Expression of retinoic acid receptor β (RARβ) is spatially and temporally restricted during embryonal development. Also during retinoic acid (RA)-dependent embryonal carcinoma (EC) cell differentiation, RARβ expression is initially up-regulated, while in later phases of differentiation expression is down-regulated, by an unknown mechanism. To gain insight into the regulation of RARβ, we studied the activity of the RARβ2 promoter and mutants thereof in various cell lines. While the RARβ2 promoter is activated by RA in a limited number of cell lines, synthetic RA-responsive reporters are activated in most cell types. We show that the expression levels of proteins that bind to the β-retinoic acid response element (RAR-retinoid X receptors and orphan receptors) and also the differential expression of a number of coactivators modulate the RA response on both natural and synthetic reporters. We further show that cell type-specific activation of the RARβ2 promoter is dependent on the promoter architecture including the spacing between retinoic acid response element and TATA-box and initiator sequence (βINR). Mutation within these regions caused a decrease in the activity of this promoter in responsive EC cells, while an increase in activity in non-EC cell lines was observed. Cell-specific complexes were formed on the βINR, suggesting that the βINR contributes to cell-specific activation of the promoter. On this basis we propose that promoter context-dependent and more general RA response-determining mechanisms contribute to cell-specific RA-dependent activation of transcription.

Growth control, differentiation, and homeostasis require a highly regulated gene expression. Inadequate gene expression may lead to tumor formation or developmental abnormalities. Regulation of gene expression is controlled by activators and repressors, as positive and negative regulators of mRNA synthesis (1). These transcription factors bind to both up- and downstream (relative to the transcriptional start site) target sites and are thought to regulate the level of transcription through an interaction with the basal transcription machinery (2), thereby increasing the formation of the preinitiation complex. Eukaryotic RNA polymerase II promoters may contain several core elements such as the TATA-box and the initiator (INR) that are recognized by general transcription factors (GTFs) (3). The TATA-box is the binding site for TFIID, consisting of the TATA-binding protein and TAFs (TATA-binding protein-associated factors). The INR, located around the transcription start site, is believed to affect TFIID recruitment and/or function, in particular within promoters without TATA-box (4). Comparison of sequences surrounding the transcription start site (5, 6) as well as random mutagenesis (7) and binding site selection experiments (6) have identified a short (weakly conserved) sequence with the consensus (T/C)A^3(G/T)(T/C) (6) or YYA^NTYY (7). In vitro transcription experiments have shown that INR-dependent basal transcription requires TAFs, since TFIID but not TATA-binding protein can enhance transcription from INR-containing promoters (8, 9). Several INR-binding factors have been described including TFIID-I, USF, YY1, RNA pol II, and E2F (10). Furthermore, an activity called CIF (cofactor of initiator function) have been purified and shown to be functionally required for INR-dependent transcription and contains multiple components including the human homolog of dTAF150 (11).

Retinoic acid (RA) has profound effects, both in vitro and in vivo, on cell growth and differentiation (12). Treatment of various cell lines e.g. embryonal carcinoma (EC) cells with RA leads to a culture condition-dependent differentiation (13) accompanied by changes in gene expression (14). This RA-dependent modulation of expression of genes implicated in early development may explain how retinoids exert their effect on differentiation. Therefore, it is important to understand the mechanisms underlying the spatio-temporal regulation of RA target genes during development.

Retinoic acid exerts its effect through two families of receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), each comprising three genes, designated RARα, -β, and -γ and RXRα, -β, and -γ. Both families belong to the steroid/thyroid hormone receptor superfamily (15). RAR and RXR together form heterodimers that bind preferentially to direct repeats with a consensus (A/G)G/G/T/TCA separated by 2 or 5 base pairs and can modulate transcription of promoters containing such binding sites (15). Upon treatment of P19-EC cells with RA, a rapid induction of the RARβ mRNA was observed (16). Cloning of the RARβ2 promoter revealed the presence of a retinoic acid response element in close proximity.
of the TATA-box (17, 18). This retinoic acid response element was shown to be required for accurate expression in vitro (19) and in vivo (20–22). Sequences upstream from this element functioning as a cyclic AMP-response element as well as putative thyroid hormone response element (TRE)-like sequences have been found that contribute to RA-dependent activation of this promoter (23). Finally, in vivo footprint experiments have identified an INR element that is occupied in a RA-dependent manner in EC cells and contributes to RARβ2 promoter activity (24).

In situ hybridization experiments have shown that RARβ is expressed in a spatio-temporally restricted pattern during embryogenesis (25–27). Furthermore, the expression of RARβ during EC cell differentiation is regulated, being maximal after 4 days, after which a decline is observed (28). Cell lines derived from RA-differentiated EC cells, such as END2 cells, express RARβ neither in the presence nor in the absence of RA (29). Interestingly, the loss of RARβ expression has been found to be correlated with tumor progression (30–33). These observations together suggest that a highly regulated expression is required for both proper development and homeostasis. Therefore, we and others have investigated the regulation of RARβ2 expression by studying the activation of its promoter in various cell lines. Northern blot analysis as well as transient transfection experiments have shown that activation of RARβ by RA is cell type-specific (29, 34–38). The mechanism underlying this cell specificity is largely unknown.

We (29) and others (38) have reported that the high activity of the RARβ2 promoter in EC cells is the consequence of the presence of E1A-like activity in these cells that is lost upon differentiation. The introduction of the adenovirus immediate early gene E1A 13S (for a review, see Ref. 39) in a differentiated derivative of P19-EC cells (END2) restores activation of the RARβ2 promoter in the presence of RA (29). This has led to the hypothesis that E1A or E1A-like activity functions as a cofactor for RARs. Recently, we have presented evidence supporting this hypothesis by showing that E1A can activate RA-dependent transcription through a direct interaction with RARs. Furthermore E1A can interact with several GTFs, thus bridging RARs with the GTFs (40).

It has been hypothesized that the inability of RARβ2 promoter activation in breast tumor and lung cancer cell lines is the consequence of limiting amounts of cofactor(s) (34, 35). This, however, was not sufficient to explain the low level of RARβ2 promoter activity in all cell lines tested, since some cell lines have a strong RA response on synthetic βRAR-containing reporters but not on the natural RARβ promoter itself (34, 36). Recently, a series of putative cofactors has been identified by their ability to interact with nuclear receptors in a ligand-dependent fashion (41–48). These cofactors/intermediary factors can inhibit squelching by overexpression of receptors, and some of these cofactors can activate transcription in transient transfection assays in mammalian cell lines. The availability of these cofactors enabled us to test the hypothesis that the lack of activation of the RARβ2 promoter is caused by limiting amounts of these proteins in nonresponsive cells.

Since the RARβ promoter is largely dependent on the RARE for transcriptional activation, factors that have been reported to influence the RA-dependent activation of RARE-containing promoters might also influence the activity of this promoter. Orphan receptors (e.g., COUP-TF) have been reported to negatively regulate the ligand-dependent induction by the vitamin D3 receptor, thyroid hormone receptors, and RARs (49–51) through several different mechanisms (52–54). Furthermore, NGF-1B/Nur77 (55), Dax1 (56), and HNF-4 (57) were shown to influence the activity of RARE-containing reporters positively.

In this paper, we investigate whether the above mentioned (transcription) factors are involved in and required for the RA response on distinct RA-responsive systems and whether they contribute to cell type-specific activation of transcription. Comparison of the RA-dependent RARβ2 promoter activity with that of other RA-dependent reporter systems in multiple cell lines indicated that these two different RA response systems are regulated cell type specifically by different mechanisms. Electrophoretic mobility shift assays and transient transfection experiments indicate that orphan receptors (COUP-TF and NGF-1B/Nur77) and cofactor expression levels contribute to RA response on both the RARβ promoter and other RA response systems. Mutational analysis further suggests that the architecture of the RARβ2 promoter, including the close proximity of the RARE to the TATA-box and INR sequence, contributes to the strong response in P19-EC cells and the poor response in many other cells.

**MATERIALS AND METHODS**

**Plasmids**—The reporters −1470/+156 Luc, 5× GAL-e1b Luc, and 3× RARE-tk CAT have been described before (40, 58). 3× RARE-tk Luc was made by cloning the RARE-containing fragments (reverse orientation) in a tk-LUC vector. All promoter mutants were generated by PCR, using a mutated forward primer and a reverse primer binding to the luciferase gene within the reporter plasmid or mutant reverse primers (−10, −20, and mtTDT) extended with a BamHI restriction site, together with a primer that recognizes the 5′ part of the RARβ promoter (−63−/45). PCR fragments were digested with BamHI and cloned into the corresponding site of pLuc. The spacer mutants were constructed by first creating an Xho site between the RARE and TATA-box using the above described PCR approach. Next, PUC DNA fragments generated by SauIIA digestion, followed by partial fill-in using T and C nucleotides, were cloned in the Xho-digested (partial fill-in using A and G nucleotides) above described reporter (spacer 8). The spacer length of 10 clones with different inserts was further analyzed by sequencing.

**Electrophoretic mobility shift assays and transient transfection experiments** indicated that these two different RA response systems contribute to cell type-specific activation of transcription. Comparison of the RA-dependent RARE-containing reporter systems and whether they are involved in and required for the RA response on distinct RA-responsive systems and whether they contribute to cell type-specific activation of transcription. Comparison of the RA-dependent RARβ2 promoter activity with that of other RA-dependent reporter systems in multiple cell lines indicated that these two different RA response systems are regulated cell type specifically by different mechanisms. Electrophoretic mobility shift assays and transient transfection experiments indicate that orphan receptors (COUP-TF and NGF-1B/Nur77) and cofactor expression levels contribute to RA response on both the RARβ promoter and other RA response systems. Mutational analysis further suggests that the architecture of the RARβ2 promoter, including the close proximity of the RARE to the TATA-box and INR sequence, contributes to the strong response in P19-EC cells and the poor response in many other cells.

**Cell Culture and Transient Transfection**—Cells were cultured as described before (59), and transient transfection experiments were performed by calcium phosphate precipitation as reported before (40). Luciferase activity was determined in a Topcount liquid scintillation counter (Packard Instrument Co.) using a Luciferase reporter gene assay kit (Packard Instrument Co.). CAT activity was determined using a mutated forward primer and a reverse primer binding to the luciferase gene within the reporter plasmid or mutant reverse primers (−10, −20, and mtTDT) extended with a BamHI restriction site, together with a primer that recognizes the 5′ part of the RARβ promoter (−63−/45). PCR fragments were digested with BamHI and cloned into the corresponding site of pLuc. All mutations were confirmed by restriction enzyme digestion and sequencing, while expression levels were confirmed by Western blot. The GAL fusion constructs have been described before (59).

**RNA isolation, Northern blotting, and RNase protection** were performed according to standard methods as described before (60), using the probes as described by Jonk or Van der Leede for the mouse or human RARs, respectively (28, 29, 60). Probes used for hybridization were as follows: p300, 2.1-kb SpeI fragment (61); CBP, −3-kb Xho fragment (62); SRLC, 900-bp EcoRI–HindIII fragment (46); Rip140, 2.3-kb HpaI fragment (41); GAPDH, 1.4-kb Psfl fragment; RARα, 1.8-kb EcoRI fragment; and RARβ, 1.4-kb SalI–BamHI fragment (60).
EMSA—Whole cell extracts (WCEs) were prepared as described before (40). Ten μg of the WCEs from the various cell lines, adjusted to 4 μl with lysis buffer (20 mM Tris (pH 7.5), 20% (v/v) glycerol, 400 mM KCl, 1 mM dithiothreitol, containing protease inhibitors) was added to the reaction mixture for EMSA experiments. When cell extracts from transiently transfected COS cells were used (prepared as described before; Ref. 40), only 0.5–1 μg of protein was added. WCE proteins were incubated for 20 min on ice in 20 μl of reaction buffer containing 25 mM Tris (pH 8.0), 50 mM KCl, 2 mM MgCl₂, 2 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 5 mg/ml bovine serum albumin, in the presence of 2 μg of dsDNA and 10⁴ to 2 × 10⁵ cpm of end-labeled double-stranded polyacrylamide gel-purified βRARE (5'-gatecgggtagGGTTCAGcggAgGGAC-3'), INR oligonucleotide (5'-TCATCTTCATTCTTGTTGCAGAAG-3'), or TDT oligonucleotide (5'-CAGCGCTTACCTCGAGAC-3') (specific activity from 3 × 10⁹ to 2 × 10¹⁰ cpm/μg). The indicated antibodies (0.5 μl) or a 100-fold molar excess of unlabeled oligonucleotides was added to the reaction mixture 10 min prior to the addition of labeled probe. Subsequently, reaction mixtures were loaded on a prerun of 4 or 5% (w/v) polyacrylamide gel for 2–3 h. Gels were vacuum-dried and exposed against Fuji RX films for 3–7 days at –80 °C using an intensifying screen.

RESULTS

Cell-specific Activation of the RARβ2 Promoter—Previously, we have reported that undifferentiated EC cells express RARβ upon RA treatment, whereas differentiated EC-cell derivatives do not (29). To increase our understanding about the mechanism underlying this cell specificity, we transfected several RA-responsive reporters, including the human RARβ promoter (−1470/+156) and an artificial RA-responsive thymidine kinase (tk) promoter containing three copies of the βRARE, joined either in sense to the CAT gene or antisense to a LUC reporter (3× RARE-tk) (giving similar -fold inductions by RA). Finally, we also used a luciferase reporter containing five GAL sites linked to a basal promoter containing a TATA box only, transfected together with a fusion protein consisting of the DNA binding domain of GAL4 and the ligand binding domain of RARβ (GAL-RAR AF2). The constructs used are schematically depicted in Fig. 1A. As shown in Fig. 1C, the activity of the RARβ2 promoter upon the addition of RA is high in some tumor cell lines (e.g. PA-1, 293) but low in others (e.g. HeLa, T47D). Furthermore, as observed before, P19-EC as well as F9 EC cells (data not shown) have a very high promoter activity upon RA treatment, whereas the differentiated P19 derivative END2 has a very poor RA response (29). Similarly, in RA-differentiated P19-EC cells, no activation of the RARβ2 promoter (or 3× RARE-tk) by RA was observed (data not shown). Differences in response between the various cell lines are not caused by differences in RAR or RXR expression, since all cell lines express comparable amounts of receptors (Fig. 1B; data not shown; see also Ref. 60). Furthermore, cotransfection of RARα or RARβ alone or in combination with RXRα only marginally increased the activity of the RARβ2 promoter (data not shown) and thus remained low in comparison with P19-EC cells, arguing that the receptor amounts are not limiting.

If, however, the activity of the 3× RARE-tk reporter was tested in the same cell lines, the pattern of activation was different (Fig. 1D). The variation in RA response between the various cell lines is smaller than with the natural promoter. Furthermore, in some cell lines in which the RARβ2 promoter is only poorly activated, the artificial promoter (3× RARE-tk) was strongly activated (e.g. COS, T47D), even better than in the cell lines with strong RARβ2 promoter activation (P19, PA-1). Finally, the activity of AF2 of RARβ is also highly cell-specific as has been reported before (59). This is illustrated by the very high activity in, for example, T47D cells, while in HeLa cells hardly any activation was observed (Fig. 1E). In comparing the three response systems, we observed several cell lines that respond to RA with all reporters (PA-1, 293, 3T3,
The indicated cell lines were transfected with the RARβ2 promoter as reporter in the presence or absence of cotransfected cytomegalovirus-driven adenovirus 5 E1A 12/13S constructs. Data are presented as the mean ± S.D. -fold activation by cotransfection of E1A 12/13S in the presence of 1 µM RA of four independent experiments.

P19-EC), while others have a poor response with all reporters (HeLa, END2), and interestingly some cell lines (COS, T47D) can activate the artificial RA response systems, while the natural RARβ2 promoter is not activated or is only marginally activated by RA.

**Cofactors Contribute to RA Response**—Since we and others have previously shown that the adenoviral protein E1A 13S can act as a cofactor in END2 or COS cells (29, 38), we investigated the effect of E1A 12/13S on the activity of the RARβ2 promoter in the cell lines. With the exception of CHO, 293, and P19-EC cells, an increase (2–6-fold) in RA-dependent activation of the RARβ2 promoter was observed in all cell lines upon cotransfection of E1A 12/13S, which permits RARβ2 promoter activation in otherwise poorly responsive cell lines (Fig. 2). Thus, an increase in the amount of this particular cofactor stimulates the RARβ2 promoter response.

The availability of recently cloned cofactors for nuclear hormone receptors (41–48) enabled us to test whether a selection of cofactors are possibly limiting. We therefore first investigated possible differences in expression levels of some of these cofactors by Northern blotting. As is shown in Fig. 3, mRNA expression levels of various cofactors were clearly different between the different cell lines. Generally, the cells with high cofactor expression show a good RA response (in particular on artificial reporters), while cell lines with low expression levels have a relatively poor RA response. PA-1 cells, for instance, expressed high levels of all cofactors tested, whereas END2 cells lack detectable expression of any of these cofactors. To directly test whether the lack of cofactors is the cause for the poor RA response in some cell lines, we used END2 cells (Fig. 3). Two concentrations of the various cofactors were transfected in END2 cells together with the above described RA-responsive reporters (Fig. 1A). Cotransfection of E1A 12/13S resulted in an increase in RA-dependent activation of all reporter systems, while cotransfection of increasing amounts of RIP140 resulted in a concentration-dependent decrease in activity (Fig. 4) as reported before (41). Cotransfection of SRC1 or p300 at the lower concentration caused a small but significant enhancement on the GAL-RAR AF2 reporter system, while a decrease was observed at the higher concentration. Interestingly, this increase was not observed on the RARβ2 promoter, but instead a decrease in activity was observed, indicating that the amount of cofactor is not limiting. Interestingly, when SRC1 and p300 were tested with these reporters in HeLa cells a 2–4-fold enhancement in activity was observed, as has been reported before (Refs. 42, 43, and 46; data not shown). These results suggest that for maximal activation an optimal amount of the appropriate cofactor is required. This finding is further strengthened by the observation that in P19-EC cells, which apparently already contain sufficient levels of cofactors, a decrease in RA response was observed upon cotransfection of cofactors on all reporters (Fig. 4; data not shown). We conclude, based on these cotransfection experiments, that cofactors can influence the RA response of all RA-responsive reporter systems both positively and negatively. Since we observed cell-specific differences in cofactor expression levels, we propose that these differences contribute to cell-specific RA response.

**Cell-specific Complexes Are Formed on the βRARE**—The βRARE is the most important element for activation of the RARβ promoter (17, 18, 24). Besides RAR-RXR heterodimers, other cell-specific proteins could bind to this element, thus contributing to cell-specific activity of this promoter. To test this hypothesis, we performed EMSA experiments using extracts from the various cell lines with a labeled βRARE oligonucleotide as probe. Different complexes were formed in the various cell lines denoted as 1–4 (Fig. 5A). The presence and amount of the individual complexes are strongly variable among the different cell lines. To designate the identity of these complexes, we overexpressed several (orphan) receptors in COS cells and used extracts from these cells in an EMSA. As shown in Fig. 5B, based on comigration we were able to designate complexes 2–4 as RAR-RXR, COUP-TFI (I or II), and NGF-1B/Nur77, respectively. Supershift experiments further confirm that complex 2 contains RAR and RXR, since in untreated P19 cells complex 2 is completely lost, and a complex with slower mobility is formed with an antibody against RARα but not with an anti-RARβ antibody (Fig. 5C). We observed that upon RA treatment complex 3 was induced specifically in P19-EC cells and was first observed after 2 days and maximally present after 3–5 days (data not shown). The RA-dependent appearance of this complex coincided with the appearance of COUP-TFI and COUP-TFII/ARP1 on a DR1 and with the induction of the COUP-TF mRNAs as measured by RNase protection (63). The identity of complex 3 was further confirmed, since this complex is partially supershifted by a COUP-TF antibody. The identity of complex 1 is unknown. Competition experiments (Fig. 5D) using a 100-fold molar excess of unlabeled oligonucleotide confirm that all four complexes are specific.

The cell type-specific expression of the different complexes indicated to us that differential orphan receptor expression might contribute to cell type-specific RA response. Transfection of increasing amounts of COUP-TFI in P19-EC cells together with either the RARβ2 promoter or the 3×ARE-tk reporter caused a concentration-dependent decrease in promoter activity in the presence of RA (Fig. 6, A and B). From these data, it is clear that overexpression of COUP-TFI and also COUP-TFII/ARP1 (data not shown) can efficiently repress both the natural RARβ2 promoter and the synthetic βRARE-containing reporter. Similar results were obtained in COS-1 cells.

Next, we investigated the role of NGF-1B/Nur77 on the activity of RARE-containing reporters. Cotransfection of increasing amounts of Nur77 in P19-EC cells resulted in a concentration-dependent increase in RARβ2 promoter activity in the absence of RA as reported before (55, 68), while in the presence of RA no further increase in activity by cotransfection was observed (Fig. 6, C and D). Possibly the high activity of this promoter in the presence of RA prevents further activation by Nur77. For reasons not understood, no increase in activity was found either in the presence or absence of RA with the RARE-tk reporter (Fig. 6D).
The observed cell type-specific differences in complexes binding to the RARE (Fig. 5) combined with the opposite results obtained by cotransfection of COUP-TF or NGF-1B suggest that these proteins may contribute to cell-specific activation of promoters by RA.

**βRARE and INR Sequences Are Required for Activation of the RARβ2 Promoter**—Both qualitative and quantitative aspects relating to cofactors and orphan receptors influence both the natural RARβ2 promoter and the 3× RARE-tk reporter and therefore cannot explain the observed differential activation of these two reporters in the panel of cell lines (Fig. 1). This suggested to us that sequences surrounding the βRARE within the RARβ2 promoter could be responsible for this cell specificity.

The position of the RARE within the RARβ2 promoter (close to the TATA-box) is unusual. To test the importance of the spacing between RARE and TATA-box, we transfected RARβ2 promoter constructs (−63/+156) with a variable spacer (PUC sequences) between the RARE and the TATA box in P19-EC cells. As shown in Fig. 7, most spacers tested were effective and altered the RA response only marginally. Two reporters were significantly less active, and three reporters with different spacers were almost completely inactive. Although we cannot rule out the presence of repressor binding sites or cryptic promoters in the introduced spacers, we propose that the orientation and/or distance of the RARE relative to the TATA-box is important for RA-dependent promoter activation. Previous experiments in P19 EC cells by Dey et al. (24) have shown that although the RARE is the most important element for activation of the RARβ2 promoter, both upstream and downstream elements (INR) also can contribute to RA-dependent activation in these cells.

We have made mutant promoter constructs and can confirm that besides the RARE also the upstream region containing CRE- and TRE-like sequences (23, 24), which also might function as a RARE (19, 64), are important for maximal activation in P19-EC cells (Fig. 8, −1470 versus −63). Furthermore, the INR region contributes to activity (Fig. 8, −63 versus −10, −20, and ΔINR). When the βINR was exchanged for a consensus initiator sequence as found in the TDT gene, activation was restored to wild type level. Finally, a reporter containing the βRARE in front of a consensus TATA-box without an INR (RARE-e1b) together with a different spacer was not as responsive as the natural promoter, while similarly the 3× RARE-tk reporter also was a weak promoter (Fig. 8). These results suggest that the specific βINR sequence fulfills an important role in RA-dependent activation. We next asked whether the above identified sequences have a role in the weak RA-dependent activation of this promoter observed in T47D cells. Again, the RARE was most important, but the upstream sequences are more important in T47D cells than in P19-EC cells (Fig. 8). Mutation of the INR within the −63 reporter construct, which is activated only four times by the addition of RA, only leads to a small decrease in activity. With the TDT INR, however, a slight increase in activation was seen as compared with the wild type reporter (−63 versus −63 TDT). With the RARE-e1b reporter, lacking the INR sequence present in the RARβ2, promoter activity is higher than with the natural promoter (Fig. 8). Together these data indicate that the RARE is most important for activation of the RARβ2 promoter, while also the specific βINR sequence contributes to cell-specific activation significantly.

**Promoter Architecture Contributes to Cell-specific Activation**—To investigate the role of the INR element and the short spacer sequence in cell-specific responses of RA-responsive RARβ2 promoter in more detail, we compared the activity of two different reporters: the natural RARβ2 promoter and the RARE-e1b reporter, which contains a βRARE directly in front of a consensus TATA box as found in the adenovirus E1B gene. In this reporter, the spacing between RARE and TATA-box was larger (25 versus 6 bp) as compared with the natural promoter. In these two promoters, we inserted either the natural RARβ2 promoter sequence up to +156 or no INR sequences. Finally, reporters were made containing a TDT INR instead of the βINR linked to the LUC reporter (without +10/+156 sequences) as schematically depicted in the left part of Fig. 9. The activity of these promoters was tested in P19 EC, T47D, and COS cells. In these three cell lines, deletion of the βINR in the context of the RARβ2 promoter caused a decrease in activity (Fig. 9). When the βINR was changed for the TDT INR, a decrease in activity was observed in P19-EC cells but not in the other cell lines. When the spacing between the RARE and a consensus TATA-box was changed (RARE-e1b context), a decrease in activity was observed in P19-EC, while in the two...
other cell lines a 2-fold increase in activity was observed. Mutation of the RARβ2 promoter TATA-box to an e1b TATA-box had no effect (data not shown). Also in the presence of other INR sequences, the activity of the promoter with the larger spacing was more active in T47D and COS cells than the corresponding reporter with the natural spacing. Interestingly, this is not the case for P19-EC cells, where for the initiator mutant with a larger spacing a decrease in activity was observed in comparison with the corresponding natural RARβ2 promoter initiator mutants. This indicates that close spacing of the RARE with respect to the TATA-box is important for strong activation by RA in P19 cells and may, together with the specific βINR sequence, prevent activation in other cell lines.

By changing both the spacing and the INR sequence, the cell specificity in the RA response in the four cell lines analyzed is mostly lost (Fig. 9, compare 2631156 with RARE-E1B TdT).

Finally, to investigate whether spacing and INR requirement are specific for RAR AF-2, we replaced the RARE with a GAL-binding site and transfected GAL-VP16 and GAL-RAR AF2. The result with both activators indicated that the INR sequence, when placed in the natural promoter context, plays an important role in determining promoter activity. Deletion of the βINR significantly decreased the activity of the activators in all cell lines. Replacement of the βINR by TDT generally permitted activation by these activators in T47D and COS cells, while again, as with the RARE-containing promoters, a decrease in activity of both VP16 and RAR AF2 was observed in P19-EC cells (data not shown). Together the results obtained with the RARE-containing reporters and the GAL-containing reporters indicate that the close spacing of the RARE to the TATA-box together with the specific βINR sequence permits RA-dependent activation in P19-EC cells, whereas activation in the other (non-EC) cell lines repressed activation.

Cell-specific complexes are formed on the βINR—Cell-specific differences between P19 and T47D and COS cells as observed upon mutation or deletion of the βINR suggest that this sequence is contributing to cell-specific activation of this reporter. We therefore performed EMSA experiments with cell extracts from the various cell lines using the βINR as a probe. As illustrated in Fig. 10A, both quantitative and qualitative differences between the various cell lines could be seen. Competition experiments using a 100-fold excess of βRARE sequence or TDT INR could not compete for binding to this probe, while an equal amount of unlabeled βINR was sufficient for complete inhibition of binding. This indicates that the observed complexes are specific and also that probably different proteins are binding to the two different INR sequences (Fig. 10B).

Interestingly, an additional slower migrating complex was
found upon RA treatment of P19-EC cells. As shown in Fig. 10C, this slower migrating complex is detectable after 1 day and is maximal after 5 days, which correlates with the period of high RARβ2 promoter activity. Finally, we performed binding assays using the TDT INR, and again different complexes were found using cell extracts from the various cell lines (Fig. 10D). Together these results indicate that various cell lines contain different protein(s) that can bind to different INR sequences and that P19-EC cells contain a specific INR-binding protein possibly involved in the strong RA-dependent RARβ2 promoter activation.

**DISCUSSION**

For a developing organism, it is important to adequately control gene expression in the proper tissues. Altered expression may lead to developmental abnormalities or tumor formation. Since retinoids fulfill important functions during embryonal development, it is not unexpected that lack or excess of retinoids can affect normal development. In this study, we have shown that cell-specific complexes are formed on the βRARE. These complexes are formed in response to RA treatment and are specific for each cell line. The formation of these complexes is dependent on the ability of the cell to bind to the βRARE sequence, as well as on the expression of specific proteins that interact with the βRARE sequence.

**Fig. 5.** Cell-specific complexes are formed on the βRARE. A, WCEs from the indicated cell lines cultured for 2 days in the presence (+) or absence of 1 μM RA (−) were used in EMSAs using a labeled βRARE as probe. At least four specific complexes were formed (denoted 1–4). In the absence of WCEs, no shift was observed (lane 1, −). Lane 2, WCEs from untransfected COS cells; lane 3, WCEs from COS cells transfected with RARβ-RXRA, which comigrated with complex 2 (C2). B, EMSA experiments with COS cells transfected with RARβ (R), RXRA (X), both (R/X), COUP-TF (coup), or Nur77 (nur) show that complex 2 comigrated with RAR-RXR. Complex 3 (C3) comigrated with COUP or COUP-related complexes, and complex 4 comigrated with Nur77. EMSA experiments using control HeLa extract and both control and RA-treated P19-EC extract were used for comparison. C, antibodies against RARα (α) or RARβ (β), RXRX (X), or COUP-TF (coup) were added to an EMSA reaction using WCEs from control or RA-treated P19-EC cells with βRARE as probe. D, competition experiments using a 100-fold excess of unlabeled βRARE, DR1, or βINR (nonspecific (ns)) showing that complexes 1–4 are specific.
retinoids as well as absence of multiple RARs or RXRs leads to developmental abnormalities (65), which is probably caused by altered expression of RA target genes. It is therefore important to understand the mechanisms involved in adequate, cell-specific control of RA-responsive genes. We show here that the RA response is regulated in two different ways: general mechanisms and promoter context-dependent mechanisms, both contributing to cell-specific activation of RARE-containing promoters. Besides the presence of RA and RARs, general activation of RAREs is influenced by the presence of cofactor orphan receptor. The cell type-specific response of the RAR\textsubscript{b2} promoter is the consequence of its architecture, including the close proximity of the RARE to the TATA-box as well as the presence of a specific INR element.

**RA Response Is Modulated by Cofactors**—In the past we and others have hypothesized that regulated cofactor expression could fulfill an important role in regulation of the RAR\textsubscript{b2} promoter (29, 34, 35, 38). The availability of some of the recently identified cofactors for the steroid/thyroid hormone receptor family enabled us to test this hypothesis. We have performed cotransfection experiments in several cell lines including END2, HeLa, T47D, 293, and P19-EC cells. Activation by these cofactors was only observed in HeLa cells, while in the other cell lines no effect or repression was observed (Fig. 4).\textsuperscript{2} By cotransfection of any cofactor used, we were unable to restore RA response in poorly RA-responsive cell lines to the levels observed in P19-EC cells. Possibly, overexpression of such proteins can titrate out other limiting proteins, thereby preventing essential cofactors to activate transcription. Based on these data, it seems that a subtle balance is required between the various cofactors for transcriptional activation, and this cannot be achieved in all cell lines by cotransfection of these cofactors individually. Therefore, we propose that variation in the expression levels of nuclear receptor-specific cofactors between various cell lines may contribute to some extent to cell-specific responses to the corresponding ligand.

Alternatively, it is possible that the cofactors tested are not the proteins that are directly involved in RAR\textsubscript{b2} promoter activation. Other cofactors like SUG1 (45, 48), SWI/SNF (66),

\textsuperscript{2} G. E. Folkers, B. van der Burg, and P. T. van der Saag, unpublished results.

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**Fig. 6. Overexpression of orphan receptors influence the activation of both the RAR\textsubscript{b2} promoter and the 3\times RARE-tk reporter.** Increasing amounts of mCOUP-TF (A and B) or Nur77 (C and D) were transfected in P19-EC cells with the RAR\textsubscript{b2} promoter (-1470+156) (A and C) or 3\times RARE-tk (B and D) as reporter. Results of two duplicate experiments are calculated as the mean ± S.D. relative luciferase activity (light units), in the presence RA or absence of RA (A and B) or the -fold induction upon cotransfection of increasing amounts of Nur77 (C and D). The amount of expression vector was kept constant by the addition of empty pSG5 vector DNA.
and TIF1 (44) could be required for maximal activation. Evidence for a distinct cofactor requirement for various receptors has come from experiments showing differential affinities of nuclear receptors for the putative cofactors mSUG1 and TIF1 (48). Furthermore, our cotransfection experiments indicate that E1A 13S enables activation of the RARβ2 promoter in cell lines, which are otherwise poorly activated by RA (Figs. 2 and 4; Refs. 40 and 58). In this case, E1A is acting as a cofactor, probably through direct interaction with RAR and GTFs (40).

This suggests that the presence of an unknown EC cell-specific cofactor, described as E1A-like activity, could be responsible for the high level of RARβ activation in EC cells, while absence of this cofactor could be responsible for the lack of activation in the differentiated derivatives (28, 38). Indeed, Okuda et al. recently described a novel putative cofactor that has an EC cell-specific expression pattern (67). Interestingly the expression of all cofactors tested was much higher in the undifferentiated P19-EC cells than in the differentiated derivative END2 (Fig. 3), suggesting that the combination of several cofactors together could act as this E1A-like activity. In order to be able to discriminate between these possibilities and to determine their relative contributions to RARβ2 promoter activation, identification of all cofactors involved, evaluation of their respective expression levels in the various cell lines, and performance of in vitro transcription experiments would be required.

**Role for Orphan Receptors in RARβ2 Promoter Activation**—By means of sequence homology within the DBD of the steroid/thyroid hormone receptor family, numerous receptors have been cloned for which a ligand is unknown. These so-called orphan receptors can also bind to hormone response elements as monomers, homodimers, or heterodimers with RXR (for a review see Refs. 68 and 69). Some of these orphans e.g. COUP-TF (49–51), DAX-1 (56), TAK1 (70), NGFI-B, and NURR1 (55) have been reported to influence the retinoid response, but these experiments were mainly performed with artificial retinoid-responsive reporters and not with natural promoters. Here we show that both synthetic reporters and the natural RARβ2 promoter containing the βRARE are repressed by overexpression of COUP-TFI or COUP-TFI/ARP-1 and, depending on the reporter used, activated by NGF-1B/Nur77 (Fig. 6). More importantly, the presence of COUP-TF as well as other proteins binding to the βRARE is cell type-specific, and these proteins were generally found to be more abundant in nonresponsive cell lines. It can be assumed that COUP-dependent repression of the RA response is occurring through competition between RAR/RXR heterodimers and COUP-TF for binding to RAREs (52, 53). Wu et al. (71) have recently shown that the retinoid response in lung cancer cells is determined by the relative expression levels of COUP-TF and Nur77. These two orphan receptors could heterodimerize, leading to either ligand-independent activation or sensitizing of the RA response when Nur77 or COUP-TFI is in excess, respectively. Although our EMSA data do not show a perfect correlation between RA response and COUP/Nur77 ratio, we cannot fully exclude this model, since we have not directly determined the NGF-1B/Nur77 expression using an antibody against this orphan receptor. We propose that the relative expression levels of RAR-RXR and the different orphan receptors are contributing to RA response. A similar competition model has been described for RA-dependent repression of the OCT-4 expression (72, 73). It is clear from our EMSA experiments (Fig. 5) that in most cell lines limiting amounts of RAR-RXR complexes are found, while the levels of the other complexes that were shown to contain orphan receptors vary considerably more.

**RARβ2 Promoter Organization Is Involved in Cell-specific Activation**—Both cofactor expression and orphan receptor expression clearly influence the RA response, but this cannot explain the observed cell-specific activation of the RARβ2 promoter completely. In vivo footprint experiments in P19-EC cells have shown that upon RA treatment first the RARE is occupied, followed by the CRE/TRE sequence, while eventually also the INR region becomes protected (24). By mutational analysis, we show that the close proximity of the RARE to the TATA-box as well as the INR sequence itself are involved in cell-specific activation. Increase of the spacing between RARE and TATA-box and/or mutation of the INR permitted activation in non-EC cells, whereas a decrease in activity in EC cells was observed (Fig. 9). Photo-cross-linking experiments using purified GTFs have indicated that TFIID, TFIIA, and possibly other GTFs can contact sequences upstream of the TATA-box, up to 46–42 (74). RAR, which is most important for activation of RARE-containing promoters (75), is occupying the 5′ half-site of the βRARE (−53 to −37) within the RARβ2 promoter (76, 77). Consequently, binding of RAR or orphan receptors could interfere with GTF binding. This might explain why this promoter is poorly activated by RA in many cell lines despite the presence of cofactors for RAR (e.g. COS, T47D). It also suggests that close proximity of the RARE to the TATA-box is an important characteristic for the cell type-specific activation of this promoter. A recent paper from Sanguedolce et al. (64) provided further support for the idea that the promoter context influences the RA response. These authors showed that by mutating the RARE(s) of the RARβ promoter to TREs this promoter remains responsive to RA, while in the context of a tk reporter this mutant had become responsive to T3. The induction of a TRE-containing RARβ2 promoter was dependent on the presence of E1A or E1A-like activity (64).

However, increased spacing alone is not sufficient for obtaining a response in non-EC cells (Figs. 8 and 9). Deletion as well as mutation of the βINR sequence influences the activity of this...
promoter positively and negatively in non-EC and EC cells, respectively (Figs. 8 and 9), suggesting a role for the INR in cell-specific regulation of transcriptional activation of this promoter. This is confirmed by two different promoters containing a RARE or a GAL response element in close proximity of the TATA-box (Fig. 9; data not shown). In both cases, the combination of close proximity and the INR was highly active in EC cells, while in other cell lines the larger distance in combination with the TDT INR was more active. Generally, however, the activation of both reporters in P19-EC cells was better or as good as in the other cells when both spacing and INR were altered. Possibly, the RARE still is positioned too close to the TATA-box to render this mutant promoter highly responsive in non-EC cells. Another explanation could be the presence of an EC cell-specific cofactor that functions efficiently when the RARE is positioned close to the transcription start site. These data together show that when the binding site for the activator is positioned close to the TATA-box, cofactor and/or INR requirements for transcriptional activation are different when compared with the promoter containing a larger spacing. Findings that cooperativity between different activators and GTFs depends on the core promoter structure including TATA sequence and INR-region (78–82) are lending further support to this hypothesis. These data further suggest that assembly of the preinitiation complex is occurring differently in various cell lines and on various promoters, possibly involving cell-specific cofactors and/or INR-binding proteins. Interestingly, both cofactor expression and the proteins binding to the two types of INRs tested are different between the various cell lines (Figs. 3 and 10, respectively).

Recently, evidence was presented for a regulatory role of INR sequences, which were found to be recognized by specific TATA-binding protein/TAF complexes, during differential promoter usage of the Adh gene during Drosophila development.
Furthermore, transcription of the terminal deoxynucleotidyl transferase gene in lymphocytes was shown to be dependent on the presence of a functional INR region (84). The requirement of INR-surrounding sequences for maximal activation of the human chorionic somatomammotropin promoter (85) and the presence of several cell type-specific complexes on this INR sequence (85) further support the hypothesis that cell type-specific proteins can bind to INR sequences and thus can contribute to cell-specific activation. A requirement for INR sequences in cell-specific activation of transcription was further shown for the FcyR1b gene, which is activated in myeloid cells by IFNγ. This activation was demonstrated on the core promoter structure, including the INR sequence (86).

Sequence comparison of the RARβ2 promoter INR sequence revealed no homology with any other known INR sequence (10). Furthermore, this sequence does not correspond to the consensus INR sequences, as found in a binding site selection assay for initiator functions (6). These experiments, revealed conser-

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**Fig. 10. Cell-specific complexes are formed on the βINR.** A, EMSA experiments were performed using WCEs from the indicated cell lines cultured in the presence (+) or absence (−) of 1 μM RA for 2 days with a labeled βINR from the RARβ2 promoter as probe. Without the addition of WCEs, no complexes are formed (first lane); the addition of various cell extracts resulted in the formation of at least three different complexes. B, competition experiments using 100-fold molar excess of βINR but not of TDT INR or βRARE indicate that the formed complexes are specific. C, cell extracts from P19-EC cells treated with RA for the indicated periods were used in EMSA experiments showing the induction of additional complexes, a fast and a slow migrating complex upon differentiation. The first complex is found in many cells including END2, whereas the slower complex is specific for differentiating P19-EC cells. D, EMSA experiments as in A but with the TDT INR as a probe.
cation of the A1 and T4 (relative to the transcription start site), which were also the important base pairs of initiators within natural promoters (5). Both base pairs are different in the βINR. The fact that the transcription start site is protected in vivo (24), the occurrence of binding of cell type-specific proteins to this site in vitro (Ref. 24; Fig. 10), and the observed contribution of this element in strong activation (Fig. 9) all support the view that this sequence is to be considered as a novel competition experiment. Further studies have shown that different proteins can bind to the βINR as compared with the TDT INR elements (Fig. 10; Ref. 24).

It is well known that the activity of RARE-containing promoters is controlled at multiple levels (e.g., through availability of retinoids (87), receptor expression levels (15, 25–27), and in vivo promoter occupancy (24)). We have shown here that there are additional mechanisms that can contribute to cell-specific promoter occupancy (24). We have provided evidence that promoter architecture, including the close proximity of the RARE to the TATA box (Fig. 7) and the presence of a unique INR (Fig. 8) and CBP were obtained from R. Eckner and R. H. Goodman, respectively. We thank M. G. Parker for COUP-TF antibodies and the RIP140 antibodies against RARα and RARβ, and CBP were obtained from R. Eckner and R. H. Goodman, respectively. We thank Patricia van Arum-Swanink for excellent technical assistance and Truus Hoeijmakers for synthesis of oligonucleotides.

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