Expression of Nuclear Retinoic Acid Receptors in Wild-type and Mutant Embryonal Carcinoma PCC4.aza1R Cells


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Abstract
Retinoic acid (RA) induces differentiation of murine embryonal carcinoma PCC4.aza1R cells. In this study, the expression of nuclear retinoic acid receptors (RARs) in PCC4.aza1R cells is examined. Analyses of [3H]RA-labeled nuclear extracts prepared from PCC4.aza1R cells by size-exclusion high-performance liquid chromatography demonstrated the presence of a specific RA-binding activity that migrated with a molecular weight of approximately 50,000. More than 95% of this binding activity was associated with the nuclear fraction. In contrast to cytosolic retinoic acid-binding protein, the RARs bound RA analogues of the Ch-series very effectively. Northern blot analyses of total RNA with complementary DNA probes specific for RARα, RARβ, and RARγ showed that PCC4.aza1R cells contain predominantly transcripts encoding RARα and RARγ; RARβ transcripts were undetectable. Treatment of PCC4.aza1R cells with RA increased the levels of RARβ mRNA in a dose- and time-dependent manner. The RA concentration for half-maximum induction of RARβ mRNA was 1 nM. An increase in RARβ mRNA was detectable as early as 2 h after the addition of RA. This increase was not abrogated by cycloheximide, suggesting that protein synthesis is not required for this response. The ability of several retinoids to increase RARβ mRNA levels in PCC4.aza1R cells correlated well with their binding affinity to the RARs but not with their binding affinity to cytosolic retinoic acid-binding protein. Two mutant cell lines, PCC4(RA)-1 and (RA)-2, which do not undergo differentiation after RA treatment, contained levels of RAR-binding activity very similar to those of the parental cells. PCC4(RA)-1 and PCC4(RA)-2 cells expressed RARα and RARγ transcripts identical in size to that of PCC4.aza1R cells. However, PCC4(RA)-1 cells expressed only one RARγ transcript of 3.1 kilobases. The loss of the expression of the 3.3-kilobase RARγ transcript in PCC4(RA)-1 cells may be causally related to their inability to differentiate in response to RA. RA treatment increased the levels of RARβ mRNA in both PCC4(RA)-1 and (RA)-2 cells, indicating that these mutant cell lines have not totally lost their responsiveness to RA.

Introduction
Embryonal carcinoma cells are tumorigenic, multipotential stem cells that are closely related to totipotent embryonic stem cells (1, 2). A variety of different chemical agents have been identified that can induce differentiation of these cells in culture (3). Retinoids, the naturally occurring and synthetic analogues of vitamin A, are among the most effective inducers of differentiation of several embryonal carcinoma cell lines (3–7). Our laboratories have been concentrating on the regulation of differentiation in embryonal carcinoma PCC4.aza1R cells (7, 8). These cells can undergo a multistage process of differentiation (reviewed in Ref. 9). RA treatment of PCC.aza1R cells causes their differentiation into mesenchymal stem cells, which can then further differentiate into preadipocytes after treatment with 5-azacytidine in a manner similar to that reported for 10T1/2 cells (10). At confluence, and in the presence of dexamethasone and insulin, these cells terminally differentiate into adipocytes as indicated by the synthesis of fat cell-specific markers (9, 11). The differentiation of PCC.aza1R cells into mesenchymal cells is accompanied by changes in cellular morphology and the induction of several biochemical and molecular markers. For example, cells start to express increased levels of epidermal growth factor receptor (12), the extracellular matrix proteins fibronectin and collagen I and the protooncogenes int-2 and c-myc (9, 13). Moreover, in contrast to embryonal carcinoma cells, the differentiated cells become permissive to viral gene expression (14, 15). At least some of the changes involved in this differentiation process appear to be regulated at the transcriptional level (1).

The molecular mechanism by which retinoids induce differentiation in embryonal carcinoma cells has yet to be established. Recently, the existence of several nuclear retinoic acid receptors, designated RARα, RARβ, and RARγ, has been demonstrated (16–20). These receptors are members of the steroid hormone/thyroid hormone superfamily and act as RA-dependent transcriptional factors. It is likely that at least some of the alterations in gene expression during differentiation of PCC4.aza1R cells are controlled by these nuclear RA receptors.

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In this study, we examine the expression of the nuclear RA receptors RARα, RARβ, and RARγ in embryonal carcinoma PCC4.aza1R cells in relation to the induction of differentiation and in two mutant cell lines, PCC4(RA)-1 and PCC4(RA)-2, which are unable to differentiate after the addition of RA (21, 22).

Results

Analysis of [3H]RA Binding. Cytosolic and nuclear extracts prepared from PCC4.aza1R cells were incubated with 5 nM [3H]RA in the absence or presence of unlabeled RA or its analogue Ch55 (1 μM), and the binding of [3H]RA was analyzed via size exclusion HPLC (Fig. 1). The HPLC profiles of the cytosolic extracts showed one single major peak of specific binding activity that eluted at 32 min (Fig. 1A) and corresponded to a molecular weight of approximately 16,000, consistent with CRABP. This radioactivity was displaced by unlabeled RA but not by unlabeled Ch55, in agreement with the previously reported binding specificity of CRABP (23). The HPLC profile of the nuclear extracts showed five peaks of radioactivity (Fig. 1B). The three minor peaks eluting at 16, 41, and 46 min represented nonspecific binding activity since there was no competition in the presence of a 200-fold excess of unlabeled retinoids, whereas the two major peaks eluting at 32 and 27 min represented specific binding. Unlabeled RA, but not Ch55, was able to compete with [3H]RA binding in the peak eluting at 32 min, the same position as CRABP. This binding activity probably represents CRABP. Most of the CRABP activity in the nuclear extract appears to be due to a contamination of the nuclear preparation with the cytosolic fraction rather than reflecting a true association of CRABP with nuclei since two more washings of the nuclei reduced CRABP levels further to 2–5% of total CRABP but caused only a slight decrease in the RAR levels (data not shown). Both unlabeled RA and Ch55 abolished the binding of [3H]RA in the peak eluting at 27 min. The elution time of these proteins corresponds to a molecular weight of approximately 50,000. We have reported previously that the RARs elute in this fraction and can bind Ch55 (24); therefore, this RA binding probably represents RAR-binding activity. This RAR receptor activity was not detectable in the cytosolic extract, suggesting that this receptor is predominantly associated with the nucleus. When HPLC analysis was carried out at room temperature instead of at 4°C, [3H]RA binding to CRABP was totally abolished, whereas the binding to RAR was only reduced by about 50% (Fig. 1C).

RAR mRNA Expression. The expression of the RARα, RARβ, and RARγ genes in PCC4.aza1R cells was examined by Northern blot analyses using total RNA isolated from these cells and 32P-labeled inserts of hRARα0, hRARβ0, and mRARγ0 as probes (Fig. 2). The hRARα probe hybridized to two RNA species of approximately 2.6 and 3.5 kb, whereas the mRARγ probe hybridized to two RNAs of 3.3 and 3.1 kb, of which the latter transcript was predominantly expressed in PCC4.aza1R cells. Taking into account the specific activity and the length of the radiolabeled probes and the length of the exposure of the autoradiogram, it was calculated that PCC4.aza1R cells contained about 4-fold higher levels of RARγ mRNAs than RARα mRNAs. Transcripts hybridizing to the hRARβ probe were undetectable.

We next analyzed the effect of RA on RAR expression. RA (1 μM) induced a small (about 3-fold) increase in the levels of RARα mRNA over a 48-h time period (Fig. 2). RARγ mRNA levels remained relatively unchanged during the first 24 h of RA treatment but decreased at longer times of exposure to RA. RARγ mRNA expression was reduced by 60% 48 h after the addition of RA (Fig. 2). No significant changes in the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA were observed after the addition of RA. Addition of 1 μM RA to PCC4.aza1R cells induced two RARβ mRNAs, 3.2 and 2.9 kb in size. The larger transcript was the most prominent. This induction occurred in a time-dependent manner (Fig. 2). An increase in RARβ mRNA could be observed within 2 h of RA addition. The induction of RARβ mRNA was dependent on the RA concentration (Fig. 3). The RA
The concentration for half-maximum stimulation was about 1 nM. Cycloheximide did not abolish the RA-induced increase in RARβ mRNA, indicating that the induction of RARβ mRNA levels by RA was not dependent on protein synthesis (Fig. 4). In fact, PCC4.aza1R cells treated simultaneously with cycloheximide and RA contained consistently higher levels of RARβ mRNA than cells treated with RA alone. Nuclear run-on experiments were carried out to determine the enhanced expression of the RARβ gene was due to increased transcription. In three independent experiments, no detectable transcription of the RARβ gene could be observed in either untreated cells or cells treated for 5 or 24 h with 1 μM RA (data not shown). However, the same experiments showed an increase in the rate of transcription of the homeobox gene Hox-1,6, another gene induced rapidly after RA addition. Examination of the stability of the RARβ mRNA is shown in Fig. 5. Densitometric analyses of the RNA stability indicated that the rate of the decrease in RARβ mRNA in cells treated for 48 h with 1 μM RA was very similar to that in cells treated for 5 h with RA (Fig. 5B). Since the level of RARβ mRNA increased about 5-fold between 5 and 48 h of RA treatment, differences in stability of this mRNA cannot account for the total increase in RARβ mRNA expression. The half-life of the RARβ mRNA in untreated cells could not be determined since the RARβ mRNA was undetectable.

**Structure-Activity Relationship.** The ability of several RA analogues to bind to nuclear RAR receptors in PCC4.aza1R cells was analyzed and compared with their binding to CRABP and their ability to increase RARβ mRNA levels. A strong qualitative correlation was found between the ability of the analogues to compete with
Fig. 4. Effect of cycloheximide on the induction of RARβ mRNA. PCC4.aza1R cells were treated with or without RA (1 μM) and/or cycloheximide (2 μg/ml) for 4 and 7 h. Total RNA isolated from these cells was analyzed via Northern blots using a 32P-labeled insert of RARβ as a probe. One h of cycloheximide (2 μg/ml) treatment inhibited protein synthesis in PCC4.aza1R cells by greater than 90% and RNA synthesis by less than 20%. Cycloheximide inhibited RNA synthesis less than 40% at 4 h of treatment. Cycloheximide did not cause any cytotoxicity in the cells during the time of treatment.

Fig. 5. Effect of RA on the stability of RARβ mRNA. Cells were treated first with 1 μM RA for 5 or 48 h and then incubated further in the presence of 250 ng/ml actinomycin D. RA-treated and control cultures were collected for RNA isolation at 0, 0.5, 1.5, and 4.0 h after the addition of actinomycin D. A, poly(A+) RNA was isolated, and 5 μg were analyzed via Northern analysis. B, a slot blot was prepared using 2.5, 0.5, and 0.1 ng of each RNA sample and then hybridized to a 32P-labeled probe for RARβ. The blot was then stripped and hybridized with 32P-labeled pGAD-28. Autoradiograms of slot blot and Northern blot were scanned with a Bio-Rad Bioimage scanner, and the level of remaining RARβ RNA relative to GPDH was calculated. Statistical evaluation was performed utilizing a two-way analysis of variance followed by Tukey’s test using the SAS program (SAS Institute, Cary, NC). •, 5 h RA treatment; O, 48 h RA treatment. * P < 0.05 versus same time point generated for the 48-h RA-treated samples. Bars, SEM.

Discussion

Previous studies have shown that RA induces differentiation of PCC4.aza1R cells into mesenchymal stem cells (5, 9). This differentiation is characterized by morphological, biochemical, and molecular changes. During the induction of this pathway of differentiation, the levels of several mRNAs are increased, including the homeobox genes...
Fig. 6. Comparison of the binding capacity of several retinoids to RAR and CRABP with their ability to induce RARβ mRNA. To examine the competition of retinoids for the binding of [3H]RA to the CRABP and the nuclear RA receptor in PCC4.aza1R cells, extracts were incubated with 5 nM [3H]RA in the absence or presence of a 200-fold molar excess of the indicated retinoid. The [3H]RA binding was analyzed as described in the legend to Fig. 1. Upper panel, the percentage competition of [3H]RA binding was calculated and plotted (solid bars, RAR competition; hatched bars, CRABP competition). Middle panel, the effect of the same retinoids (10 μM) on the induction of RARβ expression was determined by Northern blot analysis using 30 μg of total RNA. Analysis of GPDH mRNA expression was used as a control.

Hox-1.3 and -1.6, the oncogene int-2, epidermal growth factor receptor, fibronectin, and collagen I (13). We are interested in understanding the mechanisms by which RA induces differentiation and regulates the expression of the various genes in PCC4.aza1R cells.

Previous observations have revealed that PCC4.aza1R cells contain relatively high levels of the specific cytoplasmic RA-binding protein CRABP (7, 21, 22). In initial studies examining structure-activity relationships, a rather good correlation was found between the efficacy with which retinoids bind to CRABP and their ability to induce differentiation in PCC4.aza1R cells (7). These experiments suggested that CRABP was involved in this induction of differentiation by retinoids. The isolation of RA-nonspecific mutant cell lines containing low levels of CRABP supported the concept that CRABP was essential in the mechanism of action of RA (21). Later studies using retinoids of the Ch-series showed that these analogues were very effective inducers of differentiation in embryonal carcinoma F9 cells but were unable to bind to CRABP (23). The study presented here extends these findings to PCC4.aza1R cells. These observations appear to indicate that CRABP may not be essential for induction of differentiation in embryonal carcinoma cells by retinoids. However, CRABP might still participate in controlling gene expression in an indirect manner by regulating the concentration of retinoids in the cell (25), either through binding to the retinoid and/or by facilitating retinoid metabolism (26).

Recently, several RARs have been identified that function as RA-dependent transcriptional factors (16–20). PCC4.aza1R cells contain RA receptors of a molecular weight of approximately 50,000 which are associated almost solely (more than 95%) with the nucleus. These receptors probably represent the RARs. Analysis of the structure-activity relationship of several retinoids shows a good correlation between their binding to RARs and their ability to induce RARβ mRNA and differentiation. These results suggest that the induction of differentiation in PCC4.aza1R cells by RA is mediated by RARs.

PCC4.aza1R cells contain transcripts encoding RARα and RARγ, whereas RARβ transcripts were undetectable. PCC4.aza1R cells expressed two RARα and two RARγ mRNA species. RA induced a 3-fold increase in RARα and, after 48 h of treatment, decreased RARγ levels by 60%. A decrease in RARγ mRNA has also been observed in embryonal carcinoma F9 cells after treatment with RA and cyclic AMP analogues (27). Different isoforms of each RAR have been reported which differ in their NH2-terminal A-region and are generated by alternative splicing (20, 28). Conceivably, the two transcripts expressed by PCC4.aza1R cells represent RARγ-1 and RARγ-2. RARβ mRNA levels were induced after RA treatment in a time- and dose-dependent manner. Recently, RA-induced increases in RARβ mRNA were reported in other cell systems (27, 29, 30). Such an increase appeared to be regulated at least in part via a transcriptionally controlled mechanism (27, 29). An RA-responsive element has been identified in the RARβ gene (31), supporting the concept that this gene is directly controlled by an RA:RAR complex. Our studies suggest that the increase in RARβ mRNA in PCC4.aza1R cells occurs independ-
Fig 3. Comparison of the expression of RARα, RARβ, and RARγ mRNA levels in PCC4, aza1R, PCC4-RA-1, and PCC4-RA-2 cells. Total RNA was isolated from untreated and RA-treated PCC4, aza1R cells, probably due to very low rates of transcription of this gene in these cells. A similar observation has been made in F9 embryonal carcinoma cells (27). Examination of the stability of the RARγ mRNA showed no increase in the half-life of the RARγ mRNA with longer times of RA exposure, suggesting that the increase in RARγ mRNA in PCC4, aza1R cells was probably not due to enhanced mRNA stability.

The presence of multiple RARs in PCC4, aza1R cells raises the question of their roles. Recent studies by Spece et al. (32) have revealed that, in F9 embryonal carcinoma cells, the overexpression of a vector containing a truncated RARα cDNA which lacks the ligand-binding domain abrogates the induction of several, but not all, RA-inducible genes. These observations suggest that each of the RARs may control specific sets of genes and that both RARα as well as RARβ and/or RARγ may be involved in regulating gene expression during differentiation of F9 cells. RARβ may play a role in controlling the transcription of certain genes at a later stage in the differentiation process. This is certainly in agreement with the widely held view that differentiation occurs as a cascade of transcriptional and translational events. In such a scheme, then, following RA treatment, RARα and/or RARγ would modulate the expression of a set of genes including other transcription-regulatory factors, such as RARβ2 and certain homeobox genes, which would in turn alter the expression of other groups of genes.

We have examined the levels of RAR mRNA in two mutant embryonal carcinoma cell lines, PCC4(18)-1 and PCC4(18)-2, which fail to differentiate in response to RA. However, these cell lines have not totally lost their responsiveness to RA, since RARγ mRNA is still induced after RA addition. In view of the likely role of RARs in mediating RA-induced differentiation in the parental PCC4, aza1R cells, the inability of the mutant cell lines to differentiate could be related to a defect in RAR expression. We have found that, in contrast to parental cells which contain two RARγ mRNAs of 3.1 and 3.3 kb, PCC4(18)-1 cells express only one RARγ mRNA species of 3.1 kb and appear to have lost the expression of the 3.3-kb mRNA. The lack of expression of the 3.3-kb RARγ mRNA, which may represent RARγ-1, in PCC4(18)-1 cells may be related to the inability of these cells to undergo differentiation. If this is true, one would expect that the introduction of this RARγ into PCC4(18)-1 cells would enable the cells to undergo differentiation under RA addition. Experiments are under way to test this hypothesis. No changes in RAR expression were noticeable in PCC4(18)-2 cells. These cells might produce a defective RAR protein that is unable to undergo dimerization or to interact correctly with DNA and/or other regulatory proteins. Alternatively, the altered responsiveness in PCC4(18)-2 cells could be unrelated to a defect in the expression or function of one of the RARs but could be related to altered expression or function of another gene, possibly a target gene for RA activation or suppression. Paradoxically, this cell line has a greatly reduced level of CRABP, which appears not to be required in induction of differentiation by at least some retinoids (31). It is conceivable that reduced CRABP levels are not causative but indicative of this differentiation-detective phenotype; e.g., CRABP might be under the control of one or more RARs; if so, reduced expression of CRABP might be due to a defective RAR species. Recently, Wei et al. (33) have shown that in several embryonal carcinoma cells, CRABP is up-regulated by RA, supporting the concept that CRABP is either directly or indirectly under the control of RARs. Further characterization of RAR/CRABP expression in PCC4, aza1R cells and analyses of the changes in gene expression during differentiation of these cells will be necessary to determine the role of each RAR isoform and CRABP in this differentiation process.

Materials and Methods

Cell Culture and Differentiation. Characterization of the embryonal carcinoma cell line PCC4, aza1R and its mutant derivatives, PCC4(18)-1 and PCC4(18)-2, has been described previously (21). The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). All-trans-RA, RA analogues Ro 13-7410 and Ro 40-6055, and all-trans-retinol were synthesized by Hoffmann-La Roche. The analogues of the Ch-series were synthesized by K. Shudo (34). Medium, serum, and antibiotics were purchased from Gibco, Grand Island, NY. All-trans-[1H]-RA (50 Ci/mmol) was obtained from NEN-Dupont, Boston, MA. Induction of differentiation by retinoids was quantitated by the aggregate assay as described previ-
ously (5). The retinoids were dissolved in dimethyl sulfoxide. Controls received solvent only (0.1% final concentration).

**Preparation of Cytosolic and Nuclear Extracts.** The preparation of nuclear and cytosolic embryonal carcinoma cell extracts has been described previously (24, 35). Briefly, approximately 5 × 10⁶ cells were washed twice in phosphate-buffered salt solution containing 2 mM EDTA, trypsinized, and collected by centrifugation. The cells were then washed gently at 4°C in 10 ml PTG buffer consisting of 5 mM sodium phosphate (pH 7.4), 10 mM thiglycerol, 10% glycerol, and the following protease inhibitors: phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 units/ml), and leupeptin (10 units/ml). Cells were homogenized in 5 ml PTG buffer with a Dounce homogenizer (pestle B, 60–80 strokes). The homogenate was centrifuged at 4°C for 30 min at 1,000 × g. The pellet (2 ml), containing the loosely packed nuclei, was washed twice with the same buffer. To reduce the contamination of the nuclear preparation with cytosol, nuclei may be washed an additional two times. The supernatant fractions were combined and centrifuged for 30 min at 130,000 × g at 4°C. The resulting supernatant was designated as cytosolic extract. The nuclear pellet was extracted in 8 ml TKG buffer containing 10 mM Tris-HCl (pH 8.5), 1.5 mM EDTA, 10 mM thiglycerol, 10% glycerol, 0.8 M KCl, and the same protease inhibitors as in the PTG buffer. The suspension was incubated for 1 h on ice with repeated resuspension every 10 min and then centrifuged at 130,000 × g for 30 min. The resulting supernatant is referred to as the nuclear extract.

**Retinoic Acid Binding Assay.** In routine assays, cytosolic or nuclear extracts (0.3 ml) were added to 1.5 ml Eppendorf tubes containing [³H]RA to give a final concentration of 5 nM. Nonspecific binding was measured in the presence of a 200-fold excess of unlabeled RA. After 3 h incubation at 4°C, extracts were transferred to Eppendorf tubes containing dextran-charcoal pellets obtained after centrifugation of 30 µl of a charcoal-dextran suspension (3% acid-washed Norit A, 0.3% dextran C in 10 mM Tris-HCl, pH 7.4, and 0.02% sodium azide). After resuspension of the pellet, the mixture was incubated for 10 min at 4°C and then centrifuged for 15 min at 15,000 × g. The supernatant was analyzed for [³H]RA-binding activity by size exclusion HPLC (24). HPLC analysis was performed at 4 or 25°C with a Gilson liquid chromatography system. Extracts (200 µl) were fractionated over a Superose 12 HR 10/30 size-exclusion column (Pharmacia) at a flow rate of 0.5 ml/min using PTG buffer containing 0.4 M KCl as eluent. Radioactivity was monitored with a Flo-One/Beta A-200 radioflow detector (Radiomatic Instruments, Tampa, FL). In certain instances, fractions (0.5 ml) were collected, and radioactivity was determined with a scintillation counter. Binding data were calculated as the area under the curve using the trapezoidal rule or using the software provided by Radiomatic Instruments.

**Plasmid DNA.** The RAR probes, hRARα+ and mRARα+ were obtained from P. Chambon, Strasbourg, France. hRARα+ contains the full length coding sequence (1770 bp) for the human RARα protein cloned into the EcoRI site of the expression vector PSG5 (16, 18). hRARβ+ contains the human RARβ open reading frame cloned into the EcoRI and BamHI sites of the expression vector PSG5 (18). The clone mRARγ+ contains a 1.9-kb fragment of the murine RARγ sequence cloned into the EcoRI site of PSG5 (36). For Northern blot analyses, the full length hRARα sequence and hRARβ and mRARγ insertions were used as probes. The recombinant cDNA clone pGAD-28, which contains a 1261-bp cDNA insert of chicken glyceraldehyde-3-phosphate dehydrogenase (37), was digested with PstI, and an approximately 1120-bp fragment containing the coding region plus some 5’- and 3’-untranslated sequences was isolated and used as a probe. The cDNA probes were labeled with [α-³²P]dCTP (3000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by random priming using the kit and the protocols supplied by Boehringer Mannheim Biochemicals (Indianapolis, IN).

**RNA Isolation and Northern Analysis.** Total RNA from 3–5 × 10⁶ embryonal carcinoma cells was isolated by the guanidinium isothiocyanate method (38). Poly(A)⁺ RNA was selected by oligo(dT)-cellulose chromatography (39). Thirty µg total RNA or 5 µg poly(A)⁺ RNA were fractionated by electrophoresis on a 1.2% agarose/0.66 M formaldehyde slab gel in the presence of ethidium bromide and transferred to Nytran (Schleicher and Schuell, Keene, NH) membranes on a Vacuublot apparatus (American Bionetics, Hayward, CA) according to the manufacturer’s suggestion. Transferred RNA was cross-linked to the blot by UV irradiation (40, 41). Blots were prehybridized in 50% formamide, 1% SDS, 5× saline-sodium phosphate-EDTA buffer, 1× Denhardt’s solution, and 100 µg/ml denatured salmon sperm DNA at 42°C. Hybridizations were carried out with [³²P]dCTP-labeled RARα0, RARβ0, mRARα+ and pGAD-28 probes (10⁵ dpm/ml, specific activity, 5–7 × 10⁶ dpm/µg DNA) overnight at 42°C using the prehybridization buffer described above. Blots were washed twice in 2× SSC-0.1% SDS for 15 min at 25°C and once in 0.5× SSC-0.1% SDS for 30 min at 55°C. Blots probed for RARα and RARγ were washed additionally in 0.1× SSC-0.1% SDS for 15 min at 65°C. These hybridization conditions do not allow any cross-hybridization between the RAR probes.

**In Vitro Transcription.** Nuclei were isolated and [³²P]-labeled RNA transcripts were prepared by a modification of the procedure of Greenberg and Ziff (42) as described previously (43).

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**References**


