Induction of germ-cell alkaline phosphatase by butyrate and cyclic AMP in BeWo choriocarcinoma cells

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BeWo choriocarcinoma cells synthesize two alkaline phosphatase isoenzymes: germ-cell alkaline phosphatase and tissue-unspecific alkaline phosphatase. We have made use of the differential heat-stabilities of these two isoenzymes to study the induction of germ-cell alkaline phosphatase by sodium butyrate and cyclic AMP (cAMP). Sodium butyrate causes a large induction of germ-cell alkaline phosphatase activity (approx. 35-fold after 96 h) after an initial lag period of 12–24 h. We showed that butyrate increases germ-cell alkaline phosphatase mRNA. Dibutyryl cAMP also induces germ cell alkaline phosphatase (approx. 2.5-fold after 96 h). When optimal concentrations of butyrate and dibutyryl cAMP were added simultaneously to cells, they caused a synergistic induction of activity. This sug-

gested that these compounds use separate mechanisms to induce germ-cell alkaline phosphatase activity and that it is the cAMP moiety of dibutyryl cAMP that induces enzyme activity. This was confirmed by the use of two additional cAMP analogues, 8-(4-chlorophenylthio) cAMP and 8-bromo cAMP, and of two compounds, 3-methyl-1-isobutylxanthine and cholera toxin, which raise the endogenous concentration of cAMP. All four compounds caused a 2-fold increase in enzyme activity. Treatment of cells with 8-(4-chlorophenylthio) cAMP, 8-bromo cAMP and cholera toxin increased germ-cell alkaline phosphatase mRNA between 2- and 7-fold. These data suggest that this alkaline phosphatase isoenzyme is regulated at the level of its mRNA by cAMP, in a manner distinct from that of butyrate.

INTRODUCTION

In man there are four alkaline phosphatase genes: (a) tissue-unspecific (liver/kidney/bone), (b) intestinal, (c) placental, (d) germ cell [1]. The products of these genes can be distinguished on the basis of their physical, enzymic and antigenic properties, and each is expressed in a distinctive range of tissues. The regulation of the expression of the two heat-resistant isoenzymes, placental alkaline phosphatase (PLAP) and germ-cell alkaline phosphatase (GCAP), has received considerable attention. Partly this is because of the restricted number of normal cell types that express these genes, and partly because their anomalous expression has been detected in certain types of tumours. PLAP is one of the major glycoproteins of the syncytiotrophoblast membrane [2]. PLAP protein and mRNA are found in the placenta as early as 7 weeks gestation and continue to increase in amount throughout pregnancy [3]. The expression of PLAP in the placenta therefore correlates with the degree of differentiation of trophoblast cells, since it is at its lowest level when the cells are rapidly dividing and increases as their growth rate decreases. In the past it was thought that PLAP was only expressed in the placenta; however, as more sensitive methods for measuring this protein have developed, trace amounts of PLAP protein have been detected in the cervix and lung [4]. This isoenzyme has been identified in neoplasms of the testis, ovary, pancreas, breast colon, lymph tissue and bladder [5]. GCAP is expressed in small amounts in the thymus and testis [4], and is frequently expressed in germ-cell and ovarian tumours at such a high level that it is detectable in serum and is of potential use as a tumour marker [6].

The genes for PLAP and GCAP are 98% identical in their coding regions [7], but, despite their close similarity in sequence,

are expressed in a cell-type-specific fashion. The mechanism which controls the tissue-specific expression of PLAP or GCAP and the cause of the aberrant expression of these isoenzymes in tumour cells is at present not understood, and is an area of considerable interest.

The choriocarcinoma cell lines JEG3 and BeWo are useful tools for the study of the aberrant expression of germ-cell alkaline phosphatase, since these cells, being of trophoblast origin, would be expected to synthesize PLAP. In fact the cell lines express both GCAP and tissue-unspecific alkaline phosphatase at low levels [8,9]. The total alkaline phosphatase activity in these cell lines (GCAP plus tissue-unspecific) has been shown to be inducible by a variety of agents such as sodium butyrate, 5-bromodeoxyuridine, dibutyryl cyclic AMP (cAMP) and DNA-synthesis inhibitors [8–13]. However, no distinction has been made between the regulation of heat-stable GCAP and that of heat-sensitive tissue-unspecific alkaline phosphatase by these agents. Although total alkaline phosphatase in BeWo cells has been shown to be induced by dibutyryl cAMP [10], it has been unclear whether this was due to the cAMP moiety or to the butyric acid released by intracellular degradation of dibutyryl cAMP [10,14–17]. We have carried out experiments to ascertain whether cAMP does indeed induce GCAP activity in BeWo cells.

MATERIALS AND METHODS

Reagents

8-Bromo-cAMP (8-Br-cAMP), chlorophenylthio-cAMP (CPT-cAMP), *N*⁶,2'-*O*-dibutyryl cAMP and cholera toxin (CT) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3-Isobutyl-1-methylxanthine (MIX) was purchased from Aldrich

Abbreviations used: cAMP, cyclic AMP; 8-Br-cAMP, 8-bromo cyclic AMP; CPT-cAMP, 8-(4-chlorophenylthio) cyclic AMP; CRE, cAMP response element; CT, cholera toxin; GCAP, germ-cell alkaline phosphatase; hCG, human chorionic gonadotropin; MIX, 3-isobutyl-1-methylxanthine; PLAP, placental alkaline phosphatase.

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Chemical Co. (Gillingham, Dorset, U.K.). Ham's F12 medium, penicillin, streptomycin and trypsin/EDTA were obtained from Gibco BRL (Paisley, Scotland, U.K.). Foetal-calf serum was obtained from Imperial Laboratories (Europe) Ltd. (Andover, Hants., U.K.). Protein was determined with a Lowry protein-estimation kit from Sigma. Poly(A)⁺ RNA was isolated using (dT)₂₅ Dynabeads from Dynal (U.K.) Ltd. (New Ferry, Wirral, U.K.). [α -³²P]dCTP (3000 Ci/mmol) was obtained from ICN Biochemicals (Irvine, CA, U.S.A.). All other chemicals, unless stated, were purchased from Sigma.

Cell culture

BeWo choriocarcinoma cells were a gift from Professor P. M. Johnson, Department of Immunology, University of Liverpool, and were grown in Ham's F12 medium supplemented with 10% foetal-calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of air/CO₂ (19:1). For induction experiments, confluent monolayers were split 1:3 with trypsin/EDTA, and were allowed to grow to 70% confluence before addition of cAMP analogues, MIX and CT, or to confluence before addition to butyrate.

Assay of alkaline phosphatase activity in cultured cells

Cell monolayers were washed three times with ice-cold PBS and scraped into PBS with a rubber policeman. The cells were centrifuged at 1000 *g* for 5 min. The cell pellet so obtained was resuspended in 1 ml of 0.25% (w/v) sodium deoxycholate (pH 8.0), thoroughly vortex-mixed and placed on ice for 5 min. A 0.5 ml portion of this cell lysate was assayed for alkaline phosphatase activity, with phenyl phosphate as substrate. After heating the cell lysate to 65 °C for 10 min, it was cooled on ice for 5 min and then mixed with 1 ml of assay buffer (50 mM glycine/NaOH, 1 mM MgCl₂, pH 10.7) and 0.5 ml of phenyl phosphate (288 mM). The reaction mixture was incubated at 37 °C for 10 min, after which 1 ml of Folin-Ciocalteu reagent and 5 ml of 0.5 M Na₂CO₃ were added. The sample was mixed well and placed at 37 °C for a further 15 min. The resulting colour was measured at 700 nm, and the number of μ mol of phenyl phosphate hydrolysed/min was calculated from a calibration curve. Protein was estimated with a Lowry kit.

Northern blotting

Poly(A)⁺ RNA was extracted directly from cultured cells using Dynabeads (dT)₂₅ according to the manufacturer's instructions, and was electrophoresed through 1% agarose gels containing 2.2 M formaldehyde and then blotted on to nylon membranes (Hybond N; Amersham) [18]. The blots were hybridized with either a 2.8 kb PLAP cDNA isolated in our laboratory or a 1.6 kb non-muscle-actin cDNA (kindly provided by Dr. R. Barraclough, University of Liverpool). The probes were radio-labelled with [α -³²P]dCTP by using a random-primed DNA-labelling kit from Boehringer Mannheim (Mannheim, Germany). Hybridization conditions were 50% formamide, 5 $\times$ SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0), 50 mM sodium pyrophosphate, pH 6.5, 5 $\times$ Denhardt's (0.1% BSA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400, all w/v), 250 μ g/ml sonicated salmon sperm DNA, 10% (w/v) dextran sulphate at 42 °C for 18 h. Filters were washed sequentially in 2 $\times$ SSC/0.1% SDS at room temperature for 30 min, in 1 $\times$ SSC/0.1% SDS at 42 °C for 15 min and in 0.1 $\times$ SSC/0.1% SDS at 65 °C for 30 min.

Autoradiography was performed with Fuji X-ray film at -70 °C. Autoradiograms were quantified with a Shimadzu CS-900 dual-wavelength flying-spot scanner.

Labelled cDNA was stripped from membranes, before re-probing with a second cDNA, by washing them for 1-2 h in 5 mM Tris/HCl, pH 8.0, 2 mM EDTA, 0.1 $\times$ Denhardt's at 65 °C in a shaking water bath.

RESULTS

Heat-stability of alkaline phosphatase isoenzymes in BeWo cells

Since we wished to measure the induction of heat-stable GCAP by butyrate and cAMP, it was essential to characterize the heat-stability of alkaline phosphatase in BeWo cells. The results in Table 1 show that 23% of the alkaline phosphatase activity present in BeWo cells is destroyed by heating to 65 °C for 10 min and that the remaining enzyme activity is resistant to 20 min at 65 °C. Hamilton et al. [8] showed that 76% of total alkaline phosphatase activity in BeWo cells was attributable to GCAP and 24% to tissue-unspecific alkaline phosphatase activity. Thus by heating cell extracts to 65 °C for 10 min we could be certain of measuring only GCAP activity. The enzyme-activity data reported in all subsequent experiments therefore represent alkaline phosphatase activity remaining after heating deoxycholate cell extracts to 65 °C for 10 min.

Induction of GCAP activity by sodium butyrate

Figure 1(a) shows that butyrate caused a dose-dependent increase in GCAP activity which reached a maximum when cells were grown in the presence of 2.25 mM butyrate, and that when the concentration of butyrate was raised to 2.5 mM there was a slight decline in activity. This may be attributable to cell death due to the known toxic effect of butyrate [12]. The time course of induction of GCAP (Figure 1b) shows that growth of cells in the presence of butyrate does not cause any induction of activity over the first 12 h period, but after 24 h this compound caused a small increase in activity (approx. 2.5-fold). GCAP increased considerably over the next 72 h, in a nearly linear fashion, so that by 96 h the induction had reached approx. 32-fold, although activity had not yet reached a plateau.

Sodium butyrate increases the level of GCAP mRNA in BeWo cells

It has been shown previously, in another choriocarcinoma cell line [13], that butyrate causes an increase in the steady-state level of GCAP mRNA which is presumably responsible for the

Table 1 Stability at 65 °C of alkaline phosphatase activity in BeWo cells

Samples of deoxycholate extracts of BeWo cells were heated to 65 °C for the stated times, after which remaining alkaline phosphatase activity was measured as described in the Materials and methods section. This was compared with that of a sample kept on ice (0 min). Similar results were obtained in two separate experiments.

Time at 65 °C (min)	Activity (unit/mg)	Activity remaining (%)
0	0.043	100
10	0.033	77
20	0.032	74

Figure 1 Induction of GCAP activity in BeWo cells by sodium butyrate: (a) dose-response; (b) time course

BeWo cells were seeded into 75 cm² flasks at approx. 4×10^6 cells/flask and allowed to grow for 3–4 days until they formed a confluent monolayer. (a) The cells were then incubated, in either the absence (control) or the presence of sodium butyrate, at the indicated concentrations for 48 h. The cells were then harvested and assayed for heat-stable alkaline phosphatase activity. The medium was changed every 24 h. Data are presented as means $\pm$ SEM of three experiments. (b) The cells were then incubated, in either the absence (controls) or the presence of 2 mM butyrate for the time period indicated. The cells were then harvested and assayed for heat-stable alkaline phosphatase activity. The medium was changed every 24 h. Data are presented as means $\pm$ S.E.M. of three experiments.

increase in enzyme activity. We performed a Northern blot on poly(A)⁺ RNA extracted from BeWo cells to determine whether butyrate also increased GCAP mRNA in these cells. Figure 2 shows that butyrate increases the steady-state level of GCAP mRNA in BeWo cells approx. 60-fold. Because the PLAP and GCAP gene sequences are 98% identical in their coding regions [7], we were able to use a PLAP cDNA to detect GCAP mRNA in BeWo cells. Experiments in which human placental mRNA was analysed simultaneously with BeWo mRNA revealed a clear and reproducible size difference between the two mRNAs (PLAP mRNA 3.0 kb, GCAP mRNA 2.6 kb), as has previously been reported [16]. In no autoradiographs of mRNA from either control or induced BeWo cells have we been able to detect hybridization to a 3.0 kb species, suggesting that any PLAP mRNA present in these cells is below the sensitivity of our detection methods.

Figure 2 Effect of sodium butyrate on GCAP mRNA levels

BeWo cells were incubated in the absence (control) or presence of 2 mM sodium butyrate (+ butyrate) for 48 h. Poly(A)⁺ RNA was extracted from 2×10^6 cells and analysed by Northern-blot analysis for GCAP mRNA expression by using a PLAP cDNA, as described in the Materials and methods section. The blot was then re-probed with actin cDNA, to check RNA loading. The autoradiographs were examined by scanning densitometry, and induction of GCAP mRNA was corrected for hybridization to actin. Lane 1, 10 µg of control BeWo RNA; lane 2, 10 µg of + butyrate BeWo RNA.

Dibutyl cAMP and butyrate cause a synergistic induction of GCAP activity

Figures 3(a) and 3(b) show the dose-response and time course of induction of GCAP activity by dibutyl cAMP. The dose-response curve shows that the GCAP was induced to the maximally observed level of approx. 2-fold when BeWo cells were grown in the presence of 1.75 mM dibutyl cAMP. The concentrations of butyrate and dibutyl cAMP which caused maximal induction of GCAP activity (approx. 2 mM and 1.5 mM, for butyrate and dibutyl cAMP respectively) were used in combination to test whether they would cause a synergistic induction. The results shown in Table 2 illustrate that when butyrate and dibutyl cAMP are added together to BeWo cells in culture they do cause a synergistic induction of GCAP activity. They suggest, but are not definitive proof, that the induction by dibutyl cAMP is not due to the release of butyrate from the compound. These results also suggest that butyrate and dibutyl cAMP use different mechanisms to induce GCAP.

Induction of GCAP by CPT-cAMP and 8-Br-cAMP and agents which raise the intracellular level of cAMP

In order to investigate the role of cAMP as an inducer of GCAP activity, we carried out further experiments using alternative cAMP analogues and agents which raise the intracellular concentration of cAMP. We used the cAMP analogues CPT-cAMP and 8-Br-cAMP, which have the advantage over dibutyl cAMP of not being broken down to a compound which is itself an inducer of alkaline phosphatase activity. Since these were the

Figure 3 Induction of GCAP activity in BeWo cells by dibutyryl cAMP: (a) dose-response; (b) time course

(a) BeWo cells were seeded into 75 cm² flasks at approx. 3×10^6 cells/flask and allowed to grow for 2 days before growth, in either the absence (control) or the presence of dibutyryl cAMP, at the indicated concentrations for 72 h. Cells were then harvested and assayed for heat-stable alkaline phosphatase activity. The medium was changed every 24 h. The results are presented as means $\pm$ S.E.M. of three experiments. (b) BeWo cells were seeded into 75 cm² flasks at approx. 3×10^6 cells/flask and were allowed to grow for 2 days before growth, in either the absence (controls) or the presence of 1.5 mM dibutyryl cAMP (db cAMP) for the indicated time period. Cells were then harvested and assayed for heat-stable alkaline phosphatase activity. The medium was changed every 24 h. The results are presented as means $\pm$ S.E.M. of three experiments.

Table 2 Induction of GCAP by butyrate and dibutyryl cAMP in combination

BeWo cells were seeded into 75 cm² flasks at approx. 3×10^6 cells/flask and were allowed to grow for 3–4 days before addition of medium containing 2 mM butyrate or 1.5 mM dibutyryl cAMP (db cAMP) or 2 mM butyrate plus 1.5 mM dibutyryl cAMP, or no inducing compound (control). Cells were allowed to grow for 72 h in the inducing media, before harvest and assay for heat-stable alkaline phosphatase activity. The medium was changed every 24 h. Results are presented as fold induction of activity compared with control and represent means $\pm$ S.E.M. of three experiments.

Inducer	Fold induction
Butyrate	21.67 $\pm$ 3.33
db cAMP	1.67 $\pm$ 0.17
Butyrate + db cAMP	43 $\pm$ 6.24

first experiments ever to establish whether CPT-cAMP induces GCAP activity, we established a dose-response curve and time course of induction. The range of concentrations of CPT-cAMP

Figure 4 Induction of GCAP activity in BeWo cells by CPT-cAMP: time course

BeWo wells were seeded into 75 cm² flasks at approx. 3×10^6 cells/flask and were allowed to grow for 2 days before growth, in either the absence (controls) or the presence of 4 mM CPT-cAMP. Cells were allowed to grow for the indicated times, before harvest and assay for heat-stable alkaline phosphatase activity. The medium was changed every 24 h. The data are presented as means $\pm$ S.E.M. of three experiments.

used was based on that used previously in other experimental systems. It was found that CPT-cAMP at concentrations between 1 and 4 mM caused an approx. 2-fold induction of GCAP activity: 1 mM CPT-cAMP induced a 2.2-fold increase, 4 mM induced a 2.6-fold increase. The time course (Figure 4) shows that there was no measurable induction during the first 12 h of culture of BeWo cells in the presence of CPT-cAMP. After 24 h GCAP activity was induced approx. 1.5-fold, and increased again after 36 h to give a 2-fold induction of activity. Induction was still not maximal after 48 h. These results were supported by those obtained with 8-Br-cAMP (Table 3). Thus three different cAMP analogues were each able to induce GCAP activity in BeWo cells and, taken together, these results are good evidence that cAMP is an inducer of the enzyme activity. In further support of this conclusion we grew BeWo cells in the presence of compounds known to raise intracellular levels of cAMP by influencing either its rate of synthesis or its rate of degradation. We used two such compounds, MIX and CT. MIX increases intracellular cAMP by inhibiting cAMP phosphodiesterase [19]. We used 0.5 mM MIX in our experiments, a concentration which has been shown to cause a maximal, approx. 4-fold, increase in cAMP concentration in islet of Langerhans cells isolated from rats [20]. BeWo cells were grown in the presence of 0.5 mM MIX for 48 h, harvested and assayed for heat-stable alkaline phosphatase activity. The results in Table 3 show that MIX caused an increase in GCAP activity. These results suggest that the increase in endogenous cAMP level caused by the phosphodiesterase inhibitor MIX is of sufficient magnitude to induce GCAP activity. To be certain that agents which raise intracellular cAMP levels can cause induction of GCAP, we used another such agent, CT. CT increases the activity of adenylate cyclase, the enzyme which converts ATP into cAMP, by catalysing the ADP-ribosylation of the G_s α subunit of the enzyme [21] on an arginine residue which is close to the guanine-nucleotide-binding site. Such ADP-ribosylation activates G_s α (and hence adenylate cyclase) by inhibiting its intrinsic GTPase activity, giving rise to increased intracellular cAMP levels. The concentration of CT and the incubation period that we used were based on a report which showed that CT at a concentration of

Table 3 Induction of GCAP activity in BeWo cells by 8-Br-cAMP, MIX and CT

BeWo cells were seeded into 75 cm² flasks at approx. 3×10^6 cells/flask and were allowed to grow for 2 days before growth, in either the absence (control) or the presence of 0.5 mM MIX or 10 ng/ml CT or 1 mM 8-Br-cAMP for 48 h. Cells were then harvested and assayed for heat-stable alkaline phosphatase activity. The medium was changed every 24 h. Results are presented as means $\pm$ S.E.M. of three experiments.

	GCAP activity (unit/mg)	Fold induction
Control	0.077 ± 0.008	—
+ 0.5 mM MIX	0.190 ± 0.043	2.5
+ 10 ng/ml CT	0.191 ± 0.030	2.5
+ 1 mM 8-Br-cAMP	0.119 ± 0.028	1.5

Figure 5 Induction of GCAP mRNA by cAMP analogues and agents that increase endogenous cAMP levels

BeWo cells were incubated in the absence (controls) or presence of 4 mM CPT-cAMP or 1 mM 8-Br-cAMP for 48 h, or in the presence of 10 ng/ml CT for 72 h. Poly(A)⁺ RNA was extracted from 2×10^6 cells and analysed by Northern-blot analysis for GCAP mRNA by using PLAP cDNA and the re-probed with actin cDNA, to check RNA loading. The autoradiographs were examined by scanning densitometry, and induction of GCAP mRNA was corrected for hybridization to actin. Lanes 1–4 were probed together in one experiment; lanes 5–7 were analysed in a separate experiment. Lanes: 1, control; 2, + CPT-cAMP; 3, control; 4, + CT; 5, control; 6 and 7, + 8-bromo-cAMP (duplicate analyses of a single experiment).

10 ng/ml caused a maximal accumulation of cAMP in BeWo cells after 72 h in culture [22]. The results of these experiments are displayed in Table 3 and show that CT caused induction of GCAP activity. The fold induction is comparable with that observed when cells are grown in the presence of MIX. These

results establish that cAMP is an inducer of GCAP activity in BeWo cells.

cAMP increases the level of GCAP mRNA

In order to determine whether the increase in GCAP activity results from an increase in mRNA level, we performed Northern-blot analysis on poly(A)⁺ RNA extracted from cells grown in the presence of 4 mM CPT-cAMP or 1 mM 8-Br-cAMP for 48 h or 10 ng/ml CT for 72 h. Blots were probed separately with PLAP cDNA, and with actin cDNA as a non-induced control. As shown in Figure 5 the PLAP cDNA detected bands migrating at approx. 2.6 kb in mRNA from uninduced and induced cells. Quantification of the blots by densitometry indicated that 8-Br-cAMP treated cells had twice as much GCAP mRNA as uninduced cells, CPT-cAMP-treated cells had 7-fold and CT-treated cells had 3-fold more. The size of the mRNA is identical in control and cAMP-induced cells, suggesting that only GCAP is induced in these cells.

DISCUSSION

In this study we have shown that butyrate induces GCAP activity in BeWo cells by inducing GCAP transcripts [15,16]. We have demonstrated that butyrate and dibutyl cAMP cause a synergistic induction of GCAP activity, which suggests that it is the cAMP moiety of dibutyl cAMP that is the active species. However, this result does not preclude the interpretation that it is the butyric acid and cAMP, reported to be released when dibutyl cAMP is degraded intracellularly [17], that both contribute to the observed induction by dibutyl cAMP. The results of this experiment also indicate that cAMP and butyrate bring about enzyme induction by separate, complementary, mechanisms. We have produced further support for the idea that cAMP induces GCAP activity by demonstrating enzyme induction by two cAMP analogues, CPT-cAMP and 8-Br-cAMP, that cannot be degraded to yield butyrate. In addition we used MIX, a phosphodiesterase inhibitor, and CT, an adenylate cyclase activator, to cause an increase in endogenous cAMP levels and have shown these compounds also induce activity. Finally we have shown, using Northern-blot analysis, that CPT-cAMP, 8-Br-cAMP and CT increase the steady-state level of GCAP mRNA.

Our finding that cAMP induces accumulation of GCAP mRNA is supported by recent findings in JEG3 cells using 8-Br-cAMP as inducer [23]. It was shown that there was an increase in the transcription rate of the GCAP gene, but the extent of transcriptional activation by cAMP was judged insufficient to account for the full increase in mRNA. Thus it is possible that cAMP also has post-transcriptional effects, which cause increased accumulation of GCAP mRNA. Post-transcriptional effects of cAMP on gene expression have been demonstrated previously. For example, 8-Br-cAMP has been shown to enhance the half-lives of hCG β (β 3 and β 5) mRNA in JEG3 cells by 1.8- and 3.4-fold respectively [24]. However, other forms of post-transcriptional regulation are known, and involve events within the nucleus such as changes in the efficiency of RNA processing and/or transport out of the nucleus [25,26]. It is of interest that expression of the gene for human tissue-unspecific alkaline phosphatase is regulated post-transcriptionally, by a mechanism which does not involve cytoplasmic mRNA stabilization [27,28]. Therefore it would be interesting to investigate whether the gene for GCAP is regulated by changes in mRNA stability, or whether another post-transcriptional control mechanism is operational. The cAMP control element necessary for induction of the GCAP

gene is still unknown. Povinelli et al. [23] attempted to locate the sequence responsible for stimulation of GCAP transcription by linking the promoter elements of the gene to a reporter gene and measuring its activity following transfection into JEG3 cells, grown in the presence of 8-Br-cAMP. Using this procedure, they were able to show that 8-Br-cAMP induction was maintained when a GCAP promoter up to base -103 was used. The authors suggested that two overlapping 8 bp sequences, showing 69% identity with the cAMP response element (CRE) (5'-TGACGTCA-3'), are responsible for induction of cAMP-mediated transcription.

We have observed that it takes approx. 24 hours for cAMP to bring about any induction of GCAP activity. Assuming that synthesis of increased amounts of GCAP mRNA is reflected shortly afterwards in synthesis of catalytically active GCAP protein, this would tend to suggest that induction may not be due to binding of cAMP to the putative CRE described above, since all genes studied to date, whose expression is increased via binding of a transcription factor to the CRE, respond more rapidly to treatment with cAMP. The transcriptional activation of genes which are rapidly stimulated by cAMP probably involves the phosphorylation of pre-existing transcription factors [29,30]. In contrast, the activation of genes which are stimulated by cAMP with delayed kinetics is either completely or partially blocked by treatment with cycloheximide, implying that indirect mechanisms involving protein synthesis may be required for induction of these genes [24,31-33]. It is thus possible that the gene for GCAP belongs to the latter class. However, conclusive evidence for the theory would depend on testing whether induction of GCAP mRNA by cAMP is blocked by cycloheximide.

Another gene which responds slowly to treatment with cAMP is the hCG β gene. This gene has been analysed in some detail by Albanese et al. [34] in order to find the DNA sequence that confers cAMP responsiveness. They found that there was a sequence upstream of -202, 5'-GCAGGACACACC-3', which served as a protein-binding site and, in combination with several other sequences in this region, conferred cAMP stimulation. It may thus be of interest that there is a sequence at -236 in the GCAP gene which is 100% identical with the first six bases of this sequence and, when compared with the whole sequence, shows 75% identity, i.e. there are three mismatches (the GCAP sequence is 5'-GCAGGATGCACA-3'). Thus it is possible that, if these mismatches do not interfere, the same protein that binds to and causes induction of the HCG β gene might also cause induction of the GCAP gene.

In contrast with the mechanism of cAMP gene regulation, the mechanism by which sodium butyrate regulates gene expression is not so well defined. Sodium butyrate is considered a 'differentiating' agent, in that it can activate tissue-specific gene expression in several kinds of systems *in vitro* [35,36]. For instance, some colonic carcinoma cells lines differentiate or synthesize gene products characteristic of differentiated intestinal epithelium in response to sodium butyrate [37,38]. An example of such a cell line is the colonic carcinoma cell line LS174T, in which butyrate induces accumulation of an incompletely characterized alkaline phosphatase isoenzyme [39].

However, the mechanism, by which butyrate induces gene transcription has not yet been defined. This compound can cause changes in chromatin structure by a variety of mechanisms, such as histone deacetylation, increased phosphorylation of histone H3, inhibition of phosphorylation of histones H1 and H2A, acetylation of some non-histone proteins and possibly by changes in histone methylation [40,41]. Recently, Yeivin et al. [42] have claimed to have identified a butyrate-responsive element (BRE)

associated with the Moloney murine sarcoma virus (MoMSV) enhancer. They also found that enhanced transcription does not appear to be a direct effect of butyrate, but is mediated through proteins synthesized *de novo*. Some interesting research has shown that butyrate may influence cAMP levels in certain types of cell. For example, in leukaemia cells, butyrate has been shown to cause an augmented activation of adenylate cyclase, by signals transmitted from GTP-binding protein [43], raising the possibility that butyrate may exert its effects on gene expression via a cAMP response element. Recent work by Ormandy et al. [44] has shown that phorbol ester tumour promoters and sodium butyrate had similar effects on oestrogen and prolactin receptor levels. Since the oestrogen and prolactin receptors are co-ordinately expressed in human breast cancer cell lines and human breast tumour biopsies [44], this suggests that butyrate may also interact with the protein kinase C transduction pathway.

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