

# Transcriptional Regulation of the Human NRIP1/RIP140 Gene by Estrogen Is Modulated by Dioxin Signalling

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## ABSTRACT

Receptor interacting protein 140 (RIP140) is a negative transcriptional regulator of nuclear hormone receptors that is required for the maintenance of energy homeostasis and ovulation. In this study, we investigated the mechanisms by which RIP140 expression is controlled by estrogens in breast cancer cells. We first analyzed by real time reverse transcription-polymerase chain reaction the regulation of RIP140 mRNA accumulation by estrogen receptor (ER) ligands in MCF-7 cells. We showed that the induction by estradiol (E2) was rapid and did not affect the apparent stability of the mRNA, suggesting a direct transcriptional regulation. To further study the underlying regulatory mechanisms, we then characterized the human RIP140 gene. We identified several noncoding exons with al-

ternative splicing and localized the promoter region more than 100 kilobases upstream from the coding exon. Although we mapped a perfect consensus estrogen response element able to bind ER $\alpha$  in gel shift and in chromatin immunoprecipitation experiments, the effect of E2 on RIP140 gene transcription was very modest. This might result at least in part from the presence of an overlapping aryl hydrocarbon receptor (AhR) binding site, which interfered with the E2 response on both the transiently transfected reporter construct and the accumulation of the endogenous RIP140 mRNA. Altogether, our data indicate that the RIP140 gene exhibits a complex structure with several noncoding exons and supports transcriptional cross-talk and feedback involving the ER $\alpha$  and AhR nuclear receptors.

Estrogens are steroid hormones that regulate proliferation and differentiation of target tissues such as mammary glands, reproductive organs, and skeletal, cardiac, and neural cells. They act mainly by controlling the expression of a number of specific genes through binding to two distinct nuclear estrogen receptors, ER $\alpha$  and ER $\beta$ . These receptors are ligand-activated transcription factors that subsequently bind as homo- or heterodimers to estrogen responsive elements (ERE) located in the regulatory region of target gene promoters. ERs, like other nuclear receptors, stimulate transcription using both a constitutive amino-terminal and a ligand-dependent carboxy-terminal activation function (AF1

and AF2, respectively), the latter being associated with the ligand-binding domain. These activation functions act independently or synergistically, depending on the cell type and promoter context, by recruiting a number of cofactors that are able either to stabilize the transcription preinitiation complex or to alter chromatin structure through histone-modifying enzymes, thus regulating transcription factor accessibility and binding.

RIP140 was one of the first cofactors to be isolated through its recruitment by ER $\alpha$  AF2 in the presence of ligand (Cavailles et al., 1995). It has been shown to interact with many nuclear receptors such as ER $\alpha$ , thyroid hormone receptor, retinoic acid receptor, and retinoid X receptor (L'Horsset et al., 1996), adrenergic receptor (Ikonen et al., 1997), Vitamin D receptor (Masuyama et al., 1997), peroxisome proliferator-activated receptor- $\alpha$ /liver X receptor  $\alpha$  (Miyata et al., 1998), glucocorticoid receptor (Subramaniam et al., 1999), steroidogenic factor 1, and DAX-1 (Sugawara et al., 2001), and with other transcription factors like c-jun (Teyssier et al.,

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**ABBREVIATIONS:** ER, estrogen receptor; RIP140, receptor interacting protein of 140 kDa; ERE, estrogen response element; AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhRE, aryl hydrocarbon receptor core response element; bp, base pair(s); ChIP, chromatin immunoprecipitation; DTT, dithiothreitol; RT-PCR, reverse transcription-polymerase chain reaction; E2, estradiol; AF, activation function; PCR, polymerase chain reaction; RERE, RIP140 estrogen response element; DMEM, Dulbecco's modified Eagle's medium; NRIP1, nuclear-receptor-interacting protein 1; tk, thymidine kinase; kbp, kilobase pair(s); ICI182780, faslodex; SV40, simian virus 40.

2003) or the aryl hydrocarbon receptor (AhR) (Kumar et al., 1999).

Despite being recruited by agonist-liganded receptors as for coactivators, in most cases, RIP140 has been shown to inhibit target gene transcription not only by competing with coactivators (Treuter et al., 1998) but also by active repression, for instance by recruiting histone deacetylases and carboxy-terminal-binding proteins (Castet et al., 2004; Christian et al., 2004).

The RIP140 gene has been mapped to chromosome 21 in a gene-poor region. We and others have described its transcriptional regulation by estrogens (Thenot et al., 1999) or retinoids (Kerley et al., 2001), which confer to RIP140 an important regulatory role in hormone signaling.

In the present study, we have analyzed the mechanisms by which RIP140 expression is regulated by estrogens in breast cancer cells. We first investigated how the accumulation of RIP140 mRNA was controlled by the two ER isoforms and their respective ligands. To further study the regulatory mechanisms, we then cloned the RIP140 gene, characterized the promoter region, and identified a perfect consensus ERE able to bind ER $\alpha$  and support E2 regulation. We showed that RIP140 mRNA was also regulated at the transcriptional level upon activation of the AhR signaling pathway. Finally, we characterized an AhR binding site that overlapped the ERE and interfered with the E2 regulation of the RIP140 gene.

## Materials and Methods

**Plasmids.** The pSG5-ER $\alpha$  vector (HEGO) was given by P. Chambon (Institut de Génétique et Biologie Moléculaire et Cellulaire, Strasbourg, France). The expression vectors for AhR (pSG5-AhR) and aryl hydrocarbon receptor nuclear translocator were, respectively, obtained from J. F. Savouret (Institut National de la Santé et de la Recherche Médicale U530, Paris, France) and M. Daujat (Institut National de la Santé et de la Recherche Médicale U128, Montpellier, France). R900sv and R900tk were derived from the pGL3 promoter (Promega, Charbonnières, France) and pGL3-tk (P. Augereau, unpublished data) vectors by inserting between their MluI and NheI sites a promoter fragment (coordinates, 147502–148401) amplified by PCR from the 270M7 genomic clone (GenBank accession number AF127577); R900 was derived from pGL3-basic (Promega) by inserting into the NheI site, the same amplified promoter fragment. The 5' deletions  $\Delta$ Pac,  $\Delta$ PPc, and  $\Delta$ SPc were produced by removing the distal promoter fragment between a PstI site in the vector and the ApaI, PstI, or SacII site in the R900 promoter, respectively. Internal deletions  $\Delta$ AP and  $\Delta$ SP were produced by removing the ApaI-PstI and the PstI-SacII fragment from the R900 promoter fragment. The RERE-containing reporter vector has been derived from the pGL3-promoter vector by inserting in the NheI site the following double-stranded oligonucleotides: CGCGTGGGGTCAAAGTGACCCAG and CTAGCTGGGTCACTTTGACCCCA.

**RNA Extraction and RT-PCR.** Total RNA was extracted from MCF-7 cells using the TRIzol reagent (Invitrogen, Cergy Pontoise, France). RNA from normal human breast tissue was purchased from Stratagene (Amsterdam, the Netherlands). For RT-PCR, 2  $\mu$ g of total RNA was subjected to a reverse-transcription step using the Superscript II reverse transcriptase (Invitrogen). Real-time PCR quantification was then performed using a SYBR Green approach (LightCycler; Roche Diagnostics, Meylan, France). PCR was carried out in a final volume of 10  $\mu$ l using 0.5  $\mu$ l of each primer (10  $\mu$ M), 2  $\mu$ l of the supplied enzyme mix, 4.5  $\mu$ l of H<sub>2</sub>O, and 2.5  $\mu$ l of the template diluted at 1:20. After a 10-min preincubation at 95°C, runs corresponded to 45 cycles of 15 s each at 95°C (denaturation), 7 s at 57°C (annealing), and 15 s at 72°C (elongation). The RIP140 primers were

GCTGGGCATAATGAAGAGGA and CAAAGAGGCCAGTAATGT-GCTATC. PCR products were subjected to melting-curves analysis using the LightCycler system to exclude the amplification of unspecific products. For each sample, RIP140 mRNA levels were corrected for rS9 mRNA levels (reference gene) and were normalized to a calibrator sample. The primers for the rS9 mRNA are available upon request. Analysis of the 5' end of RIP140 mRNAs was performed using the F1 and R1 primers AGGACGGGGCGGCAGGCG and ACCTTCATCGCAATCAGAGAGACG.

**Cell Culture and Transient Transfection.** MCF-7 and HeLa human cancer cells were derived from stock routinely maintained in the laboratory. Monolayer cell cultures were, respectively, grown in Ham's F-12/Dulbecco's modified Eagle's medium (DMEM) (1:1) or in DMEM supplemented with 10% fetal calf serum (Invitrogen) and antibiotics. Cells were stripped of endogenous estrogens for 5 days using *N,N'*-dicyclohexylcarbodiimide-treated serum as described previously (Cavaillès et al., 1989). For transient transfection experiments, cells were plated at approximately 80% confluence ( $1 \times 10^5$  cells/15-mm diameter well) and transfected in 24-well plates using JetPEI as recommended by Qiagen (Illkirch, France), with CMV- $\beta$ Gal (0.15  $\mu$ g) as an internal control together with the indicated reporter vector (0.25  $\mu$ g) and the different receptor expression plasmids (0.15  $\mu$ g). The total DNA quantity was adjusted to 1  $\mu$ g. Cell extract preparation was carried out as recommended by Promega. Cells were lysed at 4°C for 10 min in 0.15 ml of lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 10% glycerol, and 1% Triton X-100). Luciferase activity was measured in 50- $\mu$ l supernatant aliquots during 2 s after injection of 50  $\mu$ l of luciferase detection solution using a luminometer (Labsystem, Les Ulis, France). Comparing basal levels between different cell lines, transfection data were normalized by the  $\beta$ -galactosidase activities determined as described previously (Castet et al., 2004). The results were expressed as relative luciferase activities and are presented as mean  $\pm$  S.D. Statistical analysis was performed by Student's *t* test, and a value of *p* < 0.05 was considered to be statistically significant.

*Drosophila melanogaster* SL2 cells were grown at 25°C without CO<sub>2</sub> in SF900II medium (Invitrogen) supplemented with penicillin and streptomycin sulfate. SL2 cells ( $5 \times 10^6$ ) were plated in 24-well plates, and transfection was carried out using Lipofectamine 2000. Each well was transfected with 0.25  $\mu$ g of reporter plasmid, 0.625  $\mu$ g of  $\beta$ -galactosidase plasmid, and with pPac-Sp1 (0 to 125 ng) expression vectors adjusted, when necessary, by the addition of empty vector to equalize the total DNA transfected. Twenty-four hours after transfection, the cells were harvested and assayed for luciferase activity as described above.

**Gel-Shift Assay.** Double-stranded oligonucleotides were labeled using Klenow fragment of *Escherichia coli* DNA polymerase and  $\alpha$ -[<sup>32</sup>P]dCTP (PerkinElmer Life and Analytical Sciences, Courtaboeuf, France). Sequences of oligonucleotides were the following: REREs, CGCGTGGGCGTGGGGTCAAAGTGACCCAGAGCCG; REREs, CTAGCGGCTCTGGGTCACTTTGACCCACGCCCA; EREs, CGCGTCTAGAAAGTCAGGTACAGTGACCTGATCAAG; and EREs, CTAGCTTGATCAGGTCACTGTGACCTGACTTTCTAGA.

Nuclear proteins in 25 mM HEPES, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol) were mixed with nonspecific DNA in 10 mM Tris, pH 7.5, 100 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, and 5% glycerol on ice for 30 min. Labeled probe (10<sup>5</sup> cpm) was added for 15 min at room temperature. Complexes were separated on nondenaturing 6% polyacrylamide gel electrophoresis (acrylamide/bisacrylamide, 37.5:1) in 0.5 $\times$  Tris borate-EDTA at 150 V for 2 h, gel-fixed in 40% methanol/10% acetic acid, dried, and exposed overnight.

**ChIP Analysis.** ChIP assays were performed as described by Metivier et al. (2003) with minor modifications. In brief, MCF-7 cells were synchronized by 3 days of culture in DMEM with 3% *N,N'*-dicyclohexylcarbodiimide. They were then treated with 2.5  $\mu$ M  $\alpha$ -amanitin for 2 h and followed or not by exposure to 1 nM E2 for 1 h. After cross-linking with 1.5% formaldehyde at 37°C for 5 min and

quenching with 250 mM glycine for 15 min, cells were resuspended on ice in cell buffer (100 mM Tris-HCl, pH 9.4, and 100 mM DTT) and then incubated on ice for 15 min and at 30°C for 15 min. After immunoclearing with preimmune IgG (Sigma, Lyon, France), 0.05% bovine serum albumin, and 5  $\mu$ g of salmon sperm DNA (Sigma), immunoprecipitation was performed overnight using 50  $\mu$ l of protein G Sepharose beads (Amersham, Freiburg, Germany) and 2  $\mu$ g of HC20 anti-ER $\alpha$  (Tebu-bio, Le Perray en Yvelines, France) or anti-acetyl-histone H3 (06-942) from Upstate (Mundolsheim, France). Beads were washed in buffer I (2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, and 150 mM NaCl), buffer II (2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% Triton X-100, and 500 mM NaCl), buffer III (1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 1% deoxycholate, and 0.25 M LiCl), and twice with Tris-EDTA buffer. Washed resin was resuspended in elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>, and 0.01 M DTT) with 30-min incubation and reverse-cross-linked at 65°C overnight in the presence of 0.2 M NaCl. After digestion with proteinase K, DNA samples were extracted with phenol/chloroform, and the DNA was precipitated overnight. After resuspension in 40  $\mu$ l of Tris-EDTA buffer, PCR were performed using 5  $\mu$ l of DNA solution and analyzed on agarose gel electrophoresis. ChIP primers were CTCCCAGAGTCGCTCCACACGAGT and GG-GACCCAGGCCGAATGCTC.

## Results

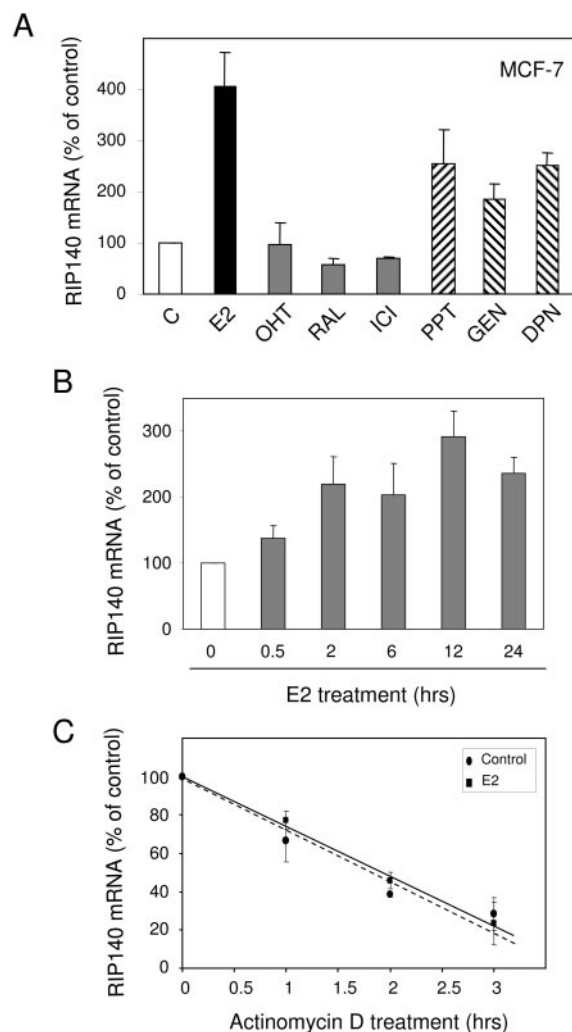
**Regulation of RIP140 mRNA Accumulation by Estrogens.** We reported previously that RIP140 gene expression was under E2 regulation in human breast cancer cells (Thenot et al., 1999). To define more precisely the mechanisms of this regulation, we tested various agonist ligands either specific for ER $\alpha$  such as 4,4',4''-(4-propyl-(1*H*)-pyrazole-1,3,5-triyl)-trisphenol or which preferentially activate ER $\beta$ , such as genistein or 2,3-bis(4-hydroxy-phenyl)-propionitrile. Figure 1A shows that in conditions in which 10 nM estradiol induced approximately 4-fold the RIP140 mRNA steady-state level, the other agonists were only 30 to 50% as potent. In contrast, ER antagonist ligands had no effect or were slightly inhibitory. A similar relative increase of RIP140 mRNA levels by estradiol was observed in two human ovary cell lines (data not shown), indicating that E2 regulation of RIP140 expression was not restricted to mammary cancer cells.

We showed previously in MCF-7 breast cancer cells that estradiol induction of RIP140 mRNA levels was independent of protein synthesis (Thenot et al., 1999); accordingly, the increase of the RIP140 mRNA level was rapid because it was observed as early as 30 min after estradiol addition to the medium (Fig. 1B). To define whether part of this regulation resulted from an increase in RIP140 mRNA stability, we performed actinomycin D chase experiments. When MCF-7 cells were treated with the transcription inhibitor, the apparent RIP140 mRNA half-life was short ( $\sim$ 2 h) and not affected by estradiol (Fig. 1C). Altogether, these results indicated that estrogen regulation of RIP140 gene expression was a primary transcriptional event.

**Characterization of the Human RIP140 Gene.** The gene encoding RIP140, which is also known as nuclear-receptor-interacting protein 1 (NRIP1), has been localized on human chromosome 21 (Katsanis et al., 1998; and Fig. 2). Although it was initially believed that this gene was monoxonic, a search for expressed sequence tags databases (<http://www.ncbi.nih.gov/EST>) identified transcripts initiated approximately 100 kbp upstream from the coding exon. Using RT-PCR with primers in putative exon 1 and in the

downstream coding exon, we confirmed the existence of three short noncoding exons. As shown in Fig. 2B, three major cDNA fragments of 304, 384, and 506 bp were detected. Sequencing of the corresponding bands revealed mRNA species containing exons 1 + 4, 1 + 2 + 4, and 1 + 2 + 3 + 4, respectively, thus indicating the existence of alternatively spliced mRNA species.

The genomic clone containing the totality of the RIP140 gene (GenBank accession number AF127577) was tested for the presence of a promoter using Promoter Inspector from the Genomatix suite (<http://www.genomatix.de>). We found only one characteristic promoter in the vicinity of exon 1. Because this DNA region seemed highly G+C-rich, we also searched for CpG island, and again, we found only one such



**Fig. 1.** Regulation of RIP140 mRNA by estrogens in MCF-7 cells. **A**, MCF-7 cells were treated for 24 h with control vehicle ethanol (C) or various ER ligands (10 nM). RIP140 mRNA levels were quantified by real-time quantitative RT-PCR as described under *Materials and Methods*. The results are expressed in arbitrary units after normalization by rS9 mRNA levels. Values are the means  $\pm$  S.D. of three independent experiments. **B**, MCF-7 cells were treated or not with E2 (10 nM) for the indicated period of time. RIP140 mRNA levels were quantified as described under *Materials and Methods*. **C**, MCF-7 cells were treated or not with E2 (10 nM) for 24 h and then incubated for different times (0, 1, 2, or 3 h) with actinomycin D (3  $\mu$ g/ml) before RNA extraction. RT-PCR was performed with total RNA to quantify RIP140 mRNA levels. The results are expressed in arbitrary units after normalization by rS9 mRNA levels. Values are the means  $\pm$  S.D. of three independent experiments.



feature overlapping the putative promoter region in the whole sequence of AF127577. Compared among human, chimpanzee, and mouse, this noncoding region (Fig. 2) seemed highly conserved, with more than 65% identity between mouse and human (Fig. 3). Then, we searched for transcription factor binding sites in this region using MatInspector from the Genomatix suite and found several putative response elements for general transcription factors such as Sp1 and CAAT binding sites (Fig. 3).

Next, we tested the 900-bp sequences upstream from exon 1, encompassing most of the conserved region (Fig. 2), for promoter activity in transient transfection experiments in various cell lines. The corresponding DNA fragment was introduced upstream from the luciferase gene in the pGL3 vector (Promega) to generate the R900 reporter construct. As shown in Fig. 4B, when tested in transient transfection in HeLa cells, this DNA fragment acted as a promoter, approximately as good as the SV40 (pGL3p) or the thymidine kinase (pGL3tk) promoters. Moreover, transcription from this promoter was efficiently stimulated by the SV40 enhancer (compare R900, pGL3E, and R900E in Fig. 4B).

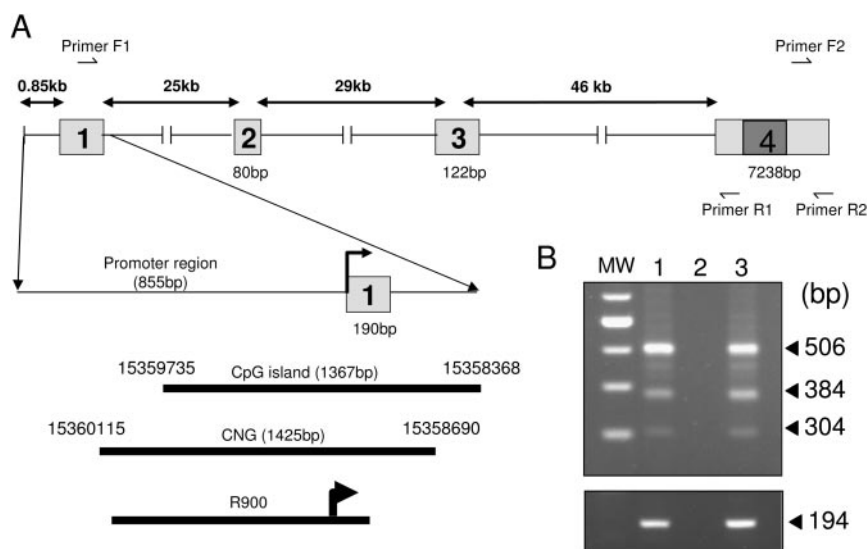
To identify important regions of the promoter, we then deleted various parts of the R900 DNA fragment and tested the resulting recombinants (described in Fig. 4A) in transient transfection in MCF-7 cells. As shown in Fig. 4C, progressive 5'-deletions identified two regions involved in promoter activity. Indeed, when deleted, the distal  $\Delta$ Pac and the central  $\Delta$ SP regions significantly decreased luciferase activity. In contrast, removal of the ApaI-PstI sequence had little effect (compare  $\Delta$ Pac and  $\Delta$ PPc). These results were supported by internal deletions that emphasized the importance of the PstI-SacII region (compare  $\Delta$ AP and  $\Delta$ SP mutants). In addition, the very proximal region contained in the  $\Delta$ PSc construct exhibited a basal promoter activity. This basal level could result from Sp1 transcriptional activity as expected from the presence of several Sp1 sites (Fig. 3B). In support of this hypothesis, the R900 recombinant was stimulated upon cotransfection in Sp1-defective SL2 insect cells with increasing doses of a plasmid expressing the Sp1 factor (Fig. 4D). The regulation was comparable with that observed on the p21<sup>WAF1/CIP1</sup> promoter used as a positive control. Altogether,

these data demonstrate that several regions contribute to the overall RIP140 promoter activity.

**Mechanism of the Transcriptional Regulation by Estrogens.** Several putative binding sites for nuclear receptors were found in the R900, and we identified a consensus palindromic ERE (GGGTCAxxxTGACCC) located in the distal part of the R900 DNA fragment (between coordinates 148309 and 148321 on the AF127577 BAC genomic clone). To investigate the functionality of this ERE, we first performed in vitro binding using gel-shift assay. As shown in Fig. 5A, ER $\alpha$  produced on the RIP140 ERE (RERE) a complex (lane 2) that was supershifted by an anti-ER $\alpha$  antibody (lane 3); moreover, this complex was titrated out either by the RERE itself (lanes 4–7) or by the ERE from the *Xenopus* vitellogenin A2 gene (vitERE, lanes 8–10), but not by an unrelated sequence (data not shown); as shown in lanes 4 to 10, approximately 4-fold more RERE was necessary to inhibit complex formation compared with the vitERE, suggesting a slightly reduced affinity of the former for ER $\alpha$ .

ER $\alpha$  binding to the RIP140 promoter region containing the RERE was also observed in intact cells, using chromatin immunoprecipitation, with the HC20 anti-ER $\alpha$  antibody. As seen in Fig. 5B, estradiol increased ER $\alpha$  recruitment to the RIP140 promoter region encompassing the RERE. In parallel, we assessed histone acetylation using an antiacetylated histone H3-K9 antibody and found that E2 stimulation increased the level of histone acetylation on the same RIP140 promoter region. Thus, both in vitro and in situ experiments demonstrated the binding of ER $\alpha$  to the RIP140 ERE after estradiol stimulation.

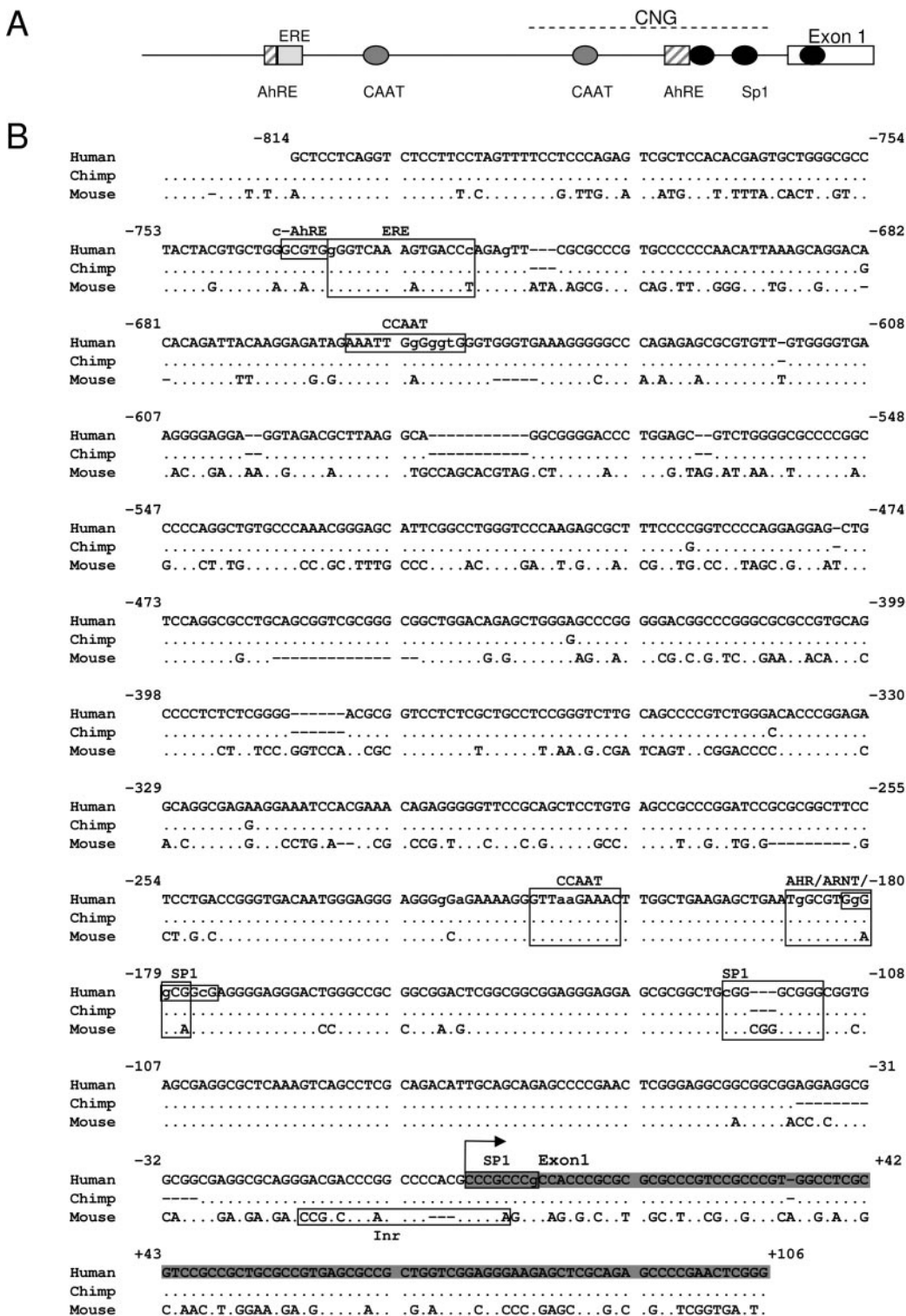
We then confirmed that the RIP140 ERE was able to sustain increased transcription when stimulated by estradiol after transient transfection in several cell lines (Fig. 6A; data not shown). When isolated in front of the SV40 early promoter of the pGL3-promoter vector, the RERE induced transcription approximately five times as efficiently as the vitERE (Fig. 6A). In contrast, the R900 construct containing the proximal promoter region of the RIP140 gene was unresponsive to estrogens (Fig. 6A). The same lack of response was observed whether this region was cloned upstream from the SV40 early promoter (R900sv compared with pGL3p as a



**Fig. 2.** Structure of the human RIP140 gene. A, the overall exon-intron structure with the corresponding sizes is shown together with the position of the two pairs of primers used in B. The promoter region is enlarged below to localize, respectively, the CpG island, the interspecific conserved region, and the position of the reference fragment used in this study. B, alternative splicing of RIP140 mRNA. Analysis was performed using RNA from MCF-7 cells (lane 1), normal breast tissue (lane 2), or in the absence of RNA (lane 3). RT-PCR was done as described under *Materials and Methods* using primers F1 and R1 (respectively, in exons 1 and 4 as shown in A). The 304-, 384-, and 506-bp PCR products corresponded to splicing using exons 1 + 4, 1 + 2 + 4, and 1 + 2 + 3 + 4, respectively. The 194-bp PCR fragment corresponded to amplification in the coding exon using primers F2 and R2 in exon 4. The molecular weight marker (MW) shown on the left was the 50-bp DNA ladder from Invitrogen.

The same R900 construct, when stably transfected in MCF-7 cells (Fig. 6B), raised a reproducible and significant regulation by ER ligands comparable with that obtained for the endogenous RIP140 gene (i.e., induction by E2 and decrease by the pure antagonist ICI182780). Altogether, these

**Cross-Talk with AhR Regulation.** To understand why the RIP140 gene was only weakly induced by estradiol despite the presence of a strong consensus ER $\alpha$  binding site, we searched for closely located or overlapping transcription binding sites. We found such a site corresponding to an AhR core response element (AhRE) immediately adjacent to the



**Fig. 3.** Sequence of the human RIP140 promoter region. A, schematic drawing of the RIP140 promoter region; boxes indicate putative transcription factor binding sites shown in B. The conserved noncoding region of 205 bp with more than 90% homology between human and mouse is shown. B, sequence comparison of the RIP140 promoter region between human, chimpanzee, and mouse; exon 1 sequences are shaded; the putative ERE and some general transcription factor binding sites are boxed; coordinates of the human sequence are given relative to the 5'-end of NRIP1 exon 1 as reported in the AceView database (<http://www.ncbi.nlm.nih.gov/IEB/Research/AceView>).

upstream ERE boundary (Figs. 3 and 7A). Another potential AhRE was present in the central PstI-SacII region, which was shown to be important for basal activity of the promoter (Fig. 4, A and D).

To demonstrate the functionality of these AhR response elements, we first tested whether AhR ligands could regulate RIP140 expression in breast cancer cells. As shown in Fig. 7B, treatment of MCF-7 cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) increased 2-fold RIP140 mRNA steady-state level. This regulation was no longer observed when TCDD effect was tested in the presence of E2. As a consequence, the amplitude of the regulation of RIP140 mRNA accumulation by E2 was significantly lower upon activation of AhR. These data therefore suggested that the two pathways could interfere in terms of regulation of RIP140 expression.

To confirm this hypothesis, we first checked that the distal AhRE (overlapping with the RERE; Fig. 7A) was responsive to dioxin treatment. Figure 7C showed that in MCF-7 cells, endogenous AhR was sufficient to mediate a significant 2-fold induction by TCDD. A similar regulation was observed in HeLa cells (data not shown). At the same time, the regulation of RERE transactivation by E2-activated ER $\alpha$  was significantly reduced. This effect was reminiscent of the regulation of RIP140 mRNA (Fig. 7B). The transcriptional interference was even more pronounced when AhR was overexpressed (Fig. 7C).

To demonstrate the role of the distal AhRE in E2 regulation of the RERE reporter construct, we introduced a single nucleotide deletion of the central T (labeled with an asterisk in Fig. 7A) in the core AhRE sequence, which changed from GCGTG to GCGG. As expected, the corresponding REREmut reporter was clearly less responsive to TCDD than was the construct containing the wild-type sequence. Concomitantly,

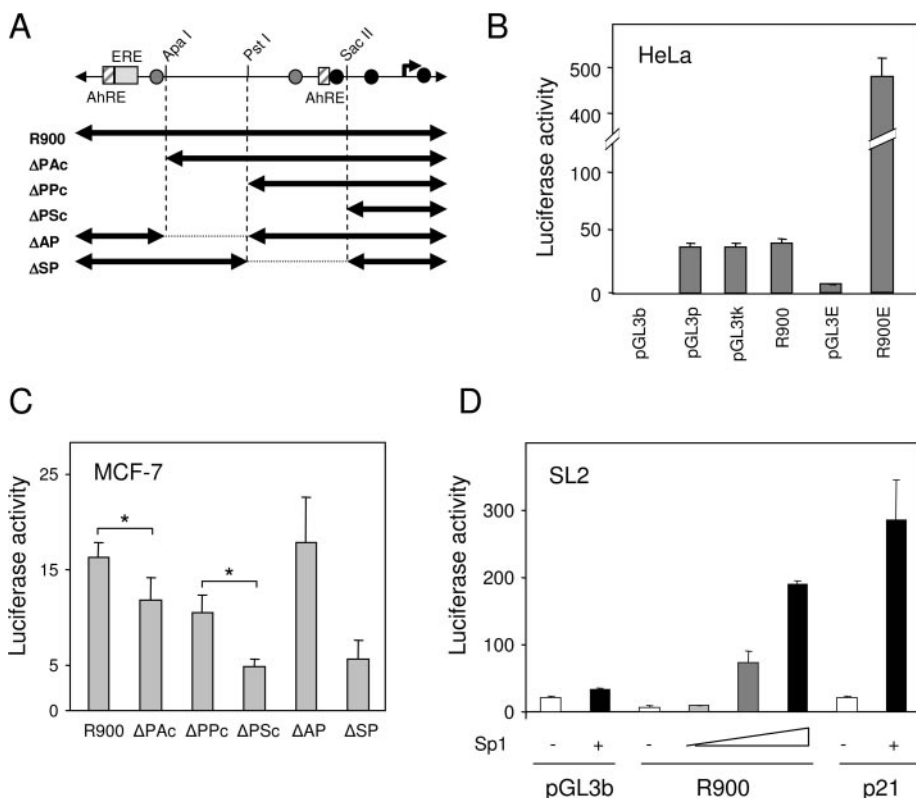
the response to E2 was severely decreased, suggesting that the AhRE is part of the E2-responsive unit of the RIP140 gene. Similar results were obtained with another mutant sequence in which the central G was deleted (data not shown). Altogether, these data demonstrated that RIP140 is a dioxin target gene and that the AhR signaling modulates its regulation by E2.

## Discussion

RIP140 is an atypical transcription regulator that could be considered an anticoactivator because it exhibits an agonist-dependent recruitment by nuclear receptors but negatively regulates their transcriptional activation. In this study, we characterized the E2 induction of RIP140 mRNA accumulation and defined at the transcriptional level the molecular mechanisms involved in this regulation.

First, our results demonstrate that the RIP140 mRNA is produced from several exons, with the downstream exon containing the complete coding sequence. The RIP140 promoter is localized 100 kbp upstream from this coding exon. This observation was unexpected because it was not consistent with results published previously (Kerley et al., 2001) that localized the RIP140 promoter approximately 5 kbp upstream from exon 4. In fact, we have tested the corresponding DNA fragment and found no promoter activity associated with this region of the gene in any of the cell lines analyzed (P. Augereau, unpublished results). The physiological relevance of the existence of several 5'-noncoding exons is currently under investigation. The alternative splicing that we evidenced could be related to the control of RIP140 mRNA stability or translation efficiency.

The characterization of the promoter region of the human



**Fig. 4.** Characterization of the RIP140 promoter. A, structure of the R900 promoter region and the various derivatives used in this study. Their sequence composition is figured by the double-headed arrows. Regulatory sites on the first line are shown as in Fig. 3A. B, promoter activity of the R900 RIP140 genomic DNA fragment compared with that of the corresponding vectors. HeLa cells were transfected as described under *Materials and Methods* with the indicated plasmid. The pGL3p and pGL3tk contained, respectively, the SV40 and the thymidine kinase promoters, whereas the pGL3b plasmid was used as a negative control. The pGL3E and R900E plasmids corresponded to the pGL3b and R900 vectors with the SV40 enhancer. Relative luciferase activity is expressed as the mean ratio of luciferase activity to that of the  $\beta$ -galactosidase produced by the CMV- $\beta$ -galactosidase internal control plasmid in the same point. C, comparison of the relative promoter activity of the R900 promoter fragment with its deleted counterparts. Transient transfection of MCF-7 cells and relative luciferase activity were performed as above (\*,  $p < 0.05$ ). D, stimulation of R900 promoter fragment activity by the Sp1 factor. SL2 cells were transfected with the indicated plasmid together with various amounts of the Sp1 expression vector.

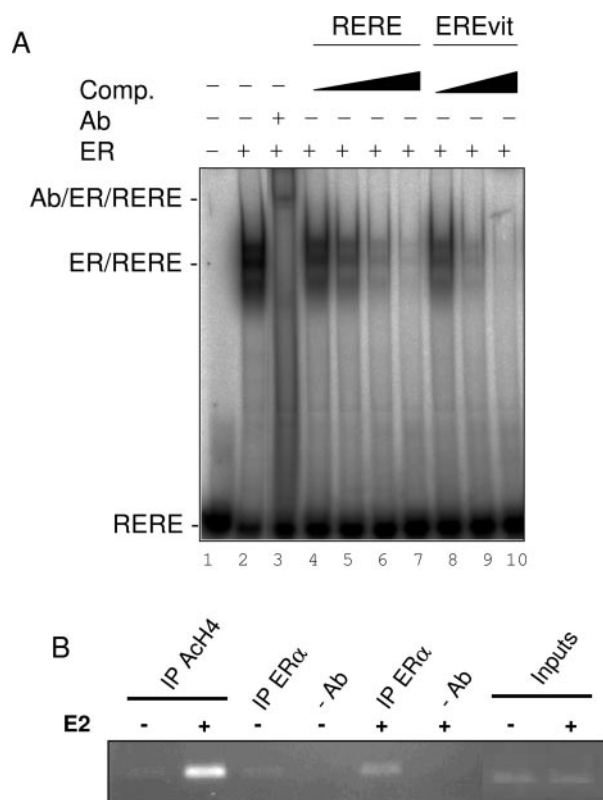


RIP140 gene also revealed the presence of a CpG island usually associated with housekeeping genes (Antequera, 2003), which is in accordance with the ubiquitous expression of the RIP140 mRNA. DNA hypermethylation in CpG-rich promoters is frequently observed in cancer (Issa, 2004), and it would be of interest to analyze whether the RIP140 gene could be methylated and epigenetically silenced in some tumors. We also noticed in the RIP140 gene promoter a non-transcribed region which exhibited an extremely high degree of interspecies conservation. Such conserved nongenic regions are single-copy sequences which represent approximately 1 to 2% of the human genome. The role of these sequences is not yet fully understood, but they could be associated with phenotypic variability and human disorders (Dermitzakis et al., 2005).

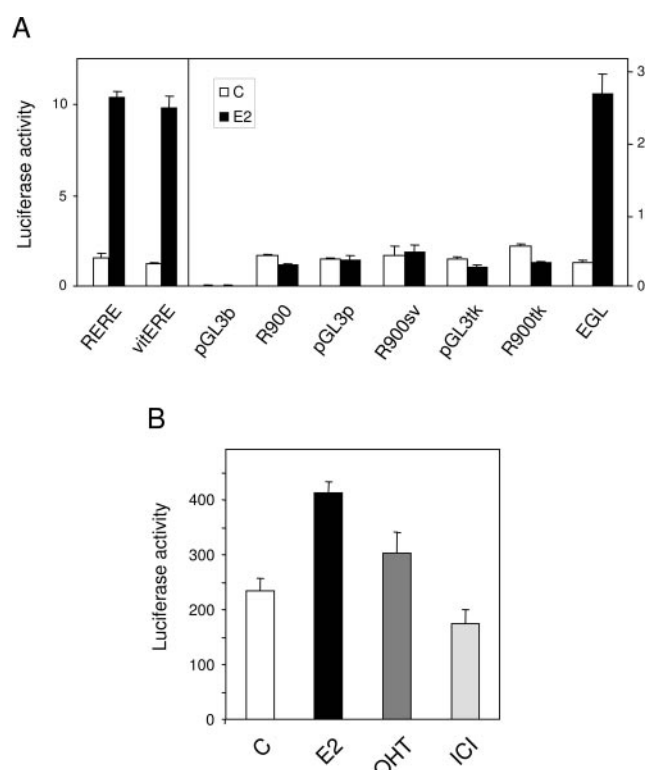
The present work indicates that, in breast cancer cells, the induction of RIP140 by estrogens occurs at the transcriptional level. The identification of an ERE with a consensus sequence, approximately 700 bp upstream from the putative initiation site, was quite unexpected because until now, only very few human genes [Efp (Inoue et al., 1993), COX7RP, or Cytos VIIa (Watanabe et al., 1998)] have been shown to

possess such a consensus element. The RIP140 ERE sequence bound the ER $\alpha$  efficiently, both in vitro and in intact cells, and allowed a mean 5-fold activation of the SV40 early promoter in transient transfection. Unexpectedly, a construct containing the entire RIP140 promoter region was completely unresponsive to estrogen induction in transient transfection. When stably introduced in the genome, the same R900 construct restored a significant regulation by ER ligands, suggesting a potential role of chromatin structure in E2 regulation of the RIP140 promoter. The regulation of the stably transfected R900 was equivalent to that of the endogenous RIP140 gene (Fig. 1), strongly suggesting that all of the regulatory elements necessary for E2 induction were located in the R900 sequence. However, an apparent discrepancy remained between the existence of a perfect ERE and the weak E2 response of the promoter, suggesting that other elements could modulate the hormonal regulation of RIP140 expression.

Upon closer examination of the sequences encompassing the RIP140 ERE, we noticed that it was bordered upstream by an AhR core binding site. Both the analysis of the endogenous RIP140 gene and transient transfection experiments using the fragment containing the AhR response element (Fig. 7) indicated that TCDD regulates by 2-fold RIP140 expression. A large number of studies have reported interferences between AhR and ER signaling, which lead to an

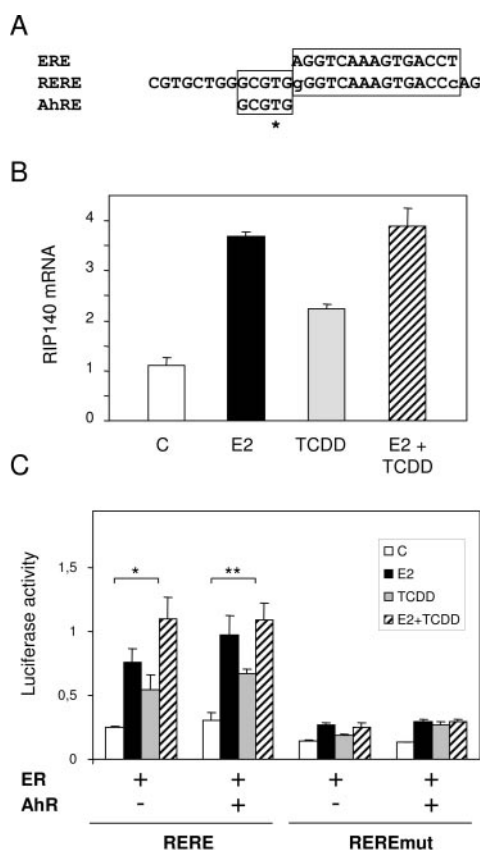


**Fig. 5.** Binding of ER $\alpha$  on the RIP140 ERE. A, electromobility shift assay. Double-stranded RERE oligonucleotide labeled with [ $\alpha$ - $^{32}$ P]dCTP using T4 DNA polymerase was incubated with the proteins indicated above the autoradiogram in 50 mM Tris HCl, pH 7.5, 2.5 mM EDTA, 2.5 mM DTT, 100 mM KCl, 2  $\mu$ g of poly(dIdC), and 24% glycerol and separated on 6% polyacrylamide, 0.5 $\times$  Tris borate-EDTA. H-184 anti-ER $\alpha$  antibody and competitor sites (equimolar amount with the probe in lanes 4 and 8, and excess of 5-fold in lanes 5 and 9, 25-fold in lanes 6 and 10, and 100-fold in lane 7) were added as indicated in the incubation mixes 5 min before the radioactive probe. Positions of free probe and DNA complexes are indicated on the left. B, chromatin immunoprecipitation. Cells were incubated with the hormone as shown, and chromatin was immunoprecipitated with the indicated antibody; PCR was performed as described under *Materials and Methods*, and the product was separated on agarose gel.



**Fig. 6.** Localization of the estrogen responsive element. A, E2 responsiveness measured after cotransfection of the recombinants in MCF-7 cells together with the HEG0 ER $\alpha$  expression vector. Relative luciferase activity is expressed as in Fig. 4. B, E2 responsiveness of the R900 promoter fragment stably transfected in MCF-7 cells. Transfected cells were treated by ethanol vehicle (C), 10 nM E2, 4-hydroxy-tamoxifen (OHT), or ICI182780 (ICI) for 24 h. Relative luciferase activity was measured as described under *Materials and Methods* and expressed as in Fig. 4. Statistical analysis performed either using Student's *t* test ( $p < 0.05$ ) or analysis of variance ( $F = 74$ ) indicated that the four means were significantly different.

inhibition of E2-induced gene expression and cell proliferation by TCDD. In the case of RIP140 gene, the cross-talk seemed slightly different because, although we failed to detect an antiestrogenic effect of TCDD, the E2 regulation of RIP140 expression was lost upon AhR activation. Based on the close vicinity of the two response elements, we believed that endogenous AhR could be involved in the low response to E2, despite the presence of a perfect consensus ERE. As shown in Fig. 7, mutation of the AhRE did not exacerbate the regulation by ER but instead decreased the amplitude of the E2 response. Because the cross-talk between the ER and AhR pathways involves protein-protein interactions between the two receptors on both EREs (Ohtake et al., 2003) and AhREs (Beischlag and Perdew, 2005), it will be important to define which complexes are formed on the RIP140 promoter (involving interactions of ER and AhR with DNA and with each other). In addition, we are currently investigating how RIP140 participates in the control of these different complexes on its own promoter.



**Fig. 7.** Effect of AhR on RIP140 regulation by E2. **A**, sequence of the region overlapping the ERE and the AhRE in the RIP140 promoter. Position of the single nucleotide deletion introduced in the RERE is indicated below the AhRE sequence. **B**, MCF-7 cells were treated for 24 h with control vehicle ethanol (C), E2 (10 nM), or TCDD (100 nM), as indicated. RIP140 mRNA levels were quantified by real-time quantitative RT-PCR as described under *Materials and Methods*. The results are expressed in arbitrary units after normalization by rS9 mRNA levels. Values are the means  $\pm$  S.D. of three independent experiments. **C**, the RERE and its mutated version RERE mutant reporter plasmids were transiently transfected in steroid-stripped MCF-7 cells either with the ER $\alpha$  (HEG0) alone or with both ER $\alpha$  and AhR/aryl hydrocarbon receptor nuclear translocator expression vectors. Cells were stimulated with the indicated hormone combination, and relative luciferase activity was measured as above (statistical analysis was performed for the RERE on the -fold induction by E2 in the absence or presence of TCDD; \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ ).

The regulation of RIP140 gene expression by TCDD thus provides another model of a regulatory loop involving RIP140. Several negative feedback regulations have been suggested that involve the induction of RIP140 expression by estrogens (Thenot et al., 1999), retinoids (Kerley et al., 2001), or, more recently, androgens (S. Carascossa, unpublished results). All of these regulations are associated with a negative control of the corresponding receptors upon overexpression of RIP140. Concerning the mechanism of retinoic acid induction, it remains questionable because our data located the promoter 100 kb upstream from the region proposed by Kerley et al. (2001), and further work is in process to determine whether the regulation by retinoids occurs through the promoter we have identified. In the case of TCDD (and contrary to what occurs for estrogens and retinoids), it seems that up-regulation of RIP140 expression could rather lead to an increase of AhR transactivation (Kumar et al., 1999). However, the TCDD-mediated increase in RIP140 expression could lead to a transrepression of nuclear receptors such as ERs and thus participate in the antiestrogenic effect of AhR (Safe et al., 1998).

Altogether, our results indicate that RIP140 is both an E2- and dioxin-induced gene and that both signals intimately interfere. Other response elements in the proximal promoter region could also attenuate ER $\alpha$  transactivation, and further work is in progress to identify such sequences. To summarize, the structure of the RIP140 gene is more complex than initially believed, and its expression seems to be subtly regulated at the transcriptional level by estrogens and dioxin. The various interferences and regulatory loops that are evidenced could have a particular significance in the regulation of breast cancer cell proliferation by hormones and environmental contaminants.

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