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Cyclic AMP Analogs and Retinoic Acid Influence the Expression of Retinoic Acid Receptor α, β, and γ mRNAs in F9 Teratocarcinoma Cells

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Retinoic acid (RA) receptor α (RARα) and RARγ steady-state mRNA levels remained relatively constant over time after the addition of RA to F9 teratocarcinoma stem cells. In contrast, the steady-state RARβ mRNA level started to increase within 12 h after the addition of RA and reached a 20-fold-higher level by 48 h. This RA-associated RARβ mRNA increase was not prevented by protein synthesis inhibitors but was prevented by the addition of cyclic AMP analogs. In the presence of RA, cyclic AMP analogs also greatly reduced the RARα and RARγ mRNA levels, even though cyclic AMP analogs alone did not alter these mRNA levels. The addition of either RA or RA plus cyclic AMP analogs did not result in changes in the three RAR mRNA half-lives. These results suggest that agents which elevate the internal cyclic AMP concentration may also affect the cellular response to RA by altering the expression of the RARs.

F9 teratocarcinoma stem cells resemble murine embryonic stem cells remarkably (7, 14). The F9 stem cells can be induced to differentiate into primitive endoderm cells by the addition of retinoic acid (RA). The addition of dibutyryl cyclic AMP (cAMP) to RA-treated F9 cells enhances their terminal differentiation into parietal endoderm cells, despite the fact that dibutyryl cAMP alone does not cause differentiation (19). Several genes that are expressed during RA-induced F9 cell differentiation have been cloned; some genes, such as laminin B1 and collagen IV(α1) (10, 22), are expressed at relatively late times after the addition of RA, and some, such as *ERA-1*/*Hox*-1.6 (11, 12) and *Hox*-1.3 (15), are expressed rapidly in response to RA.

In addition to its ability to cause teratocarcinoma cell differentiation, RA can influence cell growth (18), pattern formation in limb development and regeneration (13, 21), and fetal development (17, 20). The mechanisms by which RA exerts such striking effects on cell growth and differentiation are unclear at present, but presumably these mechanisms involve the regulation of the expression of specific genes.

Recently, the genes encoding three different human RA receptors (RARs), RARα (6, 16), RARβ (1–3), and RARγ (9), were isolated. The general structures of these RARs are very similar to those of the steroid and thyroid hormone receptors, but the DNA sequences of the three RARs differ from each other and from those of steroid hormone receptors, especially in their A, D2, and F domains (1–3, 6, 9, 16, 24). Strikingly, the human and murine RARα, RARβ, and RARγ sequences are 98 to 100% conserved (24). The existence of three RARs, i.e., RARα, RARβ, and RARγ, is intriguing. Whether all of these receptors are expressed in the same cell type, whether their expression changes during differentiation, and how the expression of the genes encoding these three receptors is regulated are questions that remain to be answered. We present data here that address some of these questions regarding the expression of these RARs in F9 teratocarcinoma cells.

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We first examined the expression of RARα mRNA over time in F9 stem cells and in F9 cells treated with RA (1 μM) alone. The F9 cells were cultured as described previously (23). Two different ^{32}P-labeled (5) probes (Fig. 1A) generated from the human RARα clone RARαO (2) were used to detect the RARα mRNA by Northern (RNA) blot analysis (22). Both probe 1 and probe 2 hybridized to only two mRNA species of 3.5 and 2.7 kilobases (kb), which indicates that there was no cross hybridization with other members of the steroid-thyroid receptor family under the stringent hybridization conditions used, i.e., hybridization for 18 h at 42°C in a buffer containing 50% formamide, followed by washing in 0.2× and 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 50°C. The presence of two species of RARα message in F9 cells is consistent with the results obtained when rat and human tissues were analyzed by Giguere et al. (6). The steady-state level of RARα mRNA in F9 stem cells remained relatively constant over time in culture, and the addition of RA did not alter the level of RARα mRNA (Fig. 2). To demonstrate that the RA-treated F9 cells had differentiated, the same blot was hybridized to the *ERA-1* and laminin B1 cDNA probes. *ERA-1* mRNA was induced within the first 8 h after the addition of RA (data not shown), as previously described by LaRosa and Gudas (11). The steady-state laminin B1 mRNA level also increased at 48 h after the addition of RA and remained elevated at 96 h, as described previously (22).

A 2.7-kb RARγ transcript was also detected in F9 stem cells (Fig. 1C for probes and Fig. 2 and 3). The steady-state RARγ mRNA was not greatly altered by the addition of RA, although the level of RARγ mRNA in both F9 stem and RA-treated cells began to decline 48 h after plating (Fig. 2).

We next analyzed the time course of RARβ mRNA expression after the addition of RA. The RARβ cDNA probes (Fig. 1B) hybridized to two RARβ mRNA species of 3.2 and 2.9 kb in F9 cells (Fig. 2 and 3). de The et al. (3) also reported that two RARβ mRNAs of 3.0 and 2.5 kb were present in different human organs and cell lines. These two different-sized RARβ transcripts could result from alternate splicing, transcriptional initiation at different sites, or poly-

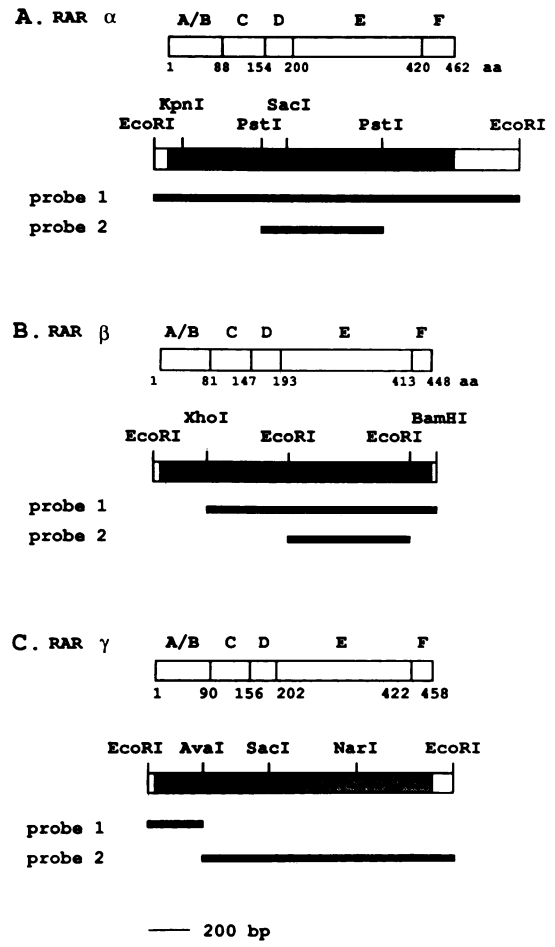

FIG. 1. Structures and restriction maps of RARα, RARβ, and RARγ cDNAs. The RARs are divided into six regions, A through F (shown on the top of the each restriction map), by analogy to the estrogen receptor (2). The A/B region may be involved in transcriptional activation. The C region represents the DNA-binding domain of the receptors, D represents the junction region, and E represents the ligand-binding domain. Numbers below the overall structure of each RAR indicate amino acid positions. Some restriction sites in the cDNA of each receptor gene are shown. (A) All three cDNAs were inserted into pSG5 (data not shown). RARα probes 1 and 2 were generated by *Eco*RI and *Pst*I digestion, respectively. (B) The *Eco*RI site at the 5′ end and the *Bam*HI site at the 3′ end of RARβ cDNA were from the polylinker region of the vector. RARβ probes 1 and 2 were made by *Xho*I-*Bam*HI and *Eco*RI digestion, respectively. (C) RARγ probes 1 and 2 were generated by *Eco*RI-*Ava*I double digestion.

adenylation at different sites. A low basal RARβ mRNA level was detected in F9 stem cells (Fig. 2). After the addition of RA, the steady-state level of RARβ mRNA was elevated within 12 h and reached a plateau about 20-fold higher than the stem cell level by 48 h (Fig. 2). The ratio of the two RARβ mRNAs remained the same during the growth of F9 stem cells in culture, but the 3.2-kb RARβ mRNA increased more than the 2.9-kb transcript after the addition of RA (Fig. 3). Thus, the addition of RA dramatically increased the steady-state level of RARβ mRNA in F9 cells.

Protein synthesis inhibitors such as cycloheximide and puromycin did not prevent the RA-associated increase in RARβ mRNA level when F9 cells were grown in the presence of these inhibitors plus RA for 12 h (data not shown). In a control experiment, cycloheximide (1 μg/ml) inhibited incorporation of [^{35}S]methionine into total protein by >90% during the 12-h incubation. Since the RA-associated increase in RARβ mRNA level occurs without concurrent protein synthesis, the RARβ gene is a primary target for RA in F9 cells.

The magnitude of the RARβ mRNA increase correlated with the concentration of exogenous RA (Fig. 3). A 20-fold increase in the steady-state level of RARβ mRNA was observed in cells grown for 48 h in the presence of 10^{-6} M RA, whereas 12-, 4-, and 2-fold increases were observed at 10^{-7}, 10^{-8}, and 10^{-9} M RA, respectively (Fig. 3).

Only a three- to fourfold increase in the steady-state RARβ mRNA level was detected in the presence of 1 μM RA plus 250 μM dibutyryl cAMP and 400 μM theophylline (Fig. 3). This change can be compared with the 20-fold

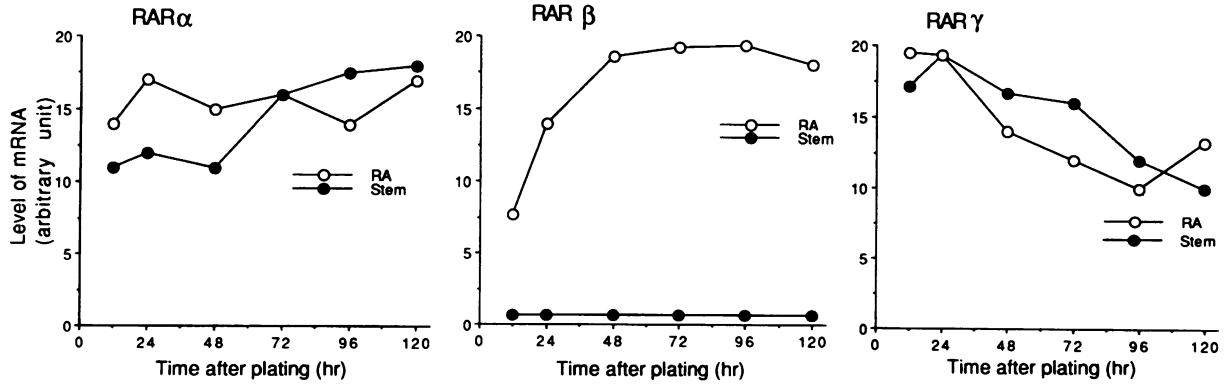

FIG. 2. Time course of RARα, RARβ, and RARγ mRNA expression in F9 cells. F9 cells were cultured in the presence or absence of RA (1 μM). Total cellular RNA was isolated at different times after the cells were plated, 30 μg of the RNA from each time was fractionated on 1% agarose gels, and the RNA was transferred to a nitrocellulose filter (22, 23). Probes used in Northern hybridizations were the 0.6-kb RARα probe 2 (Fig. 1A), the 0.6-kb RARβ probe 2 (Fig. 1B), and the 1.2-kb RARγ probe 2 (Fig. 1C). The magnitude of the hybridization was visualized by autoradiography with Kodak XAR-5 film with an intensifying screen and quantitated by scanning densitometry. Values for the levels of RAR mRNA were normalized to that of actin mRNA and plotted versus time after plating. This type of experiment was performed five times. The increases in RARβ mRNA levels at 48 h after the addition of RA ranged from 20- to 30-fold in these five different experiments. Only one of the experiments is illustrated.

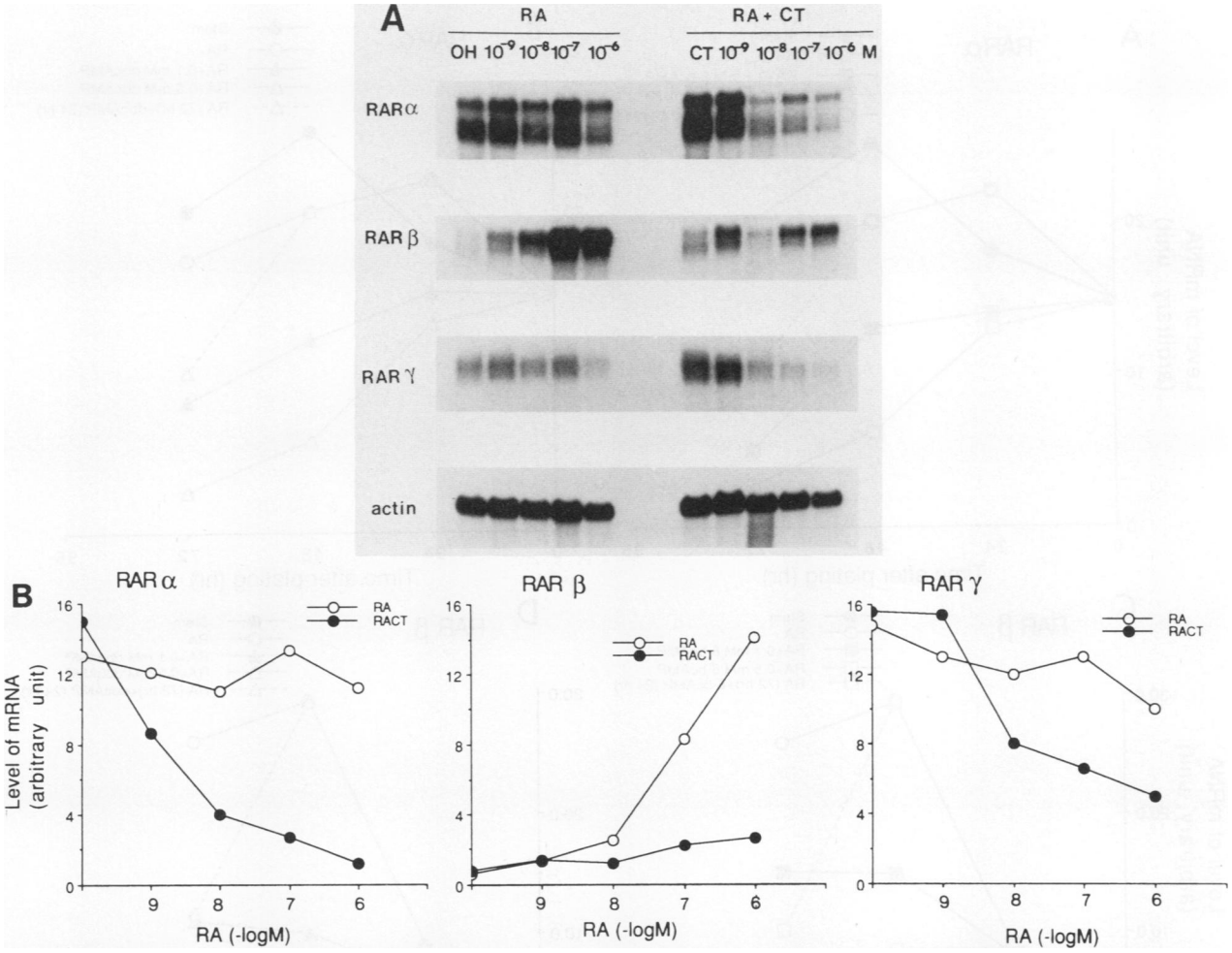

FIG. 3. Effect of RA concentration on the expression of RAR mRNAs. (A) F9 cells were plated at 5×10^6 cells per 150-mm gelatinized tissue culture dish for 48 h either in the presence of RA at the various concentrations shown (RA) or in the presence of RA at the various concentrations shown plus 250 μM dibutyryl cAMP and 400 μM theophylline (RA + CT). RNA isolated from F9 cells grown in the presence of either 0.1% ethanol (OH) or 250 μM dibutyryl cAMP plus 400 μM theophylline (CT) was used as a control. Northern blot hybridization was carried out with 5 μg of poly(A)$^+$ RNA, isolated as previously described (8). The probes were the 0.6-kb RARα (5×10^6 cpm/ml), 0.6-kb RARβ (3×10^6 cpm/ml), 0.3-kb RARγ (4×10^6 cpm/ml), and 0.6-kb actin (10^6 cpm/ml). Exposure times were 22 h for RARα, 40 h for RARβ, 24 h for RARγ, and 2 h for actin. (B) The levels of RAR mRNAs were normalized to that of the actin mRNA and plotted versus the concentration of RA. This experiment was performed twice, with very similar results (less than 10% variation). RACT, 1 μM RA plus 250 μM dibutyryl cAMP and 400 μM theophylline.

increase in the presence of 1 μM RA alone (Fig. 3). Dibutyryl cAMP plus theophylline without RA did not change the level of RARβ mRNA (Fig. 3), nor did the addition of 500 μM sodium butyrate (not shown). These results indicated that dibutyryl cAMP exerted an inhibitory effect on the RA-associated RARβ mRNA increase, and this inhibition was more clearly demonstrated by varying the dibutyryl cAMP concentration while keeping the RA concentration constant (Fig. 4D). Furthermore, another cAMP analog, 8-bromo-cAMP, also prevented the RA-associated RARβ mRNA increase, which demonstrates that this inhibitory effect could be elicited by cAMP analogs other than dibutyryl cAMP (Fig. 4C). The cAMP-analog-associated inhibitory effect was dependent on the time after the addition of both RA and the cAMP analog. For instance, no inhibitory effect was detected at 24 h after the addition of RA plus dibutyryl cAMP or of 8-bromo-cAMP, but inhibition was detected at 48 and 72 h, respectively (Fig. 4C and D).

In contrast to the RA-associated increase in RARβ mRNA, the steady-state RARα and RARγ mRNA levels were not altered by varying the concentration of RA or by adding dibutyryl cAMP plus theophylline without RA to the F9 cells (Fig. 3). However, 1 μM RA plus 250 μM dibutyryl cAMP and 400 μM theophylline reduced the levels of RARα and RARγ mRNAs 10- and 3-fold, respectively (Fig. 3), and strikingly, the magnitudes of these decreases were dependent on both the concentration of RA (Fig. 3) and the concentration of dibutyryl cAMP (Fig. 4B). 8-Bromo-cAMP also reduced the RARα mRNA level in the presence of RA to the same extent as did dibutyryl cAMP (Fig. 4A).

To determine if the cAMP-analog-associated reductions in RARα, RARβ, and RARγ mRNA levels seen in the presence of RA were mediated through a reduction in the stabilities of these messages, the relative half-lives of these messages were determined (Fig. 5). The half-lives of RARα and RARβ mRNAs in F9 stem cells, which were 120 and 100 min, respectively, were the same as those in cells treated with RA or with RA plus cAMP analog. The half-life of RARγ mRNA in RA-treated cells (180 min) was similar to that in cells treated with RA plus cAMP analog, although the RARγ

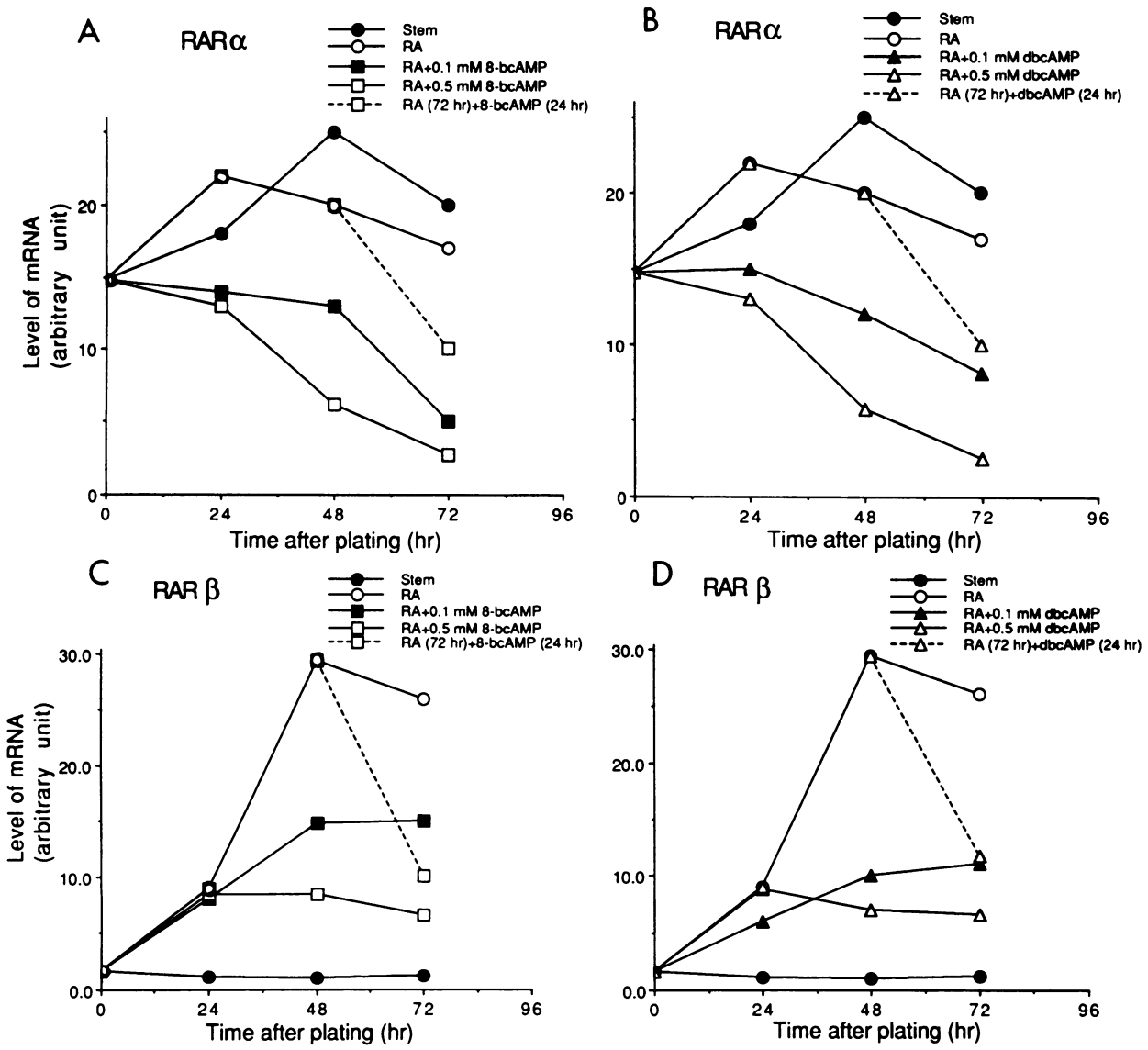

FIG. 4. Effect of cAMP analogs on the expression of RARα and RARβ mRNAs. F9 cells were plated at 4×10^6 cells per 150-mm gelatinized tissue culture dish in the absence or presence of 1 μM RA or 1 μM RA plus cAMP analogs at various concentrations (shown on the top of each panel) and 400 μM theophylline, and RNA was isolated from samples at 0, 24, 48, and 72 h. Some cells were grown in the presence of 1 μM RA for 48 h, at which time 0.5 mM dibutyryl cAMP (dbcAMP) or 0.5 mM 8-bromo-cAMP (8-bcAMP) plus theophylline was added without a medium change; samples were harvested 24 h later. Total cellular RNA (30 μg) was used in Northern hybridization analysis as previously described (23). The probes were the 0.6-kb RARα (3×10^6 cpm/ml) (A and B), the 0.6-kb RARβ (4×10^6 cpm/ml) (C and D), and the 0.6-kb actin cDNA (1×10^6 cpm/ml). Exposure times were 20 h for RARα, 48 h for RARβ, and 1 h for actin. Quantitation and plotting of the data were as described in the legend to Fig. 2. The entire experiment was performed twice, and very similar results were obtained; in addition, the levels of RAR mRNAs in the presence of the different drug combinations were determined in three different experiments for the 48-h time point only. One of these experiments is illustrated.

message was slightly more stable in F9 stem cells (half-life, 270 min) (Fig. 5). These results suggest that the RA-associated RARβ mRNA increase does not result from an increase in the stability of this message. Moreover, the inhibitory effects of cAMP analogs in the presence of RA are not the result of cAMP-associated decreases in RARα, RARβ, and RARγ mRNA stabilities. In nuclear transcription run-off assays, we have not been able to detect any signals for RARα, RARβ, or RARγ, possibly because the transcription rates of these receptor genes are very low in F9 cells. de The et al. (4) recently reported that the RARβ gene was transcriptionally regulated by RA in the human hepatoma line PCL/PRF/5, but this line has an unusually high RARβ transcription rate compared with those of other human hepatoma lines.

We have found that the steady-state levels of RARα and RARγ mRNAs are not influenced by the addition of RA to F9 cells, whereas the level of RARβ mRNA is increased 20-fold by the addition of RA. The relatively rapid increase in RARβ mRNA in response to RA occurs even in the presence of protein synthesis inhibitors and at an RA concentration that is not cytotoxic to the cells; no decrease in cell number occurs following the addition of RA, and the RA-treated F9 cells grow at the same rate as control F9 stem

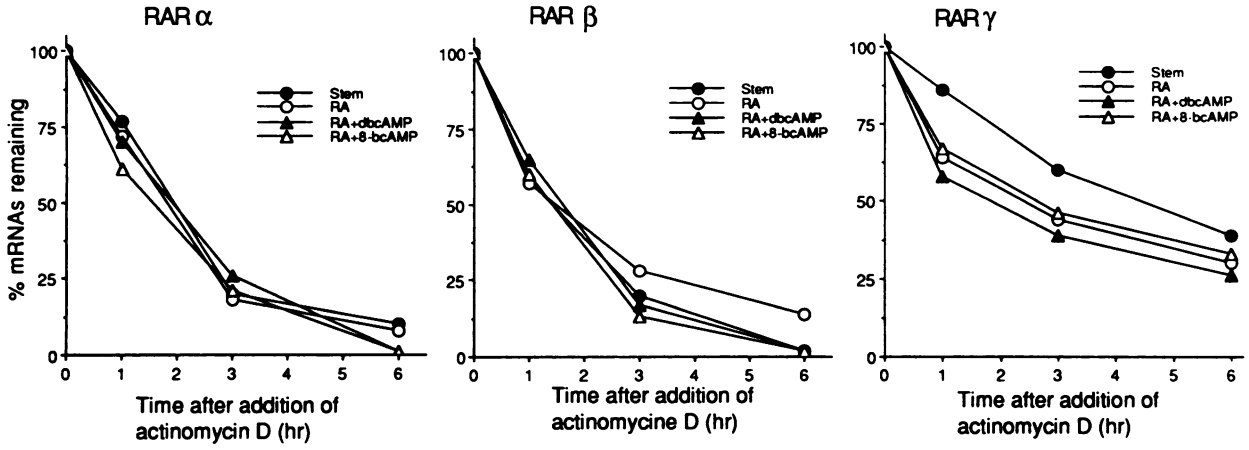

FIG. 5. Effect of RA versus RA plus cAMP analogs on the stability of RAR mRNAs. F9 cells were grown for 48 h in the presence or absence of 1 μM RA, with 1 μM RA plus 500 μM dibutyryl cAMP (dbcAMP) or with 1 μM RA plus 500 μM 8-bromo-cAMP (8-bcAMP). Dactinomycin (formerly actinomycin D) (2 μg/ml) was added at this time, and RNA was isolated after 0, 1, 3, and 6 h. Total cellular RNA (30 μg) from each sample was used in the Northern analysis. The probes used in this experiment were the same as those described in the legend to Fig. 2.

cells for at least 72 h (data not shown). The RARβ mRNA increase is therefore not likely to be the result of the selection by RA in culture of a subpopulation of F9 cells that express RARβ at a high level. We speculate that upon the addition of RA to F9 cells, the expression of the RARβ gene is directly activated by the RARα or the RARγ protein. The kinetics of the RA-associated increase in RARβ mRNA are consistent with the subsequent involvement of RARβ in the regulation of genes, such as laminin B1, induced at later times after the addition of RA. Vasios et al. (G. W. Vasios, J. D. Gold, M. Petkovich, P. Chambon, and L. J. Gudas, Proc. Natl. Acad. Sci. USA, in press) have demonstrated that all three of the RARs can increase the expression of a reporter gene downstream of the laminin B1 promoter. However, RARβ may be more effective in the activation of genes which respond to RA later during F9 cell differentiation, since it exhibits an apparent 10-fold-higher affinity for RA than does RARα (2).

cAMP analogs reduce the steady-state levels of the RARα, RARβ, and RARγ mRNAs in both a RA- and a cAMP-analog-concentration-dependent fashion (Fig. 3 and 4). Thus, this reduction may require a negative regulatory factor(s) whose expression or activation depends on the concentrations of both RA and cAMP. The cAMP inhibitory effect probably occurs at the level of transcription, since the half-lives of the RARα, RARβ, and RARγ mRNAs are similar in RA- versus RA-plus-cAMP-analog-treated cells. In contrast to the cAMP-analog-associated reductions in RARα, RARβ, and RARγ mRNA levels in the presence of RA, treatment of F9 cells with RA plus cAMP analogs is associated with the enhanced transcriptional activation of late genes, such as laminin B1, collagen IV(α1), and SPARC (J31), relative to treatment with RA alone (23; L. J. Gudas, J. F. Grippo, K. W. Kim, G. LaRosa, and C. M. Stoner, Ann. N.Y. Acad. Sci., in press). It is possible that in addition to the RARs, the enhanced expression of these later genes requires another differentiation-specific transcription factor(s), the activity of which is activated by cAMP.

There are in the literature many examples of genes that are regulated by more than one hormone or growth factor via promoter interactions. The findings presented here suggest a novel mechanism by which one signal transduction system (i.e., cAMP) can influence another (i.e., RA), allowing cAMP to modulate the effects of RA.

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