Ligand-Dependent Occupancy of the Retinoic Acid Receptor β2 Promoter In Vivo

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Retinoic acid (RA) activates transcription of the RA receptor β2 (RARβ2) gene in embryonal carcinoma (EC) cells. This activation involves binding of the RAR/retinoid X receptor (RAR/RXR) heterodimer to the RA-responsive element (βRARE). Dimethyl sulfate-based genomic footprinting was performed to examine occupancy of this promoter in P19 EC cells. No footprint was detected at the βRARE prior to RA treatment, but a footprint was detected within the first hour of RA treatment. Concomitantly, other elements in the promoter, the cyclic AMP-responsive element and tetradecanoyl phorbol acetate-like-responsive element became footprinted. Footprints at these elements were induced by RA without requiring new protein synthesis and remained for the entire duration of RA treatment but rapidly reversed upon withdrawal of RA. A delayed protection observed at the initiator site was also reversed upon RA withdrawal. The RA-inducible footprint was not due to induction of factors that bind to these element, since in vitro assays showed that these factors are present in P19 cell extracts before RA treatment. Significantly, no RA-induced footprint was observed at any of these elements in P19 cells expressing a dominant negative RXRβ, in which RXR heterodimers are unable to bind to the βRARE. Results indicate that binding of a liganded heterodimer receptor to the βRARE is the initial event that allows other elements to gain access to the factors. In accordance, reporter analyses showed that a mutation in the βRARE, but not those in other elements, abrogates RA activation of the promoter. It is likely that the RARβ2 promoter opens in a hierarchically ordered manner, signalled by the occupancy of liganded heterodimers.

Retinoic acid (RA) plays a role in the development of many vertebrate species (8, 37). RA induces differentiation of embryonal carcinoma (EC) cells, which mimics the process of early embryonic development (28, 59). A series of genes induced in P19 EC cells following RA treatment have been identified and studied (29). Many of these genes are shown to possess an RA-responsive element (RARE) in the promoter region (14, 26, 49, 61). Naturally occurring and synthetic RAREs are often composed of a direct repeat of the AG GTCA motif with a 2- to 5-bp spacer (44, 60, 62). It has been shown that RA receptor/retinoid X receptor (RAR/RXR) heterodimers bind to these elements in an asymmetrical fashion (9, 33, 35, 39, 47, 65). The critical involvement of the RAR in RA-induced differentiation in EC cells is supported by the inability of cells expressing a mutated RAR to respond to RA and to undergo differentiation (17, 50). A null mutant of RARγ also affects RA-induced differentiation (7). In support of the role of the RXR in this process, we have recently described a dominant negative RXRβ, which inhibits RA-induced gene expression in P19 EC cells (42).

Despite recent understanding of the role of RXR/RAR heterodimers in RA-mediated gene regulation, the question of how these receptors interact with the endogenous RA-responsive genes and activate transcription in vivo still remains. To gain insight into this question, we have performed genomic footprinting of the RARβ2 gene. RARβ2 is one of the immediate-early genes induced by RA in EC cells. Zelent et al. (66) have shown that RA treatment of EC cells induces RARβ2 mRNA as a major isoform in EC cells and that other

isoforms of RARβ are expressed in other cells and tissues either constitutively or after RA treatment.

Our initial goal was to determine whether this RA-responsive promoter is constitutively occupied irrespective of RA treatment or whether RA plays a role in receptor occupancy. In vivo footprinting analyses of a number of inducible promoters reported so far illustrate highly variable patterns of factor occupancy. For example, Herrera et al. (25) and Dey et al. (15) have previously noted that the c-fos promoter is fully occupied prior to growth factor stimulation, with few changes observed following stimulation. Similar constitutive occupancy has been noted for the interferon-stimulated responsive element in several interferon-inducible genes (16, 38, 43). On the other hand, footprinting of the heat shock element in the human HSP70 gene has been shown to be induced only following heat shock (1). Similarly, Garrity et al. (19) showed that activation of T cells results in induction of factor binding in the interleukin-2 promoter in vivo. In the rat tyrosine aminotransferase gene, a glucocorticoid hormone-dependent alteration of nucleosomal positioning at the glucocorticoid hormone-responsive element has been reported (12, 52). A hormone-dependent DNase I hypersensitivity noted at this site (5, 22) probably results from the alteration in the chromatin structure. Similar changes in glucocorticoid hormone-dependent factor loading and chromatin have been reported for the mouse mammary tumor virus promoter (3, 34, 54).

We show here that the RARβ2 promoter is not occupied prior to RA treatment, but RA treatment rapidly induces occupancy at multiple sites in the promoter. Further, promoter occupancy was totally absent in P19 cells expressing a dominant negative RXRβ, supporting the primary role of the RXR/RAR heterodimer for RA-induced occupancy of this promoter. The results of our study suggest a hierarchical order

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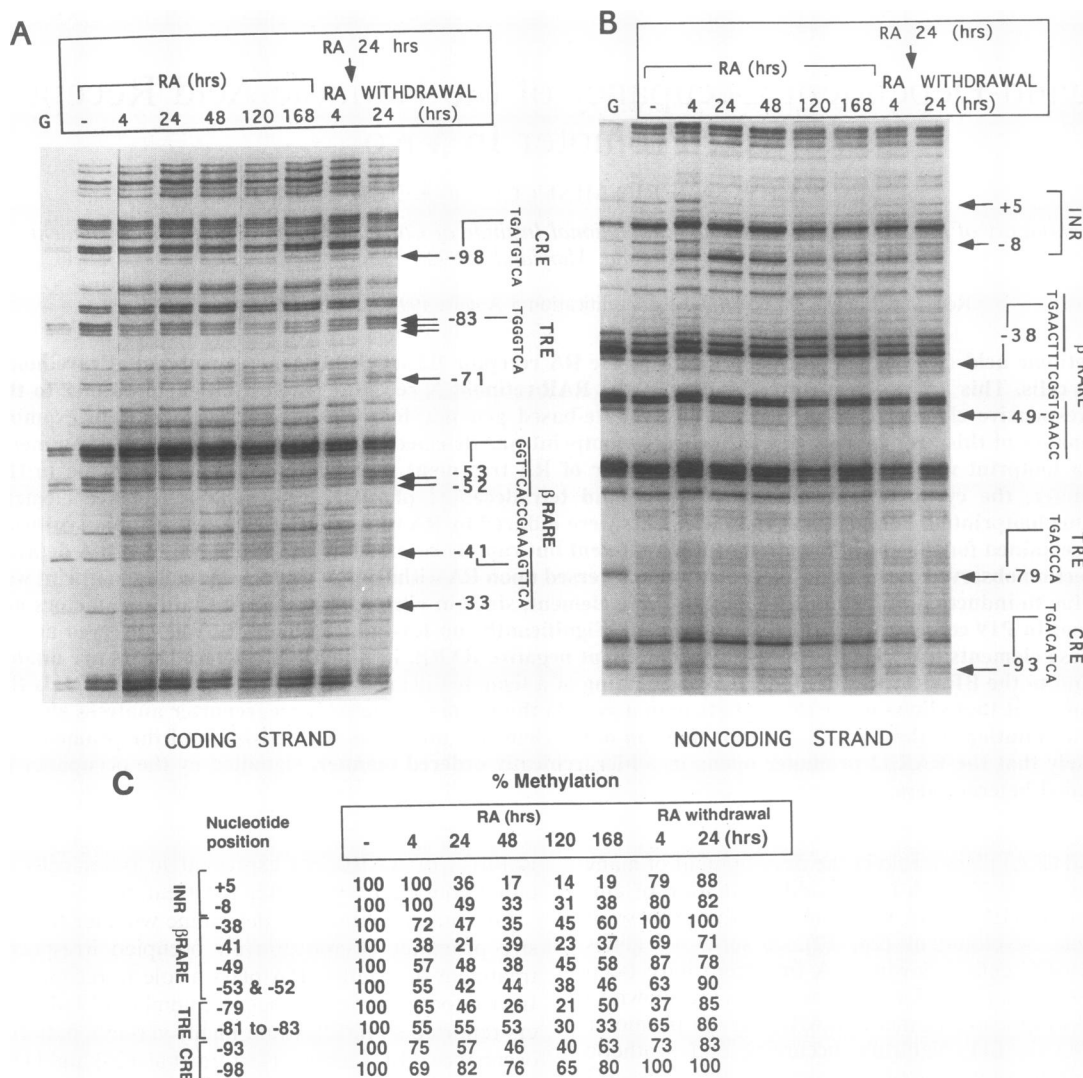
FIG. 1. In vivo footprinting of RARβ2 promoter, with induction by RA addition and reversal by RA withdrawal. LM-PCR-based footprinting analysis was performed for coding (A) and noncoding (B) strands. P19 EC cells were treated with all-*trans* RA (1 μM) for the indicated periods of time. For reversibility experiments, P19 cells were pretreated with RA (1 μM) for 24 h and then incubated without RA for the indicated periods of time. Arrows indicate the residues at which significant protection was found. (C) The level of protection at each residue was quantified by densitometry by using nt −21 and −63 as internal controls and is expressed as the percent methylation, in which the band intensity measured prior to RA treatment was taken as 100%. The level of protection obtained by using other internal controls was very similar.

MATERIALS AND METHODS

Cell culture and in vivo footprinting. P19 EC cells and P19 transfectant clones expressing a dominant negative RXRβ lacking the DNA binding domain, referred to as DBD⁻, were cultured in α minimum essential medium with supplements (42). Cells were treated with all-*trans* RA at 1 μM for the indicated periods of time. Some cells were treated with cycloheximide (CHX; Sigma) at 35 μg/ml for 30 min prior to and during the RA treatment. CHX treatment under these conditions blocked >98% of [³⁵S]methionine incorporation. Cells were then treated with 0.1% dimethyl sulfate (Kodak) for 2 min at room temperature, and high-molecular-weight DNA was extracted and cleaved with piperidine. Ligation-mediated

(LM)-PCR was done with Vent polymerase as described previously (16, 20, 38). Primers used for analysis of the coding strand were 5'-TGG CAA AGA TAT GAC-3' (+57 to +43), 5'-AGA ATA GAC CCC TCC TGC CTG CCT CGG AGC-3' (+51 to +27), and 5'-ACC CTC CTG CCT CGG AGC TCA CTT CTA-3' (+44 to +13). Those used for the noncoding strand were 5'-ATT GTT TGC AGC TGA G-3' (−210 to −194), 5'-GAC TCG CTG GCT GAA GGC TCT TGC AAG-3' (−167 to −142), and 5' GGC TGA AGG CTC TTG CAG GGC TGC TGG GAG-3' (−159 to −130). After PCR, labeled products were resolved in an 8% sequencing gel. For quantitation, autoradiograms were scanned by a laser densitometer (Molecular Dynamics Imagequant). Levels of methylation at each residue (measured as an integrated volume) were estimated relative to that at several residues outside the footprinted region chosen as internal controls (nucleotides [nt] −21, −32, and −65 for the noncoding strand; nt −45 and −63
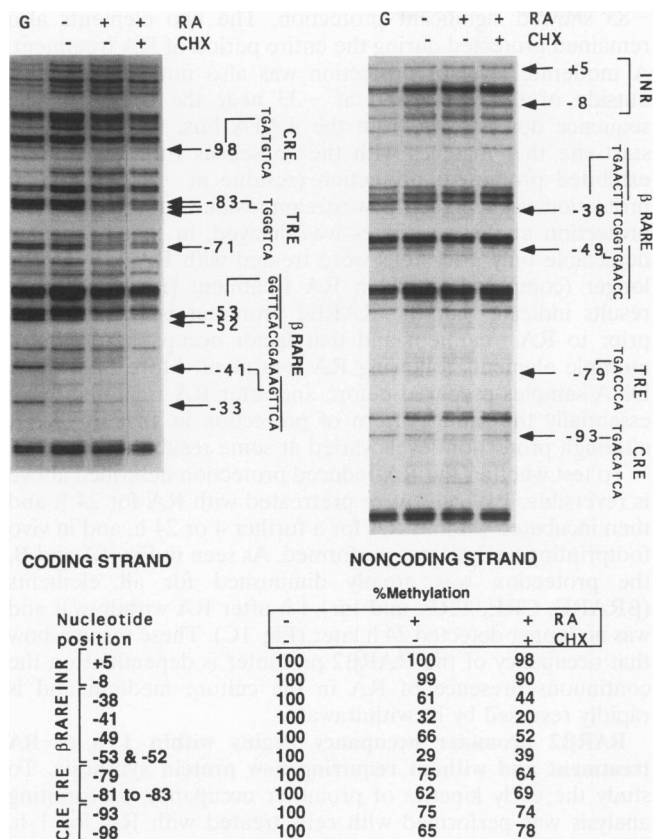
FIG. 2. Early time course of RA-inducible footprint and effect of CHX. (Top) Footprinting analysis was performed with P19 cells treated with RA alone (1 μM) or RA plus CHX (35 μg/ml) for 1 h. CHX was added 30 min prior to the addition of RA. (Bottom) The level of protection at each residue was quantified as described in the legend to Fig. 1.

FIG. 3. RA induction of RARβ2 transcription in P19 cells. (A) Nuclear run-on assay. Nuclei from P19 cells treated with RA (1 μM) for the indicated periods of time were labeled with [³²P]UTP and reacted with cDNA probes for mRARβ or mGAPDH in pBluescript or vector alone (pBluescript). The same results were obtained with cDNA fragments without the vector sequence (data not shown). (B) RNase protection assay for early kinetics of RARβ2 mRNA induction. P19 cells were treated with RA (1 μM) for the indicated periods of time, and RNase protection assays were performed with 25 μg of total RNA with probes for RARβ and GAPDH as described in reference 42. (C) Effect of CHX on RARβ2 mRNA induction. Cells were treated with CHX (35 μg/ml) for 30 min before and during 1 or 2 h of RA treatment, and RNase protection assays were performed as described above.

for the coding strand). Densitometry values were then normalized with those in the G residues obtained from the purified DNA to adjust lane-to-lane variation in DNA loading. The percent methylation was expressed relative to the levels of protection without RA treatment.

Nuclear run-on and RNase protection assays. Nuclei were isolated from P19 cells following RA treatment (all-*trans*, 1 μM) for various periods of time (23). For each reaction, 5 × 10⁷ nuclei were labeled with 100 μCi of [α-³²P]UTP. Labeled RNA was hybridized with 5 μg of the DNA probe immobilized onto the nitrocellulose paper. A murine RARβ cDNA (53) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (42) cloned in pBluescript were used as probes. In some experiments cDNA fragments without the vector sequence were also used as probes.

RNase protection assays for RARβ2 and GAPDH mRNA were performed as described previously (42) with total RNA (25 μg) prepared from P19 cells treated with RA for various periods of time.

EMSA. Electrophoretic mobility shift assays (EMSA) were performed with nuclear extracts (10 μg of protein) from P19 cells essentially as described previously (16). Oligonucleotides corresponding to the RAREβ (GATCCGCTAGCAAGG GTTCACCGAAAGTTCACTCGCATA), the initiator (Inr) (5'-17 TCAATCTTTCATTCCGTGTGACAGAAG +10), mutant Inrs, and other competitors were prepared in an
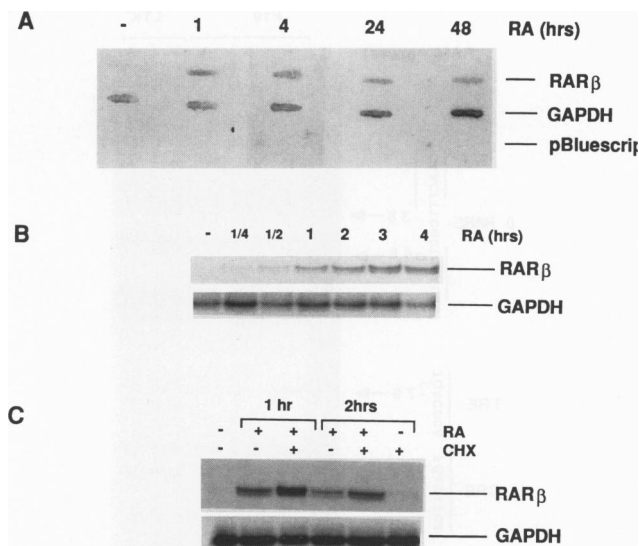
Applied Biosystem model 380B DNA synthesizer. Competitors were added at a 100-fold molar excess and incubated for 15 min before the addition of the labeled probe.

Plasmid construction and luciferase assays. Luciferase reporters driven by the RARβ2 promoters (see Fig. 8A) were constructed by inserting PCR fragments generated from the RARβ2 promoter (−124 to +14) into the SmaI and HindIII sites of the pGL2 basic vector (Promega). Mutations in the cyclic AMP-responsive element (CRE), RARE, and TATA sequences were created by a two-step PCR with internal primers carrying the desired mutations. The Inr mutant was prepared by creating a shorter fragment lacking the sequence downstream from the TATA box. The DNA sequence of these constructs was determined by the dideoxy sequencing method to verify the correct mutations. Transfection was performed by the lipofection method according to the manufacturer's instructions (GIBCO). Briefly, P19 cells were seeded in a 12-well plate (7 × 10⁴ cells per well) 12 h prior to transfection, and they were transfected with a total of 1 μg of DNA. Five hundred nanograms of reporter constructs, 200 ng of the cytomegalovirus β-galactosidase plasmid (used for transfection efficiency), and pBluescript were added per well. Cells were incubated with DNA-liposome complexes for 12 to 14 h in the absence of serum and antibiotics. Cells were then fed with fresh complete medium containing all-*trans* RA (1 μM) for 6 or 24 h. Luciferase activity was determined as described by Minucci et al. (42).
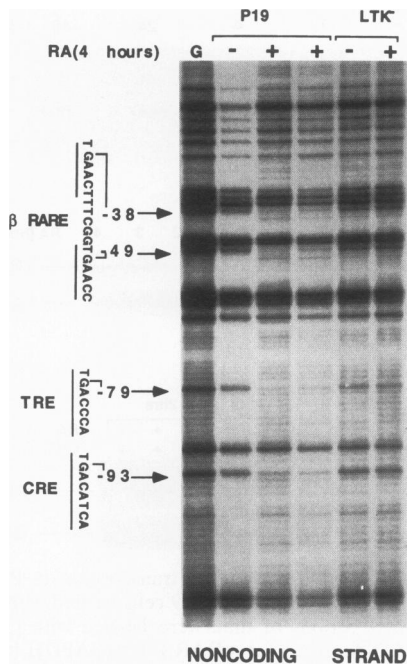
FIG. 4. Cell-type specificity of RA-inducible footprint. P19 EC cells or LTk⁻ fibroblasts were treated without (−) or with (+) RA (1 µM) for 4 h, and footprinting analysis was performed for the noncoding strand as described in the legend to Fig. 1. Arrows indicate G residues at which significant protection was detected.

RESULTS

Occupancy of the RARβ2 promoter is induced by RA addition and reversed by its withdrawal. RA induces expression of RARβ2 mRNA in P19 EC cells (32, 42, 50). LM-PCR-based genomic footprinting was performed to examine occupancy of this promoter in these cells. The primers used here allowed resolution of the sequence from −100 to +10 relative to the designated RNA start site (marked +1) (14). The upstream region includes the canonical βRARE composed of an A/GGTTCA direct repeat with a 5-bp spacer (14, 26) (Fig. 1, βRARE; see Fig. 9A). Upstream from βRARE, there are two additional elements, CRE (−99 to −92) and the tetradecanoyl phorbol acetate-like-responsive element (TRE; −84 to −78) (31). Figure 1 shows the results of in vivo footprinting of the coding and the noncoding strands obtained 4, 24, and 48 h and 5 and 7 days following RA treatment. The level of protection observed at each element was quantified by densitometric scanning and is summarized as the percent methylation (Fig. 1C). Prior to RA treatment, there was no discernible in vivo footprint in either strand. However, following 4 h of RA treatment, strong protection was noted at the βRARE. G residues exhibiting protection were nt −53, −52, and −41 in the coding strands and nt −49 and −38 in the noncoding strands, which precisely corresponded to the residues that exhibit methylation interference following binding of the RAR/RXR heterodimer to this element in vitro (33). These G residues remained protected at an approximately constant level during 7 days of RA treatment. Significant protection was also noted at the CRE and TRE, again only after RA treatment. For the CRE, residues at nt −93 and −98 were protected (the former more strongly and consistently than the latter), while for the TRE residues at nt −79, −81, −82, and

−83 showed significant protection. The two elements also remained protected during the entire period of RA treatment. A moderate level of protection was also noted at nt −71, outside of the TRE, and at −33 near the βRARE. The sequence downstream from the TATA box, near the RNA start site that matches with the consensus Inr (30, 58, 63), exhibited prominent protection (residue at −8). Prominent protection was seen at a downstream residue, nt +5. However, protection at these residues was delayed, in that it became detectable only after cells were treated with RA for 24 h or longer (compared with 4 h RA treatment [Fig. 1]). These results indicate that the RARβ2 promoter is not occupied prior to RA treatment and that factor occupancy occurs at multiple elements following RA treatment. More than three DNA samples prepared before and after RA treatment gave essentially the same pattern of protection as that in Fig. 1, although protection levels varied at some residues.

To test whether the RA-induced protection described above is reversible, P19 cells were pretreated with RA for 24 h and then incubated without RA for a further 4 or 24 h, and in vivo footprinting analysis was performed. As seen in Fig. 1A and B, the protection was greatly diminished for all elements (βRARE, CRE, TRE, and Inr) 4 h after RA withdrawal and was no longer detected 24 h later (Fig. 1C). These results show that occupancy of the RARβ2 promoter is dependent on the continuous presence of RA in the culture medium and is rapidly reversed by its withdrawal.

RARβ2 promoter occupancy begins within 1 h of RA treatment and without requiring new protein synthesis. To study the early kinetics of promoter occupancy, footprinting analysis was performed with cells treated with RA for 1 h. Some samples were treated with CHX for 30 min prior to and during RA treatment. As seen in Fig. 2, 1 h of RA treatment gave full protection at the βRARE, TRE, and CRE, irrespective of CHX treatment. No protection was seen at the Inr at this time, in agreement with data obtained with 4 h of RA treatment (Fig. 1). Levels of protection quantitated for each residue (Fig. 2, bottom panel) were similar to those found after 4 h of treatment. Thus, promoter occupancy begins rapidly after RA treatment without requiring new protein synthesis.

Nuclear run-on and RNase protection assays (Fig. 3) were performed to verify that treatment of P19 cells with RA under these conditions indeed activates RARβ transcription. In nuclear run-on assays (Fig. 3A), samples from untreated P19 cells gave no detectable signals for the RARβ probe. However, samples from cells treated with RA for 1, 4, 24, and 48 h gave strong signals, the levels of which were similar throughout. The GAPDH probe, used as a control for a constitutively transcribed gene (42), gave positive signals in all samples before and after RA treatment. The vector probe (pBluescript) used as a negative control did not give detectable signals, as expected. Thus, the rate of RARβ transcription increases within 1 h of RA treatment. In accordance, RARβ2 mRNA levels rapidly increased during the first hour and continuously increased afterwards, albeit more slowly, up to 4 h of RA treatment (Fig. 3B). Consistent with the footprinting data in Fig. 2, CHX treatment had no inhibitory effect on the mRNA levels tested either 1 or 2 h after RA treatment but rather slightly increased the RARβ2 mRNA levels (Fig. 3C). As expected, CHX alone did not induce RARβ2 mRNA. GAPDH mRNA tested as a control remained constant throughout this period. The lack of inhibition in RARβ2 mRNA induction by CHX is consistent with the report by Pratt et al. (50). These results confirm that RA treatment rapidly increases the rate of RARβ2 transcription, leading to increased mRNA levels in P19 cells.
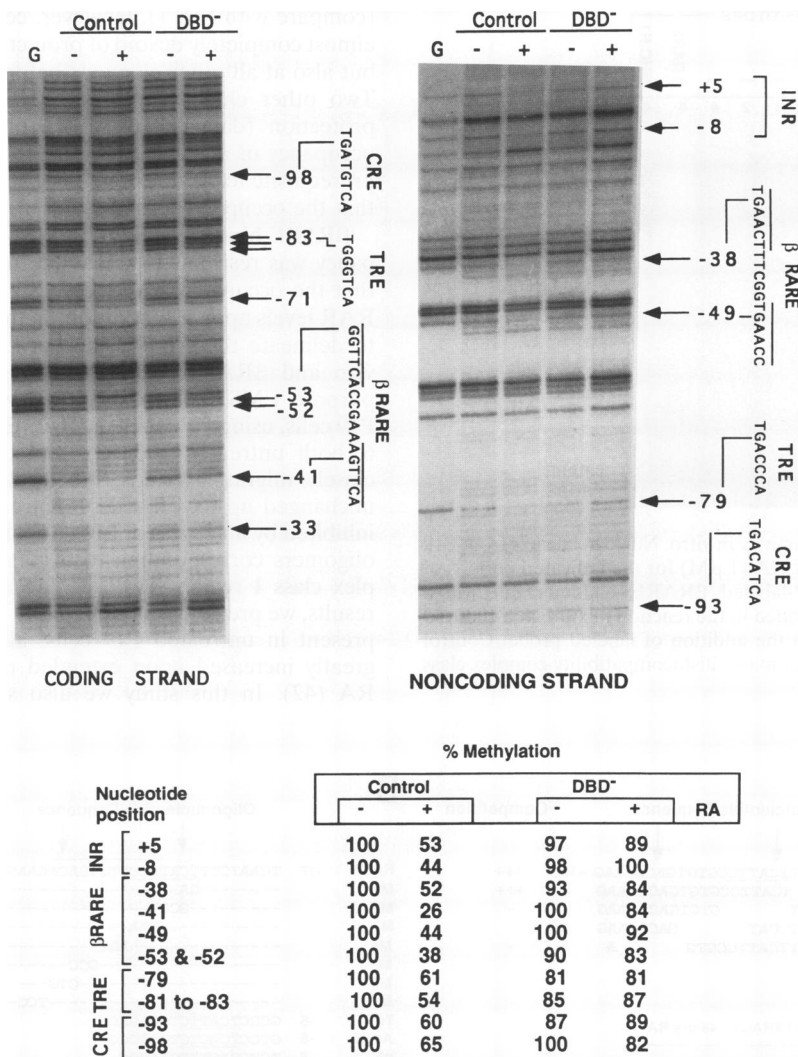
FIG. 5. Lack of footprint in P19 cells expressing dominant negative RXRβ DBD⁻. (Top) A P19 clone expressing the dominant negative RXRβ (DBD⁻) or a clone transfected with the control vector (control) was treated without (−) or with (+) RA (1 μM) for 24 h, and footprinting analysis was performed as described in the legend to Fig. 1. (Bottom) The level of protection for each residue was quantified as described in the legend to Fig. 1. Expression of the DBD⁻ protein and RA induction of the RARβ2 reporter activity in these cells have been confirmed previously (42).

Cell-type specificity of RARβ2 promoter occupancy in vivo. RA elicits biological effects in a cell-type-dependent manner. Neither morphological differentiation nor gene transcription is induced by RA in fibroblasts such as murine LTk⁻ fibroblasts (45). Furthermore, an RARβ2-luciferase reporter transiently transfected into LTk⁻ cells gave less than twofold activation upon RA addition (data not shown), far less than the levels of activation seen in P19 cells (see Fig. 8). Thus, it was of interest to examine whether RA treatment induces RARβ2 promoter occupancy in these low-responder cells. Results of footprinting analysis performed with LTk⁻ cells are shown in Fig. 4. No footprint was detected before and after·RA treatment. On the other hand, P19 cells assayed in parallel as a positive control showed strong protection at the βRARE, CRE, and TRE, as expected (the levels of protection at nt −79 in the TRE and at nt −93 in the CRE tested in these samples were somewhat greater than those found in the other P19 cell samples used in Fig. 1 and 2). These results indicate that RA induces factor occupancy in a cell-type-dependent manner, correlating with cell-type-dependent activation of the promoter.

Receptor occupancy at βRARE is required for occupancy at other elements. Factor occupancies at various elements in the RARβ2 promoter may be controlled independently of each other. Alternatively, occupancy may be coordinately regulated among elements in a mutually dependent manner or in a hierarchically ordered manner. To address this question, footprinting analysis was performed with P19 cells expressing a dominant negative receptor, RXRβ DBD⁻. This dominant negative receptor lacks the DNA binding domain but has a dimerization domain, which forms a nonfunctional heterodimer unable to bind to the RARE. P19 cells stably expressing DBD⁻ were shown to be defective in βRARE-binding activity in vivo and in vitro and unable to regulate gene expression in response to RA (42). Figure 5 compares footprinting patterns obtained from a clone expressing DBD⁻ with those obtained from a clone transfected with an empty vector. These clones were untreated or treated with RA for 24 h. The control clone exhibited strong protection at the βRARE, TRE, CRE, and Inr after RA treatment, but not before treatment, in a manner identical to that found with untransfected P19 cells
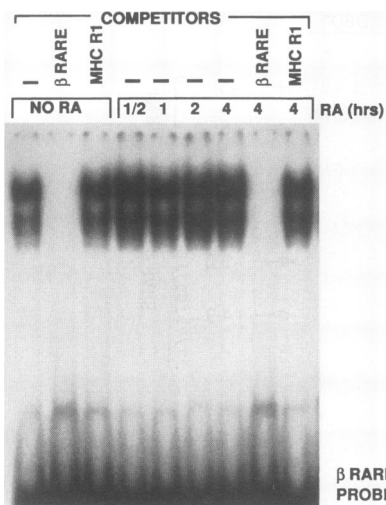
FIG. 6. βRARE-binding activity in vitro. Nuclear extracts from P19 cells treated with and without RA (1 μM) for the indicated periods of time were probed with ³²P-labeled βRARE oligomer in EMSA. Competitor oligomers were added to the reaction mixture at a 100-fold molar excess 15 min prior to the addition of labeled probe. Control oligomers corresponded to the major histocompatibility complex class I region I (κB-like [16]).

(compare with Fig. 1). However, cells expressing DBD⁻ were almost completely devoid of protection not only at the βRARE but also at all other elements before and after RA treatment. Two other clones expressing DBD⁻ also failed to exhibit protection (data not shown). These results show that the occupancy of an RXR/RAR heterodimer at the βRARE is a prerequisite for factor occupancy at other elements, suggesting that the occupancy process is hierarchical.

βRARE-binding activity in vitro. Since RA-induced occupancy was resistant to CHX treatment (Fig. 2), it is unlikely that the occupancy is induced as a result of increased RXR/RAR levels upon RA addition. In these experiments we sought to delineate the relationship between βRARE occupancy in vivo and βRARE factor-binding activity in vitro. For this purpose EMSA were performed with nuclear extracts from P19 cells, using ³²P-labeled βRARE as a probe. As seen in Fig. 6, both untreated and RA-treated P19 cells produced two closely migrating bands, the levels of which were virtually unchanged up to 4 h after RA treatment. These bands were inhibited by an excess of βRARE oligomers but not by control oligomers corresponding to a major histocompatibility complex class I region I κB site (16). In agreement with these results, we previously observed that βRARE-binding activity is present in untreated P19 cells, although the binding levels greatly increased upon extended periods of incubation with RA (42). In this study we also showed that the βRARE-
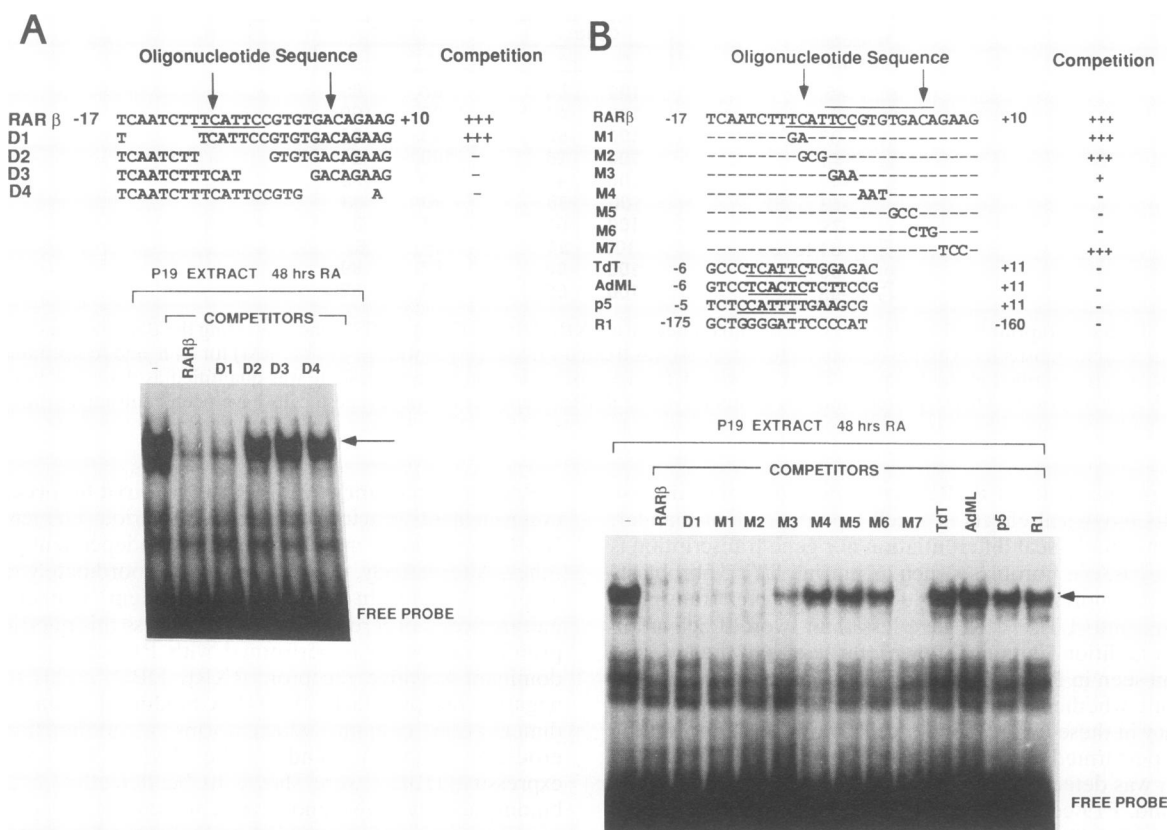

FIG. 7. Inr-binding activity in vitro. Nuclear extracts from P19 cells treated with RA for 48 h were probed with the ³²P-labeled Inr oligomer (from −17 to +10) in EMSA as described in the legend to Fig. 6. Vertical arrows above the RARβ sequence indicate the G residues at which protection was detected in vivo. The Inr consensus sequence (27) is underlined. Inr oligomers containing deletions D1 to D4 (A) or mutations M1 to M7 (B) were tested for competition. Competition was also tested with oligomers corresponding to the Inrs of the TdT, AdML, and P5 promoters (57, 58). The binding site for the RARβ2 Inr as assessed by this competition assay is indicated by a line above the sequence. R1 refers to control oligomers as described in the legend to Fig. 6. A specific retarded complex is indicated by an arrow.
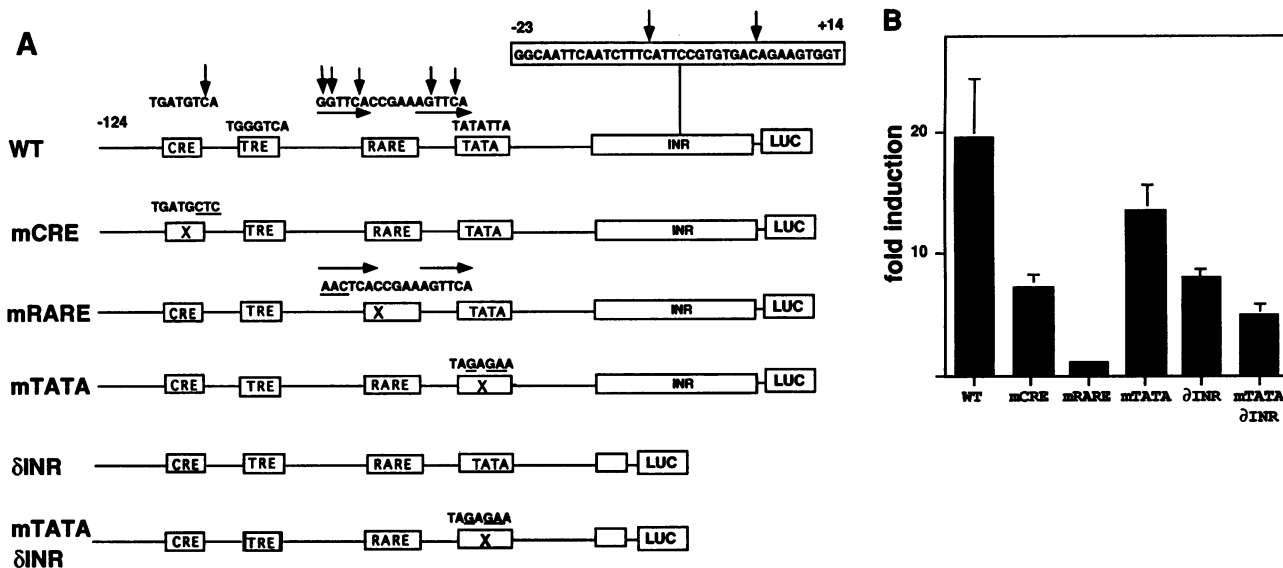
FIG. 8. Functional dominance of βRARE in RA-inducible promoter activation. (A) Schematic representation of mutations and deletions in RARβ2 promoter driving a luciferase reporter (LUC). Vertical arrows above the wild-type (WT) construct indicate the sites of in vivo protection. Mutations (shown as X, with mutated nucleotides underlined) or deletions (shown as a truncated Inr box) were introduced into the RARβ2 luciferase reporter as described in Materials and Methods. (B) P19 cells were transiently transfected with 500 ng of the indicated reporters and then treated with all-*trans* RA (1 μM) for 24 h. Fold induction was determined as luciferase activity obtained with RA relative to that without RA. Values are the averages of three assays; error bars indicate standard deviations.
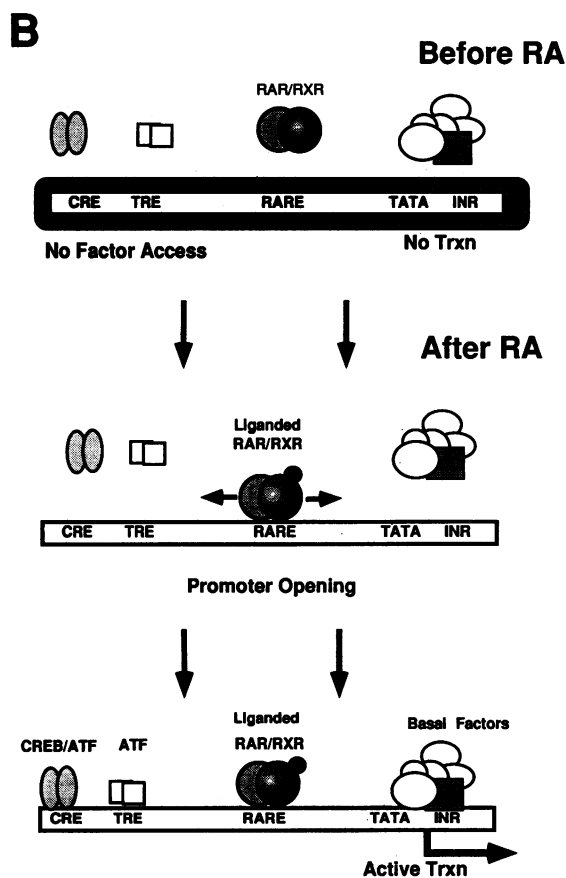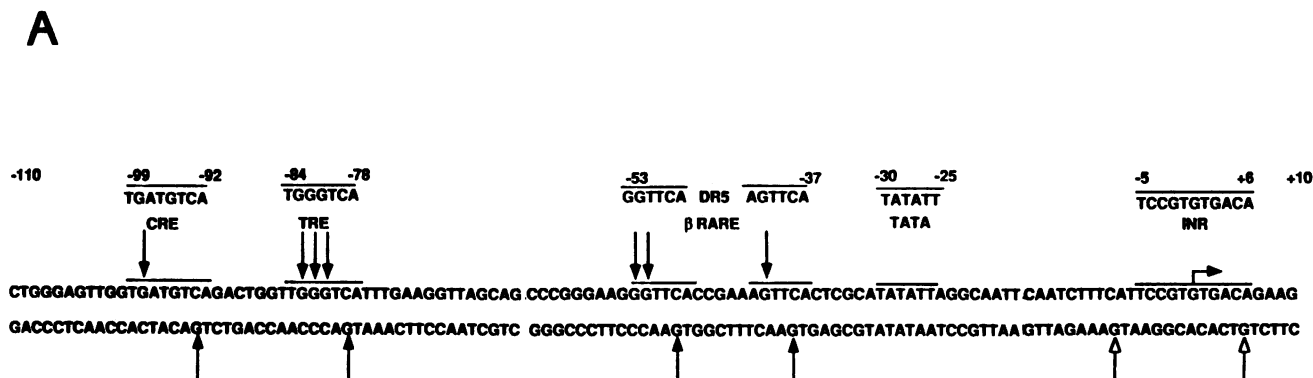
binding activity in vitro is eliminated by anti-RXRβ antibody, confirming that the binding activity contains RXRβ both before and after RA treatment. Thus, the lack of in vivo occupancy prior to RA addition was not due to the lack of receptor expression. Similarly, RA-induced occupancy in vivo was not due to the induction of receptor expression in these cells.

In agreement with the data for βRARE binding, Kruyt et al. (31) reported that the CRE-binding activity is present in untreated P19 cells as well, although this binding activity appears to increase rather rapidly after RA treatment.

Inr-binding activity in vitro. In vivo footprinting analysis (Fig. 1) revealed delayed protection at the Inr. One of the protected residues was located within the consensus Inr, TCATTCC, found in other genes (27, 30, 58, 63). Another protected residue was located downstream at +5. To our knowledge, in vivo occupancy of an initiator by dimethyl sulfate-based footprinting in vivo has not been reported. Thus, it was of interest to examine the nature of factor-binding activity for this Inr. To this end, standard EMSA were performed, using a ^{32}P-labeled probe covering a sequence from nt −17 to +10 that included in vivo-protected G residues. As seen in Fig. 7, nuclear extracts from RA-treated P19 cells gave a retarded complex that was inhibited by an excess of Inr oligomers but not by control oligomers (Fig. 7B, R1). A series of oligomers containing successive deletions (Fig. 7A) and mutations (Fig. 7B) were tested to determine the sequence requirement for this binding activity. Although the deletion of nucleotides in the 5′ end (D1) did not affect the ability to compete, deletion of the consensus Inr (D2) and of the successive 3′ sequences (D3 and D4) abolished competition. Further, while two 5′ mutations in the consensus competed well for binding (M1 and M2), a mutation in the 3′ part of the Inr consensus competed poorly (M3). Competition was abolished by successive mutations towards the 3′ ends (M4 to M6). Extracts from untreated P19 cells gave the same binding

activity (data not shown). These results suggest that this binding activity requires the consensus Inr as well as the 3′ sequence, consistent with the in vivo footprinting data in Fig. 1. Competition by M1 and M2 may suggest that binding to the Inr consensus is weak in affinity or broad in specificity (27). Pyrimidine-rich Inrs are present in the terminal deoxynucle-otidyl transferase (TdT) gene and adenovirus major late (AdML) promoters, both of which are shown to support basal transcription (11, 51, 58). These Inrs are also shown to bind sequence-specific transcription factors, TFII-I and USF (56, 63). Furthermore, a weak Inr in the P5 promoter of the adeno-associated virus binds another transcription factor, YY1 (57). To test whether the binding activity found for the RARβ2 Inr is related to the Inr-binding activities reported above, competition EMSA were performed, using oligomers for TdT, AdML, or P5 Inrs (57, 58) as competitors. None of these Inrs showed competition for binding (Fig. 7B). In reciprocal experiments in which these Inrs were tested as probes, no specific retarded complex was produced with the P19 extracts described above (data not shown). These results indicate that P19 cells express a factor(s) that binds to the RARβ2 Inr and that this binding activity is distinct from that for the TdT, AdML, and P5 Inrs.

Functional role of βRARE for RA activation of promoter. The data obtained with RXRβ DBD⁻ (Fig. 5) indicate that receptor occupancy in the βRARE is necessary for factor loading on other elements. In these experiments we sought to determine the relative significance of these elements for RA-activated transcription. Luciferase reporters driven by the RARβ2 promoter that contained mutations in these elements were constructed and tested for RA-induced activities. As seen in Fig. 8A, mutations in the CRE and βRARE included those residues that exhibited footprinting in vivo. The TATA mutant tested here was shown to abolish RA induction of the promoter activity following cotransfection with RARβ and TATA-box-binding protein (TBP) in P19-derived cells (6). δInr had a

A


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-110        -99   -92      -84   -78                        -53            -37    -30   -25           -5        +6   +10
           TGATGTCA        TGGGTCA                         GGTTCA DR5 AGTTCA      TATATT             TCCGTGTGACA
             CRE             TRE                               β RARE            TATA                    INR

CTGGGAGTTGGTGATGTCAGACTGGTTGGGTCATTTGAAGGTTAGCAG.CCCGGGAAGGGTTCACCGAAAGTTCACTCGCATATATTAGGCAATT.CAATCTTTCATTCCGTGTGACAGAAG

GACCCTCAACCACTACAGTCTGACCAACCCAGTAAACTTCCAATCGTC GGGCCCTTCCCAAGTGGCTTTCAAGTGAGCGTATATAATCCGTTAA GTTAGAAAGTAAGGCACACTGTCTTC
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B

FIG. 9. Summary of in vivo footprinting. (A) Closed vertical arrows indicate the sites at which significant protection was detected within 1 h after RA addition. Open arrows indicate the site at which delayed protection was detected. (B) Possible model for promoter activation. The RARβ2 promoter is closed prior to RA treatment. Binding of the liganded RXR/RAR to the βRARE coordinates the opening of the promoter by allowing factor loading onto other elements. Trxn, transcription.

truncation of the entire Inr sequence, as shown in Fig. 7A, while mTATA/δInr was a double mutant derived from mTATA and δInr. In this analysis the TRE was not tested, since a mutation in this element was shown to have no effect on RA activation of transient promoter activity (31). The results of reporter assays obtained with P19 cells treated with RA for 24 h are shown Fig. 8B. The wild-type RARβ2 reporter gave an about 20-fold induction by RA. In contrast, the mutation in the βRARE completely abrogated RA induction of reporter activity. The mutation in the CRE, on the other hand, reduced reporter activation by about 60% but did not totally abolish activation. The deletion in the Inr also led to reduced activation, the level of which was similar to that seen with the CRE mutation. On the other hand, the TATA mutant showed only

a minor reduction in promoter activity under these conditions. The RNA initiation site of the TATA mutant was found to be identical to that of the wild-type construct (data not shown). In agreement with the results with a single mutation, the double mutation (mTATA/δInr) led to a reduction of RA activation to a level similar to that found with δInr. The basal activity estimated without RA treatment was roughly constant among these constructs, except for δInr, whose activity was 50% lower than that with other constructs (data not shown). Essentially the same level of induction was observed when luciferase activity was measured 6 h after RA treatment (data not shown). Thus, the mutation in the βRARE abrogated RA activation of the promoter, while mutations in other elements only partially inhibited activation. These results support the primary importance of the βRARE in promoter activation, consistent with the existence of a hierarchy in promoter occupancy.

DISCUSSION

We show that the RARβ2 promoter is unoccupied in P19 EC cells before RA treatment, but factor occupancy is rapidly induced after the treatment at multiple sites in the promoter (Fig. 9A). The occupancy is maintained throughout the duration of RA treatment but reversed upon withdrawal of RA. Further, our data indicate that the occupancy of the βRARE serves as a signal for factor loading onto other sites of the promoter, supporting the primary significance of a liganded heterodimer receptor for activation of this promoter (Fig. 9B).

Ligand-dependent occupancy. The RA-dependent factor occupancy observed in this work highlights the critical role of a ligand for promoter occupancy in vivo. The strict ligand requirement observed for in vivo βRARE occupancy is in contrast to a large body of in vitro observations demonstrating that unliganded RAR/RXR heterodimers readily bind to RAREs (9, 33, 35, 39, 47, 65). The lack of factor occupancy seen prior to RA treatment is not due to the cytoplasmic partitioning of the receptors, as exemplified by the glucocorticoid hormone receptor (GR). Much of the GR is present in the cytoplasm before hormone stimulation, but the GR is translocated into the nucleus following ligand administration (48). However, RAR and RXR are localized in the nucleus even

prior to ligand addition (21, 36). RA induction of factor occupancy is unlikely to be due to increased receptor concentrations in P19 cells, since there was no significant change in the levels of βRARE-binding activity during the initial 4 h of RA treatment (Fig. 6) and since RA-induced occupancy was resistant to CHX treatment (Fig. 2). Our results suggest a mechanism active in vivo but not in vitro that allows only liganded receptors to gain access to the βRARE. The RARβ2 promoter in vivo, like other promoters in the nucleus, is likely to be associated with nucleosomes and to be under the control of a higher-order chromatin structure (24, 40, 64). An in vivo environment may impose a physical constraint on a receptor-DNA interaction not normally observed in vitro. In this regard, RARs and RXRs have been shown to undergo a conformational change upon ligand binding (2, 4; our unpublished observations). Such a change, when occurring in vivo, may confer increased access to the βRARE, although this change does not significantly affect DNA-binding affinity in vitro (4). Alternatively, it is conceivable that molecules associated with receptors such as calreticulin (10, 13) are involved in determining receptor access to the RAREs in vivo.

Hierarchy in RARβ2 promoter occupancy. P19 cells expressing the dominant negative RXRβ DBD⁻ failed to exhibit footprinting at the βRARE, as well as at all other sites of the promoter (Fig. 5). These results indicate that receptor heterodimer occupancy at the βRARE is the primary event that signals factor loading on all other elements. In agreement, the withdrawal of RA from the culture medium promptly abolished factor occupancy not only at the βRARE but also at all other elements (Fig. 1), further indicating that occupancy at these other elements is contingent upon continuous receptor occupancy in the βRARE. The primary significance of the βRARE is also supported by the total abrogation of RA-induced reporter activity upon mutation in this element. Unlike the mutation in the βRARE, mutations in other sites resulted in only a partial reduction in promoter activation (Fig. 8B). On the basis of these observations, it is likely that promoter occupancy follows a hierarchically programmed course of events. That factors specific for these sites are constitutively present (Fig. 6 and 7) but are not loaded prior to RA treatment also supports a hierarchical control of promoter occupancy.

The mechanistic basis for the ordered occupancy is not clear at present. It is possible that the receptor occupancy in the βRARE leads to reorganization of the neighboring nucleosome-chromatin milieu, allowing the opening of other elements in the promoter. A glucocorticoid hormone-induced alteration of the chromatin structure has been well documented for the mouse mammary tumor virus promoter (3, 34, 54). In the mouse mammary tumor virus, promoter binding of the liganded GR to the glucocorticoid hormone-responsive element is shown to cause factor loading on the NF-1 and octamer sites, which is associated with the displacement of a specific nucleosome (3, 54). Similarly, in the rat tyrosine aminotransferase gene binding of the GR to the GR element apparently causes an alteration in the chromatin structure (12, 52), thereby allowing the binding of hepatocyte nuclear factor 5 (HNF5) (55). These changes occur in a hormone-dependent manner. By analogy, it is possible that the RARβ2 promoter undergoes a change in its chromatin structure upon binding of a liganded RXR/RAR to the βRARE, leading to the opening of other elements.

In this context it is noteworthy that Force and Spindler (18) reported that the addition of the thyroid hormone to pituitary cells causes methylation hypersensitivity in the hormone-responsive element as well as in the adjacent Pit-1 element in the

rat growth hormone gene. The results in this report may suggest that the thyroid hormone-responsive promoters are also controlled in a ligand-dependent, hierarchically ordered manner. The opening of other inducible promoters may also be controlled by a similar mechanism. Garrity et al. (19) have recently shown that lymphocyte activation via a membrane T-cell receptor leads to multiple factor-binding events in the interleukin-2 promoter, which is otherwise totally unoccupied before activation. They showed that this induction is a coordinated event, in which factor binding in these elements occurs in concert, in an all-or-none fashion, rather than individually. However, it should be borne in mind that not all inducible promoters undergo an all-or-none change upon stimulation; it has been shown that the human HSP70 gene is occupied at the SP-1 site prior to heat shock (1). In this case the SP-1 binding may render the HSP70 promoter a constitutively open configuration, where it is poised to respond to a heat shock.

Delayed protection in RARβ2 Inr. The Inr in this promoter exhibited unusual protection that appeared only in later stages of RA treatment (Fig. 1). One of the protected G residues was found within the consensus Inr, while the other residue was downstream at +5. In accordance, the EMSA results shown in Fig. 7 revealed the presence of a constitutive factor specific for this region. Inrs have been previously shown to be involved in basal transcription by interacting with TBP and other basal factors (11, 51, 58, 63). Additionally, sequence-specific transcription factors have been shown to bind to some Inrs (41, 56, 57). We show that the binding activity for the RARβ2 Inr is distinct from sequence-specific factors reported above, since Inrs for the AdML, TdT, and P5 promoters all failed to compete for binding (Fig. 7B). Since Inrs of other genes do not readily footprint in vivo by the technique used here, it is possible that the footprinting observed in this work reflects binding of this unique factor as well as basal factors which might have facilitated detection of footprinting. If so, this factor may have been recruited later in RA treatment. At present however, neither the identity of this factor nor the functional significance of the delayed occupancy seen at this Inr is known. The transfection data in Fig. 8 indicate that the Inr is involved in RA activation of RARβ2 promoter activity, which may have a greater role than the TATA box in promoter activity. This Inr is likely to be active in transcription even in an early stage of RA treatment at which protection in vivo under these conditions has not yet been detected, since δInr inhibited reporter transcription measured within 6 h of RA treatment. The delayed recruitment of this factor may have a modulatory effect on transcription of the RARβ2 gene, which may act at the level of formation of the initiation complex, elongation, or pausing.

In summary, this work illustrates a ligand-dependent, hierarchically ordered promoter occupancy for the RARβ2 gene. It would be of importance to study how liganded receptors achieve the initial opening of the promoter.

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