Characterization of Butyrate Resistant Clones Overexpressing $p21^{Waf1/Cip1}$

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ABSTRACT

$P21^{Waf1/Cip1}$ is a potent inhibitor of cell cycle progression and functions by binding to and inhibiting cyclin-dependent kinases. Millimolar amounts of butyrate can induce cell cycle arrest in mammalian cells through induction of $p21^{Waf1/Cip1}$ expression. In butyrate resistant variants isolated from HeLa cells, clones 5.1 and 7.5 express high levels of $p21^{Waf1/Cip1}$, in regular growth medium and in medium containing butyrate. Despite this elevated levels of $P21^{Waf1/Cip1}$, the cells continue proliferating, albeit at a slower rate than the parental HeLa cells. Western blot analyses showed that other cell cycle proteins were not up regulated to compensate for the elevated expression of this inhibitor. Instead, specific enzymatic assays showed that the cdk2/cyclin E activity in these clones was not inhibited by $P21^{Waf1/Cip1}$, but remained active to allow the cells to continue to cycle in butyrate.

RESUME

La protéine cellulaire $p21^{Waf1/Cip1}$, est un puissant inhibiteur de la progression du cycle cellulaire, qui se lie aux cyclines kinases et supprime leur activité kinase. À des concentrations de l'ordre du millimolaire, le butyrate peut causer un arrêt de la division cellulaire chez les cellules animales, passant par une induction de l'expression de $p21^{Waf1/Cip1}$. Les clones 5.1 et 7.5, dérivant de la ligné cellulaire HeLa, sont résistants à l'inhibition du cycle cellulaire par le butyrate. Ces clones surexpriment la protéine $p21^{Waf1/Cip1}$ en presence comme en absence du butyrate. Mais se divisent plus lentement que les cellules parentales HeLa, malgré le niveau élevé de $p21^{Waf1/Cip1}$. Des analyses de buvardage Western indiquent qu'aucune autre protéine du cycle cellulaire n'est surexprimée, qui aurait pu compenser pour le niveau élevé de cet inhibiteur. Par ailleurs, des études enzymatiques ont démontré que dans ces clones les complexes cycline E/cdk2 ne sont pas inhibés par $p21^{Waf1/Cip1}$, mais qu'ils demeure actif, indépendamment du niveau élevé $p21^{Waf1/Cip1}$ ou du traitement au butyrate.

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INTRODUCTION

"The cell cycle describes the ordered process by which a cell executes the four essential tasks required to produce a duplicate of itself: growth (increase in mass), chromosomal duplication, chromosomal segregation, and cellular division. ... cells replicate their DNA before dividing, and then divide before re-replicating their DNA ... proliferation is not the only option open to cells ... cells must also differentiate, senesce, undergo apoptosis ... In order to decide between disparate fates, the typical somatic cell must integrate a complex array of both intra and extracellular signals to ascertain whether proliferation is desirable and feasible."[Pagano, 1998]

Over the past decades, numerous studies have been done to understand the genes that control the growth of cells. These genes were highly conserved through evolution, from the unicellular organisms such as yeast, to multicellular organisms like mammals. In humans, interests in understanding the regulation of the cell cycle stem from its link with diseases such as cancer.

Cell cycle and regulation

The four stages of the cell cycle are referred to as: Growth phase 1 (G1), DNA replication phase (S [synthesis] phase), Growth phase 2 (G2) and mitotic phase (M phase) (Fig. 1). The G1 and G2 phases were originally referred to as Gap phases, but with greater understanding of the cell cycle, they are now referred to as Growth phases. G1 phase is the growth gap between M phase and S phase. G1 phase permits the cell to

monitor its size and environment before committing to cellular replication. This is also the phase whereby gene products required for DNA replication (e.g. histones, polymerases) are expressed. G1 is the longest phase of the cell cycle, it encompasses a resting state termed G0 that cells enter and remain in as long as the signals to proliferate are not present. Differentiated and senescent cells remain in G0. G2 is the phase between S phase and M phases where the cell ensures that the genome has been duplicated without error, and where the chromosomes undergo reorganization in preparation for the M phase.

A major control mechanism of cell cycle progression includes the cyclin dependent kinases (cdk) (Fig1). As their name implies, they are partially regulated by cyclins expressed in varying levels at different phases of the cell cycle. Cyclin dependent kinase inhibitors (cki's) also play a role in kinase activity regulation [Sherr and Roberts, 1999]. In cancer cells some of these proteins are mutated, not expressed or overexpressed [Hunter and Pines, 1994].

Cyclin/cdk complexes

G1 phase

D-type cyclin expression is induced by mitogenic signals. There are three types of D cyclins, designated as: D1, D2 and D3. In early G1, D-type cyclins complex with cdk4 or cdk6. The cyclin D/cdk complexes enter the nucleus where they are phosphorylated by cdk-activating kinases, and they in turn phosphorylate their

substrates, which includes the retinoblastoma protein (pRb) [Ezhevsky *et al.*, 1997]. Other members of the pRb family phosphorylated by cdk's are p107 and p130. Taules *et al.* (1998) reported that calmodulin plays a role in the translocation and activity of the cyclin D1/cdk4 complex. Cyclin D1 has also been reported to complex with cdk2 and cdk5 to regulate their functions [Xiong *et al.*, 1992].

Phosphorylation of pRb releases the E2F transcription factor that was bound and repressed. Histone deacetylases are recruited by pRb to help in the repression of transcription [Brehm *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998]. An early gene activated by E2F is cyclin E, which then complexes with cdk2 in late G1 to promote G1-S transition. Two alternate spliced mRNAs give rise to two forms of cyclin E, with a difference of 15 amino acids in the amino terminal domain. No difference in function was determined [Ohtsubo *et al.*, 1995]. As well, another family of cyclin E was reported, and it was suggested that besides sharing certain functions, there may be some differences [Lauper *et al.*, 1998]. During S phase, cyclin E is degraded and this is dependent on ubiquination. [Sherr, 1994] Studies have shown that the cyclin E gene can functionally replace the cyclin D1 gene [Geng *et al.*, 1999].

Cdk3 has been reported to be important to cell cycle progression since the transfection of a dominant negative cdk3 arrested cells in G1. Though it shares a high sequence homology with cdk2, cdk3 plays a different role because cdk2 could not rescue the mutant cdk3 phenotype. In vitro cdk3 can bind many cyclins, however its in vivo partner is not known nor are any of its substrates. [Hengstschlager *et al.*, 1999]

S phase

Another early gene activated by E2F is cyclin A. Cyclin A enters the nucleus at S phase [Pines and Hunter, 1991] and as a complex with cdk2 maintains pRb phosphorylation [Yang *et al.*, 1999]. Another possible substrate of this complex is the initiation complex at replication origins, but this function is poorly understood. [Heichman and Roberts, 1994; Pagano, 1998]. Cyclin A/cdk2 also phosphorylates cdc6, an essential regulator of DNA replication. The phosphorylation of cdc6 relocalizes it to the cytoplasm, ensuring it does not re-initiate DNA replication [Petersen *et al.*, 1999]

G2 phase

In G2 phase, cyclin A levels are at their peak. Cyclin A is required for transition through G2 to M. Cells microinjected with antibodies against cyclin A are arrested in the cell cycle at G1 and G2 [Pagano *et al.*, 1992]. Cyclin A can also associate with cdc2 (also known as cdk1) to regulate their activities in G2 [Clark *et al.*, 1992].

Cyclin B also binds cdc2, and acts synergistically with the cyclin A complex during G2 to M transition [Knoblich and Lehner, 1993]. Cyclin B only starts to accumulate once cells are in G2 and reaches threshold levels just before mitosis [Sherwood *et al.*, 1994] and enters the nucleus just before nuclear membrane breakdown [Pines and Hunter, 1991; Hagting *et al.*, 1999].

M phase

Mitosis can be further subdivided into six subphases. Prophase is when chromosomal condensation begins. Then in prometaphase, the mitotic spindles attach to the kinetochores. At metaphase, the chromosomes are fully condensed and are lined up at the metaphase plate by the kinetochore microtubules. In anaphase, the sister chromatids are segregated to opposite poles. In telophase, there is decondensation of the daughter chromosomes and reappearance of nuclear envelope. And finally in cytokinesis, the cytoplasm is divided among the two daughter cells. [Alberts *et al*, 1994]

Cyclin A has been seen to associate with condensing chromosomes in prophase, but not with condensed chromosomes in metaphase where cyclin A starts to be degraded. On the other hand cyclin B associated with condensed chromosomes and the mitotic spindle, and is degraded at the metaphase to anaphase transition [Pines and Hunter, 1991]. In order to traverse M phase, cyclin B must go through three stages. First the cyclin B complex must be in its active state to start M phase. Then at metaphase the complex activates the cyclin degradation system that pushes the cell through anaphase. Finally inactivation of the degradation system by the G1 cyclins will restabilize cyclin B [King *et al.*, 1994].

Cdk phosphorylation

Besides having to complex with cyclins, the major control mechanism of cdk's is by phosphorylation. Some sites of phosphorylation inhibit activity, whereas others

promote it. The inhibitory phosphorylation is dominant over the activating. Cdc2 activity is inhibited through phosphorylation of Thr14 and Tyr15 by Myt1 and Wee1 kinases [Fattaey and Booher, 1997] and activated through dephosphorylation by the phosphatase Cdc25 [Borgne and Meijer, 1996]. Cdc2 is further activated through phosphorylation of Ser277 and Thr161 [Krek and Nigg, 1991] by CAK (cdc2 activating kinase) [Solomon *et al.*, 1993]. Phosphorylation of cdk2/4/6 occurs at the same regions.[Fesquet *et al.*, 1993]

Cdk inhibitors

Inhibitors also regulate cdk activities. There are two classes of inhibitors, the INK4 family of proteins and the Cip/Kip family. Known members of the INK4 family are: $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. The Cip/Kip family consists of: $p21^{WAF1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. The INK4 family is known to be specific inhibitors of cdk4/6, whereas the Cip/Kip family can inhibit most cyclin/cdk complexes [Harper and Elledge, 1996]. INK4 proteins can bind to cdk4/6 alone, but the Cip/Kip proteins require the presence of a cyclin to associate with the kinase [Hall M. *et al.*, 1995].

INK4 proteins are G1 inhibitors differentially expressed in different cell types, and p18 and p19 are mainly involved in development. G1 arrest by INK4 is dependent on functional pRb. In addition to being inhibitors, the Cip/Kip proteins can act as activators for cyclin D/cdk complexes by promoting assembly of the complex and directing it to the nucleus [Cheng *et al.*, 1999; Hiyama *et al.*, 1997; LaBaer *et al.*, 1997]. INK4 competes for cdk4/6 binding and inhibits cyclin D/cdk4/6 activity, and in the

process releases Cip/Kip proteins to inhibit the other cdk's [Sherr and Roberts, 1999; Rao *et al.*, 1998; Welcker *et al*, 1998; Hiyama *et al.*, 1997; Poon *et al.*, 1995]. Progression through S phase is achieved when cyclin A/cdk2 levels are greater than the level of $p21^{Waf1/Cip1}$ [Cai and Dynlacht, 1998].

The first cdk inhibitor discovered was $p21^{Waf1/Cip1}$ and it is the most studied [Harper *et al.*, 1993]. E2F can activate $p21^{Waf1/Cip1}$ transcription, emphasizing its role in promoting, as well as inhibiting cell cycle [Hiyama *et al.*, 1998]. Following DNA damage by irradiation, p53 up-regulates $p21^{Waf1/Cip1}$ expression, to induce cell cycle arrest, although p53-independent induction of $p21^{Waf1/Cip1}$ also exists [Bunz *et al.*, 1998; Rao *et al.*, 1998; Macleod *et al.*, 1995;]. In contrast apoptosis induced by p53, requires low levels of $p21^{Waf1/Cip1}$ [Canman and Kastan, 1998]. P53 mediates cell cycle arrest through the inhibition of cdk2 by $p21^{Waf1/Cip1}$, leading to hypophosphorylation of pRb [Brugarolas *et al.*, 1999].

Cancer

Most tumors have at least one protein, if not more, from the cell cycle regulation machinery that is not functioning properly. Disruptions of pRb, p53 and $p21^{Waf1/Cip1}$ are frequent [Topley *et al.*, 1999; Poon and Hunter, 1998; Balbin *et al.*, 1996]. Loss of p27 expression can also promote tumor cell proliferation [Saegusa *et al.*, 1999;Thomas *et al.*, 1998]. Cyclin D1 and D3 expression is elevated in breast cancers, promoting cell cycling [Russell *et al.*, 1999]. Detection of differential gene expression can be used as an indicator of tumor aggressiveness in colorectal cancer [Tortola *et al.*, 1999]. Butyrate

has long been known to offer protection against colorectal cancers, and has recently gained further interest because of its anti-neoplastic effect [Archer *et al.*, 1998a].

Butyrate

Bacteria in the colon ferment dietary fiber and produce short chain fatty acids. Butyrate is one of the fatty acids produced, and is an important energy source for normal colorectal cells [Cummings *et al.*, 1981]. Studies have shown a correlation between high fiber diets and a reduced incidence of colorectal cancer [Howe *et al.*, 1992; Jansen *et al.*, 1999]. There is also interest in butyrate for treatment of other cancers such as melanoma [Soballe and Herlyn, 1994], and myelogenous and promyelocytic leukemias [Novogrodsky *et al.*, 1983; Warrell *et al.*, 1993; Warrell *et al.*, 1998]. Because of its ability to stimulate fetal γ-globin gene expression, it also presents an interest for the treatment of beta-thalassemia and sickle cell anemia [Faller and Perrine, 1995].

Butyrate can induce cell cycle arrest [Chang and Yung, 1996; Gilbert and Weigle, 1993; Stenzel *et al.*, 1980] and cause differentiation at low concentrations [Conway *et al.*, 1995] or apoptosis at higher concentrations [Chang and Yung, 1996;Conway *et al.*, 1995]. Cell cycle arrest, differentiation and apoptosis are induced by butyrate through p53-independent mechanisms [Janson *et al.*, 1997]. Butyrate can arrests cells in G1 and/or G2 [Joensuu and Mester, 1984; Gilbert and Weigle, 1993; Heerdt *et al.*, 1997; Lallemand *et al.*, 1999]. Concentrations of butyrate needed for such growth inhibition reflect levels measured within the rat colon [McIntyre *et al.*, 1991].

Butyrate in millimolar concentrations inhibits histone deacetylases noncompetitively and induces hyperacetylation of histones [Candido *et al.*, 1978; Sealy and Chalkley, 1978]. Histone acetylation is associated with gene transcription [Bradbury, 1992; Davie and Hendzel, 1994; Grunstein, 1997; Wu, 1997]. Histone hyperacetylation by butyrate was shown to reactivate expression of virally transduced genes that were silenced in stably infected HeLa cells [Chen *et al.*, 1997]. Butyrate induces cell cycle arrest through the upregulation of certain genes and the downregulation of others [Reeves and Cserjesi, 1979; Toscani *et al.*, 1988; Gupta *et al.*, 1994]. Among the genes induced by butyrate is $p21^{Waf1/Cip1}$ [Nakano *et al.*, 1997; Archer et al., 1998b] in a p53-independent manner [Xiao *et al.*, 1997] whereas cyclin D1 is one of the genes down regulated by butyrate [Lallemand *et al.*, 1996]. Butyrate induces a hypophosphorylated state in pRb [Schwartz *et al.*, 1998; Yen and Sturgill, 1998; Yen and Varvayanis, 1995] and Vaziri *et al.* showed that this can be independent of $p21^{Waf1/Cip1}$ expression.

Thesis proposal

The aim of this project is to determine how the butyrate-resistant clones 5.1 and 7.5 can proliferate in the presence of high levels of $p21^{Waf1/Cip1}$. These butyrate-resistant cells were isolated from HeLa cells that have adapted to growth in cytotoxic concentrations of butyrate. Since the cDNA encoding $p21^{Waf1/Cip1}$ from these clones have been sequenced, and were found to contain no mutations, my research is focussed on the other cell cycle proteins that are regulated by $p21^{Waf1/Cip1}$. The $p21^{Waf1/Cip1}$ protein exerts its cell cycle control mainly by inhibiting the activity of cyclin/cdk complexes. My thesis is to examine the levels of the proteins in these complexes, and to determine if any are overexpressed to compensate for the high levels of $p21^{Waf1/Cip1}$. I will also examine the binding of $p21^{Waf1/Cip1}$ to these complexes and study the activities of these kinases

MATERIALS & METODS

Cell culture

HeLa cells and their butyrate resistant clones, 5.1 & 7.5, were isolated by Dr. J. Th'ng, and were routinely grown in Dulbecco's Modified Eagle's medium, supplemented with 10% calf serum, 100U/mL penicillin and 100μL/ml streptomycin. For experiments, cells were plated at ~80% confluence for overnight treatment with 0mM, 1mM and 5mM sodium butyrate. The cells were then collected by scrapping into the medium and pelleted by centrifugation, washed once with cold PBS (2.7mM KCl, 1.5mM KH_2PO_4, 0.14M NaCl and 8mM Na_2HPO_4).

Antibodies

Polyclonal rabbit α cyclin A serum, polyclonal rabbit α cdc2 serum and polyclonal rabbit α cdk2 serum were gifts from Dr. E.M. Bradbury (University of California, Davis). The polyclonal antibodies for cyclins D1, D2, D3, $p16^{INK4a}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and cdk6 were purchased from Santa Cruz Biotechnology, Inc. Monoclonal antibodies to PCNA and cyclin B were also purchased from Santa Cruz Biotechnology, Inc. Polyclonal antibodies to cdk4 and p53 were purchased from Calbiochem. Monoclonal antibodies to cyclin E and pRb were purchased from Pharmingen. Secondary, goat α-rabbit and goat α-mouse, antibodies conjugated with horseradish peroxidase (HRP) were purchased from Pierce.

Cell lysis and protein determination

Cell pellets were lysed in NB buffer (0.25M Sucrose, 0.2M NaCl, 10mM Tris-HCl pH 8, 2mM $MgCl_2$, 1mM $CaCl_2$ and 1% Triton) and 1-5 μL used for protein quantity determination using the Bio-Rad microassay with bovine serum albumin as the relative protein standard. The lysate was added to 800μl water and mixed with 200 μl of BioRad reagent. After allowing to sit for 5 minutes, the absorbance was read in a spectrophotometer using a wavelength of 595 nm. A control sample without lysate was used as the blank standard. The amount of protein in the cell lysates was then determined from the protein standard curve. To generate the protein standard curve, known amounts of albumin purchased from Biorad were added to the water and BioRad reagents as described. Following measurements at OD_{595}, the absorbance readings were plotted against the known concentrations of albumin. For protein gels, a 2X SDS Buffer (0.0024% bromophenol blue, 0.16mM Tris pH 6.8, 10% Glycerol, 4% SDS and 2% β-mercaptoethanol) was added to the NB lysed pellets and the volume was adjusted to a final protein concentration of 2μg/μL.

For subcellular fractionation of cells, the cells were lysed in NB buffer minus NaCl, to minimize the leaking of nuclear proteins into the cytoplasmic fraction. The suspension was centrifuged briefly at 13 000 rpm, and the supernatant used as the cytoplasmic fraction and the pellet used as the nuclear fraction. Following protein

determination of the cytoplasmic fraction, both fractions were taken up in 2X SDS-sample buffer to final equal volumes.

Protein gels

Protein samples were run on a 12% SDS- polyacrylamide gel (PAGE) in Biorad's Mini-PROTEAN II cell, according to the protocol described in Ausubel *et al.* (1989) with modifications. A stock mixture of 29% (w/v) acrylamide and 1% (w/v) bis-acrylamide was made and filtered. The separating portion of each gel is made up of: 2ml of acrylamide stock mixture, 1.25 ml of a 1.5M Tris pH 8.8 solution, 50 μL of a 10% (w/v) SDS solution and 1.7 ml of distilled water. For polymerization 8 μL each of TEMED (purchased from ICN) and 25% (w/v) APS (ammonium persulfate) are added. The stacking portion of the gel is made of: 157.5μL each of acrylamide stock mixture and 1M Tris pH6.8, 12.45μL of 10% SDS solution and 930μL of distilled water. For polymerization 5μL each of TEMED and 25% APS are added. Electrophoresis was performed at about 80 volts, with Laemmli buffer (14.4% (w/v) Glycine, 3% (w/v) Tris and 50mM SDS) as the running buffer. To separate the phosphorylated isoforms of the retinoblastoma protein (pRb) a 9% acrylamide gel was used.

Protein transfer and immunoblotting

Following electrophoretic separation of cellular proteins in the SDS-PAGE, the proteins were transferred onto a PVDF membrane (Millipore's Immobilon-P) at 100

volts for 1h (2h for pRb) in Biorad's Mini Trans-Blot cell, with transfer buffer (25mM Tris, 192mM Glycine and 20% Methanol). Ponceau red (1%) in 5% acetic acid was used for protein staining to confirm proper transfer of proteins.

Membranes were first incubated, on a shaker, in blocking buffer (TBS [25mM Tris, pH7.5 and 150 mM NaCl] plus 3% skim milk), for about an hour at room temperature. The blocking buffer was removed and the primary antibody (in TBS plus 3% skim milk, 0.05% Tween-20 and 350 mM NaCl) was added for 2h incubation. Antibody dilution was according to manufacturer's suggestion, generally 1μg/ml for purified antibodies and 1:1 000 for serum. Following primary antibody incubation, the membrane was washed (10 minutes each time) once with TBS and three times with TBS plus 3% skim milk, 0.05% Triton X-100 and 100mM NaCl. Then the secondary antibody was added for 1h, in the same buffer as the primary antibody but at a dilution of 1:10 000. The membrane was washed again as before, plus two extra washes with TBS at the end. Enhanced chemiluminescent detection was performed using Super Signal® Substrate from Pierce, following their directions. Membranes were incubated in a mixture of equal volumes of luminol/enhancer solution and stable peroxide solution. After 5 minutes, membranes were removed, wrapped in saran wrap and exposed to X-ray film for 1 second to 5 minutes, and the film developed.

Immunoprecipitation

To eliminate nonspecific binding, each sample to be studies was first incubated with 0.5 μL of pre-immune serum and mixed with protein A-Sepharose 6MB (Amersham-Pharmacia Biotech) which was washed four times and equilibrated in NB buffer. Aliquots of 75 μL of the suspension were used for each immunoprecipitation, and added to 400 μg of soluble protein extracts. After rotating in the cold room (4°C) for 1h, the samples were microcentrifuged and the supernatant was then transferred to new tubes. The antibody of choice was then added along with fresh protein A-Sepharose 6MB, and the mixture was rotated in the cold room for 2-3hrs. After 1 minute of microcentrifugation, the supernatant was removed and the beads washed six times with NB buffer. The beads were then used for a kinase assay and then resuspended in SDS buffer for electrophoresis and immunoblots.

Kinase assay

Beads with immunoprecipitate were first equilibrated with 100 μL H1 kinase buffer (50mM Tris, pH 7.4, 2mM $MgCl_2$, 1mM DTT, 100mM NaCl and 0.05mM ATP) for 10 minutes on ice. The buffer was removed and replaced with 50 μL of H1 kinase reaction buffer (same as kinase buffer plus 0.25M Na_3VO_4 and 100 nM calyculin A (Sigma) as phosphatase inhibitors, 0.1 μg/μL purified histone H1 (gift from Dr. X.W. Guo (Helix Diagnostics)), and 0.05 μCi/μL γ-^{32}P-ATP) and incubated at 30°C for 10 minutes. After the reaction, 45 μL of the reaction mixture was added to nine times the

volume of cold acetone and put at - 20 °C for precipitation of histone H1. The samples were then microcentrifuged for 5 minutes and supernatant discarded. Pellets containing histone H1 were air dried and resuspended in SDS buffer and electrophoresed in 12% SDS-PAGE gel. The gel was stained for protein with 0.3% Coomassie Blue in 10% acetic acid and 50% ethanol, then washed with 10% acetic acid and 20% ethanol. The gel was dried and exposed to X-ray film for autoradiography to determine the levels of ^{32}P-incorporation.

RESULTS

Clones

Sodium butyrate has long been known to induce cell cycle arrest in mammalian cells, and more recent studies showed that this is due to an induction in the cyclin-dependent kinase inhibitor (cki), $p21^{Waf1/Cip1}$ [Archer *et al.*, 1998b]. To further study the effects of butyrate on cells, butyrate resistant variants of HeLa cells were isolated. For this, Dr Th'ng incubated 10^8 HeLa cells per plate, in medium containing 5, 7.5 or 10 mM sodium butyrate with regular medium changes. After a week most of the cells died, and the cells that remained attached to the dishes did not show any signs of cell division. Incubation with the respective concentrations of butyrate were continued for up to 6 months, three visible colonies appeared in the plates containing medium with 5 mM sodium butyrate (designated as 5.1, 5.2 and 5.3). In the plates with 7.5 mM butyrate, one colony was recovered (designated 7.5) and no colonies were observed in the 10 mM sodium butyrate plates. The clones were then expanded in separate flasks with medium containing 5 mM sodium butyrate. For further purification the clones were subcloned again in regular growth medium without butyrate. Regular bimonthly tests revealed that the clones maintained their ability to grow in 5 mM sodium butyrate, albeit with reduced growth rates. Frozen stocks were made and clones 5.1 and 7.5 selected for further analyses. Initial studies revealed that the butyrate resistant clones had slower growth rates than the parental HeLa cells. The doubling time of HeLa was about 16 h, while that of clone 5.1 was 24 h, and that of clone 7.5 was 36 h (courtesy of Dr. H.A. Crissman, Los Alamos National Laboratories, NM). Further studies of cell growth (done

by J. Th'ng) showed that the parental HeLa cells underwent cell cycle arrest in 1 mM butyrate, and there were no increase in cell number after 3 days incubation (Fig. 2). Increasing the concentration of butyrate to 5 mM caused cells to die rapidly within 3 days. In contrast, the butyrate-resistant clones showed some increase in cell numbers after 3 days (for clone 5.1) or 6 days (for clone 7.5) incubation in 1 mM butyrate, although at a slower rate than untreated controls. When the concentration of butyrate was raised to 5 mM, the number of cells did not change over the 3 day (for clone 5.1) or 6 days (for clone 7.5) incubation period. This shows that these variants have adapted to survive in concentrations of butyrate that are normally cytotoxic to control HeLa cells. These butyrate resistant variants will gradually increase in cell density if incubation in 5 mM butyrate is continued, indicating that they are able to grow at a much reduced rate.

This approach to isolation of butyrate resistant cells differ from the methods employed by Milsted *et al.* (1985) and Chalkley and Shires (1985) who isolated butyrate resistant cells by incubation in gradual increases of butyrate concentrations. Cell cycle studies were not done for these variants because cell cycle proteins had not been identified at that time.

$P21^{Waf1/Cip1}$ protein levels

To examine the status of $p21^{Waf1/Cip1}$ in the butyrate resistant clones 5.1 and 7.5, early studies of Drs. Paul S. Wright (Hoechst Marion Roussel, NJ) and J. Th'ng by RT-PCR showed that $p21^{Waf1/Cip1}$ mRNA levels were up to eight fold higher than the

parental HeLa cells (Fig. 3). This is an interesting observation since $p21^{Waf1/Cip1}$ is a ubiquitous cell cycle inhibitor, and yet the 5.1 and 7.5 clones were still proliferating in the presence of high levels of the inhibitor. DNA sequencing, (by Dr. J. Th'ng), of the $p21^{Waf1/Cip1}$ gene showed no differences in all three cell lines. To determine if the elevated levels of message encoding $p21^{Waf1/Cip1}$ was translated into its protein product, western blot was performed. HeLa cells and the 5.1 and 7.5 clones were incubated overnight in supplemented DMEM without butyrate (control) and with 1 or 5 mM sodium butyrate. Whole cell protein samples were prepared, fractionated in a 12% SDS gel, and immunobloted. Results shown in Figure 4 confirmed the translation of the $p21^{Waf1/Cip1}$ mRNA into its protein products corresponded to the levels of transcripts synthesized. In cycling control HeLa cells, $p21^{Waf1/Cip1}$ were expressed at very low levels, and a five-fold increase was seen when the cells were treated with increasing butyrate concentrations. This resulted in arrests in G1 and G2 phases of the cell cycle (Fig.5), this is determined by the fact that the cells in S phase are cleared out increasing the peak in G2 and the G1 peak has not change since these cells do not enter into the S phase and those of G2 do not move into G1. In contrast, clones 5.1 and 7.5 have elevated levels of $p21^{Waf1/Cip1}$ protein in their respective untreated control samples. However their cell cycle profiles remained indistinguishable from that of the untreated parental HeLa cells, showing that they were able to continue with their cell cycle despite the presence of $p21^{Waf1/Cip1}$. In the presence of butyrate, the levels of $p21^{Waf1/Cip1}$ protein remained high in these cells.

Subcellular distribution of $p21^{Waf1/Cip1}$

Early studies showed that $p21^{Waf1/Cip1}$ functions by binding to proteins that regulate the cell cycle, and these include cyclin/cdk complexes. Since the activity of the complexes occurs mainly within the nucleus, an aberrant distribution of $p21^{Waf1/Cip1}$ within the cell may account for its inability to inhibit growth in the 5.1 and 7.5 clones. To study this, nuclear and cytoplasmic fractions were separated from all three cell lines following overnight incubations without (control) and with 5 mM butyrate. Western blots were then performed to determine the subcellular distribution of $p21^{Waf1/Cip1}$. As seen in Figure 6, the levels of $p21^{Waf1/Cip1}$ within the nucleus were similar to those in the cytoplasm, in all cells, indicating that the inability of the inhibitor to function is not due to its exclusion from the nuclei.

Cyclin levels in cells

One possible explanation for the ability of clones 5.1 and 7.5 to continue their cell cycle is that they overcome the elevated $p21^{Waf1/Cip1}$ by overexpressing other cell cycle proteins. To determine if this is the case, immunoblots for these cell cycle proteins were performed using samples of the three cell lines incubated without (control) and with 1 or 5 mM butyrate.

As can be seen in Figure 7, there was no overexpression of any of the cyclin proteins. Instead, most of them remained unchanged, while several proteins were expressed at lower levels. The cyclins D and E that function in G1 of the cell cycle

remained unaltered in levels. However, the decrease was seen in some of the S and G2 cyclins. Cyclin A levels decrease with increasing concentrations of butyrate in HeLa and the 5.1 and 7.5 clones which would be consistent with the slowing down of the cell cycle. On the other hand, cyclin B levels decreased with increasing concentration of butyrate in parental HeLa cells and the 7.5 clones, again consistent with the decreasing cell cycle. However, the level of cyclin B remains constant in the 5.1 clones.

PCNA, $p16^{INK4a}$ and $p27^{Kip1}$

The PCNA required for DNA synthesis is also regulated by $p21^{Waf1/Cip1}$, and again its levels are also constant in all the samples tested (Fig. 8). Two other growth inhibitor proteins were also examined. $P16^{INK4a}$ protein levels were the same in all samples from HeLa cells and the 5.1 clone (Fig. 8), levels in the 7.5 clone were constant but much lower than the other two. The levels of $p27^{Kip1}$ went down with butyrate in the HeLa cells, were about constant in the 5.1 clone, and went up slightly in the 7.5 clones (Fig. 8). These cki's have never been reported to be affected by the presence of butyrate, and all available evidence showed that butyrate induces cell cycle arrest through $p21^{Waf1/Cip1}$.

Cyclin-dependent kinases

Since there was no overexpression of cyclins to confer any growth advantage, the levels of cyclin dependent kinases (cdk's) were then examined. As seen with the

cyclins, there was no increase in the levels of the cdk's in any of the cells, in control or following butyrate-treatment (Fig. 8). The cdc2 (cdk1) protein levels were about equal in all samples and the same was true for the cdk2 protein levels (Fig. 8). These cdk's were reported to be proteins that are inhibited by $p21^{Waf1/Cip1}$.

Kinase assays

Since there was no overexpression of cdk's or cyclins to overcome the elevated $p21^{Waf1/Cip1}$ in the cells, the enzymatic activities of the cdk's were then examined. The different cdk's from each of the cell lines were immunoprecipitated and assayed for kinase activities using histone H1 as substrate and γ-^{32}P-ATP. Following the reaction, the H1 protein was electrophoresed in a 12% SDS-PAGE, stained, and the gel dried for autoradiography. An immunoblot was also done to confirm the presence and level of the cdk's that were immunoprecipitated.

As seen in Figures 9-11, the activities of cdc2 (Fig. 9), cdk4 (Fig. 10) and cdk6 (Fig. 11) on histone H1 in all the cells were not affected by the presence of butyrate. In each case, coomassie staining showed that the amounts of histone H1 substrate were evenly loaded in all the samples, and western blot analyses showed that the immunoprecipitation brought down equivalent amounts of kinases. In contrast, the results for cdk2 showed that it was the principle target for $p21^{Waf1/Cip1}$ inhibition. The phosphorylation of H1 was most pronounced in the HeLa control lysate sample (Fig. 12, panel 2), showing that the cdk2 complex that was immunoprecipitated had high

enzymatic activity. This activity was drastically diminished in HeLa cells that were exposed to 5 mM butyrate (Fig. 12, panel 2). These results were expected, since $p21^{Waf1/Cip1}$ in the 5 mM butyrate sample would inhibit the kinase activity. However in both samples from the 5.1 and 7.5 clones, the phosphorylation of histone H1 by cdk2 continued in the presence or absence of butyrate, indicating that the $p21^{Waf1/Cip1}$ was not inhibiting this kinase. The cdk2 activities in these butyrate-resistant clones were lower than that of parental HeLa cells, which agrees with their slower cell cycle. As with the other cdk's, the levels of histone H1 loaded in the gels are the same (Fig. 12, panel 1), so was the amount of cdk2 immunoprecipitated (Fig. 12, panel 3). From these results, and from the results shown in Figure 7, the cdk2 activities in the butyrate-resistant clones is from its association with cyclin E since this protein levels remained unaltered with butyrate treatment.

Phosphorylation of pRb

In vitro kinase assays showed that the cdk2/cyclin E remained enzymatically active in the presence of $p21^{Waf1/Cip1}$ (Fig. 12). To determine if this was also true in vivo, the phosphorylation of pRb was examined. It is well established that one of the common substrates for the cyclin/cdk complexes in the cell is pRb which then regulates entry into S phase [Yen *et al.*, 1998]. The proteins of the pRb family in HeLa cells have very low activity due to their binding to the E7 protein of the human papilloma virus. However, pRb can still be phosphorylated as a substrate, and was therefore used as an internal measure of kinase activity. The different isoforms of pRb were separated on a 9% SDS-PAGE and immunoblot of pRb revealed the various phosphorylated forms (Fig. 13). In

untreated control HeLa cells, the pRb were highly phosphorylated, as evident from its reduced migration in the SDS-gel. In HeLa cells that were incubated with 5 mM butyrate only the fastest migrating unphosphorylated isoform was obvious. In the 5.1 and 7.5 clones the phosphorylated isoforms were present in all samples, with or without butyrate. These results show that the endogenous cyclin-dependent kinase remained active in the butyrate-resistant cells, while it is repressed in butyrate-treated HeLa cells where the $p21^{Waf1/Cip1}$ is upregulated

Binding of $p21^{Waf1/Cip1}$ to cdk2

The absence of inhibition of cdk2 kinase in the butyrate-resistant clones could be due to its inability to associate with $p21^{Waf1/Cip1}$. To examine this possibility, immunoprecipitation of $p21^{Waf1/Cip1}$ from each of the cell lines, in the presence or absence of butyrate, were performed, and the proteins were fractionated in a 12% SDS-PAGE. Western blot analyses done on these immunoprecipitated proteins showed that little $p21^{Waf1/Cip1}$ was present in cycling HeLa cells, but that its levels were high in butyrate-treated cells, and in the butyrate-resistant clones (Fig. 14, panel 3), and this reflected the results seen in immunoblots of whole cell extracts (see Figure 4). Furthermore, the immunoblots also showed that cdk2 and cyclin E were associated with $p21^{Waf1/Cip1}$ in all the cells tested (Fig. 14, panels 4 and 5). However, enzymatic assay showed that these complexes associated with $p21^{Waf1/Cip1}$ had very low activities on histone H1 (Fig14, panel 2), even with the kinases extracted from the butyrate-resistant cells. This shows that the $p21^{Waf1/Cip1}$ in these clones retained their ability to inhibit cdk activity, and suggested that there was a sub-population of cdk2/cyclin E that was not

associated with $p21^{Waf1/Cip1}$ in the butyrate-resistant clones and remained active. This was demonstrated when the supernatant following $p21^{Waf1/Cip1}$ immunoprecipitation was then used to immunoprecipitate any remaining cdk2, and the immunoprecipitate was analyzed for kinase activity. As seen in Figure 15, panel 2, there was substantial amounts of cdk2 activity remaining unbound to $p21^{Waf1/Cip}$ in the butyrate-resistant clones. These results indicate that not all the cdk2 is bound to $p21^{Waf1/Cip1}$, in all three cell lines, but the cdk2 in the butyrate-treated HeLa cells was enzymatically inactive.

DISCUSSION

Butyrate has long been known to inhibit histone deacetylases and was routinely used for studies on chromatin structure and function. In addition to its ability to induce cellular differentiation, recent studies showed that it induces cell cycle arrest and apoptosis in a concentration-dependent manner. The report of Archer *et al.* (1998) demonstrated that this cell cycle arrest is due to the induction of $p21^{Waf1/Cip1}$, although the work of Vaziri *et al.* (1998) showed that this arrest is independent of $p21^{Waf1/Cip1}$. For further study of butyrate effects on the cell cycle, Dr. J. Th'ng isolated butyrate resistant HeLa clones capable of sustained growth in 5 mM sodium butyrate. These butyrate resistant variants have adapted to growth in the presence of elevated levels of $p21^{Waf1/Cip1}$ albeit at a slower rate. Immunoblots revealed that these clones overexpress $p21^{Waf1/Cip1}$ constitutively while parental HeLa cells only expressed high levels of $p21^{Waf1/Cip1}$ when incubated with butyrate (Fig.4). In addition to the absence of cell cycle arrest, these butyrate-resistant clones are also resistant to induction of apoptosis by butyrate. How these cells respond to other inducers of apoptosis remains to be determined. Sequencing of the $p21^{Waf1/Cip1}$ gene revealed no differences in the clones compared to the parental HeLa cells, and measurements of kinase activities after immunoprecipitation by $p21^{Waf1/Cip1}$ showed that the cdk2 remained inactive when associated with this cki (Fig.14). And immunoblots showed that the other cell cycle genes were not upregulated, in the butyrate resistant clones, to overcome this elevated increase in cki (Fig.).

Although isolated from different dishes of cells treated with different levels of butyrate, we cannot rule out the possibilities that clones 5.1 and 7.5 are the same clones. However, differences between the two clones suggest otherwise (Fig.2). In addition to their differences in growth rates, clone 5.1 also expresses elevated levels of histone H2B.Z (Dr. Th'ng, personal communication). Furthermore, clone 5.1 consistently expresses higher levels of cyclin B compared to clone 7.5 (Fig.7). These suggest a difference in genotype in the clones.

Kinase assays performed on immunoprecipitates of cdk's showed that cdk2 activity was strongly inhibited in HeLa cells incubated with 5 mM butyrate, while the cdk2 kinases from the butyrate-resistant clones remained active in spite of the presence of $p21^{Waf1/Cip1}$, although at lower activities. These in vitro studies indicated that cdk2 activity is conferring the growth advantage on both clones. Because cyclin E is the only cdk2 associated co-factor whose protein levels remain the same in the presence and absence of butyrate, it is the most likely to be involved in the active kinase complex.

Kinase assays performed on $p21^{Waf1/Cip1}$ immunoprecipitates from the butyrate-resistant variants showed that $p21^{Waf1/Cip1}$ in these cells was able to function as an inhibitor of cdk2, and that the cdk2 activities were from the unbound fraction that remained in the cell. However, similar immunoprecipitations from the parental HeLa cells that were treated with butyrate revealed a subpopulation of cdk2 that had no kinase activity, even though it was not associated with $p21^{Waf1/Cip1}$. The reason for this absence of activity is not known, implicating that other activating regulators are necessary. In cells, the activity of cyclin-dependent kinases is also regulated by phosphorylation at

specific residues. The activity of cdk2 is inhibited by phosphorylation of Thr14 and Tyr15, and activated by phosphorylation at Thr160, therefore some of these phosphorylations could be altered in the butyrate-resistant cells. Since the effect of the inhibitory phosphorylation sites dominates over that of the activating sites, there could be mutations in the Thr14 and Tyr15 of cdk2 in these clones. Alternatively, there could be a phosphatase, such as cdc25, that continuously dephosphorylates these sites or possibly the kinases, such as wee1, that phosphorylate these sites are inactivated. Future experiments will address these possibilities.

Several recent articles published after the original submission of this thesis, will direct future studies of these clones. Wang *et al.* (2000) have shown that HuR, an RNA binding protein, stabilizes $p21^{Waf1/Cip1}$ mRNA during stress induced responses. HuR activity is dependent on its elevated levels in the cytoplasm. It would be interesting to see if HuR plays a role in the high levels of $p21^{Waf1/Cip1}$ mRNA in the clones. A paper by Chai *et al.* (2000) indicates that the apoptotic effect of butyrate is dependent on $p21^{Waf1/Cip1}$ cleavage by DEVD-caspase. Therefore the elevated levels of $p21^{Waf1/Cip1}$ in the clones could be protecting them from apoptosis during incubations with butyrate at higher concentrations and longer exposures, unlike the parental HeLa cells which do undergo apoptosis. It would be of interest to look at the $p21^{Waf1/Cip1}$ levels in the parental HeLa cells and the clones, and to look for the 15Kd band that appears after DEVD-caspase activation and is thought to be a portion of the cleaved $p21^{Waf1/Cip1}$ protein.

And finally work by Estanyol *et al.* (2000) indicates that the Set protein can bind and inhibits $p21^{Waf1/Cip1}$ specifically when $p21^{Waf1/Cip1}$ is bound to the cyclin E/cdk2 complex. The inhibition of $p21^{Waf1/Cip1}$ by Set results in the cyclin E/cdk2 complex remaining active. Since this is the complex that maintains it activity in the clones, there is a possibility that Set could also be involved in this case. However, the absence of kinase activities in the cdk2 complexes when associated with $p21^{Waf1/Cip1}$, even with the kinases isolated from the butyrate-resistant clones, suggest this may not be the case. Not much is know about Set, but Estanyol *et al.* (2000) determined that it binds the carboxyl-terminal region of $p21^{Waf1/Cip1}$. Since our antibody against $p21^{Waf1/Cip1}$ also recognizes the carboxyl-terminal region, it is possible that when Set is bound to it, that a fraction of $p21^{Waf1/Cip1}$ cannot be immunoprecipitated with our antibody. However, this appears not to be the case since our immunoprecipitation method was efficient enough to deplete the cell extracts of $p21^{Waf1/Cip1}$. Further experiments will be necessary to address these questions thoroughly, and since not much is know about Set, these clones can potentially be used to elucidate its role further.

SUMMARY AND CONCLUSION

Several conclusions can be drawn from these studies. The butyrate-resistant clones continue cycling with high levels of $p21^{Waf1/Cip1}$ protein. This is due to cyclin E/cdk2 activity in the clones. The kinase activity is from the sub-population of cyclin E/cdk2 that is not associated with $p21^{Waf1/Cip1}$ since complexes that were associated with $p21^{Waf1/Cip1}$ were inhibited. Further experiments will be required to determine reason behind the ability of cdk2 to remain active in these butyrate-resistant clones.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (1994). Molecular biology of the cell. New York, NY, Garland Publishing.

Archer, S., Meng, S., Wu, J., Johnson, J., Tang, R. and Hodin, R. H. (1998a). Butyrate inhibits colon carcinoma cell growth through two distinct pathways. Surgery **124**: 248-253.

Archer, S. Y., Meng, S., Shei, A. and Hodin, R. A. (1998b). $P21^{Wafl}$ is required for butyrate-madiated growth inhibition of human colon cancer cells. Proceedings of the National Academy of Science **95**: 6791-6796.

Asada, M., Yamada, T., Ichijo H., Delia, D., Miyazono, K., Fukumuro, K. and Mizutani, S. (1999). Apoptosis inhibitory activity of cytoplasmic $p21^{Cip1/Wafl}$ in monocytic differentiaton. EMBO Journal **18**: 1223-1234.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1989). Current protocols in molecular biology. New York, NY, Greene Publication and Associates and Wiley-Interscience.

Balbin, M., Hannon, G. J., Pendas, A. M., Ferrando, A. A., Vizoso, F., Fueyo, A. and Lopez-Otin, C. (1996). Functional analysis of a $p21^{Wafl, Cipl, Sdil}$ mutant ($Arg^{94} \rightarrow Trp$) identified in a human breast carcinoma. Journal of Biological Chemistry **271**: 15782-15786.

Ball, K. L. (1997). P21: sructure and functions associated with cyclin-cdk binding. Progress in Cell Cycle Research **3**: 125-134.

Borgne, A. and L. Meijer (1996). Sequential dephosphorylation of p34(cdc2) on Thr-14 and Tyr-15 at the prophase/metaphase transition. Journal of Biological Chemistry **271**: 27847-27854.

Bradbury, E. M. (1992). Reversible histone modifications and the chromosome cell cycle. Bioessays **14**: 9-16.

Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J. and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature **391**: 597-601.

Brown, N. R., Noble, M. E. M., Lawrie, A. M., Morris, M. C., Tunnah, P., Divita, G., Johnson, L. N. and Endicott, J. A. (1999). Effects of Phosphorylation of threonine 160 on cyclin-dependent kinase 2 structure and activity. Journal of Biological Chemistry **274**: 8746-8756.

Brugarolas, J., Bronson, R. T. and Jacks, T. (1998). P21 is a critical cdk2 regulator essential fo proliferation control in Rb-deficient cells. Journal of Cell Biology **141**: 503-514.

Brugarolas, J., Moberg, K., Boyd, S. D., Taya, Y., Jacks, T. and Lees J. A. (1999). Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G1 arrest after γ-irradiation. Proceedings of the National Academy of Science **96**: 1002-1007.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W. and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science **282**: 1497-1501.

Butz, K., Geisen, C., Ullmann, A., Zentgraf, H. and Hoppe-Seyler, F. (1998). Uncoupling of $p21^{Waf1/Cip1/Sdi1}$ mRNA and protein expression upon genotoxic stress. Oncogene **17**: 781-787.

Cai, K. and B. D. Dynlacht (1998). Activity and nature of $p21^{Waf1}$ complexes during the cell cycle. Proceedings of the National Academy of Science **95**: 12254-12259.

Candido, E. P. M., Reeves, R. and Davis, J.R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. Cell **14**: 105-113.

Canman, C. E. and M. B. Kastan (1998). Small contribution of G1 checkpoint control manipulation to modulation of p53-mediated apoptosis. Oncogene **16**: 957-966.

Casini, T. and P. G. Pelicci (1999). A function of p21 during promyelocytic leukemia cell differentiation independent of cdk inhibition and cell cycle arrest. Oncogene **18**: 3235-3243.

Cayrol, C., Knibiehler, M. and Ducommun, B. (1998). P21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. Oncogene **16**: 311-320.

Chai, F., Evdokiou, A., Young, G.P. and Zalewski, P.D. (2000). Involvement of $p21^{Waf1/Cip1}$ and its cleavage by DEVD-caspase during apoptosis of colorectal cancer cells induced by butyrate. Carcinogenesis **21**:7-14.

Chalkey, R. and A. Shires (1985). The isolation of HTC variant cells which can replicate in butyrate. Journal of Biological Chemistry **260**: 7698-7704.

Chang, S. T. and B. Y. Yung (1996). Potentiation of sodium butyrate-induced apoptosis by vanadate in human promyelocytic leukemia cell line HL-60. Biochemical and Biophysical Research Communications **221**: 594-601.

Chen, W. Y., Bailey, E. C., McCune, S. L., Dong, J. Y. and Townes, T. M. (1997). Reactivtion of silenced, virally transduced genes by inhibitors of histone deacetylase. Proceedings of the National Academy of Science **94**: 5798-5803.

Chen, Y.-N. P., Sharma, S. K., Ramsey, T. M., Jiang, L., Martin, M. S., Baker, K., Adams,P. D., Bair, K. W. and Kaelin, W. G. Jr. (1999). Selective killing of transformed cells by cyclin/cyclin-dependent kinase 2 antagonists. Proceedings of the National Academy of Science **96**: 4325-4329.

Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M. and Sherr, C. J. (1999). The $p21^{Cip1}$ and $p27^{Kip1}$ cdk 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO Journal **18**: 1571-1583.

Clark, P. R., Leiss, D., Pagano, M. and Karsenti, E. (1992). Cyclin A- and cyclin B-dependent protein kinases are regulated by different mechanisms in Xenopus egg extracts. EMBO Journal **11**: 1751-1761.

Conway, R. M., Madigan, M. C., Penfold, P. L. and Billson, F. A. (1995). Induction of apoptosis by sodium butyrate in the human Y-79 retinoblastoma cell line. Oncology Research **7**: 289-297.

Cuisset, L., Tichonicky, L., Jaffray, P. and Delpech, M. (1997). The effects of sodium butyrate on transcription are mediated through activation of a protein phosphatase. Journal of Biological Chemistry **272**: 24148-24153.

Cummings, J. H. (1981). Short chain fatty acids in the human colon. Gut **22**: 763-779.

Dangond, F. and S. R. Gullans (1998). Differential expression of human deacetylase mRNAs in response to immune cell apoptosis induction by Trichostatin A and Butyrate. Biochemical and Biophysical Research Communications **247**: 833-837.

Davie, J. R. and M. J. Hendzel (1994). Multiple functions of dynamic histone acetylation. Journal of Cellular Biochemistry **55**: 98-105.

DePinho, R. A. (1998). The cancer-chromatin connection. Nature **391**: 533-536.

Estanyol, J.M., Jaumot, M., Casanovas, O., Rodriguez-Vilarrupla, A., Agell, N. and Bachs, O. (1999). The protein SET regulates the inhibitory effect of $p21^{Cip1}$ on cyclin E cyclin dependent kinase 2 activity. Journal of Biological Chemistry **274**: 33161-33165.

Ezhevsky, S. A., Nagahara, H., Vocero-Akbani, A. M., Gius, D. R., Wei, M. C. and Dowdy, S. F. (1997). Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclinD:cdk4/6 complexes results in active pRb. Proceedings of the National Academy of Science **94**: 10699-10704.

Faller, D. V. and S. P. Perrine (1995). Butyrate in the treatment of sickle cell disease and beta-thalassemia. Current Opinions in Hematology **2**: 109-117.

Fallon, R. J. and R. P. Cox (1979). Cell cycle analysis of sodium butyrate and hydroxyurea, inducers of ectopic hormone production in HeLa cells. Journal of Cell Physiology **100**: 251-262.

Fattaey, A. and Booher, R.N. (1997). Myt1: a Wee1-type kinase that phosphorylates cdc2 on residue Thr14. Progress in Cell Cycle Research **3**: 233-240.

Fesquet, D., Labbe, J. C., Derancourt, J., Capony, J. P., Galas, S., Lorca, T., Shuttleworth, J., Doree, M. and Cavadore J.C. (1993). The M015 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and its homologues. EMBO Journal **12**: 3111-3121.

Gartel, A. L., Serfas, M. S. and Tyner, A. L. (1996). P21-negative regulator of the cell cycle. Proceedings of the Society for Experimental Biology and Medicine **213**: 138-149.

Geng, Y., Whoriskey, W., Park, M. Y., Bronson, R. T., Medema, R. H., Li, T., Weinberg, R. A. and Sicinski, P. (1999). Rescue of cyclin D1 deficiency by knockin cyclin E. Cell **97**: 767-777.

Gilbert, K. M. and W. O. Weigle (1993). Th1 cell anergy and blockade in G1a phase of the cell cycle. Journal of Immunology **151**: 1245-1254.

Gomez-Lahoz, E., Liegeois, N. J., Zhang, P., Engelman, J. A., Horner, J., Silverman, A., Burde, R., Roussel, M. F., Sherr, C. J., Elledge, S. J. and DePinho, R. A. (1999). Cyclin D- and E-dependent kinases and the p57(Kip2)inhibitor: cooperative interactions in vivo. Molecular and Cellular Biology **19**: 353-363.

Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription. Nature **389**: 349-352.

Gu, Y., Rosenblatt, J. and Morgan, D. O. (1992). Cell cycle regulation of cdk2 activity by phosphorylation of Thr160 and Tyr15. EMBO Journal **11**: 3995-4005.

Gupta, S., Alpini, G., Vemuru, R. P., Hurston, E. and Shafritz, D. A. (1994). Butyrate synchronization of hepatocytes: modulation of cycling and cell cycle regulated gene expression. Growth Factors **10**: 171-180.

Hagting, A., Jackman, M., Simpson, K. and Pines, J. (1999). Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. Current Biology **9**: 680-689.

Hall M., B., S. and Peters, G. (1995). Evidence for different modes of action of cyclin-dependent kinase inhibitors: p15 and p16 bind to kinases, p21 and p27 bind to cyclins. Oncogene **11**(1581-1588).

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. and Elledge, S. J. (1993). The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75**: 805-816.

Harper, J. W. and S. J. Elledge (1996). Cdk inhibitors in development and cancer. Current opinion in genetics and development. **6**: 56-64.

Hassig, C. A., Tong, J. K. and Schreiber, S. L. (1997). Fiber-derived butyrate and the prevention of colon cancer. Chemistry & Biology **4**: 783-789.

Heerdt, B. G., Houston, M. A. and Augenlicht (1997). Short-chain fatty acid-initiated cell cycle arrest and apoptosis of colonic epithelial cells is linked to mitochondrial function. Cell Growth and Differentiation **8**: 523-532.

Heichman, K. A. and J. M. Roberts (1994). Rules to replicate by. Cell **79**: 557-562.

Hengst, L., Gopfert, U., Lashuel, H. A. and Reed S. I. (1998). Complete inhibition of cdk/cyclin by one molecule of $p21^{Cip1}$. Genes & Development **12**: 3882-3888.

Hengstschlager, M., Braun, K., Soucek, T., Miloloza, A. and Hengstschlager-Ottnad, E. (1999). Cyclin-dependent kinases at the G1-S transition of the mammalian cell cycle. Mutation Research **436**: 1-9.

Hiyama, H., Iavarone, A., LaBaer, J., and Reeves, S. A. (1997). Regulated ectopic expression of cyclin D1 induces transcriptional activation of the cdk inhibitor p21 gene without altering cell cycle progression. Oncogene **14**: 2533-2542.

Hiyama, H., Iavarone, A. and Reeves, S. A. (1998). Regulation of the cdk inhibitor p21 gene during cell cycle progression is under the control of the transcription factor E2F. Oncogene **16**: 1513-1523.

Ho, S. B., Yan, P. S., Dahiya, R., Neuschwander-Tetri, B. A., Basbaum, C. and Kim Y. S. (1994). Stable differentiation of a colon adenocarcinoma cell line by sodium butyrate is associated with mutidrug resistance. Journal of Cell Physiology **160**: 213-226.

Howe, G. R., Benito, E., Castelleto, R., Cornee, J., Esteve, J., Gallagher, R. P., Iscovich, J. M., Deng-ao, J., Kaaks, R. and Kune, G. A. (1992). Dietary intake of fiber and decreased risk of cancers of the colon and rectum: evidence from the combined analysis of 13 case-control studies. Journal of the National Cancer Institute **84**: 1887-1896.

Hunter, T. and J. Pines (1994). Cyclins and cancer II: cyclin D and cdk inhibitors come of age. Cell **79**: 573-582.

Jackson, P. K., Chevalier, S., Philippe, M. and Kirschner, M. W. (1995). Early events in DNA replication require cyclin E and are blocked by $p21^{Cip1}$. Journal of Cell Biology **130**: 755-769.

Jansen, M. C., Bueno-de-Mesquita, H. B., Buzina, R., Fidanza, F., Menotti, A., Blackburn, H., Nissinen, A. M., Kok, F. J. and Kromhout, D. (1999). Dietary fiber and plant foods in relation to colorectal cancer mortality: the seven countries study. International Journal of Cancer **81**: 174-179.

Janson, W., Brandner, G. and Siegel, J. (1997). Butyrate modulates DNA-damage-induced p53 response by induction of p53-independent diffeentiation and apoptosis. Oncogene **15**: 1395-1406.

Joensuu, T. and J. Mester (1984). Inhibition of cell cycle progression by sodium butyrate in normal rat kidney fibroblasts is altered by expression of the adenovirus 5 early 1A gene. Bioscience Reports **14**: 291-300.

King, R. W., Jackson, P. K. and Kirschner, W. (1994). Mitosis in transition. Cell **79**: 563-571.

Knoblich, J. A. and C. F. Lehner (1993). Synergistic action of Drosophila cyclins A and B during G2-M transition. EMBO Journal **12**: 65-74.

Krek, W. and E. A. Nigg (1991). Dirrerential phosphorylation of vertebrate p34cdc2 kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites. EMBO Journal **10**: 305-316.

Kruh, J. (1982). Effects of sodium butyrate, a new pharmacological agent, on cells in culture. Molecular and Cellular Biochemistry **42**: 65-82.

LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A. and Harlow, E. (1997). New functional activities for the p21 family of cdk inhibitors. Genes & Development **11**: 847-862.

Lallemand, F., Courilleau, D., Sabbah, M., Redeuilh, G. and Mester, J. (1996). Direct inhibition of the expression of cyclin D gene by sodium butyrate. Biochemical and Biophysical Research Communications **229**: 163-169.

Lallemand, F., Courilleau, D., Buquet-Fagot, C., Atfi, A., Montagne, M.-N. and Mester, J. (1999). Sodium butyrate induces G2 arrest in the human breast cancer cells MDA-MB-231 and renders them competent for DNA replication. Experimental Cell Research **247**: 432-440.

Lauper, N., Beck, A. R. P., Cariou, S., Richman, L., Hofmann, K., Reith, W., Slingerland, J. M. and Amati, B. (1998). Cyclin E: a novel cdk2 partner in the late G1 and S phases of the mammalian cell cycle. Oncogene **17**: 2637-2643.

Lee, T.-H., Chuang, L.-Y. and Hung, W.-C. (1999). Tamoxifen induces $p21^{Waf1}$ and $p27^{Kip1}$ expression in estrogen receptor-negative lung cancer cells. Oncogene **18**: 4269-4274.

Lehner, C. F. and P. H. O'Farrell (1990). The roles of cyclins A and B in mitotic control. Cell **61**: 535-547.

Lorca, T., Labbe, J. C., Devault, A., Fesquet, D., Capony, J. P., Cavadore, J. C., Le Bouffant, F. and Doree, M. (1992). Dephosphorylation of cdc2 on threonine 161 is required forcdc2 kinase inactivation and normal anaphase. EMBO Journal **11**: 2381-2390.

Ludlow, J. W., Glendening, C. L., Livingston, D. M. and DeCarprio, J. A. (1993). Specific enzymatic dephosphorylation of the retinoblastoma protein. Molecular and Cellular Biology **13**: 367-372.

Lundberg, A. S. and R. A. Weinberg (1998). Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Molecular and Cellular Biology **18**: 753-761.

Macleod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B. and Jacks, T. (1995). P53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes & Development **9**: 935-944.

Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J. P., Troalen, F., Trouche, D. and Harel-Bellan, A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature **391**: 601-604.

McBain, J. A., Eastman, A, Nobel, C. S. and Mueller, G. C. (1997). Apoptotic death in adenocarcinoma cell lines induced by butyrate and other histone deacetylae inhibitors. Biochemical Pharmacology **53**: 1357-1368.

McIntyre, A., Young, G. P., Taranto, T., Gibson, P. R. and Ward, P.B. (1991). Different fibers have different regional effects on luminal contents of rat colon. Gastroenterology **101**: 1274-1281.

Medema, R. H., Klompmaker, R., Smits, A. J. and Rijksen, G. (1998). $P21^{Waf1}$ can block cells at two points in the cell cycle, but does not interfere with processive DNA-replication or stress-activated kinases. Oncogene **16**: 431-441.

Medina, V., Edmonds, B., Young, G. P., James, R., Appleton, S. and Zalewski, P. D. (1997). Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with mitochindrial/cytochrome c-dependent pathway. Cancer Research **57**: 3697-3707.

Meijer, L. (1995). Chemical inibitors of cyclin-dependent kinases. Progress in Cell Cycle Research **1**: 351-363.

Milsted, A., Silver, B. J., Cox, R. P. and Nilson, J. H. (1985). Coordinate regulation of the messenger ribonucleic acids encoding alpha- and beta-subunits of human chorionic gonadotropin in HeLa cells and butyrate-resistant variants. Endocrinology **117**: 2033-2039.

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). Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. Journal of Biological Chemistry **272**: 2219-22206.

Novogrodsky, A., Dvir, A., Ravid, A., Shkolnik, T., Stenzel, K. H., Rubin, A. L. and Zaizov, R. (1983). Effect of polar organic compounds on leukemic cells. Butyrate-induced partial remission of acute myelogenous leukemia in a child. Cancer **51**: 9-14.

Nurse, P. (1994). Ordering S phase and M phase in the cell cycle. Cell **79**: 547-550.

Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M. and Pagano, M. (1995). Human cyclin E, nuclear protein essential for the G1-to-S phase transition. Molecular and Cellular Biology **15**: 2612-2624.

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W. and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. EMBO Journal **11**: 961-971.

Pagano, M., Ed. (1998). Cell cycle control. Results and problems in cell differentiation. New York, NY, Springer-Verlag.

Petersen, B. O., Lukas, J., Sorensen, C. S., Bartek, J. and Helin, K. (1999). Phosphorylation of mammmalian cdc6 by cyclin A/cdk2 regulates its cellular localization. EMBO Journal **18**: 396-410.

Petersen, B. O., Lukas, J., Sorensen, C. S., Bartek,J. and Helin, K. (1999). Phosphorylation of mammalian cdc6 by cyclin A/cdk2 regulates its subcellular localization. EMBO Journal **18**: 396-410.

Pines, J. and T. Hunter (1991). Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. Journal of Cell Biology **115**: 1-17.

Poon, R. Y., Toyoshima, H. and Hunter, T. (1995). Redistribution of the cdk inhibitor p27 between different cyclin.cdk complexes in the mouse fibroblast cell cycle and in cells arrested with lovastatin or ultraviolet irradiation. Molecular Biology of the Cell **6**: 1197-1213.

Poon, R. Y. C. and T. Hunter (1998). Expression of a novel form of $p21^{Cip1/Waf1}$ in UV-irradiated and transformed cells. Oncogene **16**: 1333-1343.

Rao, S., Lowe, M., Herliczek, T.W. and Keyomarsi, K. (1998). Lovastatin mediated G1 arrest in normal and tumor breast cells is through inhibition of cdk2 activity and redistribution of p21 and p27, independent of p53. Oncogene **17**: 2392-2402.

Reeves, R. and P. Cserjesi (1979). Sodium butyrate induces new gene expression in Friend erythroleukemic cells. Journal of Biological Chemistry **254**: 4283-4290.

Rousseau, D., Cannella, D., Boulaire, J., Fitzgerald, P., Fotedar, A. and Fotedar, R. (1999). Growth inhibition by cdk-cyclin and PCNA binding domains of p21 occurs by distinct mechanisms and is regulated by ubiquitin-proteasome pathway. Oncogene **18**: 4313-4325.

Russell, A., Thompson, M. A., Hendley, J., Trute, L., Armes, J. and Germain, D. (1999). Cyclin D1 and D3 associat with the SCF complex and are coordinately elevated in breast cancer. Oncogene **18**: 1983-1991.

Russo, G. L., Della Pietra, V., Mercurio, C., Della Ragione, F., Marshak, D. R., Oliva, A. and Zappia, V. (1997). Down-regulation of protein kinase CKII activity by sodium butyrate. Biochemical and Biophysical Research Communications **233**: 673-677.

Saegusa, M., Nitta, H., Hashimura, M. and Okayasu (1999). Down-regulation of p27Kip1 expression is correlated with increased cell proliferation but not expression of p21wafl and p53, and human papillomavirus infection in benign and malignant tumours of sinonasal regions. Histopathology **35**: 55-64.

Sealy, L. and C. R. (1978). The effect of sodium butyrate on histone modifiction. Cell **14**: 115-121.

Sherr, J. S. (1994). G1 phase progression: cycling on cue. Cell **79**: 551-555.

Sherr, C. J. and J. M. Roberts (1999). Cdk inhibitors: positive and negative regulators of G1-phase progression. Genes & Development **13**: 1501-1512.

Sherwood, S. W., Rush, D. F., Kung, A. L. and Schimke R. T. (1994). Cyclin B1 expression in HeLa S3 cells studied by flow cytometry. Experimental Cell Research **211**: 275-281.

Soballe, P. W. and M. Herlyn (1994). Cellular pathways leading to melanoma defferentiation: therapeutic implictions. Melanoma Research **44**: 213-223.

Solomon, M.J., Harper, J.W. and Shuttleworth, J. (1993) CAK, the p34cdc2 activating kinase, contains a protein identical or closely to p40MO15. EMBO Journal **12**: 3133-3142.

Stenzel, K. H., Schwartz, R., Rubin, A. L. and Novogrodsky, A. (1980). Chemical inducers of differentiation in Friend leukaemia cells inhibit lymphocyte mitogenesis. Nature **285**: 106-108.

Sugarman, J. L., Schonthal, A. H. and Glass, C. K. (1995). Identification of a cell-type-specific and E2F-independent mechanism for repression of *cdc2* transcription. Molecular and Cellular Biology **15**: 3282-3290.

Taules, M., Rius, E., Talaya, D., Lopez-Girona, A., Bachs, O., Agell, N. (1998). Calmodulin is essential for cyclin-dependent kinase 4 (cdk4) activity and nuclear accumulation of cyclin D1-cdk4 during G1. Journal of Biological Chemistry **273**: 33279-33286.

Taules, M., Rodriguez-Vilarrupla, A., Rius, E., Estanyol, J. M., Casanovas, O., Sacks, D. B., Perez-Paya, E., Bachs, O. and Agell, N. (1999). Calmodulin binds to $p21^{Cip1}$ and is involved in the regulation of its nuclear localization. Journal of Biological Chemistry **274**: 24445-24448.

Thomas, G. V., Szigeti, K., Murphy, M., Draetta, G., Pagano, M. and Loda, M. (1998). Down-regulation of p27 is associated with development if colorectal adenocarcinoma matastases. American Journal of Pathology **153**: 681-687.

Topley, G. I., Okuyama, R., Gonzales, J. G., Conti, C. and Dotto (1999). p21(WAF1/Cip1) fuctions as a suppressor of malignant skin tumor formation and a determinant of keratinocyte stem-cell potential. Proceedings of the National Academy of Sciences **1999**: 9089-9094.

Tortola, S., Marcuello, E., Risques, R.-A., Gonzalez, S., Aiza, G., Capella, G. and Peinado, M. A. (1999). Overall deregulation in gene expression as a novel indicator of tumor aggressiveness in colorectal cancer. Oncogene **18**: 4383-4387.

Toscani, A., Soprano, D. R. and Soprano, K. J. (1988). Molecular analysis of sodium butyrate-induced growth arrest. Oncogene Research **3**: 223-238.

Vaziri, C., Stice, L. and Faller, D. V. (1998). Butyrate-induced G1 arrest results from p21-independent disruption of retinoblastoma protein-mediated signals. Cell Growth and Differentiation **9**: 465-474.

Wang, W., Furneaux, H., Cheng, H., Caldwell, M.C., Hutter, D., Liu, Y., Holbrook, N. and Gorospe, M. (2000). HuR regulates p21 mRNA stabilization by UV light. Molecular and Cellular Biology **20**: 760-769.

Warrell, R. P. J., de The, H., Wang, Z. Y. and Degos, L. (1993). Acute promyelocytic leukemia. New England Journal of Medicine **329**: 177-189.

Warrell, R. P. J., He, L.-Z., Richon, V., Calleja, E. and Pandolfi, P. P. (1998). Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. Journal of the National Cancer Institute **90**: 1621-1625.

Welcker, M., Lukas, J., Srauss, M. and Bartek, J. (1998). $P21^{Waf1/Cip1}$ mutants deficient in inhibiting cyclin-dependent kinases (cdks) can promote assembly of active cyclin D/cdk4(6) complexes in human tumor cells. Cancer Research **58**: 5053-5056.

Wu, C. (1997). Chromatin remodeling and the control of gene expression. Journal of Biological Chemistry **272**: 28171-28174.

Xiao, H., Hasegawa, T., Miyaishi, O., Ohkusu, K. and Isobe, K. (1997). Sodium butyrate induces HIH3T3 cells to senescence-like state and enhances promoter activity of $p21^{Waf1/Cip1}$ in p53-independent manner. Biochemical and Biophysical Research Communications **237**: 457-460.

Xiong, Y., Zhang, H. and Beach, D. (1992). D type cyclins associate with multiple protein kinases and the DNA repliction and repair factor PCNA. Cell **71**: 505-514.

Yang, R., Muller, C., Huynh, V., Fung, Y. K., Yee, A. S. and Koeffler, H. P. (1999). Functions of cyclin A1 in the cell cycle and its interactions with transcription factor E2F and the Rb family of proteins. Molecular and Cellular Biology **19**: 2400-2407.

Yen, A. and S. Varvayanis (1995). Rb phosphorylation in sodium butyrate-resistant HL-60 cells: cross-resistance to retinoic acid but not vitamin D3. Journal of Cellular Physiology **163**: 502-509.

Yen, A. and R. Sturgill (1998). Hypophosphorylation of the RB protein in S and G2 as well as G1 during growth arrest. Experimental Cell Research **241**: 324-331.

Figure 1: Cyclin/cdk activity throughout the cell cycle.

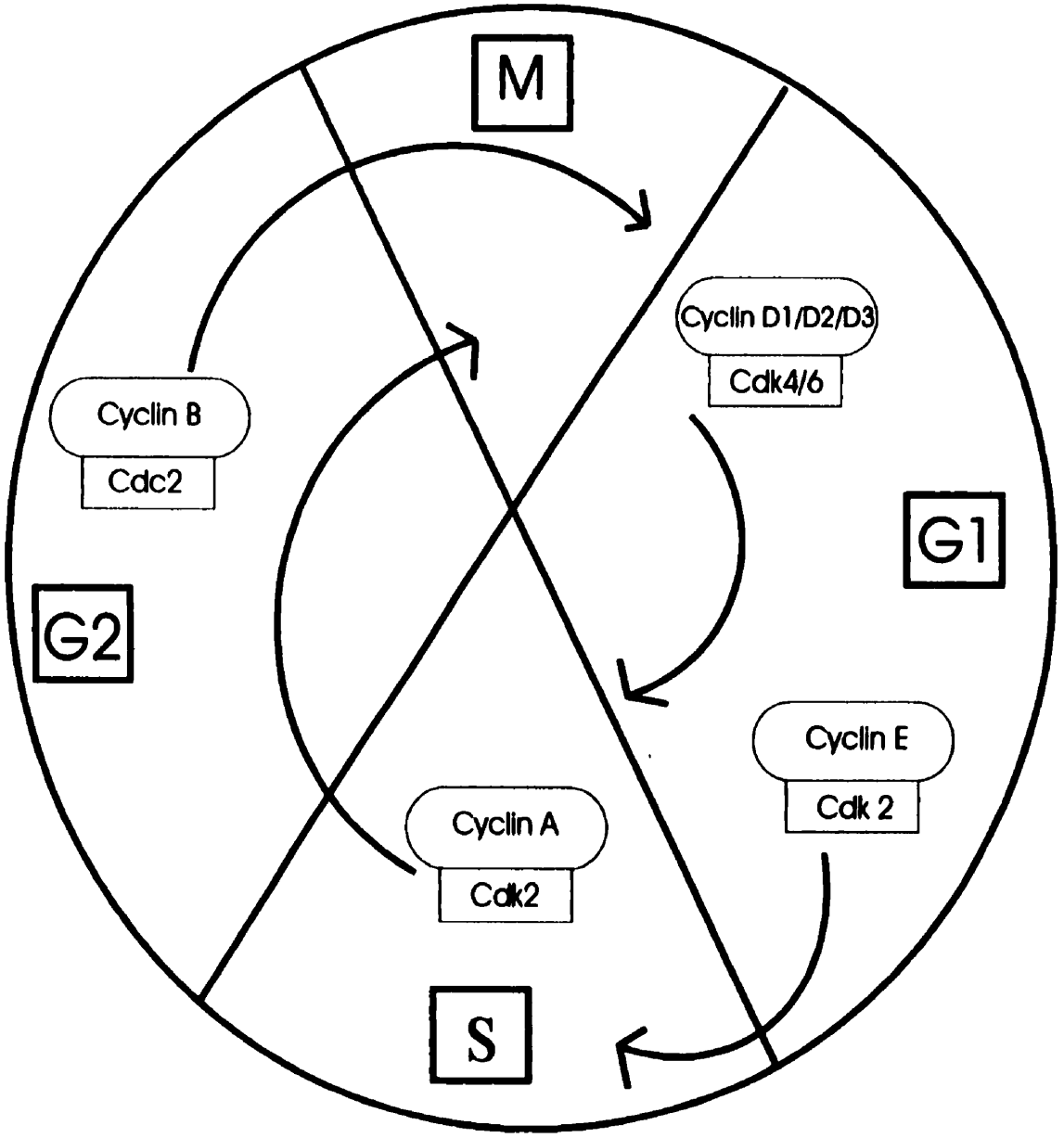
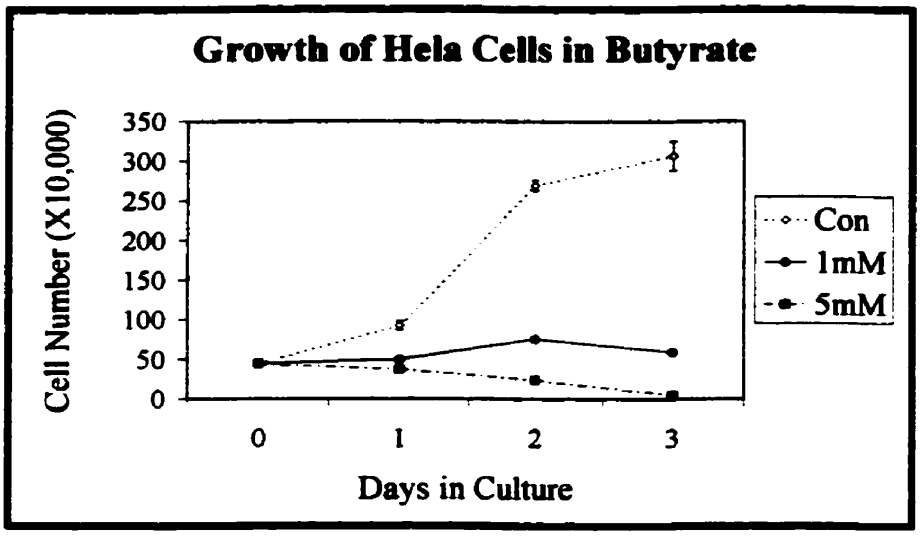
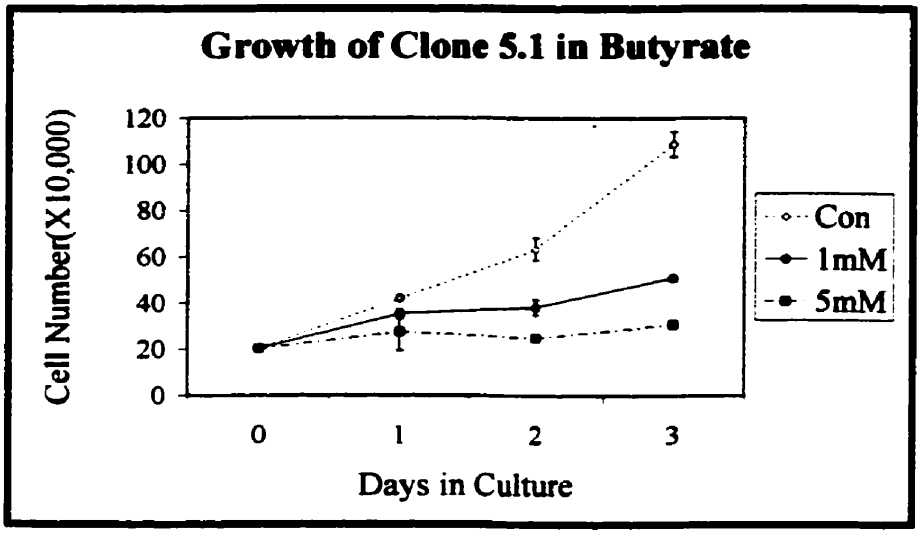

M
Cyclin D1/D2/D3
Cdk4/6
Cyclin B
Cdc2
G1
G2
Cyclin E
Cdk 2
Cyclin A
Cdk2
S

Figure 2: Growth curves. HeLa cells and the two butyrate-resistant clones were incubated in the presence and absence of butyrate. The HeLa and 5.1 clone growths were monitored for three days, whereas the 7.5 clone was monitored for six days because of its slow doubling time. HeLa growth was dramatically reduced in 1mM butyrate and all cells died after three days in 5mM butyrate. In contrast, the 5.1 clone and 7.5 clone continued to grow in butyrate, but at a slower rate.


Growth of Hela Cells in Butyrate
Cell Number (X10,000)
350
300
250
200
150
100
50
0
0
1
2
3
Days in Culture
Con
1mM
5mM


Growth of Clone 5.1 in Butyrate
Cell Number(X10,000)
120
100
80
60
40
20
0
0
1
2
3
Days in Culture
Con
1mM
5mM

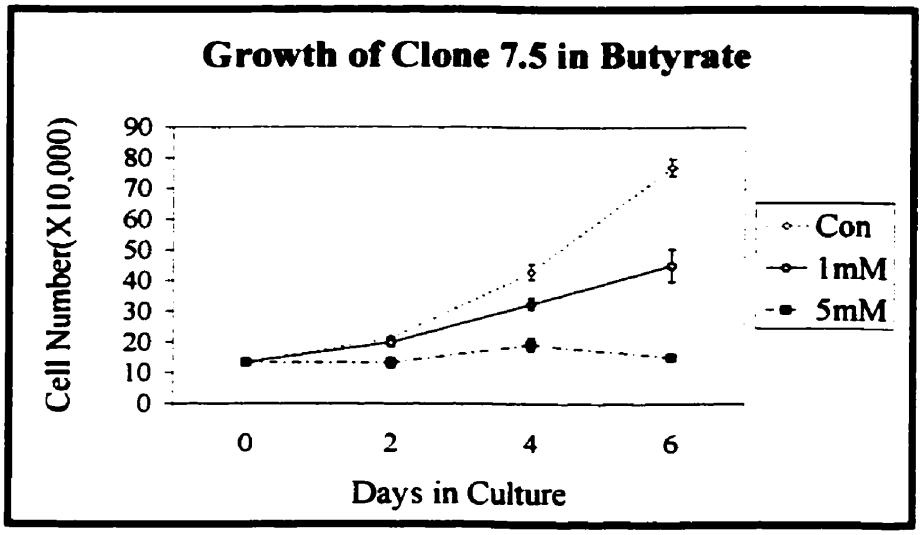

Growth of Clone 7.5 in Butyrate
Cell Number(X10,000)
90
80
70
60
50
40
30
20
10
0
0
2
4
6
Days in Culture
Con
1mM
5mM

Figure 3: P21$^{Waf1/Cip1}$ expression (courtesy of Dr. P.S. Wright). Relative levels of p21$^{Waf1/Cip1}$ mRNA levels were quantitated by RT-PCR with actin levels as an internal standard. Results were normalized against the actin, and plotted using HeLa control levels as a reference. As expected, levels increase in the HeLa cells in the presence of butyrate. Surprisingly the 5.1 and 7.5 clones also express high levels, up to 8-fold, of p21$^{Waf1/Cip1}$ in the absence of butyrate.

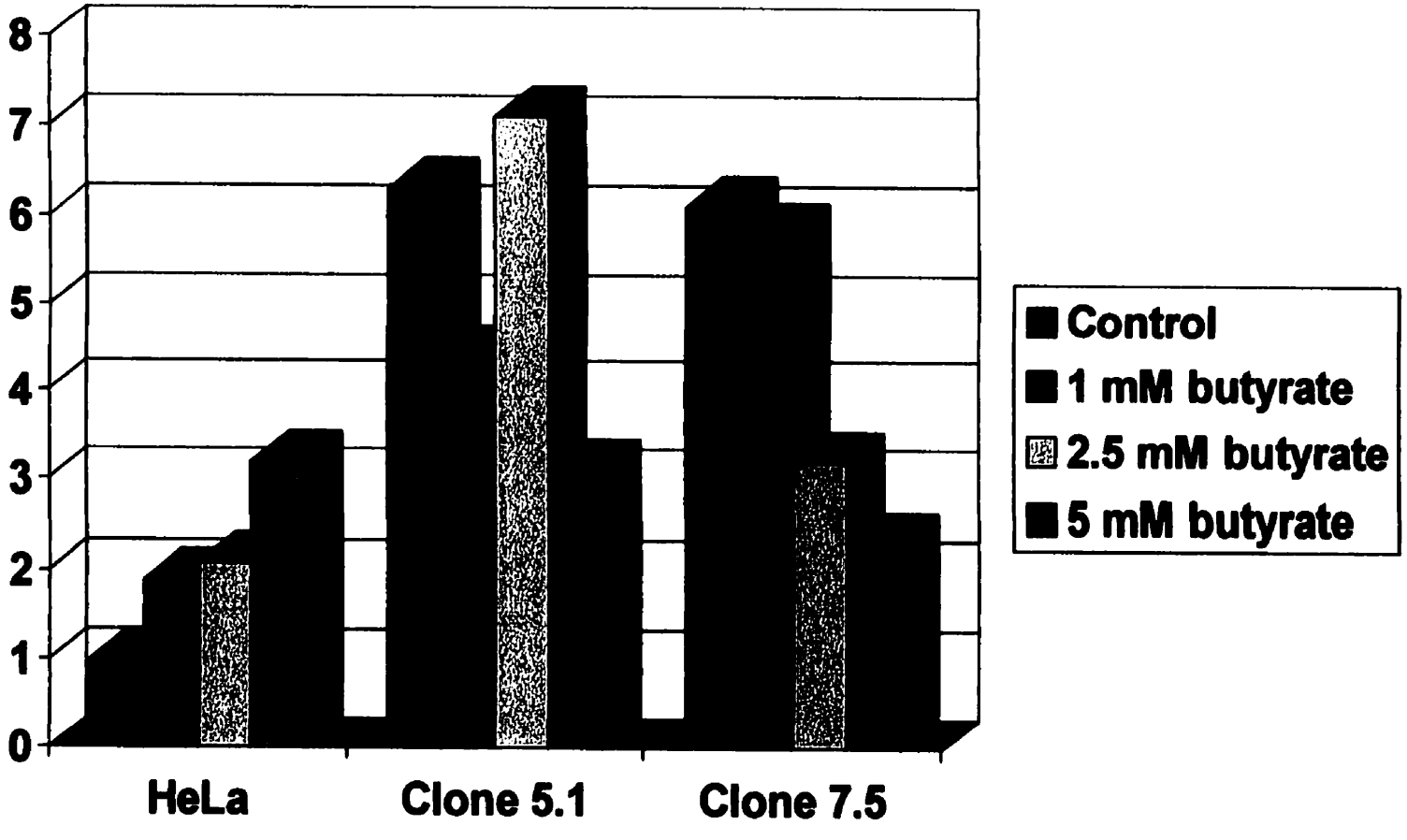

8
7
6
5
4
3
2
1
0
HeLa
Clone 5.1
Clone 7.5
Control
1 mM butyrate
2.5 mM butyrate
5 mM butyrate

Figure 4: P21$^{Waf1/Cip1}$ protein levels. Immunoblot of p21$^{Waf1/Cip1}$ protein determine its expression in HeLa and the 5.1 and 7.5 clones, with or without butyrate in overnight incubations.

P21
HeLa
5.1
7.5
Control
1 mM Butyrate
5 mM Butyrate
Control
1 mM Butyrate
5 mM Butyrate
Control
1 mM Butyrate
5 mM Butyrate

Figure 5: Cytofluorometric growth profiles (courtesy of Dr. H. Crissman). HeLa cells were incubated with 5 mM butyrate for 18 h, and fixed for flow cytometry. Cytofluorometric profiles of Clones 5.1 and 7.5 were similarly prepared.

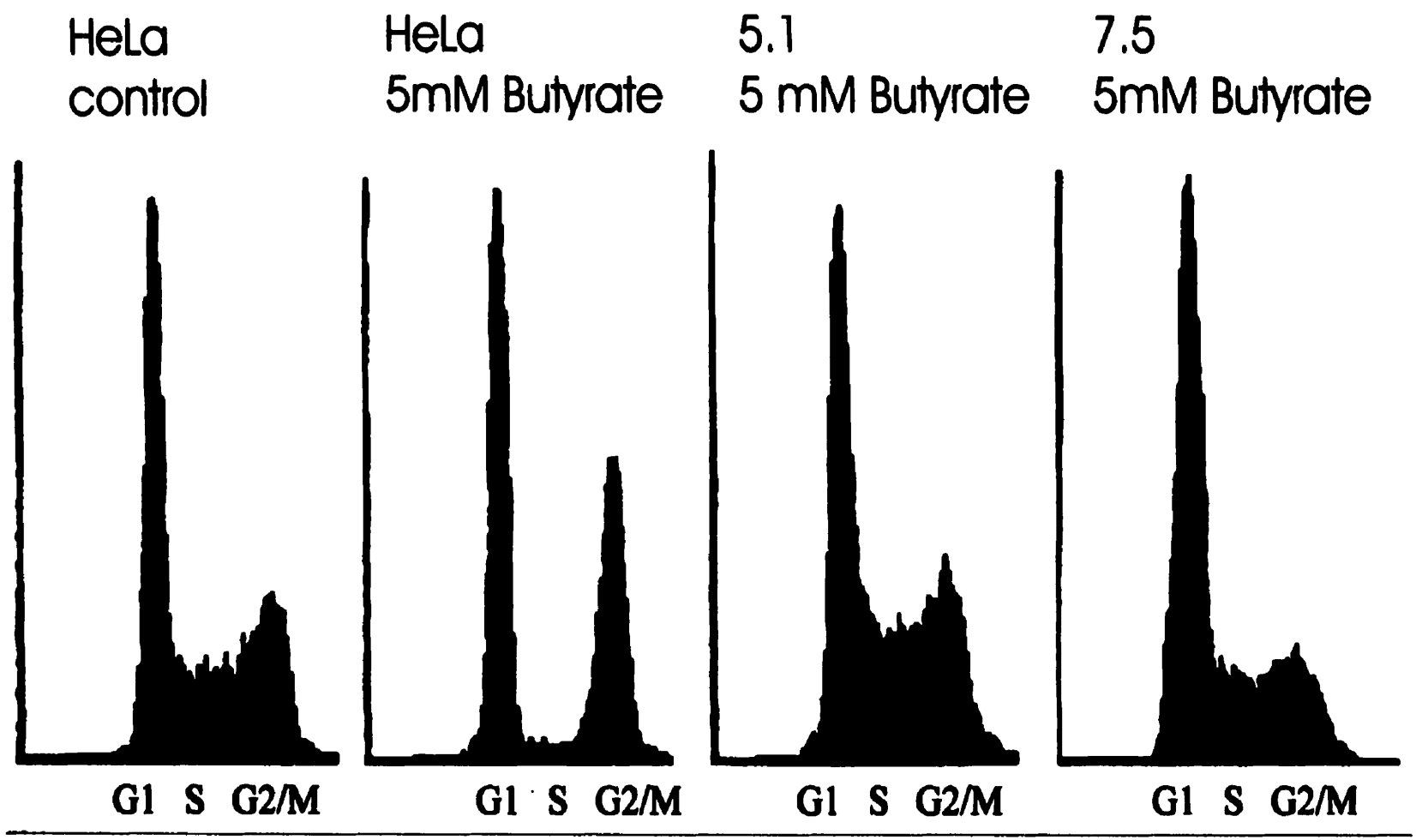

HeLa
control
HeLa
5mM Butyrate
5.1
5 mM Butyrate
7.5
5mM Butyrate
G1 S G2/M
G1 S G2/M
G1 S G2/M
G1 S G2/M

Figure 6: Nuclear and cytoplasmic levels of P21$^{Waf1/Cip1}$ protein levels. Immunoblot of p21$^{Waf1/Cip1}$ protein to compare distribution between nuclear (panel 1) and cytoplasmic (panel 2) fractions. HeLa cells and 5.1 and 7.5 clones were incubated overnight with and without 5mM butyrate.

HeLa
5.1
7.5
Control
5 mM Butyrate
Control
5 mM Butyrate
Control
5 mM Butyrate
1
2

Figure 7: Cyclin protein levels. Immunoblots of various cyclins in whole cell extracts from HeLa cells and, 5.1 and 7.5 clones incubated overnight with and without butyrate.

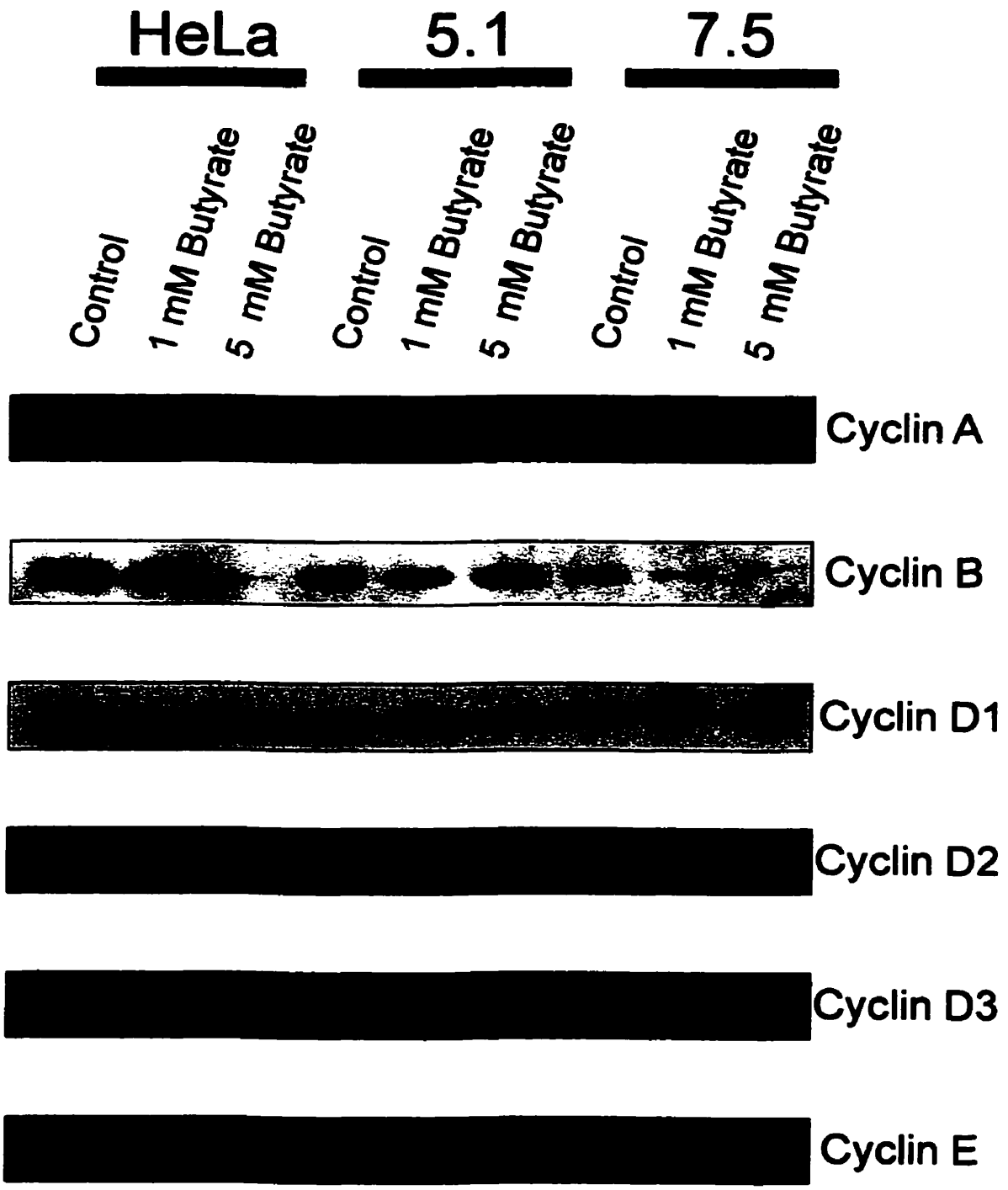

HeLa
5.1
7.5
Control
1 mM Butyrate
5 mM Butyrate
Control
1 mM Butyrate
5 mM Butyrate
Control
1 mM Butyrate
5 mM Butyrate
Cyclin A
Cyclin B
Cyclin D1
Cyclin D2
Cyclin D3
Cyclin E

Figure 8: Levels of cell cycle regulator proteins. Immunoblots of cell cycle proteins in whole cell protein extracts from HeLa cells and, 5.1 and 7.5 clones incubated overnight with and without butyrate

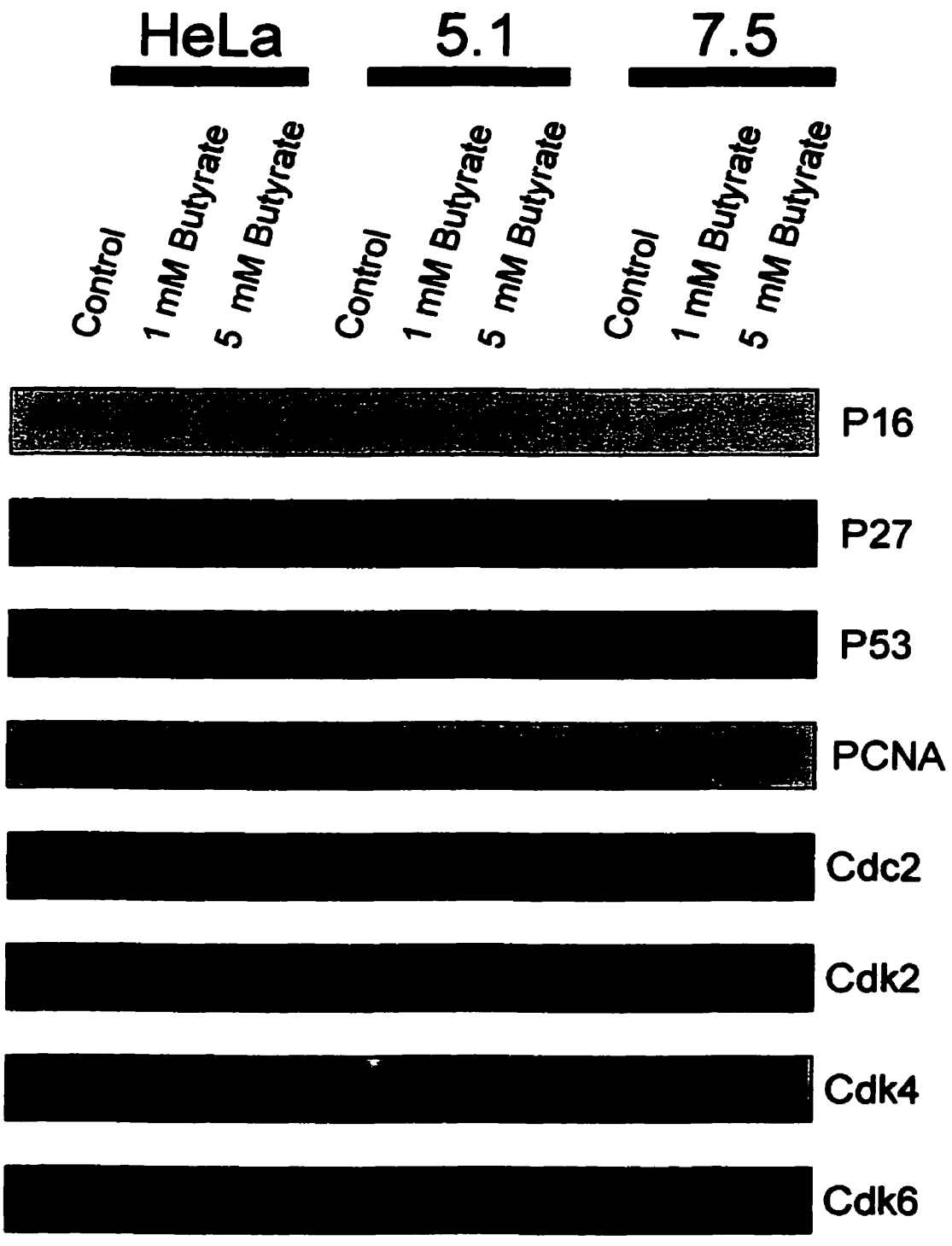

HeLa
5.1
7.5
Control
1 mM Butyrate
5 mM Butyrate
Control
1 mM Butyrate
5 mM Butyrate
Control
1 mM Butyrate
5 mM Butyrate
P16
P27
P53
PCNA
Cdc2
Cdk2
Cdk4
Cdk6

Figure 9: Kinase assay of cdc2. Cdc2 was immunoprecipitated and used in a kinase assay with histone H1 (panel 1) as the substrate. Film was exposed to determine the phosphorylation of H1 (panel 2). Immunoblot of cdc2 (panel 3) was done to confirm the presence of cdc2 in the immunoprecipitation.

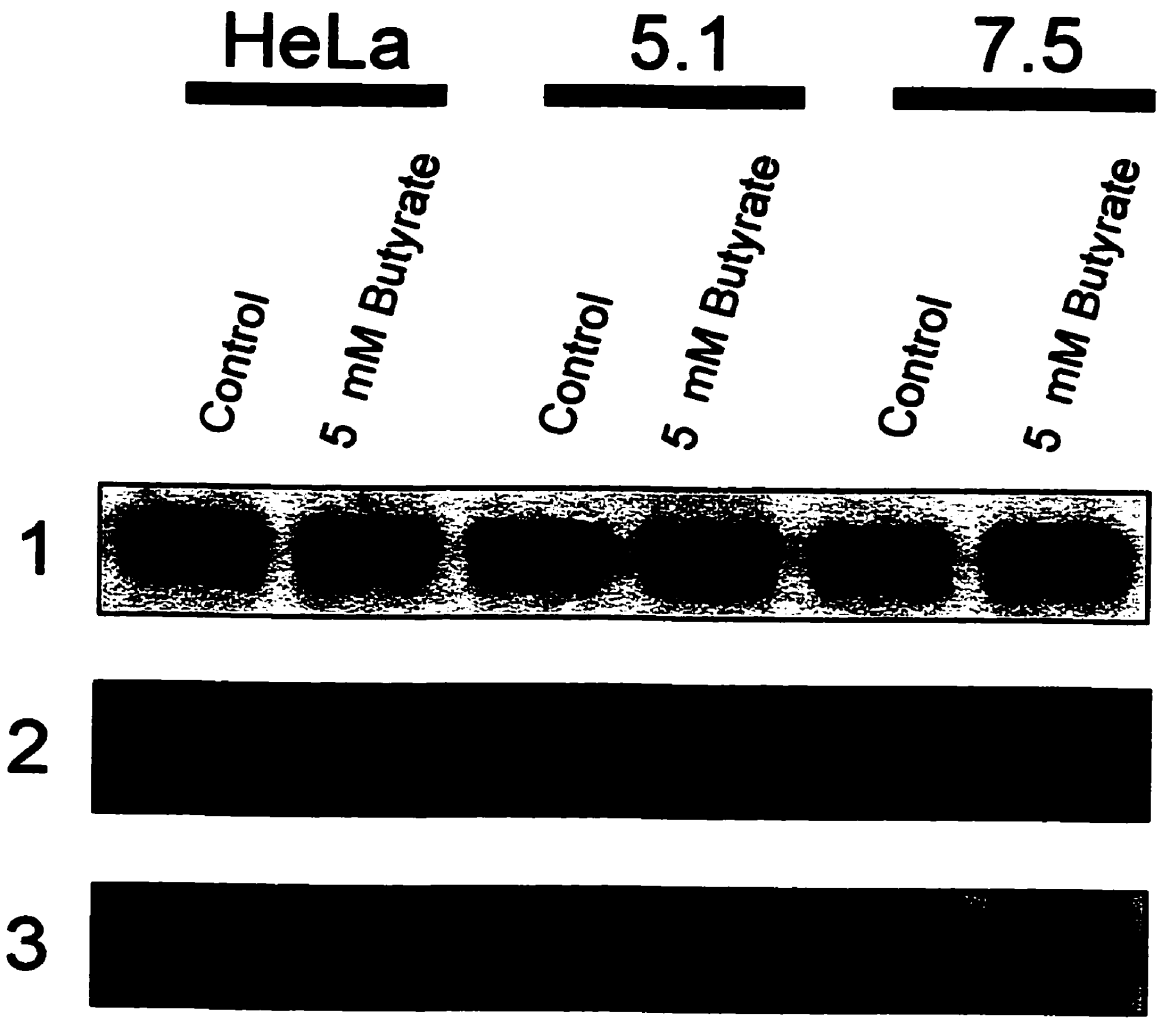

HeLa
5.1
7.5
Control
5 mM Butyrate
Control
5 mM Butyrate
Control
5 mM Butyrate
1
2
3

Figure 10: Kinase assay of cdk4. Cdk4 was immunoprecipitated and used in a kinase assay with histone H1 (panel 1) as the substrate. Film was exposed to determine the phosphorylation of H1 (panel 2). Immunoblot of cdk4 (panel 3) was done to confirm the presence of cdk4 in the immunoprecipitation.

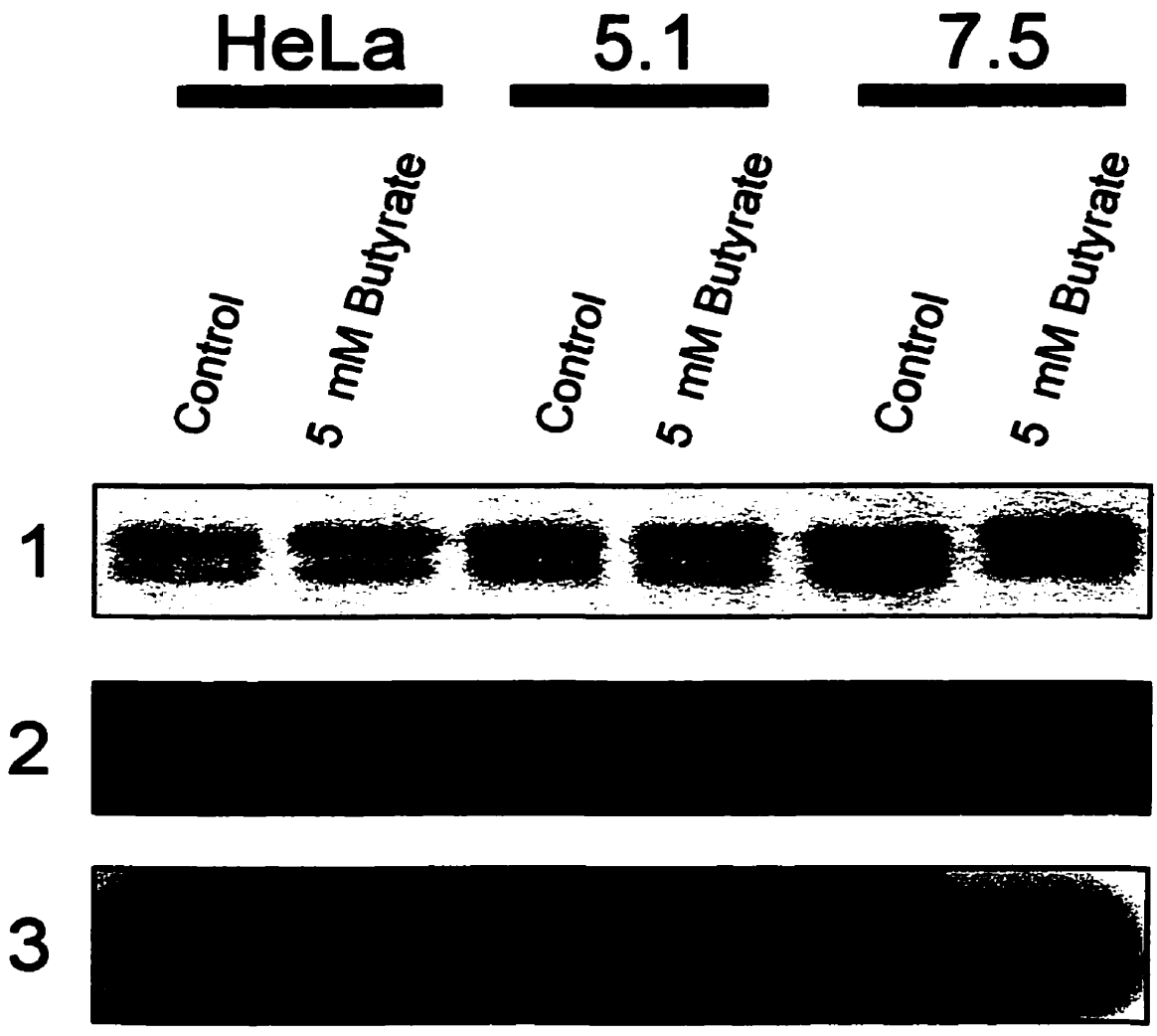

HeLa
5.1
7.5
Control
5 mM Butyrate
Control
5 mM Butyrate
Control
5 mM Butyrate
1
2
3

Figure 11: Kinase assay of cdk6. Cdk6 was immunoprecipitated and used in a kinase assay with histone H1 (panel 1) as the substrate. Film was exposed to measure the phosphorylation of H1 (panel 2). Immunoblot of cdk6 (panel 3) was done to confirm the presence of cdk6 in the immunoprecipitation.

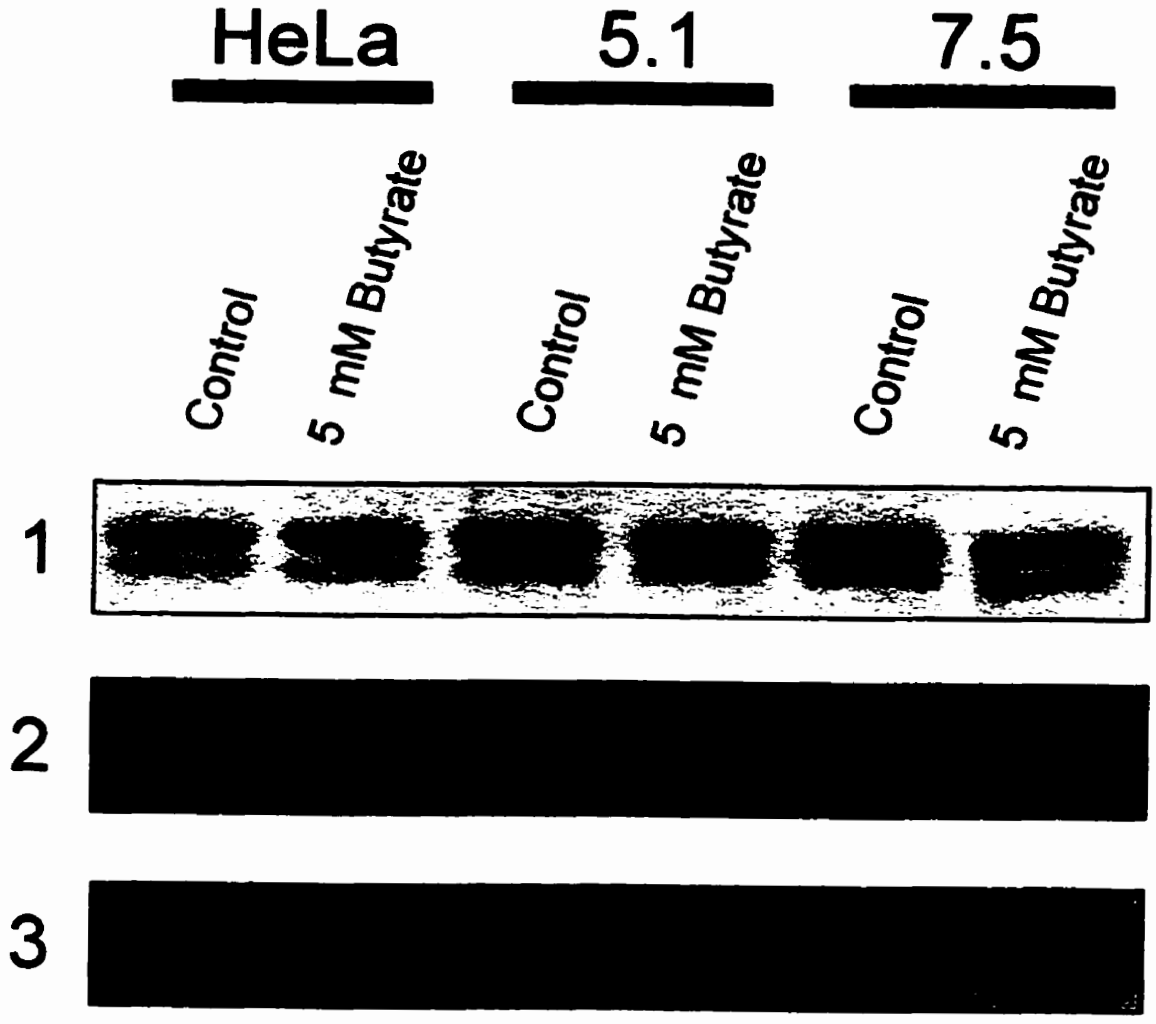

HeLa
5.1
7.5
Control
5 mM Butyrate
Control
5 mM Butyrate
Control
5 mM Butyrate
1
2
3

Figure 12: Kinase assay of cdk2. Cdk2 was immunoprecipitated and used in a kinase assay with histone H1 (panel 1) as the substrate. Film was exposed overnight to show phosphorylation of H1 (panel 2). Immunoblot of cdk2 (panel 3) was done to confirm the presence of cdk2.

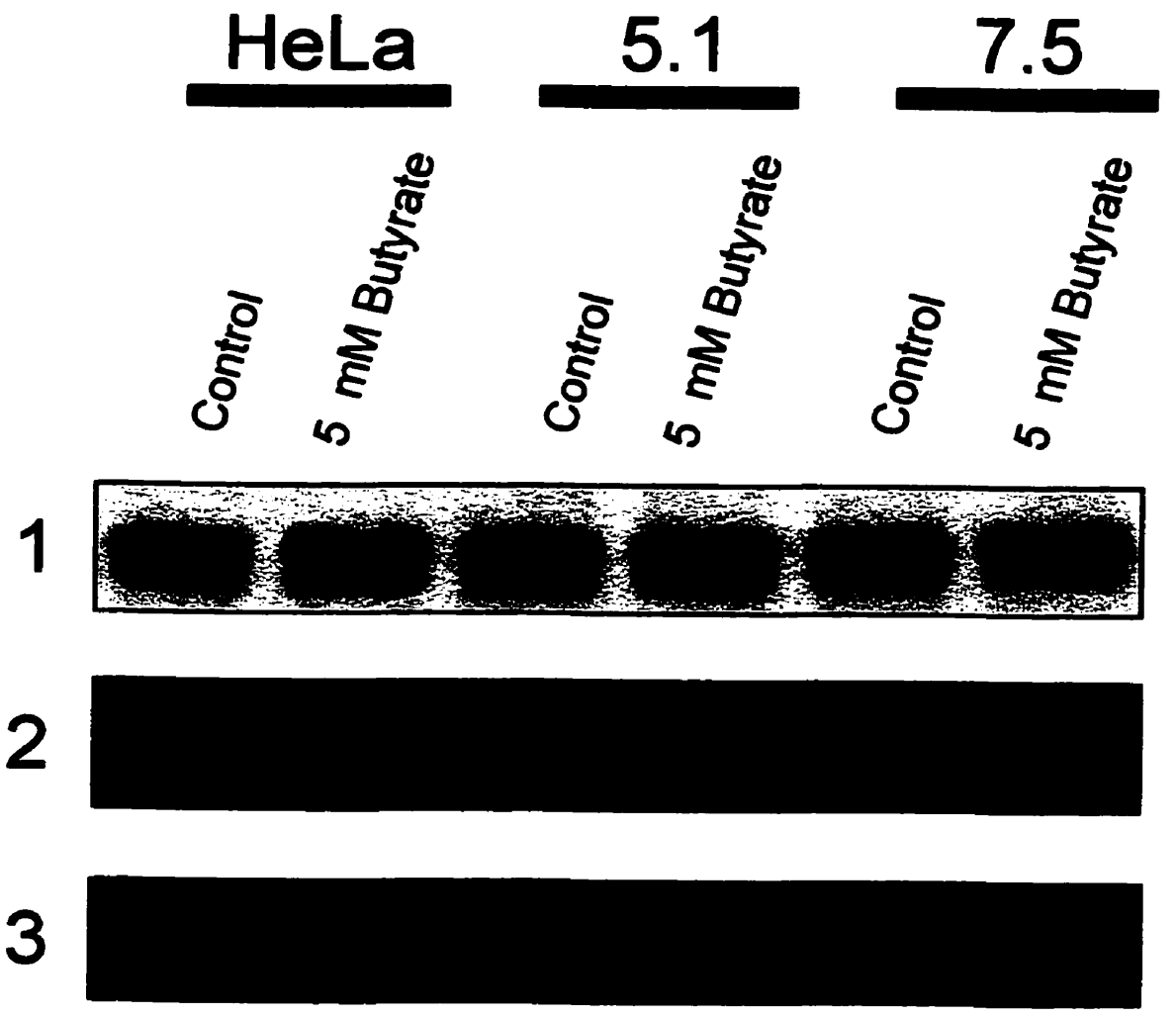

HeLa
5.1
7.5
Control
5 mM Butyrate
Control
5 mM Butyrate
Control
5 mM Butyrate
1
2
3

Figure 13: Phosphorylation of pRb. Immunoblot of phosphorylated pRb isoforms using whole cell protein extracts from HeLa cells and, 5.1 and 7.5 clones incubated overnight with and without butyrate.

Rb
HeLa
Control
1 mM Butyrate
5 mM Butyrate
5.1
Control
1 mM Butyrate
5 mM Butyrate
7.5
Control
1 mM Butyrate
5 mM Butyrate

Figure 14: Kinase assay of $p21^{Waf1/Cip1}$. $P21^{Waf1/Cip1}$ was immunoprecipitated and the complexes assayed for kinase activity with histone H1 (panel 1) as the substrate. Film was exposed overnight to show the extent of phosphorylation of H1 (panel 2). Immunoblots of $P21^{Waf1/Cip1}$ (panel 3), cdk2 (panel 4) and cyclin E (panel 5) were performed from the immunoprecipitated samples.

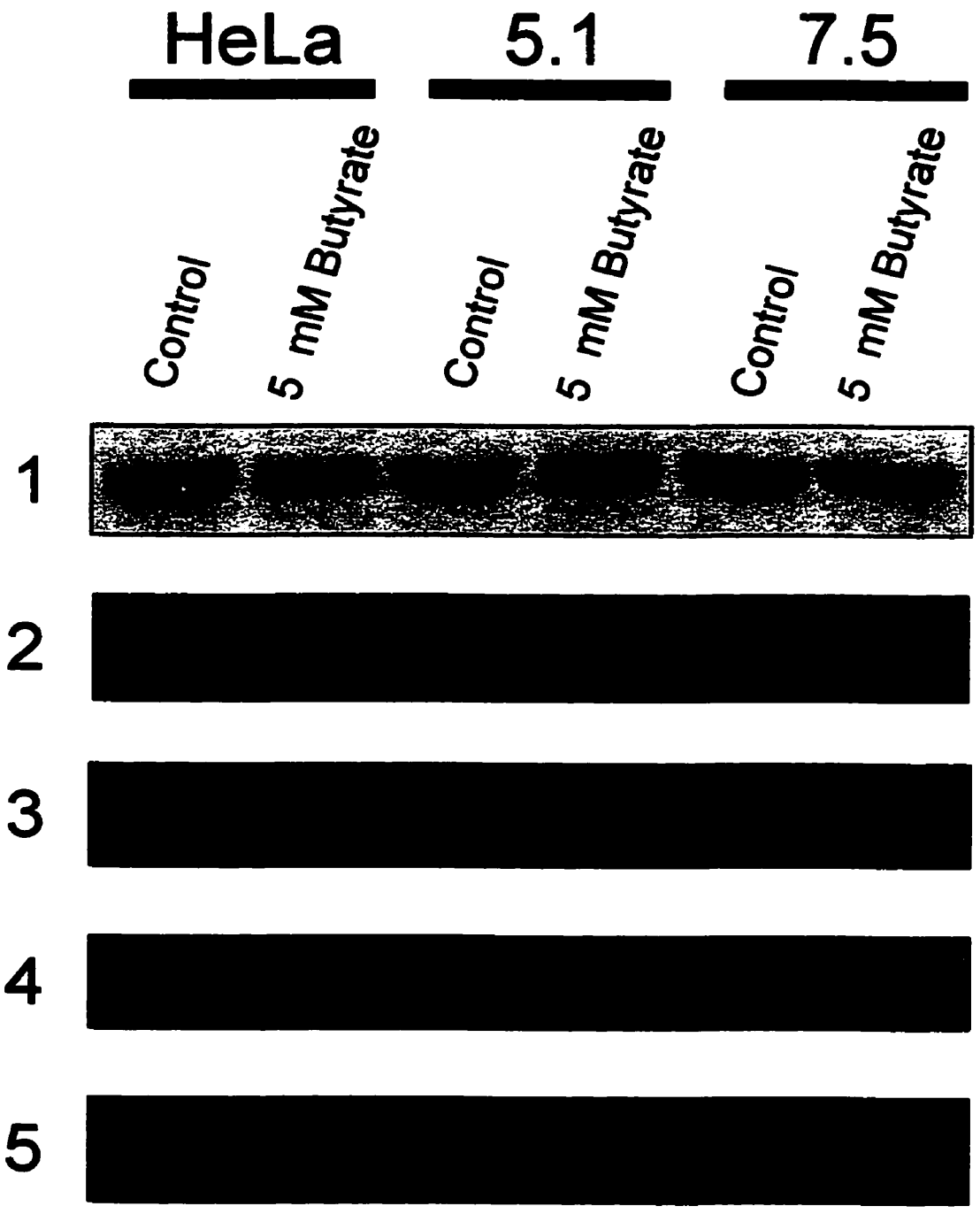
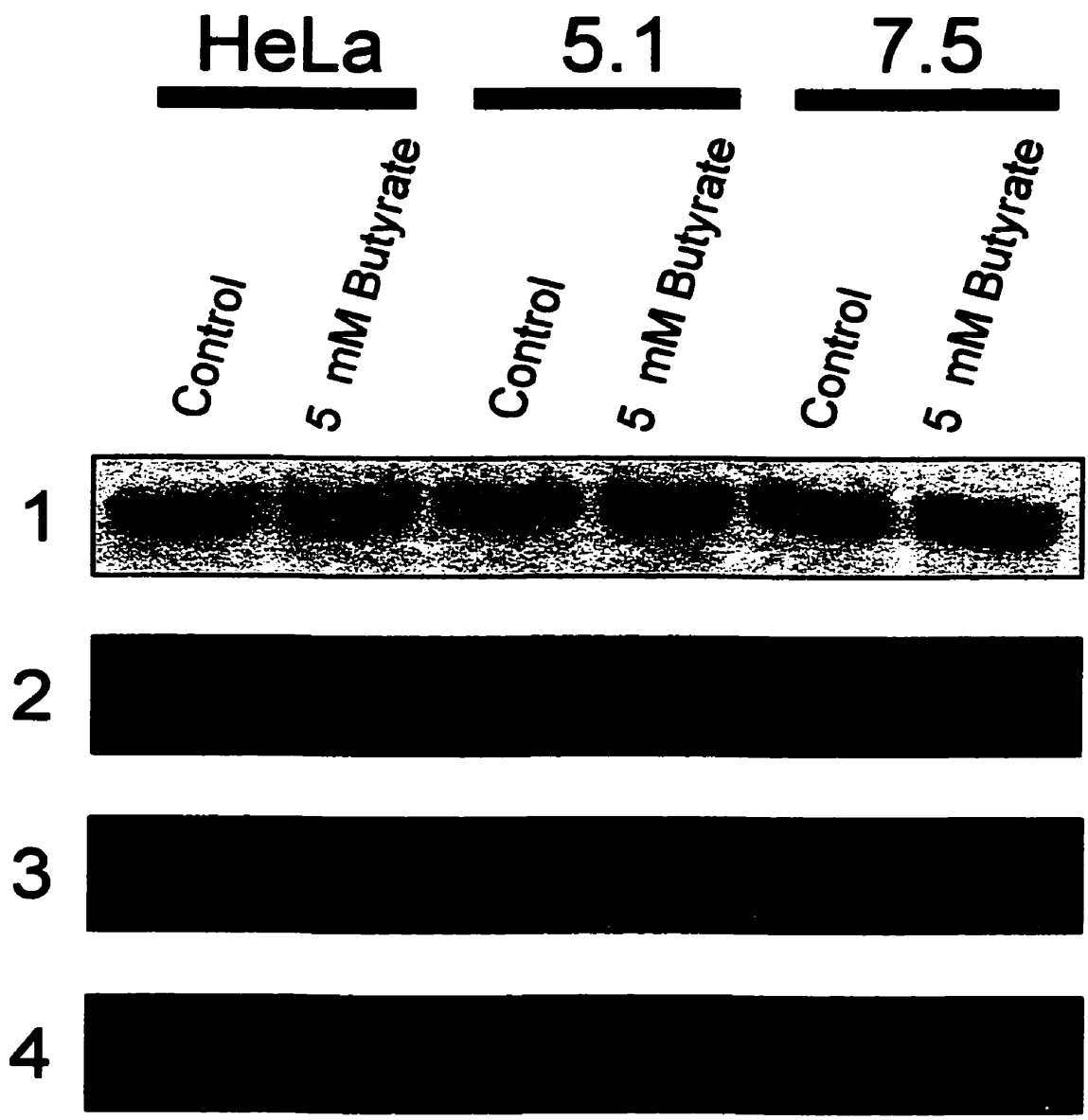

HeLa
5.1
7.5
Control
5 mM Butyrate
Control
5 mM Butyrate
Control
5 mM Butyrate
1
2
3
4
5

Figure 15: Kinase assay of cdk2 using supernatant from $p21^{Waf1/Cip1}$ immunoprecipitation. Cdk2 was immunoprecipitated from the supernatant after $p21^{Waf1/Cip1}$ immunoprecipitations, then assayed for kinase activity with histone H1 (panel 1) as the substrate. Film was exposed overnight to determine phosphorylation of H1(panel 2). Immunoblot of cdk2 (panel 3) and cyclin E (panel 4) were done on the immunoprecipitated samples.


HeLa
5.1
7.5
Control
5 mM Butyrate
Control
5 mM Butyrate
Control
5 mM Butyrate
1
2
3
4