Receptor-selective Retinoids Implicate Retinoic Acid Receptor α and γ in the Regulation of bmp-2 and bmp-4 in F9 Embryonal Carcinoma Cells

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Abstract

The effect of retinoids on malignant cells and embryos indicates that retinoids influence the expression of growth factors or alter the response of cells to growth factors. The bone morphogenetic proteins, Bmp-2 and Bmp-4, are candidates for such growth factors because retinoic acid (RA) treatment of F9 embryonal carcinoma cells induced Bmp-2 mRNA, while simultaneously repressing Bmp-4 levels. Also, recombinant Bmp-2 affected the growth and differentiation of these cells. Regulation of each gene was concentration dependent and required continuous RA treatment. The short half-lives of the Bmp-2 (75 ± 11 min) and Bmp-4 (70 ± 4 min) mRNAs suggest that their abundance is primarily controlled at the transcriptional level. To determine which RA receptor (RAR) controls bmp-2 and bmp-4 expression, F9 cells were exposed to various receptor-selective retinoids. RAR α- and γ-selective retinoids induced Bmp-2 and repressed Bmp-4 equally as well as all-trans RA. In contrast, a RAR β-selective retinoid had little effect on Bmp-2 induction but repressed Bmp-4. A RAR α-selective antagonist inhibited all-trans RA stimulation of Bmp-2, although not as dramatically as a RAR β γ-selective antagonist. No differences were observed between Bmp levels in all-trans RA and 9-cis RA-treated cells, indicating that the RXRs play little part in controlling these genes. The results are consistent with RAR α and γ-controlled Bmp-2 and Bmp-4 regulation.

Introduction

The acid form of vitamin A, RA, is an important regulator of cell proliferation and differentiation in a broad array of tissues

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. 1 This work was supported by Grant F93USF-1 from the American Cancer Society, Florida Division, Inc., the Seymour Kramer Award from the Leukemia Research Foundation, Inc., and Grant R01 HD31117 from the National Institute of Child Health and Human Development. 2 To whom requests for reprints should be addressed, at Department of Biology, LIF/136, University of South Florida, 4202 East Fowler Avenue, Tampa, FL 33620. Phone: (813) 974-2623; Fax: (813) 974-3263; E-mail: rogers@chuma.cas.usf.edu. 3 The abbreviations used are: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; Bmp, bone morphogenetic protein; RACT, a treatment of RA, dibutylryl cAMP, and theophylline; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.
ing Bmp-2 and Bmp-4 in very early mouse differentiation. F9 cells are the malignant stem cells of a mouse teratocarcinoma. Monolayer cultures of F9 cells treated with RA differentiate into benign cells resembling the primitive endoderm of the mouse blastocyst. If the cells are treated with RACT, the cells become biochemically indistinguishable from parietal endoderm and express parietal endoderm markers such as laminin and tissue plasminogen activator. This RA-responsive system was used to show that Bmp-2 and Bmp-4 are RA-regulated genes. Additionally, recombinant Bmp-2 affected the morphology, growth, and gene expression of differentiating F9 cells, suggesting that regulation of Bmp-2 and Bmp-4 may affect the loss of malignancy in these cells (10).

This work further our understanding of how bmp-2 and bmp-4 are regulated by RA in F9 cells. The mechanism by which RA regulates these growth factors was addressed by determining the half-life of the mRNAs under different treatment conditions. Finally, the effect of receptor-selective retinoids on bmp-2 and bmp-4 expression and F9 cell differentiation was determined.

**Results**

**RA Regulation of bmp-2 and bmp-4 in F9 Embryonal Carcinoma Cells.** Previous experiments showed that the abundance of Bmp-2 transcripts was increased, and the abundance of Bmp-4 transcripts was decreased in F9 cells treated with 0.5 μM all-trans RA, 250 μM dbcAMP, and 500 μM theophylline (10). To determine the optimal conditions for Bmp-2 and Bmp-4 expression, F9 cells were treated with 10^{-6} M, 10^{-7} M, 10^{-8} M, and 10^{-9} M all-trans RA with CT (250 μM dibutyryl cAMP and 500 μM theophylline) for 102 h. RNA was prepared for Northern blot analysis and Bmp-2, Bmp-4, and laminin B1 mRNA levels were measured. Fig. 1A shows the autoradiographs of these blots, and Fig. 1B shows the data normalized to the constitutive message 36B4 (18). As shown in Fig. 1, 10^{-6} M all-trans RA (Lane 6) induced Bmp-2 twice as effectively as 10^{-7} M RA (Lane 7). Bmp-4 levels were undetectable at 10^{-6} M all-trans RA, while 36% of the Bmp-4 mRNA measured in CT-treated cells remained in 10^{-7} M all-trans RA-treated cells. Higher concentrations of RA were not tested, because 10^{-6} M all-trans RA is generally considered the upper limit of physiological concentrations. Cell morphology and the abundance of the parietal endoderm marker, laminin B1, indicated that the cells were completely differentiated. Thus, high Bmp-2 RNA levels and the absence of Bmp-4 RNA correlate with the parietal endoderm phenotype.

Some transcripts are regulated very rapidly by RA (e.g., HoxA1 within 2 h; Ref. 19), whereas others require several days of RA treatment (e.g., laminin B1). A time course of Bmp-2 and Bmp-4 expression was obtained by isolating RNA from cells treated with 10^{-6} M all-trans RA and CT for 12, 24, 48, 72, and 102 h (Fig. 1, Lanes 2–6). Bmp-2 required 3 days of drug treatment to reach maximum levels. Bmp-4 was completely repressed by 2 days of treatment. This indicates that these growth factors are regulated late in the stepwise progression of differentiation.

Furthermore, continuous all-trans RA treatment was required to induce Bmp-2 RNA to maximal levels. Replacing drug-containing media with drug-free media at 12, 24, 48, or 72 h, followed by harvesting at 102 h (Fig. 1, Lanes 11–14), induced Bmp-2 to less than 40% of the amount observed in continuously treated cells. Interestingly, the Bmp-4 mRNA abundance in cells treated with 10^{-6} M RA and CT (Fig. 1, Lane 2) for 12 h was 2.5-fold higher than that observed in CT-treated cells (Fig. 1, Lane 10). This increased level was maintained for nearly 4 days following removal of drug (Fig. 1, Lane 11), suggesting that the transient Bmp-4 induction was not due to the recent change of media. The data shown in Fig. 1 indicate that optimal conditions to induce Bmp-2 and repress Bmp-4 include continuous 10^{-6} M RA and CT treatment for at least 3 days.

**Bmp-2 and Bmp-4 mRNAs Are Short-Lived.** Two main mechanisms by which RNA abundance is regulated are transcription rate and message stability. To analyze the relative stability of these transcripts under different conditions, F9 cells were grown in the absence of drug (undifferentiated), in 1.0 μM all-trans RA, or in 1.0 μM all-trans RA and CT (fully differentiated) for 3 days. The cells were then treated with the transcriptional inhibitors DRB or actinomycin D. The concentrations used, 25 μg/ml DRB or 2 μg/ml actinomycin D, have been shown to inhibit 5,6-[3H]uridine incorporation by 90–95% (20). The results of two representative experiments are shown in Fig. 2A. Since Bmp-2 mRNA is induced by RA or RACT and is undetectable in undifferentiated cells, only the data regarding the retinoid-treated cells is shown. Likewise, since Bmp-4 is detectable only in undifferentiated and RA-treated cells, only this data is shown. All transcript measurements were normalized to the level of the constitutive message 36B4, which remained constant during the 6 h of transcription inhibition. The data is plotted (Fig. 2B) as a percentage of the RNA abundance in cells not treated with inhibitor (time zero). Within 3 h of DRB or actinomycin D addition, Bmp-2 and Bmp-4 mRNA levels fell to 10% of those observed at time zero. In contrast, laminin B1 mRNA levels in RACT-treated cells were 54% of time zero levels after 6 h of DRB treatment (data not shown). A straight line was obtained when the data shown in Fig. 2B was plotted as the log of RNA amount versus time, indicating first-order decay kinetics. The Bmp-2 message half-life was 75 ± 11 min, and the Bmp-4 message half-life was 70 ± 4 min. Thus, Bmp-2 and Bmp-4 have relatively unstable messages, suggesting that the regulation of these genes occurs primarily at the transcriptional level.

**Regulation of Bmp-2 and Bmp-4 by Receptor-selective Retinoids.** Six unique retinoid receptors, RAR α, β, γ and RXR α, β, γ, mediate the transcription of RA-regulated genes. A number of natural and synthetic retinoids have been identified that specifically activate all or a subset of these receptors. All-trans RA and 9-cis-RA are naturally occurring retinoids. All-trans RA activates all three RARs, while 9-cis-RA activates all three RARs and all three RXRs. Ro13–7410 (aka TTNPB) is a nonmetabolized activator of RAR α, β, and γ. Three retinoids that preferentially activate individual RARs are Ro40–6055 (RAR α), CD2019 (RAR β), and CD437 (RAR γ). Two antagonists are available that prevent RAR α (Ro41–5253) activation or RAR β and γ (CD2665) activation.
Fig. 1. Bmp-2 mRNA induction and BMP-4 mRNA repression in F9 embryonal carcinoma cells. A, autoradiographs of Northern blots of RNA isolated from F9 cells treated with various concentrations of drugs for various times. Twenty-five μg of total RNA were loaded per lane. The same blot was probed sequentially for the Bmp-2 (top), Bmp-4 (middle), laminin B1 (bottom), and 36B4 RNAs (data not shown). Lane 1, F9 cells were treated with 10^{-5} M all-trans RA for 102 h; Lanes 2–6, cells were treated with 10^{-6} M all-trans RA and CT (250 μM dibutryl cAMP and 500 μM theophylline) for 12 h (Lane 2), 24 h (Lane 3), 48 h (Lane 4), 72 h (Lane 5), and 102 h (Lane 6); Lanes 7–9, cells were treated with 10^{-6} M (Lane 7), 10^{-5} M (Lane 8), and 10^{-6} M (Lane 9) all-trans RA and CT for 102 h; Lane 10, cells were treated with CT for 102 h, followed by RNA extraction. Lanes 11–14, cells were treated with 10^{-6} M all-trans RA and CT for 12 h (Lane 11), 24 h (Lane 12), 48 h (Lane 13), and 72 h (Lane 14), at which time drug-containing medium was replaced with drug-free medium. RNA was isolated from these cells at 102 h. B, histograms indicating the transcript levels normalized to the level of the constitutive RNA, 36B4. Bmp-2 and laminin B1 levels are expressed as a percentage of those observed in cells treated with 10^{-6} M all-trans RA and CT for 102 h. Bmp-4 levels are expressed as a percentage of those observed in cells treated with CT alone. Column numbers correspond to those shown in the autoradiographs.

To determine the relative roles that each receptor plays in regulating bmp-2 and bmp-4 expression, the receptor-selective retinoids were used to treat F9 cells for 4 days. Although 1 μM all-trans RA is optimal for Bmp-2 and Bmp-4 regulation, 0.1 μM of each retinoid agonist was chosen upon consideration of the IC\textsubscript{50}s of the nucleotide extracted, and transcript levels were analysis. Autoradiographs of this data and Table 1, Bmp-2 and laminin B1 levels.
a percentage of those observed in the all-trans RACT-treated cells included in each experiment. BMP-4 levels are expressed as a percentage of those observed in cells treated only with CT.

**RAR α-selective Retinoid Treatment.** The RAR α-selective retinoid (Ro40-6055) and CT mimicked all-trans RA and CT effectively (Fig. 3 and Table 1). BMP-2 expression was induced to 128 ± 16% of the level induced by all-trans RA and CT. The induction of BMP-2 was completely reversed by the further addition of 10 μM RAR α-selective antagonist (Ro41-5253). The RAR α agonist repressed BMP-4 to 11 ± 6% of the levels observed in CT-treated cells. This repression was partially reversed by the RAR α-selective antagonist. Thus, RAR α can regulate BMP-2 and BMP-4.

To determine if RAR α exclusively controlled BMP regulation, the RAR α antagonist was added to cells treated with all-trans RACT. All-trans RA activates RAR α, β, and γ. Ten μM antagonist had a modest inhibitory effect on BMP-2 induction (56 ± 15% of all-trans RACT levels). This effect was greater than the effect on laminin B1 induction (88 ± 15%), suggesting that RAR α may play a greater role in BMP-2 regulation. RAR α antagonist clearly boosted BMP-4 levels relative to those observed in all-trans RACT-treated cells (undetectable). However, the BMP-4 mRNA level only reached 28% of that observed in CT-treated cells. The effect of the RAR α antagonist varied between experiments. This may reflect instability of this retinoid, as alterations in the UV spectra of Ro41-5253 were observed following long-term storage in solution.

**RAR β-selective Retinoid Treatment.** The RAR β agonist and CT induced BMP-2 slightly (8%) relative to all-trans RACT and repressed BMP-4 to 10% of the abundance observed in CT-treated cells (Fig. 3). Therefore, RAR β is unlikely to be involved in BMP-2 induction but may be involved in BMP-4 repression.

**RAR γ-selective Retinoid Treatment.** The RAR γ-selective agonist and CT induced BMP-2 and repressed BMP-4 equally as well as all-trans RACT (Fig. 3). The addition of the RAR γ antagonist, CD2665, largely reversed the all-trans RACT-stimulated BMP-2 induction and BMP-4 repression (Table 1). The data from two representative experiments (Fig. 4) show that repression of the all-trans RACT effect increases with higher concentrations of antagonist. However, at the highest concentration of antagonist (10 μM), BMP-2 was still detectable (9 ± 3% of all-trans RACT levels) and BMP-4 levels reached only 61 ± 6% of those observed in CT-treated cells (Table 1). Thus, RAR γ can regulate BMP-2 and BMP-4, but other receptors may play a role.

**The Relative Role of RARs and RXRs.** To determine whether RXRs play any role in regulating BMP-2, the level of induction at 4 days by all-trans RACT was compared to that of 9-cis-RACT. 9-cis RACT is a panagonist, because it activates all six retinoid receptors. No difference was observed (Table 2). Ro13-7410, which activates RAR α, β, γ with extremely high specificity, also induced BMP-2 equally as efficiently as all-trans RACT or 9-cis-RACT (data not shown). BMP-4 mRNA was undetectable or weakly expressed. Unlike all-trans RA and 9-cis-RA, Ro13-7410 cannot be metabo-
Fig. 3. Bmp-2 mRNA induction and Bmp-4 mRNA repression in F9 embryonal carcinoma cells treated with receptor-selective agonists. A, autoradiographs of Northern blots of RNA isolated from F9 cells treated with 0.1 μM Ro40-6055 (Lane 1); 0.1 μM Ro40-6055 and 10 μM Ro41-5253 (Lane 2); 0.1 μM Ro40-6055 and CT (Lane 3); 0.1 μM Ro40-6055, 10 μM Ro41-5253, and CT (Lane 4); 0.1 μM all-trans RA (Lane 5); 0.1 μM all-trans RA and CT (Lane 6); 0.1 μM CD2019 (Lane 7); 0.1 μM CD2019 and CT (Lane 8); 0.1 μM CD437 (Lane 9); 0.1 μM CD437 and CT (Lane 10); 0.1 μM 9-cis RA (Lane 11); and 0.1 μM 9-cis RA and CT (Lane 12) for 92 h. The same blot was probed sequentially for the Bmp-2 (top), Bmp-4 (middle), laminin B1 (bottom), and 36B4 RNAs (data not shown). B, histograms indicating the transcript levels normalized to the level of the constitutive RNA, 36B4. Bmp-2 and laminin B1 levels are expressed as a percentage of those observed in cells treated with 0.1 μM all-trans RA and CT. Bmp-4 levels are expressed as a percentage of those observed in cells treated with CT (data not shown). Column numbers correspond to those in the autoradiographs.
Table 1  Effect of RAR-selective retinoids on BMP-2, BMP-4, and laminin B1 mRNA levels

RNA levels are expressed as a percentage of levels observed in trans-RACT-treated (BMP-2 and laminin B1) or CT-treated F9 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BMP-2 % ± SE</th>
<th>n</th>
<th>BMP-4 % ± SE</th>
<th>n</th>
<th>Laminin B1 % ± SE</th>
<th>n</th>
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<tr>
<td>Ro40-6055 (RAR α agonist)</td>
<td>86 ± 12</td>
<td>3</td>
<td>ND*</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ro40-6055CT (RAR α agonist)</td>
<td>128 ± 16</td>
<td>4</td>
<td>11 ± 6</td>
<td>3</td>
<td>114 ± 39</td>
<td>3</td>
</tr>
<tr>
<td>RACT &amp; Ro41-5253 (RAR α, β, γ agonist and RAR α antagonist)</td>
<td>56 ± 15</td>
<td>7</td>
<td>28 ± 10</td>
<td>5</td>
<td>88 ± 19</td>
<td>6</td>
</tr>
<tr>
<td>RACT &amp; CD2665 (RAR α, β, γ agonist and RAR γ antagonist)</td>
<td>9 ± 3</td>
<td>6</td>
<td>61 ± 6</td>
<td>6</td>
<td>16 ± 2</td>
<td>6</td>
</tr>
</tbody>
</table>

* No. of independent experiments.

Table 2  BMP-2 and BMP-4 expression in F9 cells treated with all-trans RACT (RAR α, β, γ agonist) or 9-cis RACT (panagonist).

RNA levels were normalized to the constitutive message 36B4 and are expressed as arbitrary units. Comparisons were made between the two treatments within each experiment. A test for differences between means using a paired t test revealed no significant difference (P < 0.05).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>36B4</th>
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<th>36B4</th>
<th>9-cis RACT</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1.84</td>
<td>2.11</td>
<td>0</td>
<td>0</td>
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<tr>
<td>2</td>
<td>1.77</td>
<td>2.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>2.26</td>
<td>1.85</td>
<td>0</td>
<td>0</td>
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<tr>
<td>4</td>
<td>2.87</td>
<td>4.24</td>
<td>0.054</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.74</td>
<td>0.88</td>
<td>0.005</td>
<td>0.0065</td>
</tr>
<tr>
<td>6</td>
<td>1.08</td>
<td>1.45</td>
<td>ND*</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.

Fig. 4  The effect of all-trans RA on Bmp-2 and Bmp-4 RNA levels is counteracted by the RAR γ antagonist, A, autoradiographs of Northern blots of RNA isolated from F9 cells treated with CT (Lane 1); 0.1 μM all-trans RA and CT (Lanes 2–6), and 0.1 μM (Lane 3), 1.0 μM (Lane 4), 5.0 μM (Lane 5), and 10.0 μM (Lane 6) CD2665 for 3 days. Identical blots were probed for the Bmp-2 (top), Bmp-4 (middle), laminin B1 (bottom), and 36B4 RNA (data not shown). B, the data in A were normalized to the constitutive RNA 36B4 and plotted as a percentage of the levels observed in cells treated with 0.1 μM all-trans RA and CT (Bmp-2 and laminin B1) or as a percentage of the level observed in cells treated with CT (Bmp-4). ●, Bmp-2; ■, Bmp-4; ●, laminin B1. Bars, SD.

Discussion

Retinoids affect differentiation via a staggering array of pathways. Known components include various endogenous retinoids, cytoplasmic binding proteins, and six nuclear receptors with variant isoforms. Although it is important to understand interactions between retinoids and their cytoplasmic and nuclear binding proteins, characterization of retinoid-regulated target genes is required to fully comprehend the in vivo responses elicited by retinoids. As RA-regulated growth factors, Bmp-2 and Bmp-4 may implement some of the effects RA has on differentiation and proliferation. Indeed, it was previously shown that recombinant Bmp-2 inhibits the proliferation of RA-treated F9 embryonal carcinoma cells and prevents complete terminal differentiation (10).

Complete differentiation of F9 embryonal carcinoma cells into parietal endoderm is a multistep process requiring several days. Various genes are regulated temporally following the addition of RA. Some genes, for example the transcription factors HoxAl (19) and RAR γ (21), are induced within 12 h of adding RA. Other genes, typically those characteristic of terminally differentiated cells, require 1 day or more before expression is altered. The data shown here indicate that
bmp-2 and bmp-4 fall into the latter class of genes. Bmp-2 induction and Bmp-4 repression require 2–3 days of treatment. Because numerous genes expressed in the parietal endoderm of the mouse embryo (e.g., laminin; Ref. 22) are also found in F9 cell-derived parietal endoderm (23), high Bmp-2 levels and an absence of Bmp-4 may be characteristic of terminally differentiated parietal endoderm. Likewise, since undifferentiated F9 cells share many similarities with the blastocyst inner cell mass, Bmp-4 may be expressed in the inner cell mass in vivo.

Although bmp-2 and bmp-4 are 92% identical at the amino acid level, they are oppositely regulated by RA. This is consistent with embryological analyses indicating that Bmp-2 and Bmp-4 can function similarly but have distinct embryonic expression patterns. For example, both Bmp-2 and Bmp-4 can initiate the early steps in tooth induction, but only Bmp-4 is expressed at the appropriate place and time (9). Lyons et al. (24) suggested that the coordinated expression of several different transforming growth factor βs might regulate the progression of cells through differentiation. The switch between Bmp-4 and Bmp-2 expression during parietal endoderm differentiation is consistent with this idea. Although Bmp-2 and Bmp-4 might function similarly, distinct temporal expression patterns may not allow them to compensate for each other. Indeed, bmp-2 or bmp-4 null mutations cause lethality very early in development (25).

Because regulation of Bmp-2 and Bmp-4 levels is paramount to their roles in differentiation, it is important to understand the mechanism by which these genes are regulated. As shown in Fig. 2, the half-lives of the Bmp-2 and Bmp-4 mRNAs are very short. Furthermore, the half-lives do not vary with different drug treatments. This suggests that the bmp-2 and bmp-4 genes are regulated primarily at the transcriptional level.

The transcriptional effects of RA are thought to be mediated by the RARs, and all six retinoid receptors are known to be expressed in F9 cells (21, 26, 27). Thus, receptor-selective retinoids were used to assay the role of each receptor in regulating bmp-2 and bmp-4. This pharmacological approach indicates that RAR γ is necessary for normal F9 cell differentiation. An RAR γ agonist induced the parietal endoderm markers Bmp-2, laminin B1 (Fig. 3), and tissue plasminogen activator (28) and repressed Bmp-4 (Fig. 3). The morphology of cells treated with the RAR γ agonist was indistinguishable from those treated with all-trans RA. Likewise, cells treated with all-trans RA, which activates RAR α, β, and γ, and an antagonist of RAR β and γ maintained an undifferentiated morphology and expression pattern. These observations are consistent with the phenotype of F9 cells containing a null mutation for RAR γ (29), in which most differentiation markers, including bmp-2, did not respond to all-trans RA.

Although RAR γ function may be required for normal differentiation, RAR α can regulate the bmp-2 and bmp-4 genes. A RAR α agonist induced Bmp-2 mRNA and repressed Bmp-4 mRNA, and a RAR α antagonist counteracted this effect (Table 1). The RAR α agonist can also induce laminin B1 (Table 1), tissue plasminogen activator (28), and the parietal endoderm morphology. Consistent with the ability of RAR γ to mediate differentiation, the RAR α antagonist incompletely repressed the effect of all-trans RA (Table 1), and the RAR α null mutant F9 cells regulated Bmp-2 and Bmp-4 RNAs normally (29). These observations are consistent with an early requirement for one or more genes regulated specifically by RAR γ. Inhibition of this gene(s) arrests differentiation at an early stage. Late genes, such as bmp-2 and bmp-4, can be regulated by either RAR α or γ. This work and the study of Boylan et al. (29) detected no difference between all-trans RA and 9-cis RA in controlling differentiation, indicating that the RXRs play a minor role.

Since all-trans RA regulates hundreds of known genes (30), the receptor-selective retinoids can help identify the subsets of genes regulated by each receptor. For example, the RAR β agonist was ineffective at inducing Bmp-2 or laminin B1 but did repress the Bmp-4 RNA to 10% of the level observed in undifferentiated cells. Thus, it might be possible to specifically inhibit Bmp-4 expression. Further characterization of genes that are specifically regulated by the receptor-selective retinoids may identify therapeutic activities while avoiding the toxic effects caused by activating several receptors.

**Materials and Methods**

**Drugs.** All-trans RA (RAR α, β, γ agonist; Ref. 31), dibutyryl cAMP, theophylline, DRB, and actinomycin D were obtained from Sigma Chemical Co. Ro40-6655 (also known as Ams80; RAR α agonist; Ref. 32), 9-cis RA (RAR α, β, γ, RXR α, β, γ paragonist; Ref. 31), Ro13-7410 (RAR α, β, γ agonist; Ref. 31), and Ro41-5253 (RAR α agonist; Ref. 32) were obtained from Hoffman-LaRoche. CD2019 (RAR β agonist; Ref. 28), CD437 (RAR γ agonist; Ref. 28), and CD2665 (RAR βγ agonist; Ref. 33) were obtained from CIRD Galderna. The Kₚ of CD2665 are 2250, 306, and 110 nM for RAR α, β, γ, respectively (Serge Michel, CIRD Galderna Valbonne, France). Values for the remaining retinoids may be found in the cited references.

**F9 Cell Culture and Differentiation.** F9 embryonal carcinoma cells were grown in DMEM supplemented with 10% heat-inactivated calf serum, 2 mM glutamine, and 0.1 mM mercaptoethanol. For differentiation experiments, cells were trypsinized and replated at 5.6 × 10⁶ cells/cm². After cells attached, the medium was replaced with drug-containing medium. On the third day of long experiments, the medium was replaced with fresh medium containing drugs.

**Northern Analysis.** RNA was isolated by the guanidinium isothiocyanate gradient method (34) and electrophoretically separated on 1% agarose/2.2 M formaldehyde gels, transferred to nylon (BioBlot-N; Costar), and hybridized to ³²P-labeled probes. Double-stranded DNA probes were labeled with [³²P]CTP by the random primer method (34). The Bmp-2 and Bmp-4 probes (plasmids Bmp2–68 and Bmp4–70) have been described in (10). The laminin B1 probe (pC56) was described by Rogers et al. (35). The probe for the constitutive RNA, 36B4, was described by Rio et al. (18). Transcript signals were measured using a Molecular Dynamics Phosphorimager.

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**References**


