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Retinoic acid induces liver/bone/kidney-type alkaline phosphatase gene expression in F9 teratocarcinoma cells

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All-trans retinoic acid (RA) induces alkaline phosphatase (ALP) activity by 3–8-fold in murine F9 teratocarcinoma cells, in parallel with their differentiation towards primitive endoderm. The elevation of ALP activity is associated with increases in the amounts of liver/bone/kidney-type ALP protein and the respective transcript. These effects of RA are due to activation of ALP gene transcription rather than to an increase in the half-life of the mRNA. Induction of ALP mRNA does not require *de novo* protein synthesis, since it is not blocked by treatment with cycloheximide. Dibutyryl cyclic AMP, which is known to induce further differentiation of F9 cells from the primitive to the parietal endoderm, blocks the induction of ALP mRNA by RA.

INTRODUCTION

Alkaline phosphatases (ALPs) [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] constitute a family of isoenzymes capable of hydrolysing organic monophosphate esters *in vitro*. The physiological function of ALPs, however, is not known, in spite of thorough knowledge on the gene structure of many members of the enzyme family in various animal species (Kam *et al.*, 1985; Weiss *et al.*, 1986; Berger *et al.*, 1987; Terao & Mintz, 1987; Garattini *et al.*, 1987; Misumi *et al.*, 1988).

In the adult mouse at least two different ALP isoenzymes are known (Goldstein *et al.*, 1980; Wilcox, 1983), i.e. liver/bone/kidney-type ALP (LBK-ALP) and intestinal ALP. The former is expressed mainly in liver, bone, kidney and placenta, whereas the expression of the latter is limited to the intestine (McComb *et al.*, 1979). ALP enzymic activity is also found in various embryonal cells (Chiquoine, 1954; Mintz & Russel, 1957); however, the isoenzymic form in these cells has not yet been identified. Particularly high levels of ALP expression are observed in primordial germ cells, whose migration from the yolk sac towards the genital ridges can be traced by using ALP activity as a marker (Mintz & Russel, 1957). The importance of ALP during embryogenesis has been further substantiated by a report suggesting that ALP might influence embryonic cell migrations in the axolotl (Zackson & Steinberg, 1988).

Mouse teratocarcinoma cells resemble closely embryonic stem cells (Graham, 1977; Martin, 1980), and provide a simple means for the study of cell differentiation *in vitro* (Strickland & Mahdavi, 1978). One of these murine cell lines, F9 tetratocarcinoma, has been used more extensively than any other. F9 cells differentiate into primitive, parietal and visceral endoderm depending on the culture conditions employed (Martin, 1980; Strickland & Sawey, 1980; Strickland *et al.*, 1980; Hogan *et al.*, 1981, 1983). Differentiation towards the primitive endoderm is obtained by treatment of adherent undifferentiated F9 cells with various organic compounds, such as retinoic acid (RA) and its derivatives (Strickland & Mahdavi, 1978; Sherman, 1986; Schindler, 1986). F9 tetratocarcinoma cells as well as other embryonal cells are known to constitutively express ALP enzymic activity in their undifferentiated state (Strickland & Mahdavi, 1978; Terao & Mintz, 1987). It has been shown that the isoenzyme form expressed in these cell lines is the LBK-type ALP (Terao & Mintz, 1987; Hahnel & Schultz, 1990). Differentiation of F9 cells induced by RA is accompanied by induction of ALP activity as well as by increased levels of several other gene products, such as tissue plasminogen activator (Strickland & Mahdavi, 1978), laminin B1 and collagen type IV (Wang & Gudas, 1983; Wang *et al.*, 1985). An elevation in ALP activity is observed after 48 h of treatment with RA, and this is closely correlated with the maximal morphological and biochemical differentiation of F9 cells into primitive endoderm (Strickland & Mahdavi, 1978). F9 cells thus constitute a good *in vitro* model with which to study the mechanism of induction of ALP by RA and the relationship between increased expression of ALP and induction of a differentiated phenotype in embryonic cells.

The data presented in this report help in understanding the molecular mechanisms underlying ALP induction by RA in F9 teratocarcinoma cells. They may also be useful, in more general terms, in comprehending the action of RA, which is currently the only recognized morphogen (Thaller & Eichele, 1987).

MATERIALS AND METHODS

Cell culture and reagents

F9 teratocarcinoma cells, a gift from Dr. B. Terrana (Sclavo Laboratories, Siena, Italy), were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. The cells were seeded at a concentration of 10^5 cells/ml in 25 cm^2 Falcon culture flasks (Becton Dickinson, Lincoln Park, NJ, U.S.A.) and allowed to attach to the plastic substrate for 4–6 h before treatment with RA or dibutyryl cyclic AMP. Control cultures were always run in parallel. Unless otherwise stated, the concentration of RA used throughout the experiments was 1 μM, a concentration known to induce differentiation of F9 cells towards the primitive endoderm. Differentiation of F9 cells was evaluated by measuring the induction of tissue plasminogen activator (Erickson *et al.*, 1984). COS cells were obtained from the

Abbreviations used: ALP, alkaline phosphatase; LBK-type ALP, liver/bone/kidney-type ALP; RA, retinoic acid; RAR, RA receptor; RABP, RA-binding protein.

American Type Culture Collection and were cultured in the same conditions used for F9 cells.

Cultures were free from mycoplasma, as assessed using the Hoechst 33258 fluorescent dye system (Farbwerke Hoechst AG, Frankfurt, Germany). RA, cycloheximide, actinomycin D and dibutyryl cyclic AMP were from Sigma (St. Louis, MO, U.S.A.).

Measurement of ALP activity

Cell monolayers from a 25 cm^2 dish were washed twice with 0.9% NaCl, harvested using a rubber policeman and pelleted by centrifugation at 400 *g* for 10 min. Cells were resuspended in homogenization buffer (1 mM-$MgCl_2$/1 mM-$CaCl_2$/20 μM-$ZnCl_2$/0.1 M-NaCl/0.05 M-Tris/HCl, pH 7.4) and disrupted by sonication using a Branson sonifier at its maximum setting for 2 × 2 s at 4 °C. The total homogenate was used for ALP assays, which were performed using *p*-nitrophenol phosphate (Sigma) as substrate, according to the instructions of the manufacturer.

ALP activity was normalized for the content of protein in the sample. Proteins were measured according to the Bradford (1976) method using BSA as standard. One unit is defined as the amount of enzyme capable of transforming 1 μmol of substrate in 1 min at 37 °C.

Western blotting analysis

Western blotting analysis was performed as described previously using a polyclonal antibody raised against the *N*-terminal peptide of human LBK-type ALP (Garattini *et al.*, 1986). The specificity of this antibody as well as its cross-reactivity with mouse LBK-type ALP have been described previously (Terao & Mintz, 1987). Quantification of the ALP protein band was performed by laser scanning of the negative film obtained from a photograph of the Western blot. COS cell protein extracts were used as a negative control, as preliminary experiments showed that this cell line does not express ALP activity.

Northern blotting analysis

Total RNA was prepared from F9 teratocarcinoma cells according to a modification of the guanidium isothiocyanate/CsCl method (Rambaldi *et al.*, 1987). RNA (10 or 15 μg) was then fractionated on a 1.2% agarose gel with 6% formaldehyde and blotted on to synthetic nylon membranes (Gene Screen Plus; New England Nuclear, Boston, MA, U.S.A.). These membranes were hybridized with a 2 kb *Eco*RI–*Bgl*II fragment of mouse placental cDNA (Terao & Mintz, 1987), mouse actin cDNA (Minty *et al.*, 1981) and histone H4 cDNA (Seiler-Tuyns & Birnstiel, 1981). The various probes were labelled to a specific radioactivity of (1–2) × 10^9 c.p.m./μg by using hexanucleotide primers and [^{32}P]dCTP (Feinberg & Vogelstein, 1983). Hybridization was performed at 60 °C overnight in a solution containing 1 M-NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate (Sigma), 100 μg of salmon-sperm DNA/ml (Boehringer, Mannheim, Germany) and (1–2) × 10^6 c.p.m. of labelled probe/ml. The membranes were washed twice with 2 × SSC/1% SDS (1 × SSC = 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0) for 1 h at 65 °C and with 0.1 × SSC for 30 min at room temperature. The membranes were dried and exposed to Kodak X-Omat X-ray films with two intensifying screens (Dupont Cronex) at −70 °C.

Nuclear transcription run-on assay

Nuclear transcription run-on assays were performed as described by Greenberg & Ziff (1984) with some modifications. Briefly, nuclei were prepared by lysing cells with 4 ml of lysis buffer [0.5% (w/v) Nonidet P-40/0.01 mM-NaCl/3 mM-$MgCl_2$/0.01 M-Tris, pH 7.4]. After washing with ice-cold lysis buffer, nuclei were resuspended in glycerol buffer [40% (w/v) glycerol/5 mM-$MgCl_2$/0.1 mM-EDTA/0.05 M-Tris, pH 8.0] and incubated at 30 °C for 30 min in run-on buffer containing 5 mM-Tris, pH 8.0. 2.5 mM-$MgCl_2$, 0.15 M-KCl, 1.25 mM each of ATP, CTP and GTP (Pharmacia), and 100 μCi of [^{32}P]UTP (Amersham). Nuclei were then resuspended in 4 M-guanidium isothiocyanate and nascent RNA was recovered by centrifugation through CsCl and ethanol precipitation. Labelled elongated RNAs (minimum 1 × 10^6 c.p.m./ml) were hybridized to 5 μg of each of the cDNA plasmids immobilized on nitrocellulose membranes after denaturation by heat and alkali treatment. The filters were washed at a stringency of 2 × SSC at 55 °C for 1 h and then treated with 10 μg of RNAase A/ml (Sigma) in 2 × SSC for 30 min at 37 °C. cDNAs used for these experiments were mouse placental ALP cDNA (Terao & Mintz, 1987), histone H2a cDNA (Hatch & Bonner, 1988) and actin cDNA (Minty *et al.*, 1981). Autoradiograms of both Northern blotting analysis and nuclear run-on assays were quantified by laser scanning densitometry using a laser beam densitometer (300 A computing densitometer Fast Scan; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

The differentiation of F9 teratocarcinoma cells towards primitive endoderm induced by RA was accompanied by an increase in ALP activity, as expected from other reports (Strickland & Mahdavi, 1978). Under our experimental conditions the kinetics of induction of ALP activity reached a maximum at 48 h and was maintained for at least 72 h; a statistically significant increase compared with control cells was observed after only 16 h. A

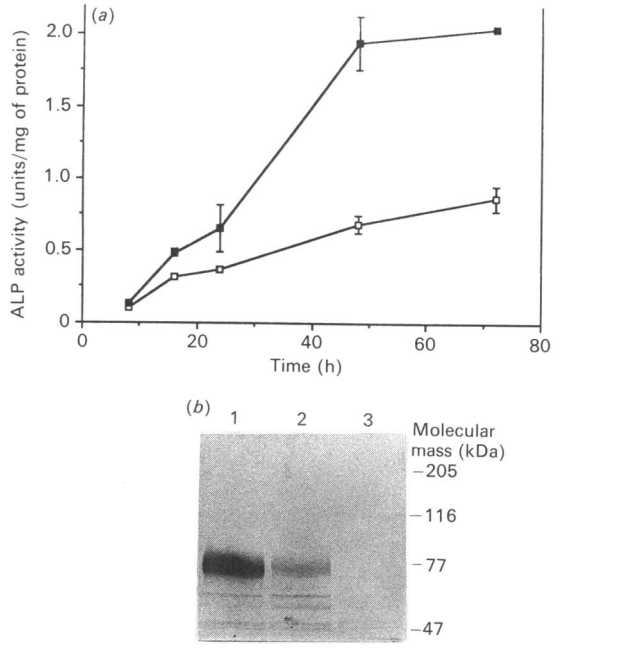

Fig. 1. Induction of ALP during RA-induced differentiation of F9 teratocarcinoma cells towards the primitive endoderm

(*a*) Time course of the induction of ALP activity. ALP activity was measured in cell homogenates obtained from F9 teratocarcinoma cells cultured in the presence (■) or absence (□) of RA for the indicated amounts of time. Each experimental value is the mean ± S.D. from three separate culture dishes. (*b*) Western blotting analysis of ALP protein after 48 h of induction with RA. Proteins (115 μg), extracted from RA-treated (lane 1) and undifferentiated (lane 2) F9 teratocarcinoma cells, and from COS fibroblasts (lane 3) as a negative control, were loaded in each lane. Molecular mass markers are indicated (ovalbumin, 47 kDa; BSA, 77 kDa; β-galactosidase, 116 kDa; myosin, 205 kDa).

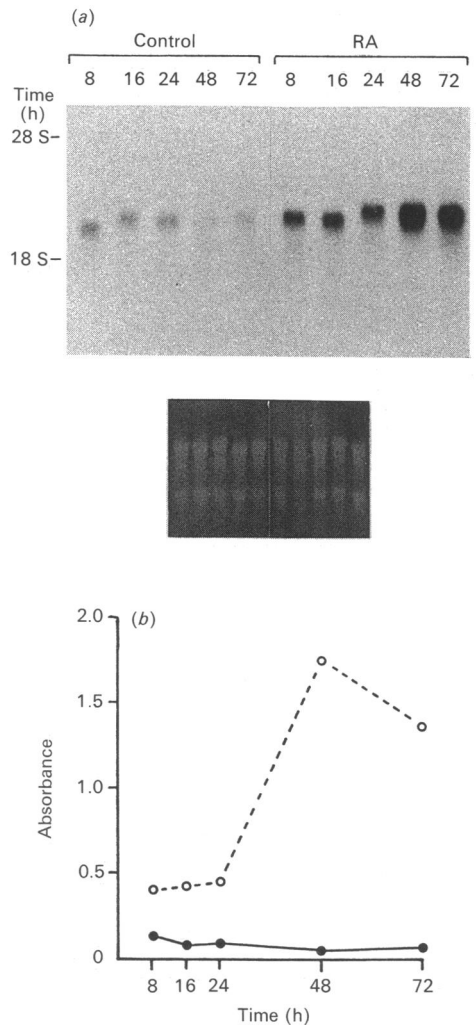

Fig. 2. ALP mRNA induction by RA treatment

(*a*) Total RNA (20 μg for each lane) was extracted for Northern blotting analysis from F9 teratocarcinoma cells incubated with or without RA for the indicated amounts of time. The positions of the size markers (28 S and 18 S rRNAs) are indicated. The lower Figure represents an ethidium bromide staining of the gel before transfer to the nylon membrane to show the amount of RNA loaded in each lane. (*b*) Quantitative representation of the results shown in (*a*). Densitometric analysis was performed as reported in the Materials and methods section and is expressed in absorbance units.

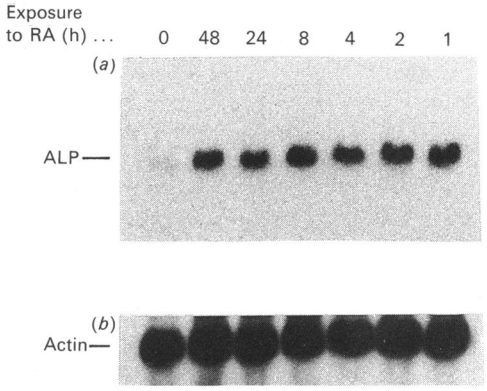

Fig. 3. Effect of time of exposure of RA to F9 teratocarcinoma cells on ALP mRNA induction

(*a*) Cells were treated with RA for the indicated amounts of time, washed and incubated with fresh medium without RA for up to 48 h. RNA was extracted and used for Northern blotting analysis. (*b*) The same filter was then rehybridized with actin cDNA.

typical time course of induction is shown in Fig. 1(*a*). After 48 h the level of induction was increased by about 4-fold in the experiment shown, although this value varied from 3- to 8-fold under our experimental conditions. The elevation in ALP activity was also evident when the data are expressed per cell number basis (results not shown).

The induction of ALP activity was accompanied by a quantitatively similar increase (4.8-fold by densitometry) in a protein band of the expected size (80 kDa), as demonstrated by Western blotting analysis using a specific polyclonal antibody which recognizes the *N*-terminus of the mouse LBK-type ALP isoenzyme (Fig. 1*b*).

To determine whether the increase in the amount of ALP protein is due to more efficient translational elongation or to an increase in specific mRNA levels, Northern blotting analysis was performed. Fig. 2(*a*) shows a typical time course of induction of ALP mRNA. Quantification by densitometry of the signals demonstrated an 8-fold increase in the transcript after 48 h of treatment with RA (Fig. 2*b*). The maximum induction was attained between 24 and 48 h; however, the elevation of ALP mRNA was already significant after 8 h. The increase in ALP mRNA temporally preceded the increase in ALP activity, being evident at 8 h, at which time the enzyme activity had not yet been significantly induced (see Fig. 1*a*).

To assess whether the continuous presence of RA is required for ALP mRNA induction, F9 teratocarcinoma cells were cultured in the presence of the morphogen for different amounts of time and ALP transcript levels were measured at 48 h. Fig. 3 demonstrates that RA does not need to be in continuous contact with F9 cells for maximal induction of ALP mRNA at 48 h. In fact, even 1 h of contact of RA with F9 cells is capable of inducing the transcript to its maximal level (compare 1 h and 48 h lanes), consistent with the fact that long-lasting changes in ALP gene expression are taking place very early during the induction period.

Nuclear run-on experiments (Fig. 4*a*) performed on cells after 48 h of treatment with RA demonstrated that there was an increase in the transcriptional rate of the ALP gene compared with the control (2-fold by densitometric analysis). Nuclear run-on assays were also performed using histone H2a and actin cDNAs as control plasmids. Consistent with the slight decrease in the growth rate of F9 cells after RA treatment (results not shown), the transcriptional rate of the histone H2a gene (a gene whose expression is regulated by DNA synthesis) (Wu *et al.*, 1982) was decreased by 3-fold. The transcriptional rate of the actin gene, on the contrary, was not changed by RA treatment, consistent with the fact that its mRNA is not regulated by retinoids (see also Fig. 3).

To assess the kinetics of induction of ALP gene transcription, run-on experiments were conducted at different times after continuous treatment of F9 cells with RA. Fig. 4(*b*) shows that induction became evident after 8 h of treatment. Quantification of these ALP run-on signals by densitometric analysis of the autoradiography demonstrated a 2–3.5-fold increase between 8 and 24 h after RA treatment. The greatest increase in transcriptional rate was observed at 24 h (3.5-fold increase compared with control). At earlier time points the increase in transcriptional rate was rather constant (2.3-fold at 8 h and 2.5-fold at 16 h). At 4 h after RA treatment the ALP gene transcriptional rate was decreased by a factor of 2. The experiment shown in Fig. 4(*b*) was reproduced in another independent experiment.

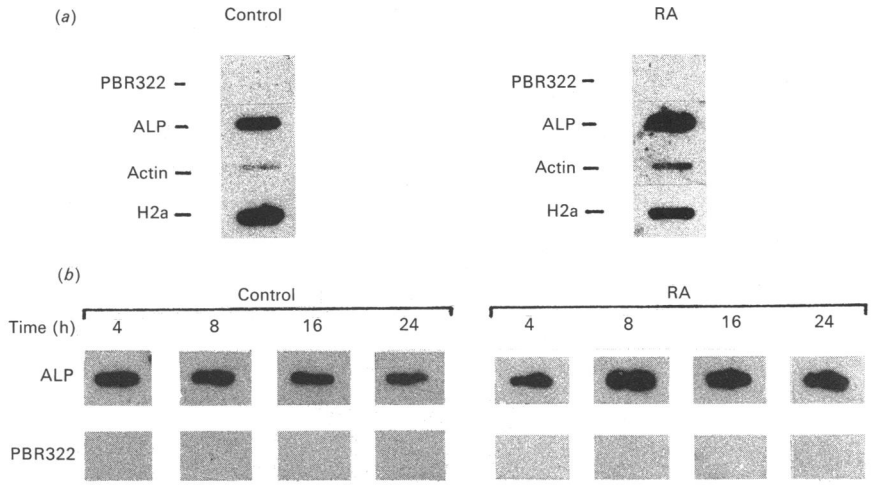

Fig. 4. Effect of RA on the transcription of the ALP gene

(*a*) Nuclear run-on assays were performed on F9 cells after 48 h of incubation in the absence (control) or in the presence (RA) of RA. The probes used in this experiment are indicated. The plasmid pBR322 was used as a negative control. (*b*) Nuclear run-on assays were performed at the indicated times after incubation of F9 cells in the absence or presence of RA.

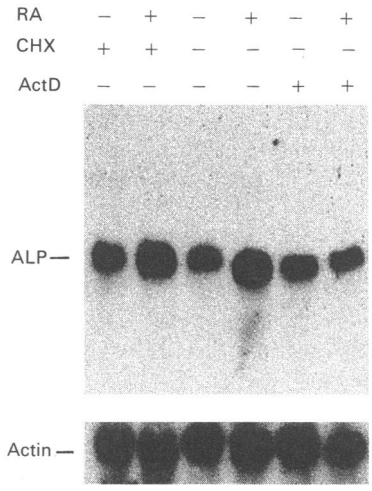

Fig. 5. Effects of cycloheximide and actinomycin D on the induction of ALP mRNA by RA

F9 teratocarcinoma cells were cultured in the absence or presence of RA in medium alone, or in medium containing 10 μg of cycloheximide/ml (CHX) or 5 μg of actinomycin D/ml (Act D). Total RNA was extracted and used for Northern blotting analysis (20 μg/lane). The same filter was sequentially hybridized with ALP and actin cDNAs.

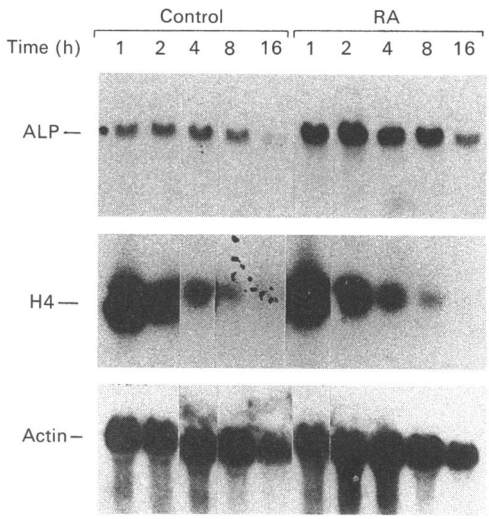

Fig. 6. Effect of RA on the stability of ALP mRNA

Cells were cultured in the presence or absence of RA. After 48 h fresh medium containing 10 μg of actinomycin D was added. Total RNA was extracted at the indicated times after actinomycin D addition and 20 μg was used to perform Northern blotting analysis. The same filter was sequentially hybridized with ALP, histone H4 (H4) and actin cDNAs.

To further support the hypothesis that the increase in ALP mRNA is associated with an increase in the transcriptional rate of the gene, RA treatment was performed in the presence of actinomycin D, a known inhibitor of RNA polymerase II. The RNA synthesis inhibitor was added along with RA, and kept in contact with the cells for 30 h. It is evident from Fig. 5 that ALP mRNA induction by RA at 30 h was completely blocked by the addition of actinomycin D.

The effect of cycloheximide on the induction of ALP mRNA by RA was also illustrated in the same experiment. This inhibitor of protein synthesis did not block the response of the ALP gene to the retinoid, demonstrating that protein synthesis *de novo* is not required for the induction of ALP mRNA. Furthermore, the increase in ALP mRNA at 30 h was also blocked by treatment of the cells with actinomycin D and RA for 1 h only (results not shown), as expected from the results presented in Fig. 3.

To study whether the stability of ALP mRNA is also affected by RA, the half-life of ALP mRNA was evaluated after inhibition of RNA synthesis by actinomycin D. Densitometric analysis of the autoradiogram presented in Fig. 6 demonstrated that ALP mRNA is very stable, with a half-life of about 14 h. This half-life was not changed by treatment with RA. The long half-life of ALP mRNA is not due to a lack of activity of actinomycin D, since the calculated half-life of histone H4 (a short-lived mess·ge) and actin (a long-lived message) mRNAs are 2.5 h and more than 21 h respectively. Again, these values were not altered by RA treatment.

The reported inhibitory effect of dibutyryl cyclic AMP on the

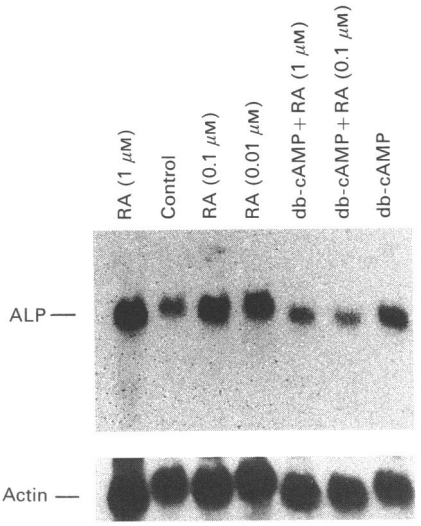

Fig. 7. Effect of dibutyryl cyclic AMP on the induction of ALP mRNA by RA

Northern blotting analysis of ALP mRNA was performed using 20 μg of total RNA. Cells were incubated for 48 h in the presence of RA, dibutyryl cyclic AMP (db-cAMP; 1 mM) or a combination of the two. The same filter was used for rehybridization with actin cDNA.

RA induction of ALP activity (Strickland & Mahdavi, 1978) prompted us to study the ALP mRNA levels under these conditions. Fig. 7 shows that dibutyryl cyclic AMP, in the presence of two different concentrations of RA (0.1 and 1 μM), decreased ALP mRNA to control levels. Paradoxically, dibutyryl cyclic AMP alone caused a slight but reproducible increase in mRNA levels (1.5-fold); this was reflected by a quantitatively similar increase in ALP activity (results not shown).

DISCUSSION

The data presented in this paper demonstrate that the increase in ALP activity observed after RA treatment of undifferentiated F9 teratocarcinoma cells is accompanied by a concomitant increase in the amounts of ALP protein and mRNA. This rules out the possibility that the increase in ALP activity is due either to unmasking of enzyme active sites, as reported for neutrophil ALP after treatment with fMetLeuPhe (Borregaard *et al.*, 1987), or to an increase in the translational rate of the transcript.

Recently a new 5′ untranslated region, resulting from a differential splicing event of the LBK-type ALP gene, has been demonstrated in cyclic-AMP-treated mouse L cells (Brown *et al.*, 1990). However, S1 mapping experiments showed that the 5′ untranslated region of ALP mRNA expressed and regulated by RA in F9 cells is identical with the mouse placental ALP mRNA, but different from that expressed in L cells (results not shown).

The induction of ALP mRNA is relatively slow and the peak is observed at 48 h. This is similar to what is observed for the laminin B1 gene as well as for some other genes that are induced during differentiation of F9 cells (Strickland & Mahadavi, 1978; Wang & Gudas, 1983; Wang *et al.*, 1985). ALP mRNA accumulation is primarily a consequence of an increase in the transcriptional rate of the gene and is not associated with increased stability of the mRNA induced by RA treatment. However, it should be noticed that we have never been able to demonstrate more than a 2–3-fold increase in ALP transcriptional rate by nuclear run-on experiments, whereas the elevation of ALP mRNA levels is 3–10-fold. The studies described in this report thus cannot rule out the participation of other, as yet unknown, mechanisms, such as increased movement of mature ALP mRNA from the nucleus to the cytoplasm, in the induction process. Similar discrepancies between transcriptional rate and mRNA accumulation were reported for the increased expression of the ALP gene induced by cyclic AMP in mouse L-cells (Brown *et al.*, 1990). Furthermore, mechanisms other than increased transcription of the gene have been suggested to explain the remarkable induction of human LBK-ALP mRNA after granulocyte-colony-stimulating factor treatment in neutrophils (Rambaldi *et al.*, 1990).

Induction of ALP mRNa does not require continuous contact of the F9 cells with RA. A 1 h exposure to RA was enough to trigger maximal up-regulation of ALP gene expression at 48 h, suggesting that induction of ALP mRNA is a consequence of events taking place very early during the differentiation process caused by the morphogen. It would be interesting to know whether this observation is typical of the ALP gene or whether it can be generalized to other RA-regulated genes as well.

The negative effect of dibutyryl cyclic AMP on RA induction of ALP mRNA accumulation is also remarkable. Although at present we do not understand the mechanism underlying this phenomenon, we can rule out the possibility that dibutyryl cyclic AMP is acting at the translational or post-translational level.

A crucial question in the understanding of the molecular mechanisms regulating the induction of ALP mRNA by RA in F9 cells is whether the phenomenon is mediated through one of the known nuclear RA receptors (RARs) (Petkovich *et al.*, 1987; de The' *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988; Krust *et al.*, 1989), its cytoplasmic RA-binding protein (RABP) (Stoner & Gudas, 1989) or other second messengers (Tanaka *et al.*, 1990). Both the nuclear receptors and the cytosolic binding protein are known to be expressed in F9 teratocarcinoma cells (Stoner & Gudas, 1989; Hu & Gudas, 1990). In particular, RAR-α and -γ mRNAs are constitutively expressed in undifferentiated F9 cells, whereas the expression of RAR-β is induced during differentiation towards the primitive endoderm caused by RA treatment (Hu & Gudas, 1990; Martin *et al.*, 1990). It is thus possible that ALP gene expression is regulated by RAR-β, whose kinetics of induction (at least at the mRNA level) are compatible with this hypothesis (Hu & Gudas, 1990; Martin *et al.*, 1990). This speculation is also consistent with the fact that dibutyryl cyclic AMP blocks the induction of both ALP and RAR-β mRNAs by RA (Martin *et al.*, 1990; see also Fig. 6). Furthermore, the RA-induced increases in the steady-state levels of ALP (see Fig. 5) and RAR-β mRNAs (Martin *et al.*, 1990) are not blocked by cycloheximide, demonstrating that in both cases *de novo* protein synthesis is not required. However, the scenario might be more complex, since antisense mRNAs to RAR-α and RABP transcripts block the induction of ALP activity by RA in human skin keratinocytes (Cope & Wille, 1989).

Nuclear RARs are similar to corticosteroid receptors, insofar as they contain two distinct domains, i.e. a DNA-binding domain and a ligand-binding domain (Petkovich *et al.*, 1987; de The' *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988; Krust *et al.*, 1989). Once loaded with their specific ligand, they are thought to mediate the action of RA through direct binding to specific consensus sequences located on the target genes. It would thus be important to establish whether ALP is one such target gene, or whether its regulation by RAR(s) is mediated through other trans-acting factors switched on by RA treatment. The ALP gene seems to be, at least in part, directly regulated by RAR(s), since the mouse ALP gene promoter contains two GGTCAC sequences (starting at nucleotides −1138 and −848) (Terao *et al.*, 1990) that are known to be involved in oestrogen, thyroid hormone and retinoid receptor binding to DNA (Evans, 1988; Beato, 1989; Sucov *et al.*, 1990).

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