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Retinoic Acid Increases *zif268* Early Gene Expression in Rat Preosteoblastic Cells

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In this study we demonstrate that retinoic acid (RA) increases the expression of transcription factor *zif268* mRNA in primary cultures of fetal rat calvarial cells and in simian virus 40-immortalized clonal rat calvarial preosteoblastic cells (RCT-1), which differentiate in response to RA, but not in the more differentiated RCT-3 and ROS 17/2.8 cells. The increased expression of *zif268* mRNA is rapid (maximal within 1 h), transient (returns to basal levels by 3 h), detectable at RA doses of 10^{-12} M, and independent of protein synthesis. The relative stimulation of *zif268* mRNA by RA was much larger than that of other early genes, including c-*fos*, c-*jun*, and *junB*. The rate of transcription of RA-stimulated RCT-1 cells, estimated by nuclear run-on assays, was elevated, suggesting that RA regulation of *zif268* gene transcription was at least in part transcriptional. Moreover, RA stimulated the transcriptional activity of a Zif268CAT (chloramphenicol acetyltransferase) plasmid containing 632 bp of *zif268* 5′ regulatory sequences in RCT-1 cells but not in the more differentiated RCT-3 cells. These in vitro data support the in vivo observations which localize *zif268* and RA receptor-γ transcripts to bone and cartilage during development, suggesting that both RA and *zif268* may play a role in osteoblast differentiation.

The growth and differentiation of mammalian cells is regulated by many factors, one of which is the putative morphogen retinoic acid (RA). However, the mechanism by which RA induces cell growth and differentiation is at present unclear. The identification of specific RA receptors (RARs), which are structurally related to the steroid-thyroid hormone receptor superfamily (1, 2, 14, 19, 20, 31, 34, 48), suggests that RA effects are mediated by the interaction of activated RARs with certain *cis*-acting DNA sequences which regulate the expression of particular target genes.

A number of genes, including the proto-oncogenes c-*myc*, c-*jun*, and c-*fos*, which have the properties of transcription factors and are localized in the nucleus, show rapid transcriptional activation in response to certain exogenous factors and were proposed to play a role in the regulation of gene expression during growth and differentiation (8, 11, 37, 38, 48, 60). The transcription factor *zif268* (8), also referred to as *Krox-24*, *NGFI-A*, and *Egr-1* (39, 48, 61), is a member of this family of inducible early-response genes. *zif268* encodes a protein of 533 amino acids containing three DNA-binding zinc finger regions (8, 48, 61) which bind to a specific sequence in genomic DNA (9). This gene was originally identified as an early-response gene following stimulation by mitogens (8, 48, 61) and serum proteins (8, 39, 60). *zif268* induction is not inhibited by cycloheximide (CHX), and the kinetics of its activation are similar to those described for c-*fos* (26, 60). Given the broad spectrum of stimuli which induce *zif268* expression, the resulting protein is likely to have an important function in many biological processes (47).

Transcripts of *zif268* were shown to be predominantly expressed in bone and cartilage during murine embryogenesis (47). Interestingly, these are also the known sites of c-*fos* expression (5, 49). In addition, RAR-γ transcripts have been localized to similar sites within the developing embryo (54), thus implicating *zif268* and RA in osteoblast differentiation in vivo. This colocalization of *zif268* and RAR-γ to developing bone and cartilage raises the possibility that in the early phases of bone development, *zif268* expression is linked to the physiological role of RA in bone.

We therefore examined whether *zif268* expression is regulated by RA in vitro in bone-derived cells. In this study, we demonstrate that in a cell line in which RA induces osteoblastic features and in primary cultures of embryonic osteoblastic cells, RA rapidly and transiently increases *zif268* mRNA. However, RA had no effect on *zif268* mRNA expression in differentiated cells, supporting the in vivo observations that *zif268* and RA may be involved in osteoblast differentiation.

MATERIALS AND METHODS

Cell culture. RCT-1 cells (28) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 μg of glutamine and 200 μg of G418 (GIBCO, Grand Island N.Y.) per ml, and RCT-3 cells (28) were maintained in F12 medium supplemented with 5% FBS and 200 μg of G418 (GIBCO) per ml. At appropriate times before harvest for RNA extraction, cells were treated with 1 μM all-*trans* RA (Sigma, St. Louis, Mo.), 1 μg of CHX (Sigma) per ml, and/or 2 μg of actinomycin D (Act D) (dactinomycin; Sigma) per ml. Primary rat calvarial osteoblasts (16) and ROS 17/2.8 cells (43) were prepared and cultured as described previously. Prior to harvest and extraction of total RNA, the cells were treated with either 1 μM all-*trans* RA or 10^{-10} M thyroid hormone (Sigma) in the presence of 10% charcoal-treated FBS.

RNA isolation and Northern (RNA) gel analysis. Total cellular RNA was isolated by guanidinium isothiocyanate and phenol extraction as previously described (7). Poly(A)$^+$ RNA was obtained by oligo(dT) cellulose chromatography (44). Total RNA (20 μg) or poly(A)$^+$ RNA (2 μg) was electrophoresed through 1% agarose gels containing 0.22 M

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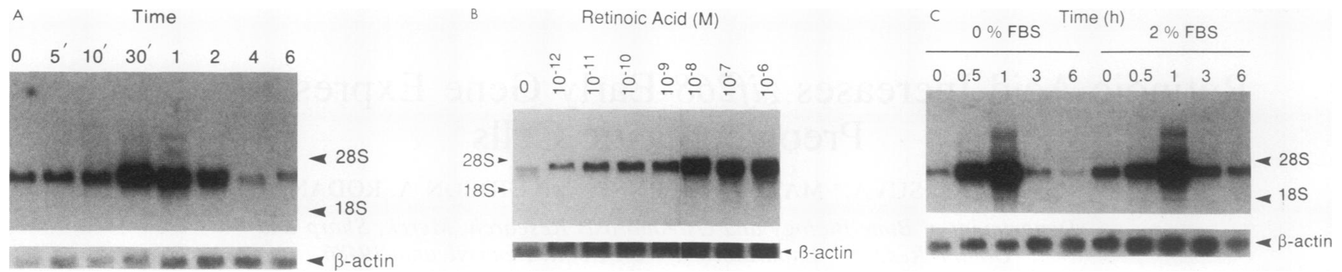


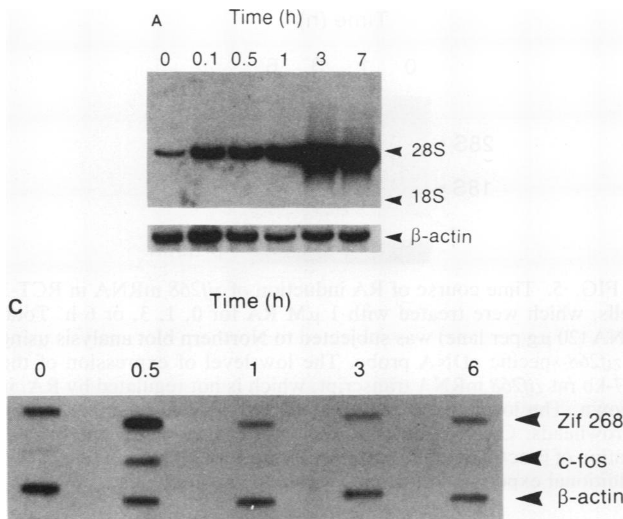
FIG. 1. Induction of *zif268* in RCT-1 cells. Total RNA (20 μg per lane) was subjected to Northern blot analysis using a *zif268*-specific cDNA probe which hybridized to the 3.7-kb rat *zif268* mRNA transcript as shown. The locations of 28S and 18S rRNAs are indicated by arrowheads. Comparison of RNA loading is shown by the hybridization of β-actin to the same filter. (A) Time course of induction by RA. RCT-1 cells were treated with 1 μM RA for the indicated times, from 0 to 6 h. Results are representative of four additional experiments, which yielded similar results. Densitometry measurements normalized relative to β-actin mRNA showed that *zif268* mRNA levels were 30-, 25-, and 12-fold above the time zero level at 30 min, 1 h, and 2 h, respectively. At 4 and 6 h, *zif268* levels consistently dropped below those of time zero controls. (B) Dose-dependent induction by RA. RCT-1 cells were treated with increasing concentrations of RA, from 10^{-12} to 10^{-6} M, for 1 h. Results are representative of three additional experiments, which yielded similar results. Densitometric analysis showed a 2- to 3-fold increase in *zif268* mRNA expression with 10^{-12} M RA and a 30-fold increase with 10^{-8} M RA. (C) Serum induction. RCT-1 cells were cultured in the presence of medium containing 0 or 2% FBS for 18 h, and then fresh medium containing 10% FBS was added for 0 to 6 h. Results are representative of two additional experiments, which yielded similar results.

formaldehyde and transferred to nitrocellulose (BRL, Bethesda, Md.) for screening as described previously (62).

cDNA probes and hybridization. cDNA probes for *zif268*, c-*jun*, *junB* (kindly provided by D. Nathans), chicken β-actin cDNA (Oncor, Gaithersburg, Md.), c-*fos*, (American Type Culture Collection, Rockville, Md.), and RAR-α, -β, and -γ (kindly provided by P. Chambon) were labeled with [α-^{32}P] deoxy-CTP (Amersham Corp, Arlington Heights, Ill.) by using a random primer DNA-labeling kit (Pharmacia, Piscataway, N.J.). The filters were prehybridized in a buffer containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM sodium phosphate (pH 7.0), 5× Denhardt's solution, 1% skim milk powder, 100 μg of sonicated salmon sperm DNA per ml, and 0.1% sodium dodecyl sulfate (SDS) at 42°C, hybridized in the same buffer containing ^{32}P-labeled cDNA probes at 10^6 cpm/ml for 18 h, and washed twice at 50°C in 0.1× SSC–0.1% SDS. Changes in RNA levels were quantified by normalization relative to β-actin levels by using an LKB Ultroscan XL enhanced-laser densitometer.

In vitro transcription assay. Isolation of nuclei, in vitro transcription, and hybridization were carried out essentially as described previously (46) with minor modifications. Approximately 2×10^7 to 3×10^7 nuclei were isolated by gentle homogenization of cells on ice in a buffer containing 10 mM KCl, 10 mM Tris-HCl (pH 7.4), 1.5 mM $MgCl_2$, and 0.25% (vol/vol) Nonidet P-40. The isolated nuclei were incubated for 20 min at 25°C in a buffer containing 50 mM Tris-HCl (pH 7.4); 100 mM ammonium sulfate; 1.8 mM dithiothreitol; 1.8 mM $MnCl_2$; 80 U of RNasin; 0.3 mM each ATP, GTP, and CTP; and 100 μCi of [α-^{32}P]UTP (800 Ci/mmol; Amersham) and then subjected to sequential digestions with DNase I and proteinase K. RNA was extracted by phenol-chloroform and ethanol precipitated. Following centrifugation at 12,000 × *g* for 10 min, the pellets were dissolved in 6 M guanidinium hydrochloride, and then 0.5 volume of ethanol was added and precipitation was done at −20°C overnight. After centrifugation at 12,000 × *g* for 10 min at 4°C, the pellets were rinsed with 80% ethanol, dried, and suspended in TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA). Prehybridization (16 h) and hybridization (96 h) were carried out in 1 ml of a solution containing 50% formamide, 5× SSC, 5× Denhardt's solution, 10 mM EDTA, 100 mM Tris-HCl (pH 7.4), 20 μg of tRNA per ml, 0.1% SDS, and 10 μg of sonicated salmon sperm DNA per ml at 42°C. Isolated ^{32}P-labeled transcripts (2×10^6 cpm/ml) were hybridized to 2 μg of plasmid cDNA insert bound to nitrocellulose (BRL). Filters were washed three times at room temperature in 2× SSC for 5 min per wash and twice in 0.1× SSC–0.1% SDS at 65°C for 20 min per wash. Filters were then autoradiographed at −70°C with intensifying screens and quantitated with an LKB Ultroscan XL enhanced-laser densitometer.

***zif268*-CAT construction and transient transfection.** A bacteriophage containing the *zif268* mRNA sequences and genomic flanking sequences from both the 5′ and 3′ regions was isolated from a Sprague-Dawley adult liver DNA library (Clontech, Palo Alto, Calif.) by using a cDNA probe. A *Sac*II fragment of 632 bp (−532 to +100) derived from the 5′ regulatory region of the *zif268* gene, which necessarily includes the transcriptional start site, was inserted in the sense orientation upstream of the promoterless chloramphenicol acetyltransferase (CAT) reporter gene coding sequence in the plasmid pCAT-Basic (Promega, Madison, Wis.) (pCAT0) to yield plasmid pZIFCAT1. This regulatory region was shown previously to be functional and regulated by nerve growth factor in transient transfection assays (6). The fragment endpoints were determined by dideoxynucleotide sequencing. RCT-1 or RCT-3 cells, grown as described above and seeded at 50,000 cells per cm^2, were transfected with pZIFCAT1 (10 μg), pCAT0 (10 μg), or pACTINCAT (2.5 μg) (55) by the DEAE-dextran-chloroquine procedure (40) for 1 h. Immediately after transfection, cells were treated with either control medium or medium supplemented with 1 μM RA for 48 h. Cell extracts were prepared according to a standard protocol (24), and CAT activities were determined (24). The protein concentrations in the cell extracts were determined (58), and the CAT activities were normalized relative to constant amounts of cell extract, usually 25 to 50 μg of protein per assay point. All assays were visualized by autoradiography using [^{14}C]chloramphenicol (50 Ci/mmol; Amersham), and acetylated products were quantitated by scintillation spectrometry.



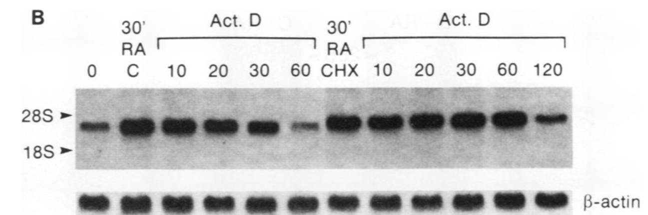


FIG. 2. (A) Effect of CHX on RA induction of *zif268* mRNA. RCT-1 cells were treated with 1 μM RA plus 1 μg of CHX per ml for the indicated times, from 0 to 7 h. Total RNA (20 μg per lane) was subjected to Northern blot analysis using a *zif268*-specific cDNA probe which hybridized with the specific 3.7-kb rat *zif268* mRNA transcript as shown. The locations of 28S and 18S rRNAs are indicated by arrowheads. Comparison of RNA loading is shown by the hybridization of β-actin to the same filter. Results are representative of two additional experiments, which yielded essentially identical results. (B) Transcriptional activation of *zif268* mRNA by RA in RCT-1 cells. RCT-1 cells were treated with either 1 μM RA or 1 μM RA plus 1 μg of CHX per ml for 30 min, and then both groups of cells were treated with 2 μg of Act D per ml for the indicated times from 10 to 120 min. Total RNA (20 μg per lane) was subjected to Northern blot analysis using a *zif268*-specific cDNA probe which hybridized with the 3.7-kb rat *zif268* mRNA transcript as shown. The locations of 28S and 18S rRNAs are indicated by arrowheads. Comparison of RNA loading is shown by the hybridization of β-actin to the same filter. Lane 0, Unstimulated RCT-1 cells; lane 30′ RA C, RCT-1 cells after 30 min of treatment with 1 μM RA, lanes 10, 20, 30, and 60, RNA from RCT-1 cells treated with 2 μg of Act D per ml for 10, 20, 30, or 60 min after 30 min of treatment with 1 μM RA; lane 30′ RA CHX, RCT-1 cells treated with 1 μM RA plus 1 μg of CHX per ml for 30 min; lanes 10, 20, 30, 60, and 120, RNA from RCT-1 cells treated with 2 μg of Act D per ml for 10, 20, 30, 60, or 120 min after 30 min of treatment with 1 μM RA plus 1 μg of CHX per ml. Results are representative of two additional experiments, which yielded similar results. (C) Transcriptional regulation of *zif268* and c-*fos* by RA. Transcriptional activity of the genes encoding *zif268* and c-*fos* was evaluated by nuclear run-on assays at 0, 0.5, 1, 3, and 6 h after stimulation with 1 μM RA. A positive, unresponsive control is represented by β-actin. Results are representative of one additional experiment, which yielded similar results.

RESULTS

Kinetics of *zif268* mRNA induction by RA in preosteoblastic cells. RCT-1 cells are clonal simian virus 40-immortalized rat calvarial preosteoblasts that, in the presence of RA, acquire phenotypic characteristics of more mature osteoblasts, including up-regulation of type I collagen mRNA, parathyroid hormone-responsive adenylate cyclase, and alkaline phosphatase activity (28). RNA prepared from untreated cultures of RCT-1 cells grown in the presence of 10% FBS contain a single *zif268* mRNA species of approximately 3.7 kb. Stimulation of RCT-1 cells with 1 μM RA resulted in a rapid and transient increase in *zif268* mRNA expression (10- to 30-fold), reaching a maximum at 30 min and returning to below basal levels within 3 h (Fig. 1A). The increased expression of *zif268* mRNA by RA is dose dependent, being first detectable at concentrations as low as 10^{-12} M (2- to 3-fold increase) and reaching a plateau at 10^{-8} M (30-fold increase) (Fig. 1B).

It has been shown previously that *zif268* is rapidly induced by serum in quiescent mouse fibroblasts (8, 39, 61). To compare the induction of *zif268* by RA with that by serum, RCT-1 cells were made quiescent by growth overnight in the absence or in a low concentration of FBS (2%) and then stimulated by the addition of fresh medium containing 10% FBS. Serum stimulation of RCT-1 cells also resulted in a rapid and transient increase of *zif268* mRNA expression (10- to 20-fold) (Fig. 1C).

Effect of protein synthesis inhibitors on *zif268* mRNA stability. It has been shown that the transcription of the c-*fos* and c-*myc* genes can be prolonged in the presence of protein synthesis inhibitors such as CHX (3, 25, 26, 32). This phenomenon has also been observed for many other early-response genes, including *zif268*, which is superinduced in the presence of CHX (38). In RCT-1 cells treated with RA alone, *zif268* mRNA levels increased 30-fold by 30 min and were substantially reduced by 3 h (Fig. 1A). The presence of CHX (1 μg/ml) did not alter *zif268* mRNA levels 30 min after the addition of RA; however, at 3 h, *zif268* mRNA levels were significantly higher and remained elevated for at least 7 h (Fig. 2A). Thus, CHX inhibits transcriptional shutdown and stabilizes *zif268* mRNA, suggesting that protein synthesis is required to degrade *zif268* mRNA and/or repress *zif268* gene transcription in RCT-1 cells.

Characteristically, the mRNAs of early-response genes have short half-lives (3, 26, 32, 38). To examine the effect of CHX on the stability of *zif268* mRNA in the absence of transcription, RCT-1 cells were treated with 1 μM RA either in the absence or presence of 1 μg of CHX per ml for 30 min and then treated with 2 μg of Act D per ml for increasing times from 0 to 2 h. The addition of Act D, following RA, caused a rapid decay of *zif268* mRNA, which decreased to basal levels by 1 h (Fig. 2B). However, when RCT-1 cells were treated with both RA and CHX followed by Act D, levels of *zif268* mRNA fell to basal level within 2 h (Fig. 2B). These observations indicate that RA acts, at least in part, at the level of gene transcription.

Transcriptional regulation of *zif268* by RA. It has been shown that the RA regulation of gene expression occurs at both the transcriptional (36, 59, 64), and posttranscriptional (23) levels. We examined the effect of 1 μM RA on the transcription rate of the *zif268* gene by in vitro nuclear transcription assays. *zif268* in vitro transcription was stimulated fourfold within 30 min of RA addition (Fig. 2C), indicating that RA directly stimulates the transcription of the *zif268* gene. In the same experiment, the transcriptional rate of c-*fos* was also rapidly stimulated by approximately five-fold within 30 min. In addition, the transcriptional rates of other genes unresponsive to RA, such as β-actin, which served as a control, were relatively unaffected (Fig. 2C).

RA regulation of other immediate-early genes in preosteo-

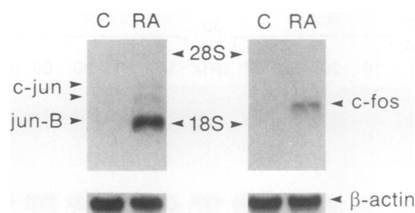


FIG. 3. Induction of c-*fos*, c-*jun*, and *junB* by RA in RCT-1 cells. RCT-1 cells were treated with 1 μM RA for 1 h and then screened for early-gene induction. Poly(A)$^+$ RNA (2 μg per lane) was subjected to Northern blot analysis using three specific early-gene cDNA probes. Lanes C, untreated RCT-1 cells; lanes RA, RCT-1 cells treated with RA for 1 h. Arrowheads indicate the positions of two c-*jun* mRNA transcripts, the single *junB* transcript, and the single c-*fos* transcript. The positions of the 28S and 18S rRNAs are also shown. Comparison of mRNA loading is shown by the hybridization of β-actin to the same filter. Results are representative of one additional experiment, which yielded similar results.

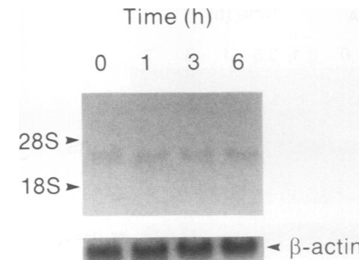


FIG. 5. Time course of RA induction of *zif268* mRNA in RCT-3 cells, which were treated with 1 μM RA for 0, 1, 3, or 6 h. Total RNA (20 μg per lane) was subjected to Northern blot analysis using a *zif268*-specific cDNA probe. The low level of expression of the 3.7-kb rat *zif268* mRNA transcript, which is not regulated by RA, is shown. The locations of the 28S and 18S rRNAs are indicated by arrowheads. Comparison of RNA loading is shown by the hybridization of β-actin to the same filter. Results are representative of two additional experiments, which yielded identical results.

blastic cells. To determine the relative extent of *zif268* mRNA induction by RA in RCT-1 cells, we examined the response of c-*fos*, c-*jun*, *junB*, and *era-1* mRNAs to RA. Using 30 μg of total RNA, we failed to detect transcripts encoding these early genes in RA-treated RCT-1 cells. Poly(A)$^+$ RNA was therefore prepared, and 1 h after treatment with 1 μM RA (the time point for a 20- to 25-fold elevation in *zif268* mRNA expression), c-*jun* transcripts of 2.7 and 3.2 kb and a *junB* transcript of 2.1 kb were detected in this preparation (Fig. 3). The mRNA for the RA-responsive gene *era-1*, a gene first characterized as an early RA-responsive gene in F9 teratocarcinoma stem cells (32), was undetectable in poly(A)$^+$ RNA from these cells (data not shown). Since *zif268* mRNA could be easily detected in total RNA, the mRNA levels of the other early genes examined following RA stimulation (1 h) were considerably lower than that of *zif268*.

Induction of *zif268* mRNA by RA in other osteoblastic cells. To corroborate the observations made with simian virus 40-immortalized RCT-1 cells in nontransformed cells, we examined the effect of RA on *zif268* mRNA in primary cultures of fetal rat calvarial cultures prepared and maintained as described previously (16) and stimulated with 1 μM RA. RA treatment of calvarial cultures resulted in a rapid and transient increase in the level of *zif268* mRNA (40- to 50-fold) (Fig. 4), with kinetics very similar to those seen in RCT-1 cells.

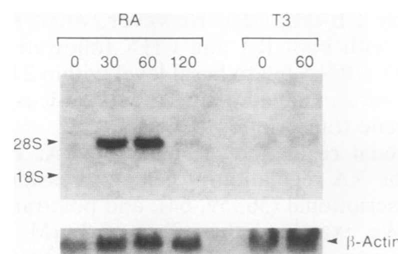


FIG. 4. Time course of *zif268* mRNA induction in fetal rat calvarial cultures, which were treated with either 1 μM RA or 10^{-10} M thyroid hormone (T3) for the indicated times (minutes). Total RNA (20 μg per lane) was subjected to Northern blot analysis using a *zif268*-specific cDNA probe. The expression of the 3.7-kb rat *zif268* mRNA transcript is shown. The locations of 28S and 18S rRNAs are indicated by arrowheads. Comparison of RNA loading is shown by the hybridization of β-actin to the same filter. Results are representative of three additional experiments, which yielded similar results.

Calvarial cells possess thyroid hormone receptors (33), and both RA and thyroid hormone were reported to regulate gene expression via the same genomic sequence (22). In addition, RARs have been shown to interact with a number of other regulatory sequences (4, 13, 59, 64). Treatment of these cells with 10^{-10} M thyroid hormone did not increase *zif268* mRNA levels (Fig. 4), suggesting that the induction of *zif268* gene expression by RA in these cells is not mediated by the RA-thyroid hormone-responsive element.

We next examined the induction of *zif268* by RA to determine whether it was characteristic of the preosteoblastic stage of the RCT-1 cells. Immortalized RCT-3 cells (28), which constitutively express many osteoblastic markers, including alkaline phosphatase, type I collagen, osteocalcin, and osteopontin (28) as well as RAR-α and RAR-γ mRNAs (data not shown) and which are thought to represent a more mature and differentiated population of osteoblasts, were treated with 1 μM RA and showed no change in the low basal level of *zif268* mRNA (Fig. 5). Another cell line, ROS 17/2.8, which represents a more-differentiated osteoblastic phenotype, also possesses RAR-α and RAR-γ (see Fig. 7B) and was shown to respond to RA by a decrease in alkaline phosphatase activity (30, 43, 52). These cells have a very low basal level of *zif268* mRNA which is not regulated by RA (data not shown).

Differentiation-related regulation of pZIFCAT by RA. To further investigate the nature of RA regulation of the *zif268* gene, we examined the effects of 1 μM RA on pZIFCAT1 following transfection of this plasmid into RCT-1 and RCT-3 cells. As shown in Fig. 6A, treatment with 1 μM RA resulted in an average 7.6-fold increase in *zif268* reporter activity in preosteoblastic RCT-1 cells. In contrast, when pZIFCAT1 was transfected into more-differentiated RCT-3 cells, there was no basal or RA-regulated reporter activity (Fig. 6B). The transfection of pACTINCAT into RCT-3 cells clearly demonstrated that other promoter regions were functional in these cells (Fig. 6B).

Taken together, these data suggest that the increased level of *zif268* mRNA expression resulting from treatment with RA is a direct effect of RA on transcription of the *zif268* gene. In addition, these data suggest that regulation of the *zif268* gene in osteoblastic cells may be differentiation related.

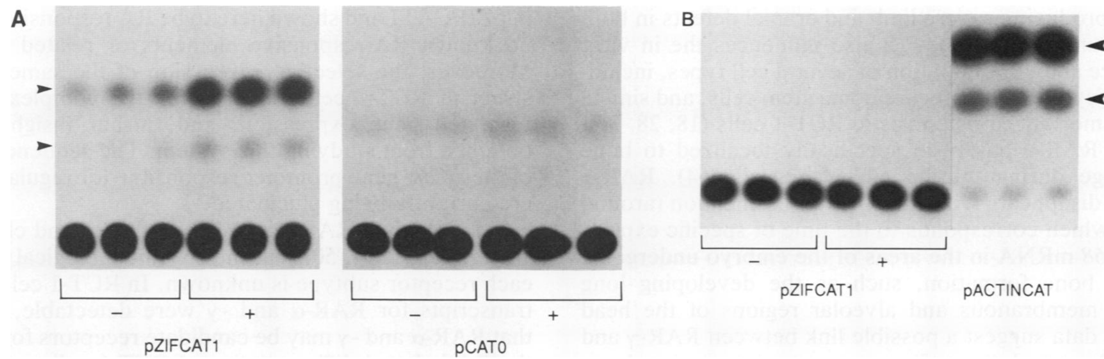


FIG. 6. Effect of RA on CAT activity. After transfection, cells were either treated with 1 μM RA for 48 h (+) or left untreated (−). Equivalent amounts of protein in the extracts were assayed for CAT enzyme activity. Products were chromatographed by thin-layer chromatography and analyzed for acetylated [^{14}C]chloramphenicol in the autoradiographs shown. (A) Induction in RCT-1 cells transfected with pZIFCAT1 or pCAT0. Arrowheads indicate the positions of acetylated chloramphenicol produced by pZIFCAT1. Results are representative of three additional experiments, which yielded similar results. (B) No induction in RCT-3 cells transfected with pZIFCAT1, pACTINCAT, or pCAT0. Results are representative of two additional experiments, which yielded similar results.

RARs in osteoblastic cells. We next examined the RAR status of the RA-responsive cells used in this study.

Poly(A)$^+$ RNAs prepared from untreated preosteoblastic RCT-1 cells and from RCT-1 cells exposed to 1 μM RA for 2 h were screened for transcripts of three different RARs: α (19, 50), β (1, 2, 14), and γ (20, 31, 34) (Fig. 7A). The RAR-α cDNA hybridized to two mRNA transcripts of approximately 3.5 and 2.7 kb (19). The very low constitutive level of RAR-α was increased after 2 h of treatment with 1 μM RA (Fig. 7A). Similarly, a single 2.7-kb RAR-γ mRNA transcript which was detected in RCT-1 cells (Fig. 7A) was increased after 2 h with RA. In contrast, RAR-β mRNA was undetectable under the hybridization conditions employed in the absence or presence of RA in RCT-1 cells. These results suggest the possible involvement of RAR-α and/or RAR-γ in mediating the effects of RA on RCT-1 cells, which, at least in the initial stages, presumably involves the induction of transcription factor genes such as *zif268*. In longer time course experiments (72 h), RAR-β mRNA was not detected either (data not shown).

The presence of 1 μg of CHX per ml for 2 h did not prevent the increase of RAR-α and -γ mRNA transcripts, suggesting that protein synthesis is not required for increased RAR gene expression (40). Since the RA-stimulated increase in RAR-α and -γ mRNA levels occurs in the absence of protein synthesis, the RAR-α and -γ genes may be primary targets for RA action on RCT-1 cells, although the involvement of low levels of RAR-β cannot be excluded.

Poly(A)$^+$ RNA from the more differentiated osteoblastic ROS 17/2.8 cells was also screened for the three different RAR subtypes (Fig. 7B). RAR-α cDNA hybridized to two specific mRNA transcripts, and RAR-γ cDNA hybridized to a single mRNA species, as seen in RCT-1 cells. There was also a complete absence of RAR-β mRNA under these hybridization conditions in ROS 17/2.8 cells. Unlike the results with RCT-1 cells, the addition of 1 μM RA had no effect on the level of RAR in these cells. The pattern of RAR gene expression observed in ROS 17/2.8 cells was identical to that seen in other osteoblastic cells, such as RCT-3, with the expression of RAR-α transcripts of 3.5 and 2.7 kb and a single RAR-γ mRNA transcript of 2.7 kb (data not shown). Under the hybridization conditions employed, no RAR-β mRNA was detectable in any osteoblastic cell line tested.

DISCUSSION

RA exerts profound effects in many different biological systems and is considered to act as a natural morphogen in limb development and neuron-polarized growth (15, 42, 63, 66). In vivo, RA has striking effects on the regenerating amphibian limb bud, causing duplication along the proximodistal axis (41). RA has also been shown to be a powerful

FIG. 7. RAR status of osteoblastic cells. Poly(A)$^+$ RNA (2 μg per lane) was subjected to Northern blot analysis using three specific cDNA probes for RAR-α, -β, and -γ. Panels α, β, and γ correspond to the probe used on each filter. Arrowheads indicate the positions of the two RAR-α transcripts and the single RAR-γ transcript detected. The locations of the 28S and 18S rRNAs are shown. Comparison of RNA loading is shown by the hybridization of β-actin to the same filters. (A) RCT-1 cells, which were treated with 1 μM RA for 2 h. Lanes: C, untreated RCT-1 cells; RA, RCT-1 cells treated with RA for 2 h. Results are representative of three additional experiments, which yielded similar results. (B) ROS 17/2.8 cells, from which poly(A)$^+$ RNA was prepared. Results are representative of two additional experiments, which yielded similar results.

teratogen, producing severe limb and cranial defects in both mice and humans (35, 53). RA also influences the in vitro maintenance and differentiation of several cell types, including keratinocytes, F9 teratocarcinoma stem cells, and simian virus 40-immortalized osteoblastic RCT-1 cells (18, 28, 34). Moreover, RAR-γ has been specifically localized to bone and cartilage during murine embryogenesis (54). RAR-γ expression disappears with the onset of ossification (around day 14.5), which corresponds to the time of specific expression of *zif268* mRNA in the areas of the embryo undergoing substantial bone formation, such as the developing long bones and membranous and alveolar regions of the head (47). These data suggest a possible link between RAR-γ and *zif268* in the regulation of gene expression during bone development in vivo.

In this study we showed that RA causes rapid induction of the transcriptional regulatory gene *zif268* in preosteoblastic RCT-1 cells, in which RA induces differentiation, and in embryonic calvarial cells, suggesting its possible involvement in the differentiation process. Recent observations suggest that *zif268* expression may play a similar role in B-cell development (56, 57). The increased expression of *zif268* mRNA caused by RA in RCT-1 cells is similar to *zif268* induction by mitogens (8, 48, 60) and suggests that *zif268* may mediate early cellular events which follow RA treatment. The induction of other early-response genes, such as c-*fos*, was shown to be an important mechanism for initiating cellular responses, including growth and differentiation (60, 65).

In cell lines which represent a more-differentiated osteoblastic phenotype, such as RCT-3 (Fig. 5; 23) and ROS 17/2.8 (43, 52), basal levels of *zif268* are low and not regulated by RA, suggesting that RA regulation of *zif268* mRNA may be related to the state of differentiation of these cells. This difference was also apparent in the expression of the pZIF CAT1 construct, which was up-regulated by RA only in preosteoblastic RCT-1 cells (Fig. 6A) and not in the more-differentiated RCT-3 cells (Fig. 6B) or ROS 17/2.8 cells (data not shown), suggesting phenotypic differences in the transcriptional regulation of this gene between related cell types. These differences could reflect the role of coregulator proteins, which were recently shown to participate in RA regulation of gene expression (21).

Different mechanisms can account for the effect of RA on individual target genes. Since RA regulation of gene expression was shown to occur at both the transcriptional (4, 13, 36, 59, 64), and posttranscriptional (23) levels, the increased expression of *zif268* mRNA produced by RA could be the result of an increased transcription rate of the *zif268* gene, stabilization of the newly synthesized mRNA transcripts, or a combination of both. In vitro nuclear transcription assays (Fig. 2C) showed that *zif268* transcription was stimulated approximately fourfold within 30 min of RA addition. This effect was smaller than the 10- to 30-fold increase in steady-state mRNA levels observed in whole cells. The difference could be due to RNA accumulation in vivo as a result of mRNA stabilization. It could also reflect differences in the sensitivity of the assays, due, for example to the requirement for RA coregulator proteins (21) during RA-stimulated transcription.

Unlike other members of the steroid-thyroid hormone receptor family, no clear consensus sequence has emerged for RAR interaction with genomic DNA. In various genes, several sequences were found to be essential for RA-stimulated gene expression (4, 14, 22, 59, 64). Interestingly, within the 5′ regulatory sequences of the *zif268* gene (6) contained in pZIFCAT1 and shown here to be RA responsive, there are no known RA-responsive elements or related sequences. Moreover, the selective expression of the same CAT construct in RCT-1 cells underscores the complexity of RA-regulated gene expression, and further insights may be obtained from studying this system. The sequence elements of the *zif268* gene promoter responsible for regulation by RA are currently being elucidated.

Many distinct RARs have been isolated and cloned (1, 2, 14, 19, 20, 31, 34, 50), but the specific biological function of each receptor subtype is unknown. In RCT-1 cells, only the transcripts for RAR-α and -γ were detectable, suggesting that RAR-α and -γ may be candidate receptors for mediating the RA-induced differentiation of RCT-1 cells, although the involvement of very low levels of RAR-β in this process cannot be excluded. RAR-α has been implicated in the terminal granulocytic differentiation of HL-60 cells (12), although these cells express mRNA transcripts for all three RARs (12, 27). The rapid increase of RAR mRNA levels in RCT-1 cells (within 2 h) occurs many hours before the induction of osteoblast marker genes such as alkaline phosphatase and may reflect a very early cellular commitment toward the onset of differentiation. In contrast to results with RCT-1 cells, the addition of RA to murine F9 teratocarcinoma stem cells does not alter the levels of RAR-α and -γ, whereas the level of RAR-β is increased within 12 h (29, 45). This may reflect not only a species and tissue difference, but possibly a mechanism by which RA can exert different effects through the use of alternate receptor subtypes.

zif268, like c-*fos*, is transiently expressed in many cell types in response to different ligands, suggesting the existence of multiple pathways of induction (10, 47). It is likely that the same regulatory proteins regulate different responses in different cells or in a ligand-specific manner in the same cell (9, 10) or that combinations of regulatory proteins function to bring about the required regulation of gene expression (17, 51). At present, the possible role of *zif268* in bone cells is unclear; however, the characteristics of *zif268* up-regulation in preosteoblastic cells suggest that *zif268* may be an immediate-early osteoblast differentiation response gene. Since zif268 is a transcription factor able to specifically bind genomic DNA and potentially activate target gene expression, further studies of the RA-induced differentiation of preosteoblastic RCT-1 cells may provide insights into the molecular mechanisms of the genetic events leading to the differentiation of the mature osteoblastic phenotype.

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