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ALTERATIONS IN PEA ROOT MERISTEM PROTEINS AFTER BUTYRATE EXPOSURE

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Key Word Index—*Pisum sativum*; Leguminosae; root meristems; cell cycle; sodium butyrate; PAGE; protein profiles.

Abstract—One-dimensional PAGE has shown that butyrate upregulates two proteins of M_r 25 and 38 k in comparison with controls in pea roots. Two-dimensional electrophoresis further supported these results by displaying an increase of two groups of basic proteins, one group at M_r 24–26 k and the other group at about M_r 35 k. The eukaryotic cell cycle is controlled by a family of cyclin dependent kinases (CDKs), as well as many other regulatory proteins. Since the mode of action of these proteins and their interactions serve as regulators at various points in the cell cycle, butyrate may control gene expression and cell cycling. © 1997 Elsevier Science Ltd

INTRODUCTION

Butyrate is a natural four carbon fatty acid formed by the hydrolysis of ethyl butyrate [1]. In mammalian cells in culture a variety of cell cycle alterations occur after exposure to butyrate [2, 3]. Recent evidence has implicated butyrate in causing the differentiation of rat liver progenitor cells [4]. Butyrate also caused the selective hypermethylation of nucleosomal DNA in cultured fibroblasts [5].

Studies on the action of butyrate have revealed that it acts similarly in plants and animal cells in culture. In the root meristems of *Allium cepa* bulbs, butyrate at 4 mM and above arrested the meristem cells in mid-G1 or close to the S/G2 border [6]. In *Lactuca sativa* meristems, butyrate causes a marked decrease in the incorporation of [^{3}H]-thymine deoxyribose (TdR). The effect is reversible, and cells return to normal when treatment with butyrate ceases [7].

Early studies on root meristems of *Pisum sativum*, demonstrated that cells accumulated in both G1 and G2 when exposed to 5 mM butyrate. The percentage of cells arrested in G2 was higher than that of cells arrested in G1 [8]. Experiments showed that butyrate halted cell proliferation in root meristems of *P. sativum* as determined by the decrease in mitotic index. Roots exposed to lower concentrations of butyrate showed a less drastic decrease in the mitotic index, suggesting that these roots may have some ability to adapt to low concentrations of butyrate, perhaps undergoing a partial synchronization [9].

Since the effects of butyrate were reversible, experiments determined that butyrate had a similar effect on cells in the root meristem of *P. sativum*. Roots exposed to butyrate for 12 hr and then transformed to fresh medium, provided evidence that pea cells recovered from the effects of butyrate and resumed cycling [9]. Other short-chain fatty acids, propionic and valeric acid, showed similar effects in inhibiting mitosis, lowering the mitotic index to near zero after 12 hr. Following 12 hr exposure to either propionic or valeric acid, meristematic cells resumed normal cycling 12 hr after acid removal, with a burst of mitosis 8 hr after transfer, a result similar to butyrate [10]. A causative role of pH in blocking mitosis is improbable since both the addition of 1 mM caprylic acid and 1 mM benzoic acid fail to reduce the mitotic index of pea meristems grown in culture [10]. Isolated pea root meristems can grow in a broad pH culture change from 3.0–8.0.

Experiments confirmed that butyric acid and other short-chain fatty acids were incorporated into the root meristem, and eventually the nucleus. The results of the incorporation of [1-^{14}C] butyrate and [1-^{14}C] propionate, respectively, into root meristems of *P. sativum* over a 6 hr exposure period show similar uptake patterns [11]. The results of the incorporation of [1-^{14}C] butyrate into the root meristems of *P. sativum* determined by autoradiography complemented these

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results. After an initial plateau lasting for 6 hr, there was a steady increase of labelled nuclei which signified an increase in butyrate uptake with time and movement of the isotope to the nucleus [11].

The effects of various concentrations of butyrate on the mitotic index of root meristem cells of soybean, pinto bean, and broad bean, are all similar. After a 24 hr butyrate exposure, the mitotic indices were significantly lower than controls. Higher concentrations of butyrate showed the greatest decline in overall mitotic indices. Of the three species, pinto beans were most sensitive to the increase in butyrate concentrations, essentially eliminating mitotic figures at 4 mM exposure [12].

Since butyrate, propionate and valerate all demonstrated identical patterns of mitotic inhibition and repression of DNA synthesis, experiments were undertaken to determine if the polypeptide profiles of root meristem cells were altered in the presence of butyrate. Many cell division cycle proteins (CDCs) regulate the orderly transition of eukaryotic cells through the cell division cycle and evidence has shown the presence of the product of the cdc2 gene, p34, in pea meristems [13]. Therefore, extracts from butyrate treated roots were subjected to both one and two-dimensional gel electrophoresis and compared with control (non-treated) extracts.

RESULTS AND DISCUSSION

Figure 1 shows a Coomassie Blue stained one-dimensional protein profile of cultured pea root meristems. The addition of 1 mM sodium butyrate to the culture medium altered the protein profile of the meristem. Enhanced M_r bands at *ca* 25 and 38 k were evident in the butyrate treated meristems. All other bands were identical between the two samples.

A fluorograph of the treatments shown in Fig. 1 is presented in Fig. 2. The newly synthesized protein pattern also varies with butyrate exposure. An enhanced band at M_r *ca* 25 k is viewed when butyrate is present. Similar M_r bands are evident at 15, 30–40, 65 and 94 k.

In order to confirm the presence of butyrate-influenced polypeptides, two-dimensional gel electrophoresis was employed. Figures 3 and 4 show the protein profiles of control and butyrate treated meristems, respectively. In the butyrate treated meristems two significant groups of basic proteins, indicated by arrows, appear to be up-regulated, one group at M_r 24–26 k, a second group at about M_r 35 k. This result supports the data from the one-dimensional gels. Two-dimensional gel electrophoresis of propionate treated meristems showed an identical protein pattern as butyrate with up-regulated groups at M_r 24–26 k and 35 k. Stationary phase meristems, those grown in media lacking sucrose, showed protein patterns identical to controls. This is important since stationary phase meristems also do not cycle, but are halted due to sucrose deprivation. Since the two groups of peptides only appear in butyrate or propionate treated meristems, the mechanisms for halting the cell cycle are probably different.

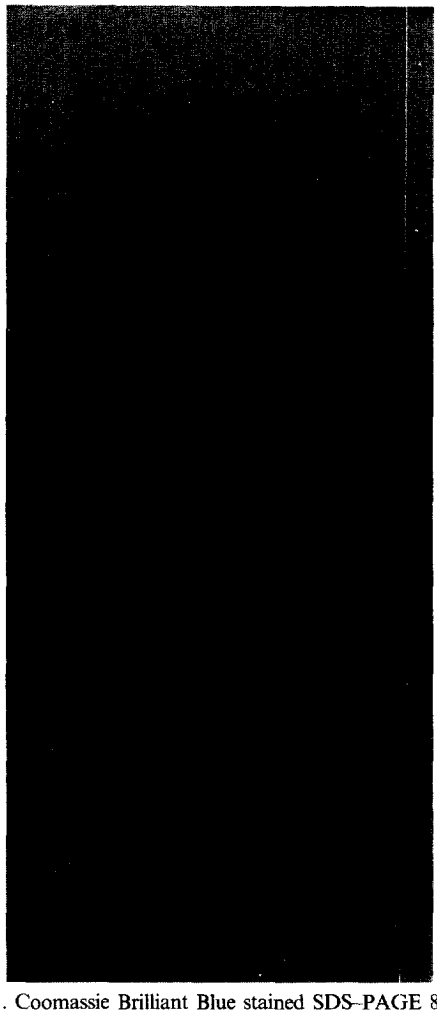

Fig. 1. Coomassie Brilliant Blue stained SDS–PAGE 8–16% gradient gels of proteins extracted from cultured pea root meristems (0–2 mm terminus) which have been grown in White's medium with sucrose for 24 hr. Lane 1: Medium without 1 mM sodium butyrate. Lane 2: 1 mM sodium butyrate added. M_r markers: phosphorylase B (97 k), bovine serum albumin (66 k), ovalbumin (45 k), carbonic anhydrase (29 k), lysozyme (15 k). Arrows indicate up-regulated butyrate proteins.

Prior results from this laboratory have shown that butyrate and other short-chain fatty acids effectively block mitosis and DNA synthesis in root meristems of peas and other legumes [9–12]. The expression of a unique set of proteins in a dormant (non-cycling) pea axillary bud meristem has been shown [14]. Therefore, it is conceivable that non-cycling cells express a different set of proteins as do cycling cells. Many cell division kinases (CDK) and other important proteins needed for continual cycling have been demonstrated in plants [15–16]. A cdc2 homologue (M_r 34 k) has been isolated in peas, corn and *Arabidopsis* [17–19], while cyclins have been analysed in *Arabidopsis* [20].

Since only root meristems exposed to the short-

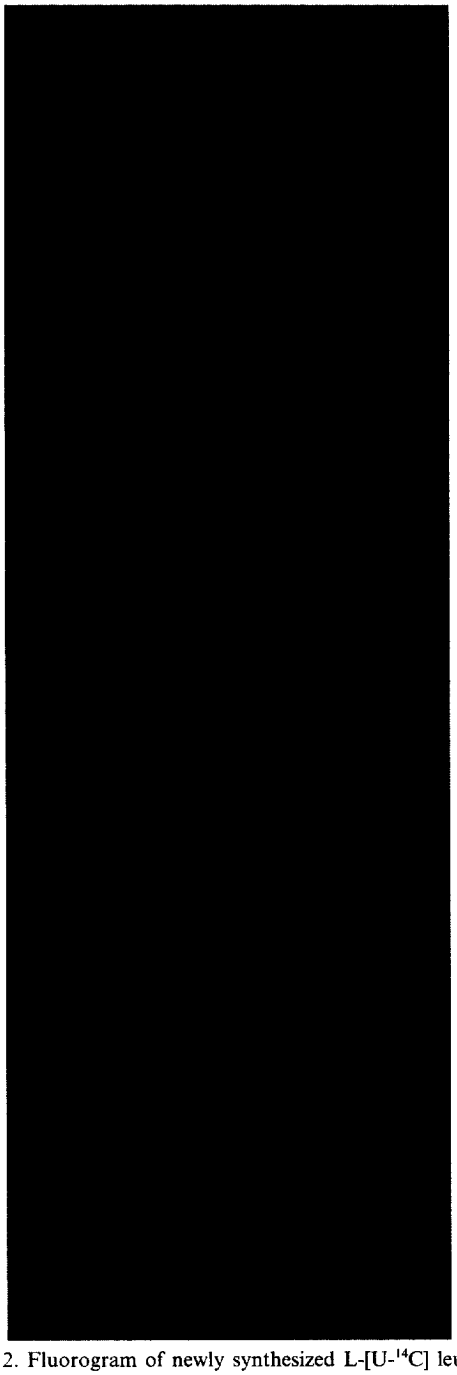

Fig. 2. Fluorogram of newly synthesized L-[U-^{14}C] leucine polypeptides from cultured pea root meristems (0–2 mm terminus). Level 1: Medium without 1 mM sodium butyrate. Level 2: 1 mM sodium butyrate added. M_r markers indicated at left. Arrows indicate up-regulated butyrate proteins.

chain fatty acids demonstrated the additional proteins, their appearance may be correlated with the elimination of mitosis and reduced DNA synthesis and the subsequent halt in the cell cycle. Given this information, these proteins may be the consequence of butyrate addition.

EXPERIMENTAL

General culture conditions. Seeds of *Pisum sativum* L. Cv. Alaska 2B (garden peas) were surface sterilized in a sterilized laminar flow hood with undiluted Clorox for 10 min, washed with sterile H_2O and germinated in the dark at 23° in sterile vermiculite.

Isolation of labelled proteins. Excised 3-day-old roots were aseptically cultured in White's medium +/− 1 mM Na butyrate for 24–48 hr. The 0–2 mm root tips were excised and placed into fresh media containing L-[U-^{14}C] leucine (Amersham; spec. act. 342 mCi mmol^{-1}) for 6 hr after which they were harvested, weighed and the total protein was extracted by grinding the samples in Webster Buffer A (0.05 M Tris buffer (pH 8.0) containing 0.25 M sucrose, 1 mM EDTA, 0.7% HOC_2H_5SH (ME), 0.005% PMSF, 1% Triton X-100) followed by centrifugation at 400 *g* for 30 min. The supernatant was added to an equal vol. of Webster Buffer B (6 M urea, 1 M sucrose, 0.125 M Tris (pH 6.8), 2.5% SDS, 5% HOC_2H_5SH (ME), and boiled for 3 min [21].

Protein assay. The Pierce BCA Protein Assay was employed (Pierce) to determine the amount of protein present. Samples were incubated at 37° for 1 hr, and protein was determined spectrophotometrically.

1-*Dimensional protein separation.* Samples were added to an equal vol. of SDS gel electrophoresis loading buffer (3 ml glycerine, 1.7 ml H_2O, 0.2 ml HOC_2H_5SH (ME), 2 ml 10% SDS, 0.025% bromophenol blue) and loaded onto a 8–16% SDS gradient gel. Pre-cast gradient gels (NOVEX) were used to separate polypeptides in the 10–150 kDa range. Samples were electrophoresed using the Xcell II Mini-Cell gel apparatus (NOVEX), run with a Trisglycine–SDS buffer, pH 8.3 at 125 V, until the tracking dye reached the bottom of the gel. The gel was fixed in HOAc–MeOH–H_2O (1:4:5).

Protocol for polyacrylamide gel staining. Following fixation, the gels were stained either with 0.2% Coomassie Brilliant Blue C for 1 hr and then destained with the fixative, or the Bio-Rad Silver Stain Kit (Bio-Rad Laboratories). The gel was fixed in 10% HOAc–40% MeOH ×2 for 30 min each, oxidized with 10% Bio-Rad oxidizing reagent and washed ×3 times with H_2O. A 10% Ag reagent soln stained the polypeptides. The gels were developed with Ag developer and placed in a 5% HOAc 'stop' soln, and dried at 80° under vacuum.

Fluorography protocol. After staining with Coomassie Brilliant Blue C, the gel was washed ×3 for 10 min each in a new fixative, HOAc–*iso*-PrOH–H_2O (1:1:18). Excess fixer was removed with a continuous flow of H_2O for 15 min. The gel was immersed in Amplify (Amersham) in a vol. ×3 the gel thickness, agitated for 2 hr, placed directly on filter paper and dried at 80° in vacuum. After drying, the gel was exposed to Hyperfilm MP at −50° for one week, developed and fixed.

2-*Dimensional protein separation.* Isoelectric focus-

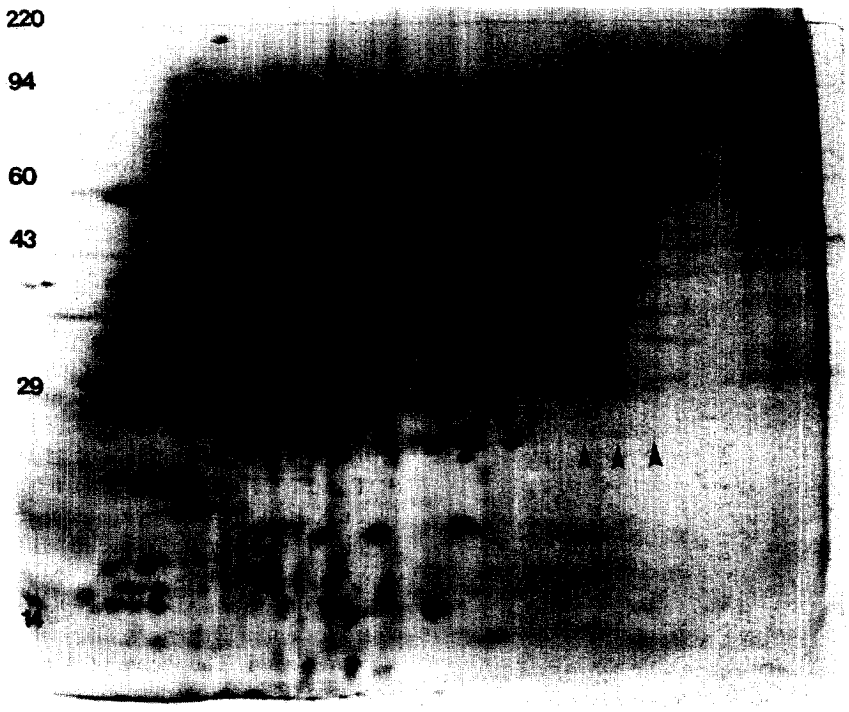

Fig. 3. Silver-stained two-dimensional PAGE of proteins extracted from cultured pea root meristems (0–2 mm terminus) which have been grown in White's medium with sucrose for 48 hr. *M*, markers listed on the left. Arrows indicate areas where up-regulated butyrate proteins would be present. Double arrowhead indicates 33 k tropomyosin internal standard.

Fig. 4. Silver-stained two-dimensional PAGE of proteins extracted from cultured pea root meristems (0–2 mm terminus) which have been grown in White's medium with sucrose, with 1 mM sodium butyrate for 48 hr. *M*, markers listed on the left. Arrows indicate up-regulated butyrate proteins. Double arrowhead indicates 33 k tropomyosin internal standard.

ing was carried out on the protein samples in glass tubes of inner diam. 2 mm using pH 4–8 ampholines for 9600 V hr^{-1}. Fifty ng of an int. standard, tropomyosin, was added to each sample. Tropomyosin migrates to an M_r of 33 k with a pl of 5.2 [22]. After equilibration for 10 min in buffer, the tube gel was sealed to the top of a 10% acrylamide gel. SDS slab gel electrophoresis was carried out for 4 hr at 12.5 mA gel^{-1}. Gels were fixed in a soln of 10% HOAc–50% MeOH overnight. M_r standards were added to the tubes and the gel was Ag stained and dried [23]. All 2-D gel electrophoresis was carried out by Kendrick Labs in Madison, WI. Kendrick analysed the gels and determined any differences between the protein profiles in control vs treated root meristem extracts.

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