Retinoic acid and cyclic AMP synergistically induce the expression of liver/bone/kidney-type alkaline phosphatase gene in L929 fibroblastic cells

Maurizio GIANNÌ,* Mineko TERAO,* Silvano SOZZANI† and Enrico GARATTINI*‡

*Molecular Biology Unit, Centro Catullo e Daniela Borgomainerio
and †Laboratory of Human Immunology, Istituto di Ricerche Farmacologiche 'Mario Negri', via Eritrea 62, 20157 Milano, Italy

In L929 mouse fibroblastic cells, liver/bone/kidney type alkaline phosphatase (L/B/K-ALP) enzymic activity is induced by all-*trans*-retinoic acid at concentrations between 10^{-6} and 10^{-5} M. At lower concentrations, retinoic acid is incapable of inducing this enzymic activity *per se*, but increases cyclic AMP (cAMP)-mediated induction. This effect is observed after incubation of the retinoid with dibutyryl cAMP, 8-bromo cAMP or forskolin. The synergism is dependent on the order of addition of retinoic acid and the activator of the cAMP pathway. Contemporaneous addition of the two agents, or addition of cAMP prior to retinoic acid (but not addition of retinoic acid before cAMP), is necessary to produce this synergistic interaction. The synergism results in increased steady-state levels of L/B/K-ALP mRNA and it is the consequence of increased transcriptional activity of the gene. The expression of the mouse L/B/K-ALP gene is regulated by the presence of two leader exons, 1A and 1B, resulting in the synthesis of two alternatively spliced mRNAs that are different only in part of their 5′ untranslated region [Studer, Terao, Giannì and Garattini (1991) Biochem. Biophys. Res. Commun. **179**, 1352–1360]. PCR amplification and nuclear run-on experiments performed using probes specific for each leader exon demonstrate that treatment of these cells with retinoic acid, forskolin or dibutyryl cAMP, and with the combination of the retinoid and one of the cAMP-elevating agents, leads to the accumulation of nascent and mature L/B/K-ALP mRNA containing exon 1B. The synergistic induction of the transcription of the L/B/K-ALP gene is well correlated with quantitative and qualitative changes of retinoic-acid-receptor mRNAs mediated by cAMP.

INTRODUCTION

All-*trans*-retinoic acid (RA) is one of the most powerful differentiating agents so far known. RA controls the differentiation of various cell types, including promyelocytes [1], keratinocytes [2] and embryonal stem cells [3–5]. This compound exerts most of its pleiotropic effects through the transcriptional activation or repression of responsive genes [6,7]. The regulation of target genes is believed to be largely due to the mediation of nuclear receptors (RARs) [8–11] that are capable of binding to particular DNA sequences known as RA-responsive elements (RAREs) [12,13]. Three classes of RARs, i.e. α, β and γ, are known and each of these classes is subdivided into multiple members differing in their primary structure at the N-terminal portion of the protein [11,14,15]. The RA-related receptors (RXRs), belonging to the same superfamily of nuclear proteins as RARs, have also been shown to play a role in the cellular response to RA [16–19]. Furthermore, it is known that RA interacts with other cellular targets whose functionai significance is still obscure [20–25]. The intracellular effectors involved in the activity of RA are potentially modulated by many other second-messenger molecules. Of particular interest is the interaction between the RA and the cAMP pathways. In fact, compounds that increase intracellular levels of cAMP have been demonstrated to synergize with the retinoid in terms of cellular differentiation [26,27]. The molecular mechanisms underlying the cross-talk between RA and cAMP are not yet known, especially at the level of single responsive genes [28–32].

Alkaline phosphatases [orthophosphoric-monoester phosphohydrolase (alkaline optimum), E.C. 3.1.3.1] are enzymes that hydrolyse phosphomonoester bonds contained in various organic compounds. Their physiological function and their natural substrates are not yet known, even though they seem to play a role in the mineralization of the extracellular matrix in the bone and they have been suggested to perform various functions in other tissues [33–35]. The liver/bone/kidney-type alkaline phosphatase (L/B/K-ALP) is the product of one [36–39] of the ALP genes [40–45]. The mouse L/B/K-ALP gene is ubiquitous in its expression, being present in the kidney, osteoblasts, placenta and ovary at high levels and in many other tissues at low levels [46]. The mouse [46–48] as well as the rat [49] and the human [42,50] L/B/K-ALP genes contain two leader exons, 1A and 1B, and produce two alternatively spliced mRNAs, differing only in their 5′ untranslated region. In the mouse, the transcript containing exon 1A is present in most tissues, whereas the mRNA containing exon 1B is detected only in the heart and in the diaphragm [46]. In general, the expression of the two mRNAs is mutually exclusive in the various tissues and cell lines so far investigated. The factors involved in the tissue-specific expression, as well as the possible endogenous and exogenous regulators of the levels of the two transcripts, are not yet known.

The expression of L/B/K-ALP enzymic activity is under the control of various stimuli [51–54]. In particular, the enzyme is inducible by all-*trans*-retinoic acid (RA) in mouse teratocarcinomas [53–56] as well as in other cell lines [57,58]. Nuclear run-on experiments have demonstrated that the control of ALP gene expression by RA in F9 [53] and in P19 [56] teratocarcinomas is mainly transcriptional. The induction is

Abbreviations used: L/B/K-ALP, liver/bone/kidney-type alkaline phosphatase; cAMP, cyclic AMP; RA, all-*trans*-retinoic acid; RARs, nuclear RA receptors; RAREs, RA-responsive elements; RXRs, RA-related receptors; db-cAMP, dibutyryl cyclic AMP; 8Br-cAMP, 8-bromo cyclic AMP; CHX, cycloheximide; Act-D, actinomycin D; SSC, saline/sodium citrate (see the text for composition); PKA, protein kinase; CREBP, cAMP-response-element-binding proteins.

‡ To whom correspondence should be sent.

blocked by co-treatment with dibutyryl cyclic AMP (db-cAMP) [53]. Furthermore, in F9 cells, RA induces the expression of the L/B/K-ALP transcript containing exon 1A [46]. These latter findings prompted us to study whether RA and its combination with cAMP is modulating the expression of the message containing exon 1B as well. For this purpose, we selected the L929 fibroblastic cell line, since it is the only one so far tested that expresses the ALP mRNA containing exon 1B [46,48]. This cell line does not express L/B/K-ALP mRNA and the corresponding encoded protein under basal conditions, but the transcript is dramatically induced both by stable cAMP analogues and classical inducers of the cyclic nucleotide second-messenger pathway [46,48,51].

Here we show that the L/B/K-ALP mRNA induced by RA contains only exon 1B. We also demonstrate a strong synergistic interaction between RA and cAMP in the induction of L/B/K-ALP gene expression. Both RA and the combination of RA and cAMP act at the level of gene transcription and the synergism is well correlated with qualitative and quantitative changes in the expression of RAR mRNAs.

MATERIALS AND METHODS

Cell lines and reagents

L929 is a fibroblastic cell line and it was obtained from the American Type Culture Collection (A.T.C.C.), Rockville, MD, U.S.A. The cells were routinely passaged in RPMI 1640 containing 10% (v/v) fetal-calf serum. Cells were seeded at a concentration of 10^5 cells/ml in 25 or 75 cm^2 Falcon culture flasks (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and grown for 24 h before appropriate treatments were performed. Treatments were started when cells had reached confluency. The number of cells and their viability were not altered by treatment with db-cAMP, forskolin, 8-bromo cAMP (8Br-cAMP), RA or the combination of the retinoid with the three other compounds separately. F9 teratocarcinoma cells were seeded (10^5 cells/ml) and grown in DMEM containing 10% (v/v) fetal-calf serum. Cultures were free from mycoplasma as assessed using the Hoechst 33258 fluorescent dye system (Farbwerke Hoechst AG, Frankfurt, Germany). RA, db-cAMP, 8-Br-cAMP, forskolin, cycloheximide (CHX) and actinomycin D (Act-D) were from Sigma (St. Louis, MO, U.S.A.). Stock solutions of RA (10^{-2} M) were prepared in dimethyl sulphoxide under dimmed light and stored at −80 °C protected from light until use.

Measurement of ALP activity and Western-blot analysis

Cell monolayers 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 homogenate was used for the ALP assay, which was performed with *p*-nitrophenyl 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 by the Bradford method [59] using BSA fraction V (Sigma) as a standard. One unit of ALP activity is defined as the amount of enzyme capable of transforming 1 μmol of substrate in 1 min at 37 °C. Enzyme assays were performed in conditions of linearity relative to the substrate and to the concentration of proteins.

For Western-blot analysis the homogenate was extracted with n-butanol (25%, v/v) at room temperature for 1 h. The aqueous phase containing ALP enzymic activity was separated by centrifugation and immediately subjected to cold acetone (50%, v/v) precipitation. Treatment of L929 cells with the various compounds used throughout the study did not influence the recovery of ALP enzymic activity (approx. 85%) from the homogenate by this extraction procedure. The resulting protein pellet was resuspended in an appropriate volume of homogenization buffer and subjected to SDS/PAGE under reducing conditions, after measurement of ALP enzymic activity and determination of the protein content. Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) as described by Towbin et al. [60] and subjected to Western-blot analysis using a polyclonal antibody raised against the N-terminal peptide of human L/B/K-ALP [39] and ^{125}I-labelled protein G (Amersham International, Little Chalfont, Bucks., U.K.) according to standard procedures [61]. The specificity of the ALP antibody, as well as its cross-reactivity with mouse L/B/K-ALP, have been described previously [39,53].

Northern-blot analysis and PCR amplification

Total RNA was prepared from L929 fibroblastic and F9 teratocarcinoma cell lines according to a modification of the guanidinium isothiocyanate/CsCl method [62] and used for Northern-blot analysis and PCR amplification. For Northern-blot analysis, RNA samples (10 μg) were fractionated on 1%-agarose gels containing 6% formaldehyde and blotted on to synthetic nylon membranes (GeneScreen Plus; New England Nuclear Corp., Boston, MA, U.S.A.). These membranes were hybridized with a 2 kb *Eco*RI–*Bgl*II fragment of mouse placental (L/B/K-type) ALP cDNA [39], mouse α-actin [63], human histone H4 [64] and mouse RAR α1, β2 and γ1 c-DNAs [10]. 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 [65]. 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 (du Pont Cronex; du Pont de Nemours, Bad Homburg, Germany) at −70 °C.

PCR amplifications of the ALP transcripts were carried out from total RNA after reverse transcription using the gene AMP kit (Cetus/Perkin–Elmer, Norwalk, CT, U.S.A.) according to the instructions of the manufacturer. The antisense downstream oligonucleotide was common for the amplification of both exon 1A and 1B transcripts and is contained in exon 2 (5′ TGTACCCTGAGATTCGT 3′ complementary to nucleotides 164–180 of mouse placental ALP cDNA) [39], whereas sense upstream oligonucleotides were specific for exon 1A (5′ CGCCAGAGTACGCTCCCGCC 3′; nucleotides 1–20 of the placental ALP cDNA) [40] and 1B (5′ GACATAGGGG ACAGGGACCTGT 3′; nucleotides 92–113 of the mouse L/B/K-ALP gene exon 1B) [46] respectively. For β-actin amplification, two oligonucleotides specific for the β-actin gene (5′ CACCACACCTTCTACAA 3′, nucleotides 181–197; and 5′ CGGTCAGGATCTTCATGA 3′, complementary to nucleotides 488–505 of the mouse β-actin cDNA) [66] were used. The samples were subjected to 30 and 25 cycles of amplification (94 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min) for ALP exons 1A and 1B, and β-actin respectively. Amplifications were

carried out simultaneously on one-third of the same reverse-transcription reaction. The assays were performed under conditions of linearity in terms of RNA concentration and during the logarithmic phase of the amplification by *Taq* polymerase. After amplification, the samples were run on a 1.5%-agarose gel. In the case of β-actin, amplified bands were revealed after staining with ethidium bromide, whereas ALP products were transferred on to nylon membranes and detected with the use of ^{32}P-labelled oligonucleotides specific for exon 1A (5′ GTCTGTTCCGGCTCGCG 3′; complementary to nucleotides 45–61 of placental ALP cDNA) [39] and exon 1B [5′ TCGATCCAGATGCTGAA 3′; nucleotides 122–138 of the mouse L/B/K-ALP gene exon 1B) [46] according to Wood et al. [67].

Nuclear transcription run-on assay

Nuclear transcription run-on assay was performed as described by Eick and Bornkamm [68] with minor modifications. Briefly, nuclei (approx. 5×10^6) were prepared by lysing L929 or F9 cells in 4 ml of lysis buffer [0.5% (w/v) Nonidet P40/0.01 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 7.5, 2.5 mM $MgCl_2$, 0.15 M KCl, 0.35 mM each of ATP, CTP and GTP (Pharmacia, Uppsala, Sweden) and 200 μCi of [^{32}P]UTP (> 800 Ci/mmol; Amersham International). Labelled elongated RNAs (minimum 3×10^6 c.p.m./ml) were hybridized to 10 μg each of the cDNA plasmids immobilized on nitrocellulose membranes after denaturation by heat and alkali treatments. Filters were prehybridized in a solution containing 5 × SSC, 1 × Denhardt's, 1% SDS, 50 mM phosphate buffer, pH 6.5, and 100 μg of salmon sperm DNA/ml at 65 °C for 4 h. Hybridization was carried out in 0.3 M NaCl, 1 × Denhardt's, 0.2% SDS, 10 mM Tes, pH 7.4, 10 mM EDTA and 3×10^6 c.p.m. of ^{32}P-labelled nuclear RNA/ml for 2 days at 65 °C. Filters were washed for 30 min twice at 37 °C in 2 × SSC/0.1% SDS, once in 2 × SSC and twice in 2 × SSC containing 10 mg/ml RNAase A (boiled for 2 min before use). Filters were air-dried and exposed to a Kodak X-Omat AR film using an intensifying screen. Plasmids used for the experiments were pBluescript (Stratagene, La Jolla, CA, U.S.A.), histone H4 cDNA [64] in pBR322, mouse α-actin cDNA [63] in pBR322 and the two ALP genomic fragments containing exon 1A (*Eco*RI fragment containing 1.8 kb of 5′ flanking region, the entire exon 1A and 1 kb of intronic sequences) and 1B (*Hin*cII–*Pst*I fragment containing 0.7 kb of 5′ flanking region, the entire exon 1B and 0.4 kb of intronic sequences) respectively in pBluescript [46,69].

Autoradiograms of both Northern-blot analysis and nuclear run-on assays were quantified by laser-scanning densitometry with a laser-beam densitometer (300 A computing densitometer Fast Scan; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Intracellular cAMP levels and cAMP-dependent protein kinase (PKA) activity

L929 cells were incubated for 5 min, 1 and 24 h with medium alone or medium containing forskolin, RA or the combination of the two compounds. In the case of the determination of the levels of intracellular cAMP, all treatments were performed in the presence of a mixture of compounds inhibiting phosphodiesterase, isobutylmethylxanthine (Sigma) and Ro20-1724 (Hoffmann–La Roche, Basel, Switzerland), at final concentrations of 0.2 and 0.02 mM respectively.

cAMP was extracted from cell monolayers (1.4×10^6 cells/plate) twice with 0.5 ml of ice-cold 70% (v/v) ethanol in water. The two combined extracts were evaporated under vacuum and resuspended in 100 μl of distilled water. Total intracellular cAMP was measured with a specific radioimmunoassay (Amersham International) according to the instructions of the manufacturer. Assays were run in duplicate on 10 μl of the extract. For PKA assays, 4×10^6 cells were sonicated in 500 μl of extraction buffer [50 mM Tris/HCl (pH 7.5)/50 mM 2-mercaptoethanol/1 mM phenylmethanesulphonyl fluoride/2 mM EGTA]. The supernatant obtained after a 150000 *g* centrifugation was used as the source of PKA. PKA activity was measured as previously described [70]. Briefly, the reaction mixture [35 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$/50 μM ATP (containing 10^6 c.p.m. of ^{32}P[ATP] labelled in the γ-position) and 160 μg/ml of histone type 2A; final volume 250 μl] was preincubated at 30 °C for 5 min before the addition of 50 μl of the cytosol fraction. The incubation was terminated after 5 min by the addition of 1 ml of 20% trichloroacetic acid. The precipitates were passed through 0.45 μm-pore-size nitrocellulose filters (Millipore, Vimodrone, Italy) and the associated radioactivity was measured in a Beckman liquid-scintillation counter. Results are expressed as the difference of the incorporated radioactivity in the presence and in the absence of cAMP exogenously added to the reaction mixture (10 μM final concn.). Under these experimental conditions the stimulation of phosphotransferase activity in the presence of cAMP is about 7-fold for control samples. A unit of PKA enzymic activity is defined as 1 pmol of phosphate incorporated into histone 2A/min per mg of protein.

Statistical analysis

Statistical significance of the experimental data was evaluated by one-way analysis of variance and *post hoc* comparison by Duncan's test [71,72] or by two-way analysis of variance and *post hoc* comparison according to Tukey's test in the case of the results shown in Figure 1 (below) [71,73].

RESULTS

Effects of cAMP and RA on ALP enzymic activity

Figure 1(a) shows that incubation of L929 cells for 72 h with various concentrations of RA results in a dose-dependent induction of ALP enzymic activity. ALP activity is barely detectable under basal conditions. Induction by RA is evident at 10^{-6} M and is maximal at 10^{-5} M, whereas the retinoid at concentrations between 10^{-10} and 10^{-7} does not lead to levels of ALP activity significantly higher than those in control cells. db-cAMP at 5×10^{-3} M induces ALP activity, as expected [48]. When L929 cells are concomitantly incubated with the two compounds, a strong synergistic effect is observed. RA at concentrations between 10^{-10} and 10^{-6} M, in the presence of db-cAMP, gives rise to a 2–5-fold increase in ALP activity relative to db-cAMP alone. The synergism is not statistically significant at concentrations of 10^{-5} M because of the strong inducing effect of RA alone on ALP activity. As shown in Figure 1(b), db-cAMP induces ALP enzymic activity in a dose-dependent manner and it is maximally active at 5×10^{-3} M. If cells are incubated with various concentrations of cAMP in the presence of RA at 10^{-8} M, a synergistic interaction between the two compounds is again observed. The lowest concentration at which db-cAMP is capable of producing synergism with RA is 1.5×10^{-3} M, and the synergistic effect ranges from 2- to 5-fold. The synergism observed with the combination of RA and db-cAMP is not an artifact due to the butyrate moiety of the latter compound, since a similar effect is observed after

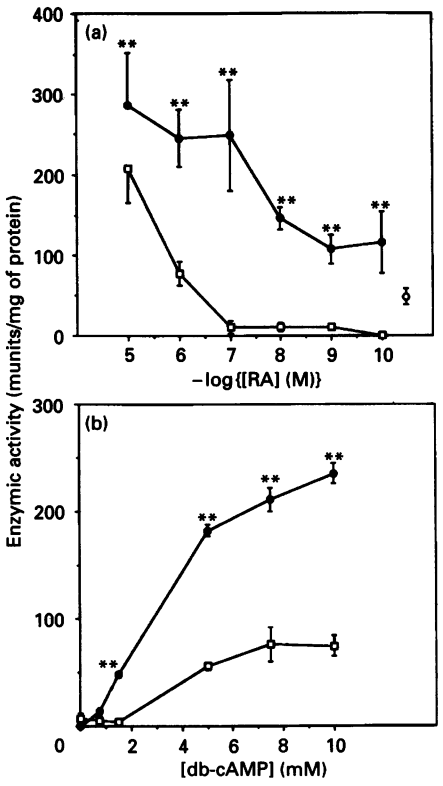

Figure 1 Synergistic effect between RA and db-cAMP in the induction of ALP enzymic activity in L929 cells

(**a**) ALP activity was measured in cell homogenates obtained from L929 cells cultured with the indicated concentration of RA either in the presence (●) or in the absence (□) of 5×10^{-3} M db-cAMP for 72 h. The open circle on the lower right side of the Figure indicates the amount of ALP enzymic activity determined after 72 h of treatment with db-cAMP alone. Each experimental value is the mean ± S.D. for three separate culture dishes. (**b**) ALP activity was measured in cell homogenates obtained from L929 cells grown with the indicated amount of db-cAMP either in the presence (●) or in the absence (□) of 10^{-8} M RA for 72 h. Each experimental value is the mean ± S.D. for three culture dishes. **, Significantly higher relative to db-cAMP alone ($P < 0.01$ according to Duncan's test). Notice that, for (**a**), except for the data obtained in the presence of 10^{-5} M RA, the values for the combination of db-cAMP and RA are also significantly higher relative to the sum of the values of db-cAMP and RA separately. The F of interaction was highly significant for all the experimental points between 10^{-6} and 10^{-10} M RA after two-way analysis of variance ($P < 0.01$ according to Tukey's test).

Table 1 Synergistic effect of db-cAMP, forskolin and 8Br-cAMP in combination with RA on ALP enzymic activity

L929 cells were treated for 72 h with the indicated compounds. ALP enzymic activity was measured in cell homogenates. Each experimental value is the mean ± S.D. for three separate culture dishes. RA was present at 10^{-8} M, db-cAMP at 5×10^{-3} M, 8Br-cAMP at 1×10^{-3} M and forskolin at 1×10^{-4} M.

Treatment	ALP activity (munits/mg of protein)
Control	< 10*
RA	15 ± 1
db-cAMP	118 ± 18
db-cAMP + RA	205 ± 44†
Forskolin	225 ± 50
Forskolin + RA	1013 ± 87‡
8Br-cAMP	473 ± 10
8Br-cAMP + RA	1213 ± 155§

* Below the limit of detection of the enzymic assay.

†, ‡, § Significantly higher relative to db-cAMP (†), to forskolin (‡) or to 8Br-cAMP (§) alone respectively ($P < 0.01$ according to the Duncan's test).

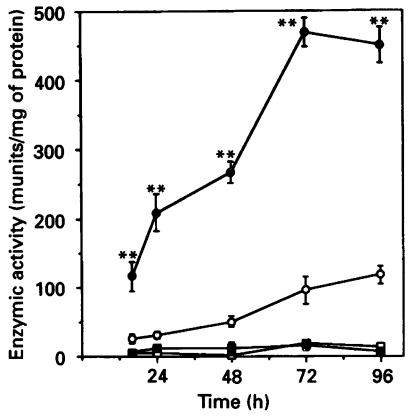

Figure 2 Time course of the synergism between db-cAMP and RA in the induction of ALP enzymic activity

L929 cells were treated with medium alone (□), medium containing 10^{-8} M RA (■), 5×10^{-3} M db-cAMP (○) and 10^{-8} M RA + 5×10^{-3} M db-cAMP (●) respectively for the indicated amount of time. At the end of each treatment, cells were collected, homogenized and ALP enzymic activity was measured. Each experimental value is the mean ± S.D. for three separate culture dishes. **, Significantly higher relative to db-cAMP alone ($P < 0.01$ according to Duncan's test).

treatment of L929 cells with another stable analogue of the cyclic nucleotide, 8Br-cAMP, and with a direct inducer of adenylate cyclase, forskolin (Table 1). At the concentrations used, 8Br-cAMP is a stronger inducer of ALP activity than forskolin or db-cAMP. In the experiment shown, the synergistic effect (approx. 4-fold) between forskolin and RA is more marked than that between db-cAMP or 8Br-cAMP and RA. However, in separate experiments the level of induction varies and it is independent of the stimulus used to increase the intracellular levels of the cyclic nucleotide. Inhibition of the catabolism of cAMP does not alter the synergism between cAMP and RA. In fact, in the presence of phosphodiesterase inhibitors (a combination of 0.2 mM isobutylmethylxanthine and 0.02 mM Ro-1724), the ALP activity in the cells treated with forskolin alone is 272 ± 18 munits/mg of protein, whereas the combination of forskolin and RA increases the ALP specific activity to 925 ± 21 munits/mg protein. These results are not significantly different from those obtained in the absence of the phosphodiesterase inhibitors (32 ± 51 after forskolin, and 720 ± 68 munits/mg of protein after forskolin + RA treatment respectively). Similar results are obtained if forskolin is replaced by db-cAMP (results not shown).

The kinetics of the induction of ALP enzymic activity by db-cAMP and db-cAMP + RA are almost parallel and relatively slow (Figure 2). After treatment with db-cAMP, an increased level of enzymic activity is observed after 16 h and tends to level off after 3 days. The synergistic effect between the two compounds is again significant after 16 h, but it is maximal after 3 days. Similar kinetics of induction are observed with forskolin, 8Br-cAMP and the combinations of one of the two compounds with RA (results not shown).

The order of addition of db-cAMP and RA to the culture medium is important for the synergistic interaction between the two compounds. As expected, Table 2 shows that the synergism

Table 2 Influence of the order of addition of the compounds on the synergism between db-cAMP and RA

L929 cells were grown in the absence or in the presence of 10^{-8} M RA, 5×10^{-3} M db-cAMP, and the combination of the two, for 36 h. Cells were washed with fresh medium and further incubated for 36 h as indicated. The solidus under 'Treatment' represents the removal of the medium after the first 36 h of incubation. For example, db-cAMP/RA means that the cells were grown in the presence of 5×10^{-3} M db-cAMP for the first 36 h, washed and incubated for another 36 h in the presence of 10^{-8} M RA. Control cultures were grown for 72 h in medium alone (medium). At the end of each treatment, cells were collected, homogenized and ALP enzymic activity was measured. Each experimental value is the mean ± S.D. for three separate culture dishes.

Treatment	ALP activity (munits/mg of protein)
Medium	< 10*
Medium/RA	< 10*
RA/medium	< 10*
db-cAMP/medium	101 ± 1
Medium/db-cAMP	35 ± 1
Medium/db-cAMP + RA	102 ± 4†
RA/db-cAMP	34 ± 1
db-cAMP/RA	158 ± 6‡

* Below the limit of detection of the enzymic assay.

†, ‡ Significantly higher relative to medium/db-cAMP and RA/db-cAMP (†) or relative to db-cAMP/medium, medium/db-cAMP, RA/db-cAMP and medium/db-cAMP + RA (‡) ($P < 0.01$ according to Duncan's test).

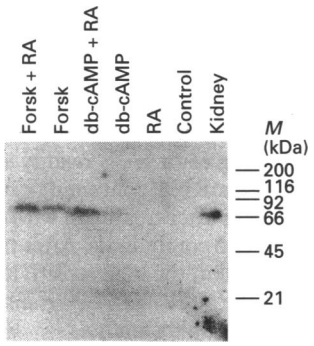

Figure 3 Effects of db-cAMP, forskolin (Forsk) and RA on the levels of ALP protein

The same amount of protein (100 μg) extracted from L929 cells in the absence of any treatment (control) or after treatment for 72 h with the indicated compounds was loaded in each lane and subjected to Western-blot analysis using an antibody specific for the L/B/K-type ALP and ^{125}I-labelled protein G. As a positive control for the experiment, a protein extract derived from C57/Bl mouse kidney (equivalent to 0.5 unit of ALP activity) was used, and it is shown on the right. The positions of molecular-mass (*M*) markers are indicated on the right (trypsin inhibitor, 21 kDa; ovalbumin, 45 kDa; BSA, 66 kDa; phosphorylase *b*, 92 kDa; β-galactosidase, 116 kDa; myosin, 200 kDa).

is evident if RA and db-cAMP are simultaneously added to the medium and left in contact with the cells for 36 h (the ratio of the values for the columns marked as medium/db-cAMP + RA and medium/db-cAMP is 2.9). If cells are left in contact with RA for 36 h prior to washing, addition of fresh medium containing db-cAMP and further incubation for 36 h, the synergistic effect between the two compounds is abolished (the ratio of the values indicated by RA/db-cAMP and medium/db-cAMP is 1.0). Conversely, when db-cAMP is added before RA, the synergistic effect is maintained, albeit reduced in its level (the ratio of the values indicated as db-cAMP/RA and db-cAMP/medium is 1.6). The absolute amount of ALP enzymic activity is higher in the experimental groups where db-cAMP is added at the beginning of the experiment relative to those where the cyclic nucleotide is added in the last 36 h of incubation (compare db-cAMP/medium and medium/db-cAMP, db-cAMP/RA and medium/db-cAMP + RA respectively). This can be explained by the fact that the cAMP analogue is accumulating in the intracellular compartment, resulting in stimulation of the cells and consequent induction of ALP activity for a total of 72 h.

The induction of ALP enzymic activity by RA in combination with db-cAMP or forskolin is due to an increased level of L/B/K-ALP protein. As shown in Figure 3, the amount of the ALP protein was measured by Western-blot analysis. The intensity of the immunoreactive band correlates with enzymic activity. In fact, in basal conditions as well as after treatment with 10^{-8} M RA alone, no immunoreactive band is observed. After db-cAMP or forskolin treatment, a specific band with an apparent molecular weight of 75 kDa is evident. In the presence of RA, this band is increased 4-fold after db-cAMP and 3-fold after forskolin treatment respectively.

Effects of cAMP and RA on L/B/K-ALP mRNA accumulation

As shown in Figure 4, the L/B/K-ALP mRNA is undetectable in basal conditions. The transcript is induced after 6 h of treatment with db-cAMP and increases up to 72 h. Treatment with db-cAMP + RA leads to an approximately 3-fold induction of the mRNA relative to db-cAMP alone, starting from 24 h. Similar results are obtained if db-cAMP is replaced by forskolin (only data at 24 h are shown).

The effects of CHX, a protein-synthesis inhibitor, on the accumulation of L/B/K-ALP transcript were examined, and they are shown in Figure 5. In the presence of db-cAMP, treatment of L929 cells with CHX for 16 h leads to a marked decrease in the induction of the mRNA. In contrast, the protein-synthesis inhibitor superinduces the expression of the message

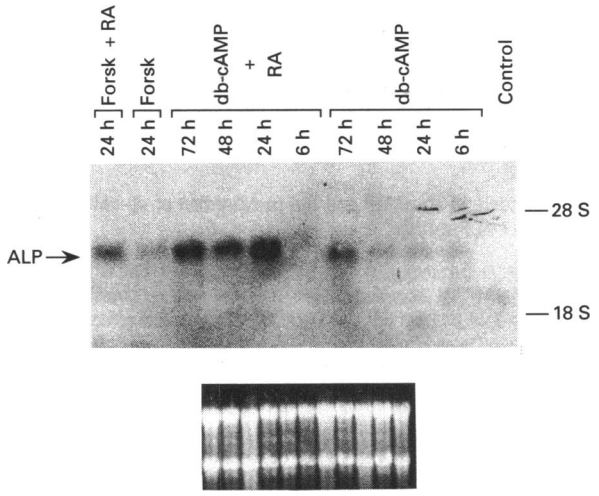

Figure 4 L/B/K-ALP mRNA induction by db-cAMP and RA

Total RNA (10 μg for each lane) was extracted for Northern-blot analysis from L929 cells incubated in the presence of db-cAMP (5×10^{-3} M), RA + db-cAMP (10^{-8} M and 5×10^{-3} M respectively), forskolin (Forsk; 10^{-4} M) and forskolin + RA (10^{-4} M and 10^{-8} M respectively) for the indicated amount of time. RA alone did not induce the ALP transcript at any time point, and this result is not shown. Control RNA was extracted after incubation of the cells for 72 h in medium alone. The position of the size markers (28 S and 18 S rRNAs) is indicated. A picture of the ethidium bromide staining of the RNA is shown at the bottom and demonstrates that approximately equal amounts of RNA were added in each lane.

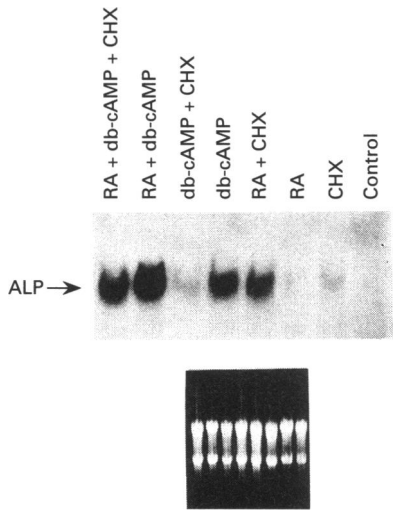

Figure 5 Effects of CHX on the induction of ALP mRNA by db-cAMP and RA

L929 cells were cultured in the presence of the indicated compounds for 48 h. In these experimental conditions CHX (10 μg/ml) is not significantly toxic, as tested by the Trypan Blue-exclusion test, while it is inhibiting protein synthesis over 80%. Total RNA was extracted and used for Northern-blot analysis (10 μg/lane). A picture of the ethidium bromide staining of the RNA is shown at the bottom and demonstrates that approximately equal amounts of RNA were added in each lane.

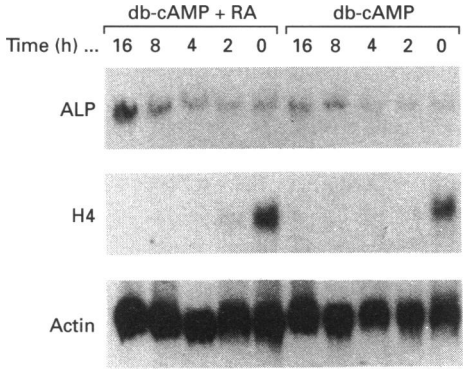

Figure 6 Effects of db-cAMP and the combination of db-cAMP and RA on the stability of ALP mRNA

Cells were cultured in the presence of db-cAMP (db-cAMP, 5×10^{-3} M) or db-cAMP and RA (5×10^{-3} and 1×10^{-8} M respectively). After 72 h, fresh medium, containing 5 μg of Act-D/ml, was added. Total RNA was extracted at the indicated times after actinomycin D addition, and 10 μg was used to perform the Northern-blot analysis. The same filter was sequentially hybridized with ALP, histone H4 and α-actin cDNAs.

both under basal conditions (the superinduction is evident only after longer exposure of the autoradiography; results not shown) and after treatment with 10^{-8} M RA. As a result of these two opposing effects, the levels of the L/B/K-ALP transcript appear almost unchanged after treatment with the combination of db-cAMP and RA relative to those observed with db-cAMP alone. In fact, if the intensity of the L/B/K-ALP mRNA signal after treatment with db-cAMP is taken as 100%, the results of the densitometric analysis of the autoradiography are: RA + CHX, 104%; db-cAMP + CHX, 22%; RA + db-cAMP, 322%; RA + db-cAMP + CHX, 210%; CHX, 24%. This experiment was independently repeated a second time with similar results.

If the cells are contemporaneously treated with db-cAMP or the combination of db-cAMP and RA in the presence of Act-D (5 μg/ml), an RNA synthesis inhibitor, for 16 h, no L/B/K-ALP transcript is observed (results not shown). This indicates that the cyclic nucleotide, alone as well as in combination with RA, is inducing the expression of the mRNA through a transcriptional mechanism.

In studies aimed at determining the half-life of the L/B/K-ALP transcript, cells were treated with Act-D after maximal induction of the message was obtained with db-cAMP or with the combination of the cyclic nucleotide and RA. The mRNA is extremely long-lived (the half-life of the message cannot be calculated accurately because the toxicity of Act-D prevents treatments longer than 16 h) in the presence of both db-cAMP and db-cAMP + RA (Figure 6). The presence of the retinoid does not alter the decay curve of the transcript. Act-D treatment causes a reproducible increase in the levels of the transcript at 8 and 16 h. The decay curves of a short-lived (histone H-4) and of a long-lived message (α-actin) in the same experimental conditions are shown for comparison.

cAMP and RA are modulating the levels of the L/B/K-ALP transcript containing exon 1B through a transcriptional mechanism

The presence of two leader exons in the mouse L/B/K-ALP gene [46,47], giving rise to two transcripts differing in their 5′ untranslated region, prompted us to investigate whether RA is up-regulating the expression of the same mRNA as cAMP in L929 cells. For this purpose, PCR amplification of the cDNA after reverse transcription of the message (RT/PCR) was performed using two couples of oligonucleotides specific for the L/B/K-ALP message containing exons 1A and 1B. The data shown in Figure 7(a), albeit semi-quantitative, demonstrate that the mRNA containing exon 1A is readily detectable in F9 cells, as expected [46]. The mRNA containing exon 1B is the only one expressed in L929 cells after treatment with db-cAMP, RA or the combination of the two compounds. After treatment with 10^{-8} M RA, it is noteworthy that the high sensitivity of RT/PCR allows the detection of the L/B/K-ALP transcript, which is not detectable by Northern-blot analysis.

To demonstrate that cAMP and RA are interacting at the transcriptional level (as suggested by the inhibition of this cross-talk by Act-D) and to determine the transcriptional activity of the L/B/K-ALP gene in L929 cells, nuclear run-on assays were performed (Figure 7b). The experiments were conducted after 4, 16 and 72 h of treatment with forskolin, RA or the combination of the two compounds. The plasmids used as probes were two L/B/K-ALP genomic clones specific for leader exons 1A and 1B respectively. At all time points, and under all the experimental conditions, exon 1A is not transcribed in the nucleus. This is not due to technical problems, for a strong signal is observed in F9 teratocarcinoma cells, which are known to express only the mRNA containing leader exon 1A [46]. Exon 1B is not transcribed in L929 nuclei after incubation with medium alone or with medium containing 10^{-8} M RA, at 4 and 72 h, whereas it shows a weak, but significant, signal at 16 h. A much stronger signal corresponding to the transcription of exon 1B is readily observed after treatment of L929 cells with 10^{-6} M RA for 16 h (M. Giannì, M. Terao and E. Garattini, unpublished work). Exon 1B is not transcribed after treatment with forskolin for 4 h, but it shows a strong hybridization signal at 16 and 72 h. When RA is present, the signal induced by forskolin is stimulated 4-fold at 16 and 2-fold at 72 h. The transcriptional activity of the genes coding for α-actin and histone H4 were also measured for

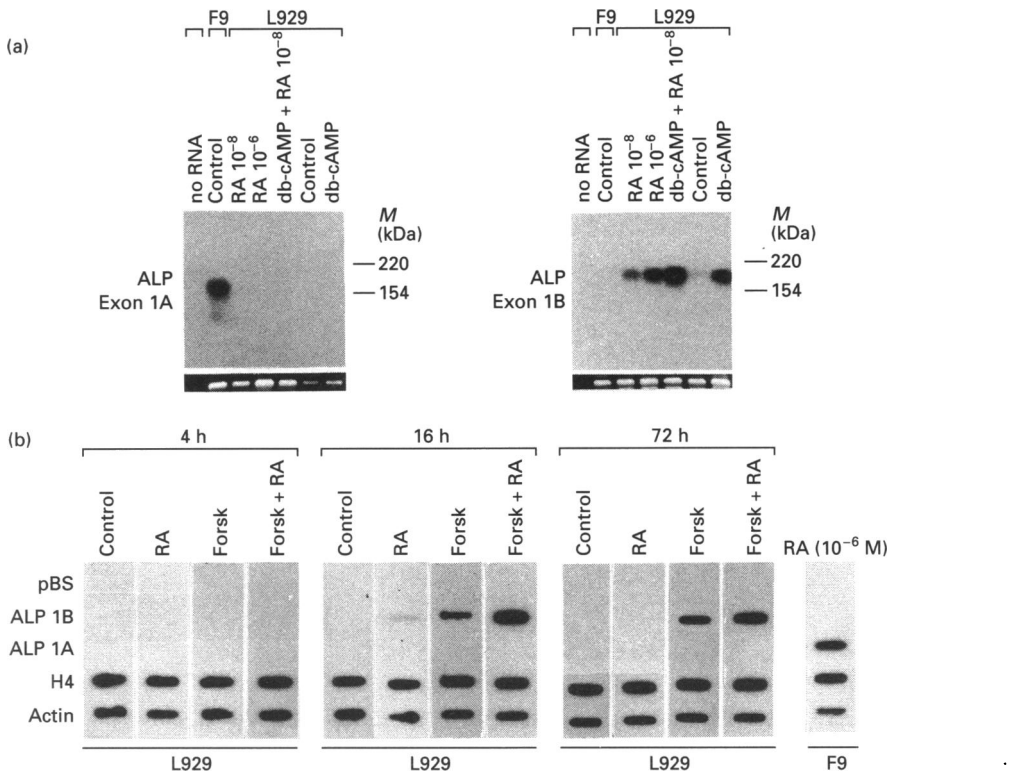

Figure 7 Expression of exons 1A and 1B in L929 cells after treatment with RA, db-cAMP or the combination of the two compounds

(**a**) Total RNA (1 μg) from L929 cells treated with the indicated compounds for 72 h was reverse-transcribed, and the resulting cDNA mixture was divided into three equal portions and PCR-amplified using oligonucleotide couples specific for ALP exons 1A and 1B, and for β-actin cDNA respectively. The products of the amplification were run on a 1.5%-agarose gel and directly photographed after ethidium bromide staining (β-actin; shown on the bottom) or blotted on nylon membranes (ALP exons 1A and 1B) and hybridized to ^{32}P-labelled synthetic nucleotides specific for each exon. The oligonucleotides employed for the hybridization were distinct and internal to the couple of oligonucleotides used for the amplification step. The expected size of the amplified products is 180, 186 and 324 bp for L/B/K-ALP exon 1A, exon 1B and β-actin respectively. The position of the molecular-mass (*M*) markers are indicated on the right-hand side. (**b**) Nuclear run-on assay was performed at the indicated times after incubation of L929 cells in the presence of the indicated compounds. As a positive control for the expression of nascent pre-mRNA containing ALP exon 1A, a run-on experiment was conducted on nuclei isolated from F9 teratocarcinoma cells incubated with 1×10^{-6} M retinoic acid for 48 h (F9). The probes used in the experiments are indicated. Plasmid pBluescript (pBS) was used as a control for non-specific hybridization. ALP 1A, pBS containing a genomic insert encompassing exon 1A of the ALP gene; ALP 1B, pBS containing a genomic insert encompassing exon 1B of the ALP gene; H4, pBR322 containing histone H4 cDNA; Actin, pBR322 containing mouse α-actin cDNA.

comparison. As expected on the basis of Northern-blot analysis (see Figure 5), the transcriptional rate of these two genes is high in basal conditions and it is not affected by any treatment.

RA does not influence the intracellular levels of cAMP and PKA

To assess the effect of RA on the amount of intracellular cAMP, L929 cells were treated in the presence of phosphodiesterase inhibitors for 5 min, 1 and 24 h with medium alone, medium containing RA, forskolin or the combination of the two compounds. As shown in Table 3, the levels of the cyclic nucleotide are significantly increased by forskolin at all the three time points. Maximal stimulation is observed at 24 h, demonstrating that forskolin is producing a long-lasting activation of adenylate cyclase that leads to progressive accumulation of cAMP. RA does not modulate the levels of intracellular cAMP either in the absence or in the presence of forskolin.

The levels of PKA were also determined after treatment with RA, forskolin or the combination of the two compounds at 5 min, 1 and 24 h. In Table 3, PKA activity is expressed as the difference between phosphotransferase activity before and after addition of 10 μM cAMP to the assay mixtures. PKA is decreased relative to control levels after treatment with forskolin for 1 and 24 h. This is not unexpected [30], and it results from increased basal levels of the phosphotransferase activity in the absence of cAMP exogenously added to the reaction mixture. RA is not altering the amounts of PKA either in the absence or in the presence of forskolin. Taken together, these data indicate that RA treatment is not affecting the two most upstream components of the cAMP second-messenger cascade.

Increased intracellular cAMP induces the expression of the mRNAs coding for RAR α, β and γ isoforms

Figure 8 demonstrates that L929 cells express the three classes of the RAR mRNAs in basal conditions. In particular, two separable bands specifically hybridizing with the RARα cDNA and one band each, hybridizing with RAR β and γ cDNAs are evident. Their levels do not change if cells are kept in normal growth medium up to 72 h. Treatment of L929 cells with db-cAMP increases the levels of the two RAR α mRNAs at 6 and 24 h. At 48 and 72 h of treatment, the amounts of RARα mRNAs are decreased to those observed under basal conditions. The co-treatment of L929 cells with db-cAMP and RA does not substantially alter the amounts of the two RAR α transcripts relative to db-cAMP alone. Starting from 6 h of treatment, db-cAMP induces the expression of novel and more-rapidly-migrating molecular forms of the RAR β mRNAs. After db-cAMP

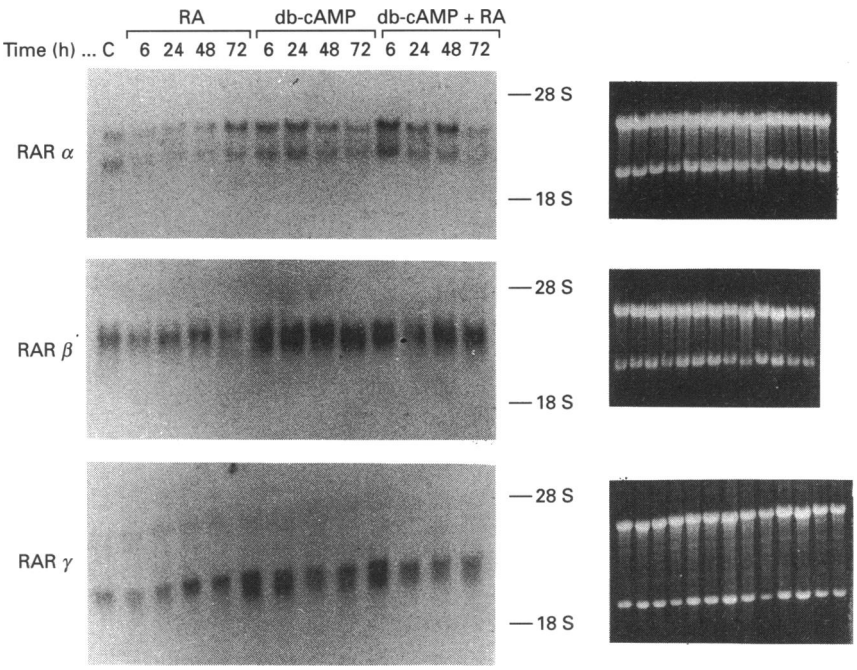

Figure 8 Effect of db-cAMP, RA and the combination of the two compounds on the steady-state levels of RAR α, β and γ mRNAs

Total RNA was extracted from L929 cells treated as indicated. The same RNA preparation (10 μg for each) was used to perform Northern-blot analysis. The three membranes were hybridized with probes specific for RAR α, β and γ, respectively. The ethidium bromide staining of each gel is shown on the right side and demonstrates that an equal amount of RNA was added in each lane. C, control.

treatment, the induced mRNAs are incompletely separated and consistently result in a smeared hybridization signal. New and separable band(s) hybridizing to RAR γ cDNA are observed after treatment with the cAMP analogue. The induction of both RAR β and γ mRNAs is long lasting and it is observed up to 72 h. The combination of RA and db-cAMP only minimally influences the levels of RAR β and γ transcripts relative to db-cAMP alone. Collectively taken, these results demonstrate that the increase in intracellular cAMP levels alters the expression of RAR mRNAs both qualitatively and quantitatively.

DISCUSSION

The mouse [46,47,69], human [42,50] and rat L/B/K-ALP [49] genes have a very similar structural organization and contain two leader exons, 1A and 1B, separated by a long first intron. We have suggested that the tissue-specific expression of the two alternatively spliced mRNAs containing exon 1A and 1B respectively is controlled by the activity of two distinct promoters [46], similar to what is observed for other genes with more than one leader exon [74,75]. In the mouse L/B/K-ALP gene, the 5′ flanking region of exon 1A has strong promoter activity in various cellular contexts ([69]; M. Giannì, M. Terao and E. Garattini, unpublished work), whereas promoter activity in the 5′ flanking region of exon 1B has not yet been demonstrated.

In mouse F9 teratocarcinoma cells we have previously shown that RA induces L/B/K-ALP protein by increasing the amount of the relative mRNA containing exon 1A through augmentation of the transcriptional activity of the gene [46,69]. This effect is probably the consequence of an activation of the putative promoter placed in front of leader exon 1A. However, sequences that are structurally [69] or functionally (M. Giannì, M. Terao and E. Garattini, unpublished work) similar to an RARE do not seem to be present in the 5′ flanking region of this exon. This is in contrast with what has been reported for the human L/B/K-ALP gene [76], where a RARE (albeit atypical) has been described.

In L929 cells, we observed that RA is capable of inducing the expression of the L/B/K-ALP transcript containing exon 1B through a transcriptional mechanism. However, a sequence structurally or functionally similar to a typical RARE is not present in the 5′ flanking region of this exon ([46]; M. Giannì, M. Terao and E. Garattini, unpublished work). This finding has important implications for the regulation of the L/B/K-ALP gene by RA, since it demonstrates that the transcription of both leader exons is responsive to RA. We suggest that the cellular context is playing an important role in determining which exon is transcribed as a consequence of the treatment with the retinoid. It is tempting to speculate that the chromatin environment around exon 1A or 1B in different cell types is dictating the accessibility of the transcription factors involved in the basal as well as in the induced expression of the L/B/K-ALP gene.

The major point of the present study is represented by the observation of a strong synergism between cAMP and RA in the induction of the expression of the L/B/K-ALP gene in L929 fibroblastic cells. This is different from what we observed in F9 teratocarcinoma cells, where cAMP antagonizes the induction of L/B/K-ALP activity by RA [53]. The synergism is at the level of the transcriptional activity of the gene and results in the up-regulation of the transcript containing exon 1B.

The onset of this synergistic effect is slow, requiring more than 16 h to be apparent at the level of enzymic activity. This finding is similar to what was observed for two other gene products, tissue transglutaminase [27] and the urokinase-type tissue plasminogen activator [30], whose expression is synergistically

Table 3 Effect of forskolin, RA and the combination of the two compounds on the intracellular levels of cAMP and cAMP-dependent PKA

To measure intracellular levels of cAMP, L929 cells, which were grown to confluency in 12 cm^2 culture dishes, were treated with forskolin (100 μM), RA (10^{-8} M) or the combination of the two compounds in the presence of the phosphodiesterase inhibitors isobutylmethylxanthine (0.2 mM) and Ro 20-1724 (0.02 mM) for the indicated amount of time. Control cultures were grown in the presence of phosphodiesterase inhibitors alone. To measure cAMP-dependent PKA, L929 cells were grown to confluency in 25 cm^2 culture flasks and treated as above in the absence of phosphodiesterase inhibitors. PKA is measured as the difference in the incorporation of radiolabelled ^{32}P into histone 2A in the presence and in the absence of exogenously added cAMP (10 μM). Each value is the mean ± S.D. for three separate cultures. Statistical significance: * significantly lower relative to control ($P < 0.01$ according to Duncan's test); ** significantly lower relative to control ($P < 0.05$ according to Duncan's test).

		cAMP (pmol/1 × 10^6 cells)			PKA activity (units/mg of protein)		
Treatment	Time ...	5 min	1 h	24 h	5 min	1 h	24 h
Control		0.9 ± 0.2	0.8 ± 0.1	2.0 ± 0.6	163 ± 15	171 ± 11	177 ± 27
RA		0.9 ± 0.1	0.8 ± 0.1	2.3 ± 0.1	172 ± 15	159 ± 5	171 ± 26
Forskolin		12.0 ± 0.6*	17.4 ± 0.9*	30.0 ± 0.6*	154 ± 16	143 ± 19**	127 ± 13**
Forskolin + RA		13.4 ± 1.2*	16.6 ± 0.3*	29.1 ± 0.9*	136 ± 19	143 ± 11**	134 ± 6**

augmented by cAMP and RA in a cervical adenocarcinoma cell line and in macrophages respectively. The slow induction of the expression of the L/B/K-ALP gene suggests that the synthesis of intermediary transcriptional factors is required. Transcriptional factors belonging to the family of Hox gene products, whose synthesis is known to be increased by cAMP and RA in F9 teratocarcinoma cells [77,78], might be implicated in the up-regulation of L/B/K-ALP mRNA in L929 fibroblasts. If intermediary transcriptional factors are indeed synthesized before the induction of the expression of the L/B/K-ALP gene, protein-synthesis inhibitors should block the response. However, protein-synthesis inhibition has complex outcomes on the induction of L/B/K-ALP transcript in L929 cells. In basal conditions or after treatment with RA, CHX decreases the levels of one or more short-lived proteins involved either in the transcriptional repression of the L/BK-ALP gene or in the catabolism of the L/B/K-ALP mRNA. After treatment with cAMP elevating agents, CHX blocks the synthesis of protein factors involved in the induction of gene expression. Thus the net effect of CHX on L/B/K-ALP mRNA after induction with cAMP and RA is practically null and it is the result of two opposing interactions that tend to counterbalance each other.

Some of the factors potentially involved in the synergistic interaction between RA and cAMP have been studied. Theoretically, RA could render cells more susceptible to the effects of cAMP or, by contrast, cAMP could sensitize them to the action of retinoids. Regarding the first possibility, if RA is sensitizing L929 cells to cAMP, this does not involve the priming effect suggested in other cellular models [30]. Indeed, the incubation of cells with the retinoid prior to the addition of cAMP leads to a complete loss of the synergism between the two compounds. Furthermore, our data clearly demonstrate that RA does not affect the first two steps in the cascade of events triggered by cAMP. The retinoid alters neither the intracellular levels of cAMP nor those of PKA. It has been observed that RA is inducing the activity of protein kinase C [20]. Indeed, protein kinase C may be involved in the cross-talk between RA and cAMP second-messenger pathways through the phosphorylation and activation of relevant substrates involved in the cascade of events triggered by protein kinase A. Experiments performed with tetradecanoyl phorbol acetate, a direct activator of protein kinase C, in the presence of db-cAMP, do not support this hypothesis (M. Giannì, M. Terao and E. Garattini, unpublished work). At present we cannot rule out the possibility that RA stimulates the synthesis or reduces the turnover of protein factors such as CREBPs (cAMP-response-element-binding proteins) [79,80] that act as substrates for the phosphorylation of PKA. RA might also modify the CREBPs or other protein factors post-translationally, rendering them more susceptible to the activation of cAMP. It is known that RA induces the retinoylation of several proteins [24,81–83]; however, this kind of post-translational modification has never been proved to have any functional consequence.

Taken together, the evidence presented here suggests that cAMP is sensitizing the cells to the action of RA. Firstly, synergism between db-cAMP and RA is observed when L929 cells are concomitantly treated with the two compounds or when cells are treated with the cyclic nucleotide before the addition of the retinoid. Secondly, cAMP induces the mRNA accumulation of the two transcripts coding for the RAR α isoforms already present under basal conditions in L929. Thirdly, mRNA isoforms of the RAR β and γ that are not expressed in L929 cells under basal conditions appear as a result of treatments elevating intracellular cAMP. The kinetics of these effects on RAR transcripts are consistent with the induction of L/B/K-ALP and could entirely explain the observed synergistic interaction between cAMP and RA. It would be of considerable interest to know whether the RAR β and γ mRNA forms induced by cAMP represent novel or already described molecular species. In the case of the RAR γ transcripts expressed in L929 cells, the electrophoretic pattern is consistent with constitutive expression of the γ2 form and cAMP-induced expression of the γ1 form [84]. However, it remains to be demonstrated that these quantitative and qualitative changes in the RAR mRNAs is associated with translation of the transcripts into active proteins. Until this point is experimentally verified, other possibilities should be considered to explain the synergism between retinoids and cAMP. Mouse RARs type α, β and γ contain several amino acid sequences that might be phosphorylated by cAMP-dependent PKA [11,14,15]. Moreover, phosphorylation of the various forms of RAR has been proved in cell culture [85]. Up-regulation of the level of phosphorylation might render RARs more responsive to RA and thus increase the transcription of responsive genes. It is also possible that cAMP is inducing the expression or causing post-translational modifications of RXRs, which are known to stimulate the transactivation ability of RARs in co-transfection experiments with appropriate reporter genes [12,17–19]. Finally the interaction between cAMP and RA might be at the level of binding and activation of the regulatory elements on the L/B/K-ALP gene. The analysis of such an interaction calls for the determination of the sequences important for the regulation of the L/B/K-ALP gene by cAMP and RA respectively.

Experiments are in progress to define responsive elements to RA and cAMP outside the 5′ flanking regions of exons 1A and 1B. Moreover, we are currently investigating whether selective stimulation of the α, β and γ type RARs or RXRs by specific

agonists [86,87] or by 9-*cis*-retinoic acid [88,89] respectively are also capable of reproducing the synergism with cAMP.

This work was supported in part by grants from the Consiglio Nazionale delle Richerche, Progetto Finalizzato 'Biotecnologie e Biostrumentazione', 'Ingegneria Genetica', 'Chimica Fine' and the Associazione per la Ricerca contro il Cancro (AIRC). We thank Professor P. Chambon for kindly providing the cDNA clones coding for the retinoic acid receptors type α, β and γ. We are also grateful to Professor S. Garattini and Dr. M. Salmona for critical reading of the manuscript and to Dr. E. Ghibaudi and Mr. F. Passerini for the synthesis of the oligonucleotides.

REFERENCES

1 Breitman, T. R., Selonick, S. E. and Collins, S. J. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2936–2940
2 Lotan, R. (1980) Biochim. Biophys. Acta **605**, 33–91
3 Thaller, C. and Eichele, G. (1987) Nature (London) **327**, 625–628
4 Sherman, M. I. (1986) in 'Retinoids and Cell Differentiation' (Sherman, M. I., ed.), pp. 161–186, CRC Press, Boca Ratón, FL
5 Schindler, J. (1986) in 'Retinoids and Cell Differentiation' (Sherman, M. I., ed.), pp. 137–159, CRC Press, Boca Ratón, FL
6 La Rosa, G. J. and Gudas, L. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 329–333
7 Nicholson, R. C., Mader, S., Nagpal, S., Leid, M., Rochette-Egly, C. and Chambon, P. (1990) EMBO J. **9**, 4443–4454
8 Petkovich, M., Brand, N. J., Krust, A. and Chambon, P. (1987) Nature (London) **330**, 444–450
9 Giguere, V., Ong, E. S., Sequi, P. and Evans, R. M. (1987) Nature (London) **330**, 624–629
10 Zelent, A., Krust, A., Petkovich, M., Kastner, P. and Chambon, P. (1989) Nature (London) **339**, 714–717
11 Krust, A., Kastner, P., Petkovich, M., Zelent, A. and Chambon, P. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5310–5314
12 Mangelsdorf, D. J., Umesono, K., Kliever, S. A., Borgmeyer, U., Ong, E. S. and Evans, R. M. (1991) Cell **66**, 555–561
13 Umesono, K., Murakami, K. K., Thompson, C. C. and Evans, R. M. (1991) Cell **65**, 1255–1266
14 Leroy, P., Krust, A., Zelent, A., Mendelsohn, C., Garnier, J.-M., Kastner, P., Dierich, A. and Chambon, P. (1991) EMBO J. **10**, 59–69
15 Zelent, A., Mendelsohn, C., Kastner, P., Krust, A., Garnier, J.-M., Ruffenach, F., Leroy, P. and Chambon, P. (1991) EMBO J. **10**, 71–81
16 Kliewer, S. A., Umesono, K., Mangelsdorf, D. J. and Evans, R. M. (1992) Nature (London) **355**, 446–449
17 Zhang, X. K., Hoffmann, B., Tran, P. B.-V., Graupner, G. and Pfahl, M. (1992) Nature (London) **355**, 441–446
18 Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J-Y., Staub, A., Garnier, J.-M., Mader, S. and Chambon, P. (1992) Cell **68**, 377–395
19 Bugge, T. H., Pohl, J., Lonnoy, O. and Stunnenberg, H. G. (1992) EMBO J. **11**, 1409–1418
20 Chytil, F. and Ong, D. E. (1984) in The Retinoids (Goodman, D. S., ed.), vol. 2, pp. 89–123, Academic Press, San Diego
21 Bailey, J. S. and Siu, C.-H. (1988) J. Biol. Chem. **263**, 9326–9332
22 Kitamoto, T., Momoi, T. and Momoi, M. (1988) Biochem. Biophys. Res. Commun. (1988) **157**, 1302–1308
23 Giguere, V., Lyn, S., Yip, P., Siu, C. H. and Amin, S. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6233–6237
24 Takahashi, N., Liapi, C., Anderson, W. B. and Breitman, T. R. (1991) Arch. Biochem. Biophys. **290**, 293–302
25 Gruber, J. R., Ohno, S. and Niles, R. M. (1992) J. Biol. Chem. **267**, 13356–13360
26 Strickland, S., Smith, K. K. and Marotti, K. R. (1980) Cell **21**, 347–355
27 Olsson, I. L. and Breitman, T. R. (1982) Cancer Res. **42**, 3924–3927
28 Murtaugh, M. P., Moore, W. T. Jr. and Davies, P. J. A. (1986) J. Biol. Chem. **261**, 614–621
29 Hu, L. and Gudas, L. J. (1990) Mol. Cell. Biol. **10**, 391–396
30 Mira-Y-Lopez, R. (1991) J. Cell. Physiol. **147**, 46–54
31 Vasios, G. W., Gold, J. D., Petkovich, M., Chambon, P. and Gudas, L. J. (1992) Proc. Natl. Acad. Sci. U.S.A. **86**, 9099–9103
32 Weiler-Guettler, H., Yu, K., Soff, G., Gudas, L. J., Rosenberg, R. D. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 2155–2159
33 McComb, R. B., Bowers, G. N., Jr. and Posen, S. (1979) Alkaline Phosphatase, Plenum Publishing Corp., New York
34 Swarup, G., Cohen, S. and Garbers, D. L. (1981) J. Biol. Chem. **156**, 8197–8201
35 Makyia, R. and Stigbrand, T. (1991) Eur. J. Biochem. **205**, 341–345
36 Weiss, M. J., Henthorn, P. S., Lafferty, M. A., Slaughter, C., Raducha, M. and Harris, H. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7182–7186
37 Garattini, E., Hua, J. C., Pan, Y. E. and Udenfriend, S. (1986) Arch. Biochem. Biophys. **245**, 331–337
38 Garattini, E., Hua, J. C. and Udenfriend, S. (1987) Gene **59**, 41–46
39 Terao, M. and Mintz, B. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7051–7055
40 Henthorn, P. S., Raducha, M., Edwards, Y. H., Weiss, M. J., Slaughter, C., Lafferty, M. A. and Harris, H. H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1234–1238
41 Berger, J., Garattini, E., Hua, J. C. and Udenfriend, S. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 695–698
42 Weiss, M. J., Ray, K., Henthorn, P. S., Lamb, B., Kadesch, T. and Harris, H. (1988) J. Biol. Chem. **263**, 12002–12010
43 Knoll, B. J., Rothblum, K. N. and Longley, M. (1988) J. Biol. Chem. **263**, 12020–12027
44 Millan, J. L. and Manes, T. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3024–3028
45 Manes, T., Glade, K., Ziomek, C. A. and Millan, J. L. (1990) Genomics **8**, 541–554
46 Studer, M., Terao, M., Giannì, M. and Garattini, E. (1991) Biochem. Biophys. Res. Commun. **179**, 1352–1360
47 Toh, Y., Yamamoto, M., Endo, H., Misumi, Y. and Ikehara, Y. (1990) Biochem. Int. **22**, 213–218
48 Brown, N. A., Stofko, R. E. and Uhler, M. D. (1990) J. Biol. Chem. **265**, 13181–13189
49 Toh, Y., Yamamoto, M., Endo, H., Misumi, Y. and Ikehara, Y. (1989) Eur. J. Biochem. **182**, 231–237
50 Matsuura, S., Kishi, F. and Kajii, T. (1990) Biochem. Biophys. Res. Commun. **168**, 993–1000
51 Firestone, G. L. and Heath, E. C. (1981) J. Biol. Chem. **256**, 1396–1403
52 Green, E., Todd, B. and Heath, D. (1990) Eur. J. Biochem. **188**, 147–153
53 Giannì, M., Studer, M., Carpani, G., Terao, M. and Garattini, E. (1991) Biochem. J. **274**, 673–678
54 Zernik, J., Kream, B. and Twarog, K. (1991) Biochem. Biophys. Res. Commun. **176**, 1149–1156
55 Strickland, S. and Mahdavi, V. (1978) Cell **15**, 393–403
56 Scheibe, R. J., Moeller-Runge, I. and Mueller, W. H. (1991) J. Biol. Chem. **266**, 21300–21305
57 Reese, D. H. and Politano, V. A. (1981) Biochem. Biophys. Res. Commun. **102**, 322–327
58 Reese, D. H., Larsen, R. A. and Hornicek, F. J. (1992) J. Cell. Physiol. **151**, 239–248
59 Bradford, M. (1976) Anal. Biochem. **72**, 248–254
60 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
61 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
62 Rambaldi, A., Young, D. C. and Griffin, J. D. (1987) Blood **69**, 1409–1413
63 Minty, A. J., Caravatti, M., Robert, B., Cohen, A., Daubas, P., Weydert, A., Gros, F. and Buckingham, M. E. (1981) J. Biol. Chem. **256**, 1008–1014
64 Seiler-Tuyns, P. and Birnstiel, M. L. (1981) J. Mol. Biol. **157**, 607–625
65 Feinberg, A. P. and Vogelstein, B. (1983) Anal. Biochem. **132**, 6–13
66 Alonso, S., Minty, A., Bourlet, Y. and Buckingham, M. (1986) J. Mol. Evol. **23**, 11–22
67 Wood, W. I., Gitschier, J., Lasky, L. A. and Levin, R. M. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1585–1588
68 Eick, D. and Bornkamm, G. W. (1986) Nucleic Acids Res. **14**, 8331–8346
69 Terao, M., Studer, M., Giannì, M. and Garattini, E. (1990) Biochem. J. **268**, 641–648
70 Sozzani, S., Agwu, D. E., McCall, C. E., O'Flaherty, J. T., Schmitt, J. D., Kent, J. D. and McPhail, L. C. (1992) J. Biol. Chem. **267**, 20481–20488
71 Rocchetti, M. and Recchia, M. (1982) Comput. Prog. Biomed. **14**, 7–20
72 Duncan, D. B. (1975) Biometrics **31**, 339–359
73 Linton, M. and Gallo, P. S., Jr. (1975) The Practical Statistician Simplified Handbook of Statistics, Brooks/Cole, Monterey, CA
74 Hagenbuche, O., Tosi, M., Schibler, U., Bovey, R., Wellauer, P. and Young, R. A. (1981) Nature (London) **289**, 643–646
75 Nabeshima, Y., Fujii-Kuriyama, Y., Muramatsu, M. and Ogata, K. (1984) Nature (London) **308**, 333–339
76 Heath, J. K., Suva, L. J., Yoon, K., Kiledjian, M., Martin, T. J. and Rodan, G. A. (1992) Mol. Endocrinol. **6**, 636–646
77 Colberg-Poley, A. M., Voss, S. D., Chowdhury, K. and Gruss, P. (1985) Nature (London) **314**, 713–718
78 Breier, G., Bucan, M., Francke, U., Colberg-Poley, A. M. and Gruss, P. (1986) EMBO J. **5**, 2209–2215
79 Hoeffler, J. P., Meyer, T. E., Yungdae, Y., Jameson, J. L., Habener, J. F. (1988) Science **242**, 1430–1433
80 Gonzalez, G. A., Yamamoto, K. K., Fischer, W. H., Karr, D., Menzel, P., Biggs, W., Vale, W. W. and Montminy, M. R. (1989) Nature (London) **337**, 749–752
81 Takahashi, N. and Breitman, T. R. (1989) J. Biol. Chem. **264**, 5159–5163

82 Takahashi, N. and Breitman, T. R. (1989) *In vitro* Cell. Dev. Biol. **25**, 1199–1200
83 Takahashi, N. and Breitman, T. R. (1991) Arch. Biochem. Biophys. **285**, 105–110
84 Kastner, P., Krust, A., Mendelsohn, C., Garnier, J. M., Zelent, A., Leroy, P., Staub, A. and Chambon, P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2700–2704
85 Gaub, M. P., Rochette-Egly, C., Lutz, Y., Ali, S., Matthes, H., Scheuer, I. and Chambon, P. (1992) Exp. Cell Res. **201**, 335–346
86 Delescluse, C., Cavey, M. T., Martin, B., Bernard, B. A., Reichert, U., Maignan, J., Darmon, M. and Shroot, B. (1991) **40**, 556–562
87 Bernard, B. A., Bernardon, J. M., Delescluse, C., Martin, B., Lenoir, M. C., Maignan, J., Charpentier, B., Pilgrim, W. R., Reichert, U. and Shroot, B. (1992) Biochem. Biophys. Res. Commun. **186**, 977–983
88 Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberg, M., Lovey, A. and Grippo, J. F. (1992) Nature (London) **355**, 359–361
89 Heyman, R. A., Mangelsdorf, D. J., Dick, J. A., Stein, R. B., Eichele, G., Evans, R. M. and Thaller, C. (1992) Cell **68**, 397–406

Received 17 February 1993/3 June 1993; accepted 25 June 1993