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Retinoid-Induced Chromatin Structure Alterations in the Retinoic Acid Receptor β2 Promoter

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Transcription of the retinoic acid receptor β2 (RARβ2) gene is induced by retinoic acid (RA) in mouse P19 embryonal carcinoma (EC) cells. Here we studied RA-induced chromatin structure alterations in the endogenous RARβ2 promoter and in an integrated, multicopy RARβ2 promoter in EC cells. RA markedly increased restriction site accessibility within the promoter, including a site near the RA responsive element (RARE) to which the nuclear receptor retinoid X receptor (RXR)-RAR heterodimer binds. These changes coincided with RA-induced alterations in the DNase I hypersensitivity pattern in and around the promoter. These changes became undetectable upon removal of RA, which coincided with the extinction of transcription. Analyses with receptor-selective ligands and an antagonist showed that increase in restriction site accessibility correlates with transcriptional activation, which parallels the RA-induced in vivo footprint of the promoter. Despite these changes, the micrococcal nuclease digestion profile of this promoter was not altered by RA. These results indicate that concurrent with the binding of the RXR-RAR heterodimer to the RARE, the local chromatin structure undergoes dynamic, reversible changes in and around the promoter without globally affecting the nucleosomal organization.

It has been shown that retinoic acid (RA) induces expression of many genes in different cell types, while repressing expression of other genes (14, 34). One of the immediate-early genes induced in P19 embryonal carcinoma (EC) cells by RA is the retinoic acid receptor β2 (RARβ2) gene (49, 64). The promoter region of this gene contains an RA responsive element (RARE) to which the nuclear hormone receptor heterodimer RAR-retinoid X receptor (RXR) binds (14, 34). Previous in vivo footprinting analyses showed that the RARβ2 promoter is not occupied in P19 cells prior to RA addition, but after RA addition, occupancy is induced in the RARE and other *cis*-acting elements that coincides with transcriptional activation of the promoter (7, 17, 37, 38). Ligand-dependent occupancy of the RARβ2 promoter has been recently confirmed by others in NB4 cells (16). In contrast, in vitro the RXR-RAR heterodimer can bind to the RARE in the absence of ligand. Binding of the heterodimer to DNA in vitro has been demonstrated even in the presence of corepressors, such as N-CoR, RIP13, and SMRT (15, 24). The striking differences observed between in vitro and in vivo studies suggest that accessibility of transcription factors to the promoter in vivo is governed by a mechanism that is not readily reconstituted in vitro and which may involve regulation by chromatin.

It has been shown that chromatin structure plays an important role in gene activation (1, 29, 33, 45, 57, 60). Several hormone-responsive promoters have been shown to undergo chromatin structure alterations following hormone addition. For example, a glucocorticoid-hormone-dependent alteration of nucleosomal positioning as well as a hormone-dependent DNase I hypersensitivity site has been observed for the rat tyrosine aminotransferase promoter (6, 20, 46). Zaret and Yamamoto (63) have demonstrated that glucocorticoids can induce reversible changes in the chromatin structure of the mouse mammary tumor virus (MMTV) promoter. These observations have been extended by others (3, 41, 48, 53). Alterations of chromatin structure in the MMTV promoter are thought to be initiated by binding of the glucocorticoid hormone receptor to its element. Although the mechanisms of chromatin structure alterations are not fully understood, several recent reports indicate that local histone acetylation may play a role (13, 23, 42).

The present work shows that RA treatment leads to reversible alterations in the chromatin structure of the RARβ2 promoter which are coupled with RA-induced occupancy and transcriptional activation of the promoter. Our results are analogous to changes noted in other hormone-responsive promoters, indicating a common mechanism of ligand-mediated chromatin structure regulation.

MATERIALS AND METHODS

Retinoids and luciferase assays in P19 cells. Mouse P19 EC cells were treated with RA (all-trans; Sigma) at a concentration of 1 μM or as previously indicated (17). Synthetic retinoids SR11237 and TTNPB and the RARα antagonist Ro 41-5253, provided by A. Levine, were solubilized in ethanol and added at the indicated concentrations. Establishment of P2 cells, a stable clone containing the RARβ2-luciferase gene, was described previously (25). Briefly, parental P19 cells were transfected with an equimolar mixture of the mouse RARβ2 promoter (−124 to +14) connected to the luciferase gene (17) in the pGL2-Basic vector and the SV40-neo plasmid (36) and selected for G418 resistance for 10 to 14 days. Colonies were propagated in separate dishes and tested for luciferase activity following RA addition as previously described (36). Individual colonies showing RA-responsive luciferase activity were recloned, propagated again, and frozen in multiple ampules. After several months of in vitro culture, P2 cells were replaced with freshly thawed ampules to insure the stable presence of the integrated gene. Southern analysis of P2 cell DNA indicated that the luciferase gene

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was integrated into the P19 genome as a tandem repeat of 25 to 30 copies per cell (data not shown).

In vivo footprinting of the RARβ2 promoter in the P2 clone. Dimethyl sulfate (DMS)-based in vivo footprinting for the integrated, multicopy RARβ2-luciferase gene was performed according to the procedure described for the endogenous RARβ2 gene (17) except for the use of different primers. Ligation-mediated PCR (LM-PCR) was performed with the following primers designed from the coding strand: 5′-TTT GTC CAA ACT CAT CAA TGT ATC TTA-3′ (nucleotide [nt] 5546 to 5572 from the pGL2-Basic vector [Promega, Madison, Wis.]), 5′-TGT ATC TTA TGG TAC TGT AAC TGA GCT AAC-3′ (nt 5564 to 5593), and 5′-GTA ACT GAG CTA ACA TAA CCC GCG CTG TGA G-3′ (nt 5580 to 3 and nt −123 to −114 from the mouse RARβ2 promoter). PCR products were run on a 6% sequencing gel. In vivo footprinting of the endogenous RARβ2 promoter was performed as previously described (17). Autoradiographs were scanned and quantified by using the PhosphorImager IQ analysis package. The percent methylation was calculated as described in reference 17.

Nuclei preparations and restriction site accessibility assays. Nuclei were isolated according to a previously described method (21). Untreated and RA-treated P19 or P2 cells were cultured in α-modified essential medium containing 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, Md.). Cells were suspended in ice-cold lysis buffer (10 mM Tris [pH 7.4], 3 mM $CaCl_2$, 2 mM $MgCl_2$). Swollen cells were resuspended with equal volumes of lysis buffer and Nonidet P-40 (NP-40) lysis buffer containing 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40, homogenized in a Dounce homogenizer, and centrifuged at 500 × *g* for 10 min at 4°C. Nuclear pellets were stored in buffer (25% glycerol, 5 mM Mg-acetate, 50 mM Tris [pH 8.0], 0.1 mM EDTA, and 12 mM β-mercaptoethanol) at −70°C. Equal amounts of nuclei (10 to 20 μg of DNA equivalent) were digested with restriction enzymes in buffer containing 10 mM Tris (pH 7.4), 15 mM NaCl, 60 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 5% glycerol, and 1 mM dithiothreitol at 37°C for 15 min. The following enzymes (Promega) were used: *Sma*I (60 U/μl), *Hph*I (12 U/μl), *Xba*I (80 U/μl), and *Hin*cII (60 U/μl). Purified deproteinated P2 and P19 genomic DNAs were also isolated and were digested with the same enzyme in each case and used as the control. Digestion was terminated by adding proteinase K buffer (10 mM Tris [pH 7.4], 5 mM EDTA, 0.25% sodium dodecyl sulfate, 100-ng/ml proteinase K [Boehringer Mannheim, Indianapolis, Ind.]) at 65°C overnight. Purified DNAs from these nuclei were subjected to the secondary digestion by enzymes specified in the text. Digested samples were resolved on a 2.2% agarose gel or on indicated gels and were hybridized with the randomly primed ^{32}P-labeled probes indicated in each experiment. The intensity of the digested bands was quantified in a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Percent cut was calculated as (intensity of digested bands/intensity of undigested and digested bands) × 100.

DNase I hypersensitivity assays. These assays were performed according to Mirkovitch and Darnell (40). Nuclei (25 to 50 μg of DNA equivalent) were digested with DNase I (0 to 20 U; GIBCO-BRL) in 50 μl of buffer at 37°C for 1 min. Reactions were stopped by the addition of proteinase K buffer as described above. DNAs were digested with restriction enzymes and probed with ^{32}P-labeled fragments as specified in Fig. 8.

MNase digestion assays. Nuclei (50 μg of DNA equivalent in each case) in micrococcal nuclease (MNase) buffer (35) supplemented with 3 mM $CaCl_2$ were preincubated at 37°C for 1.5 min and digested with MNase (Worthington Biochemical Corporation, Freehold, N.J.) (0 to 8 U) for 2 min at 37°C. Purified DNAs were further digested by *Pst*I, resolved on a 1.6% agarose gel, and hybridized with a ^{32}P-labeled *Pst*I-*Nae*I fragment (47).

RESULTS

RA-induced activation of the integrated RARβ2 promoter. P2 cells, a clone from P19 cells, harboring an integrated multicopy RARβ2 promoter-luciferase fusion gene were established to study chromatin structure alterations (25) (see Materials and Methods). In P2 cells the reporter luciferase gene is under the control of a 138-bp long RARβ2 promoter that includes the RARE and other *cis*-acting elements (17). These cells were used as a model similar to that of the integrated high-copy-number MMTV promoter with which hormone-dependent chromatin structure alterations have been extensively studied (3, 4, 52, 53).

RA responsiveness of the integrated promoter was confirmed by the RNA blot and reporter analyses shown in Fig. 1A and B, where reporter mRNA and luciferase activity were induced following addition of RA. To test whether RA induces an in vivo footprint in the integrated promoter, as was observed in the endogenous RARβ2 promoter (16, 17), DMS-based genomic footprinting was performed. As can be seen in Fig. 1C, no protection was observed in the promoter without RA treatment (lane 2). However, addition of RA led to protection of G residues in the RARE, the cyclic AMP-like response element, the auxiliary RARE, and the initiation site (INR) (Fig. 1C, lane 3) which was identical to those protected in the endogenous promoter after RA addition. These results show that the integrated promoter is transcriptionally active and that factor occupancy is induced following RA addition.

RA-induced restriction site accessibility in the integrated RARβ2 promoter. In vivo restriction site accessibility assay has been widely used as a quantitative tool to study chromatin structure alterations (3, 54, 58, 59). To assess whether the RARβ2 promoter changes accessibility to restriction enzymes upon RA treatment, P2 cells were treated with RA for 24 h and nuclei were digested with increasing amounts of *Sma*I. DNAs were then purified and further digested with *Hin*cII and *Xba*I, and the resultant fragments were detected by Southern blot analysis using a probe covering the entire RARβ2 promoter. *Sma*I cuts at the −57 nucleotide just outside of the RARE in the promoter and generates fragments of 211 and 156 bp (Fig. 2A). Results shown in Fig. 2A (upper) show a marked increase in *Sma*I accessibility following RA treatment at all three enzyme concentrations tested. The percentage of accessibility (estimated as described in Materials and Methods) was increased from about 16% to more than 50% (compare lanes 3 to 5 with lanes 7 to 9). Increased *Sma*I accessibility was also seen at lower concentrations of *Sma*I (data not shown). As depicted in Fig. 2B, another endonuclease, *Hph*I, which also cuts within the RARβ2 promoter (see the cutting position in Fig. 1C), also showed increased accessibility upon RA addition, although to a lesser extent than that observed with *Sma*I. The amount of *Hph*I products (the 173-bp and 144-bp fragments shown in Fig. 2B) increased from 11 to 14% to 20 to 26%. An internal 50-bp fragment expected to be produced by *Hph*I was not detected in these blots, perhaps due to low sensitivity. To examine whether increases in restriction site accessibility are localized to the RARβ2 promoter or spread to other regions, accessibility was tested with two additional enzymes, *Xba*I and *Hin*cII. *Xba*I cuts outside the 3′ boundary of the integrated promoter (66 bp downstream from the promoter and within the luciferase gene), whereas *Hin*cII cuts outside the 5′ boundary (147 bp upstream from the promoter). As shown in Fig. 2C and D, RA addition did not change the accessibility to the two enzymes. Similarly, another enzyme, *Bam*HI, which digests approximately 2.2 kb upstream from the promoter, produced no difference after RA treatment (data not shown). These results show that RA treatment increases a localized restriction site accessibility in the RARβ2 promoter.

Time course analysis and RA requirement. As shown in Fig. 3A, an increase in *Sma*I accessibility was observed within 15 min of RA treatment. Accessibility peaked at 3 h and persisted for the entire 24 h of RA treatment. In these experiments the amount of *Sma*I product in untreated cells was somewhat higher than in other experiments, most likely due to the assay-to-assay variability. As can be observed in Fig. 3B, luciferase activity also increased after 15 min of RA addition and was followed by a further increase after 24 h of treatment. An increase seen after 24 h of treatment may reflect accumulation of the luciferase protein. To determine whether increased *Sma*I accessibility is reversed after RA withdrawal, P2 cells were treated with RA for 24 h, and the cells were washed and incubated in the absence of RA for an additional 24 h. As depicted in Fig. 4A, RA-induced *Sma*I accessibility (increase in percent digestion from 15 to 42%) was completely abolished after the removal of RA (14%). Similarly, accessibility was no longer detectable when tested 10 h after removal of RA (data not shown). In agreement, RA-induced luciferase activity (22-

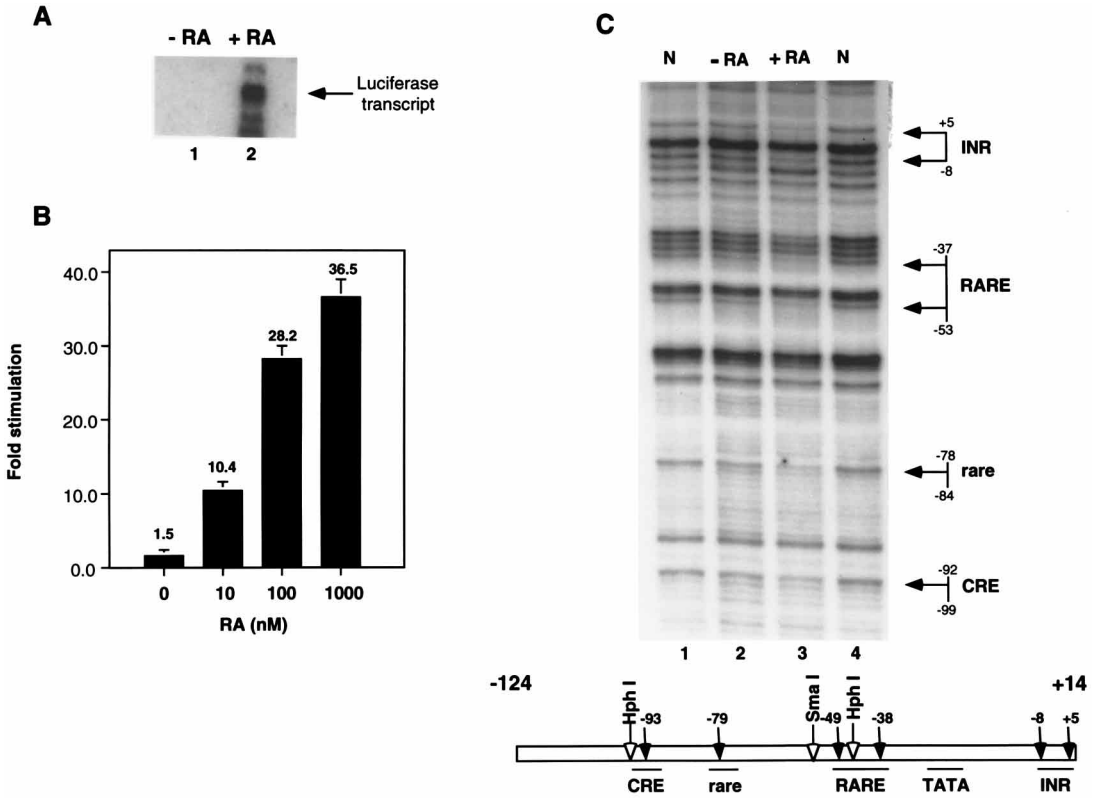

FIG. 1. RA-induced transcription and in vivo footprinting of the integrated, multicopy RARβ2-luciferase gene promoter. (A) Northern blot analysis. The P2 cells were treated without (−RA) or with (+RA) 1 μM RA for 24 h, and total RNA (20 μg) was probed with a luciferase gene fragment. (B) Luciferase activity. Luciferase activity of RA-treated P2 cells was measured as previously described (36) and normalized by protein concentration. Values (fold induction) represent the averages of four assays ± standard deviations. (C) In vivo footprinting. P2 cells treated without RA (lane 2) or with 1 μM RA (lane 3) for 24 h as described above were subjected to DMS-based in vivo footprinting. Deproteinated DNAs from P2 cells were used as controls (lanes N). Protected G residues in the regulatory elements (see Results) and their corresponding nucleotide positions are marked by arrows. Nucleotide positions are shown at the bottom of the panel and the restriction sites within the promoter region are indicated by open arrows. rare, auxiliary RARE; CRE, cyclic AMP-like response element.

fold induction) fell to almost basal levels after RA removal. These results indicate that increased *Sma*I accessibility is dependent on the continuous exposure of cells to RA, which correlates with the transcriptionally active state of the promoter. Paralleling these results, RA-induced footprint in this promoter is reversed upon RA withdrawal (17).

Effect of an RAR antagonist. Ro 41-5253 is a specific antagonist of RARα (2) and has been shown to inhibit in vitro transcription from the RARβ2 promoter (56). We examined whether the antagonist inhibits RA-induced RARβ2 transcription in vivo and restriction site accessibility. The antagonist reduced RA-induced luciferase activity by more than 70% (Fig. 5A). The antagonist also reduced *Sma*I accessibility from 50 to 23% for 10 nM RA and from 65 to 45% for 100 nM RA (Fig. 5B). It has previously been shown that Ro 41-5253 has no effect on the in vitro binding of the RAR-RXR heterodimer to the RARE (56). To test whether Ro 41-5253 alters the binding of the RAR-RXR heterodimer to the RARE in vivo, DMS-based footprinting was performed. The RA-induced footprint was inhibited in the presence of the antagonist (Fig. 5C). Quantitation by PhosphorImager scanning showed that two residues in the RARE (nt −38 and nt −49) and a residue in the INR (nt −8) were less protected in samples treated with Ro 41-5253 than in those without treatment (Fig. 5C, bottom), indicating that the antagonist interferes with the binding of the heterodimer to the RARE in vivo.

Effects of RAR- and RXR-specific ligands on restriction site accessibility. Some of the recently developed synthetic retinoids act selectively on RAR or RXR (2, 31) and thus have been used to define the roles of each receptor in the heterodimer in activating transcription. Several studies have shown that RAR-selective ligands alone can stimulate transcription from the RARβ2 promoter, while RXR ligands do not (31, 38). The differential ability of these ligands to stimulate transcription correlates with their ability to induce promoter occupancy in vivo; only the RAR ligands but not the RXR ligands cause in vivo protection in the RARβ2 promoter (16, 38). To examine whether these ligands differ in their abilities to induce restriction site accessibility, P2 cells were treated with TTNPB and SR11237, which are selective for RAR and RXR, respectively (38). As shown in Fig. 6, DNAs from *Sma*I-digested nuclei were cut by *Xba*I and *Bgl*I, which created a *Sma*I fragment of 386 bp. Treatment with TTNPB caused a marked increase in *Sma*I accessibility (44 and 50% digestion for 1 nM and 1 μM TTNPB, respectively, over 28% digestion in untreated cells). The levels of *Sma*I products observed with TTNPB were comparable to those with RA (55%). In contrast, 1 μM of RXR ligand SR11237, which has been shown to synergize with RAR ligands in transcription (38), failed to increase *Sma*I accessibility; the digestion levels by this ligand were 24%, which is comparable to that by untreated samples. A similar, increase in *Sma*I accessibility was observed when DNAs were digested

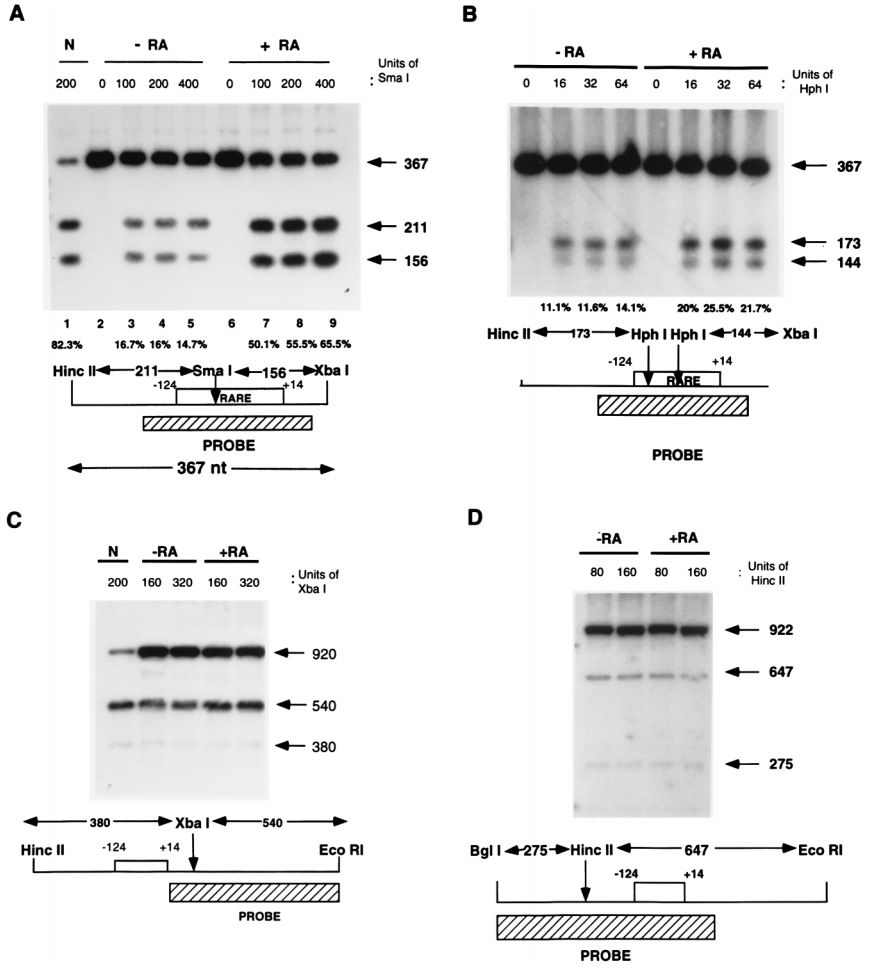

FIG. 2. Specificity of restriction site accessibility. (A) *Sma*I digestion. Nuclei (10 μg of DNA) isolated from the untreated (−RA) and RA-treated (1 μM RA for 24 h) (+RA) P2 cells were digested with increasing amounts of *Sma*I. DNAs were purified for a secondary digestion with *Hin*cII and *Xba*I and then subjected to Southern blot hybridization using a probe covering the entire promoter (diagrammed at the bottom of the panel). The undigested (367 bp) and digested bands (211 and 156 bp) are marked by arrows. N, deproteinated DNA. The percent digestion calculated according to the formula described in Materials and Methods is shown below each lane. The schematic diagram depicted at the bottom of the panel indicates the position of digested bands in the RARβ2-luciferase gene. The open box indicates the position of the 138-bp RARβ2 promoter (nt −124 to nt +14). Note that *Sma*I cuts near the RARE within the promoter. (B) *Hph*I digestion. Assays were performed with the same procedure as described above except that nuclei were digested with increasing amounts of *Hph*I. *Hph*I cutting sites are shown at the bottom of the panel and also at the bottom of Fig. 1C. (C) *Xba*I digestion. Nuclei were digested with *Xba*I, and purified DNAs were then digested with *Eco*RI and *Hin*cII and probed with a fragment corresponding to the luciferase gene. The position of the cutting site is shown at the bottom of the panel. (D) *Hin*cII digestion. Nuclei were digested with *Hin*cII, and DNAs were then digested with *Bgl*I and *Eco*RI and probed with a fragment corresponding to the upstream sequence and the promoter. The cutting sites are shown at the bottom of the panel.

with *Hin*cII and *Xba*I, as was also been done in the preceding experiments (data not shown). These results further support a link among restriction site accessibility, RA-induced in vivo footprint, and transcriptional activation.

RA-induced restriction site accessibility in the endogenous RARβ2 promoter. We then examined whether RA treatment also induces *Sma*I accessibility in the endogenous RARβ2 promoter. Nuclei from parental P19 cells were digested with *Sma*I, and DNAs were digested with *Alu*I and probed with a fragment containing the RARβ2 promoter (Fig. 7A). *Alu*I digestion generates a 314-bp fragment, which is cleaved by *Sma*I in the promoter, yielding a 232-bp fragment. RA treatment markedly increased *Sma*I accessibility in the endogenous promoter (from 22 to 41% digestion). A similar, marked increase was observed when DNAs from *Sma*I-digested nuclei were cut with *Pst*I or *Eco*RI and probed as described above (data not shown). These results suggest that RA treatment induces chromatin structure alterations in the endogenous promoter which are similar to those induced in the integrated promoter.

To ascertain whether RA induces increased restriction site accessibility only in the promoters responsive to RA, we studied *Sma*I accessibility in the RA nonresponsive promoter of the interferon consensus sequence binding protein (ICSBP) gene (28). The ICSBP gene is silent in P19 cells and is not induced by RA. As shown in Fig. 7B, *Sma*I digests a site which is 350 bp upstream from the initiation site and where several regulatory elements are present nearby (28). No RA-induced changes in *Sma*I accessibility were observed in this promoter (Fig. 7B). The high background that appears in the autoradiogram was probably due to the high G + C content of the probe and of the tested region of the promoter (28). These results suggest that RA-induced changes in restriction site accessibility are limited to RA-responsive promoters.

RA-induced DNase I hypersensitivity in the integrated and endogenous RARβ2 promoters. In some genes transcriptional

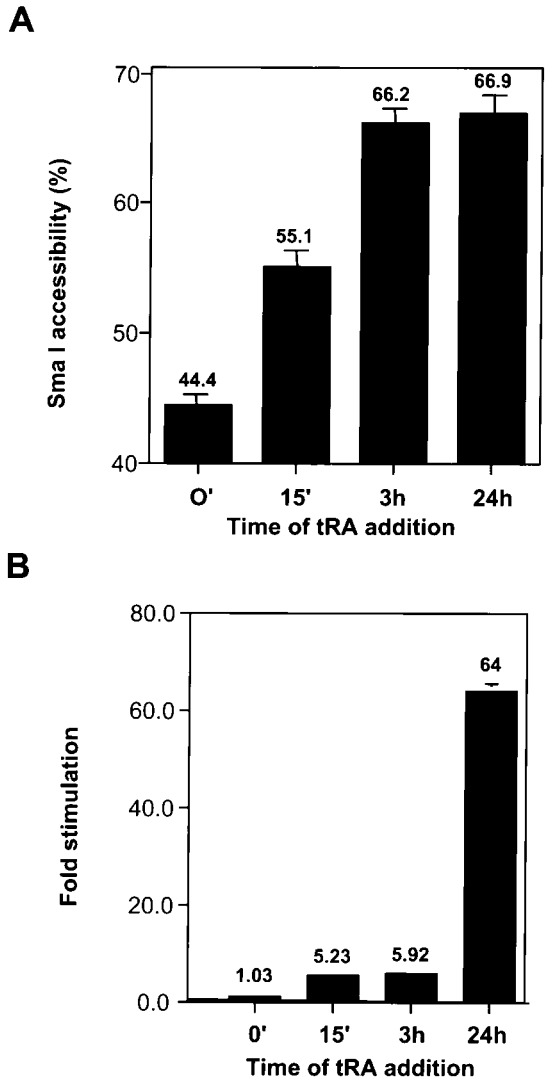

FIG. 3. Time course analysis of RA induction. P2 cells were treated with RA (1 μM) for the indicated periods of time and assayed for *Sma*I accessibility (A) (200 U of *Sma*I was used in each case). Values are averages of three assays ± standard deviations. (B) Luciferase activity. The procedures used for these experiments were the same as those described for in Fig. 1 and 2A.

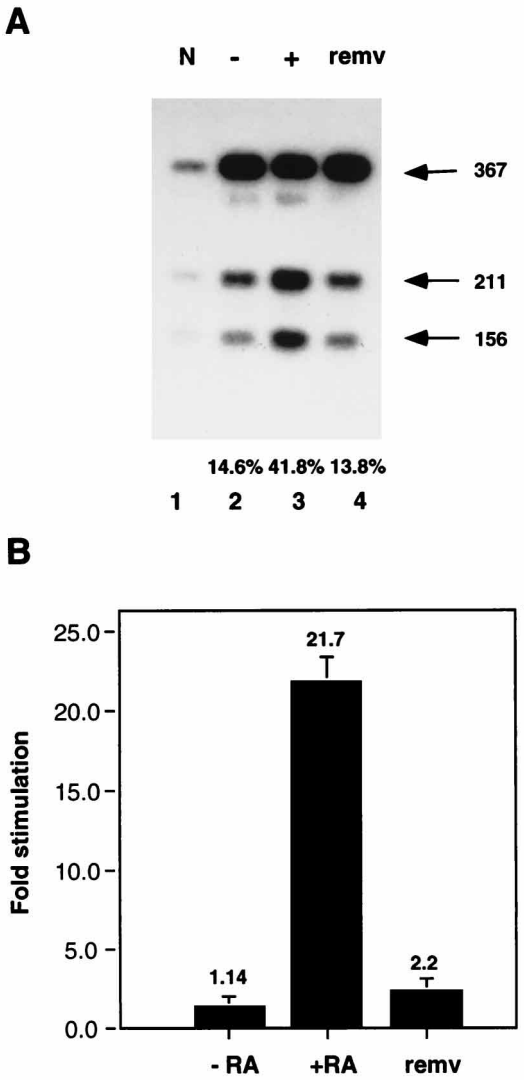

FIG. 4. Effect of RA removal on *Sma*I accessibility. P2 cells were treated with RA (1 μM) for 24 h and were washed extensively and then incubated in the absence of RA for 24 h. These samples were assayed for *Sma*I accessibility (A) (200 U of *Sma*I was used in each case) and luciferase activity (B). (A) Lane 1, deproteinated P2 DNA; lane 2, untreated; lane 3, cells treated with RA; lane 4, RA-treated cells subsequently cultured without RA.

activation is associated with changes in DNase I hypersensitivity (6, 10, 18, 22, 32, 40, 52). We investigated whether RA treatment causes alterations in DNase I hypersensitivity in or near the promoter. As shown in Fig. 8A, several constitutively hypersensitive sites were detected in the integrated promoter, as revealed by bands of about 2.2, 2.0, and 1.0 kb and of smaller sizes. The 2.0- and 1.0-kb bands may represent sites intrinsically sensitive to DNase I, since deproteinated DNA revealed bands of similar size (Fig. 8A, lanes 1 to 3). There were two hypersensitive sites of approximately 650 and 750 bp, which showed a marked increase in intensity following RA treatment. These sites were localized within the promoter (Fig. 8A, bottom). Similar to the reversal of restriction site accessibility (Fig. 4), the RA-induced DNase I hypersensitivity was reversed after RA removal (Fig. 8A, lanes 14 to 18).

DNase I hypersensitivity of the endogenous promoter was investigated with the 4-kb region that contained the RARβ2 gene and the flanking sequences at both sides (49) (Fig. 8B, bottom). A prominent constitutive band of 2.9 kb was present in every sample, in addition to weaker bands of 2.6 and 2.3 kb which were variable in intensity (Fig. 8B, right). No intrinsic hypersensitivity to DNase I was detected within this region, as judged by digestion of deproteinated DNA (Fig. 8B, left). Treatment with RA led to the generation of an intense 2.7-kb band. The intensity of this band was much weaker in samples without RA treatment. While the intensity of this band was increased that of the 2.6- and 2.3-kb bands was significantly reduced following RA addition, indicating that RA alters DNase I hypersensitive sites. Both constitutive and RA-altered sites were localized 5′ upstream to the promoter, which may suggest the presence of regulatory sequences beyond the 124-bp promoter. These results indicate that RA treatment alters DNase I hypersensitivity patterns in both the integrated and endogenous promoters. A clear DNase I hypersensitivity was not readily detected within the promoter region for the

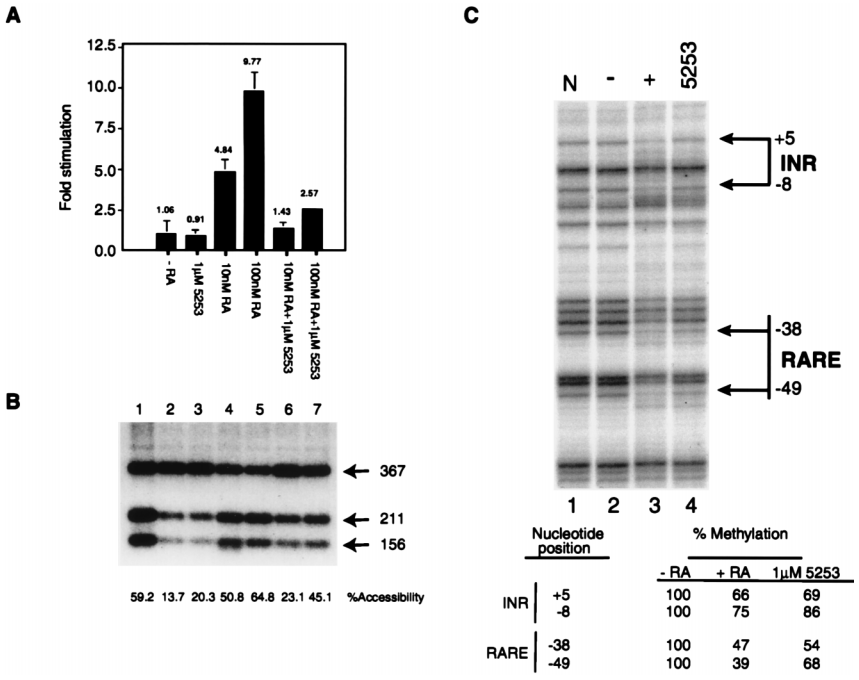

FIG. 5. Effect of the RARα antagonist. P2 cells were treated with RA or RARα antagonist Ro 41-5253 (5253) at the indicated concentrations, alone or in combination, for 24 h. Cells were tested for luciferase activity (A) and *Sma*I accessibility (B) (200 U in each case). Lane 1, deproteinated DNA; lane 2, no ligand; lane 3, 1 μM Ro 41-5253; lane 4, 10 nM RA; lane 5, 100 nM RA; lane 6, 10 nM RA and 1 μM Ro 41-5253; lane 7, 100 nM RA and 1 μM Ro 41-5253. Arrows indicated undigested (367 bp) and digested (211 and 156 bp) bands. (C) DMS-based in vivo footprinting was performed in P19 cells (17). Lane 1, deproteinated DNA (G ladder); lane 2, untreated cells; lane 3, cells treated with 1 μM RA; lane 4, cells treated with 1 μM Ro 41-5253 (5253). The levels of protection in the residues of the INR and RARE were quantitated by PhosphorImager scanning (17) and are expressed as percents methylation. The band intensity measured for the untreated cells (−RA) was considered 100%.

endogenous gene. Improvement of resolution may allow detection of intrapromoter hypersensitive sites.

MNase digestion profile of the RARβ2 promoter. Studies with the albumin enhancer (35) and the MMTV long terminal repeat (19) showed that transcriptional activation occurs in the promoter with stably positioned nucleosomes. Other studies have indicated that transcriptional activation is associated with destabilization and/or disruption of nucleosomes in the promoter (57, 58). By MNase digestion assay we examined whether nucleosomal positioning in and around the RARβ2 promoter is affected by RA treatment. DNA preparations from MNase-digested nuclei were cut with *Pst*I and probed with a fragment corresponding to a region upstream from the promoter (Fig. 9). MNase digestion generated five fragments of approximately 960, 620, 510, 430, and 260 bp (±25 bp) (Fig. 9, lanes 7 to 16). The ~960-bp fragment corresponded to the coding region of the RARβ2 gene, while fragments of ~620 and ~510 bp corresponded to the promoter region. Control deproteinated DNAs were digested evenly with few bands, which did not match with the MNase-digested fragments obtained from the nuclei samples (Fig. 9, lanes 1 to 5), indicating that the digestion pattern seen in vivo was dependent on nucleosomes. The MNase digestion pattern was not affected by RA treatment (Fig. 9, right). No major changes were observed with a shorter RA treatment (4 h) either (data not shown). Similarly, no discernible difference was detected in the MNase digestion profiles of the integrated promoter following RA treatment (data not shown). In these experiments, MNase digestion produced smaller than expected bands with intervals less than 180 to 200 bp (see 620-, 510-, and 430-bp bands in Fig. 9), which may suggest multiple positioning of nucleosomes in the promoter. A similar, closer than expected MNase cutting was observed for the yeast SUC2 promoter (9) and in the chicken erythroid-specific globin gene enhancer (10). LM-PCR-based high-resolution analysis (see Materials and Methods) performed with the above-described MNase-digested

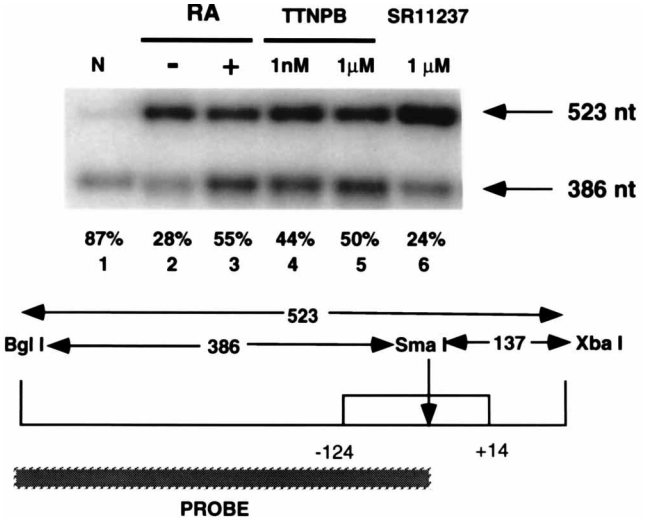

FIG. 6. Ligand specificity. P2 cells were treated with RA (1 μM), TTNPB (at indicated concentrations), or SR11237 (1 μM) for 24 h. Nuclei were digested with *Sma*I (200 U in each case). DNAs were then digested with *Bgl*I and *Xba*I and probed with a 386-bp fragment, which is shown in the diagram at the bottom of the panel. The undigested band (523 bp) and the *Sma*I-*Bgl*I fragment (386 bp) are marked by arrows.

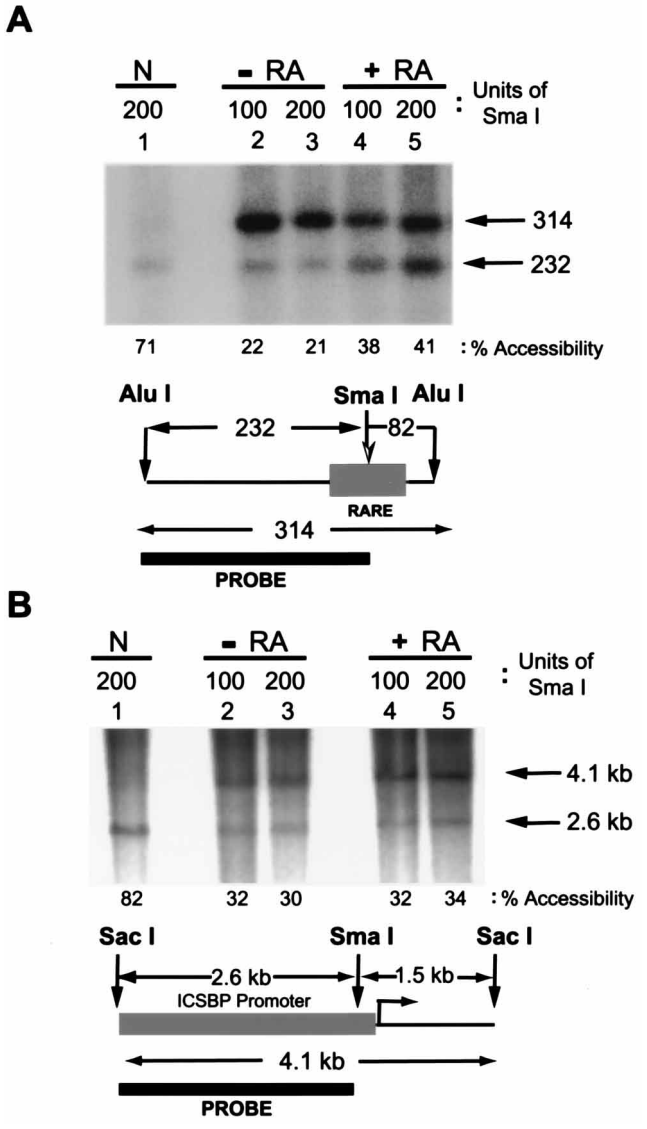

FIG. 7. *Sma*I accessibility in the endogenous RARβ2 (A) and the RA nonresponsive ICSBP (B) promoters in P19 cells. (A) Nuclei (20 μg of DNA) prepared from parental P19 cells treated with (+RA) or without (−RA) RA (1 μM) for 24 h were digested with *Sma*I. Purified DNAs were then digested with *Alu*I and probed with a 232-bp fragment, which is depicted in the diagram at the bottom of the panel. The undigested (314 nt) and digested (232 nt) bands are marked by arrows. The position of the *Sma*I site in the promoter (box) is indicated by an open arrow. The percentage of *Sma*I digestion is presented below each lane. (B) DNAs from *Sma*I-digested nuclei in panel A were digested with *Sac*I and probed with a 2.6-kb ICSBP promoter fragment. The undigested (4.1 kb) and digested (2.6 kb) bands are marked. The position of the *Sma*I site within the ICSBP promoter (28) is indicated. N, deproteinated DNA.

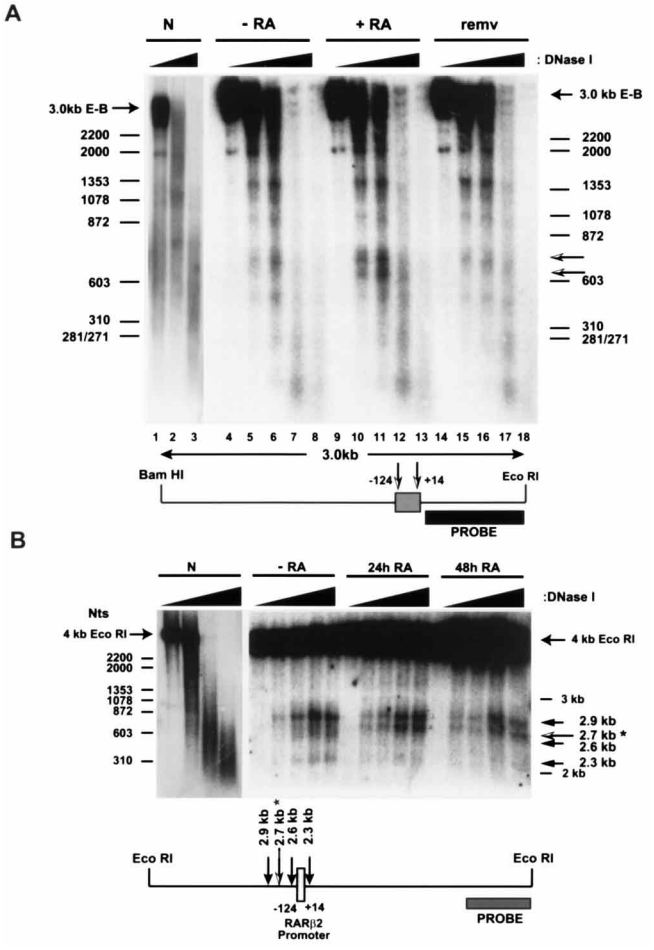

FIG. 8. DNase I hypersensitivity assays. (A) The integrated multicopy RARβ2 promoter. P2 cells were treated with RA as described for Fig. 4, and nuclei were digested with increasing amounts of DNase I. Purified DNAs were then digested with *Bam*HI and *Eco*RI and probed with a 0.65-kb *Eco*RI-*Hin*dIII fragment (17), which is shown in the diagram at the bottom of the panel. The positions of the RA-induced bands are shown by open arrows. N, deproteinated DNA samples. The shaded box in the diagram indicates the position of the RARβ2 promoter. (B) The endogenous RARβ2 promoter. Nuclei from parental P19 cells were digested with increasing amounts of DNase I. Purified DNAs were digested with *Eco*RI and probed with the 3′-end-labeled 0.7-kb *Eco*RI-*Pvu*II (49) fragment as a probe, which is shown at the bottom of the panel. The positions of the constitutively hypersensitive bands are marked by closed arrows. The position of the RA-inducible hypersensitive is marked with an open arrow and an asterisk.

samples did not reveal a discernible difference between RA-treated and untreated samples (data not shown).

DISCUSSION

RA-induced restriction site accessibility, DNase I hypersensitivity, and changes in the local chromatin structure. The present work shows that RA causes alterations in restriction site accessibility and DNase I hypersensitivity in both the endogenous and integrated RARβ2 promoters in P19 cells. Results in this work are consistent with previous findings with the MMTV promoter (53, 63) and strengthen the idea that ligands plays a critical role in altering chromatin structure in and around the promoter. The increased restriction site accessibility was a rapid event, occurring within 15 min of RA addition, and was reversed upon ligand withdrawal (Fig. 2 to 4). RA-induced DNase I hypersensitivity was also rapid and was reversed upon RA withdrawal (Fig. 8A). The accessibility was confined within the promoter region and was not found in a promoter unresponsive to RA. Since the accessibility was increased rather than decreased by RA addition, it is not likely to be attributable to steric hindrance caused by binding of transcription factors to the promoter. Rather, similar to the results of previous studies with isolated nuclei and in vitro-assembled chromatin, these changes are likely to reflect RA-induced alterations of chromatin structure. Changes in DNase I hypersensitivity observed in and near the promoter following RA

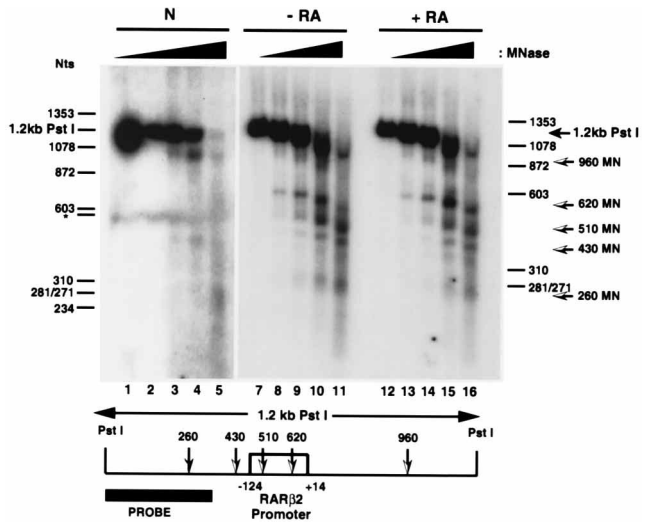

FIG. 9. MNase digestion profile. P19 cells were treated with (+RA) or without (−RA) RA (1 μM) for 24 h, and nuclei were digested with increasing amounts of MNase. DNAs were further digested with *Pst*I and probed with a 5′-end-labeled 350-bp *Pst*I-*Nae*I (47) fragment as a probe, which is shown in the diagram at the bottom of the panel. The position of the −124 to +14 nt promoter region is indicated by an open box. The positions of the MNase cuts are marked by open arrows. The band indicated by an asterisk represents a nonspecific noise in the autoradiogram of the purified DNA used as a control.

treatment (Fig. 8) also support this explanation (3, 10, 18, 22, 40, 53, 54, 58, 60).

The RA-induced alterations correlated with the transcriptionally active state of the promoter, as observed in experiments using the RARα antagonist Ro 41-5253 and receptor-selective ligands and after RA withdrawal (Fig. 4 to 6). Furthermore, these changes also occurred in good correlation with the ligand-induced in vivo footprint of the RARβ2 promoter, observed again with respect to ligand specificity (16, 38), inhibition by antagonist, and extinction after ligand withdrawal (17). These results are consistent with previous reports (3, 45, 50, 63) indicating that binding of transcription factors to the promoter is a prerequisite for chromatin structure transition.

Previous in vivo footprinting studies performed with P19 cells expressing dominant negative receptors (7, 17) indicated that RA-induced promoter occupancy is initiated by the binding of liganded heterodimer RXR-RAR to the RARE, which might in turn stimulate recruitment of other transcription factors and facilitate the establishment of an "open" chromatin configuration of the promoter. Thus, RA-induced chromatin structure transition in this promoter may be a consequence of the recruitment of the heterodimer to the RARE. In agreement with this possibility, the importance of ligand-bound progesterone receptor in eliciting chromatin transition has been demonstrated for the MMTV promoter, in which displacement of the progesterone receptor from the promoter by an antagonist results in the "closing" of the previously open chromatin (41, 52). Although resembling the MMTV promoter in many aspects, the RARβ2 promoter seems to differ in one aspect in that it has constitutive DNase I hypersensitivity sites, which are apparently absent in the former (48, 53). The basis of this difference is not clear from the present study.

Possible mechanisms. Several possible mechanisms can be envisaged for the RA-mediated chromatin structure alterations in this promoter. One possibility is the depletion of the linker histone H1, which might have occurred following RA treatment. Linker H1 is thought to play a role in the maintenance of a repressive chromatin; for example, binding of upstream stimulatory factor to the nucleosomal DNA is inhibited by H1 in vitro (26). Similarly, linker histone H1 represses nucleosome mobility in the assembled chromatin template (55). Conversely, glucocorticoid-hormone-induced activation of the MMTV promoter is associated with the depletion of histone H1 (11). Depletion of linker histone is also observed with the active β-globin gene locus (27). Another possibility is increased histone acetylation, which has been linked to transcriptionally active chromatin (51, 61). Several histone acetylases have been recently isolated and were found to be homologous to the yeast transcriptional coactivator GCN5, further reinforcing the link between histone acetylation and transcriptional activation (12, 62). Studies with histone acetylase inhibitors such as trichostatin A have provided additional support for the role of histone acetylases in transcriptional activation (51). For example, these inhibitors exert, among other effects, dramatic effects on glucocorticoid hormone induction of the MMTV promoter (5). We have recently observed that treatment of P2 cells with trichostatin A, alone or in combination with RA, significantly increases *Sma*I accessibility in the RARβ2 promoter, concomitant with a marked increase in the promoter activity, indicating that histone acetylation contributes to alterations of chromatin structure and promoter activation (39). More recent findings in this laboratory show that the RXR-RAR heterodimer bound to the RARE recruits a histone acetylase PCAF upon ligand addition (8). Ligand-bound heterodimers apparently recruit additional histone acetylases, CBP and p300, that serve as transcriptional coactivators (13, 43). Ligand-induced recruitment of histone acetylases by the RXR-RAR heterodimer may be coupled with a reciprocal event where ligand-induced release of corepressors such as SMRT and N-CoR could stimulate dissociation of the histone deacetylase-Sin3 complex bound to the corepressor (23, 42). On the other hand, chromatin alterations in this promoter could be mediated by other non-histone nuclear factors, some of which might directly or indirectly interact with RAR-RXR heterodimer. Candidates for such factors include the conserved SWI-SNF complexes (30), nucleosome-remodeling factors (54), and a high-mobility group protein, HMG17 (44). Identification of such factors and detailed descriptions of their mechanisms of their action await further investigations performed in vivo and in vitro.

Nucleosomal organization of the RARβ2 promoter. Although evidence indicates that the organization of nucleosomes is affected during gene activation (57, 58), the MNase digestion data shown Fig. 9 suggest that RA treatment does not disrupt the overall nucleosome architecture nor does it cause major changes in nucleosomal positioning in and around the RARβ2 promoter.

These findings are remarkably similar to those reported for the chromatin transition induced in the MMTV promoter by glucocorticoid and progesterone receptors (11, 19, 41, 52, 53). In the MMTV promoter an ordered nucleosomal protein structure exists prior to induction; upon hormone stimulation the MNase pattern is essentially unchanged, while access for restriction enzymes increases dramatically. Based on studies with a metal-responsive promoter in yeast, it has also been reported that the positioning of nucleosomes remained unchanged during transactivation (65). This feature also appears to be shared by the MMTV promoter, as Fragoso et al. (19) showed that addition of hormone does not lead to an alteration or loss of nucleosomes in the promoter, including those located at the glucocorticoid and progesterone response element. Together, our results show that the RARβ2 promoter undergoes

a reversible chromatin structure transition after ligand addition. This transition is coupled with transcriptional activation and occurs without grossly altering the nucleosomal organization in the promoter. The close resemblance of this event to that in the MMTV promoter suggests that the mechanism of ligand-induced chromatin transition may be conserved among hormone-responsive promoters.

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