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TRANSCRIPTIONAL REGULATION OF THE HUMAN NRIP1/RIP140 GENE BY ESTROGEN IS MODULATED BY DIOXIN SIGNALLING[#]

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Running title: Human RIP140 gene regulation by estrogens and dioxin

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Non-standard abbreviations:

RIP140, receptor interacting protein of 140 kDa; ER, estrogen receptor;

ERE, estrogen response element; CNG, conserved non-coding region;

AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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ABSTRACT

Receptor interacting protein 140 (RIP140) is a negative transcriptional regulator of nuclear hormone receptors which is required for the maintenance of energy homeostasis and ovulation. In this study, we have investigated the mechanisms by which RIP140 expression is controlled by estrogens in breast cancer cells. We first analyzed by real time RT-PCR 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 non-coding exons with alternative splicing and localized the promoter region more than 100kb upstream from the coding exon. Although we mapped a perfect consensus estrogen response element able to bind ERα 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 AhR binding site which interfered with the E2-response both on the transiently transfected reporter construct and on the accumulation of the endogenous RIP140 mRNA. Altogether, our data indicate that the RIP140 gene exhibits a complex structure with several non coding exons and supports transcriptional cross-talk and feed-back involving the ERα and AhR nuclear receptors.

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Estrogens are steroid hormones that regulate proliferation and differentiation of target tissues such as mammary glands, reproductive organs, as well as skeletal, cardiac and neural cells. They act mainly by controlling the expression of a number of specific genes through the binding to two distinct nuclear estrogen receptors, ERα and ERβ. These receptors are ligand-activated transcription factors which subsequently bind as homo- or hetero-dimers 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 cofactor to be isolated through its recruitment by ERα AF2 in the presence of ligand (Cavailles *et al.*, 1995). It has been shown to interact with many nuclear receptors such as ERα, TR, RAR and RXR (L'Horset *et al.*, 1996), AR (Ikonen *et al.*, 1997), VDR (Masuyama *et al.*, 1997), PPARα/LXRα (Miyata *et al.*, 1998), GR (Subramaniam *et al.*, 1999), SF1 and DAX-1 (Sugawara *et al.*, 2001), and with other transcription factors like c-jun (Teyssier *et al.*, 2003) or the aryl hydrocarbon receptor (AhR) (Kumar *et al.*, 1999).

In spite of 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

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instance by recruting histone deacetylases (HDACs) and carboxy-terminal-binding proteins (CtBPs) ((Christian *et al.*, 2004), (Castet *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 signalling.

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 ERα and support E2-regulation. Interestingly, 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 which overlapped the ERE and interfere with the E2 regulation of the RIP140 gene.

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MATERIALS AND METHODS

Plasmids

The pSG5-ERα vector (HEGO) was given by P Chambon (IGBMC, Strasbourg, France). The expression vectors for AhR (pSG5-AhR) and ARNT were respectively obtained from JF Savouret (INSERM U530, Paris) and M Daujat (INSERM U128, Montpellier). R900sv and R900tk were derived from the pGL3-promoter (Promega corp.) and pGL3-tk (unpublished) vectors by inserting between their Mlu I and Nhe I sites a promoter fragment (coordinates : 147502-148401) amplified by PCR from the 270M7 genomic clone (Accession number: AF127577); R900 was derived from pGL3-basic (Promega corp.) by inserting into the Nhe I site, the same amplified promoter fragment. The 5' deletions ΔPAc, ΔPPc, and ΔPSc were produced by removing the distal promoter fragment between a Pst I site in the vector and the Apa I, Pst I or Sac II site in the R900 promoter respectively. Internal deletions ΔAP and ΔSP were produced by removing the Apa I-Pst I and the Pst I-Sac II fragment from the R900 promoter fragment. The RERE-containing reporter vector has been derived from the pGL3-promoter vector by inserting in the Nhe I 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 µg of total RNA was subjected to a reverse transcription step using the Superscript II reverse transcriptase (InVitrogen, Cergy Pontoise, France). Real-time PCR quantification was then performed using a SYBR Green approach (Light Cycler; Roche Diagnostics, Meylan, France). PCR was carried out in a final volume of 10µl using 0.5µl of each

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primer (10µM), 2µl of the supplied enzyme mix, 4.5µl of H2O and finally 2.5µl of the template diluted at 1:20. After a 10 min pre-incubation at 95°C, runs corresponded to 45 cycles of 15s at 95°C (denaturation), 7s at 57°C (annealing) and 15s at 72°C (elongation). The RIP140 primers were GCTGGGCATAATGAAGAGGA and CAAAGAGGCCAGTAATGTGCTATC. PCR products were subjected to melting curves analysis using the light cycler system to exclude the amplification of unspecific products. For each sample, RIP140 mRNA levels were corrected for rS9 mRNA levels (reference gene) and 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 ACCTTCCATCGCAATCAGAGAGAGACG.

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 (1:1) (F12/DMEM) or in DMEM supplemented with 10% foetal calf serum (FCS) (InVitrogen, Cergy-Pontoise, France) and antibiotics. Cells were stripped of endogenous estrogens for 5 days using DCC treated serum as described (Cavailles *et al.*, 1989). For transient transfection experiments, cells were plated at about 80% confluence ($1x10^5$ cells/15mm-diameter well) and transfected in 24-well plates using JetPEI as recommended by Qbiogene (Illkirch, France), with CMV-βGal (0.15µg) as an internal control together with the indicated reporter vector (0.25µg) and the different receptor expression plasmids (0.15µg). The total DNA quantity was adjusted to 1µg. Cell extract preparation was carried out as recommended by Promega Corporation (Charbonnieres, France). Cells were lysed at 4°C for 10mn in 0.15ml of lysis buffer (25mM Tris pH7.8, 2mM EDTA, 10% glycerol, 1% Triton-X100). Luciferase activity

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was measured in 50µl supernatant aliquots during 2 seconds after injection of 50µl of luciferase detection solution using a luminometer (Labsystem, Les Ulis, France). When comparing basal levels between different cell lines, transfection data were normalised by the β-galactosidase activities determined as described (Castet *et al.*, 2004). The results were expressed as relative luciferase activities and presented as mean ± SD. Statistical analysis was performed by Student's *t*-test and a value of $p < 0.05$ was considered to be statistically significant.

Drosophila SL2 cells were grown at 25°C without CO_2 in SF900II medium (InVitrogen, Cergy-Pontoise, France) supplemented with penicillin and streptomycin sulfate. SL2 cells (5×10^6) were plated in 24-well plates and transfection was carried out using Lipofectamin 2000. Each well was transfected with 0.25µg of reporter plasmid, 0.625µg of β-Gal plasmid and with pPac-Sp1 (0 to 125ng) 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 *E. coli* DNA polymerase and α[^{32}P]dCTP (PerkinElmer, Courtaboeuf, France). Sequences of oligonucleotides were as follow:

RERE*s* : CGCGTGGGCGTGGGGTCAAAGTGACCCAGAGCCG

RERE*as* : CTAGCGGCTCTGGGTCACTTTGACCCCACGCCCA

ERE*s*: CGCGTCTAGAAAGTCAGGTCACAGTGACCTGATCAAG

ERE*as*: CTAGCTTGATCAGGTCACTGTGACCTGACTTTCTAGA

Nuclear proteins in (25mM Hepes pH 7.6, KCl 40mM, EDTA 0.1mM, DTT 1mM, 10% glycerol) were mixed with non-specific DNA in 10mM Tris pH 7.5, 100mM KCl, 0.5mM EDTA, 0.5mM DTT, 5% glycerol on ice for 30 min. 10^5 cpm of labeled probe

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was added for 15 min at room temperature. Complexes were separated on non-denaturing 6% PAGE (acrylamide/bisacrylamide 37.5:1) in 0.5xTBE at 150 V for 2 hours, gel fixed in 40% methanol/10% acetic acid, dried and exposed overnight.

ChIP analysis

Chip assays were performed as described in Metivier *et al.* (Metivier *et al.*, 2003) with minor modifications. In brief, MCF-7 cells were synchronized by a 3 days of culture in DMEM 3% DCC. They were then treated with 2.5µM α-amanitin for 2hr, and followed or not by exposure to 1nM E2 for 1h. After crosslinking with 1.5% formaldehyde at 37°C for 5 min and quenching with 250mM glycine for 15 min, cells were resuspended on ice in Cell buffer (100mM Tris-HCl pH 9.4, 100mM 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 % BSA, and 5µg salmon sperm DNA (Sigma), immunoprecipitation was performed overnight using 50µl protein G sepharose beads (Amersham, Freiburg, Germany) and 2µg of HC20 anti-ERα (Tebu-bio, Le Perray en Yvelines, France) or anti-acetyl-histone H3 (06-942) from Upstate (Mundolsheim, France). Beads were washed in buffer I (2mM EDTA, 20mM Tris-HCl (pH 8.0), 0.1% triton X-100, 150mM NaCl), buffer II (2mM EDTA, 20mM Tris-HCl (pH 8.0), 0.1% SDS, 1% triton X-100, 500mM NaCl), buffer III (1mM EDTA, 10mM Tris-HCl (pH 8.0), 1 % NP-40, 1 % deoxycholate, 0.25M LiCl), and twice with TE buffer. Washed resin was resuspended in elution buffer (1% SDS, 0.1M NaHCO3, 0.01M DTT) with 30 min incubation, and reverse crosslinked at 65°C overnight in the presence of 0.2M NaCl. After digestion with proteinase K, DNA samples were extracted with phenol:chloroform and the DNA precipitated overnight. After resuspension in 40µl of TE buffer, PCR were performed using 5µl of DNA solution

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and analyzed on agarose gel electrophoresis. ChIP primers were CTCCCAGAGTCGCTCCACACGAGT and GGGACCCAGGCCGAATGCTC.

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RESULTS

Regulation of RIP140 mRNA accumulation by estrogens

We previously reported 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 ERα such as 4,4',4''-(4-propyl-(1H)-pyrazole-1,3,5-triyl)-trisphenol (PPT) or which preferentially activate ERβ such as genistein or 2,3-bis (4-hydroxy-phenyl)-propionitrile (DPN). Figure 1A shows that in conditions where 10^{-8} M estradiol induced about 4-fold the RIP140 mRNA steady state level, the other agonists were only 30-50 % as potent. By 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 previously showed in MCF-7 breast cancer cells that estradiol induction of RIP140 mRNA levels was independent of protein synthesis (Thenot *et al.*, 1999); accordingly, increase of RIP140 mRNA level was rapid since observed as early as 30 min after estradiol addition to the medium (figure 1B). In order 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 (~2 hours) and not affected by estradiol (figure 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.*,

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1998) and figure 2). Whereas it was initially thought that this gene was monoexonic, search for expressed sequence tags databases (http://www.ncbi.nih.gov/EST) identified transcripts initiated about 100 kbp upstream from the coding exon. Using RT-PCR with primers in putative exon1 and in the downstream coding exon, we confirmed the existence of three short non-coding exons. As shown in figure 2B, three major cDNA fragments of 304, 384 and 506bp 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 (accession number AF127577 in GenBank) 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. Since this DNA region appeared highly G+C rich, we also searched for CpG island, and again, we found only one such feature overlapping the putative promoter region, in the whole sequence of AF127577. Interestingly, when compared between human, chimpanzee and mouse, this non-coding region (figure 2) appeared highly conserved with more than 65% identity between mouse and human (figure 3). Then, we searched for transcription factor binding sites in this region, using Mat-Inspector from the Genomatix suite and found several putative response elements for general transcription factors such as Sp1 and CAAT binding sites (figure 3).

Next, we tested the 900 bp sequences upstream from exon 1, encompassing most of the conserved region (figure 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 Corp.) to generate the R900 reporter construct. As shown in figure 4B, when tested in transient

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transfection in HeLa cells, this DNA fragment acted as a promoter, about 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 figure 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 figure 4A) in transient transfection in MCF-7 cells. As shown in figure 4C, progressive 5'-deletions identified two regions involved in promoter activity. Indeed, when deleted, the distal ΔPAc and the central ΔSP regions significantly decreased luciferase activity. By contrast, removal of the Apa I-Pst I sequence had little effect (compare ΔPAc and ΔPPc). These results were supported by internal deletions which emphasized the importance of the Pst I-Sac II region (compare ΔAP and ΔSP mutants). In addition, the very proximal region contained in the ΔPSc construct exhibited a basal promoter activity. This basal level could result from Sp1 transcriptional activity as expected from the presence of several Sp1 sites (figure 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 (figure 4D). The regulation was comparable to that observed on the $p21^{WAF1/CIP1}$ 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 identified in the R900 and very interestingly, 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).

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To investigate the functionality of this ERE, we first performed *in vitro* binding using gel shift assay. As shown in figure 5A, ERα produced on the RIP140 ERE (RERE) a complex (lane 2) that was supershifted by an anti-ERα antibody (lane 3); moreover, this complex was titrated out either by the RERE itself (lanes 4–7) or by a 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 4–10, about 4 fold more RERE was necessary to inhibit complex formation compared to the vitERE, suggesting a slightly reduced affinity of the former for ERα.

ERα binding to the RIP140 promoter region containing the RERE was also observed in intact cells, using chromatin immunoprecipitation, with the HC20 anti-ERα antibody. As seen in figure 5B, estradiol increased ERα recruitment to the RIP140 promoter region encompassing the RERE. In parallel, we assessed histone acetylation using an anti-acetylated 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 ERα to the RIP140 ERE following 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 (figure 6A and data not shown). When isolated in front of the SV40 early promoter of the pGL3-promoter vector, the RERE induced transcription about 5-times, as efficiently as the vitERE (figure 6A). By contrast, the R900 construct containing the proximal promoter region of the RIP140 gene was unresponsive to estrogens (figure 6A). The same lack of response was observed whether this region was cloned upstream from the SV40 early promoter (R900sv compared to pGL3p as a control) or upstream from the HSV tk proximal promoter (R900tk compared to

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pGL3tk). In the same experiment, the EGL reporter which contains the vitERE in front of the β-globin promoter was strongly induced by E2.

Interestingly, the same R900 construct when stably transfected in MCF-7 cells (figure 6B) raised a reproducible and significant regulation by ER ligands comparable to that obtained for the endogenous RIP140 gene (*i.e.* induction by E2 and decrease by the pure antagonist ICI182780). Altogether, these results demonstrated the presence of a functional ERE in the RIP140 promoter and suggested that its regulation by estrogens requires a proper chromatin configuration.

Cross-talk with AhR regulation

To understand why the RIP140 gene was only weakly induced by estradiol despite the presence of a strong consensus ERα 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 upstream ERE boundary (figure 3 and 7A). Another potential AhRE was present in the central Pst I-Sac II region which was shown to be important for basal activity of the promoter (see figure 4A and D).

In order 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 figure 7B, treatment of MCF-7 cells by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) increased two-fold RIP140 mRNA steady state level. Interestingly, 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 term of regulation of RIP140 expression.

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To confirm this hypothesis, we first checked that the distal AhRE (overlapping with the RERE; figure 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). Interestingly, the regulation of RERE transactivation by E2-activated ERα was significantly reduced. This effect was reminiscent of the regulation of RIP140 mRNA (figure 7B). Interestingly, the transcriptional interference was even more pronounced when AhR was overexpressed (figure 7C).

In order 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 * in figure 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 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 where 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.

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DISCUSSION

RIP140 is an atypical transcription regulator which could be considered as an anti-coactivator since it exhibits an agonist-dependent recruitment by nuclear receptors but negatively regulates their transcriptional activation. In this study, we have 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 since it was not consistent with previously published results (Kerley *et al.*, 2001), that localized the RIP140 promoter about 5 kpb 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 (our unpublished results). The physiological relevance of the existence of several 5' non-coding 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 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 inter-species conservation. Such conserved non-genic regions are single copy sequences which represent approximately 1-2% of the human genome.

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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, about 700 bp upstream from the putative initiation site was quite unexpected since until now, only very few human genes [Efp (Inoue *et al.*, 1993), COX7RP or Cytox VIIa (Watanabe *et al.*, 1998)] have been shown to possess such a consensus element. The RIP140 ERE sequence bound the ERα 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. Interestingly, the regulation of the stably transfected R900 was equivalent to that of the endogenous RIP140 gene (figure 1) strongly suggesting that all 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 (figure 7), indicated that TCDD regulates by 2-fold RIP140 expression. Interestingly, a large number of studies have reported interferences between AhR and ER signaling which lead for instance to an

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inhibition of E2-induced gene expression and cell proliferation by TCDD. In the case of RIP140 gene, the cross-talk appeared slightly different since, 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 thought that endogenous AhR could be involved in the low response to E2 despite the presence of a perfect consensus ERE. As shown in Figure 7, mutation of the AhRE did not exacerbate the regulation by ER but instead decreased the amplitude of the E2 response. Since 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.

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 which involve induction of RIP140 expression by estrogens (Thenot *et al.*, 1999), retinoids (Kerley *et al.*, 2001) or, more recently, androgens (Carascossa *et al*., submitted for publication). All 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 since our data located the promoter 100kb upstream from the region proposed by Kerley *et al.* (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 appears that up-regulation of RIP140 expression could rather lead to an increase of AhR

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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 signaling intimately interfere. Other response elements in the proximal promoter region could also attenuate ERα transactivation and further work is in progress to identify such sequences. To summarize, the structure of the RIP140 gene is more complex than initially thought and its expression appears to be subtlety 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 cells proliferation by hormones and environmental contaminants.

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Footnotes

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LEGENDS FOR FIGURES

Figure 1 – Regulation of RIP140 mRNA by estrogens in MCF-7 cells

A. MCF-7 cells were treated for 24h with control vehicle ethanol (C) or various ER ligands (10^{-8}M). RIP140 mRNA levels were quantified by real-time quantitative RT-PCR as described in Materials and Methods. The results are expressed in arbitrary units after normalization by rS9 mRNA levels. Values are the means ± SD of three independent experiments. **B.** MCF-7 cells were treated or not with E2 (10^{-8}M) for the indicated period of time. RIP140 mRNA levels were quantified as described in Materials and Methods. **C.** MCF-7 cells were treated or not with E2 (10^{-8}M) for 24h and then incubated for different times (0, 1, 2 or 3h) with actinomycin D (3 µg/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 ± SD of three independent experiments.

Figure 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 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 3) or in the absence of RNA (lane 2). RT-PCR were done as described in 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

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exon using primers F2 and R2 in exon 4. The molecular weight marker (MW) shown on the left was the 50bp DNA ladder from InVitrogen (Cergy-Pontoise, France).

Figure 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 non-coding region (CNG) 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 AceView database (Aug 2004).

Figure 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 figure 3A.

B. Promoter activity of the R900 RIP140 genomic DNA fragment, compared to that of the corresponding vectors. HeLa cells were transfected as described in 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 β-galactosidase produced by the CMV-β-gal internal control plasmid in the same point. **C.** Comparison of the relative promoter activity of the R900 promoter fragment with

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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.

Figure 5 – Binding of ERα on the RIP140 ERE

A. Electromobility shift assay. Double-stranded RERE oligonucleotide labeled with [α^{32}P]dCTP using T4 DNA polymerase, was incubated with the proteins indicated above the autoradiograms in 50 mM Tris HCl, pH 7.5, 2.5 mM EDTA, 2.5 mM DTT, 100 mM KCl, 2 µg polydIdC and 24% glycerol, and separated on 6% polyacrylamide, 0.5 X TBE. H-184 anti-ERα 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 in Materials and Methods, and the product separated on agarose gel.

Figure 6 – Localization of the estrogen responsive element

A. E2-responsiveness measured after cotransfection of the recombinants in MCF-7 cells together with the HEG0 ERα expression vector. Relative luciferase activity is expressed as in figure 4. **B.** E2-responsiveness of the R900 promoter fragment stably transfected in MCF-7 cells. Transfected cells were treated by ethanol vehicle (C), 10^{-8}M estradiol (E2), 4-hydroxy-Tamoxifen (OHT) or ICI 182780 (ICI) for 24 h. Relative luciferase activity was measured as in Materials and Methods, and

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expressed as in figure 4. Statistical analysis performed either using Student t-test ($p<0.05$) or Anova ($F=74$) indicated that the four means were significantly different.

Figure 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 24h with control vehicle ethanol (C) estradiol (E2, 10^{-8}M), or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, 10^{-7} M), as indicated. RIP140 mRNA levels were quantified by real-time quantitative RT-PCR as described in Materials and Methods. The results are expressed in arbitrary units after normalization by rS9 mRNA levels. Values are the means ± SD of three independent experiments. **C.** The RERE and its mutated version REREmut reporter plasmids were transiently transfected in steroid-stripped MCF-7 cells either with the ERα (HEG0) alone or with both ERα and AhR/Arnt expression vectors. Cells were stimulated with the indicated hormone combination, and relative luciferase activity measured as above (statistical analysis was performed for the RERE, on the fold induction by E2 in the absence or presence of TCDD; *$p<0.05$ and **$p<0.005$).

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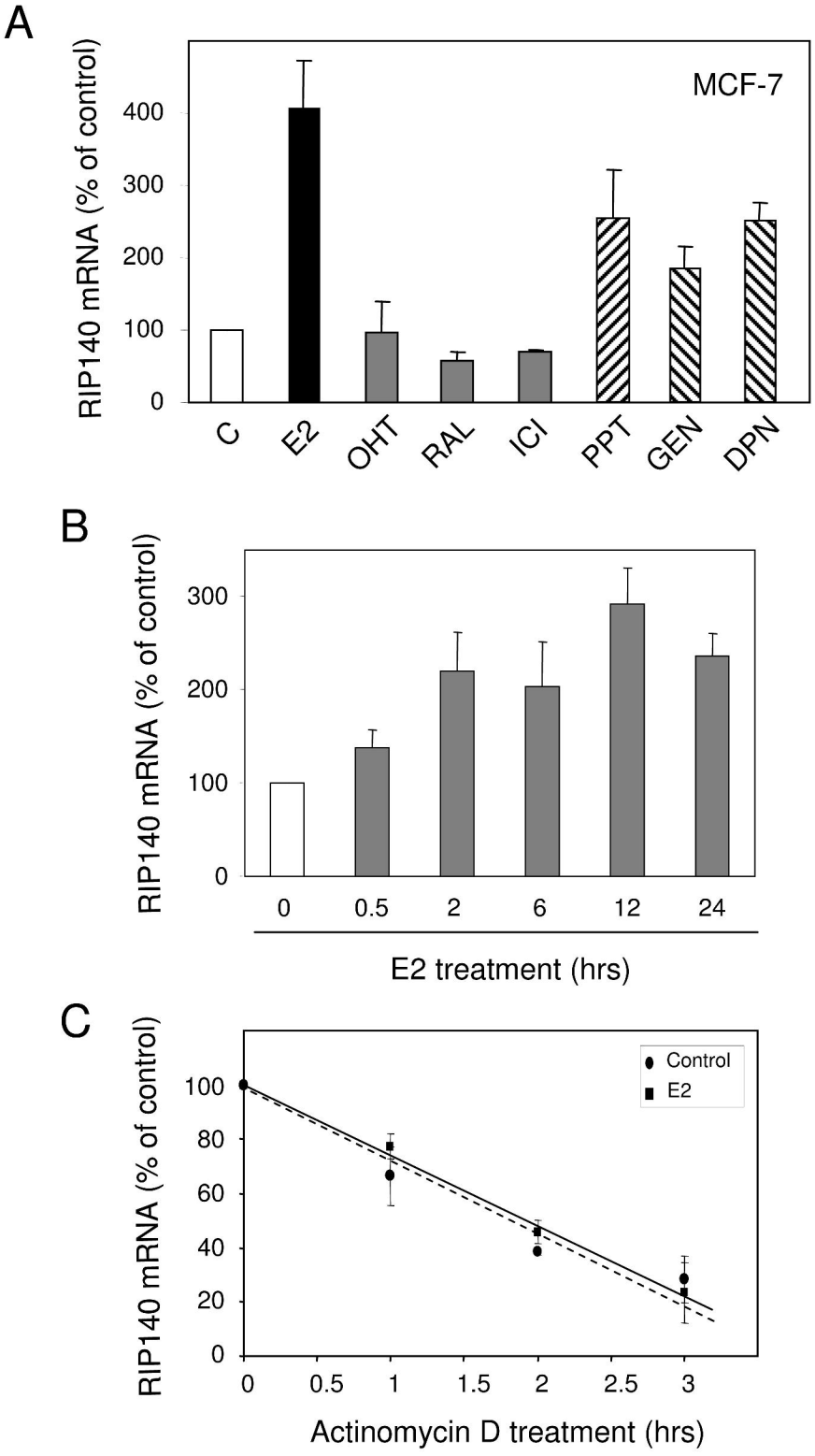

Figure 1

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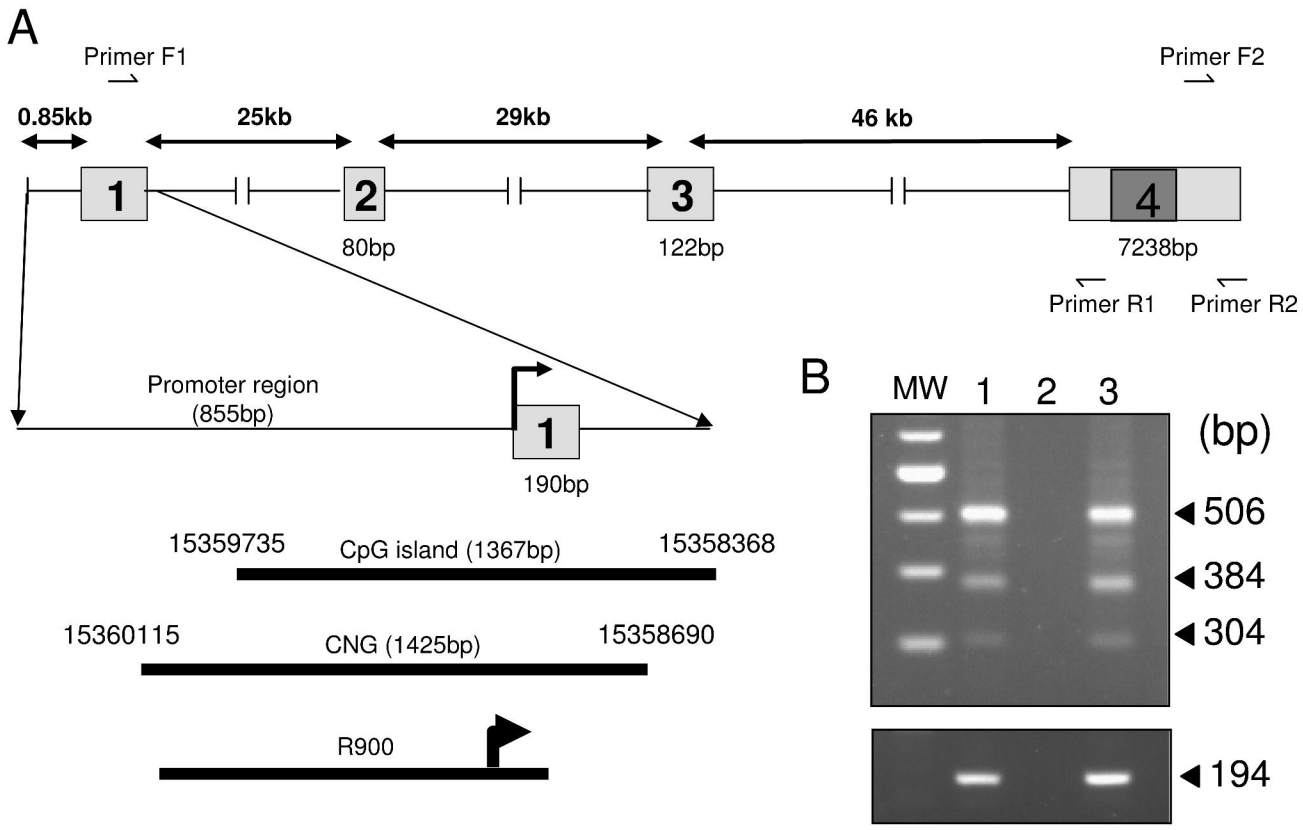

Figure 2

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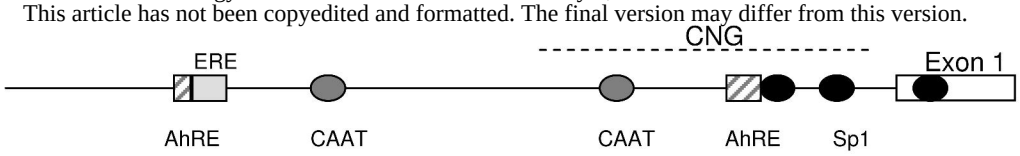
A



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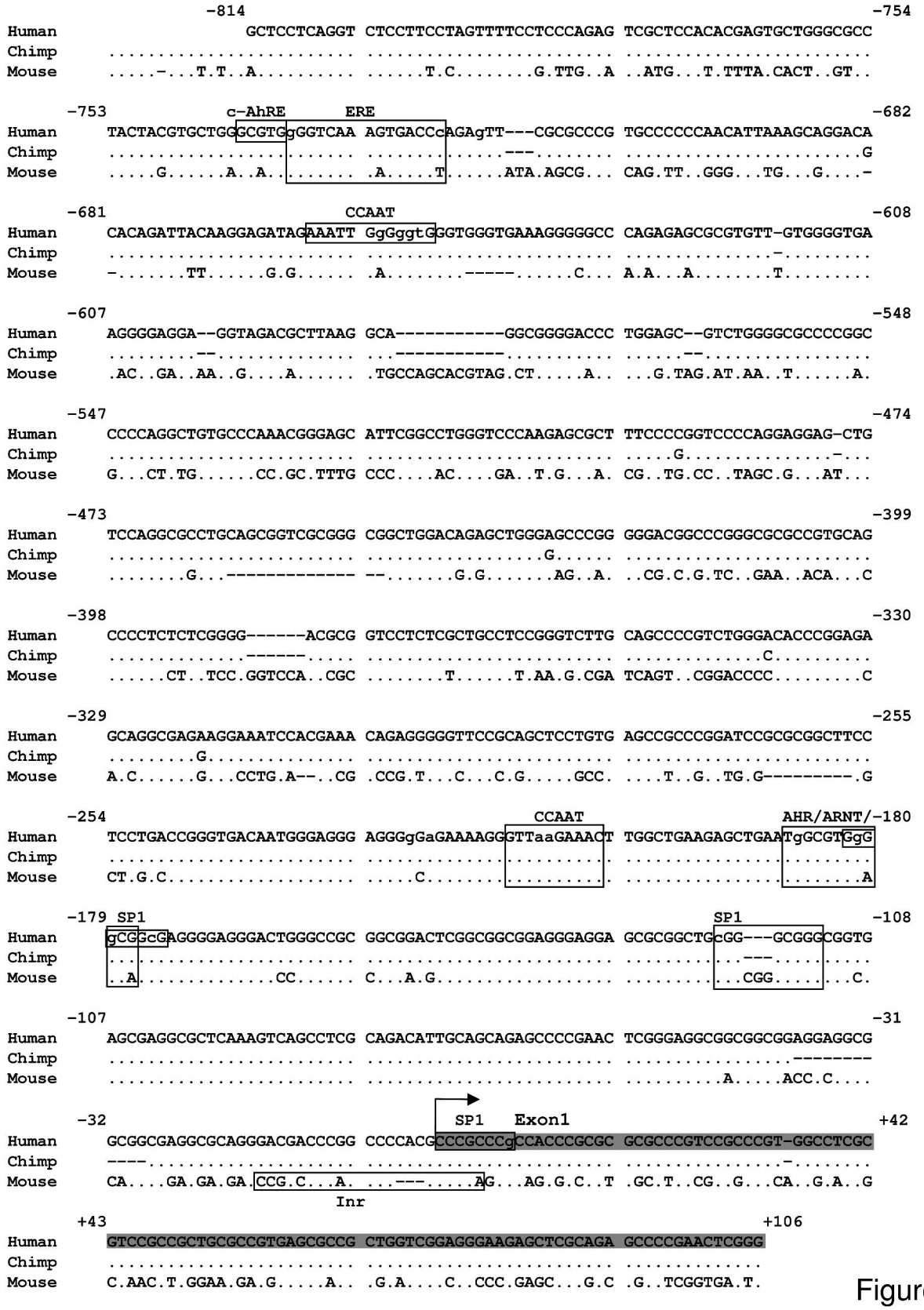

Figure 3

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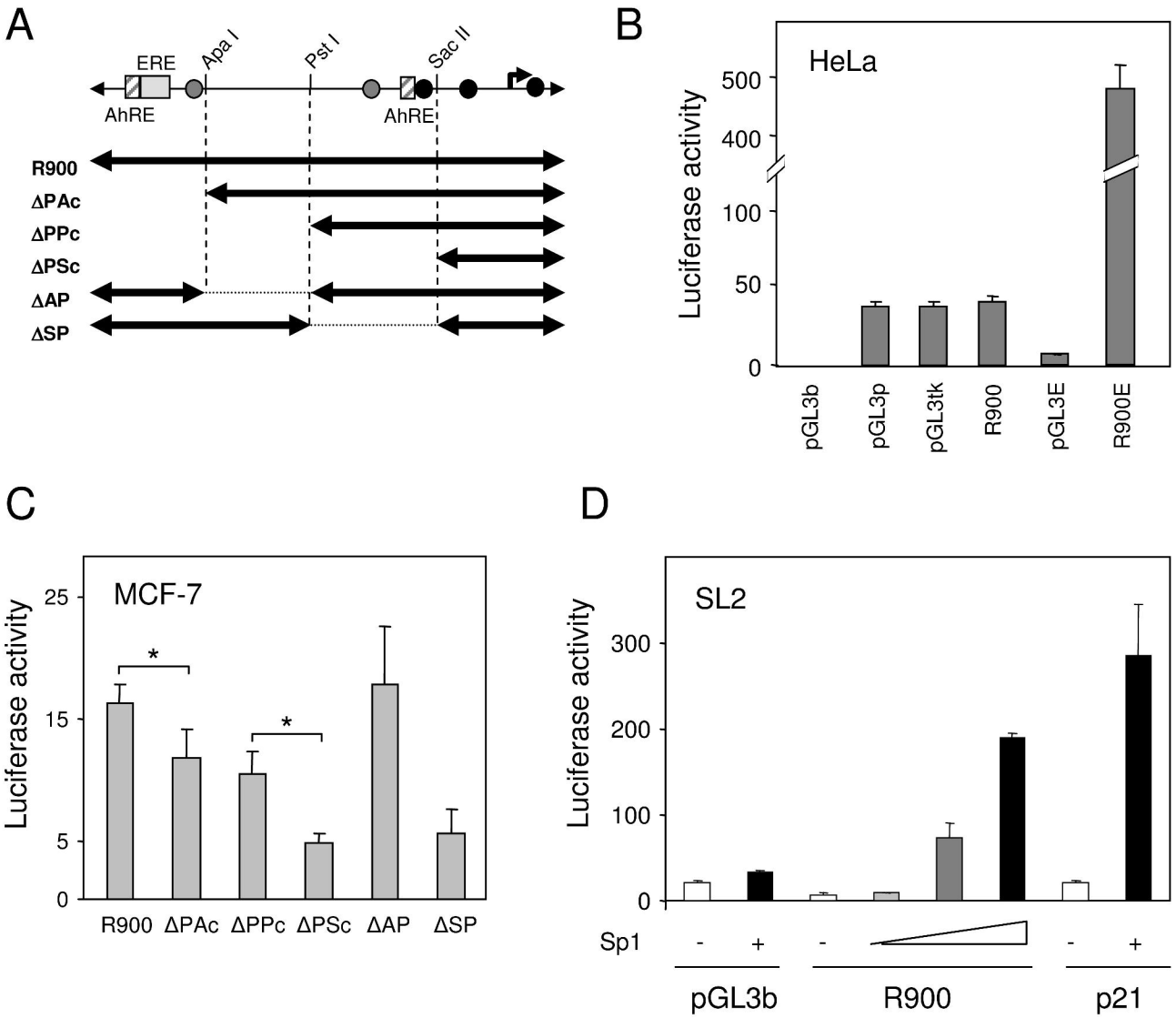

Figure 4

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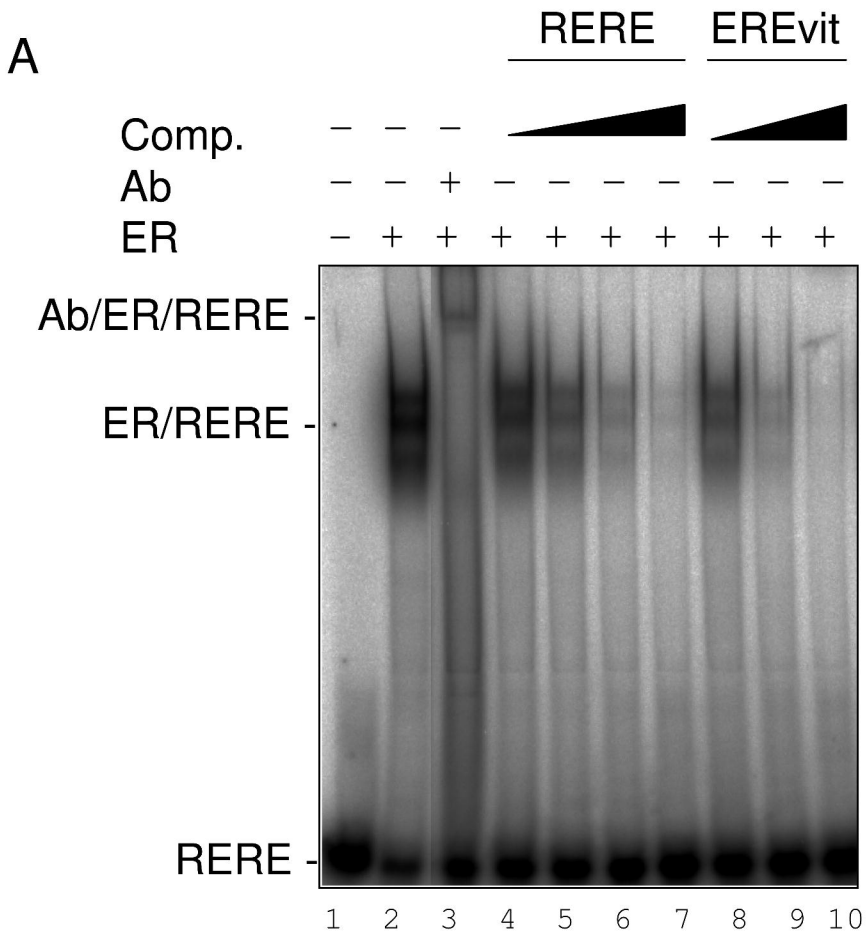

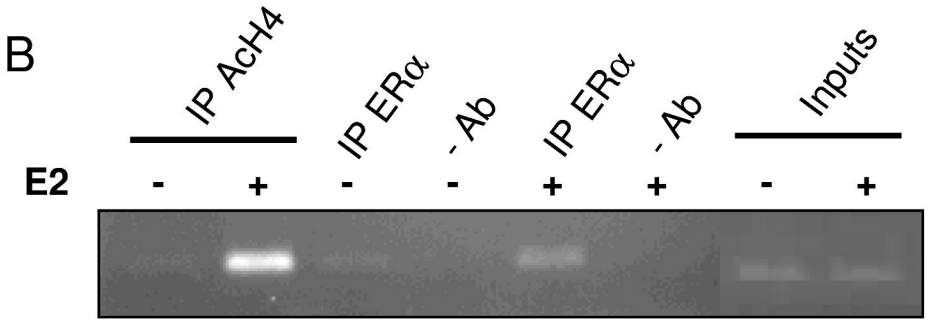

Figure 5

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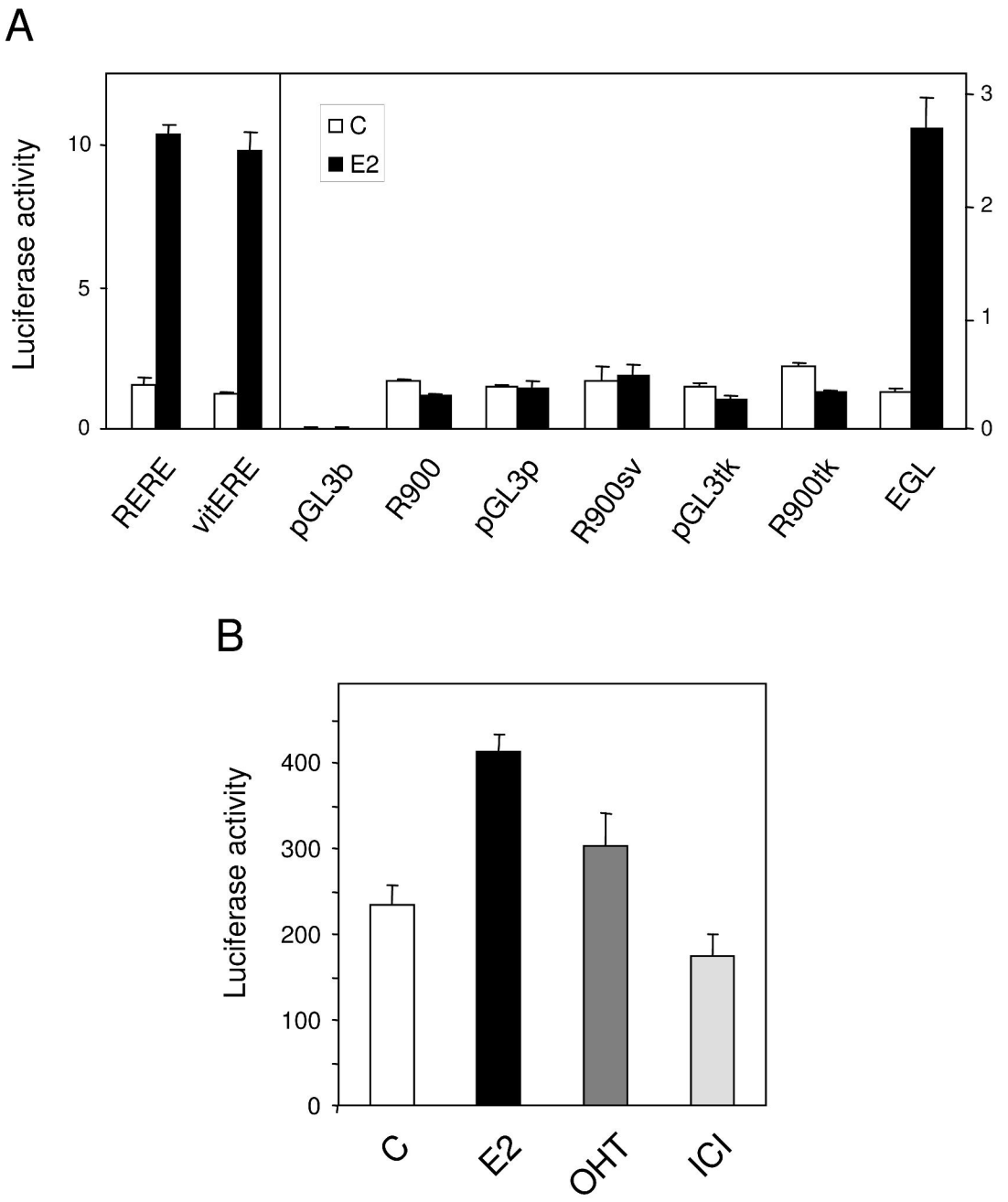

Figure 6

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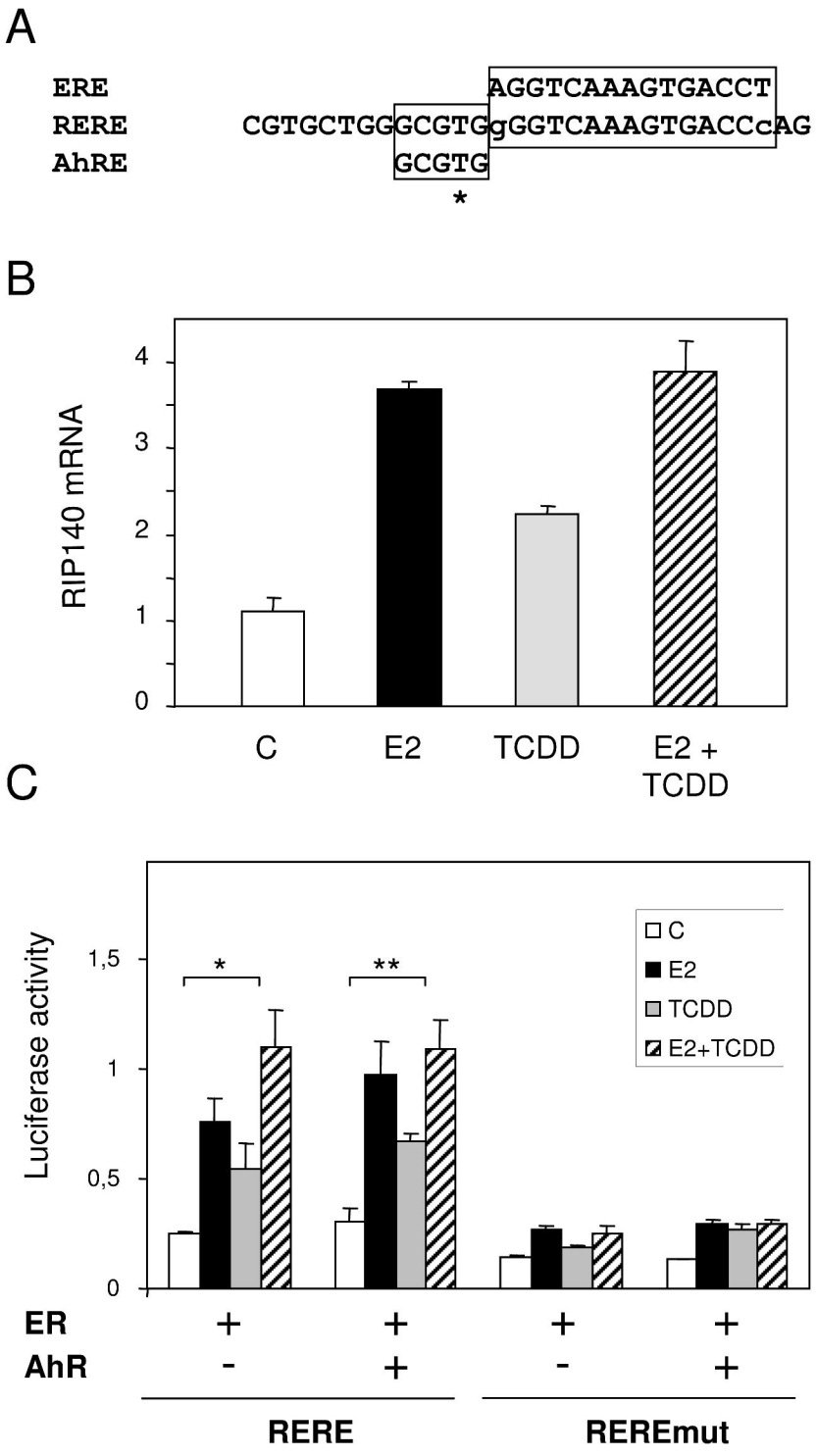

Figure 7