Regulation of the expression of alkaline phosphatase in a human breast-cancer cell line

Tsu-Chung CHANG,*§ Jehng-Kang WANG,* Mei-Whey HUNG,‡ Chih-Hung CHIAO,* Lai-Chen TSAI‡ and Gu-Gang CHANG*

*Department of Biochemistry, National Defense Medical Center and ‡Department of Medical Research, Veterans General Hospital, Taipei, Taiwan, Republic of China

Treatment of the cultured human breast-cancer cells BC-M1 with dexamethasone induced a placental-type alkaline phosphatase (ALP). Both the ALP activity and the mRNA level in the cells were increased. The induction of ALP activity by dexamethasone was time- and dose-dependent. The accumulation of ALP mRNA was inhibited by both actinomycin D and cycloheximide, indicating that its induction is a complex event and may involve other regulatory proteins. Retinoic acid showed opposing effects with dexamethasone on the expression of alkaline phosphatase. Retinoic acid (RA) and phorbol 12-myristate 13-acetate also substantially reduced the dexamethasone-induced expression of ALP. Studies on thermostability and

sensitivity to various amino acid inhibitors indicated that the BC-M1 ALP is most similar to the placental form. Northern hybridization analysis also revealed that ALP mRNA transcripts in BC-M1 and term placenta are similar in size and are distinct from that of the placental-like mRNA transcript in choriocarcinoma cells. Analysis of the degradation of BC-M1 ALP mRNA showed a similar half-life of 27 h in the untreated and in dexamethasone- or RA-treated cells. These findings demonstrated that the induction of ALP in BC-M1 cells by dexamethasone is mainly due to the increase in the transcription of the ALP gene.

INTRODUCTION

Alkaline phosphatase [ALP; orthophosphoric monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1] is a group of phosphatidylinositol-linked membrane proteins with wide substrate specificity [1,2]. These enzymes are widely distributed in Nature, being found in prokaryotes and higher eukaryotes. In humans, at least four distinct classes of this enzyme were identified by genetic and biochemical analysis [3,4]. The isoenzymes from placenta or intestine are tissue-specific and relatively thermostable. The thermolabile isoenzyme characteristic for liver/bone/kidney is non-tissue-specific and present in most cell types. The fourth isoenzyme is a placental-like form and present at low levels in adult germ cells. The placental isoenzyme is of particular interest because of its increased incidence of ectopic or enhanced expression associated with some human cancers [1,5,6]. However, the significance and regulation of their expression have yet to be elucidated. Studying the mechanism that controls the expression of alkaline phosphatase genes should therefore enable us to understand more about the transformation processes.

ALP genes are among the genes that can be modulated by steroid hormones, although the detailed mechanisms are not completely understood. Steroid hormones are known to produce diverse effects and regulate gene expression in a variety of tissues and cell lines [7]. The hormone binds to the receptor protein, followed by accumulation of hormone–receptor complex which interacts with specific DNA sequences adjacent to the regulated genes. Glucocorticoids were shown to induce ALP activity in HeLa and osteoblast cells [8–10]. The placental ALP activity was also induced by oestrogen in human endometrial adenocarcinoma cells [11]. The induction of ALP by progesterin in the oestrogen-receptor-positive breast-cancer cells T47D has also

been studied [12]. In addition to steroids, a variety of agents, such as sodium butyrate and retinoic acid (RA) were shown to regulate ALP activity in various cells [13,14]. Because of the diverse effects of glucocorticoids and RA upon cell differentiation and gene expression, it is important to study the mechanism by which these agents regulate the expression of ALP in different cells. The purpose of the present study was to characterize the isoenzyme form of ALP that was expressed in the cultured oestrogen-receptor-negative human breast-cancer cells, BC-M1 [15]. The changes in expressed enzyme activities in response to glucocorticoids and other potential agents were also investigated. We demonstrated that dexamethasone (Dex) induced the BC-M1 cells to express a placental-type ALP, which was modulated by glucocorticoid and RA through the changes in the ALP mRNA level.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and foetal-bovine serum (FBS) were obtained from Gibco (Grand Island, NY, U.S.A.). Culture plasticware was purchased from Costar (Cambridge, MA, U.S.A.). Human term placental ALP and bovine intestinal ALP (both freeze-dried powders), Dex, RA and phorbol 12-myristate 13-acetate (PMA; 'TPA') were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Term placenta was obtained freshly from the delivery room of the Tri-Service General Hospital, Taipei, Taiwan, and preserved in liquid nitrogen immediately. 5'-[α -³²P]dCTP (6000 Ci/mmol), [5,6-³H]uridine (3000 Ci/mmol) and [³⁵S]methionine (1000 Ci/mmol) were obtained from du Pont/NEN (Boston, MA, U.S.A.). All

Abbreviations used: Dex, dexamethasone; RA, retinoic acid; RAR, RA receptor; PMA, phorbol 12-myristate 13-acetate ('TPA'); ALP, alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal-bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

§ To whom correspondence should be sent at the following address: Department of Biochemistry, National Defense Medical Center, P.O. Box 90048-501, Taipei, Taiwan, Republic of China.

other chemicals used were purchased either from Sigma or E. Merck (Darmstadt, Germany).

Cell culture

Human breast-cancer cells, BC-M1, originally cultured by Dr. Ching-Liang Meng, were supplied from the cell bank of Tri-Service General Hospital, Taipei, Taiwan. The cell line was established from a clinical specimen of breast cancer of a Chinese patient and characterized for anchorage-independent proliferation, chromosome number, and tumorigenicity in nude mouse [15]. The cells expressed polymorphism, prominent nuclei and grown as monolayer cultures with a stable cell proliferation rate. The concentration of oestrogen receptor in BC-M1 cells was determined by a receptor-binding assay [16] and was demonstrated to be less than 10 fmol/mg of protein (S. P. Dan and C. F. Chao, unpublished work). The level of glucocorticoid receptor and RA receptor (RAR)- β were also low in BC-M1 cells, as analysed by Northern-blot analysis. Three different molecular sizes (4.8, 3.5 and 2.6 kb) of RAR- α mRNA transcripts and a single 3.2 kb RAR- γ mRNA transcript were detected in the cells (T.-C. Chang, unpublished work). In the present study, JAR choriocarcinoma cells (from A.T.C.C.) and BC-M1 cells were cultured at 37 °C in DMEM medium supplemented with 15 mM Hepes, 26 mM sodium bicarbonate, 2 mM L-glutamine, 100 μ g/ml streptomycin, 100 units/ml penicillin and 10% (v/v) charcoal/dextran-treated FBS (Gibco) with 5% CO₂. The growth curve of cells was determined by counting the viable cells by Trypan Blue exclusion every 24 h using a haemocytometer. The doubling time of the cell line was calculated while cells were grown exponentially. Cells in the exponential growth phases were used in the present study. The cells were seeded into culture dishes and allowed to attach for 24 h prior to treatment. Hormones and PMA were added to the medium as a concentration stock in ethanol. At the time indicated, the cells were harvested. Control cells were fed with medium containing the same amount of ethanol.

Measurement of ALP activity

Cells were washed with PBS and harvested by scraping with a rubber policeman into the resuspension buffer [20 mM Tris/HCl (pH 8.0)/1 mM MgCl₂/150 mM NaCl]. The cells were solubilized by adding Triton X-100 to give a final concentration of 1%, and the cell extracts were prepared by centrifugation at 12000 *g* for 15 min. The supernatants were assayed for the ALP activity by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at pH 9.8 and 37 °C as described previously [17]. The enzyme specific activity was expressed as $\Delta A_{410}/\text{min}$ per μ g of protein. Protein was determined by the method of Bradford [18].

Characterization of the BCM-1-cell ALP

The stability of the enzyme molecule at 56 °C was determined by incubating the enzyme solution in a constant-temperature water bath. Aliquot enzyme samples were drawn and assayed for enzyme activity. First-order kinetics of the enzyme activity loss were analysed by plotting of $\ln(E_t/E_0)$ versus time, where E_0 and E_t were the enzyme activity at zero time and at time *t* respectively. The slopes of the resulting lines represented the inactivation rate constant (*k*).

An inhibition study was performed by adding the inhibitor into the reaction mixture, and the resulting enzyme activity was compared with that of the control without inhibitor added. Inhibition with L-phenylalanine was further analysed by varying the concentration of *p*-nitrophenyl phosphate from 0.25 to 2 mM and L-phenylalanine from 0 to 3.4 mM. Concentrations of other components were held constant. Fitting of experimental data was carried out by a curve-fitting microcomputer program EZ-FIT [19].

Treatment of BC-M1 cells with RNA and protein-synthesis inhibitors

For experiments in which the effect of actinomycin D or cycloheximide on Dex induction of ALP expression was determined, BC-M1 cells were plated into 100 mm-diameter culture dishes at a seeding density of 10⁶ cells/dish for 24 h. Cells were incubated with 0.01 μ g/ml actinomycin D or 0.5 μ g/ml cycloheximide, either alone or simultaneously with Dex (10⁻⁷ M) for 24 h before being harvested for assay of enzyme activities and mRNA levels. For measurement of the effect of actinomycin D on RNA synthesis, BC-M1 cells were plated into 24-multiwell plates at a density of 2 $\times$ 10⁴ cells/well for 24 h and then incubated with 0.01 μ g/ml actinomycin D for 24 h in triplicate. [5,6-³H]Uridine (2 μ Ci/well) was then added to the culture medium, which was incubated for a further 1 h. The cells were washed twice with PBS and three times with cold 5% trichloroacetic acid and then twice with 100% ethanol. The dried cells were dissolved in 200 μ l of 0.2 M NaOH at 37 °C for 10 min with gentle shaking. Radioactivity incorporated in 100 μ l sample were determined by liquid-scintillation counting. The effect of cycloheximide on protein synthesis was determined by a similarly designed experiment. BC-M1 cells were incubated with 0.5 μ g/ml cycloheximide for 24 h and then labelled with 2 μ Ci/well of [³⁵S]methionine for 1 h. The cells were washed twice with PBS and then solubilized in 200 μ l of cell-lysis buffer [10 mM Tris/HCl (pH 8.0)/150 mM NaCl/10 mM EDTA/0.5% BSA/0.5% Nonidet P40/5 mM iodoacetamide/1 mM phenylmethanesulphonyl fluoride] at 4 °C for 1 h. Insoluble material was removed by centrifugation at 12000 *g* for 5 min at 4 °C, and 100 μ l aliquots of supernatant were precipitated with 10% trichloroacetic acid. The acid-insoluble material was pelleted by centrifugation at 12000 *g* at 4 °C for 10 min and then dissolved in 0.1 ml of 10% SDS. Radioactivity incorporated in acid-insoluble material was determined by liquid-scintillation counting. Cell viability was determined by Trypan Blue exclusion.

Isolation of RNA and Northern-blot analysis

Total RNA was isolated with guanidine isothiocyanate procedure [20]. The fresh placenta or cultured cells were lysed, and proteins were denatured with 1.2 ml of 4 M guanidine isothiocyanate/0.5% sodium dodecylsarcosine/2.5 mM sodium citrate (pH 7.0)/0.1 M 2-mercaptoethanol. The DNA was sheared, and total RNA was pelleted by centrifugation through a 5.7 M CsCl cushion. The RNA concentrations were determined from the absorbance at 260 nm. For Northern analysis, 20 μ g of total RNA were treated with glyoxal/dimethyl sulphoxide at 50 °C for 1 h and size-fractionated on 1.2% agarose gel. The RNA was transferred to nylon filters and baked at 80 °C in a vacuum oven. The filters were prehybridized in 50% formamide/50 mM NaH₂PO₄/Na₂HPO₄ (pH 6.8)/0.5% SDS/0.1 $\times$ Denhardt's solution/1% Ficoll/1% polyvinylpyrrolidone/1% BSA/0.1 mg/ml salmon sperm DNA at 42 °C for 4 h. Labelled probe was added to a concentration of 2 $\times$ 10⁶ c.p.m./ml, and the

incubation continued for 16 h at 42 °C. The filters were washed with 0.1 % SDS in 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) for 10 min at 55 °C and then washed twice with 0.1 × SSC containing 0.1 % SDS at 65 °C for 30 min. After washing, the filters were exposed to X-ray film with intensifying screen at −70 °C for 3–5 days. The cDNA probes were prepared by random primer extension with [α - 32 P]dCTP to a specific radioactivity of about 5×10^8 c.p.m./ μ g of DNA according to the manufacturer's instructions (Boehringer Mannheim). The full-length human placental ALP cDNA was generously given by Dr. José Luis Millán [21]. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) full-length cDNA template was the 1.25 kb *Pst*I fragment of the plasmid pHcGAP [22]. Autoradiography results were normalized with respect to GAPDH mRNA as determined by hybridization analysis. A Molecular Dynamics computing laser densitometer with Image Quant software was used to quantify the autoradiographic results.

RESULTS

Effect of Dex, RA and PMA on the growth of BC-M1 cells

Treatment of the BC-M1 cells with Dex or RA did not significantly alter the cell number (Figure 1). The growth-inhibitory effect of RA was observed only at 10^{-6} M, as shown in Figure 1. RA treatment had no significant effects on the cell morphology (results not shown). The population doubling time during the exponential growth phase was calculated to be 18 h. PMA and RA were also shown to inhibit the growth of many other tumour cells [23–25]. The viability of BC-M1 cells treated with Dex, RA or PMA showed no significant difference, indicating that the inhibition of cell growth by both RA and PMA was not due to the toxic effects of these agents.

Effects of Dex on the ALP activity

The kinetics of Dex-induced alteration of ALP activity were measured for BC-M1 cells. Triplicate cultures were grown in the presence or absence of various concentrations of Dex and harvested after 48 h for the assay of ALP activity. As shown in Figure 2, the specific activity of ALP was induced gradually as the cells were treated with an increasing concentration of Dex. The maximum induction in ALP activity was about 3–4-fold at a concentration of 10^{-7} M.

The time course of induction of ALP activity in BC-M1 cells was determined by treating the cells with or without 10^{-7} M Dex for the time indicated (Figure 3). The enzyme activity reached a plateau after 36 h treatment, with an average 3-fold induction. The mean values of enzyme activity in Dex-treated samples were significantly different from those of the untreated controls after 6 h of Dex incubation.

Characterization of ALP expressed in BC-M1 cells

Several biochemical methods, including thermostability of the enzyme and sensitivity of the enzyme to specific inhibitors, were used to differentiate the various isoenzymes of ALP [3,4]. As shown in Table 1, the ALP from BC-M1 cells behaves more like the thermostable placental or placental-like form when we compared the first-order inactivation rate constant (k) of the enzyme activity loss at 56 °C. The ALPs from Dex-treated and untreated BC-M1 cells have similar thermostability. The inactivation rate constant for the Dex-treated, untreated BC-M1 samples, placental isoenzyme and intestinal isoenzyme were

found to be 0.011, 0.012, 0.006 and 0.073 min $^{-1}$ respectively (Table 1b). The sensitivity of the enzyme to specific inhibitors was also investigated. The BC-M1 enzyme, like the placental isoenzyme, was sensitive to L-phenylalanine and less sensitive to L-leucine, but resistant to L-homoarginine and EDTA (Table 1a). Since the germ-cell ALP has been shown to be more sensitive to EDTA and L-leucine than is the placental isoenzyme [26,27], our results indicated that the BC-M1 enzyme is more likely to be the placental form. L-Phenylalanine was a specific uncompetitive inhibitor for the placental ALP, but was insensitive to the non-tissue-specific ALP [1,2]. Inhibition by L-phenylalanine was thus analysed with cancer-cell ALP. As shown in Figure 4, with L-

Figure 1 Effect of Dex, RA and phorbol ester on the proliferation of BC-M1 cells

BC-M1 cells were subcultured into 24-well dishes in DMEM containing 10% charcoal/dextran-stripped FBS at a seeding density of 2×10^3 cells/well. Cells were treated for 96 h with the indicated concentrations of Dex, RA and PMA. The open circle (○) indicates the untreated samples. Cells were harvested on day 5, and cells numbers were counted with a haemocytometer. The culture medium was changed every 48 h. Results are means $\pm$ S.E.M. ($n = 3$).

Figure 2 Dose-response of the Dex-induced ALP activity in BC-M1 cells

BC-M1 cells were subcultured into 24-well dishes in DMEM containing 10% charcoal/dextran-treated FBS at a seeding density of 1×10^5 cells/well. After 12 h the cells were incubated in the absence (○) or presence (●) of various concentrations of Dex. After 48 h the cells were harvested, and crude cell extract was assayed for ALP activity. Results are means $\pm$ S.E.M. ($n = 3$).

Figure 3 Time course of the Dex-induced ALP activity in BC-M1 cells

BC-M1 cells were incubated in the absence (○) or presence (●) of 10^{-7} M Dex. At designated times, individual cultures were harvested and their ALP activity was assayed. Results are means $\pm$ S.E.M. ($n = 3$).

Table 1 Effects of specific inhibitors and heating on BCM-1 ALP activity

ALP from human placental (PLAP), bovine intestinal (IALP), and BC-M1 cells were incubated in the absence (control) or presence of Dex (10^{-7} M) for 48 h. Crude cell extracts were assayed for enzyme activity. In (a), each inhibitor was added to the standard assay mixture. The final concentration was 5 mM for L-leucine, L-phenylalanine and L-homoarginine and 0.1 mM for EDTA. Percentage activity remaining was calculated relative to the control samples containing no inhibitor. Results are means $\pm$ S.E.M. ($n = 3$). In (b), enzyme samples were precipitated at 56 °C in assay buffer for 0, 10, 20, 40 and 60 min. The enzyme activity loss followed first-order kinetics with a rate constant of k , which was the average for two experiments.

	Remaining activity (% of control)			
	BCM-1 ALP			
	Control	Dex	PALP	IALP
(a) Inhibitor				
EDTA	102 $\pm$ 3	95 $\pm$ 8	72 $\pm$ 8	12 $\pm$ 1
L-Leucine	50 $\pm$ 3	48 $\pm$ 2	55 $\pm$ 3	76 $\pm$ 5
L-Phenylalanine	38 $\pm$ 2	32 $\pm$ 2	21 $\pm$ 4	71 $\pm$ 2
L-Homoarginine	98 $\pm$ 7	87 $\pm$ 6	84 $\pm$ 8	86 $\pm$ 14
(b) Heat-inactivation				
k (min^{-1}) ...	0.012	0.011	0.006	0.073

phenylalanine as the inhibitor and *p*-nitrophenyl phosphate as the varied-concentration substrate, a series of parallel lines were obtained, conforming to an uncompetitive inhibition pattern. From the inset Figure, the K_i value was determined to be 1.16 ± 0.06 mM, almost identical with that of the human placental ALP [28]. Since Ovitt et al. [29] showed that the placental-like ALP mRNA transcript in choriocarcinoma cells were about 0.4 kb shorter than that of the 3.0 kb placental mRNA transcript, we performed Northern hybridization analysis in order to compare the size of ALP mRNA transcripts in term placenta, BC-M1 and JAR choriocarcinoma cells. Total RNA isolated from the placenta and cultured cells were electrophoresed and hybridized with the full-length human placental ALP cDNA. As shown in Figure 5, the size of ALP mRNA transcripts in BC-M1 cells and placenta are identical (approx. 3.0 kb) and are distinct

Figure 4 Inhibition of human breast-cancer cell ALP by L-phenylalanine

Enzymic activity was assayed as described in the Materials and methods section, except that *p*-nitrophenyl phosphate and L-phenylalanine concentrations were varied as indicated. From bottom to top, the L-phenylalanine concentrations were: ○, 0 mM; ●, 0.8 mM; □, 2.0 mM; and ■, 3.5 mM. The inset shows the vertical intercept replotted against [L-phenylalanine].

Figure 5 Northern analysis of ALP mRNA in placenta, BC-M1 and JAR cells

Total RNA (10 μ g) isolated from term placenta, BC-M1 and JAR choriocarcinoma cells were fractionated, transblotted, hybridized with 32 P-labelled DNA probes of human placental ALP and GAPDH and then autoradiographed. The total RNAs analysed were as follows: lane 1, term placenta; lane 2, BC-M1; lane 3, JAR. ALP mRNA from term placenta and BC-M1 cells migrate with an apparent size of 3.0 kb relative to 18 S and 28 S rRNAs. The JAR choriocarcinoma ALP mRNA is about 2.6 kb in size. The exposure time was 5 days.

from the 2.6 kb mRNA transcript in JAR choriocarcinoma cells. Taken together, these results demonstrated that ALP expressed in BC-M1 cells is likely to be the placental isoenzyme and the ALP activity and mRNA expressed is identical in the Dex-treated and untreated control cells.

Effects of Dex, RA and PMA on BC-M1 ALP activity and mRNA levels

The effect of Dex, RA and PMA on ALP mRNA levels was measured by Northern hybridization. Table 2 summarizes the

Table 2 Effects of steroid hormones and various agents on activity and mRNA level in BC-M1 cells

BC-M1 cells were treated in the absence (control) or presence of hormones and agents for 48 h. ALP activity and mRNA levels were measured as described in the Materials and methods section. The data shown are ratios of specific activity or densitometric units of the treated and untreated control samples. Results for enzyme activity are means $\pm$ S.E.M ($n = 3$) and for mRNA are the average for two experiments that were normalized with respect to GAPDH.

Treatment	Dose (M)	ALP activity	ALP mRNA
Control	0	100	100
Dex	10^{-6}	342 ± 7.7	292
Dex	10^{-7}	353 ± 5.3	432
RA	10^{-7}	72 ± 3.8	63
PMA	10^{-7}	121 ± 7.8	137
Dex + RA	$10^{-7} + 10^{-7}$	75 ± 4.6	65
Rex + PMA	$10^{-7} + 10^{-7}$	181 ± 5.4	141

relative abundance of cytoplasmic mRNA for ALP quantified by densitometric analysis and normalized to the amount of GAPDH mRNA. In agreement with the enzyme-activity alterations, Dex increased the steady-state levels of BC-M1-cell ALP mRNA by about 3–4-fold. Maximum induction of both enzyme activity and mRNA level was achieved at 10^{-7} M Dex (Figure 2 and Table 2). While the protein kinase C activator PMA showed no significant effect on BC-M1 ALP activity, RA decreased both the enzyme activity and mRNA level. Since the alterations in the level of ALP mRNA closely matched the enzyme activity, the effects of Dex and RA on ALP activity in BC-M1 cells were primarily at the level of gene transcription. The results shown in Table 2 also indicate that RA and PMA abolished the Dex-induced mRNA level.

Effect of actinomycin D and cycloheximide on the induction of ALP in BC-M1 cells

In order to determine whether the induction of ALP expression in BC-M1 cells was dependent on *de novo* RNA or protein synthesis, treatment of the cells with actinomycin D or cycloheximide was performed. BC-M1 cells were incubated with 0.01 μ g/ml actinomycin D or 0.5 μ g/ml cycloheximide alone or in combination with 10^{-7} M Dex for 24 h before being harvested for assay of ALP activities and mRNA levels. The concentrations of actinomycin D and cycloheximide used in these experiments were chosen because the preliminary results indicated that higher concentrations of either agent showed significant toxic effect on the cells after 24 h incubation as assessed by the Trypan Blue exclusion for the viable cells. In this experiment, RNA synthesis was inhibited by an average of 90% at the concentration of actinomycin D used over the period of 24 h as measured by the incorporation of [3 H]uridine into the cells. Treatment of the cells with 0.5 μ g/ml cycloheximide inhibited protein synthesis by about 90% over a period of 24 h, as measured by the incorporation of [35 S]methionine into cell extract precipitable by 10% trichloroacetic acid. Results shown in Figure 6 indicate that the ALP activity and mRNA level are closely matched in most samples. Incubation with Dex alone induced ALP expression levels by approx. 200% over the period of 24 h. In the absence of Dex, actinomycin D or cycloheximide treatment decreased control levels of ALP expression by about 50 and 80% respectively. When incubated simultaneously with Dex, both actinomycin D and cycloheximide completely blocked ALP activity and mRNA induction. These results indicate that the induction

Figure 6 Effects of actinomycin D and cycloheximide on Dex-induced ALP expression

BC-M1 cells were subcultured into 100 mm-diameter dishes at a seeding density of 10^6 cells/dish for 24 h. After a change to fresh medium, the cultures were continued and treated as follows: untreated control for 24 h (lane 1); 10^{-7} M Dex for 24 h (lane 2); 0.01 μ g/ml actinomycin D for 24 h (lane 3); 0.5 μ g/ml cycloheximide for 24 h (lane 4); 10^{-7} M Dex plus 0.01 μ g/ml actinomycin D for 24 h (lane 5); 10^{-7} M Dex plus 0.5 μ g/ml cycloheximide for 24 h (lane 6). (a) ALP activity and mRNA levels. The data shown are percentage of specific activity or densitometric units of treated relative to untreated control samples. Results for the enzyme activity are means for three experiments. Values for mRNA levels are the average of results for two experiments that were normalized with respect to the level of GAPDH mRNA. (b) Autoradiogram of the same experiment as that described above. Total RNA (20 μ g) from each sample was fractionated, transblotted, hybridized with 32 P-labelled DNA probes of human placental ALP and GAPDH and autoradiographed. The exposure time was 3 days.

of ALP by Dex in BC-M1 cells occurs primarily at the transcription level and is dependent on RNA and protein biosynthesis.

Stability of ALP mRNA in the control and treated cells

To assess the mechanism of the effect of Dex and RA on the ALP mRNA level, the half-life of the mRNA for the treated and untreated cells was measured. The Dex- or RA-treated cells were exposed to 25 μ g/ml 5,6-dichloro-1 β -D-ribofuranosylbenzimidazole for 0, 14, 24 and 37 h, and the decay of ALP mRNA was detected by Northern hybridization (Figure 7a). The half-life of ALP mRNA was calculated by using the level of internal-control GAPDH mRNA for normalization. 5,6-Dichloro-1 β -D-ribofuranosylbenzimidazole is known to selectively inhibit the synthesis of heterogeneous nuclear RNA without interfering with synthesis of ribosomal or transfer RNA [30]. In both control and the hormone-treated cells the ALP mRNA was found to decrease with a similar half-life (~ 27 h) (Figure 7b). These results indicated that there is no apparent difference in ALP mRNA stability in either Dex- or RA-treated and untreated control BC-M1 cells.

DISCUSSION

The results obtained from this study indicate that human breast-cancer cell BC-M1 expresses a placental-type ALP which is inducible by Dex. The specific activity of ALP increased to approx. 3-fold after 36 h treatment with Dex (Figure 3). The Dex

Figure 7 ALP mRNA stability in the RA- or Dex-treated cells

BC-M1 cells were cultured in the absence (control) or presence of 10^{-7} M Dex or RA (results not shown in **a**) for 48 h with medium replacement. 5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole (DRB; 25 μ g/ml) was then added and the cells were incubated for additional 14, 24 or 37 h. **(a)** At the time indicated, total RNA (20 μ g) was fractionated, transblotted, hybridized with 32 P-labelled DNA probes of human placental ALP and human GAPDH, and autoradiographed. The exposure time was 5 days. **(b)** Densitometric quantification of the ALP mRNA stability in BC-M1 cells. Values for the levels of ALP mRNA from two experiments were normalized with respect to GAPDH mRNA. Results are expressed as the percentage of initial mRNA levels.

concentration giving a maximum induction of ALP activity was approx. 100 nM (Figure 2). This concentration is comparable with the induction of ALP by steroid hormones in other cells [10,31,32]. The changes in ALP activity in response to Dex treatment closely matched its mRNA levels (Table 2). An increase in enzyme activity was detected at about 12 h after the hormone treatment (Figure 3). Since a primary hormone effect should be much more rapid, the delayed response to Dex suggests that the glucocorticoid induction of ALP is a secondary effect and other uncharacterized regulatory proteins are probably involved [32,33]. The fact that Dex-induced rising of ALP mRNA level was completely abolished by co-incubation of the cells with the protein-synthesis inhibitor cycloheximide also suggests the involvement of *de novo* protein biosynthesis in the action of Dex on ALP. In addition, our results indicate that the induced ALP activity is also the result of an increased gene transcription by glucocorticoid, as co-incubation of the cells with actinomycin D also abolished the Dex-induced increase in mRNA (Figure 6). These results demonstrate that induction of ALP activity in BC-M1 cells by Dex is dependent on both protein and mRNA biosynthesis.

Human ALPs are membrane-bound proteins that exist as homodimers [3,4]. In this study we demonstrate that the BC-M1 ALP is similar to the placental isoenzyme in terms of the sensitivity to specific inhibitors, thermostability, inhibition constant of L-phenylalanine and the apparent size of ALP mRNA transcript (Table 1, Figures 4 and 5). Previous studies indicated that the germ-cell ALP is more sensitive to L-leucine and EDTA [26,27]. Since the BC-M1 and placental isoenzymes exerted comparable sensitivity toward L-leucine and EDTA, we believe that the BC-M1 ALP is likely to be the placental type rather than the germ-cell isoenzyme. The identical size (3.0 kb) of ALP mRNA transcripts in BC-M1 cells and placenta further demonstrate that BC-M1 isoenzyme is the placental form, since the placental-like mRNA transcripts are approx. 0.4 kb shorter in length [29]. The immunological analysis using isoenzyme-specific antibodies should be a conclusive method to characterize the ALP isoenzyme. Experiments designed to allow purified ALP from the BC-M1 cells to react with the isoenzyme-specific monoclonal antibodies is currently underway in our laboratory. Several growth factors and hormones were shown to modulate the expression and activity of ALP in various tissues and cultured cells [8–14]. The results presented in Table 2 demonstrate that the transcriptional activation of the ALP by Dex was inhibited by PMA. There is a clear correlation between enzyme activity and mRNA level of ALP. Phorbol esters, such as PMA, are known to influence cell functions mainly through the effector molecule Jun/AP-1 of the protein kinase C pathway [34]. The mutual antagonism of glucocorticoid and phorbol esters in the regulation of ALP expression is consistent with previous findings in many genes such as the collagenase and proliferin genes [35,36]. Although RA showed no significant effect on the proliferation of BC-M1 cells, the expression of ALP in BC-M1 cells was substantially inhibited when the cells were treated with RA, either in the absence or in the presence of Dex. The opposite effects of RA and glucocorticoids have been described previously for the expression of ALP, collagen, catecholamine receptor and G-proteins in different cultured cells [13,14]. The mechanism of action of RA is similar to that of steroid hormones inasmuch as it involves binding to specific nuclear receptors and regulating the gene transcription. The interaction between RA and Dex at the transcriptional level was demonstrated in the expression of osteonectin and ALP in the preosteoblasts [13]. Alterations in the mRNA level and in *de novo* protein biosynthesis were also described for the regulation of ALP by steroids or RA [9–11,13,14,31]. Considering that RA and phorbol esters modulate gene expression through different pathways, it is possible that the inhibition of Dex-induced ALP mRNA by these agents resulted from different mechanisms in cultured BC-M1 cells. These findings again suggest that mediator proteins are involved in the regulation of ALP expression in BC-M1 cells. Regulation of ALP gene expression by hormones and various agents occurred in different cells or tissues by a variety of mechanisms. In HeLa cells, it was shown that glucocorticoids induced the ALP activity by altering the kinetic parameters of pre-existing enzyme molecules, although evidence also supports an enzyme-induction mechanism [8,9,37]. Inhibition of protein glycosylation by 2-deoxyglucose or tunicamycin also significantly reduced the ALP activity [38]. While steroids act primarily by altering the rate of transcription of specific genes, post-transcriptional factors, such as mRNA stability, might also play a significant role. In this study, experiments using the RNA-synthesis inhibitor 5,6-dichlororibofuranosylbenzimidazole demonstrated that neither Dex nor RA has significant effect on the stability of BC-M1 ALP mRNA (Figure 7). Our results are thus consistent with other reports that the ALP mRNA stability in the osteoblast-like cells

or teratocarcinoma cells was not affected by Dex or RA respectively [32,39]. The results indicated that regulation of ALP mRNA in BC-M1 cells by either Dex or RA occurred primarily at the transcriptional level and not due to the stabilization of pre-existing mRNA. Taken together, the results in this study demonstrated that the alterations in ALP-gene expression under various conditions are complex events and due to modulation of gene transcription. Further work is required to study the basis for the regulation of ALP-gene expression during hormonal induction.

This work was supported by a grant from the National Science Council (NSC 80-0412-B016-15 to T.C.C.) and Academia Sinica (to G.G.C.), Taiwan, Republic of China. We thank Dr. Jose Luis Millán for the placental ALP cDNA probe.

REFERENCES

- 1 Fishman, W. H. (1987) *Adv. Cancer Res.* **48**, 1–35
- 2 Harris, H. (1989) *Clin. Chim. Acta* **186**, 133–150
- 3 Harris, H. (1982) *Harvey Lect.* **76**, 95–124
- 4 Stigbrand, T., Millán, J. L. and Fishman, W. H. (1982) *Isoenzymes: Curr. Top. Biol. Med. Res.* **6**, 93–117
- 5 Wahren, B., Holmgren, P. A. and Stigbrand, T. (1979) *Int. J. Cancer* **24**, 749–753
- 6 Lange, P. H., Millán, J. L., Stigbrand, T., Vessella, R. L., Ruoslahti, E. and Fishman, W. H. (1982) *Cancer Res.* **42**, 3244–3247
- 7 Yamamoto, K. R. (1985) *Annu. Rev. Genet.* **19**, 209–252
- 8 Ghosh, N. H., Rukenstein, A., Baltimore, R. and Cox, R. P. (1972) *Biochim. Biophys. Acta* **286**, 175–185
- 9 Chou, J. Y. and Takahashi, S. (1987) *Biochemistry* **26**, 3596–3602
- 10 Majeska, R. J., Nair, B. C. and Rodan, G. A. (1985) *Endocrinology (Baltimore)* **116**, 170–179
- 11 Albert, J. A., Sundstrom, S. A. and Lyttle, C. R. (1990) *Cancer Res.* **50**, 3306–3310
- 12 Lorenzo, D. D., Albertini, A. and Zava, D. (1991) *Cancer Res.* **51**, 4470–4475
- 13 Ng, K. W., Manji, S. S., Young, M. F. and Findlay, D. M. (1989) *Mol. Endocrinol.* **3**, 2079–2085
- 14 Imai, Y., Rodan, S. B. and Rodan, G. A. (1988) *Endocrinology (Baltimore)* **122**, 456–463
- 15 Meng, C. L., Peng, F. K. and Shan, T. Y. (1985) *Bull. Chin. Oncol. Soc.* **1**, 27–28
- 16 Zava, D. T. and McGuire, W. L. (1977) *J. Biol. Chem.* **252**, 3703–3708
- 17 Chang, T. C., Huang, S. M., Huang, T. M. and Chang, G. G. (1992) *Eur. J. Biochem.* **209**, 241–247
- 18 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 19 Perrilla, F. W. (1988) *Anal. Biochem.* **174**, 437–447
- 20 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 21 Millán, J. L. (1986) *J. Biol. Chem.* **261**, 3112–3115
- 22 Tso, J. Y., Sun, X. H., Kao, T. H., Reece, K. S. and Wu, R. (1985) *Nucleic Acids Res.* **13**, 2485–2502
- 23 Fraker, L. D., Halter, S. A. and Forbes, J. T. (1984) *Cancer Res.* **44**, 5757–5763
- 24 March, C., Bock, G. and Daxenbichler, G. (1984) *Biochem. Pharmacol.* **33**, 2217–2221
- 25 Marth, C., Bock, G. and Daxenbichler, G. (1985) *J. Natl. Cancer Inst.* **75**, 871–875
- 26 Nakayama, T., Yoshida, M. and Kitamura, M. (1970) *Clin. Chim. Acta* **30**, 546–548
- 27 Sakiyama, T., Robinson, J. C. and Chou, J. Y. (1978) *Arch. Biochem. Biophys.* **191**, 782–791
- 28 Chang, G. G., Pan, F. and Yeh, M. J. (1981) *Proc. Natl. Sci. Counc. Repub. China* **85**, 190–197
- 29 Ovitt, C. R., Strauss, A. W., Alpers, D. H., Chou, J. Y. and Boime, I. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3781–3785
- 30 Sehgal, P. B., Darnell, J. E., Jr. and Tamm, I. (1976) *Cell* **9**, 473–480
- 31 Mulkins, M. A. and Allison, A. C. (1987) *J. Cell. Physiol.* **133**, 539–545
- 32 Green, E., Todd, B. and Heath, D. (1990) *Eur. J. Biochem.* **188**, 147–153
- 33 Diamond, D. J. and Goodman, H. M. (1985) *J. Mol. Biol.* **181**, 41–62
- 34 Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P. and Karin, M. (1987) *Cell* **49**, 729–739
- 35 Schule, R., Rangarajan, P., Kliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M. and Evans, R. M. (1990) *Cell* **62**, 1217–1226
- 36 Diamond, M. I., Miner, J. N., Yoshinaga, S. K. and Yamamoto, K. R. (1990) *Science* **249**, 1266–1272
- 37 Hanford, W. C., Kottel, R. H. and Fishman, W. H. (1981) *Biochem. J.* **200**, 461–464
- 38 Pan, C. J., Sartwell, A. D. and Chou, J. C. (1991) *Cancer Res.* **51**, 2058–2062
- 39 Scheme, R. J. R., Moeller-Runge, I. and Muller, W. H. (1991) *J. Biol. Chem.* **266**, 21300–21305