the effects of butyrate; that is, WI-38 HDF were found to be highly sensitive to propagation in the presence of trichostatin but exhibited no alteration in growth rate so long as they had remained under conditions that should ensure complete quiescence during treatment with this agent (Fig. 6C and D).

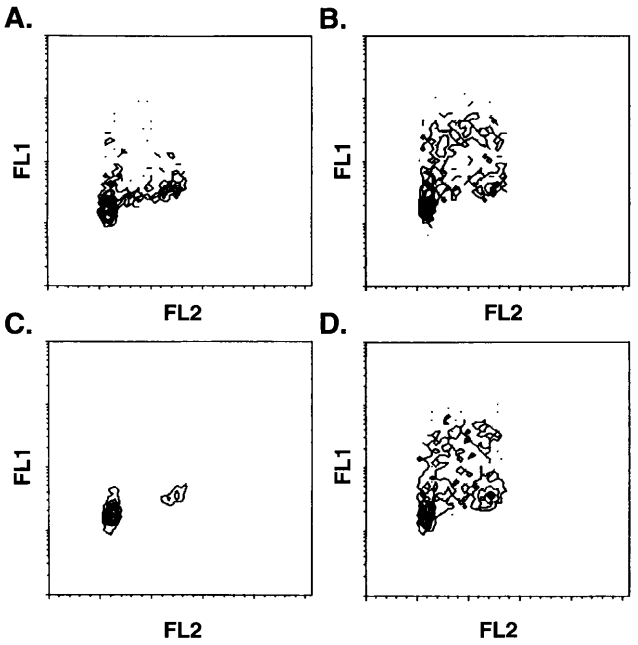

FIG. 5. G_1 arrest and responsiveness to T antigen. HDF were transfected with pCMV-IL2R (5 μg), with either the control vector pCMV.3 (30 μg) (54) or pCMV-TAg (30 μg), and maintained for 48 h in medium containing 10% fetal bovine serum (FBS). Cultures were incubated in 10 μM BrdUrd for 6 h to label cells in S phase and then subjected to affinity sorting. Nuclei prepared from sorted cells were stained with fluorescein-coupled anti-BrdUrd (FL1) and propidium iodide (FL2). Abscissa and ordinate values represent fluorescence intensity (arbitrary units). (A) PBA HDF (mean population-doubling [MPD] level, 33); (B) PBA HDF plus pCMV-TAg; (C) senescent HDF; (D) senescent HDF plus pCMV-TAg.

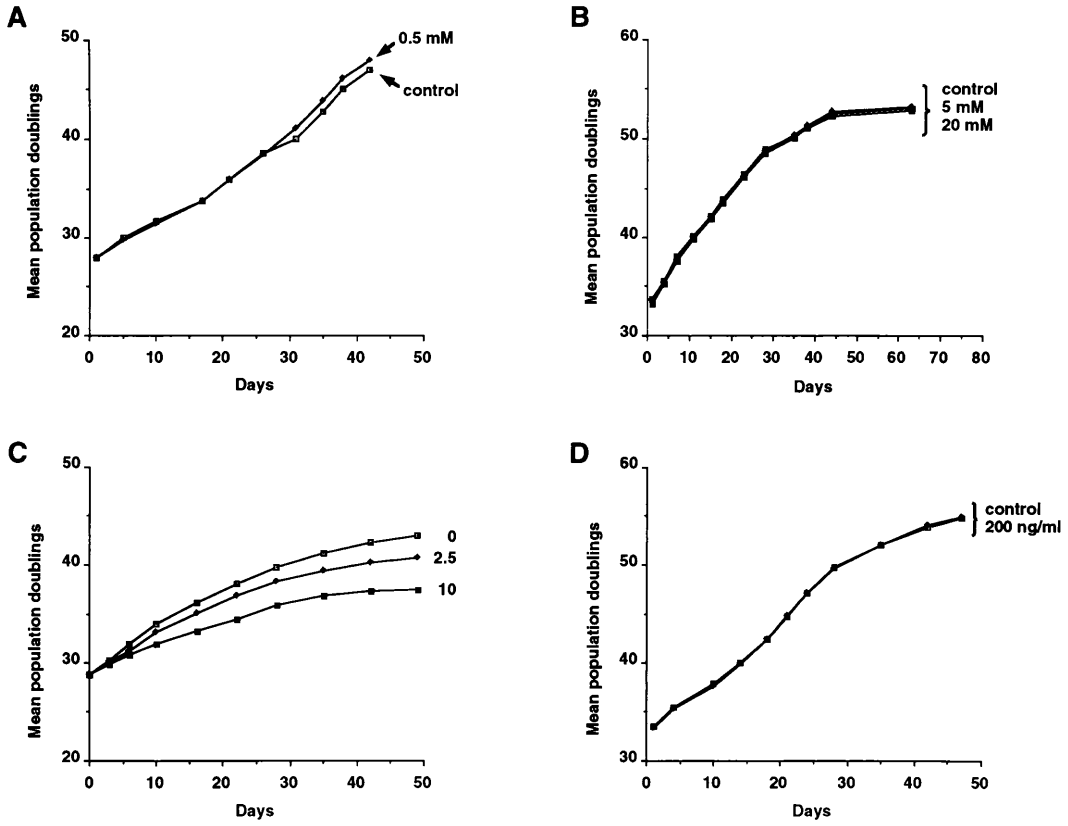

FIG. 6. Cell cycle coupling and growth curves in trichostatin. (A) WI-38 cells were propagated in medium containing 10% fetal bovine serum (FBS) until confluence and then shifted to medium containing 0.3% FBS for 5 days. Thereafter, such confluent, serum-deprived cells were maintained in medium containing 0.3% FBS and 0.5 mM sodium butyrate, as indicated to the right of the curves, with refeeding at 5- to 6-day intervals, for a total of 36 days. After removal of butyrate, cells were kept at confluence in medium containing 0.3% FBS for 4 days and then split to a density of 10^4 cells per cm^2 for determination of growth curves. Control cells were treated identically except for the omission of butyrate. (B) WI-38 cells were treated as described for panel A, except that treatment of confluent, serum-deprived cells consisted of maintenance in 5 or 20 mM sodium butyrate for 24 h. (C) WI-38 cells were propagated as described in the legend to Fig. 2, except that media contained the indicated concentrations of trichostatin A (numbers to the right of the curves; nanograms per milliliter). (D) Cells were treated as described for panel A, except that confluent, serum-deprived cultures were maintained in 200 ng of trichostatin A per ml for 24 h.

Role of Rb family proteins. In senescent HDF, efficient stimulation of DNA replication by T antigen requires binding to pRb and/or other Rb family proteins (8a, 65, 70). We next asked whether PBA or post-trichostatin-arrested fibroblasts, i.e., cells committed to persistent growth arrest following removal of these agents, would exhibit a similar pattern of T-antigen responsiveness. Such cells, together with quiescent and senescent controls, were transiently transfected with vectors expressing either wt T or Rb^- T and monitored for DNA synthesis under restrictive growth conditions. As reported earlier (65), control senescent cells differed from young cells in requiring an intact pRb-binding domain in order to respond to the mitogenic action of T antigen (Fig. 7A and B). A very similar situation was observed with both PBA and post-trichostatin-arrested cells in that such cells were stimulated much more efficiently by wt T than by Rb^- T (Fig. 7C and D). These data further support the notion that propagation of HDF in histone deacetylase inhibitors induces a growth arrest state that simulates cellular senescence.

Telomere length in PBA cells. There is currently strong support for the telomere-shortening model of cellular senescence (1, 33, 87), and it seems intuitively clear that, in the absence of other mechanisms, loss of telomeres will eventually supervene to limit the proliferative life span. In consideration of the possibility that histone deacetylase inhibitors might interfere with telomere integrity, we examined the lengths of telomere-containing restriction fragments in PBA HDF. Interestingly, no evidence for accelerated telomere loss was found (Fig. 8). This is in contrast with the premature growth arrest induced by mild hyperoxia, for which a concomitant premature shortening of telomeres is evident (83). It should be noted that failure to detect premature telomere shortening in PBA HDF constitutes strong evidence against increased cell turnover (i.e., cryptic toxicity) being the mechanism by which butyrate reduces population doubling potential.

DISCUSSION

There has been considerable recent progress in identifying the effector pathways by which HDF are held in the senescent state (3, 5, 21, 31, 32, 52, 62, 68, 70, 76, 77). These pathways are not unique to senescence but rather represent the coordinated action of gene products that are also believed to play critical roles in quiescence, response to DNA damage, and terminal differentiation. By contrast, information concerning a cell division counting mechanism(s) that limits maximum HDF proliferative potential remains more circumscribed, although the prevailing view accords to two senescence-associated changes, telomere shortening (33, 34, 55, 87) and increasing oxidative stress (12, 13), the status of likely causal factors.

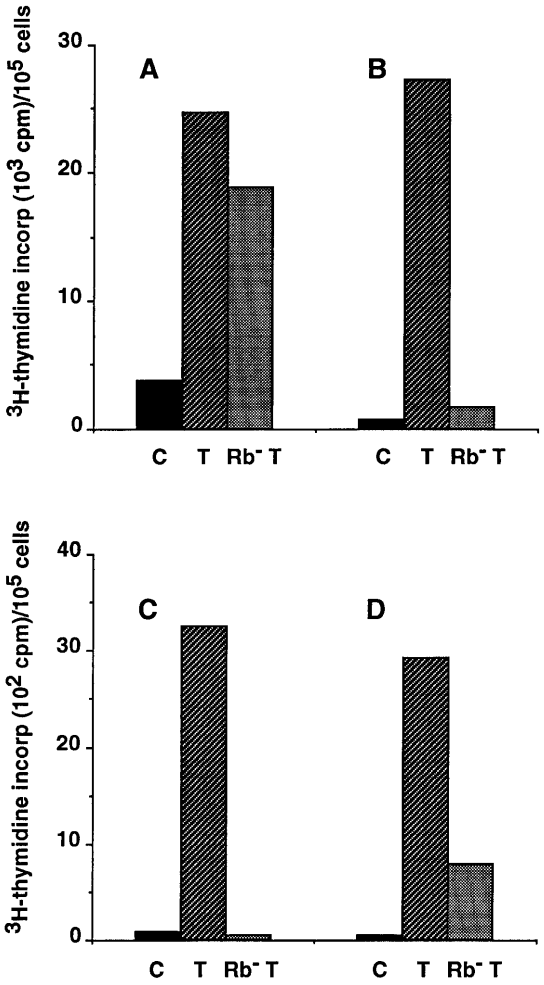

FIG. 7. T-antigen versus Rb^- T-antigen responsiveness as a measure of commitment to growth arrest. WI-38 HDF were transfected with pCMV-IL2R (5 μg) alone (control [C]) or with pCMV-TAg (30 μg) (T) or pCMV-TAg(Rb^-) (30 μg) (Rb^-T) and maintained for 48 h under the indicated conditions. Cultures were incubated in 10 μCi of [^{3}H]thymidine for 120 min to label cells in S phase and then subjected to affinity sorting. (A) WI-38 cells in 0.2% FBS plus 0.5 mM sodium butyrate; (B) senescent WI-38 cells in 10% FBS; (C) PBA cells in 0.2% FBS; (D) post-trichostatin-arrested cells in 0.2% FBS. The mitogenic response profile of cells shown in panel A is indistinguishable from that of cells in 0.2% FBS without butyrate (unpublished results).

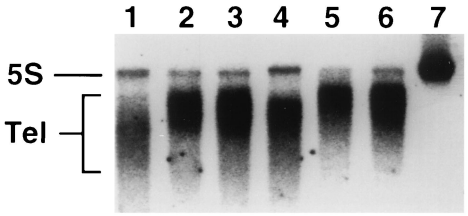

FIG. 8. Telomere loss is not accelerated by propagation in sodium butyrate. Shown is in-gel hybridization of genomic DNAs from HDF cultures. Products from *Pvu*II or *Xba*I digests (lanes 1 to 3 and 4 to 6, respectively) were fractionated and hybridized with a probe directed against the basic telomere repeat unit $[(TTAGGG)_5]$. Lanes 1 and 4, senescent HDF; lanes 2 and 5, PBA HDF; lanes 3 and 6, young HDF; lane 7, undigested control DNA. As an internal control for DNA loading and mobility, a probe against the tandemly repeated 5S rRNA gene array was used. The 5S rRNA sequence lacks internal *Xba*I or *Pvu*II sites, and so upon digestion the array runs as a band unresolved from undigested DNA. This band does not hybridize with the telomere probe (data not shown). Tel, telomere.

Here the possibility has been explored that a third, differentiation-related component of senescence can be accentuated and hence more readily studied. In support of this idea, we report that even low concentrations of histone deacetylase inhibitors can profoundly reduce the proliferative life span in HDF. While it remains to be determined whether these agents induce a full phenocopy of the senescent state, strong similarities between senescent HDF and PBA or post-trichostatin-arrested cells have been demonstrated in terms of cell cycle distribution, pRb phosphorylation level, and response to transient expression of SV40 T antigen. Quite striking in the last-mentioned respect is an apparent alteration in cell cycle regulation in each of these cell types that renders sequestration of Rb family proteins essential to obtain efficient mitogenic stimulation. PBA and post-trichostatin-arrested cultures exhibit the cell morphological changes and reduced saturation density typical of senescent cultures; in addition, expression of pH 6 β-galactosidase, a specific marker for HDF senescence, is clearly evident in PBA and post-trichostatin-arrested cells (Fig. 3 and unpublished results). Lastly, and in some ways most importantly, the influence of histone deacetylase inhibitors on HDF proliferative potential is strongly coupled with the exposure of cells to these agents during cell cycle transit. Taken together, these results lead us to suggest that histone deacetylase inhibitors directly perturb at least one of the counting mechanisms that limit the HDF life span.

Candidate mechanisms. The immediate question that arises from the above proposal is how butyrate and trichostatin A might act to induce commitment to a senescence-like growth arrest state. While these agents may not act solely by inhibition of histone deacetylase, it is likely that altered deacetylase activity is an important contributory factor in the observed premature growth arrest. In this regard, there are several interrelated possibilities to be considered, the first being that these agents act locally to modulate the expression of cell cycle-regulatory genes. Butyrate treatment can either induce or block induction of gene expression, depending on the target gene studied (see reference 18 for a review). When transcription is activated, the mechanism is thought to involve hyperacetylation of histones H3 and H4, perhaps facilitating enhancer function (28). Importantly, histone hyperacetylation is reversible after butyrate is removed (67); furthermore, in the cells examined, transcription of butyrate-induced genes returns to its basal level after the cessation of treatment (50). The simplest way to reconcile the reversibility of the effect of histone deacetylase inhibitors (i.e., on acetylation and local transcription) with the apparently irreversible cell cycle exit that these agents can induce is to postulate that cell doubling in the presence of these agents alters a second, as yet unidentified, control mechanism. DNA methylation, for example, is a candidate for such a coupled mechanism, particularly since loss of methyl-CpG residues has been correlated with cell senescence (10, 23, 86). According to this suggestion, DNA replication in the presence of butyrate or trichostatin could induce irreversible loss of DNA methylation at critical regulatory loci (with such consequences as, e.g., activation of tumor suppressor gene expression). Effects of butyrate on the level of 5-methyl-CpG modification have been reported; however, the patterns of change include both increased and decreased DNA methylation levels (6, 15, 47, 56).

Chromatin-mediated epigenetic inheritance. DNA methylation is thought to be involved in epigenetically inherited silencing. A more direct way in which butyrate and trichostatin A might affect epigenetic silencing (and perhaps thereby acti-

vate a cell cycle checkpoint) would be by impairing the stability of heterochromatin-like chromatin structures. Such structures are implicated in the developmental control of differentiation and, importantly, in specific cases appear to persist by virtue of a memory mechanism(s) (44, 58, 59, 63, 69, 71). A striking feature of heterochromatin-like regions in mammalian cells is the underacetylation of selected lysine residues in histone H4 (40); accordingly, proper function of histone deacetylase is assumed to be required for the reformation of these regions during the latter part of each cell cycle (2). While no experiments directly pertaining to heterochromatin structure have been described here, it has been shown that in *Drosophila melanogaster* sodium butyrate acts as a suppressor of position effect variegation (51) for euchromatic genes and conversely behaves as an enhancer of position effect variegation for heterochromatic genes (36). In addition, this agent enhances the phenotypic effects of a certain suppressor of position effect variegation mutants (72). Since position effect variegation is generally believed to reflect spreading or influence of heterochromatin domains (14, 37, 61), such results strongly suggest that butyrate affects those structures. Interestingly, the proposal that heterochromatin-like domains may play a role in aging is not without precedent. Correlative evidence consistent with selective heterochromatin instability as a contributory factor in senescence derives from reports of age-dependent recession of a subset of heterochromatin domains in vivo (11, 49, 81, 84) and in vitro (66). Results from a study on aging in *Saccharomyces cerevisiae* have also led to the suggestion that the stability of repressive chromatin domains may be a strong determinant of proliferative potential (41).

Retinoblastoma gene function and chromatin structure. Our results indicate that pRb function is enhanced by histone deacetylase inhibitors, both as a direct response and, in committed cells resembling senescent cells, after removal of these agents. While mechanistic links between altered chromatin structure and pRb action remain unknown, it is noteworthy that pRb interacts with BRG1, a homolog of the *Drosophila brahma* gene product and of the yeast transcriptional activator SNF2/SW12 (22). Recent evidence indicates that the latter may act cooperatively with the histone acetyltransferase-containing complexes to modify chromatin structure (8). A tempting speculation prompted by these findings is that histone deacetylase inhibitors potentiate the transcriptional regulatory action of pRb by rendering it less dependent on the cooperative action of targeted histone acetyltransferase-containing complexes. To the extent that the growth-inhibitory action of pRb is linked to its transcription-regulatory activity, this could partially explain the enhanced pRb activity in histone deacetylase-treated cells.

With respect to trichostatin A- and butyrate-induced commitment, it is conceivable that irreversible alterations in higher-order repressive chromatin domains in some way activate pRb-dependent growth arrest pathways. This might involve unmasking and reactivation of growth-regulatory genes initially embedded in heterochromatin (87) or an as-yet-unrecognized mechanism(s) in which abnormal euchromatin-heterochromatin boundaries are more directly detected by cell cycle checkpoint controls. Interestingly, the human retinoblastoma-binding protein RBP-1 contains a putative chromo domain and thus may be associated with heterochromatin-like regions (42); moreover, pRb is preferentially localized within the nucleus at euchromatin-heterochromatin interfaces (78).

Telomere shortening. Whether or not butyrate and trichostatin A reduce proliferative potential by one of the above-described mechanisms, a notable finding reported here is that cells prematurely arrested by these agents do not display the telomere shortening normally seen in senescent HDF. Telomere length is closely correlated with proliferative potential in human fibroblasts (33), and cells from individuals with premature-aging syndromes have shorter-than-expected telomeres (1). Moreover, the accelerated growth arrest induced by mild hyperoxia is purportedly associated with abnormal reductions in telomere length (83). To the extent that the premature arrest state induced by histone deacetylase inhibitors shares attributes with conventional senescence, our results bolster the view that several counting mechanisms act in concert to limit HDF proliferative potential. This in turn implies that HDF senescence has several components and that pharmacologic or genetic manipulations which accentuate one component relative to the others are possible.

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