Universitat Pompeu Fabra

Facultad de Ciencias de la Salud y de la Vida

TESIS DOCTORAL

Regulation of the 11beta-hydroxysteroid dehydrogenase type 2 promoter by steroid hormones in breast cancer cells. Convergence of progesterone receptor binding to DNA and JAK / STAT pathway activation.

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Abril de 2007

Regulation of the 11beta-hydroxysteroid dehydrogenase type 2 promoter by steroid hormones in breast cancer cells.
Convergence of progesterone receptor binding to DNA and JAK / STAT pathway activation.

Trabajo presentado por Alicia Subtil Rodríguez para optar al grado de Doctora. Este trabajo ha sido realizado bajo la dirección del Dr. Albert Jordan Vallès en el Programa de Regulación Génica del Centre de Regulació Genòmica (CRG) de Barcelona.

Dr. Albert Jordan Vallès
Firma del Director de Tesis

CONTENTS

ABBREVIATIONS

11β-HSD2	11 beta-hydroxysteroid dehydrogenase type 2
A	Adenosine
AF	Activation function
AR	Androgen receptor
ARE	Androgen responsive element
ATP	Adenosine triphosphate
bp	Base pain
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
DAPI	4',6'-diamidino-2-phenyliondole, dihydrochloride
DBD	DNA binding domain
Dexa	Dexamethasone
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylene diaminetetraacetic acid
ER	Estrogen receptor
ERID	ER interaction domain
EtBr	Ethidium bromide
FCS	Fetal calf serum
G	Guanosine
GR	Glucocorticoid receptor
HAT	Histone acetyltransferase
HDAC	Histone deacetyltransferase
HEPES	(2-Hydroxyethyl)-1-piperazineethanesulphonic acid
JAK	Janus kinase
K	Lysine
Kb	Kilobase pair
KDa	Kilodalton
LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinase

MEM	Minimum Essential Medium
MMTV	Mouse mammary tumour virus
NaCl	Sodium chloride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIPES	1,4-Piperazinediethanesulfonic acid
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcription PCR
S	Serine
SDS	Sodium-dodecyl-sulphate
STAT	Signal transducers and activators of transcription
T	Thymine
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)-amino-methane
wt	Wild type
YY-1	Ying and Yang 1

SUMMARY

Steroid hormone receptors regulate gene expression interacting with target DNA sequences but also activating cytoplasmic signalling pathways. Using the human 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) gene as a model, we investigated the contribution of both effects on a human progesterone responsive promoter in breast cancer cells. The expression of the 11β-HSD2 gene is up-regulated by progestins and requires progesterone receptor (PR) activation in T47D cells. Deletion analysis of the 11β-HSD2 promoter to define the minimal part of the promoter regulated by hormones showed that the distal region between nucleotides -1778 and -1345 upstream transcription start site is involved in most of the hormone dependent activation. Computational analysis of the promoter sequence showed the presence of potential PR binding sites along the entire promoter region, and a putative STAT5A binding site at the distal 11β-HSD2 promoter region. Chromatin immunoprecipitation has shown hormone-dependent STAT5A recruitment to the distal region and PR recruitment to the distal and proximal 11β-HSD2 promoter regions. Our results suggest two different mechanisms of hormone-induced PR recruitment, since T47D cells stably expressing PR containing a mutated DNA binding domain have affected hormone-dependent PR recruitment to proximal promoter, and JAK/STAT pathway inhibition blocks PR recruitment to distal promoter. 11β-HSD2 gene expression after hormone stimulation was partially decreased by MAPK and PI3K / AKT pathway inhibitors and totally blocked by JAK/STAT pathways inhibitors, indicating that cytoplasmic PR effects, and specially JAK / STAT activation, are involved in progestin-induced 11β-HSD2 expression. Expression of constitutive active or dominant negative STAT5A forms have confirmed that JAK/STAT pathway and STAT5A are functionally important for PR recruitment in response to progesterone and hormone-induced 11β-HSD2 gene expression. Therefore, coordination of direct recruitment of PR and cytoplasmic signalling PR effects, in particular progestins-induced JAK/STAT pathway, are critical to regulate endogenous 11β-HSD2 gene expression in breast cancer cells. Moreover, microarray experiments using a breast cancer customized cDNA array show that a subset of progesterone-regulated genes are affected by JAK / STAT pathway inhibition. Importantly, characterization of 11β-HSD2 promoter upon hormone induction has shown that

active form of RNA polymerase II is recruited from the distal promoter region and a minimal promoter including nt –1778 to –1345 has hormone-responsiveness by itself, suggesting that progesterone-dependent 11β-HSD2 expression starts upstream the previously characterized transcription start site.

INTRODUCTION

I.1. Steroid hormones and their receptors

The nuclear receptors are ligand activated transcription factors that regulate the expression of target genes. The nuclear receptor superfamily is typically subdivided into three families (Chawla et al. 2001):

i) the steroid receptor family;

ii) the thyroid / retinoid family, that includes thyroid receptor (TR), vitamin D receptor (VDR), retinoic acid receptor (RAR), and peroxisome proliferator-activated receptor (PPAR);

iii) the orphan receptor family, defined by a set of proteins identified by comparative sequence analysis as belonging to the nuclear receptor superfamily, but which ligands are unknown.

The gonads and adrenal gland produce five major groups of steroid hormones (SHs): estrogens, progestins, androgens, glucocorticoids and mineralocorticoids. SHs have importantly regulatory roles in a wide variety of biological processes including reproduction, differentiation, development, cell proliferation, apoptosis, inflammation, metabolism, homeostasis and brain function (Mangelsdorf et al. 1995, Tsai, O'Malley 1994).

SHs are small lipophilic molecules that can pass the cell membrane by simple difusion and, into the target cells, bind to steroid hormone receptors (SHRs). Unliganded SHRs are associated with a large multiprotein complex of chaperones, including heat shock protein 90 (Hsp90) and immunophilin Hsp56. The chaperone-interaction phase, that normally would be transitory during protein folding, is extended indefinitely in the case of SHRs folding, to repress SHRs transcriptional activity while the receptor is not activated by SHs (Pratt 1993, Smith 2000). Thus, SHs activate SHRs in order to act as transcription factors to modulate gene expression.

I.1.1. Structure of SHRs

Following the cloning of the receptors for glucocorticoids (GR) and estrogens (ERα), receptor for androgens (AR), progestins (PR) and mineralocorticoids (MR), were identified and characterized. Later, a second

estrogen receptor (ERβ) and two estrogen-related receptor (ERRα/ERR1 and ERRβ/ERR2) have been characterized (Beato, Klug 2000).

SHRs contain two structural subunits (Fig. I.1):

i) a C-terminal ligand binding domain (LBD);

ii) a centrally located DNA-binding domain (DBD)

Figure I.1: Steroid hormone receptor (SHR) structure.
General schematic representation of the different SHRs, including the structural domains with critical functions. AF-1 *(green box)*, activation function 1; DBD *(yellow box)*, DNA binding domain; Hinge *(dark blue box)*, short amino acid sequence that connects DBD with LBD; LBD AF-2 *(clear blue box)*, comprises ligand binding domain and activation function 2. N-terminal domain *(grey box)*, varies between the different SHRs.

LBD has a number of critical functions. Firstly, it contains an interior binding pocket specific for its corresponding hormone. Secondly, the LBD contains a ligand-regulated transcriptional activation function (AF-2) necessary for recruiting co-activating proteins. These coactivators interact with chromatin remodelling proteins and the transcriptional machinery. Finally, the LBD is the primary mediator of dimerization, necessary for DNA response element binding (Kumar, Chambon 1988).

The DBD is the responsible of the binding to the hormone response elements (explained in more detail below), located in SHRs-regulated promoters. DBD is also an allosteric transmitter of information to other regions of the receptor molecule (Beato, Klug 2000).

The DBD is connected to the LBD via a short amino acid sequence termed the hinge. The complete functional properties of the hinge are still unclear, although it can be phosphorylated and this phosphorylation is coupled to increased transcription activation (Knotts et al. 2001, Vicent et al. 2006).

The amino acid sequence N-terminal to DBD contains a transcriptional activation function termed AF-1. AF-1 sequence shows weak conservation between the SHRs family members. This could explain how closely related SHRs can bind to similar response elements in vitro, but differentially regulate gene promoters containing those sequences in vivo (Takimoto et al. 2003)

I.1.2. Progesterone receptor (PR)

The PR belongs to the steroid receptor family of nuclear receptors. PR mediates the action of the hormone progesterone, a key ligand in reproduction and pregnancy. PR was the first SHR shown to exist in two isoforms generated by differential promoter usage (Kastner et al. 1990). One promoter initiates transcription at positions +1 and +15 of the *Pgr* gene which gives the longer human isoform, PRB. The second promoter initiates human PR transcripts between +737 and +842 encoding the shorter human isoform, PRA, that lacks 164 amino acids at its N-terminal domain (Fig. I.2). Although this was considered as an exception, alternative splicing and differential promoter usage are now the rule for all SHRs family members (Beato, Klug 2000).

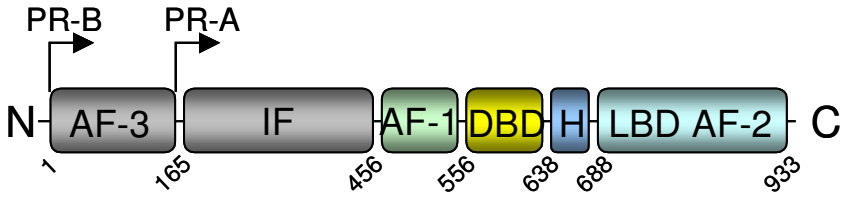

Figure I.2: Progesterone receptor (PR) structure.
The numbers refer to amino acid positions. Arrows indicate the amino acid initiation of PR isoform A (PRA) and isoform B (PRB). AF-3 (only presents in PRB) and IF *(grey boxes)*, activation function 3 and inhibition function; AF-1 *(green box),* activation function 1; DBD *(yellow box),* DNA binding domain; H *(dark blue box);* hinge, short amino acid sequence that connects DBD with LBD; LBD AF-2 *(clear blue box)*, comprises ligand binding domain and activation function 2.

I.1.2.1. PR Ligand-Binding Domain (LBD)

Progesterone binds in the lower half of the LBD, forming highly specific hydrogen bonds and van der Waals contacts. Additional hydrophobic interactions between the ligand and the walls of the binding pocket contribute to the stability of the binding reaction (Williams, Sigler 1998). LBD structure shows that is necessary for binding coactivating proteins (Tanenbaum et al. 1998). Thus, LBD includes a ligand-dependent activation function (AF-2) that is able to recruit proteins such as the steroid receptor coactivator (SRC) family (Xu, Li 2003).

I.1.2.2. PR DNA-Binding Domain (DBD)

The PR DBD folds into a globular domain made up of two different zinc-finger structures (Fig. I.3). Each zinc atom is coordinated by four cystein residues. The atoms are necessary to stabilize the structure and function because removal of the zinc ion leads to protein unfolding and loss of DNA-binding activity (Freedman et al. 1988). PR DBD suffers DNA-induced dimerization upon binding an inverted repeat (palindrome) response element (named hormone response element -HRE- or progesterone response element -PRE-). The residues that define the dimer interface are located into the C-terminal zinc-finger and constitute the D-Box (Fig. I.3). The sequence-specific DNA binding residues are defined as the P-Box (Fig. I.3) (Beato, Klug 2000).

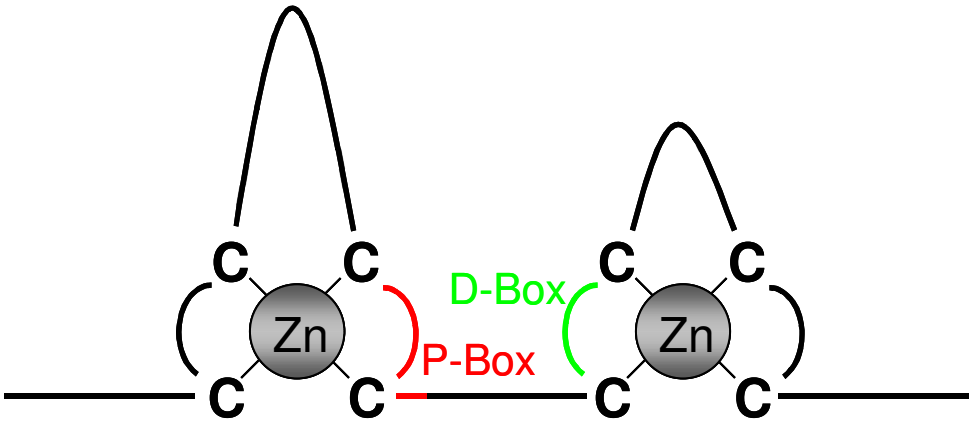

Figure I.3: SHR-specific zinc fingers of the PR DBD.
The DNA binding domain of human PR is characterized by the presence of two SHR-specific zinc fingers. Four cysteines each tetrahedrally coordinates two zinc ions *(grey)*. The proximal box (P-box), responsible for specific DNA recognition is shown in *red*; and the distal box (D-box), mediating DBD dimerization is shown in *green*.

PR binds the DNA as a dimer, using a head-to-head orientation. Analysis of the protein-DNA interaction reveals that amino acid chain contacts with bases in the major groove are almost identical to those defined for GR and AR. However, relative the minor groove, PR binding is highly different to that of the GR or AR-DNA complexes (Roemer et al. 2006).

I.1.2.3. N-terminal domain

Regarding N-terminal domain functional importance, remarkably, DBD can stabilize and influence N-terminal structure. PR fragments lacking the DBD are immediately degraded by proteases (Bain et al. 2000, Bain et al. 2001).

Upon binding to a palindromic response element, PRA and PRB N-terminal domains suffer changes in conformation. These changes were localized into the AF-1 region, but also into the hinge, demonstrating that DBD allosterically transmits structural transitions. These changes seem to be necessary for recruitment of coactivators to the target promoter (Kumar, Thompson 2005).

Comparing their N-terminal domains, both PR forms, PRA and PRB, are identical except that PRA lacks 164 amino acids contained at the N-terminal end of PRB. The region of the protein that is unique to PRB contains a transcription activation function, AF-3 (Sartorius et al. 1994b). Biochemical analysis of PRA and PRB isoforms N-terminal domains revealed that their structure were almost identical. However, analyses have shown that there are differences at the level of secondary or tertiary structure. This could explain why PRB is stabilized in a more functionally active conformer that PRA (Bain et al. 2000, Bain et al. 2001), since, in addition to the fact that AF-3 is unique to PRB, the PRB–specific region has a distinct conformation and is likely to mask an inhibitory domain that is active in the N-terminus of the PRA protein (Huse et al. 1998).

In addition to its relevance in functional differences between PRB and PRA, N-terminal half of PRB is remarkably important in the progesterone cytoplasmic-signalling mediated effects. An interaction between PRB and ERα has been identified in breast cancer cell lines (Migliaccio et al. 1998). PRB interacts with ERα through two domains located in the N-terminus of PRB, ERID I and ERID II (ER interaction domains I and II), that are required for the interaction with the LBD of ERα and for efficient activation of the Src / Ras / Erk cascade (Ballare et al. 2003) (Fig. I.4).

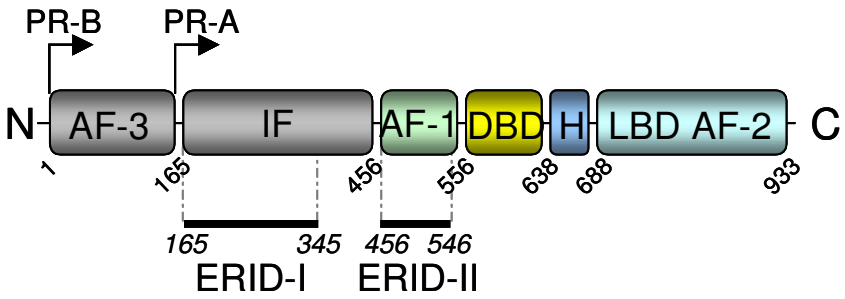

Figure I.4: ER interaction domains I and II (ERID I and II)
PRB interacts with ERα through two domains located in the N-terminus of PRB, ERID I and ERID II (ER interaction domains I and II), that are required for the interaction with LBD of ERα and for efficient activation of the Src/Ras/Erk cascade (Ballare et al. 2003). The numbers refer to amino acid positions.

I.1.2.4. Functional differences between PRB and PRA isoforms

Although PRB and PRA isoforms show a high degree of sequence identity, they display significant different functional properties on the regulation of target promoter:

i) PRB is a much stronger transcriptional activator than PRA (Sartorius et al. 1994b);

ii) PRA gene knock-out mice develop uterine dysplasia and abnormal ovaries, whereas PRB gene knock-outs have affected the mammary glands, causing incomplete lobular-alveolar differentiation (Mulac-Jericevic et al. 2000);

iii) Microarray studies showed that the two isoforms regulate different subset of genes (Richer et al. 2002).

I.1.3. SHRs effects

I.1.3.1. Direct effects (Fig. I.5)

Nuclear translocation of the SHRs is a SHs dependent process mediated by several nuclear localization signals (NLSs) located in the C-terminus of the second zinc finger, in the hinge region and in the LBD. At equilibrium, the majority of ER, AR and PR is in the nucleus due to the presence of these NLSs required for nuclear pore recognition (Guiochon-Mantel et al. 1991).

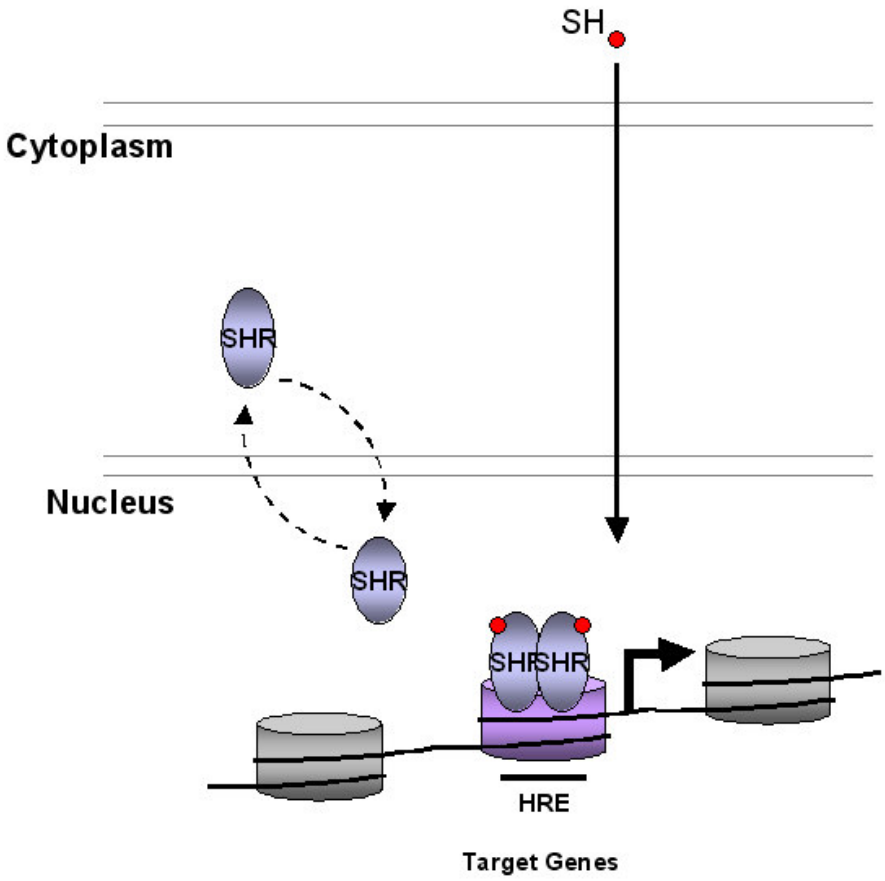

Figure I.5: SHRs direct effects.
SHs are lipophilic molecules that difuse into the cells and bind SHRs. Ligand activated SHRs dimerize and translocate into the nucleus. There SHRs bind to specific sequences (hormone response elements, HREs) located in the promoter of hormone regulated target genes.

In the nucleus, PR, GR, MR and AR bind to the same HREs, which were described as glucocorticoid responsive elements (GREs) (Karin et al. 1984, Scheidereit et al. 1983). HREs are composed of hexanucleotides (TGTTCT) arranged as inverted repeats (palindromes) and separated by three non-conserved base pairs (Beato 1989), although the sixth base pair of each half-palindrome is not well conserved and is not essential for specific binding (Truss et al. 1991) (Fig. I.6). Each half-site is recognized by one receptor monomer (Luisi et al. 1991). GR and PR discriminate their half-site (GRE and PRE) by a hydrophobic interaction with the methyl group of thymine in position 3 of each half-site (TG**T**TCT), that is not present in other HREs (Truss, Chalepakis & Beato 1990).

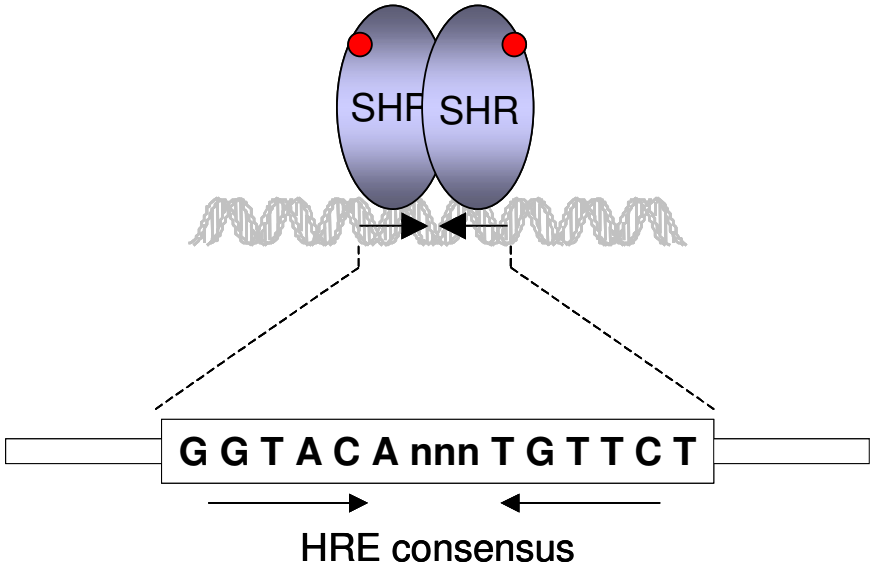

Figure I.6: Schematic representation of a ligand activated SHR homodimer binding to its correspondent hormone response element (HRE).
The consensus HRE of the SHRs PR, GR, AR and MR, is an inverted repeat or palindrome. The half-sites (arrows) are separated by three unspecific nucleotides (n).

SHRs bind to HREs organized in chromatin since the region of the major groove contacted by the receptor is exposed on the surface of nucleosomes (Pina, Bruggemeier & Beato 1990). Therefore, the DNA nucleotide sequence and its specific packaging in chromatin determine the interaction of hormone regulatory sites with their correspondent SHR (Beato, Klug 2000).

In order to regulate transcription, agonist-liganded SHRs have to interact with the members of the general transcription machinery which have to be recruited to the chromatin-organized promoters to form the transcription pre-initiation complex at the transcription start site. SHRs can interact directly with general transcription factors or by coactivators or mediators (Beato, Klug 2000). In this way, SHRs can also activate genes lacking HREs by interaction with other sequence-specific transcription factors bound to their target sequences (Beato, Herrlich & Schutz 1995).

I.1.3.2. Signalling-mediated effects

In addition to the genomic effects, SHs are known to induce rapid responses of kinase cascades activated by cytoplasmic events. Rapid, steroid “non-genomic effects”, often occur via second messenger cascades, which in turn originate from signalling complexes located at membranes. Consequently,

receptors for signalling-mediated actions are widely believed to be membrane-associated or integrated into the membrane (Wehling, Losel 2006).

To explain the relevance of SHRs signalling-mediated effects could be interesting to briefly review some related examples. Estrogens activate the Src/Ras/Erk and the PI3K/Akt pathways via direct interaction of ER with SH2 domain of c-Src and the regulatory subunit of PI3K, respectively (Fig. I.7A) (Castoria et al. 2001, Migliaccio et al. 1996). These pathways are determinant for estrogen induction of cell proliferation in breast cancer cells.

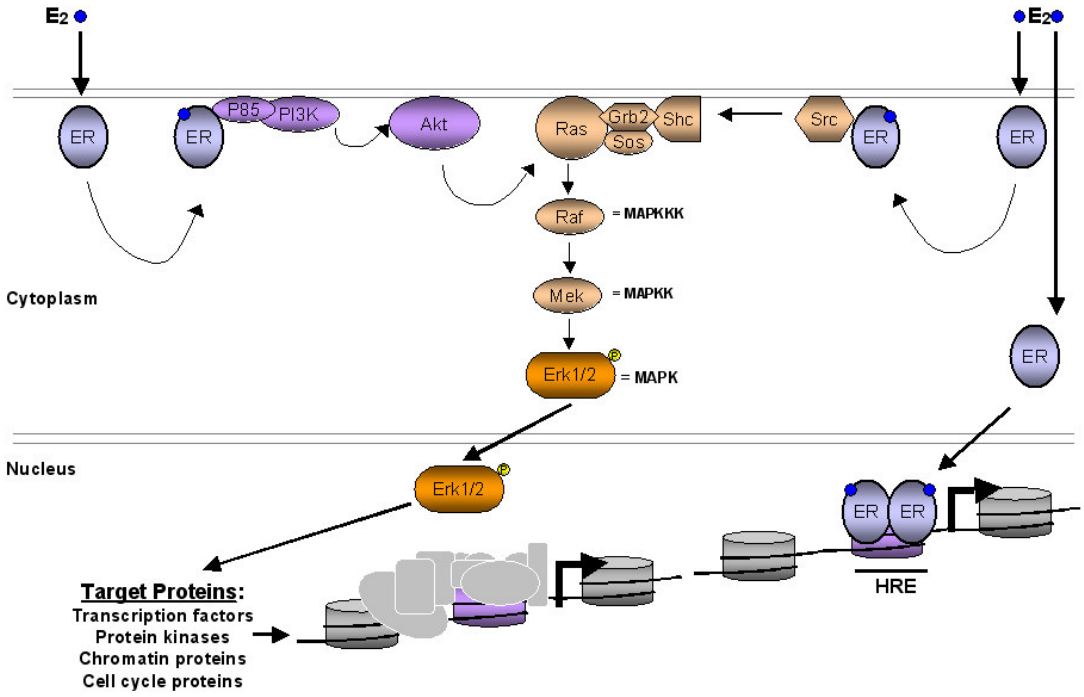

Figure I.7A: Signalling-mediated effects activated by estrogens.
Estrogens activate the Src/Ras/Erk and the PI3K/Akt pathways via direct interaction of ER with SH2 domain of c-Src and the regulatory subunit of PI3K, respectively. Erk might be responsible of regulation / activation of different target proteins within the nucleus (including protein kinases, transcription factors, chromatin remodelling complexes proteins and cell cycle regulating proteins) that may affect gene expression and cell cycle progression.

Progesterone can also crosstalk to kinase cascades through an interaction of PR with SH3 domain of c-Src (Ballare et al. 2003, Boonyaratanakornkit et al. 2001). In breast cancer cells, containing ER, approximately 5% of cellular PR is located in the cytoplasm, and the progesterone effect on the Src/Ras/Erk pathway is mediated by the interaction of two N-terminal PR domains (ERID I and II) with LBD of ERα which itself triggers the activation of the cascades (Ballare et al. 2003, Migliaccio et al. 1998) (Fig. I.7B). The targets of the activated kinase cascades might be

transcription factors and corregulators involved in DNA synthesis and cell proliferation.

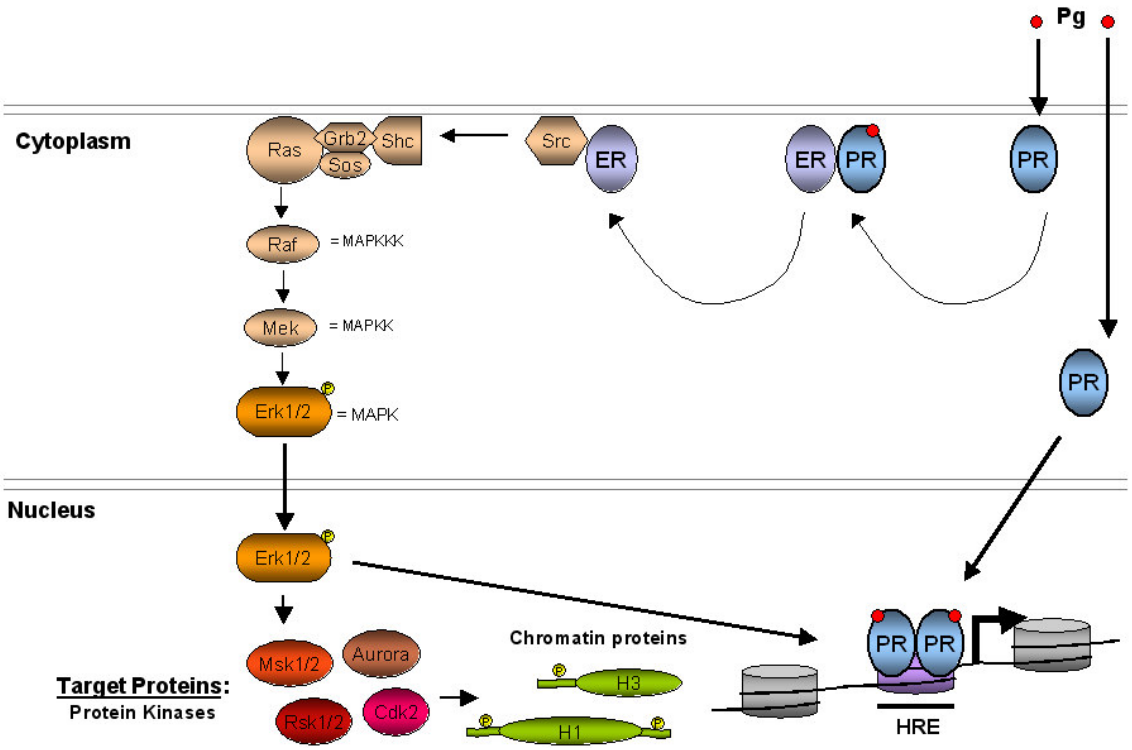

Figure I.7B: Signalling-mediated effects activated by progestins.
In breast cancer cells, containing ER, 5% of cellular PR is located in the cytoplasm, and the progesterone effect on the Src/Ras/Erk pathway is mediated by the interaction of PR-ERID I and II with LBD of ERα, which itself triggers the activation of the cascades. Erk may be responsible of regulation / activation of diferent protein kinases involved in events related with chromatin remodelling and transcriptional activation, such as histone tails phosphorylation.

I.1.4. SHRs and transcription (Fig. I.8)

How SHRs bound to DNA activate gene transcription in response to hormone remains an essential and incompletely understood question. *In vitro* transcription assays showed that ligand-activated receptor increase the rate of transcription by recruiting and stabilizing the pre-initiation transcription complex at the promoter of hormone responsive target genes (Beato, Sanchez-Pacheco 1996). Thus, *in vitro* binding of SHRs to general transcription factors that associate with RNA polymerase II has been described as a possible mechanism for recruitment of the pre-initiation complex. *In vivo*, the fact that SHRs regulate transcription through interaction with HREs that function as enhancers, suggests the existence of other mechanisms for receptor communication with basal transcriptional machinery.

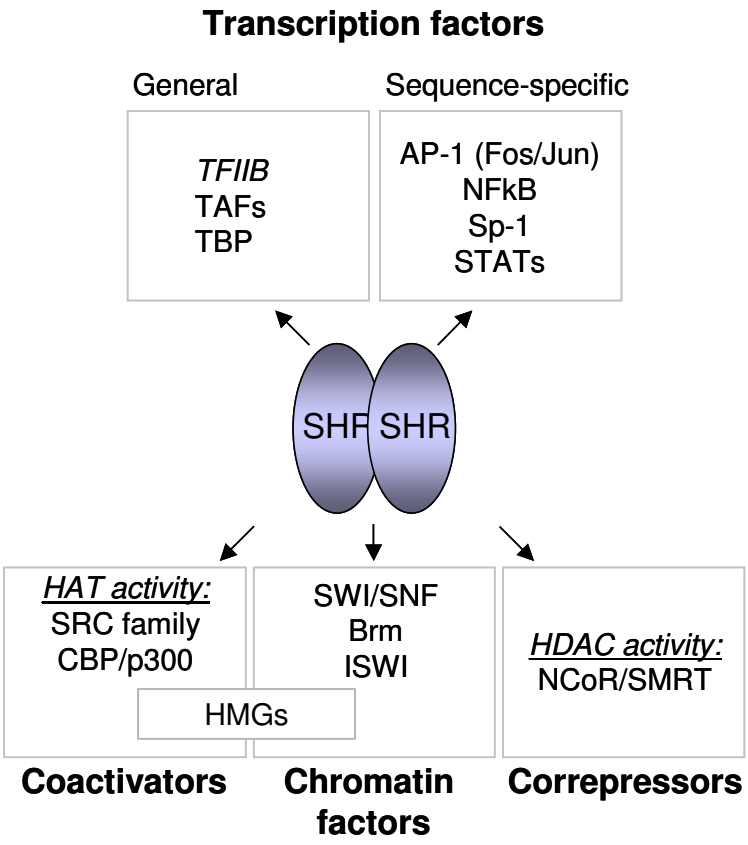

Figure I.8: Overview of nuclear partners of SHRs required for activation or repression of transcription.
For each class (general transcription factors, sequence specific transcription factors, coactivators, correpressors and chromatin factors), typical examples are shown.

I.1.4.1. Coactivators

SHRs coactivators bridge between DNA-bound sequence-specific transcription factors and general transcription factors. Requirement of coactivators was clear since an excess of receptor amount can inhibit its own transactivation as well as transcription by other transactivators (Wright et al. 1991). This phenomenon is due to the fact that additional factors (present in limited amounts) are trapped by excess of SHRs in unproductive complexes. AF-2 of the SHRs LBD is responsible of the major ligand-dependent transcriptional activity. The family of p160 proteins binds in a hormone-dependent manner to the AF-2 region (Beato, Klug 2000).

p160 family was identified using biochemical isolation of proteins that bound *in vitro* to the SHRs LBD and by yeast two hybrid screening (McKenna, Lanz & O'Malley 1999). Also termed steroid receptor coactivators (SRC), the family consists of three closely related members:

i) SRC-1 (or NCoA-1), was firstly identified as a protein that interacts with AF-2 of PR (Onate et al. 1995)

ii) SRC-2 (or GRIP-1, NCoA-2, or TIF-2 –transcriptional intermediary factor 2-), identified as a GR interacting protein (Voegel et al. 1996)

iii) SRC-3, also termed p/CIP, RAC3, ACTR, TRAM-1 or AIB1.

The p160/SRC coactivators do not have DNA binding activity but are recruited to promoters of steroid responsive target genes via protein-protein interaction with nuclear receptors. This interaction is dependent on hormone agonist and the integrity of AF-2. SRC-1 also enhance the transcriptional activity of Sp-1, GAL-4, AP-1, Serum response factor (SRF), NFkB and STAT proteins (Xu, Li 2003).

The C-terminal domains of SRC-1 and SRC-3 contain histone acetyl-transferase (HAT) activities, so SRC can play a direct role in chromatin remodelling during the process of SHR-directed initiation of transcription. However, SRC HAT activity is much weaker than those in CBP, p300 or PCAF (Spencer et al. 1997).

SRCs may play important roles in the chromatin remodelling and the assembly of general transcription factors through direct and indirect recruitments of other coactivators (Fig. I.9). Thus, SRC pre-existing complex with CBP, p300, PCAF, CARM-1 and PRMT-1 are recruited to chromatin by ligand-triggered interactions between SHRs and SRCs (Li et al. 2003). In the same way, SWI/SNF chromatin-remodeling complex is recruited to chromatin through direct or indirect interaction with CBP/p300. The SWI/SNF complex causes specific histone acetylations in an ATP-dependent manner, which results in changes of DNA topology (Huang et al. 2003). Finally, the process of SHR-induced coactivator recruitment, asembly of transcription machinery and initiation of transcription is dynamic and may occur in a cyclic manner (Shang et al. 2000).

In addition to coactivators that interact directly with transcriptional activation domains of SHRs, proteins that modulate sequence specific DNA binding SHRs have been identified. The best characterized of these factors are the nuclear high mobility group proteins HMG-1, and related HMG-2, that enhance the affinity of the SHRs for their specific target HREs.

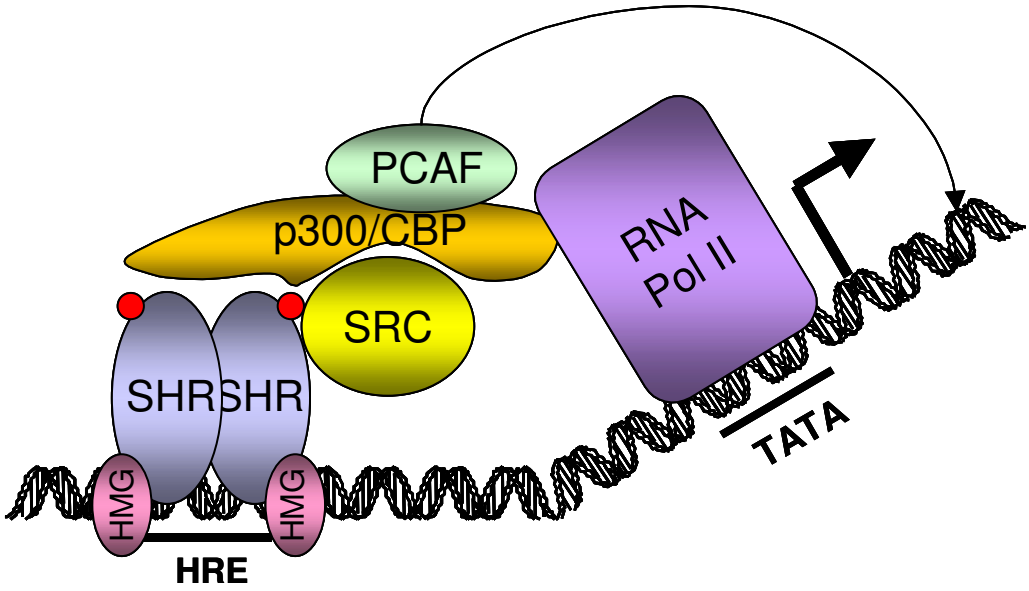

Figure I.9: Mechanism of action of SHRs coactivators.
A mechanism for how p160 (SRC) proteins mediate SHRs transcriptional activation in two steps: firstly, histone acetyl transferase (HAT) activity of the recruited p160 coactivator complex modulates chromatin structure locally resulting in general transcription factors access to DNA at the promoter. Later, RNA pol II complex is recruited through direct or undirect binding of coactivators with general transcription factors (p300/CBP and PCAF) associated with RNA pol II. HMG-1 and HMG-2 enhances transcription by promoting SHRs recruitment to specific hormone response elements (HREs) and stabilizing SHR-DNA complex.

HMG-1 and HMG–2 in mammalian cells enhance the hormone-dependent transcriptional activity of several different SHRs including PR, AR, GR and ER. HMG-1 and HMG–2 are sequence-independent proteins that recognize DNA structure. They do not interact with HREs, but form a weak transient protein interaction with PR and is found associated with PR-DNA complex by establishing protein-protein and protein-DNA contacts (Melvin, Edwards 1999). Therefore, HMG-1 and HMG–2 contribute to the stability of the receptor-DNA complex (Fig. I.9).

I.1.4.2. Correpressors

Several groups have discovered that antagonists of ER and PR promote receptor association with the correpressor NCoR (nuclear receptor correpressor) and SMRT (silencing mediator of retinoid and thyroid receptor) (Wagner et al. 1998). NCoR and SMRT are multiprotein complexes that exhibit deacetylase activity (HDAC), indicating that targeted deacetylation of core histones is necessary for the silencing activity of these proteins (Edwards

2000). Thus, NCoR and SMRT have opposite effect on chromatin structure to HATs and repress access of general transcription factors to DNA.

Specially relevant is the correpressor activity when SHRs are occupied by hormone antagonist. Tamoxifen (antagonist of estrogens) and antiprogestin RU 486 can act as partial agonist / antagonists or complete antagonists. Both steroid analogs inactivate AF-2, whereas the partial agonist activity is mediated by AF-1 (Leonhardt, Edwards 2002). Under partial agonist / antagonist activity conditions, overexpression of NCoR or SMRT inhibits partial agonist activity, indicating that the level of expression and cellular availability of coactivators or correpressors determines the activity or inactivity of an specific promoter (Jackson et al. 1997).

I.1.4.3. General transcription factors

SHRs not only regulate transcription by HRE-mediated effects, but also control the activity of promoter through positive and negative interactions with other sequence-specific transcription factors. Specially interesting is the relation between SHRs and their transcriptional partners AP-1 and Sp-1, that will be illustrated later with different representative SHR-promoter regulation examples.

Transcription factor cross-talk is not limited to SHRs and AP-1 or Sp-1, but also occurs between SHRs and the NFkB factor, CREB, the octamer transcription factor (OTF-1) and STATs (Kutoh, Stromstedt & Poellinger 1992, Scheinman et al. 1995, Stocklin et al. 1996). Briefly, transcriptional cross-talk depends on the interaction of proteins whereas interaction with the DNA in many cases is secondary since only a recognition motif in the target gene for one of the factors is sufficient.

I.1.4.4. Chromatin remodelling factors

Interaction of SHRs with DNA, general transcription machinery, correpressors and coactivators and general transcription factors takes place in the nucleus with DNA compacted into chromatin. Modulation of the chromatin structure and dynamics of the nucleosomes is an important regulatory mechanism of DNA transcription. Changes in chromatin structure affect the binding of transcription factors and the transcription machinery complex by restricting their access.

The cell has multiple strategies to use chromatin structure to regulate DNA-related processes. The two more important are ATP-dependent chromatin remodelling and enzimatic complexes that post-translationally modify the histones. On the other hand, the histones can incorporate core histone variants to alter the structure and dynamics of chromatin.

ATP-dependent chromatin remodelling complexes use ATP hydrolysis to modify histone-DNA and histone-histone interactions. ATP-dependent chromatin remodelling complexes are multisubunit complexes with an ATPase enzimatic activity as a catalytic center. ATPase subunits can be classified in three families depending on the presence of functional domains (Eisen, Sweder & Hanawalt 1995):

i) SWI/SNF-ATPases are characterized by the presence of a bromodomain which binds acetylated histones. This family includes mammalian BRM and brahma-like 1 (BRG-1) (Hassan et al. 2002)

ii) The Imitation SWI (ISWI) family has a SANT domain, which is thought to act as a histone-binding domain to recognize specifically modified histones. This family includes mammalian SNF2H and SNF2L (Boyer, Latek & Peterson 2004)

iii) CHD-ATPases are the chromodomain and helicase-like domain family and have two amino-terminal chromodomains that interact with methylated histone tails (Flanagan et al. 2005)

Post-translational modifications of the histone-tails modify chromatin to regulate accesibility to DNA and nuclear factors recruitment. The modifications are summarized on Fig. I.10. Briefly, they consist in acetylation of lysines, methylation of lysines or arginines, phosphorylation of serines and threonines, as well as ubiquitynation and sumoylation of lysines (Jenuwein, Allis 2001).

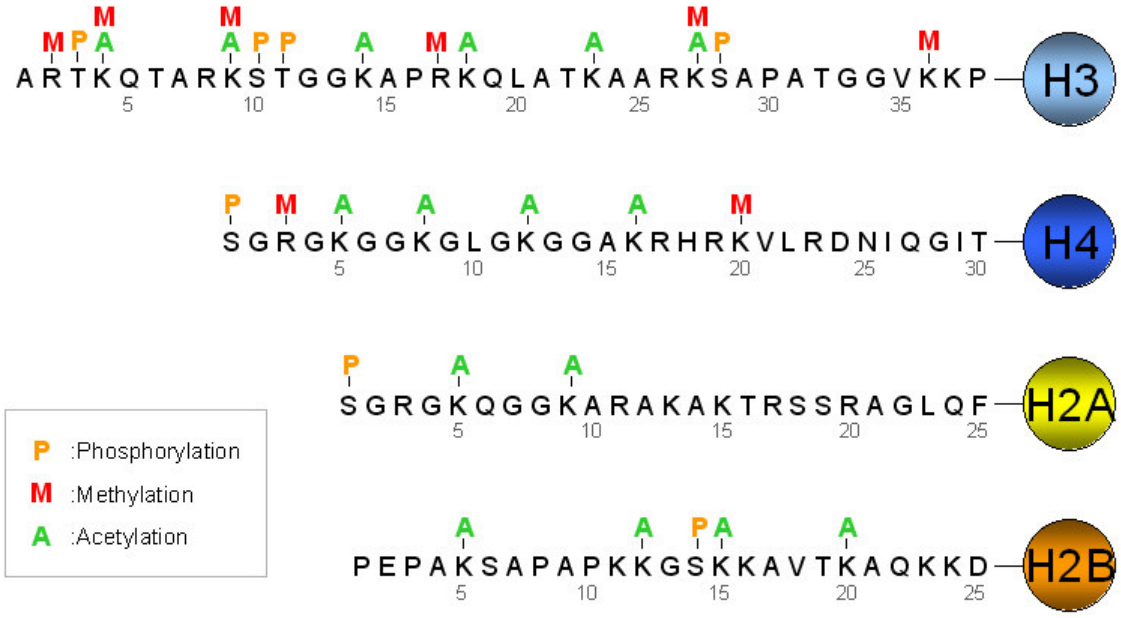

Figure I.10: Post-translational modifications of the core histones.
The colored letters represent the known post-translational modifications of the core histones (H3, H4, H2A and H2B). The histone tails can be phosphorylated at serines or threonines (orange P); methylated at lysines and arginines (red M); and acetylated at lysines (green A).

I.1.5. Reviewing three SHs-regulated promoter models

I.1.5.1 PR regulation of the MMTV promoter

Many regulatory regions of hormone-responsive genes are organized in positioned nucleosomes, which are remodelled in the context of hormone induction. One of the more extensively studied model systems is the hormonal regulation of the Mouse Mammary Tumour Virus (MMTV) promoter.

After hormone stimulation, rapid changes in its chromatin structure occur since it was reported the appearance of one DNase I hypersensitive site in a promoter region in which there are HREs. The MMTV HREs were firstly identified in experiments with the GR (Scheidereit et al. 1983), although later it was shown that PR also could bind them with high affinity and mediated progestone-dependent promoter transcription (Cato, Henderson & Ponta 1987, Chalepakis et al. 1988).

The MMTV promoter is organized in positioned nucleosomes, with the nucleosome B covering the HREs region and a binding site for NF1 (Fig. I.11) (Richard-Foy, Hager 1987). For the hormonal activation of the promoter is required not only the HREs but also the NF1 binding site, indicating that both

factors, GR or PR and NF1, synergize *in vivo*. However, the nucleosomal organization of the promoter seems to be very important, since NF1 competes with PR for binding and transactivation in *in vitro* experiments using naked DNA templates(Kalff, Gross & Beato 1990). In intact cells, PR and NF1 occupy their binding sites at the same time after hormone induction on the surface of the nucleosome (Fig. I.11) (Truss et al. 1995).

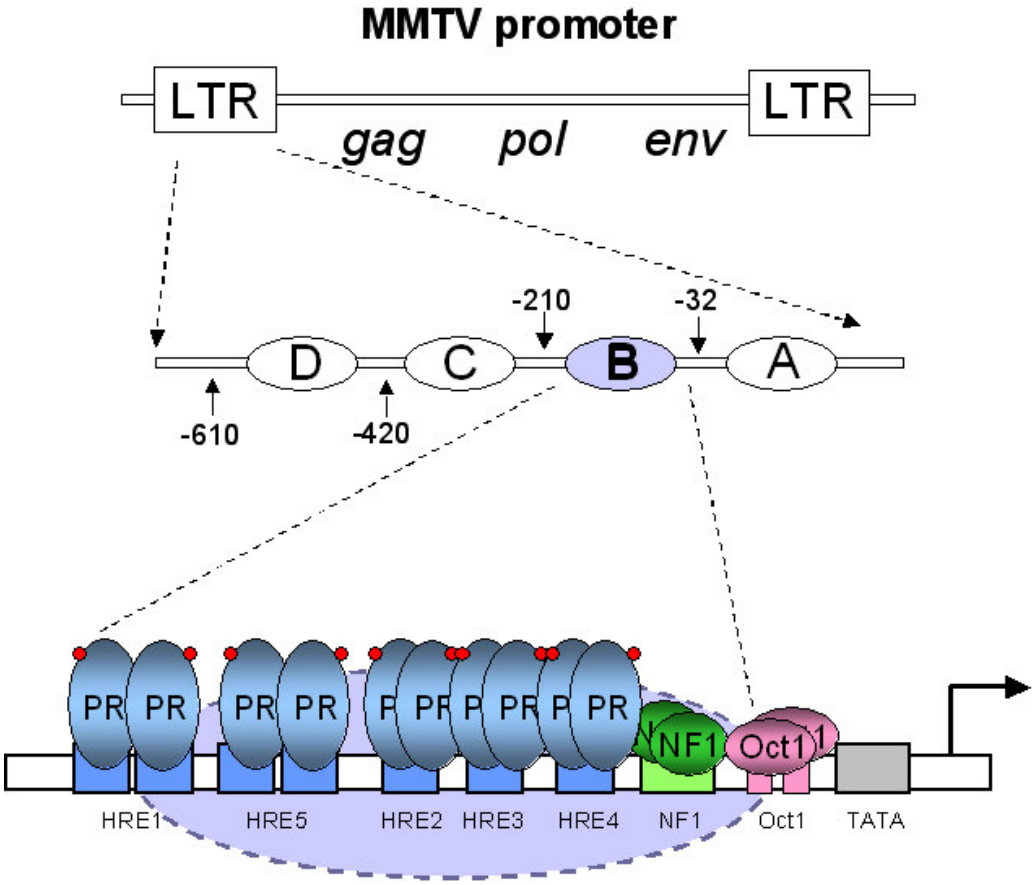

Figure I.11: Schematic representation of the main elements in the MMTV promoter. MMTV promoter is found at the 5'-LTR of the viral genome, where several nucleosomes (A – D) are positioned when integrated at the host genome. Nucleosome B has a regulatory role covering HREs and binding sites for transcription factors (NF1 and Oct 1). The positions covered by the nucleosome B are indicated by the blue oval. The positions occupied by PR and transcription factors represented correspond with the *in vivo* situation after hormone induction. The numbers refer to the distance in nucleotides from the transcription start site.

The octamer transcription factor 1 (Oct-1) also participates in MMTV promoter regulation. In fact, Oct-1 shows transcriptional synergism with PR in transient transfection experiments and *in vitro*. But the role of Oct-1 in chromatin-organized MMTV promoter has not been confirmed *in vivo*.

In vitro nucleosomes assemblies with MMTV promoter DNA, showed that the promoter adopts a precise rotational orientation on the surface that exposes

HRE-1 and HRE-4, but leaves inaccessible the central HREs 2, 3 and 5, essential for hormone inducion. In the same way, NF-1 cannot bind to the MMTV promoter (Eisfeld et al. 1997, Pina, Bruggemeier & Beato 1990). These evidences showed that the nucleosome may undergo changes during hormone induction to enable the binding of PRs and NF-1 and their synergism.

In T47D-MTVL cells (T47D cells carrying a single copy of the MMTV promoter integrated in the chromatin), a DNase I hypersensitive site appears after progesterone treatment in the HREs promoter region (Truss et al. 1995). The same DNase I hypersensitive site appears after treatment with tricostatin A (TSA), an inhibitor of histone deacetylases, reflecting that the changes of the chromatin could be started by an increase in histone acetylation (Bartsch et al. 1996).

To study the interaction between PR and chromatin-organized MMTV promoter sequences, arrays of nucleosomes were generated with chromatin assembly systems. In particular, extracts from pre-blastodermic Droshophila embryos were used for chromatin assembly, because of the abundance in core histones and the presence of the machinery needed in those extracts (Venditti et al. 1998). Minichromosomes assembled in these extracts exhibit the same behaviour that nucleosomes detected in chromatin of breast cancer cells. In a cell-free transcription system, addition of PR and NF-1 to these minichromosomes causes a strong synergistic transcriptional activation, which is dependent on the presence of ATP, suggesting that ATP-dependent chromatin remodelling complexes are needed (Di Croce et al. 1999). DNA footprinting experiments indicated that under physiological conditions, not only PR helps NF-1 to bind, but NF-1 is needed for optimal PR binding (Di Croce et al. 1999). NF-1 seems to be necessary to stabilize the “open” conformation of the nucleosome and facilitate the access of PR.

Chromatin immunoprecipitation (ChIP) assay in T47D-MTVL cells showed that after progesterone treatment with synthetic analogue R5020, PR is bound to the MMTV promoter. The coactivator SRC-1 and the chromatin remodelling complexes BRG-1 and SNF2H were also detected (Truss et al. 1995, Vicent et al. 2004). ChIP assays also showed that there is a loss of histones H2A and H2B from the promoter nucleosome B, but not from the nucleosomes C or D (Vicent et al. 2004). *In vitro* experiments confirmed that in

the presence of ATP, SWI/SNF could displace H2A and H2B from MMTV promoter nucleosomes (Vicent et al. 2004). These results suggested that the nucleotide sequence leads nucleosome positioning and the remodelling process.

The dynamics of the nucleosome could be also regulated by the linker histone H1 due to its interaction with nucleosomal DNA. H1 is a structural component of chromatin that functions as a general repressor of transcription. In the presence of bound H1, SWI/SNF complex cannot remodel nucleosomes *in vitro* (Horn et al. 2002). H1 binds asymmetrically to MMTV nucleosomes, with preference for the distal 5' end (Vicent, Melia & Beato 2002). MMTV promoter transcription and induction by PR and NF-1 were enhanced in H1 containing minichromosomes, due to the better positioning of nucleosomes in the presence of H1 and a better binding of PR (Koop, Di Croce & Beato 2003). In the presence of bound PR, H1 is phosphorylated and removed from the promoter on transcription initiation (Koop, Di Croce & Beato 2003).

Progestins stimulate proliferation of human breast cancer cell lines and a rapid activation of the Src/Ras/Erk pathway that requires PR and ER (Ballare et al. 2003). Progestin activation of the PR/ER complex triggers ER-mediated activation of the Src/Ras/Erk cascade and accumulation of active Erk1/2 in the nucleus (Fig. I.12)(Vicent et al. 2006). Ligand binding to the complex of PR in the nucleus, leads to the formation of PR homodimers that are phosphorylated by activated Erk1/2, which also phosphorylates Msk1 (Mitogen- and stress-activated protein kinase-1). The three activated proteins form a ternary complex that is recruited to the nucleosome B, due to the affinity of PR for the exposed HREs. Once bound to the promoter, Msk1 phosphorylates histone H3S10 generating a signal that leads to displacement of a repressive complex containing HP1γ. Thus, ATP-dependent chromatin remodelling complexes are recruited by PR and removal of H2A/H2B dimers from nucleosome B allows binding of NF-1, coactivators and the basal transcriptional machinery including RNA polymerase II (Fig. I.12) (Vicent et al. 2006).

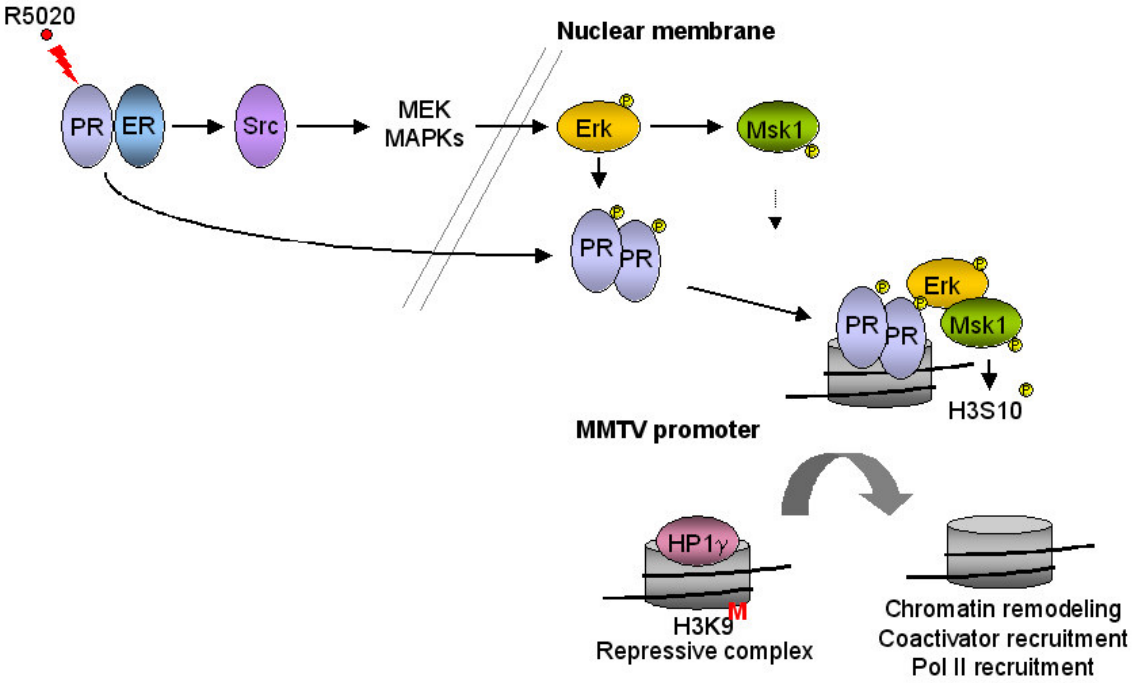

Figure I.12: Model for the MMTV promoter induction by progestins
Progesterone binds to cytoplasmic PR / ER complexes and activate the Src/Ras/Erk pathway leading to nuclear accumulation of activated Erk. PR is phosphorylated by pErk, which also phosphorylates Msk1. A complex of pPR/pErk/pMsk1 is recruited to the promoter and catalyzes phosphorylation of H3S10 and displacement of a HP1γ-containing repressive complex. Subsequently, an ATP-dependent chromatin-remodelling complex is recruited to the promoter and catalyzes displacement of H2A/H2B dimers, leading to further transcription factors recruitment, remodelling events and RNA pol II recruitment.

Thus, the MMTV promoter represents a well-known model of link between rapid kinase activation and gene induction by progesterone.

I.1.5.2. PR regulation of p21 promoter

Progesterone has effects on mitosis and cell cycle regulation in breast cancer cells (Groshong et al. 1997, Musgrove et al. 1997). Treatment with progestins induces biphasic effects:

i) progestin induces entry of cells into S-phase and increases cyclin D1 and cyclin-dependent kinase 4 (cdk-4)

ii) this first cycle is followed by growth arrest at the G1/S boundary of the second cycle (Groshong et al. 1997, Musgrove et al. 1997). After the first cycle, cells enter a period of resistance to growth effects, characterized by hypophosphorylation, down-regulation of pRb, loss of cyclins D, A and B, and increases in

the level of the cdk inhibitor p21 ($p21^{Waf1,\ Cip1,\ Kip1,\ Sdi1}$) and p27 (Groshong et al. 1997).

Elevated p21 levels are associated with differentiation and transcription activation of p21 promoter in a p53-independent manner, suggesting that integration of different signals occurs at the p21 promoter (Xiong et al. 1993).

p21 promoter lacks a canonical PRE, however, in transient transfections progesterone activates about 2-3 fold-induction of p21 promoter, and a second dose of hormone rises this activation to 12-fold (Owen et al. 1998). The progesterone-dependent minimal promoter described by deletion analysis maps to the Sp1-3 and Sp1-4 sites located between –84 and –65 nt (Fig. I.13), whereas the p53 binding sites are located at –1.4 and –1.9 kb, suggesting that progesterone-regulation of p21 activation is a p53-independent process (Owen et al. 1998).

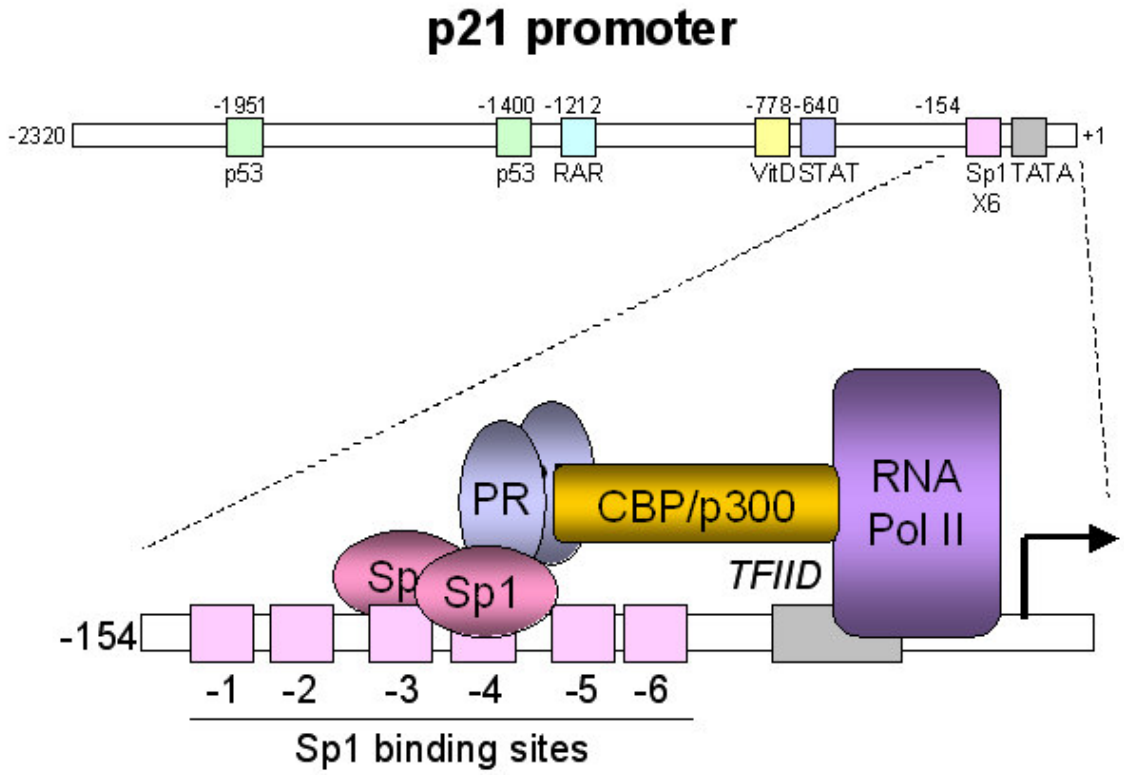

Figure I.13: Progesterone regulation of the p21 promoter.
Upper panel, the proximal 2.3 Kb of *CDKN1A* (p21) promoter showing key regulatory regions and factor binding sites. The numbers refer to the distance in nucleotides from the transcription start site. RAR, retinoic acid receptor; Vit-D, vitamin D receptor.
Lower panel, model for the progesterone-regulated p21 promoter in which PRs are indirectly bound to the promoter through Sp1 proteins covering Sp1 sites 3 and/or 4, in a multiprotein complex that also includes CBP/p300 and TFIID. Binding of these proteins to the TATA box is not required. *Adapted from* (Owen et al. 1998).

Proximal p21 promoter contains six Sp1 binding sites and a TATA box. Footprinting studies have shown that Sp1 is constitutively bound to DNA (Biggs, Kudlow & Kraft 1996), and deletion of TATA box does not abolish the response to progesterone (Datto, Yu & Wang 1995, Owen et al. 1998). Interestingly, the PR DBD interacts with dTAF110 (TATA-binding protein associate factor) at its C-terminus in the TFIID complex and Sp1 is known to interact with the N-terminus of dTAF110 (Mitchell, Tjian 1989, Schwerk et al. 1995). Thus, PR and Sp1 could bind dTAF110 suggesting a mechanism for PR regulation of the p21 promoter (Owen et al. 1998).

On the other hand, same studies identified that CBP/p300, a corregulator of many transcription factors, including SHRs, can bind to PR and, together with Sp1, p300 is required for progesterone induction of p21 promoter (Owen et al. 1998). Therefore, p300 is an integral component of the basal transcriptional machinery of p21 promoter or PR recruits p300 to the transcription complex (Fig. I.13).

More recent studies described that minimal region of p21 promoter, containing from –60 to +40bp promoter region, is essential and sufficient for the induction of p21 promoter by an inhibitor of histone deacetylases, trichostatin A (TSA), and that Sp1 and Sp3 are the functional activators of this region (Xiao, Hasegawa & Isobe 1999). Remarkably, p300 possess intrinsic histone acetyltransferase activity (Smith et al. 1996), suggesting the linkage between p300 and TSA-induced p21 promoter activation. Thus, the functional collaboration of p300 and Sp1 in p21 expression may rely on the effect of p300 on the activity of the transcriptional initiation complex and the interaction of this complex with Sp1, which is the base for Sp1-mediated transcription in p21 activation. Some reports have indicated that p300 is the indispensable factor for the p21 promoter activation, not only in the p53-mediated process, but also in the Sp1-mediated (Xiao, Hasegawa & Isobe 2000).

I.1.5.3. ER regulation of Cyclin D1 promoter

Cyclin D1 (CCND1) is part of the family of cyclins D, which regulate G1/S cell cycle progression. CCND1 acts by activation of cyclin-dependent kinases (cdks) that phosphorylate and inactivate the retinoblastoma protein (Rb). CCND1 could also promote cell cycle progression by the interaction with

transcription factors (Arnold, Papanikolaou 2005). Interestingly, CCND1 is overexpressed in human breast cancer and, remarkably, CCND1 expression is induced in mammary epithelial cells by estrogens and progesterone (Sutherland et al. 1998). Transcriptional activation of CCND1 in breast cancer by factors such as ERα could be an important mechanism to explain its overexpression. No EREs or ERE half-sites are present along CCND1 gene/promoter regions (Sabbah et al. 1999)

Recently, it was identified a 24 bp regulatory element, located about 940 nt upstream CCND1 transcription start site, which is responsible of the estrogen regulation (Cicatiello et al. 2004). This site includes an AP-1 element, which binds the c-Jun / c-Fos heterodimer in breast cancer cells, and overlapping binding sites for Oct-1 and YY-1 (Cicatiello et al. 2004).

In hormone starved cells, where CCND1 transcription is minimal, the site is occupied *in vivo* by a complex of YY-1 and HDAC-1. YY-1 is a zinc finger protein that shows activation or inhibitory behaviour on gene expression (Thomas, Seto 1999). YY-1 forms repressing complexes with histone-modifying enzymes, in particular, HDAC-1. Thus, YY-1 competes for binding of positive acting factors to the same DNA, including AP-1 and Oct-1 (Mizuno et al. 2003, Ye et al. 1996). YY-1 binding site of the CCND1 promoter overlaps with those for AP-1 and Oct-1, therefore, its presence is mutually exclusive with that of RNA polymerase II on the promoter (Fig. I.14)(Cicatiello et al. 2004).

At 10 – 15 min after cell stimulation with estrogens, YY-1 / HDAC-1 complex is replaced by AP-1 and Oct-1, which, in turn, interact with transcriptionally active RNA polymerase II. cFos / cJun proteins are present in the nucleus in increased concentrations because their corresponding genes are activated by the hormone during the early cell cycle phase. On the other hand, binding of Oct-1 to the site might exert different functions. First, it may help in replacing YY-1 by AP-1. Second, the presence of Oct-1 may help to establish interactions between the binding site and activators bound to different sites in CCND1. Third, DNA looping and protein bridges that connect the binding site to the basal transcriptional machinery assembled at the transcription start site might be reinforced by Oct-1 (Boulon et al. 2002, Nakshatri, Nakshatri & Currie 1995, Pfeuffer et al. 1994).

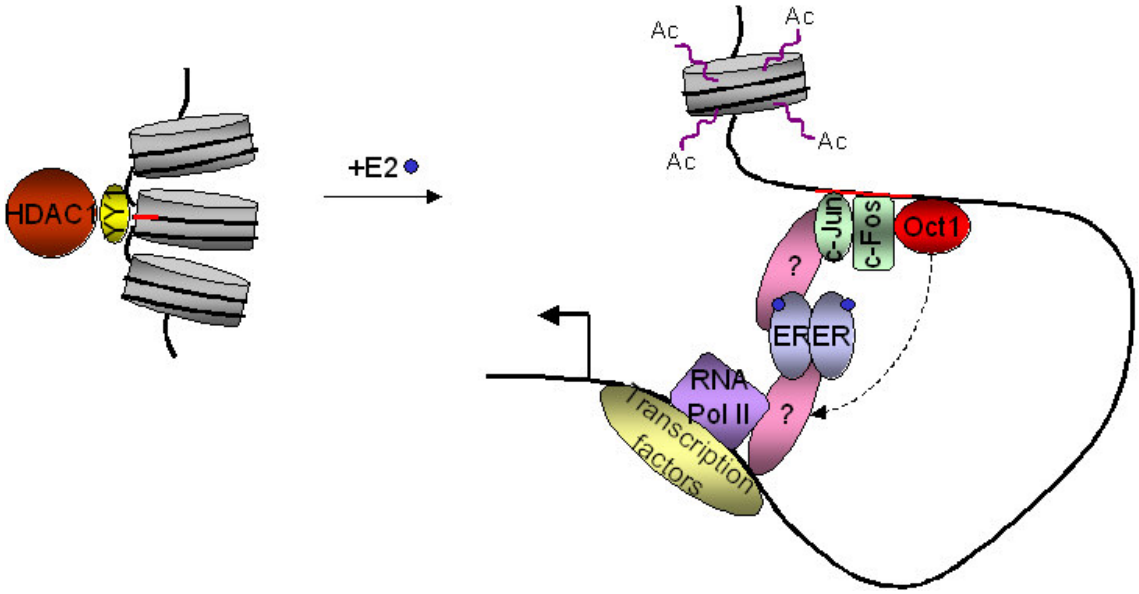

Figure I.14: Estrogen-dependent regulation of the *CCND1* gene promoter.
In hormone deprived cells, HDAC1 is present in a complex with YY-1 on the estrogen regulated site of the *CCND1* gene promoter (left panel). Upon estrogen stimulation, these trans-repressors are displaced from DNA by an AP-1/ER complex and Oct-1, inducing physical interactions of the ER element-bound complexes with the basal transcriptional machinery (right panel). *Ac,* acetylated histone tails.

The increased binding of both AP-1 and Oct-1 to their binding site in cells stimulated by estrogens is accompanied of ERα recruitment to the promoter (Fig. I.14), which is required to act as a transcriptional corregulator for the AP-1 complex (Cicatiello et al. 2004). ERα plays a determinant role in recruiting coactivators to the AP-1 complex.

More recently, it has been reported the presence of two enhancers in the CCND1 gene: an enhancer element was located between –2.2 and –2 kb, whereas estrogens induction of CCND1 expression in breast cancer cells depends on a cell-type-specific enhancer downstream from the CCND1 coding region (Eeckhoute et al. 2006). ERα acts directly at the CCND1 gene through a FOXA1-dependent interaction with this second enhancer and collaborating with other transcription factors (Eeckhoute et al. 2006). FOXA1 is required for estrogen induction of cell cycle progression and breast cancer cells growth. There are different evidences about FOXA1 importance for ERα recruitment to a subset of its target gene sequences (Laganiere et al. 2005). FOXA1 could be involved in chromatin remodelling activities, indeed, FOXA1 recruitment is able to induce remodelling of compacted chromatin *in vitro* and *in vivo* (Eeckhoute et al. 2006).

I.2. STATs

I.2.1. JAK / STAT pathway overview: family members and structure

STAT (for Signal transducers and activators of transcription) family of transcription factors exist within the cytoplasm in an inactive state. STATs are activated by phosphorylation on a single tyrosine located around residue 700 (Darnell, Kerr & Stark 1994). Ligand-activated receptors that catalyze this phosphorylation include receptors with intrinsic tyrosine kinase activity (i.e., epidermal growth factor), as well as receptors that lack intrinsic tyrosine kinase activity but to which Janus Kinases (JAKs) are non-covalently associated (i.e. prolactin receptor) (Ihle et al. 1995).

JAK family (Jak1 to 3 and Tyk2) includes four large tyrosine kinases (~1200 amino acids) characterized by a COOH-located kinase and an adjacent domain that resembles but is not an active kinase. JAKs are rapidly activated by autophosphorylation. In turn, activated JAK kinase phosphorylates the receptor to which it is bound, and receptor-associated signaling proteins (Fig. I.15). Receptor phosphorylation enables STAT docking to this complex, via binding of a STAT SH2 domain to a receptor phosphotyrosine residue. STAT phosphorylation of the tyrosine residue present in the STAT C-terminus triggers its release from receptor and homodimerization. Dimerized STATs rapidly translocate to the nucleus, where binding to gene promoter through cognate DNA-binding sequences occurs. Then, STATs engage several elements of the transcriptional apparatus, stimulating gene expression (reviewed in (Clevenger 2004).

Seven mammalian STAT genes have been identified in three chromosomal clusters (Copeland et al. 1995). The genes encoding STATs 1 and 4 map to human chromosome 2; STATs 3, 5A and 5B map to chromosome 12; and STATs 2 and 6 to chromosome 17. STATs 1, 3, 4, 5A and 5B are between 750 and 795 amino acids long, whereas STATs 2 and 6 are ~850 amino acids long.

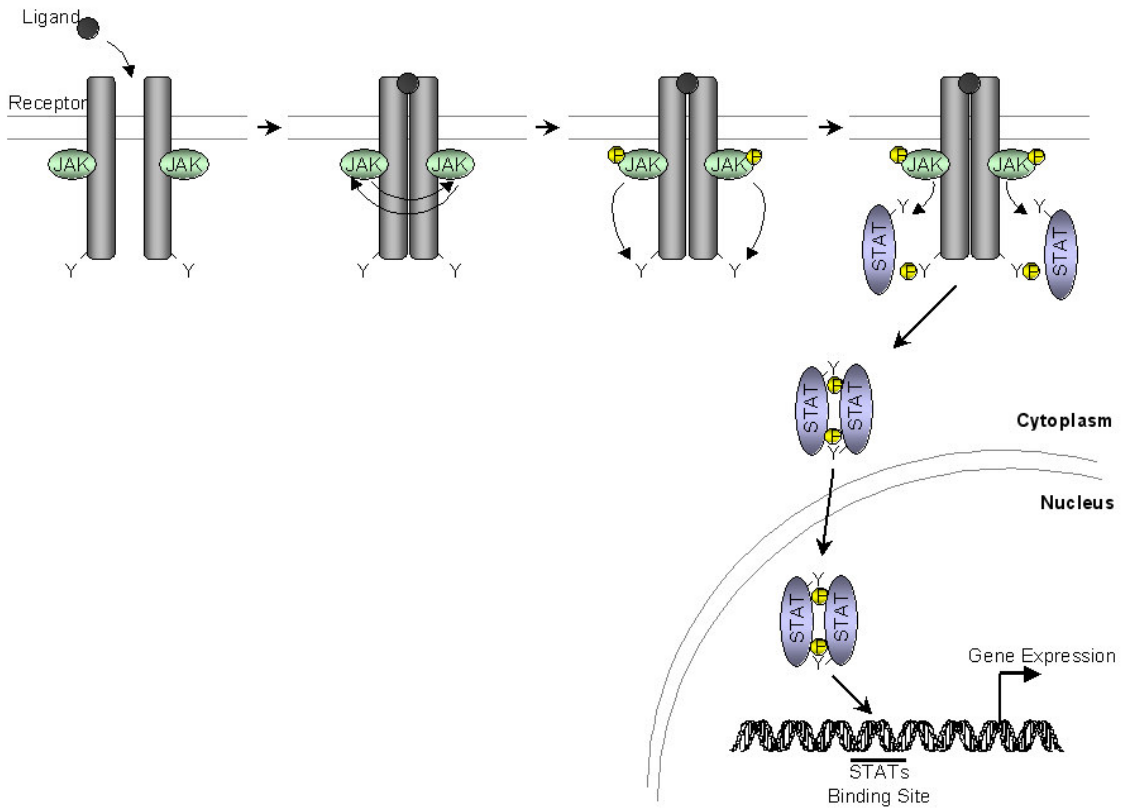

Figure I.15: Generalized mechanism of STAT tyrosine phosphorylation by JAK.
After ligand-induced receptor dimerization, activated JAK sequentially autophophorylates, phosphorylates the receptor and STAT. This induces the release and dimerization of phosphorylated STAT, enabling its translocalization into the nucleus and subsequent DNA binding to target promoter. Receptors involved in JAK activation could be those with intrinsic tyrosine kinase activity (i.e., epidermal growth factor receptor, EGFR), as well as receptors that lack intrinsic tyrosine kinase activity but to which JAKs are non-covalently associated (i.e. prolactin receptor, PRLR).

STAT family was first identified related with interferon (IFN) triggered gene expression (Darnell 1996). Analysis of the promoter regions of these IFN-activated genes revealed that specific motifs with a consensus DNA sequence of

TT(C/A)NNN(G/T)AA

were required for this expression.

More recently, with the recognition of STATs as transcription factors, came the identification and characterization of the JAK family of tyrosine kinases. Using mutagenesis and complementation approaches, cell clones defective in IFN-induced signalling regained IFN-responsiveness when JAKs and STATs were reintroduced, establishing linkage between these two families (Darnell 1996).

STAT proteins consist of numerous distinct functional domains (Bromberg, Darnell 2000) (Fig. I.16):

i) the N terminal domain has been found necessary for the interaction of STATs 3 and 5 with a number of coactivators such as p300 and c-Jun

ii) the central DNA-binding domain is required for recognition of binding sequences

iii) coupled with DBD, there is a flexible linker to a SH2 domain that is necessary for the recognition of phospho-tyrosine residues that contribute to STAT dimerization

iv) between the SH2 motif and the C-terminal transactivation domain residues, a conserved tyrosine residue which phosphorylation is required for the dimerization of all STAT family members

v) the transactivation domain has a critical role in coordination with the transcriptional machinery and also contains a serine residue(s) that regulates the activity of this domain.

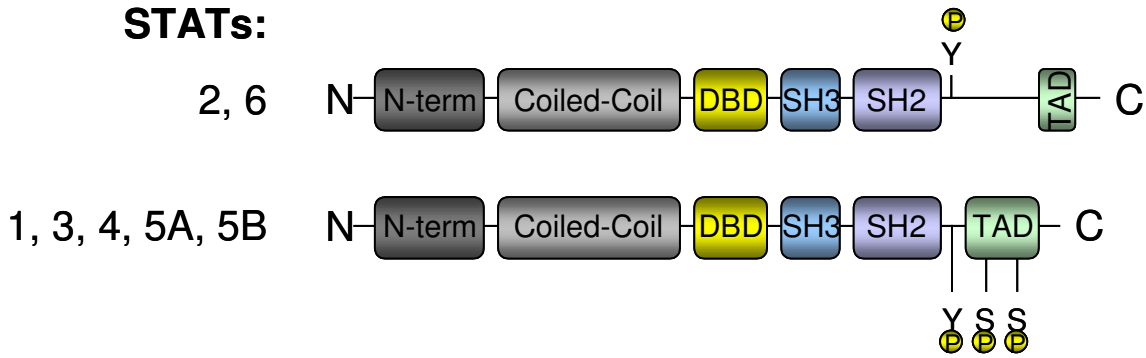

Figure I.16: STAT proteins structure.
General schematic representation of the different STATs, including the structural domains with critical functions. The universally shared regions and their boundaries are indicated with the different coloured boxes. Phosphotyrosine (pY) is present in all activated STATs; phosphoserine (pS) is present in activated STATs 1, 3, 4, 5A and 5B. Transactivation domain (TAD) is shown in green.

The structure of DNA-bound STAT dimers has been resolved at the crystallographic level (Becker, Groner & Muller 1998, Chen et al. 1998). Crystal structures resemble a nutcracker, with the interactions between the respective STAT phosphotyrosine/SH2 domains serving as the hinge and the DNA acting

as the nut. Interestingly, the engaged DNA is bent by 40º as a result of this interaction.

I.2.2. STATs regulation

I.2.2.1. STAT DNA binding

Selection of optimum binding sites for STATs resulted in the recovery of the consensus oligonucleotide sequence

TT(C/A)nnn(G/T)AA,

or generically, TTN5AA, where N represents any nucleotide (Horvath, Wen & Darnell 1995, Xu, Sun & Hoey 1996).

STATs molecules interact with DNA as dimers. Mutants of STATs that lack the phosphorylated tyrosine residue critical for dimerization do not bind DNA (Gouilleux et al. 1994). Mutational analysis of the DNA binding motif of STAT and analogous substitution to alanines of a conserved sequence motif within this, abolished STAT binding to specific promoter sites (Herrington et al. 1999). More detailed homology analysis and extrapolation from crystal structure models defined a central DNA-binding motif of STATs that corresponds to amino acid 331 to 496 (Chen et al. 1998). This motif is highly conserved between human STAT5A and 5B with only six moderate amino acid deviations. However, the natural sites from genes regulated in response to particular ligands show clear preferential binding affinities to the different STATs. Thus, selective gene activation by the various STATs could be attributable to differential STAT dimer binding to DNA (Schindler, Darnell 1995).

I.2.2.2. Tyrosine phosphorylation

STAT activation requires C-terminal tyrosine phosphorylation by a receptor-associated Jak kinase. This phosphorylation occurs adjacent to the transcriptional activation domain within STATs (Y694 in STAT5A) and induces dimerization / multimerization and nuclear translocation of the STAT complex when it binds to its specific DNA binding sequence, resulting in promoter transactivation under appropriate conditions (Darnell 1996).

Tyrosine phosphorylation is necessary for STAT activity; replacement of this tyrosine residue results in an inactive STAT incapable of nuclear

translocation or transactivation. However, other tyrosine kinase-signalling pathways may also impact STAT activation because both STAT3 and 5 can be phosphorylated by Src family members (Kazansky et al. 1999). In spite of this, only Src-induced phosphorylation of STAT5B, but not STAT5A, promoted nuclear translocation (Kazansky et al. 1999). It is clear, however, that STATs are downstream of Src-family-mediated signalling because transcriptionally inactive STAT3 blocks Src-mediated transformation (Garcia et al. 2001). Remarkably, enhanced levels of STAT3 and STAT5 tyrosine phosphorylation have been noted in human breast cancers (Cotarla et al. 2004).

I.2.2.3. Serine phosphorylation

All STAT family members with the exception of STAT2 undergo serine phosphorylation after receptor-mediated signalling. Serine phosphorylation at residue 727 of STAT1 and 3 results in a significant up-regulation of the transcriptional activity of these STATs (Decker, Kovarik 2000). This event appears to be mediated by several converging kinases including MAPK, p38, JNK and protein kinase Cd (Decker, Kovarik 2000).

In contrast, serine phosphorylation at positions 725 and 779 in STAT5A and position 730 in STAT5B, down-regulated the transcriptional activity of STAT5 (Park et al. 2001). The phosphorylation of serine 725 and 779 of STAT5A is cooperative and mediated by both MAPK and non-MAPK signalling pathways; however, the suppressive effects of this phosphorylation appears to be mitigated in part by co-stimulation of GR in MCF7 breast cancer cells (Park et al. 2001).

I.2.2.4. Dephosphorylation

Several phosphatases, notably SHP1, SHP2, CD45, PRP1B and TCPTP, have been demonstrated to regulate Jak kinase activity, and many of these have also been found in association with the STAT family (Shuai, Liu 2003).

Tipically, receptor-associating phosphatases contain tyrosine-binding SH2 motifs; a phosphatase domain; and regulatory tyrosine residues that undergo variable phosphorylation.

TCPTP has been found to associate with STAT5A and 5B within the nucleus and induce their dephosphorylation and inactivation. The phosphatase

activity of TCPTP, however, is not required for the inactivation of STAT5-mediated gene expression: the association of TCPTP may be sufficient (Aoki, Matsuda 2002).

PTP1B also has similar effects on STAT5A and 5B phosphorylation and activity, however, this phosphatase is found within the cytoplasm (Aoki, Matsuda 2000)

SHP2 has been found in association with STAT5A and migrates with it as a complex into the nucleus. Interestingly, the intact phosphatase activity of SHP2 is required for STAT5 phosphorylation, suggesting that this phosphatase is not directly involved in the dephosphorylation / deactivation of STAT5, but instead indirectly implicated with the activation of this STAT (Chughtai et al. 2002).

I.2.2.5. SOCS proteins

The SOCS (supressors of cytokine signalling) family consists of eight members comprised of SOCS1 to SOCS7 and CIS (cytokine-inducible SH2 domain protein). Each family member exhibits three domains: a poorly conserve N terminus; a central phospho-tyrosine-binding SH2 domain; and a conserved, C-terminal SOCS-box motif, that may mediate post-translational ubiquitination (Starr, Hilton 1999).

In resting cells, SOCS proteins are expressed at low levels; after receptor-mediated STAT family signalling, SOCS levels increase. This increase is principally mediated at the transcriptional level although SOCS phosphorylation contributes to protein stability (Naka et al. 1997).

SOCS proteins act as classic negative regulatory inhibitors. SOCS1 blocks STAT phosphorylation and activation by directly binding to phosphorylated Jak. SOCS3 inhibits Jak activity by first binding receptor. CIS blocks STAT activation by blocking STAT-binding sites on receptors. In addition, the ability of the SOCS box to engage elements of the ubiquitination pathway may significantly contribute to down-regulation of JAK / STAT signalling (Zhang et al. 1999).

I.2.2.6. PIAS / Sumoylation

The PIAS (peptide inhibitors of activated STATs) family of proteins (PIAS1, PIAS3, PIASx and PIASy) have been found to bind STAT family members and block their binding to DNA and / or transcriptional activity (Shuai 2000).

PIAS proteins contain three regions of protein homology:

i) a N-terminal LXXLL motif, thought to contribute to nuclear receptor interactions

ii) a central ring-finger domain, related with sumoylation activity

iii) and a serine-rich C-terminus.

PIAS proteins are constitutively expressed within the nucleus and appear to act as constitutive repressors of STAT activity (Shuai 2000). Each PIAS member associates and modulates the function of distinct subset of transcription factors, for example PIAS3 associates with STAT3, 5A and 5B and PIASx with the AR. Evidences indicated that the N-terminal domain of STAT1 binds to the region between the ring-finger and serine / threonine-rich domains of PIAS1, a region termed the linker domain (Liao, Fu & Shuai 2000).

PIAS proteins serve as small ubiquitin-like modifier (SUMO) E3 ligases (Jackson 2001). Sumoylation is the process by which one of three SUMO peptides (termed SUMO1 to SUMO3), consisting of ~100 amino acids is added to a consensus sumoylation site within a peptide. Sumoylation requires an E1-activating enzyme and an E2 conjugase. Although the process can occur without an E3 ligase, it is not efficient (Muller et al. 2001). Unlike ubiquitin conjugation, sumoylation appears to modify protein function not through degradation but by altering function, localization or extent of ubiquitination (Muller et al. 2001). Interestingly, the functional effects of PIAS association and PIAS-induced sumoylation seem to be distinct. Some data suggest that functional modulation of PIAS proteins may result as a consequence of PIAS interaction, subsequent sumoylation, or both, in a given cellular context.

I.2.2.7. STATs corregulators

a) BRCA1 and BRCA2

The breast-cancer susceptibility genes BRCA1 and BRCA2 were identified in studies of families with inherited susceptibility to breast cancer.

Mutations in these genes create a predisposition to the disease. In MCF7 breast cancer cells, BRCA1 and BRCA2 interacted with STAT5 after stimulation with prolactin (PRL) and suppressed its transcriptional activity on β–casein promoter by a mechanism that remained unknown (Vidarsson et al. 2002). Since BRCA 1 and BRCA2 do not affect STAT5 levels or DNA-binding activity, they may affect STAT5 capacity to regulate transcription by forming a multimerized complex in which the BRCAs serve as bridging proteins. An example is the interaction of BRCA1 with Nmi (an N-myc interactor) which binds STAT5 to form an Nmi-BRCA1-c-myc complex (Li, Lee & Avraham 2002). On the other hand, BRCA1 also binds the POU homodomain protein Oct-1, which interacts with STAT5 C-terminus (Wang, Yu & Deng 2004). Deregulation of STAT5 interaction with BRCA1 could indirectly affect any of these processes.

b) SMRT

STAT5 binds to SMRT (silencing mediator for retinoic acid receptor and tryroid hormone receptor), a protein that recruits histone deacetylase activity to a promoter sequence and participates in gene silencing (Nakajima et al. 2001). SMRT binds to both STAT5A and STAT5B and strongly represses STAT5-dependent transcription *in vitro*.

c) CBP

STAT5 interacts with CBP protein with intrinsic histone acetylase activity (Pfitzner et al. 1998). It has been shown that CBP enhances PRL-induced transcriptional activation by interacting with the transactivation domain of STAT5. This correlates with the notion that histone acetylase activity causes a more relaxed chromatin structure and higher accessibility of transcription factors to the promoter region.

d) NCoA-1 / SRC-1

SRC-1 is a member of a family of coactivators that interact with many transcriptional factors. Gene inactivation of NCoA-1 results in developmental defects in the mammary gland (Xu et al. 1998). In HEK293T cells, SRC-1 co-immunoprecipitates with STAT5A. The transactivation domain of STAT5A mediates this interaction (Litterst et al. 2005). CBP and SRC-1 cooperatively

enhance PRL-induced activation of β–casein minimal promoter in transfected HEK293 cells. In HeLa cells, the same authors showed that SRC-1 is able to further enhance the cooperative action of GR and STAT5A (Litterst et al. 2005).

e) Oct-1

Cyclin D1 promoter encompasses a STAT5 binding site (Brockman, Schroeder & Schuler 2002). In megakaryocytes, its activation involves interaction of STAT5 with the POU homodomain protein Oct-1. *In vitro*, the formation of STAT5-Oct-1 complex does not depend on the presence of DNA. However, DNA mutagenesis experiments have showed that Oct-1, by binding to both the DNA and STAT5, enhances the stability of complexes between STAT5 and weaker binding sites (Magne et al. 2003).

I.2.3. Role of STATs in mammary gland and breast cancer

Gene targeting studies have revealed phenotypes for STAT3 and 5A during mammary differentiation. These data suggest that STAT5 plays a critical role in lobulo-alveolar proliferation, differentiation and expansion; whereas STAT3 regulates lobulo-alveolar apoptosis during pregnancy, lactation and involution (Watson 2001).

STAT3 tyrosine phosphorylation and DNA-binding activity of several breast cancer lines has been found to be elevated. Conversely, dominant-negative or pharmacological inhibition of STAT3 activity have been found to block the proliferation and survival of breast cancer cells (Yu, Jove 2004). In the same way, repression of STAT3 DNA-binding activity by PIAS3 inhibits breast cancer cell growth (Yu, Jove 2004).

Several lines of evidence indicate that STAT3 and 5 can activate the transcription of genes associated with cell-cycle progression, cell survival, transformation, and angiogenesis. STAT3 and STAT5 activity are implicated in the up-regulation of expression of the cell cycle-regulatory proteins cyclin D1 and D2, in several nonmammary cell lines, and some data have suggested that may have similar effects in breast epithelial cell (Brockman, Schroeder & Schuler 2002). Regulation of anti-apoptotic members of the Bcl-2 family,

specifically Bcl-XL, has also been associated with STAT3 and 5 activity (Yu, Jove 2004). STAT3- and 5- induced transcription has also been involved in c-myc oncogene overexpression (Yu, Jove 2004), as well as, STAT3 activity seems to be involved in up-regulated vascular endothelial growth factor (VEGF) expression, an event that may further serve to promote tumour progression (Niu et al. 2002).

All these data suggest a significant role of STATs in breast cancer proliferation, survival and differentiation.

I.2.4. STATs and SHRs cooperation

I.2.4.1. STAT5 and GR

Prolactin and glucocorticoid hormone are signals which regulate the transcription of milk protein genes in mammary epithelial cells. Both hormones activate latent transcription factors in the cytoplasm of mammary epithelial cells. Prolactin exert its effect through binding to the extracellular domain of the prolactin receptor (PRLR) and through receptor dimerization. This leads to the activation of a protein tyrosine kinase (Jak2), which is non-covalently associated with the cytoplasmic domain of the PRLR. Jak2 phosphorylates STAT5 which causes its dimerization and nuclear translocation where STAT5 specifically binds to sequence elements in the promoter regions of milk protein genes (Doppler 1994). This is the case of β–casein promoter that contains binding sites for several nuclear factors, including STAT5, but does not contain a GRE consensus sequence (Welte et al. 1993). STAT5 and GR form a molecular complex which cooperates in the induction of transcription of the β–casein gene (Stocklin et al. 1996). STAT5 response element within the β–casein gene promoter is sufficient to elicit the cooperative action of STAT5 and GR on transcription (Stoecklin et al. 1997). Activation of STAT5 through tyrosine phosphorylation is an absolute prerequisite for transcription. Deletion of the transactivation domain of STAT5 results in a molecule which cannot mediate transactivation by itself but can still cooperate with GR. Deletion of the ligand binding domain of GR does not abolish cooperation with STAT5, whereas GR N-terminal truncated form prevents this cooperation (Stoecklin et al. 1997).

Control of apoptosis by glucocorticoids is exerted by modulation of a few genes, such as *bcl-2*, *bcl-X*, *bax* and *NFkB*, in a cell type-specific manner. *bcl-X*, plays a critical role in the control of programmed cell death. The large isoform Bcl-X_L protects cells against death, while the short isoform Bcl-X_S, antagonizes cell death inhibition by interacting with Bcl-X_L and Bcl-2 (Boise et al. 1993).

The 5' upstream region of the *bcl-X* gene contains five different promoters (P1-P5), which exhibit a tissue-specific pattern of promoter usage. Two HREs located immediately upstream of P4 bind GR and confer hormone responsiveness to the core promoter (Viegas et al. 2004).

Glucocorticoids-repressed *bcl-X_L* gene in T lymphocite derivative cells correlates with the recruitment of STAT5B protein to a STAT5 binding site located upstream of P4 TATA box (Rocha-Viegas et al. 2006). STAT5B is phosphorylated independently of the presence of dexamethasone and it does not seem to interact with the ligand-activated GR (Rocha-Viegas et al. 2006). Thus, both factors seem to bind to the P4 region independently.

The differences in the GR and STAT5B binding observed suggest that chromatin structure plays a role in P4 regulation. Transcriptional repression of *bcl-XL* P4 by Dexa in thymocytes follows a rapid recruitment of GR and a combination of factors involved in transcriptional activation: SRC-1, BRG-1 and RNA polymerase II (Rocha-Viegas et al. 2006). These indicators, however, are bound for only a short time, insufficient to result in transcription activation. GR could be helping recruitment of STAT5B to the promoter region in an indirect process: transient recruitment of BRG-1 by RG could be promoting a wave of chromatin remodelling that would allow STAT5B to gain access to its target sequence. Later, after STAT5B binding, SMRT correpressor and HDAC-3 are also recruited to the promoter, and histone H3 is partially deacetylated (Rocha-Viegas et al. 2006). SMRT recruitment could be an active competition between GR and SMRT for binding STAT5B, preventing further GR binding.

I.2.4.2. STATs and ER

By GST pull-down assays, an interaction between the C-terminus of STAT5A and ERα has been shown in HEK293 cells. In these cells, ERα

expression suppressed STAT5A phosphorylation, nuclear translocation and DNA binding (Wang, Cheng 2004).

Direct association between STAT5A and ERα has also been shown in breast cancer MCF7 and T47D cell lines. ER activation enhanced PRL signalling in MCF7, but not in T47D cells. In both MCF7 and T47D cells PRLR activation leads to attenuation of the ER signalling (Wang, Cheng 2004). All these results suggest that the biological consequences of STAT-ERα interaction depend on the type of the cell line investigated.

I.2.4.3. STATs and AR

Recently it has been reported the synergistic stimulatory action of dihydrotestosterone (DHT) and PRL on PRL-inducible protein/gross cystic disease fluid-15 (PIP/GCDFP-15) gene transcription in human breast cancer cells, mediated by a functional interaction between activated STAT5 and activated AR (Carsol, Gingras & Simard 2002). PIP/GCDFP-15 is a glycoprotein secreted by the mammary gland that has been also reported as an aspartyl proteinase that might play a role in the proteolysis associated with invasive breast cancer lesions (Caputo et al. 2000). Its expression is increased by DHT, Dexa and PRL, and is down-regulated by 17β–estradiol.

Using carboxyl-truncated STAT5A and 5B mutants, which lack the major transcriptional activation domain region but still retain their DNA-binding capacity and are able to be tyrosine phosphorylated, it was determined that the transactivation domain of STAT5 is crucial for the transcriptional synergy of DHT and PRL (Carsol, Gingras & Simard 2002). PRL-induced tyrosine phosphorylation in STAT5A and 5B is also required. AR mutated in the ligand-binding domain that has not affected any ligand-binding property but has altered the transactivation function, abolished the synergism of androgens and PRL. The integrity of both half AREs and STAT5-binding sites in the PIP/GCFP-15 gene promoter was required for gene responsiveness to DHT and PRL. Although the physical interaction between AR and STAT5, if it exists, was impossible to be detected, AR mutated in the DBD, that leads to an AR form that is transcriptionally inactive and unable to bind DNA, confirmed that AR

binding to DNA was involve in the synergistic stimulation of DHT and PRL (Carsol, Gingras & Simard 2002).

I.2.4.4. STATs and PR

Progestins modulated STAT expression in breast cancer cells was demonstrated since the treatment of T47D cells with R5020 resulted in up-regulation of STAT3, 5A and 5B protein levels (Lange et al. 1998). In addition, constitutive association between STAT5 and PRB was observed in HeLa cells transfected with PRB (Richer et al. 1998).

More recently, it has been reported that progestins induce rapid STAT3, Jak1 and Jak2 tyrosine phosphorylation (Proietti et al. 2005). Progesterone stimulates association between PR and STAT3, STAT3 nuclear translocation, binding to DNA, and transcriptional activation. All these effects were abrogated by RU 486, indicating involvement of the classical intracellular PR. Moreover, the same authors described that progestins induce STAT3 tyrosine phosphorylation by a Jak-dependent pathway and the role of c-Src in progestin-induced STAT3 activation. Abolishment of Jak or c-Src activity using dominant negative forms or specific inhibitors resulted in inhibition of progesterone-induced STAT3 tyrosine phosphorylation and Jak1 / Jak2 tyrosine phosphorylation, respectively (Proietti et al. 2005).

Since Src, activated by progestins binding to the classical nuclear PR, acts as the upstream kinase for phosphorylation of Jak1 and Jak2, STAT3 activation could be explained by two molecular mechanisms: first, both Src and Jaks might act as kinases for STAT3; second, activated Jaks might serve to recruit STAT3 to Src, which in turn could directly phosphorylate STAT3 (Proietti et al. 2005).

Interestingly, blockage of STAT3 activation by a dominant negative STAT3 form resulted in inhibition of *in vivo* breast tumour growth in a model of immunocompetent mice (Proietti et al. 2005).

I.3. 11 beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2)

I.3.1. 11β-HSD function

I.3.1.1. Biochemistry of cortisol metabolism

Plasma cortisol is bound with high affinity to corticosteroid-binding globulin, which protects it from degradation. The normal half-life of cortisol is between 60 and 80 min, in contrast to other steroids such as aldosterone that have half-lives of less than 20 min, due, in large part, to lower affinity for plasma proteins (White, Mune & Agarwal 1997).

The liver and the kidney are the principal organs involved in metabolizing glucocorticoids and clearing them from the circulation. Metabolism decreases the biological activity of these hormones and increases their water solubility by converting them to hydrophilic compounds that can be excreted in urine (White, Mune & Agarwal 1997).

In the metabolism of cortisol, the C-4,5 double bond is reduced; if the hydrogen at the 5 position is added in the β–orientation, the product is 5β–dihydrocortisol, whereas 5α-reduction yields 5α-dihydrocortisol (Fig. I.17). Under normal circumstances, 5β–reduction predominates. The 3-oxo group may also be reduced; 3α-reduction is strongly favoured over 3β–reduction. The products of these reductions are tetrahydrocortisol and allo-tetrahydrocortisol. Cortisol or its reduced metabolites may be oxidized at the 11-hydroxy position to cortisone, dihydrocortisone or tetrahydrocortisone. The enzymatic activity catalyzing this conversion is termed 11beta-hydroxysteroid dehydrogenase (11β-HSD, E.C.1.1.1.140). The oxydation by 11β-HSD of cortisol to cortisone represents the conversion of an active ligand to a relatively inactive agonist for mineralocorticoid receptor (MR). 11β-HSD plays a pivotal role in mineralocorticoid target tissues by allowing aldosterone access to the MR, which binds cortisol with equal affinity. Aldosterone is not a substrate for the enzyme and is thus able to bind to MR, maintaining salt-water homeostasis (White, Mune & Agarwal 1997).

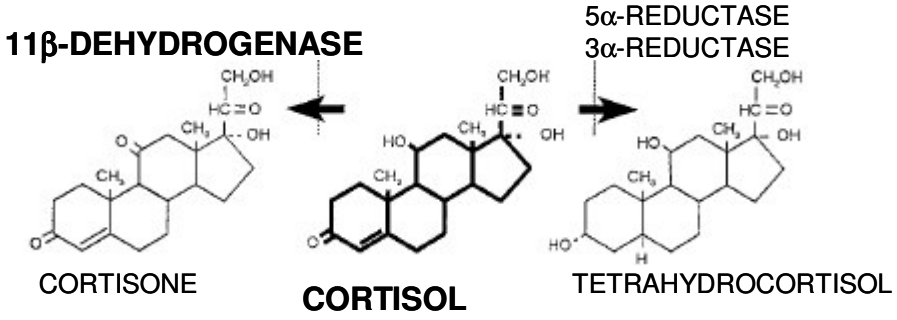

Figure I.17: Biochemistry of cortisol metabolism
In the metabolism of cortisol, 5α-reduction or 3α-reduction yields tetrahydrocortisol, an active steroid; whereas 11β-hydrogenation yields cortisone, a relatively inactive agonist of the mineralocorticoid receptor.

I.3.1.2. 11β-HSD isoforms

Two isoforms of 11β-HSD are known. The liver isoform (11β-HSD L or type 1) is widely expressed with the highest expression in the liver. It utilizes NADP+ as a cofactor, catalyzes both 11β-dehydrogenation and reverse 11-reduction and has Km values for steroids in the micromolar range. In humans, a 1.5 kb mRNA was observed in samples from liver, testis, lung, fore-skin fibroblasts, ovary, colon and kidney, predicting a protein of 292 amino acids and 30 KDa (Tannin et al. 1991). Of the tissues tested, by far the highest level of expression was in the liver, in which 11β-HSD1 has a main function to catalyze reduction of cortisone to cortisol, thus modulating the concentration of active glucocorticoids in the circulation (Fig. I.18) (White, Mune & Agarwal 1997).

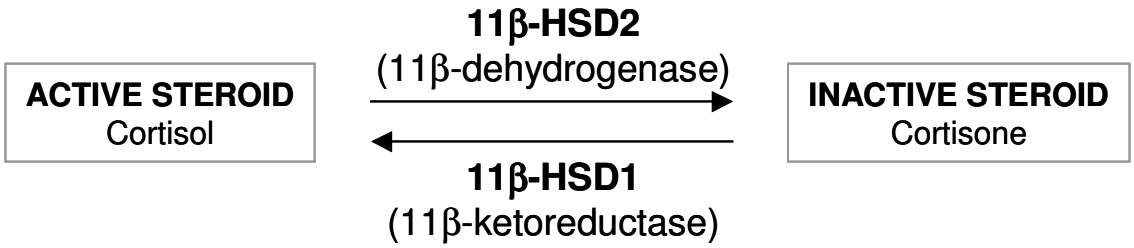

Figure I.18: 11beta-hydroxysteroid dehydrogenase isoforms
Although the two isoforms can have both activities, their predominant activity is shown, with the consequence of having opposite effects on the conversion between active / inactive steroids.

The second isoform (11β-HSD type 2), cloned from kidney, is more tissue restricted in its expression, being found at high levels in mineralocorticoid target tissues including kidney, colon and placenta, and is also present in ovary,

prostate and testis (Albiston et al. 1994). This isozyme uses NAD+ as a cofactor and has Km values for steroids in the 1 to 100 nM range (White, Mune & Agarwal 1997). Unlike the 11β-HSD1 isozyme, 11β-HSD2 isozyme only catalyzes dehydrogenation (Fig. I.18). The protein is predicted to contain 405 amino acids residues with a total molecular mass of 41 KDa.

Regions of sequence similarity between the two isozymes include part of the putative binding site for the nucleotide cofactor (residues 85–95 in 11β-HSD2) and the absolutely conserved tyrosine and lysine residues (Y232 and K236 in this enzyme) that function in catalysis. The region immediately to the N-terminal side of the catalytic residues forms part of a putative steroid-binding pocket that has been analysed by x-ray crystallography. This region is notably well conserved (10/18 identical residues) between the two isozymes of 11β-HSD, consistent with a role in binding the substrate.

I.3.2. *HSD11B2* gene

The corresponding gene that encodes for 11β-HSD2 enzyme, termed *HSD11B2* gene, is located on chromosome 16q22 and contains five exons spaced over approximately 6.4 kb (Fig. I.19). The putative binding site for the NAD+ cofactor (including the core sequence, GnnnGnG) is split between exons 1 and 2, whereas the putative catalytic residues, Y232 and K236, are in exon 4 (Agarwal et al. 1995).

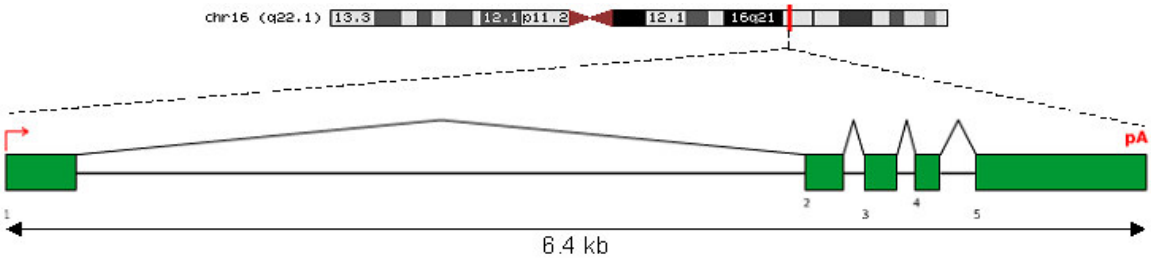

Figure I.19: Schematic representation of *HSD11B2* gene.
Upper panel, chromosomal localization of *HSD11B2* gene within the chromosome 16. *Lower panel,* schematic representation of *HSD11B2* gene that spans 6.4 kb and contains five exons.

Ribonuclease protection analysis showed that the coding protein in the human kidney begins at +117 (nt). This site is used to a minor extent in the placenta, in which the coding protein begins predominantly at +74 (nt). There are no TATA elements upstream or either cap site (Agarwal et al. 1995).

I.3.3. 11β-HSD2 promoter structure

The region ~1.8 kb of 5' upstream sequence, relative to the initial ATG codon, was identified as the promoter sequence for basal transcription of *HSD11B2* in human JEG-3 human choriocarcinoma cells (Agarwal, White 1996). Using luciferase reporter constructs, it was determined that along the promoter there are regions with positive or negative regulatory effects for basal transcription (Fig. I.20).

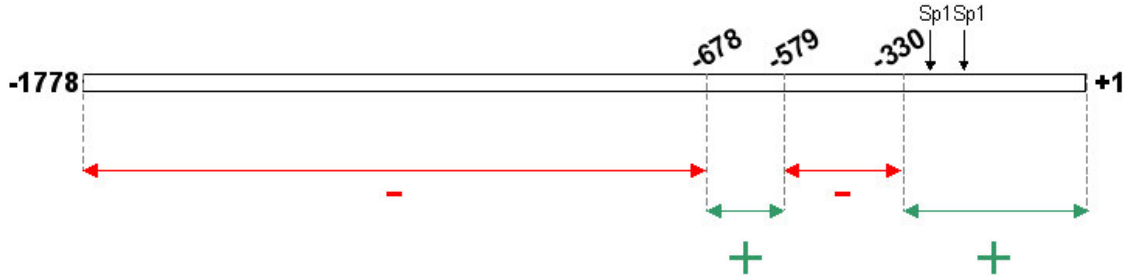

Figure I.20: Schematic representation of 11β-HSD2 promoter characterization in JEG-3 choriocarcinoma cancer cells.
Negative and positive regulatory regions involved in basal promoter activation in JEG-3 cells in the context of transfected reporter constructs. Arrows indicate Sp1 binding positions characterized *in vitro* by gel shift. *Adapted from Agarwal, White 1996.*

Two segments of the promoter, nt from -278 to –257, and from –215 to –194, were protected in DNase I footprinting analysis. Both segments have consensus binding sites for the Sp1 transcription factor (Fig. I.20). Gel shifts assays of these segments showed several DNA-protein complexes using JEG-3 nuclear extracts. Only the slowest migrating complex was competed by an antiserum to Sp1. These results suggested that the Sp1 sites, either alone or in combination, were important for transcription of *HSD11B2* gene in JEG-3 cells (Agarwal, White 1996).

Using *in vivo* genomic footprinting, important regions for *HSD11B2* gene regulation were identified in human cell lines: two GC-rich regions in the first exon and two upstream elements at nt –213 to –178 and nt –122 to –75 (Nawrocki et al. 2002). The footprints suggested a correlation between the extent of *in vivo* protein occupancy at three of these regions and the rate of gene transcription in cells with high (SW620, colon); intermediate (HCD and HK-2, kidney; and MCF7, breast); or low (SUT, lung) 11β-HSD2 mRNA levels.

Gel shifts assays revealed that decreased 11β-HSD2 expression is related to a decreased binding activity with the putative regulatory elements. Antibody supershifts identified the majority of the components of the binding complexes as the transcription factors Sp1 and Sp3 (Fig. I.21) (Nawrocki et al. 2002). Later investigations revealed the presence of a nuclear factor 1 (NF1) binding site in the 11β-HSD2 promoter (nt –419 to –397) (Fig. I.21) (Alikhani-Koopaei et al. 2004).

Figure I.21: Schematic representation of 11β-HSD2 promoter characterization by *in vivo* footprinting.
Arrows indicate NF-1, Sp1 and kB1 binding sites characterized by *in vivo* DNA footprinting in different cell lines including MCF7 breast cancer cells, SW620 colon cancer cells, SUT lung cancer cells, HCD and HK-2 kidney cancer cells. *Adapted from Nawrocki et al. 2002.*

Recently, it was elucidated whether an epigenetic mechanism, DNA methylation, controls the expression of 11β-HSD2 (Alikhani-Koopaei et al. 2004). Using different cell lines it was determined that different CpG islands cover the promoter and exon 1 of *HSD11B2* gene (Fig. I.22). These CpG islands were found to be densely methylated in tissues and cell lines with low expression but not in those with high expression of the gene. Methylation of recognition sequences for transcription factors, including those for Sp1 / Sp3 and NF1, diminished their DNA-binding activity *in vitro*. Moreover, *in vitro* experiments showed that methylated CpG binding protein complex 1 (MeCP1) was bound to densely methylated regions of 11β-HSD2 promoter bound (Alikhani-Koopaei et al. 2004). MeCP1 is a complex that discriminates between methylated and unmethylated DNA and represses transcription through preferential binding to methylated nucleosomes.

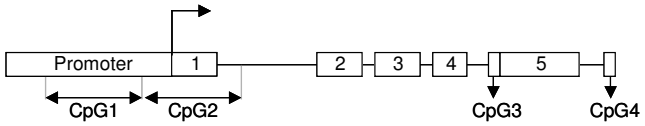

Figure I.22: CpG islands of the human *HSD11B2* gene.
Schematic representation of the putative CpG islands along the gene, defined as a region in which the calculated percentage of CpGs over an average range was over 50% and the calculated versus the expected CpG distribution higher than 0.6. *Adapted from Alikhani-Koopaei et al. 2004*.

More recently, it was determined that 11β-HSD2 promoter contains several binding sites for NFkB transcription factors in the region between nt –185 and –88 (Fig. I.21) (Kostadinova et al. 2005). In unstimulated cells, NFkB is bound within the cytoplasm to its inhibitor protein IkB. Upon stimulation with tumour necrosis factor a (TNFα) or phorbol 12-myristate 13-acetate (PMA), IkB is phosphorylated, ubiquitinated and degraded (Chen et al. 1995). Previous works in different cell lines had determined that TNFα downregulates 11β-HSD2 activity (Takahashi et al. 1999). Released NFkB translocates to the nucleus and modulates the expression of target genes. Various NFkb dimers act as transcriptional activators and repressors. Homodimers of NFkB lacking transcriptional activation domains, such as p50 / p50, mediate transcriptional repression (Grundstrom et al. 2004). Chromatin immunoprecipitation (ChIP) experiments and gel shift demonstrated the relevance of NFkB binding to 11β-HSD2 promoter in human colon SW620 cells (Kostadinova et al. 2005). After long treatments with TNFα and PMA, 11β-HSD2 expression is downregulated because a switch of binding to NFkB sites from active p65 / p50 heterodimers to inactive p50 / p50 homodimers (Kostadinova et al. 2005).

I.3.4. 11β-HSD2 and cancer

I.3.4.1. 11β-HSD isozymes and cell proliferation

The mechanisms by which glucocorticoids regulate cell proliferation are related with the fact that amongst the most prominent glucocorticoid target genes are the cyclin-dependent kinases (CDKs) and their corresponding CDK inhibitor (CDIs), such as the Cip / Kip family of CDIs, particularly p57Kip2 (Kato et al. 1994). p57Kip2 is rapidly regulated by glucocorticoids and is central to the glucocorticoid-induced accumulation of cells in G1-phase of the cell cycle (Samuelsson et al. 1999). Glucocorticoids may also alter cell cycling by modulating growth factor-mediated changes in tyrosine kinase signalling, either by direct effects on membrane receptor expression or by indirect regulation of protein phosphorylation (Croxtall, Choudhury & Flower 2000). Furthermore, GR-mediated transrepression of NFkB signalling is also important in controlling cell cycle progression (McKay, Cidlowski 1999).

The enzymes 11β-HSD type 1 and 2 have well-defined roles in the tissue-specific metabolism of glucocorticoids. However, different studies have shown that the effects of 11β-HSD1 and 2 are not restricted to distinct tissue-specific hormonal functions. Studies of normal adult tissues as well as their tumour equivalents, have shown a further differentiation in 11β-HSD expression and activity. Specifically, most normal GR-rich tissues express 11β-HSD1, whereas their tumor equivalents express 11β-HSD2 (Rabbitt et al. 2003).

It has been postulated that the ability of 11β-HSD1 to generate cortisol acts as an anti-proliferative, anti-differentiation stimulus in normal adult tissues (Fig. I.23). Cells expressing 11β-HSD1 showed lower rates of proliferation whereas cells expressing 11β-HSD2 showed much higher proliferation than control (Rabbitt et al. 2002). Crucially, the effects of 11β-HSD1 and 2 on cell proliferation were independent of any change in GR expression, emphasising the pivotal role of these isozymes as determinants of cell proliferation. This indicates that the pro-proliferative effects of 11β-HSD2 are due to increased capacity for the local inactivation of cortisol (Fig. I.23) (Rabbitt et al. 2003).

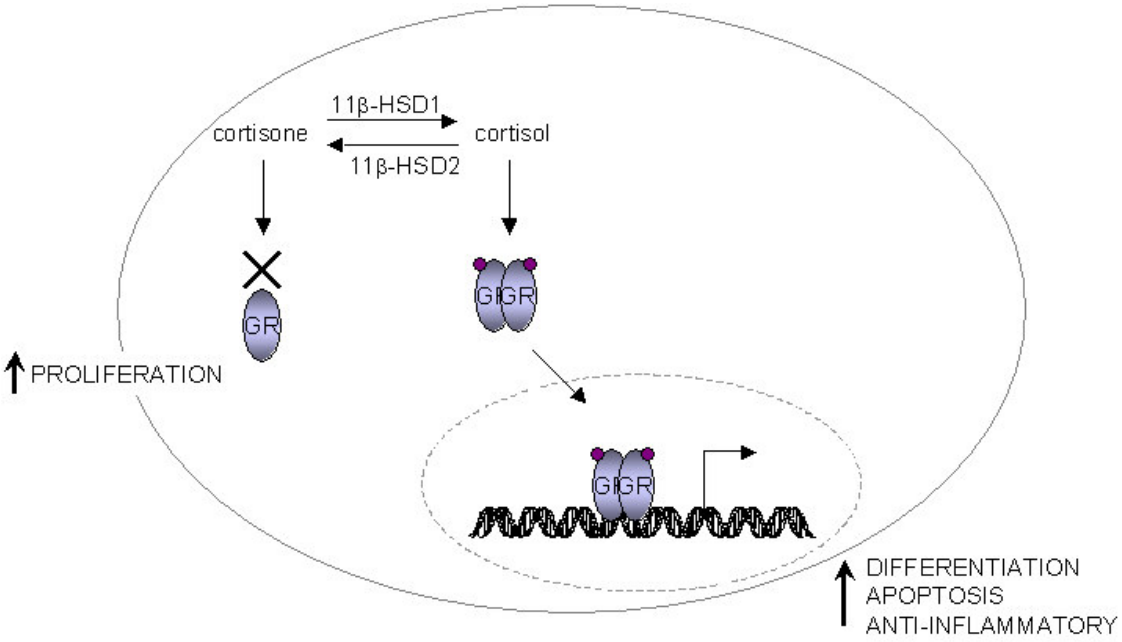

Figure I.23: 11β-HSD isozymes and the pre-receptor regulation of GR-mediated transactivation.
Interconversion of cortisol and cotisone catalysed by 11β-HSD1 and 2 determines the availability of ligand for GR. Liganded GR modulates target gene transcription. Glucocorticoid inactivation via the dehydrogenase activity of 11β-HSD2 decreases availability of active cortisol facilitating proliferation. Reductase activity of 11β-HSD1 increases local cortisol levels and leads to increased differentiation and, possibly, apoptosis.

I.3.4.2. 11β-HSD expression in malignant tissue

In different cancer types (including breast, prostate and endometrial cancer), the relationship between expression of nuclear receptors (ER, AR and PR) and the local availability of ligand for these receptors has been shown to be crucial for tumour development (Wiseman, Duffy 2001). In contrast, it has been difficult to define a cancer lesion that is specifically associated with GR function. By showing that differential regulation of 11β-HSD1 and 2 is a key determinant of cell proliferation, a mechanism independent of GR expression, its disregulation could be involved in tumorigenesis.

In normal tissues (such as bone), a switch from 11β-HSD2 activity in fetal tissue to 11β-HSD1 activity in adult tissue, has been described. In tumours this appears to reverse and tissues normally expressing 11β-HSD1 show expression of 11β-HSD2. An example of this situation is in the adrenal gland. In the human fetal adrenal gland, 11β-HSD2 is detected whereas in normal adult adrenal glands 11β-HSD2 does not appear to be expressed. However, in adrenal cortical carcinoma and adenoma, 11β-HSD2 mRNA and protein are present (Coulter et al. 1999). In addition, elevated 11β-HSD2 expression has been described in leukemic and breast cancer cell lines (Hundertmark et al. 1997). Other reports describing high levels of 11β-HSD2 expression in different malignant tissues have showed that, in each of these cases, the upregulation of 11β-HSD2 in neoplastic tissues and cell lines contrasts with their normal tissue equivalents (Table. I.24). Significantly, the presence of 11β-HSD2 in many of these systems is associated with GR rather than MR expression, suggesting an alternative function for the isozyme that is distinct from its classical role in MR-rich tissues such as the colon and kidney.

Study model	11β-HSD1	11β-HSD2
Osteosarcoma cell lines (TE-85, MG-63, SaOS-2)	X	✔
Fibrosarcoma cell lines (Hs913)	X	✔
Endometrial cancer cell line (Ishikawa)	X	✔
Breast cancer cell lines (MCF7, ZR-75-1, PMC42, T47D)	X	✔
Myelomonocytic cell line (U937)	X	✔
Adrenal cortical carcinoma / adenoma	X	✔
Colon carcinoma	X	✔
Colono carcinoma cell lines (Caco-2, Ht-29)	X	✔
Breast tumour specimens	X	✔
Pituitary adenomas	X	✔

Table I.24: Summary of 11β-HSD isozyme expression in neoplastic tissues and cell lines

I.3.4.3. 11β-HSD2 as a model in the regulation by SHs in breast cancer cells

Microarray analysis of gene expression in the human breast cancer cell line T47D to identify genes that are differentially regulated by glucocorticoids and progestins, have shown that 31 genes (of a total 5600 full-length human genes) are regulated by the two hormones by more than 3-fold (Wan, Nordeen 2002). Of particular interest, this study showed that 11β-HSD2 gene expression was clearly upregulated in response to glucocorticoids and progestins. The levels of fold change over vehicle showed that dexamethasone (Dexa) treatment led to 7.4- and 27.1-fold induction after 2 and 6 h, respectively. Levels of 11β-HSD2 gene expression were increase by progesterone analogous R5020 up to 6.9- and 21-fold induction after 2 and 6 h of incubation, respectively (Wan, Nordeen 2002). These results showed that *HSD11B2* gene was the most robustly regulated gene by glucocorticoids and progestins in T47D cells, and could provide an excellent endogenous promoter model to study the steroid hormones regulation.

MATERIALS AND METHODS

M.1. Reagents

R5020 was purchased from PerkinElmer Life Sciences; Dexamethasone (Dex) and RU 486 (RU) were from Sigma. ICI 182,780 (ICI) was from Tocris, and PD 98059 (PD), Wortmannin (WM) and AG 490 (AG) inhibitors were from Calbiochem. The antibody against 11β-HSD2 protein was purchased from Binding Site. Antibodies against FLAG-tag and α-tubulin were purchased from Sigma. Antibodies against STAT5A and YY-1 were from Santa Cruz. Antibodies against polymerase II (Pol II – 8WG) and phospho-polymerase II (pPol II – H14) were obtained from Covance. Antibodies against AcH4 and H3S10p were from Upstate. All other antibodies (against H3K4Me3, HDAC-1, SRC-1 and LSD-1) were purchased from Abcam.

M.2. Oligonucleotides

Table M.1: *Oligonucleotides used for RT-PCR*

Oligonucleotide	*Sequence*
11β-HSD2 (exon 3)	5'-ACGCAGGCCACAATGAAGTAG-3'
11β-HSD2 (exon 4)	5'-GCAGCCAGGCTGGATGATG-3'
CCND1 up	5'-CCCTCGGTGTCCTACTTCAA-3'
CCND1 low	5'-AGGAAGCGGTCCAGGTAGTT-3'
CXCR4 up	5'-GGTGGTCTATGTTGGCGTCT-3'
CXCR4 low	5'-TGGAGTGTGACAGCTTGGAG-3'
DUSP1 up	5'-CAGCTGCTGCAGTTTGAGTC-3'
DUSP1 low	5'-AGAGGTCGTAATGGGGCTCT-3'
GAPDH up	5'-TTGGTCGTATTGGGCGCCTGG-3'
GAPDH low	5'-CAAAGTTGTCATGGAT-3'
JUN up	5'-GCCTCAGACAGTGCCCGAGATGCC-3'
JUN low	5'-TGCCACCTGTTCCCTGAGCATGTTGG-3'
Luc +49 (up)	5'-CTAGAGGATGGAACCGCTGG-3'
Luc +255 (low)	5'-ACACCGGCATAAAGAATTGAAGA-3'
MAP3K3 up	5'-GCCTAGGCTGCATTGAAAAG-3'
MAP3K3 low	5'-TTTGGACACAGCTGGTGGTA-3'
MYC up	5'-TCGGATTCTCTGCTCTCCTC-3'
MYC low	5'-CCTGCCTCTTTTCCACAGAA-3'
p21 up	5'-CAGGGGACAGCAGAGGAAGA-3'
p21 low	5'-GGGCGGCCAGGGTATGTA-3'
STAT5A up	5'-CCCCAGGCTCCCTATAACAT-3'
STAT5A low	5'-CGGGAGTCAAGACTGTCCAT-3'

Table M.2: *Oligonucleotides used for ChIP PCRs*

	Oligonucleotide	*Sequence*
11β-HSD2	*-1778*	5'-GGGGTGCTGTGTCTGCCTCCAAG-3'
	-1596	5'-GCCATGACCCTGTGTGTGCAAGT-3'
	-1542	5'-AAGTGCTTACAAACACAGGC-3'
	-1362	5'-GGGAGAGTCTCTGGGTGGAG-3'
	-1367	5'-TCTCCCTTTAGGTGGGTCTG-3'
	-1216	5'-GCCCTGTTGCTCATTCTCTC-3'
	-1219	5'-GGGCTAGGAAGTGTGACAGG-3'
	-1048	5'-ACTGGGAGTTGGTGCCAGTA-3'
	-802	5'-GGGCCATAAGTAATGGGAGA-3'
	-641	5'-CTCCCGGTTCTTGGAGTCT-3'
	-611	5'-TCCCAGGCAGGTTTTGTG-3'
	-427	5'-CAAGCCTGCAGGAACACC-3'
	-336	5'-ACCTGAGCGCGGCGGCTTGG-3'
	-219	5'-CCTGGCTGCGGGCGGTGCTT-3'
	-203	5'-GCAGAGAAAGCGAGTGTCCC-3'
	+6	5'-AGAGGGACACTCGCTTTCTCTGCT-3'
	+423	5'-GCGGGACTGGACACTCAACA-3'
	+620	5'-GCCCACTCCCTGTCTCACTT-3'
MMTV	*Nuc B up*	5'-GGGCTTAAGTAAGTTTTTGGTTACA-3'
	Nuc B low	5'-TTTACATAAGATTTGGATAAATTCC-3'
β-globin	*up*	5-ACACAACTGTGTTCACTAGC-3'
	low	5'-CAACTTCATCCACGTTCACC-3'

M.3. Cell culture and hormone treatments

T47D breast cancer cells and T47D-MTVL cells (carrying one stably integrated copy of Luciferase reporter gene driven by the MMTV promoter;(Truss et al. 1995)) were routinely grown in DMEM or RPMI 1640 medium, respectively, supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

T47D-YV cells (PR negative clonal derivative cell line of T47D; Sartorius, 1994), were used to generate TYML cells (T47D-YV-derived cell lines with one integrated copy of MMTV-Luciferase) expressing either the wild-type PRB or the PRB DBD mutant, as previously described (Knuesel et al. 2003). All T47D-YV-derived cell lines were routinely grown in MEM medium supplemented with 7% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

For the experiments, cells were plated in medium without phenol red supplemented with 10% dextran-coated charcoal-treated FBS (DCC/FBS), and 24 h later medium was replaced by fresh medium without serum. After 2 days in

serum-free conditions, cells were treated with R5020 (10 nM) or ethanol for different times at 37ºC. When indicated, RU (1 μM) or ICI (10 μM) were added simultaneously, or PD (50 μM), WM (0.1 μM) or AG (50 μM) were also added 1 h before hormone treatment.

M.4. Stable cell lines expressing shRNA against STAT5A

Infectious viral stocks were generated by transfecting pVSVG (encoding for an envelope gene) and pLKO.1 vector (containing MISSION^{TM} shRNA directed against STAT5A or control irrelevant shRNA from Sigma) into the packaging cell line GP2293 (gag & pol genes) by calcium phosphate transfection, recovered the supernatant for 72 hours and concentrated the viral particles by ultracentrifugation in a bottom of 20% sucrose. Media with viral particles at different MOIs was added to 35mm-plates containing 3 X 10^5 TYML cells expressing FLAG-tagged wild-type PRB, which had been plated the day before. The cells were submitted to spinoculation, by centrifugation for 2 hours at 1200 rpm. The medium with the virus was maintained for 24 h and then replaced by fresh medium containing puromycin at a final concentration of 2μg/ml.

M.5. Plasmids

pGL3 –1778 11βLuc reporter vector, was a gift from Lewis P. Rubin (Agarwal, White 1996). The entire promoter construct pGL3 –1778+117 11βLuc was obtained adding 117 bp of exon 1 obtained by PCR amplification of human genomic DNA with primers –368: 5'-GTGTCCCGAACAAGCGTGAGTGGC-3', and +608: 5'-TCTCACTTTCCCTCCAACACTCCC-3', followed by digestion of the product with *ApaI* and *NcoI*. The unique restriction sites *NcoI*, *AatII* and *BssHII* were used to obtain the deletion constructs starting at positions -1551, -839 and -571, respectively. The deletion construct –1345, was generated by amplifying from pGL3 –1778+117 11βLuc plasmid, with the oligonucleotide 5'-TTTGGTACCTCCCAGCCTCCCCTGAGATT-3', corresponding to nucleotides from –1345 to –1326 plus a *KpnI* restriction site and the oligonucleotide 5'-CAGTGGAGGGTGGGGTGTCAG-3', corresponding to the nucleotides –748 to –769, as forward and reverse primers, respectively. The PCR product was cut

with *KpnI* and *AatII* and then cloned into *KpnI* and *AatII* sites of pGL3 –839+117 11βLuc construct.

The vector pGL3 –1778–1345 11βLuc was generated by amplifying from pGL3 –1778+117 11βLuc plasmid, with the oligonucleotide 5'-ATTTCTCTATCGATAGGTACC-3', that includes the *KpnI* restriction sequence of pGL3 reporter vector multiple cloning site, and oligonucleotide 5'-AAAGGTACCGCCAGACCCACC-3' corresponding to the nucleotides from –1345 to –1336 plus a *KpnI* restriction site added to perform the cloning, as forward and reverse primers, respectively. The PCR product was blunted, cut with *KpnI* and then cloned into *KpnI* site of pGL3 reporter vector. The vector pGL3 –1778–1345 / -368+117 11βLuc, was generated cloning the same PCR product into *KpnI* site of pGL3 –368+117 11βLuc construct.

pSG5-PRB and pSG5-PRA, were a gift from Pierre Chambon (Kastner et al. 1990). pSTAT5A-WT and pSTAT5A*6 (STAT5A constitutive active mutant) were kindly provided by Toshio Kitamura (Ariyoshi et al. 2000). pSTAT5AΔ749 (STAT5A dominant negative mutant) was a gift from Fabrice Gouilleux (Moriggl et al. 1996).

M.6. Immunofluorescences

T47D cells were seeded onto coverslips in 35-mm dishes at 1.2X105 cells/cm2 as described above for hormone treatment experiments in the absence of serum. After the treatment with ethanol or R5020, cells were washed, fixed by incubation in 4% paraformaldehyde in PBS for 15 min at room temperature, and permeabilized by incubation in 0.2% Triton X-100 in PBS for 10 min at room temperature. After rinsing three times for 5 min in PBS, the coverslips were incubated for 1 h with 3% BSA in PBS at room temperature to reduce non-specific staining. To detect 11β-HSD2 protein, cells were incubated with specific antibody against 11β-HSD2 diluted 1:1000 in 3% BSA in PBS for 1 h at room temperature. After several washes in PBS, coverslips were exposed to biotinilated-secondary antibody antigoat (Molecular Probes) diluted 1:200 in 3% BSA in PBS for 1 h at room temperature. Then coverslips were washed in PBS and exposed to streptavidin-conjugated antibody ALEXA 488 (Molecular Probes), diluted 1:1000 in 3% BSA in PBS for 1 h at room temperature.

Coverslips were mounted on slides with VectaShield – DAPI mounting medium (Vector Laboratories) and subjected to Leica DM IRBE inverted research microscope.

M.7. RNA extraction and RT-PCR

Total RNA was prepared by using Tryzol Reagent (Invitrogen) as described in the manufacturer's instructions. Cells are lysed in a solution of guanidine isothiocyanate and phenol. Addition of chloroform generates a second organic phase into which DNA and proteins are extracted, leaving RNA in the aqueous supernatant. RNA was precipitated with isopropyl alcohol and washed with 75% ethanol. The RNA pellet was dissolved in RNase-free water and stored at –80ºC.

The cDNA was generated from 100 ng of total RNA by using Superscript First Strand Synthesis System (Invitrogen). 1 µl of cDNA was used as template for RT-PCR. Indicated gene products were analysed by PCR. When indicated, quantification of gene products was performed by Real-Time PCR using SYBR green (Roche). Each value was corrected by the human GAPDH and expressed as relative units. Primer sequences are summarized in the Tables M.1 and M.2.

M.8. Microarrays and data analysis

Samples where process by the Microarray Unit at Genomic Regulation Center (CRG). Raw data was processed using MARGE, an in house developed web implementation of LIMMA, a microarray statistical analysis package of Bioconductor (http://www.bioconductor.org) that is run in the R programming environment. Discriminant factor analysis was run using an application of the software FADA. Gene intensities were background subtracted (taking mean of channel intensities and median of background). Spots with intensities <2 times the local background in either or both dye filter channels (Cy3 or Cy5) as well as controls were excluded from normalization, and were referred as "not reliable". An intensity dependent normalization algorithm (global lowess) was applied using a smoothing factor f=0.2 for all experiments. Normalized Log_2Ratios (Intensity Cy5/Intensity Cy3) were scaled so that they all had the same median absolute standard deviation across all the arrays, to give the same weight to each gene, and not only due the magnitude of the expression ratio. The

computed B statistic rank value from all replicate hybridizations was used to determine the genes with significant changes. We considered genes that showed a 1.4-fold gene up or down-regulation relative to control sample with a B–rank value above the 90th percentile as significant. The value of fold change or copy number relative change was calculated as $2^{Log2Ratio}$, if the value of the ratio was >0, or $2^{-1/Log2Ratio}$, if it was <0.

In order to do the statistical analysis of the data, we have used the open-source, freely available software package for microarray data management and analysis TM4 obtained from TIGR (http://www.tigr.org/software/).

M.9. Transfection assays

For transient transfections, 24 h before transfection, exponentially growing cells were harvested by trypsination and replated at a density of 2 X 10^5 cells plated in 35-mm plates in growth medium without antibiotics. The transfection was performed with Lipofectamine 2000 (Invitrogen) following the instructions of the manufacturer. A total amount of 3 μg of reporter and expression vectors were used for each well. Six h later, the medium was replaced by medium with antibiotics and without phenol red. After two days in serum-free conditions cells were incubated with R5020 for 16 h. After incubation, cells were harvested in 300 μl of ice-cold lysis buffer (Promega), cell lysates were collected in 1.5 ml tubes and cleared by centrifugation at 13.000 g for 2 min at 4ºC. Protein amount was determined by Micro BCA protein assay (Pierce). Lysates were adjusted to equivalent protein concentrations with lysis buffer and luciferase activity was determined with luciferase assay kit (Promega) according to the manufacturer's instructions. Briefly, 30 μl of equivalent protein concentrations (in a range of 10-30 μg) of each cell lysate were added into individual luminometer tubes. Light was measured with an AutoLumat 953 luminometer (Berthold) by injecting 100 μl of Luciferase assay solution into the sample tubes and measuring light output over 10 sec.

For deletion analysis of 11β-HSD2 promoter, the vectors expressing luciferase under the control of the different constructs of the promoter were

transfected in T47D or T47D-YV cells, co-transfected with empty pSG5, pSG5-PRB or pSG5-PRA expression vectors.

For expression analysis of 11β-HSD2 promoter under the control of wild type STAT5A or mutant STAT5A forms, pGL3 –1778+117 11βLuc reporter vector or the indicated deletion constructs of the promoter were co-transfected in T47D-YV cells or T47D cells with pSG5-PRB expression vector and pSTAT5A-WT, pSTAT5A*6 or pSTAT5AΔ749 expression vectors.

M.10. Chromatin immunoprecipitation assays

ChIP assays were performed as described (Strutt, Paro 1999) by using chromatin from TYML cells expressing wt PRB or, when indicated, PRB DBD mutant, cultured and treated as described before.

The cells were treated with R5020 10 nM for the appropriate time or, when indicated, AG 490 inhibitor was added 1 h before hormone treatment. After R5020 treatment, medium was replaced with fresh medium (serum-free medium without phenol red) and proteins were crosslinked to DNA by adding C*rosslinking solution*, containing formaldehyde, directly to culture medium to a final concentration of 1% formaldehyde, and incubating for 10 min at 37ºC. Then the crosslinking reaction was stopped by adding Glycine to a final concentration of 0.1M and incubating for 5 min at room temperature. The medium was removed and the cells were washed twice using ice cold PBS containing protease and phosphatase inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1μg/ml aprotinin, 1μg/ml pepstatin A, 1μM sodium ortovanadate, 20mM β-Glycerophosphate and 1X Protease inhibitors cocktail (Roche)). The cells were scrapped in PBS containing inhibitors and pelleted for 5 min at 4000 rpm at 4ºC. Cell pellets were resuspended in 2.5 ml of *Lysis Buffer* containing inhibitors and incubating for 10 min on ice. After lysis, cells were pelleted for 5 min at 4000 rpm at 4ºC and then resuspended in 1 ml of *Nuclei Lysis Buffer*.

Lysate was sonicated on ice to shear DNA to lenghts between 300 and 500 bp. After sonicated matherial was centrifugated 5 min at 4000 rpm at 4ºC, to recover the supernatant (chromatin) and discard the cell debris. An aliquot of the chromatin was treated with Proteinase K and the DNA was recovered by

phenol / chloroform extraction, to quantified DNA concentration and to see the sizes of the sheared DNA in a 1.2% agarose gel.

To perform the chromatin immunoprecipitation, 20-30 μg per sample of quantified chromatin was diluted 10-fold in *ChIP Buffer*. To reduce non-specific background, the diluted chromatin was pre-cleared with 15 μl of Salmon Sperm DNA / Protein A or G Agarose – 50% Slurry (Upstate) for 4 h at 4ºC with rotation. The pre-cleared chromatin was collected by brief centrifugation and the corresponding immunoprecipitating antibody was added to the supernatant fraction (1 – 5 μg; the amount will vary per antibody), and was incubated over night at 4ºC with rotation. Control for non-specific interaction of DNA was performed by using as non-specific antibody normal rabbit or mouse IgG (Sigma). For input control, an aliquot of the pre-cleared chromatin was recovered before antibody incubation. 30 μl of Salmon Sperm DNA / Protein A or G Agarose – 50% Slurry were added for 2 h at 4ºC with rotation, to collect the antibody/proteins/DNA complexes. The agarose was pelleted by gentle centrifugation (1 min at 3600 rpm at 4ºC) and supernatant containing unbound inespecific DNA was discarded. The agarose with bound antibody/protein/DNA complexes was washed for 5 min at 4ºC with rotation with each of the *Washing Buffer 1, 2* and *3*, and then twice with TE1X. The DNA was eluted by incubating twice the washed agarose with *Elution Buffer* for 15 min at room temperature with rotation. The supernatant was recovered by centrifugation for 5 min at 3600 rpm at room temperature. 0.2M NaCl was added and samples were incubated overnight at 65ºC to reverse crosslinking, followed by treatment with proteinase K and DNA recovered by phenol / chloroform extraction. DNA was precipitated with 100% ethanol, 10% sodium acetate and 0.1% glycogen, washed with 70% ethanol, and finally, the DNA pellet was dissolved in DNase-free water.

For each experiment, PCRs were performed with dilutions of input DNA to determine the linear range of amplification. The human β–globin gene was used as a control. Primer sequences are summarized on Tables M.1 and M.2.

ChIP Solutions:

Crosslinking Solution: 50mM Hepes pH8.0; 0.1M NaCl; 1mM EDTA pH8.0; 0.5mM EGTA pH8.0.

Cell Lysis Buffer: 5mM Pipes pH8.0; 85mM KCl; 0.5% NP-40

Nuclei Lysis Buffer: 1% SDS; 10mM EDTA pH8.0; 50mM Tris-HCl pH8.1

ChIP Buffer: 0.01% SDS, 1.1% Triton X-100; 1.2 mM EDTA pH8.0; 16.7 mM Tris-HCl pH8.1; 167mM NaCl

Washing Buffer 1: 0.1% SDS; 1% Triton-X100; 2mM EDTA pH8.0; 20mM Tris-HCl pH8.1; 150mM NaCl

Washing Buffer 2: 0.1% SDS; 1% Triton-X100; 2mM EDTA pH8.0; 20mM Tris-HCl pH8.1; 500mM NaCl

Washing Buffer 3: 0.25M LiCl; 1% NP-40; 1% Sodium Deoxicholate; 1mM EDTA pH8.0; 10mM Tris-HCl pH8.1

Elution Buffer: 1% SDS, 0.1M $NaHCO_3$

M.11. *In silico* analysis

Screening for potential transcription factor binding sites was performed by weβ–based prediction of regulatory elements using ConSite ((Sandelin, Wasserman & Lenhard 2004); http:/www.phylofoot.org/consite) and Transfac ((Wingender et al. 1996); http://www.gene-regulation.com/pub/databases.html#transfac).

M.12. Statistical analysis

Results were analysed by Student's *t* test. Differences between two means with a $p<0.05$ were regarded as significant.

RESULTS

R.1. 11β-HSD2 is expressed in breast cancer cells.

In Northern blot analyses using poly (A) RNA from multiple human tissues, 11β-HSD2 gene was shown to be strongly expressed in kidney, colon, pancreas and placenta, and is also present in ovary, prostate and testis (Albiston et al. 1994). Breast cancer cells were analysed by RT-PCR showing strong expression of 11β-HSD type 2, whereas 11β-HSD2 type 1 showed no expression (Arcuri et al. 2000). Microarray analysis of gene expression in the human breast cancer cell line T47D had shown that *HSD11B2* gene was the most robustly regulated gene by glucocorticoids and progestins (Wan, Nordeen 2002). In order to find an endogenous promoter model for studying expression regulated by progesterone at the molecular level, we checked 11β-HSD2 protein expression in T47D cells (Fig. R.1A). Immunofluorescence analysis using specific antibody against 11β-HSD2 protein showed that the protein is expressed in T47D cells growing exponentially (Fig. R.1A, left panel).

R.2. 11β-HSD2 promoter expression in T47D cells is induced by progestins and depends on the classical PR activation.

To check the hormone responsiveness of 11β-HSD2 in T47D cells, R5020-induced 11β-HSD2 protein levels after 24 h of incubation (Fig. R.1A, middle and right panels) and 11β-HSD2 mRNA expression after 16 h of cell treatment (Fig. R.1B, lanes 1 and 3) were analysed by immunofluorescence and RT-PCR, respectively. R5020 was used at a physiological concentration (10nM), and after 48 h of serum deprivation in order to minimize basal 11β-HSD2 expression. R5020-dependent 11β-HSD2 protein and mRNA induction was observed in both cases.

In order to estimate if this expression is PR-specific, we analysed the effect of RU-486 antiprogestin action (Fig. R.1B, lanes 2 and 4). 11β-HSD2 expression induced by R5020 treatment was totally abolished by RU incubation. These results indicated that the promoter expression is specifically induced by progestins through the activation of PR in T47D cells.

To analyse the kinetics of hormone activation of 11β-HSD2 promoter we performed a time course with T47D cells growing in absence of serum and treated with R5020 for different times, between 15 min and 16 h.

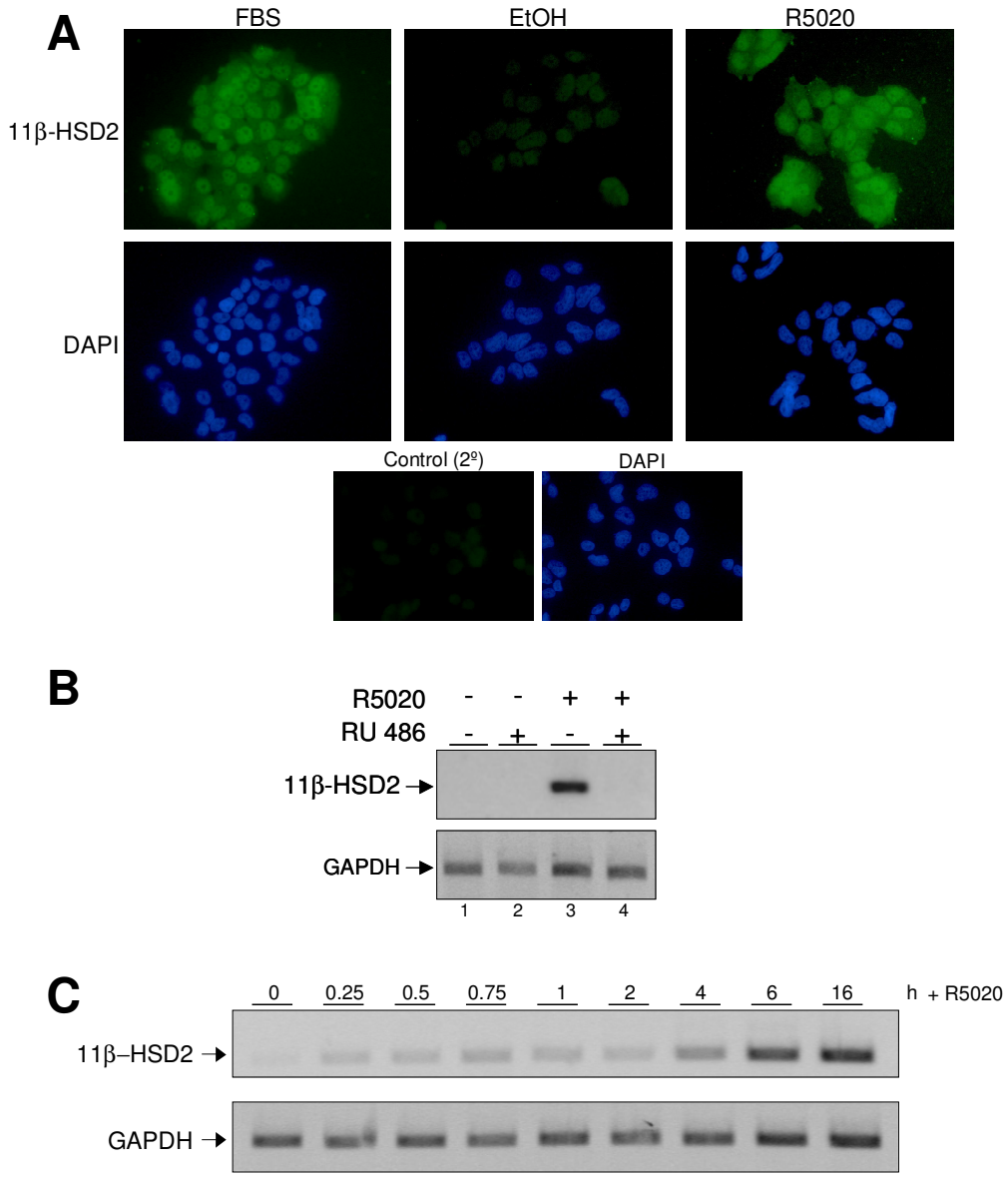

Figure R.1: 11β-HSD2 promoter expression in T47D cells is induced by progestins and depends on the classical PR

(A) ***11β-HSD2 expression detected by immunofluorescence.***

Upper panel, T47D cells were cultured over coverslips in 35-mm dishes in rich medium with 10% FBS (FBS) or in medium deprived of steroids and serum-free conditions for 48 h before the addition of R5020 10 nM or ethanol (EtOH), for 24 h. Then the cells were fixed, impermeabilized and incubated with antibody against 11β–HSD2 followed by biotinilated-conjugated secondary antibody, then streptavidin-conjugated ALEXA 488 and, finally, DAPI to stain nucleic acids (as described in Material and Methods).

Lower panel. For negative control of immunocytochemistry, T47D cells were analysed as previously described in the absence of the primary antibody (-11β–HSD2).

(B) ***Progestins induce 11β–HSD2 transcription***

T47D cells were cultured in medium deprived of steroids and serum-free conditions for 48 h, before the addition of R5020 10 nM and/or RU 486 1 μM, or ethanol, for 16 h. Cells were harvested and total RNA was extracted. The 11β–HSD2 mRNA expression was analysed by RT-PCR with specific primers. GAPDH cDNA specific primers were used as a control. PCR products were run on a 1.2% Agarose gel and visualized with ethidium bromide.

(C) ***11β–HSD2 expression along time after hormone addition.***

T47D cells cultured as in Figure R.1B were untreated (0) or treated with R5020 10 nM for the times indicated. 11β–HSD2 expression was analysed as in (B), and GAPDH expression was used as a control.

Then we extracted total RNA and 11β-HSD2 and GAPDH expression were analysed by RT-PCR (Fig. R.1C). The results showed an increase in the transcript levels after 15-30 min of treatment with R5020, and then a more sustained increase, after 4 h of incubation.

R.3. *In silico* analyses of 11β-HSD2 promoter reveals several potential HREs along the promoter.

Since 11β-HSD2 expression is hormone dependent in T47D cells, we focused the next step of our work in to define the minimal promoter responsible for the expression induced by progestins.

Previous works had determined that the 11β-HSD2 promoter activity was confined between nt -1778 preceding the transcription start and part of the first exon to nt +117 (Agarwal, White 1996, Nawrocki et al. 2002). To perform an *in silico* analysis we used the gene annotation from Ensembl and University of California at Santa Cruz (UCSC) genome browsers (Fig. R.2A). Possible transcription factor binding were located using Consite (http:/www.phylofoot.org/consite) and Transfac (http://www.gene-regulation.com/pub/databases.html#transfac) computational tools (Sandelin, Wasserman & Lenhard 2004, Wingender et al. 1996). The computational analysis of 11β-HSD2 promoter revealed several potential progesterone responsive elements (PREs) along the promoter, compared with described consensus PRE (GGTACA nnn TGTTCT) (Figs. R.2B and C).

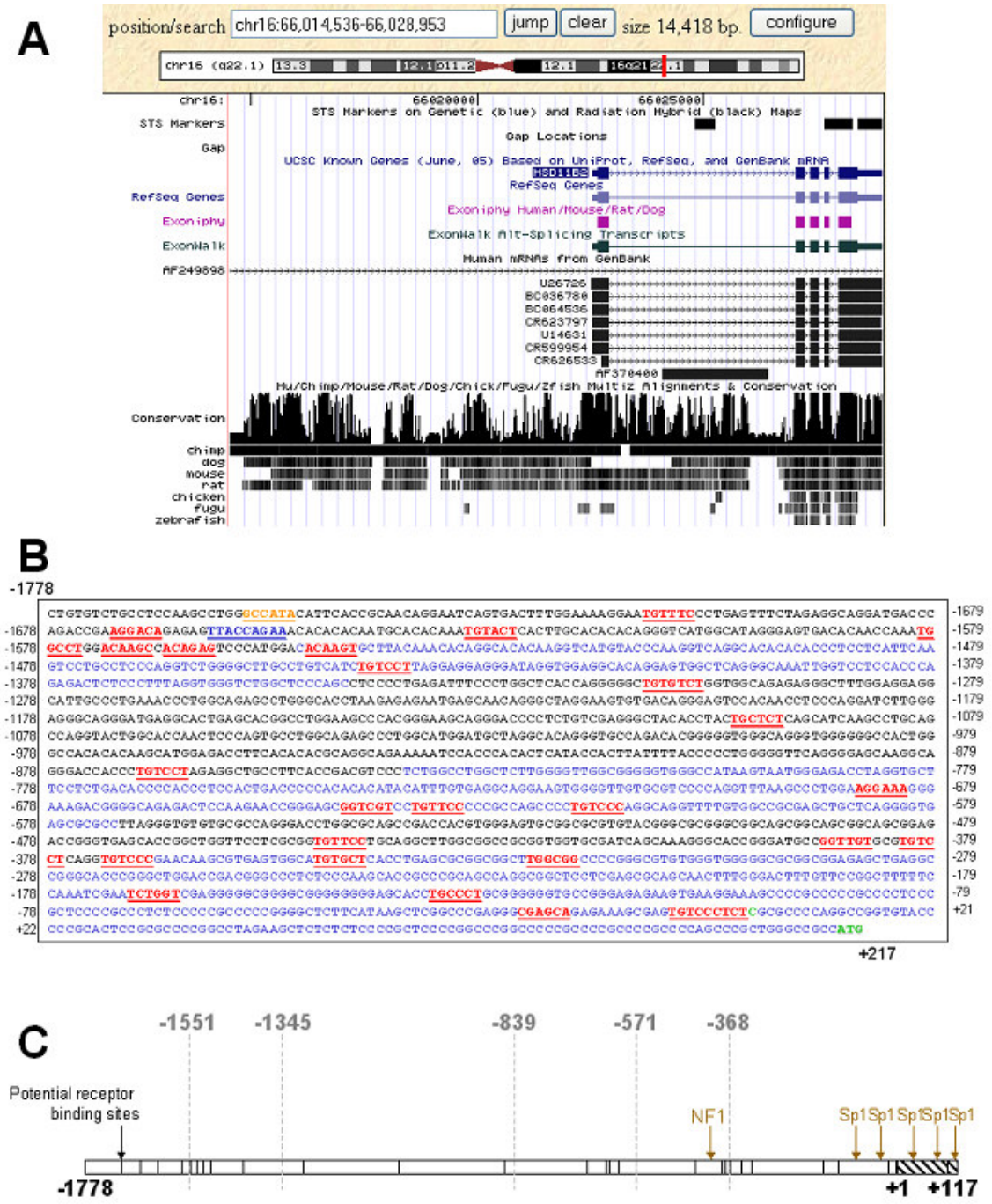

Figure R.2: ***In silico*** **analyses of 11β–HSD2 promoter reveals several potential HREs along the promoter**

(A) ***HSD11B2 is located at chromosome 16 and spans 5 exons.***

Graphical representation of *HSD11B2* gene chromosomal localization and gene information available obtained with the University of California at Santa Cruz (UCSC) genome browser.

(B) ***In silico analysis reveals potential HREs along 11β–HSD2 promoter and putative binding sites for STAT5A and YY-1 proteins in the distal promoter region***

Computational analysis of 11β–HSD2 proximal promoter sequence between nts –1778 and +117. The potential progesterone receptor binding sites predicted by Consite and Transfac software are indicated in red, as well as the putative STAT5A (blue) and YY-1 (orange) binding sites. The nucleotide at the position +1 and ATG at the position +117 are indicated in green. The sequence corresponding to each deletion construct is indicated in blue or black.

(C) ***Predicted HREs are distributed along the 11β–HSD2 promoter***

Human 11β–HSD2 promoter region structure. The brown arrows indicate the position of NF1 and Sp1 binding sites. The vertical bars along the 11β–HSD2 promoter region indicate the position of potential progesterone receptor elements predicted by computational analysis with Consite and Transfac software. The deletion endpoints used in the deletion analysis are indicated in grey.

R.4. The distal promoter (-1778 to –1345) of 11β-HSD2 gene is necessary for hormone-regulated promoter expression

R.4.1. The 11β-HSD2 promoter region between -1778 and –1345 is necessary for progesterone-induced promoter expression

In order to define more precisely the critical region responsible of 11β-HSD2 promoter induction by progestins in T47D cells, promoter deletions were cloned in front of the luciferase reporter gene (Fig. R.3 – R.6). The entire promoter was obtained fusing the part of the first exon (from nt +1 to nt +117), amplified by PCR from T47D cells, to the construct –1778 +1 (Agarwal, White 1996) kindly provided by Dr. Lewis P. Rubin. As deletion end points were chosen –368, -571, -839, -1345 and –1551, respectively (see chapter Materials and Methods). All constructs were sequenced from the luciferase gene and from the 5'-end into the insert for verification. Constructs were transiently transfected into T47D cell-derivatives, and hormonal response was evaluated as indicated in Material and Methods. Transfection efficiency was checked by co-transfecting a GFP expression construct. For luciferase activity measurement, protein was determined by BCA protein assay.

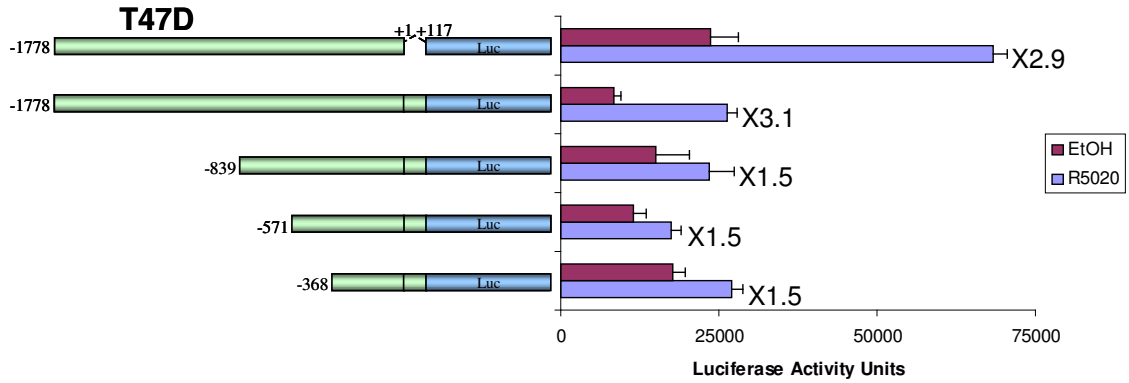

Figure R.3: Deletion analysis of 11β–HSD2 promoter fused to the luciferase reporter in T47D cells in response to progestins

T47D cells transfected with 3 μg of each 11β–HSD2 promoter construct (-1778+1; -1778+117; -839; -551 and –368) in pGL3 vector were serum-starved for 48 h and then treated with ethanol or R5020 10 nM for 16 h and luciferase activity was measured. For normalization equal amounts of cellular extract of each sample were used. The values represent the mean luciferase arbitrary units ±SD of two experiments performed in duplicate. Fold induction in response to hormone compared to ethanol is shown for each construct.

Transient transfection results in T47D cells are shown in Fig. R.3. Firstly, we tested hormone-induced luciferase expression under the control of the entire region (between nt –1778 and +117); the construct lacking +1 to +117

(previously reported) and the deletion fragments –839, -571 and –368. The basal levels of luciferase activity obtained with the different constructs were similar to the previously reported due to the presence of negative and positive regulatory regions (Agarwal, White 1996). Although the basal level obtained with the construct that lacks part of the first exon was higher than in the case of the entire 11β-HSD2 promoter, the response to progesterone was in both cases ~3-fold induction. The response obtained with the 11β-HSD2 deleted fragments was 1.5 fold-induction, indicating that the promoter region upstream nucleotide –839 is important in the hormone-dependent activity.

Because fold change was lower than the one obtained on the endogenous 11β-HSD2 expression, we decided to test whether in transient experiments PR levels were limiting. In order to enhance the hormone-response of the reporter constructs, we overexpressed PRB and PRA isoforms in T47D cells, co-transfecting PRB or PRA expression vectors with the different Luc constructs (Fig. R.4). PRB overexpression resulted in a better hormone-dependent response of the entire constructs (12 – 13 fold induction) while the deleted constructs showed a response of 2-3 fold induction (Fig. R.4A). This last result indicates that the proximal promoter region (still present in deletion –368) retains a residual promoter activity and hormone response in the optimal experimental circumstances (i.e. T47D cells overexpressing PRB).

To compare these results with PRA overexpression, we performed the same experiment with the Luc constructs (Fig. R.4B). PRA increased the hormone-induced response of entire constructs to ~7 – 8 fold-induction. The deletion constructs had a response of 1.5 – 2 fold, while in the same experiment, the activation with PRB overexpression and with the endogenous PR was of 20-fold induction and 3-fold induction, respectively (Fig. R.4B).

These results suggested that PRB and PRA isoforms have different functionality in the regulation of 11β-HSD2 promoter expression mediated by progesterone. However, in both cases the results confirmed that the region upstream nt –839 was important in the hormone induction.

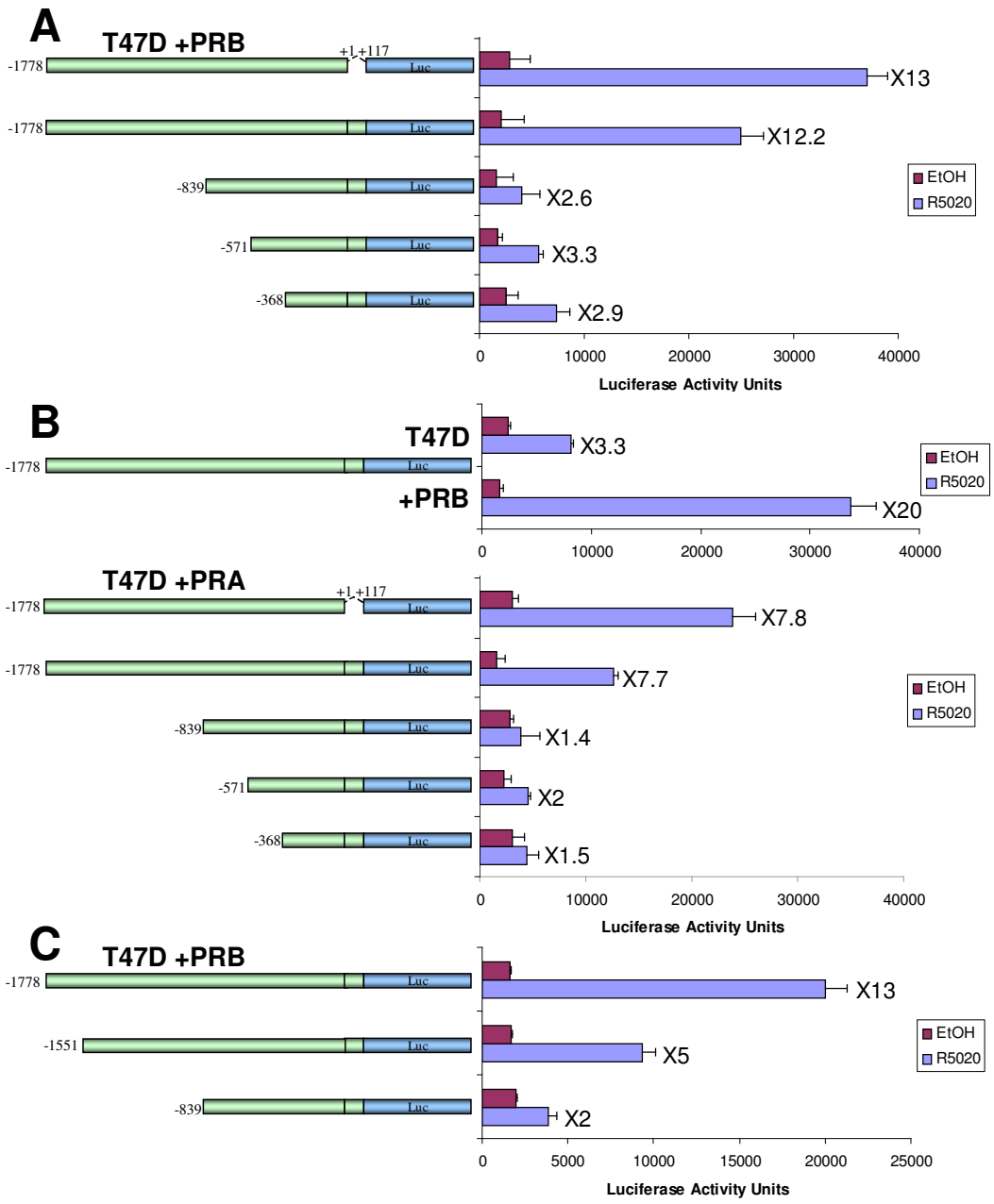

Figure R.4: Deletion analysis of 11β–HSD2 promoter deletions in T47D cells overexpressing PRA or PRB.
(A-C) Constructs –1778, -839, -571 and –368 were compared in T47D cells co-transfected with PRB (A) or PRA (B). In C, construct –1551 was added co-trasnfected with PRB. For normalization equal amounts of cellular extract of each sample were used. The values represent the mean luciferase arbitrary units ±SD of two experiments performed in duplicate. Fold induction in response to hormone compared to ethanol is shown for each construct.

To analyse with more detail the region between nts –1778 and –839, we performed a deletion construct that contains the promoter region between nt –1551 and +117 (Fig. R.4C). R5020 treatment of T47D cells co-transfected with PRB isoform and –1551 deletion construct showed 5-fold induction, that was ~40% of the response obtained with the entire promoter construct in the same

conditions (Fig. R.4C), indicating that this deletion construct has partially affected the hormone responsiveness.

In order to better define the involvement of PRA and PRB isoforms, we repeated the experiments in T47D-YV cells, that is a PR-negative clonal derivative cell line of T47D cells (Sartorius et al. 1994a) (Fig. R.5). The entire 11β-HSD2 promoter transfected in T47D-YV cells was not able to induce Luc activity by R5020 due to the lack of endogenous PR isoforms (Fig. R.5A, upper panel). Co-transfection of entire 11β-HSD2 promoter with PRB isoform exhibited a robust response to R5020, around 10-fold induction. Deletion of nts –1778 to –1551 led to a partial reduction in R5020-induced 11β-HSD2 promoter activity. As in T47D cells (Fig. R.3), -839, -571 and -368 deletion constructs reduced totally its hormone-dependent induction (Fig. R.5A, middle panel).

Co-transfections with PRA isoform showed the same behaviour although the levels of hormone activation reached lower fold induction (Fig. R.5A, lower panel), correlating with the results obtained in T47D cells (Fig. R.4B) and with previous works in which PRA isoform has a less regulatory effect than PRB in breast cancer cells (Jacobsen et al. 2002).

To define more accurately the participation of the different portions of the promoter to the hormone responsiveness between nts –1551 and –859, we performed another deletion construct from the –1778 to –1345 region (Fig. R.5B). Deletion of this region showed a substantial reduction of 11β-HSD2 promoter expression mediated by R5020. (Fig. R.5B).

Taken together, these results indicate that the distal 11β-HSD2 promoter region between –1778 and –1345 bp is indispensable for R5020-induced 11β-HSD2 promoter activation in breast cancer cells, and deletion at position –1551 partially affects this hormone-responsiveness identified at the distal portion of the promoter.

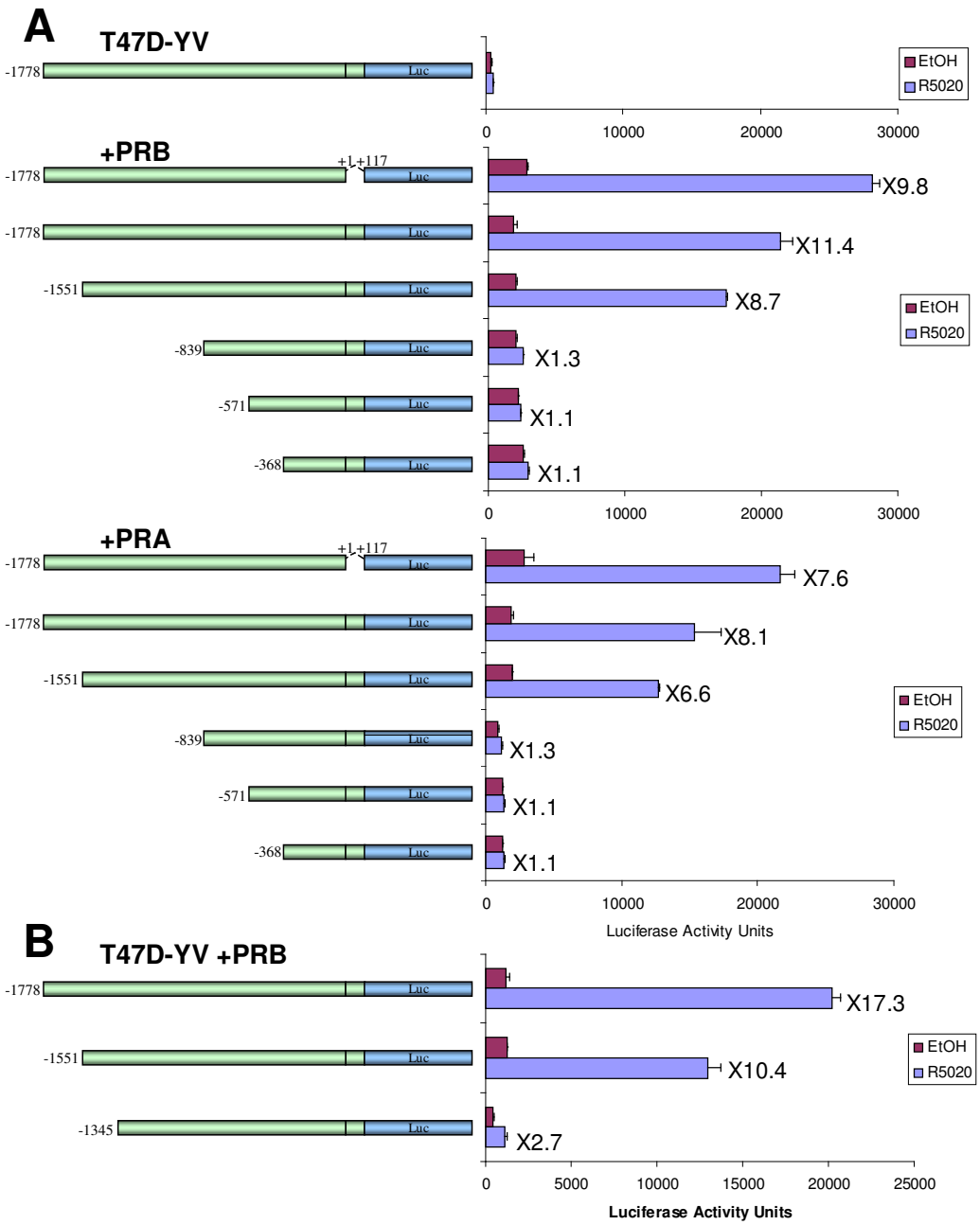

Figure R.5: Deletion analysis of 11β–HSD2 promoter deletions in T47D-YV transfected with PRA or PRB

(A-B) Constructs –1778, -1551, -839, -571 and –368 were compared in T47D cells co-transfected with PRB or PRA as indicated (A). In B, construct –1345 was added co-transfected with PRB. For normalization equal amounts of cellular extract of each sample were used. The values represent the mean luciferase arbitrary units ±SD of two experiments performed in duplicate. Fold induction in response to hormone compared to ethanol is shown for each construct.

R.4.2. The promoter region between –1778 and –1345 is also necessary for glucocorticoids-dependent promoter expression

Previous work has reported the activation of 11β-HSD2 expression in response to dexamethasone (Dexa) (Wan, Nordeen 2002). Because PR and GR use the same HREs, we asked whether in response to Dexa we observe

the same differences among deletion constructs (Fig. R.6). Our results showed that the levels of 11β-HSD2 promoter activation are comparable with those reached after R5020 induction in T47D cells without overexpressing PRB or PRA (Fig. R.3). Dexa is able to up-regulate 11β-HSD2 promoter ~2.5-fold induction in the case of entire and –1551 promoter constructs (Fig. R.6). –1345, -839, -571 and –368 deletion constructs showed a total abolishment of Dexa-mediated promoter activation (Fig. R.6).

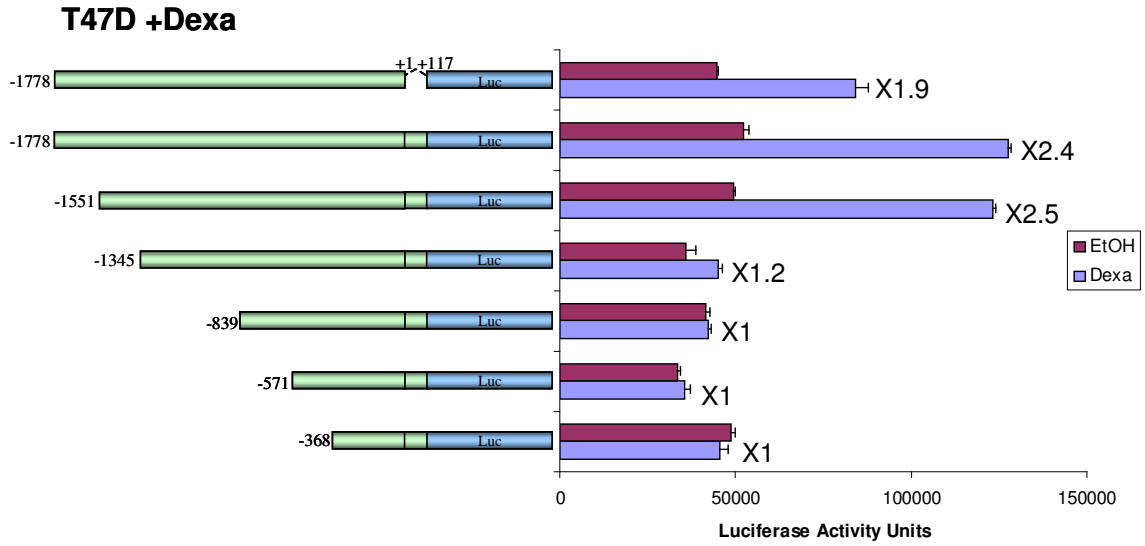

Figure R.6: Response to glucocorticoids of the 11β–HSD2 promoter constructs in transfected T47D cells

T47D cells transfected with 3 μg of each 11β–HSD2 promoter construct were serum-starved for 48 h and then treated with ethanol or Dexa 10 nM for 16 h and luciferase activity was measured. For normalization equal amounts of cellular extract of each sample were used. The values represent the mean luciferase arbitrary units ±SD of two experiments performed in duplicate. Fold induction in response to hormone compared to ethanol is shown for each construct.

These results indicate that also in the case of the Dexa-induced 11β-HSD2 promoter activation, the region between nt –1778 and –1345 is necessary for hormone-dependent promoter expression. A particularity is that deletion at –1551 does not partially abolish responsiveness as it happened in response to R5020 in T47D overexpressing PRB or PRA.

R.5. PR binds to two different regions of the 11β-HSD2 promoter after hormone activation

Deletion analysis showed that region upstream nt –1345 is responsible of the regulation of 11β-HSD2 promoter in response to R5020 and Dexa. However, inspection of promoter region using Consite and Transfac predicted the

presence of potential HREs along the entire promoter. In order to define whether PR is recruited to the promoter in response to hormone and where, chromatin immunoprecipitation (ChIP) analysis of PR recruitment was performed (Fig. R.7). In a parallel work we have used the T47D-YV cell line deprived of endogenous PRA and PRB expression, to stably express FLAG-tagged variants of PRB (wild-type and mutated at different functional domains). In this work we have used the cell line expressing the FLAG-tagged wild-type PRB for our ChIP experiments. This cell line, in addition, contained an integrated copy of the MMTV promoter, fused to the luciferase reporter gene (Quiles *et al*, manuscript in preparation).

We performed ChIP assays in cells that were hormone-starved for 2 days and then stimulated with R5020 for 5, 10 and 30 min, and using antibody against FLAG-tag (for details, see chapter Materials and Methods). We found that PR was recruited at the distal region of the 11β-HSD2 promoter following R5020 stimulation (Fig. R.7A, panels A and B). Surprisingly, PR was also recruited in response to hormone at the proximal 11β-HSD2 promoter region (Fig. R.7A, panels G and H), although this region had shown only reduced induction by R5020 in the promoter deletion analyses in the best situation, i.e. when PRB was overexpressed in T47D cells (Figs. R.3 – R.6). No recruitment of PR was observed in the rest of the 11β-HSD2 promoter regions tested along the promoter (Fig. R.7A, panels C-F), nor in the coding region (Fig. R.7A, panel I). As a control we checked the recruitment of PR after hormone treatment to the MMTV promoter nucleosome B (Fig. R.7B). As controls, we used normal mouse IgG as non-related antibody, checked a non-related genomic region (β–globin) and PCR-amplified the input samples for comparison (Fig. R.7A and B).

The results demonstrate that R5020 treatment induces PR recruitment to the distal and the proximal regions of the 11β-HSD2 promoter.

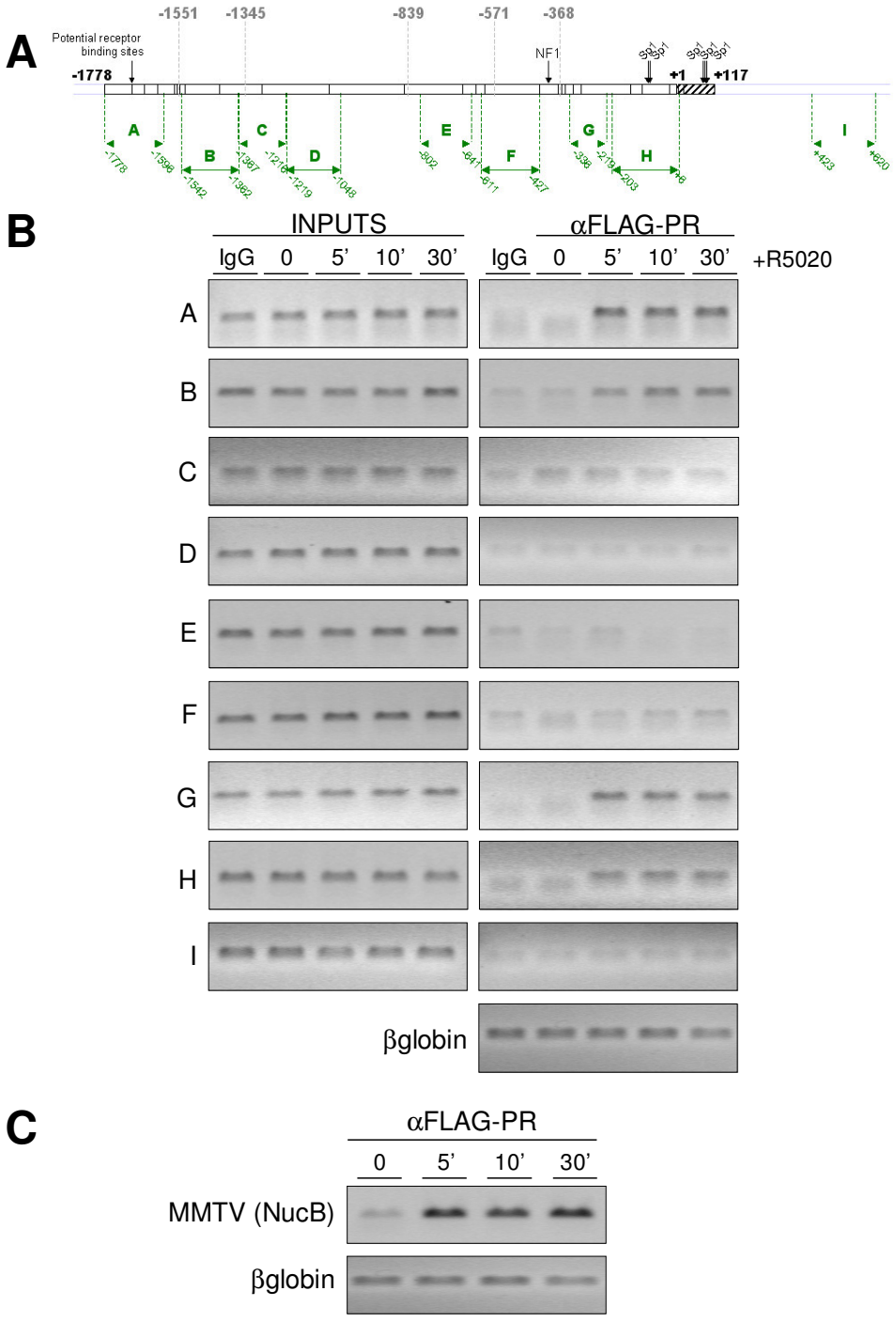

Figure R.7: PR binds to two different regions of the 11β–HSD2 promoter after hormone activation
(A) ***Schematic representation of the 11β–HSD2 promoter showing the location of PCR amplicons used to analyse ChIP material***
(B – C) ***Chromatin immunoprecipitation identifies two regions of PR binding to the 11β–HSD2 promoter after hormone activation***
TYML cells (T47D-YV cell line carrying an integrated copy of the progesterone reporter MMTV-Luciferase), expressing wild-type PRB (PRB wt) tagged with FLAG, were cultured as in Figure R.1B, untreated (0) or treated with R5020 10 nM for 5, 10 or 30 min, harvested, and used for ChIP experiments with α-FLAG antibody as indicated in *Material and Methods*. The precipitated DNA fragments were subjected to PCR with primers for the indicated 11β–HSD2 promoter and the β–globin gene as a control. (C) The MMTV nucleosome B region was also amplified to detect PR recruitment as a control of the experiment**.** Input material is shown for comparison. PCR products were run in a 1.2% Agarose gel and visualized with ethidium bromide.

R.6. Hormone-dependent PR recruitment to distal and proximal 11β-HSD2 promoter involves two different mechanisms

R.6.1. Progesterone-induced PR recruitment to proximal promoter is affected by mutations in the DNA binding domain (DBD) of the receptor

To investigate the mechanisms involved in hormone-responsive PR recruitment to the two 11β-HSD2 promoter regions, we have used another TYML-derived cell line, expressing a FLAG-tagged version of PRB isoform with three point mutations in the first Zinc finger of the DNA binding domain (PRB–DBDm) (Quiles *et al*, manuscript in preparation).

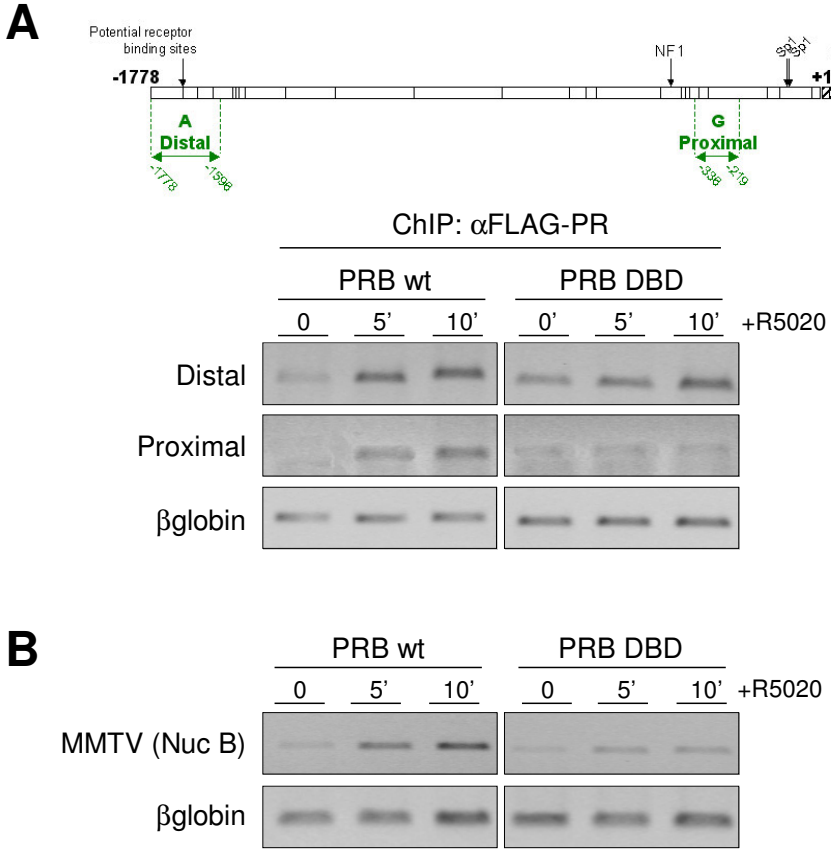

Figure R.8: Progesterone-induced PR recruitment to proximal promoter is affected by mutations in the DNA binding domain (DBD) of the receptor
(A - B) TYML cells expressing PRB wt tagged with FLAG or mutant DBD PRB (PRB DBDm) tagged with FLAG, cultured as in Figure R.1B, were untreated (0) or treated with R5020 10 nM for 5 or 10 min, harvested, and used for ChIP experiments with antibody against FLAG-tag. The precipitated DNA fragments were subjected to PCR analysis with specific primers corresponding to the distal or proximal 11β–HSD2 promoter regions (A), MMTV nucleosome B (B) and the β–globin gene as a control (A and B).

ChIP analysis of PR recruitment in response to progesterone showed that point mutations in the DBD of the PRB isoform did not affect its recruitment

to the distal 11β-HSD2 promoter region (Fig. R.8A, upper panel), while there was no hormone-dependent PRB–DBDm recruitment to the proximal 11β-HSD2 promoter region (Fig. R.8A, middle panel). To confirm the unability of the DBD mutant to bind to HREs, we tested its recruitment to the MMTV promoter by ChIP (Fig. R.8B). As expected point mutations abolished PR recruitment to MMTV promoter in response to hormone. (Fig. R.8B).

To explore with more detail the relevance of hormone-dependent PR recruitment to the proximal 11β-HSD2 promoter region, we checked the R5020-activation of 11β-HSD2 promoter mediated by PRB–DBDm (Fig. R.9). Firstly, we performed transient transfections in T47D-YV cells, co-transfecting 11β-HSD2-Luc reporter (pGL3 –1778+117 11β Luc) and wt PRB or PRB–DBDm (Fig. R.9A). 11β-HSD2-Luciferase activation by R5020 and wt PRB (~21-fold induction) was partially blocked in the case of PRB–DBDm transient transfection (~7-fold induction) (Fig. R.9A). As control, we also performed transient co-transfections in T47D-YV cells with MMTV-Luc reporter vector and wt PRB or DBD mutant PRB (Fig. R.9B). As expected, MMTV promoter activation by R5020 and wt PRB (10-fold induction), was totally blocked in the case of the PRB–DBDm (Fig. R.9B).

Next we explored the effect of PRB–DBDm on the endogenous expression of 11β-HSD2 gene, using TYML cells stably expressing FLAG-tagged wt-PRB or PRB–DBDm (Fig. R.9C). Surprisingly, 11β-HSD2 expression in response to hormone was no affected in TYML cells expressing PRB–DBDm (Fig. R.9C). Again, as control, we checked in the same experiment Luciferase mRNA expression due to hormone-induced MMTV promoter activation (Fig. R.9D). We confirmed that R5020 activation of MMTV promoter was totally abolished by DBD point mutations (Fig. R.9D), showing that the hormone regulation of 11β-HSD2 promoter is different to the classical studied MMTV promoter.

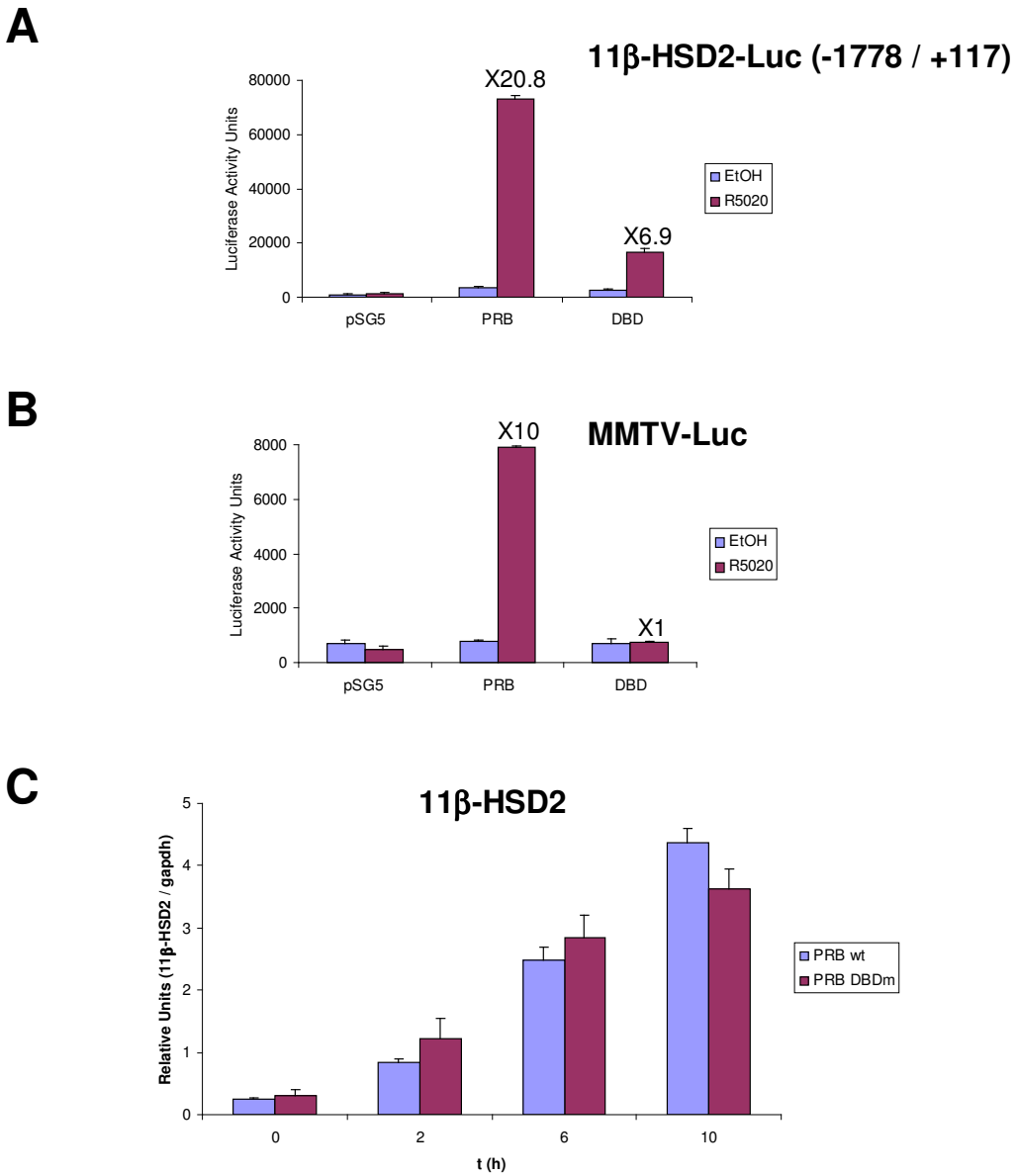

Figure R.9: Effect of PR mutations at the DBD on the progesterone-induced 11β–HSD2 promoter activity

(A – B) ***Progesterone-induced 11β–HSD2 promoter activity in T47D-YV cell transiently co-transfected with PRB wt or PRB DBDm***

T47D-YV cells co-transfected with pSG5-PRB wt, pSG5-PRB DBDm or pSG5 empty vector and 11β–HSD2-Luc (A) or MMTV-Luc (B) reporter vectors, were treated with R5020 10 nM for 16 h and luciferase activity was determined. Fold induction in response to hormone is shown for each construct. The values represent the mean ±SD of two experiments performed in duplicate.

(C) ***Progesterone-induced 11β–HSD2 mRNA expression in TYML cells expressing PRB wt or PRB DBDm***

TYML cells expressing PRB wt or PRB DBDm were cultured as in Figure R.1B, before the addition of R5020 10 nM for 6 h. Then the cells were harvested and total RNA was extracted. The 11β–HSD2 mRNA expression was analysed by RT and Real Time PCR with specific primers.

R.6.2. STAT5A is recruited to the distal region of the 11β-HSD2 promoter in response to progestins

A sequence analysis of the distal region of 11β-HSD2 promoter revealed the presence of a putative STAT5A binding site, 5'-TTAccaGAA-3' (Fig. R.10A),

which is well conserved when compared with a consensus STAT5A binding site (Clevenger 2004, Ihle 1996). This potential STAT5A binding site is located between nt –1654 and –1646 (Fig. R.10A).

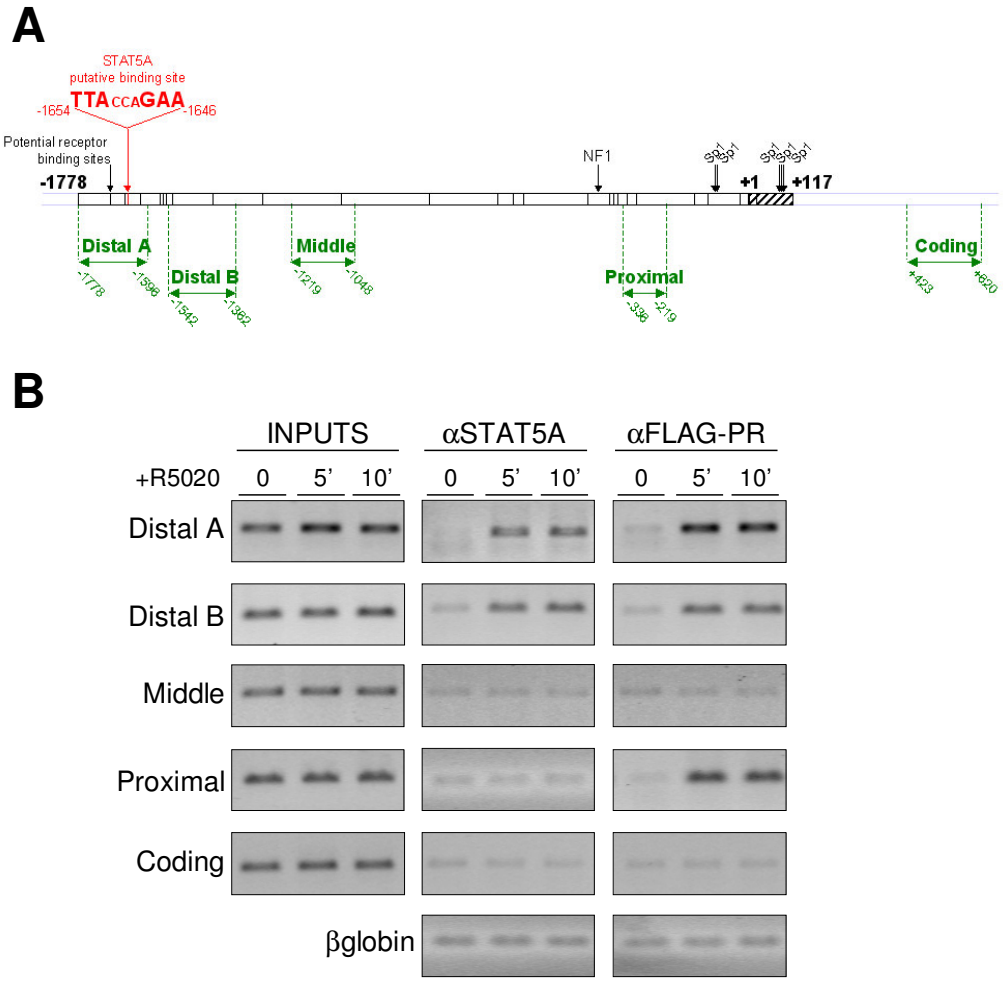

Figure R.10: STAT5A is recruited to the distal region of the 11β–HSD2 promoter in response to progestins
(A) Schematic representation of 11β–HSD2 promoter region with the location and sequence of the potential STAT5A binding site predicted by Consite and Transfac software, as well as location of amplicons for ChIP analysis.
(B) TYML cells expressing PRB wt tagged with FLAG, were cultured as in Figure R.1B, untreated (0) or treated with R5020 10 nM for 5 or 10 min, harvested, and used for ChIP experiments using antibodies against STAT5A (middle panel) or FLAG-tag (right panel). The precipitated DNA fragments were subjected to PCR analysis with primers corresponding to the indicated 11β–HSD2 promoter regions and the β–globin gene. Input material is shown for comparison. PCR products were run in a 1.2% Agarose gel and visualized with ethidium bromide.

Therefore, we next investigated by ChIP assay STAT5A recruitment to the 11β-HSD2 promoter in TYML cells expressing FLAG-tagged wt PRB isoform (Fig. R.10B). After hormone treatment, STAT5A was found bound to the 11β-HSD2 distal promoter region (Fig. R.10B, middle panels). Independently of

progesterone treatment, no STAT5A was bound to the 11β-HSD2 middle or proximal promoter regions nor to the coding region (Fig. R.10B, middle panels). As a control, we confirmed in the same experiment that PR was recruited in response to hormone to the distal and the proximal promoter regions but not to the middle and coding regions (Fig. R.10B, right panels). β–globin was used as internal loading control in all the cases (Fig. R.10B, lower panels).

These results confirmed that STAT5A is recruited to the distal 11β-HSD2 promoter region, in which a putative STAT5A binding site was predicted.

R.6.3. STAT5A recruitment after hormone activation is involved in PR recruitment to 11β-HSD2 distal promoter region

Since mutations at DBD do not affect hormone-dependent PR recruitment to the distal 11β-HSD2 promoter region, we next explored the possibility that STAT5A could be involved in the mechanism by which PR is recruited to this region. Previous works have related STAT factors with the regulation of target genes by steroid hormone receptors (Buser et al. 2007, Carsol, Gingras & Simard 2002, Stocklin et al. 1996). Recently, it has been published that progestins are able to induce STAT-mediated transcriptional activation via rapid JAK and STAT tyrosine phosphorylation in T47D cells (Proietti et al. 2005). On the other hand, it has been reported that STAT factors are phosphorylated by activated JAK to go to the nucleus and to be recruited to promoters (Cella, Groner & Hynes 1998). Moreover, STAT5 and GR form a molecular complex which cooperates in the induction of transcription of the β–casein gene by GR recruitment to the promoter mediated by the STAT protein (Stocklin et al. 1996).

Thus, we analysed by ChIP assay the influence of the JAK / STAT pathway inhibitor AG 490 on hormone-dependent recruitment of PR to 11β-HSD2 promoter region (Fig. R.11A). As expected, AG inhibitor affected STAT5A recruitment to the distal 11β-HSD2 promoter region (Fig. R.11A, left panel). No differences in STAT5A recruitment to the proximal 11β-HSD2 promoter region were observed upon hormone and AG inhibitor treated or untreated cells (Fig. R.11A, left panel).

Importantly, in the presence of the JAK / STAT pathway inhibitor we found that PR was remarkably less recruited in response to R5020 to the distal promoter region (Fig. R.11A, right panel), but not to the proximal promoter region. As control, we tested if AG inhibitor treatment affected PR recruitment to another hormone-regulated promoter (Fig. R.11B). Using nucleosome B specific primers, ChIP experiment showed that PR was normally recruited in response to hormone to the MMTV promoter (Fig. R.11B, right panel), in both cases, AG-treated and untreated cells. As expected, ChIP also confirmed that STAT5A was not recruited to the MMTV promoter nucleosome B (Fig. R.11B, left panel).

These findings support the idea that hormone-dependent recruitment of PR to distal 11β-HSD2 promoter region is mostly regulated by JAK / STAT pathway. Importantly, STAT5A activation by JAK and recruitment to the distal promoter region are mechanisms involved in R5020-dependent PR binding to the same region.

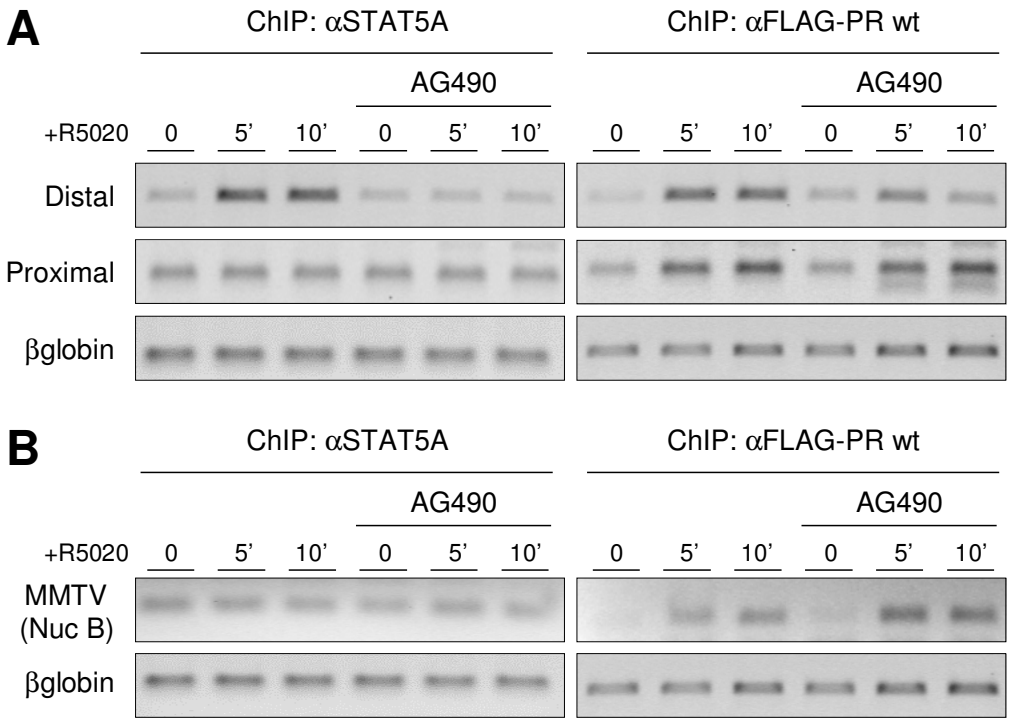

Figure R.11: Inhibition of JAK / STAT pathway interferes with STAT5A and PR recruitment to the distal 11β–HSD2 promoter region
(A – B) TYML cells expressing PRB wt tagged with FLAG cultured as in Figure R.1B, were untreated (0) or treated with R5020 10 nM for 5 or 10 min, harvested, and used for ChIP experiments with antibodies against STAT5A or FLAG-tag. When indicated, cells were pre-treated with AG490 50 μM for 1 h. The precipitated DNA fragments were subjected to PCR analysis with specific primers corresponding to the distal and proximal 11β–HSD2 promoter regions (A) or MMTV nucleosome B (B), and the β–globin gene as a control. PCR products were run in a 1.2% Agarose gel and visualized with ethidium bromide.

R.7. STAT5A is functionally important for hormone-dependent 11β-HSD2 promoter activation

R.7.1. JAK2 / STAT5A pathway activation is involved in the hormonal induction of 11β-HSD2 promoter

As it has been explained before, recent reports have described that progestins induce activation of JAK / STAT pathway and STAT protein phosphorylation in breast cancer cells (Proietti et al. 2005). In the same way, treatment of breast cancer cells with R5020 results in up-regulation of STAT3, STAT5A and STAT5B expression (Lin et al. 2003, Richer et al. 1998). Consequently, this pathway could be involved in changes of gene expression caused by progestins treatment.

To test the functional relevance of STAT5A in 11β-HSD2 gene expression, we performed transient transfection assays in T47D-YV cells with the PRB expression vector and the 11β-HSD2-Luc reporter vector, and tested the effect of the JAK inhibitor AG 490 (Fig. R.12A). Hormonal-dependent increase of 11β-HSD2 promoter activity was totally decreased in the presence of AG inhibitor (Fig. R.12A).

To test the effect of JAK / STAT pathway activation on the hormone-dependent induction of endogenous 11β-HSD2 promoter, progesterone-induced 11β-HSD2 mRNA expression was analysed in the presence of AG (Fig. R.12B). Normal induction of 11β-HSD2 expression after hormone treatment for 2 and 6 h was abolished when cells were treated with the JAK inhibitor (Fig. R.12B).

n order to compare the results obtained with the hormone-inducible MMTV promoter, we performed these analyses in the T47D-MTVL cell line. MMTV promoter was normally activated after R5020 induction in both AG treated and untreated cells (Fig. R.12C). Another two hormone-inducible genes were checked in the same experiment, Cyclin D1 and p21, confirming that AG-dependent inhibition of the progestin-induced 11β-HSD2 mRNA expression is not a general effect of the inhibitor over cellular hormone response gene expression. (Fig. R.12C).

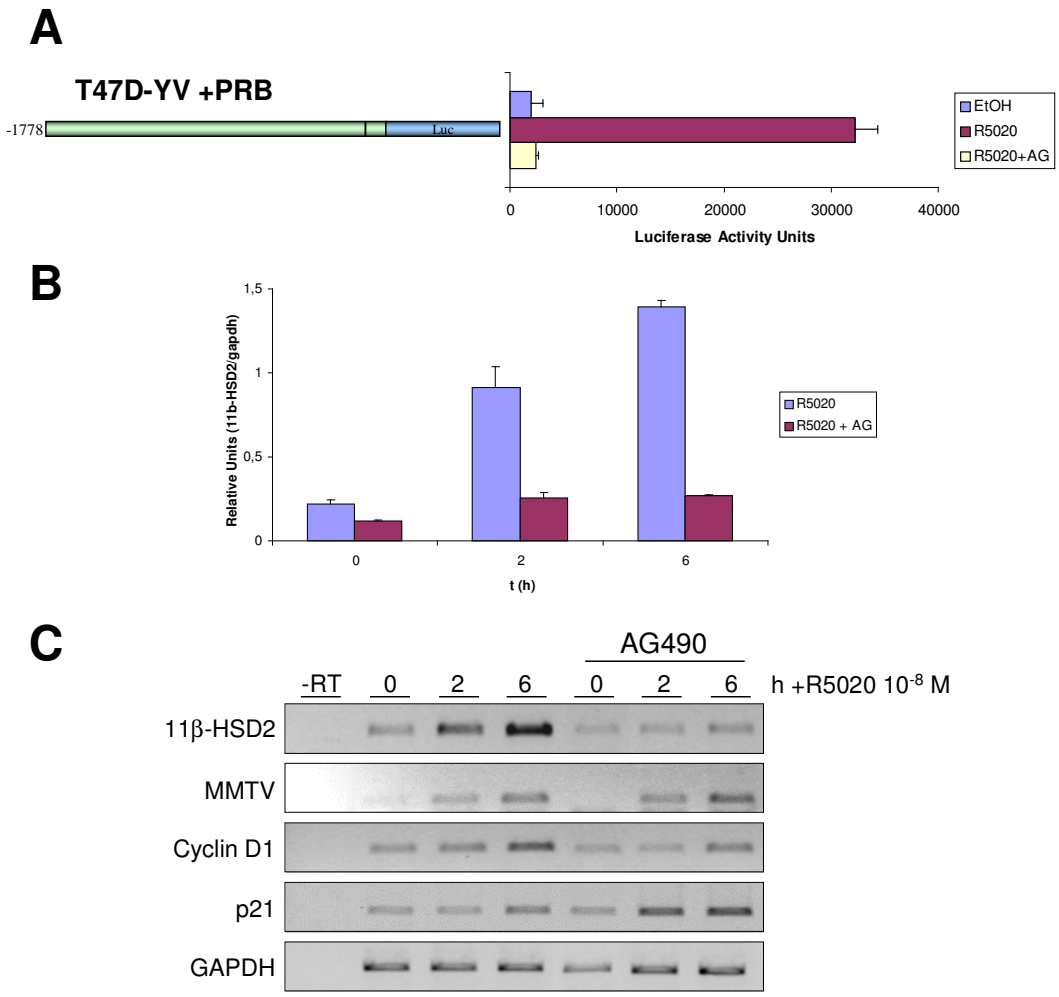

Figure R.12: JAK2 / STAT5A pathway activation is involved in the hormonal induction of 11β–HSD2 promoter
(A) T47D-YV cells co-transfected with pSG5-PRB wt and 11β–HSD2-Luc reporter vectors, were treated with R5020 10 nM for 16 h and luciferase activity was determined. When indicated, cells were pre-treated with AG490 50 μM for 1 h. For normalization equal amounts of cellular extract of each sample were used. The values represent the mean luciferase arbitrary units ±SD of two experiments performed in duplicate.
(B) T47D cells cultured as in Figure R.1B, were treated with R5020 10 nM for 6h. When indicated, cells were pre-treated with AG490 50 μM for 1 h. The cells were harvested and total RNA was extracted. The 11β–HSD2 mRNA expression was analysed by RT and Real Time PCR with specific primers. GAPDH cDNA specific primers were used as a control.
(C) T47D cells cultured as in Figure R.1B, were treated with R5020 10 nM for 6h. When indicated, cells were pre-treated with AG490 50 μM for 1 h. Then the cells were harvested and total RNA was extracted. The 11β–HSD2, Luciferase, Cyclin D1 and p21 mRNA expression were analysed by RT-PCR with specific primers. GAPDH cDNA specific primers were used as a control. PCR products were run on a 1.2% Agarose gel and visualized with ethidium bromid

These results suggested that JAK / STAT pathway activation has an important role on hormone-induced 11β-HSD2 expression.

R.7.2. Expression of constitutive active or dominant negative STAT5A forms affects the hormone activation of 11β-HSD2 promoter

To better characterize the functional role of STAT5A in R5020 induction of 11β-HSD2 promoter activation, we transiently co-transfected T47D-YV cells with the full-length 11β-HSD2-Luc reporter vector, the PRB expression vector, and expression vectors for the wild-type STAT5A (wt-STAT5A), a constitutively active (CA-STAT5A), or a dominant negative (DN-STAT5A) STAT5A forms (Fig. R.13). Overexpression of wt-STAT5A or expression of CA-STAT5A showed an increase (~32-fold induction) over the normal hormone-mediated 11β-HSD2-Luc activity reached with the endogenous STAT5A (~24-fold induction) (Fig. R.13). In contrast, expression of DN-STAT5A remarkably decreased not only the hormone response of the promoter (5-fold induction remained), but also its basal activity (Fig. R.13).

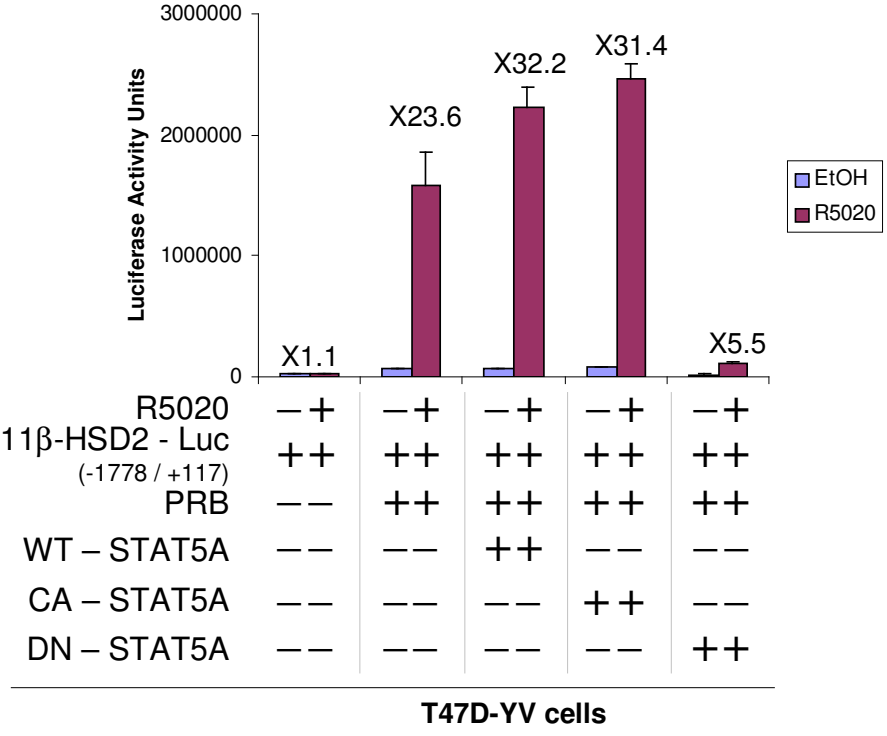

Figure R.13: Expression of dominant negative STAT5A affects the hormone activation of 11β–HSD2 promoter

T47D-YV cells co-transfected when indicated with 1 μg of 11β–HSD2-Luc (-1778 / +117) reporter vector, 1 μg of pSG5-PRB and 1 μg of wild-type STAT5A form (wt-STAT5A); constitutive active form of STAT5A (CA-STAT5A); or dominant negative form of STAT5A (DN-STAT5A), were treated with ethanol or R5020 10 nM for 16 h and luciferase activity was measured. For normalization equal amounts of cellular extract of each sample were used. The values represent the mean luciferase arbitrary units ±SD of two experiments performed in duplicate. Fold induction in response to hormone is shown for each construct.

In another set of experiments we studied the effect of wt-STAT5A or DN-STAT5A expression, on the entire 11β-HSD2 promoter construct compared to the –1551 construct (Fig. R.14A). This deletion construct includes nt –1551 to +117, which does not contain the predicted putative STAT5A binding site. Co-transfections of entire promoter construct or –1551-11β-Luc with PRB expression vector and wt-STAT5A or DN-STAT5A in T47D-YV cells resulted in a similar behaviour of both promoter constructs (Fig. R.14A), although as shown before (Fig. R.5), the –1551 construct has a lower activity than the full-length promoter. Thus, overexpression of wt-STAT5A increased R5020 induction of –1551-11β-Luc (~19-fold induction and ~27-fold induction for endogenous STAT5A and wt-STAT5A overexpression, respectively). However, DN-STAT5A expression resulted in a clear decrease of the Luciferase activity after hormone treatment (Fig. R.14A), comparable to the effect on the entire promoter construct.

In order to check the effects of DN-STAT5A expression over the entire promoter deletion constructs, transient co-transfections were performed with the entire promoter construct or the promoter –1551, -1345, -839 and -368 deletions, and the DN-STAT5A form (Fig. R.14B). In order to be able to see clearly the activity of the shortest constructs, (see chapter R.4 and Fig. R.4A and C), this experiment was performed in T47D cells co-transfected with the PRB expression vector. After hormone addition, only the longest constructs (-1778 and –1551-11β-Luc) decreased their fold-induction with DN-STAT5A co-transfections (Fig. R.14B). The shortest constructs (-1345, -839 and –368-11β-Luc) were not affected by DN-STAT5A co-transfection and retained the weak level of induction (~2-3-fold induction) (Fig. R.14B). Notice that all basal expression levels where lower when DN-STAT5A was co-transfected.

Taken together, these results showed that STAT5A has a functional role in the hormone-induced 11β-HSD2 promoter activation. Moreover, these results suggested that although the predicted putative STAT5A binding site is located upstream nt –1551 (-1654 to –1646), the functional involvement of STAT5A in the progesterone-dependent 11β-HSD2 promoter regulation requires not only this region but also another region upstream nt –1345. In addition, the reduced

promoter activity present still in deletion –368 (proximal promoter region), where PR also binds *in vivo*, is independent of JAK / STAT activation.

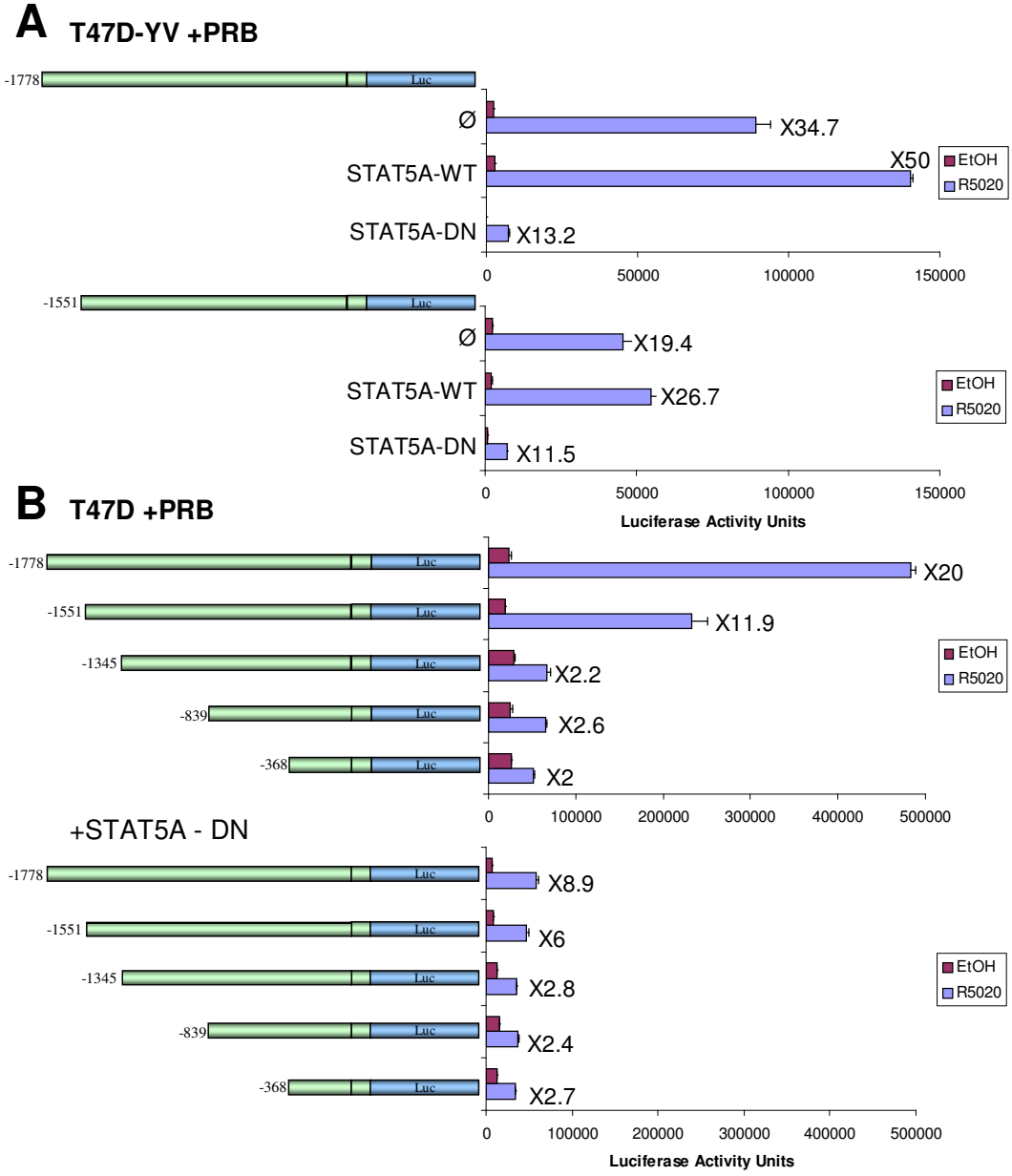

Figure R.14: Analysis of 11β–HSD2 promoter deletions constructs affected by the dominant negative form of STAT5A

T47D-YV (A) or T47D (B) cells co-transfected with 1 µg of indicated 11β–HSD2 promoter constructs, 1 µg of pSG5-PRB and 1 µg of wild-type STAT5A form (wt-STAT5A) or dominant negative form of STAT5A (DN-STAT5A), were treated with ethanol or R5020 10 nM for 16 h and luciferase activity was measured. Fold induction in response to hormone is shown for each construct. The values represent the mean ±SD of two experiments performed in duplicate.

R.7.3. Partial inhibition of STAT5A expression by specific shRNAs is not sufficient to affect neither progestins-dependent 11β-HSD2 expression nor PR and STAT5A recruitment to the distal promoter

To test the involvement of STAT5A on the endogenous 11β-HSD2 gene expression, we decided to perform RNA interference to knock-down STAT5A expression (Fig. R.15). For that, we used commercial shRNA-expression lentivirus directed against STAT5A. After viral infection and selection with puromycin, STAT5A expression was analysed by RT-PCR. We tested five different shRNA clones, and we found that two of them reached a considerable silencing effect (clones A10 and A12) (Fig. R.15A). Real Time PCR showed that shRNA clones A10 and A12 were able to inhibit STAT5A mRNA expression up to 73% and 68%, respectively.

We next analysed whether the STAT5A silencing reached affected endogenous R5020-induced 11β-HSD2 mRNA expression (Fig. R.15B). Unexpectedly, we observed similar (or slightly weaker) levels of hormone-dependent 11β-HSD2 expression in both cell lines with decreased STAT5A expression compared with control cells (Fig. R.15B).

Consequently, we performed ChIP experiments to search an explanation for this observation (Fig. R.15C). ChIP assays showed that partial STAT5A expression inhibition by shRNAs was not sufficient to block R5020-dependent STAT5A recruitment to the distal 11β-HSD2 promoter region (Fig. R.15C, left panels). As a result, hormone-dependent PR recruitment to the same region was also not affected (Fig. R.15C, right panels).

With these results, we concluded that partial STAT5A mRNA inhibition reached by specific shRNAs is not sufficient to affect STAT5A functional role in R5020-dependent 11β-HSD2 promoter regulation.

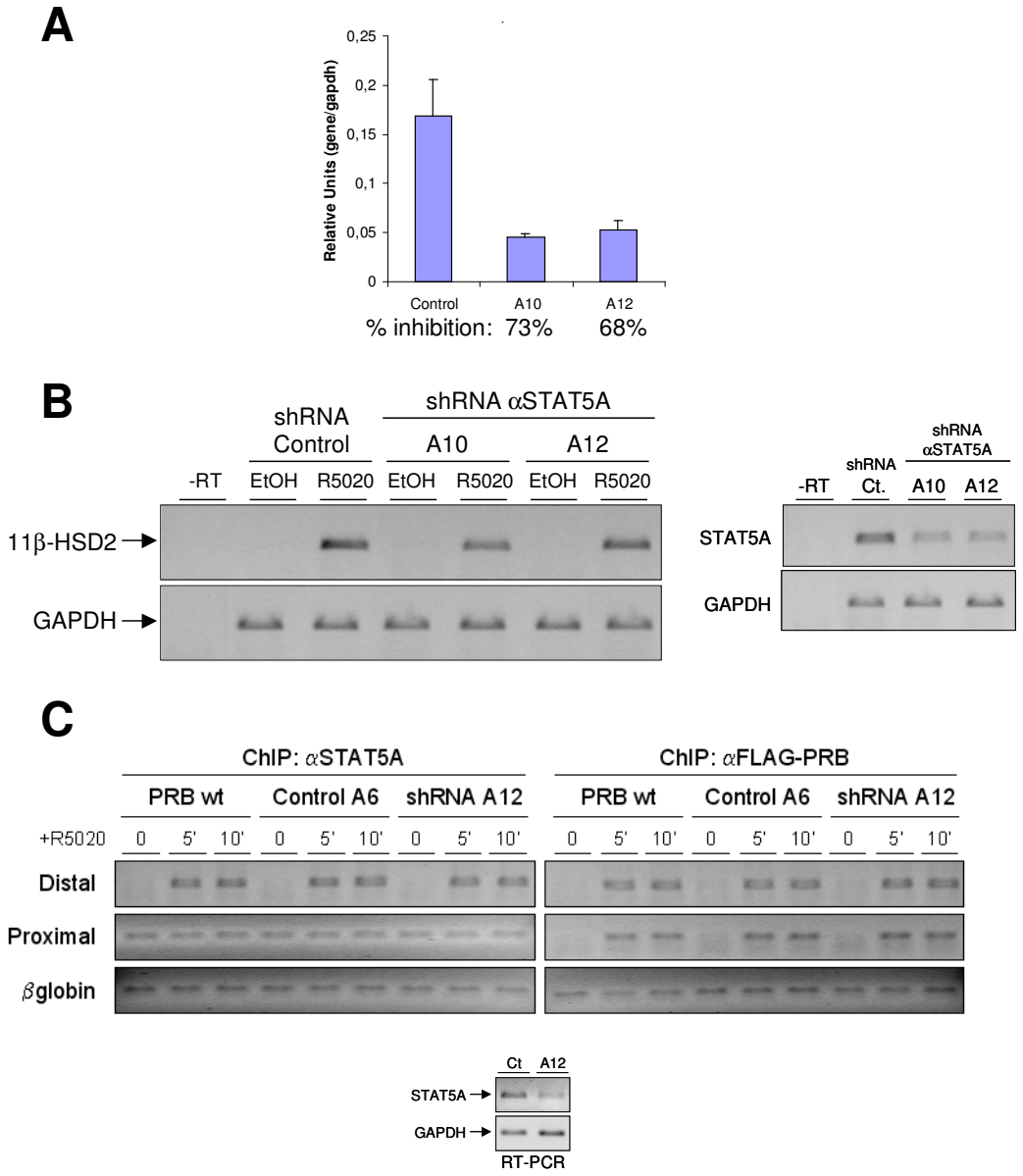

Figure R.15: Effect of STAT5A knock-down on 11β–HSD2 expression
(A) ***Inhibition of STAT5A expression with stable shRNA expression***
TYML cells expressing PRB wt tagged with FLAG and control shRNA or two different shRNAs against STAT5A (called A10 and A12) were harvested and total RNA was prepared. cDNA was generated and used as a template for Real Time PCR with STAT5A and GAPDH specific primers. Percentage of STAT5A expression inhibition is shown in each case.
(B) ***11β–HSD2 expression after knocking-down STAT5A***
TYML cells expressing PRB wt tagged with FLAG and control shRNA or two different shRNAs against STAT5A cultured as in Figure 1B were harvested or treated with ethanol or R5020 10 nM for 6 h. Total RNA was prepared and cDNA was generated and used as a template for PCR with 11β–HSD2 (left panel), STAT5A (right panel) specific primers. GAPDH was used as a control (left and right panels).
(C) ***Recruitment of STAT5A and PR to 11β–HSD2 promoter on STAT5A knocked-down cells***
TYML cells expressing PRB wt tagged with FLAG and control shRNA or shRNAs against STAT5A cultured as in Figure 1B were untreated (0) or treated with R5020 10 nM for 5 or 10 min, harvested, and used for ChIP experiments with antibodies against STAT5A or FLAG-tag. The precipitated DNA fragments were subjected to PCR analysis with specific primers corresponding to the distal and proximal 11β–HSD2 promoter regions and the β–globin gene. In parallel, STAT5A expression was measured again by RT-PCR as described in (B) (lower panel).

R.8. Inhibition of JAK / STAT pathway affects progestins induction of other target genes

A customized human cDNA microarray containing about 750 genes of interest in breast cancer and steroid hormone regulation was used to identify the subset of progesterone-induced genes dependent on activation of the JAK / STAT pathway cascade. Previous kinetic experiments performed in T47D cells on this array platform had shown that at 6 h of R5020 treatment, an extensive number of genes changed their expression (unpublished results). T47D-MTVL cells were serum-starved for 48 h and then were pre-treated with AG 490 1 h prior treatment with hormone or vehicle for 6 h. Cell were harvested and RNA was extracted for microarray hybridization.

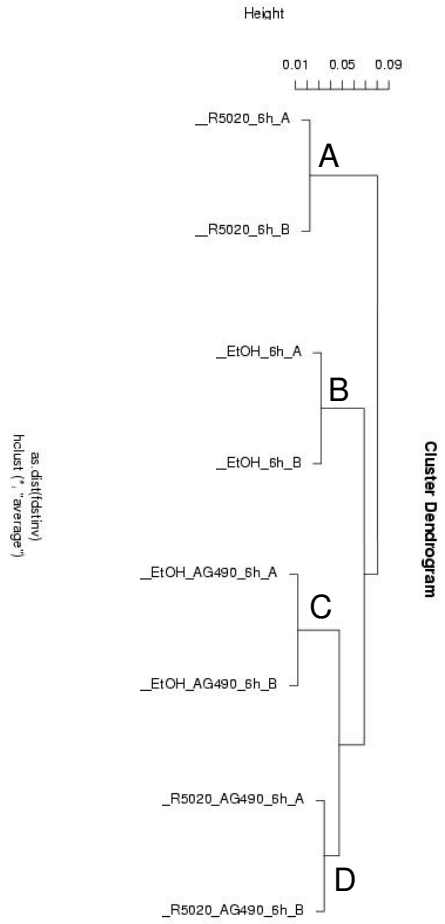

Figure R.16: Cluster tree for grouping of samples submitted to microarray hybridization
Cluster tree representation of the data set obtained with a customized human cDNA microarray containing about 750 genes of interest in breast cancer and steroid hormone regulation. T47D-MTVL cells were serum-starved for 48 h and then were pre-treated with AG 490 1 h prior treatment with ethanol or R5020 10 nM for 6 h. Cells were harvested and RNA was extracted for microarray hybridization. Cluster tree represents the information concerning which observations are grouped together because of their level of similarity.

The data sets were scaled so that they could be plotted for cluster tree visualization (Fig. R.16). Cluster tree represents the information concerning which observations are grouped together because of their level of similarity. Each sample is considered its own cluster and is connected to the correspondent replicate sample (Fig. R.16, A, B, C and D). In this way, the R5020-treated cell replicates are connected because the level of similarity between them is very high (Fig. R.16A), as well as in the case of EtOH-treated cells replicates (Fig. R.16B). On the other hand, these two clusters are well separated from the EtOH-plus-AG and R5020-plus-AG treated cells clusters (Fig. R.16C and D). This indicates that the results differ on a higher extent due to the AG treatment, than to the EtOH / R5020 treatment.

To identify statistically significant changes in expression, we applied Significance Analyses of Microarrays (SAM). This method assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of the duplicate measurement (Tusher, Tibshirani & Chu 2001) (Fig. R.17, left panels). We identified a set of 35 genes in which progesterone induction was higher than 1.4-fold and statistically significant with a p=0.05, in the absence of AG. (Fig. R.17 A, left panel). Our goal was to identify which of these genes had an altered response to hormone in the presence of AG. Average R5020 / EtOH fold induction of duplicate experiments, in the absence or presence of AG inhibitor, was calculated for these genes (Fig. R.17 A, middle panel). The results showed that a subset of progesterone target genes is dependent on JAK / STAT pathway activation.

The effect of AG on the basal expression of the genes reported was also calculated as fold change of the EtOH + AG / EtOH –AG samples and is shown in Fig.17, A and B, right panel.

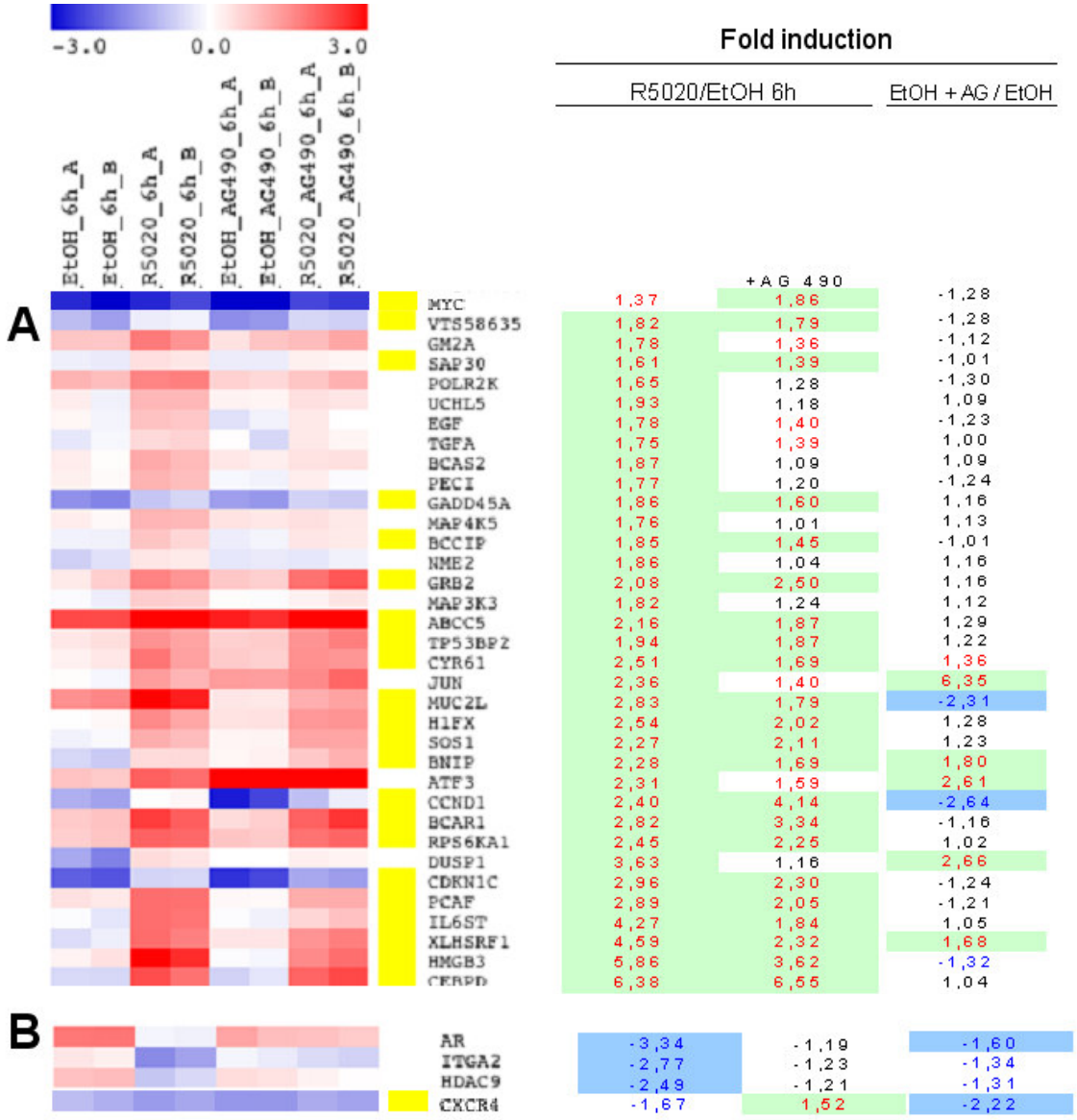

Figure R.17: Effect of AG 490 on the gene expression profile in response to R5020
Set of genes in which progesterone induction (A) or repression (B) was higher than 1.3-fold and statistically significant with a p<0.05, applying the Significance Analyses of Microarrays (SAM) method (see *Materials and Methods)*, in the absence of AG (graphical representation in the left panel). Average R5020 / EtOH fold induction of duplicate experiments, in the absence or presence of AG inhibitor, is indicated for these genes (middle column). In the right column is also shown the effect of AG 490 on the basal expression of these genes, represented as fold change EtOH + AG / EtOH –AG. Fold changes higher than 1.3 are in red, lower than –1.3 in blue, boxed when statistically significant with p<0.05.

We next studied with more detail the hormone response in the presence of AG by using RT-PCR and Real Time PCR to validate the microarray experiments results obtained for some representative genes (Fig. R.18). Before of that, 11β-HSD2 mRNA expression was analysed as a control for the experiment, obtaining similar results as previously explained (Fig. R.12B), meaning a strong effect of AG on the hormonal response of 11β-HSD2,

although basal expression is also affected. In the case of the *cyclin D1* gene (Fig. R.18), the AG inhibitor caused a decrease on the basal expression, although hormone fold induction was not affected. After 6 h of progestin treatment, the induction of *Dusp 1* was not observed in the presence of AG treatment. As in the *cyclin D1* gene, *Dusp1* mRNA basal levels were affected by AG, in this case increasing its basal expression. On the contrary, the induction of *Map3k3* by progestins was a bit reduced by AG treatment and *Myc* gene hormone-dependent induction was slightly increased by AG. In the case of the *Jun* gene, the induction after progesterone treatment was reduced in cells treated with AG, and basal expression was higher in the presence of AG inhibitor. Finally, the *Cxcr4* is a hormone-dependent repressed gene with decreased basal levels in cells treated with AG. Progestins-dependent gene expression inhibition was reverted in the presence of AG inhibitor, and the *Cxcr4* induction was increased as a consequence.

Thus, our results allow us to conclude that only a small subset of cellular genes showing progesterone regulation are affected by JAK / STAT pathway inhibition, but still this is not unique to the 11β-HSD2 gene.

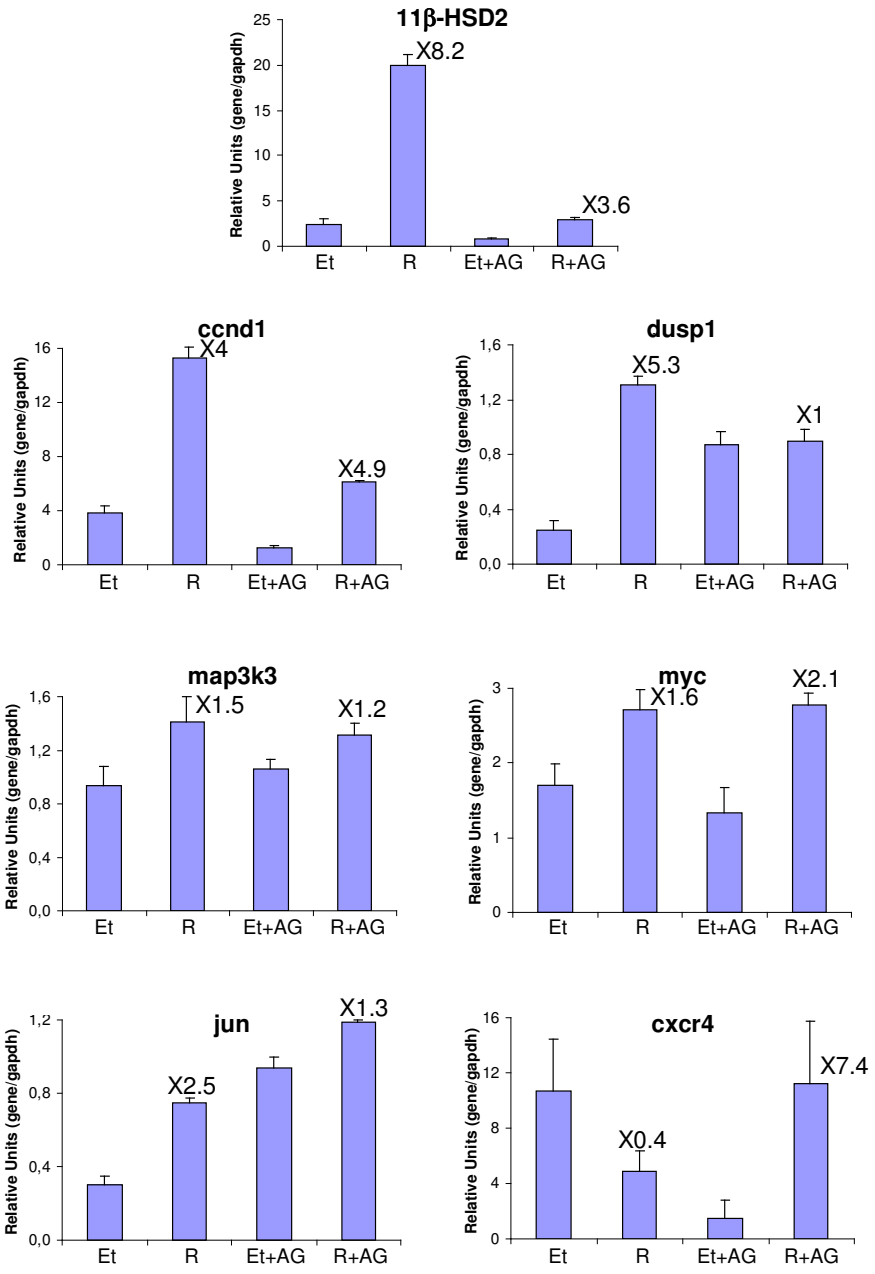

Figure R.18: Validation of microarray results by RT-PCR
T47D cells cultured as in Figure R.1B were treated with ethanol or R5020 10 nM for 6 h. When indicated cells were pre-treated with AG 490 1 h. Then cells were harvested and total RNA was prepared. cDNA was generated and used as a template for Real Time PCR with 11β–HSD2, Ccnd1, Dusp1, Map3k3, Myc, Jun, Cxcr4 and GAPDH specific primers. Data shown is corrected by GAPDH expression. Fold-changes in response to hormone compared to vehicle are shown, both in the absence or presence AG.

R.9. MAPK and PI3K / Akt pathways are partially involved in hormone activation of 11β-HSD2 promoter expression

Previous works indicated that progestins stimulate proliferation of T47D cells and induce a rapid and transient activation of Src / Ras / Erk pathway, and PR is required for this stimulation (Ballare et al. 2003, Migliaccio et al. 1998). Moreover, rapid Erk activation by progestins participates in induction of target

genes as MMTV promoter activation (Vicent et al. 2006). To investigate the possible involvement of signalling-mediated PR effects on 11β-HSD2 promoter induction, firstly we used PD 98059 (PD), a specific inhibitor of mitogen activated protein kinase kinase 1 (MKK1) (Fig. R.19A). In T47D cells transiently co-transfected with PRB expression vector and entire 11β-HSD2 promoter construct, -1551-11β-Luc or –368-11β-Luc deletion constructs, we observed in all the cases that Erk pathway inhibition by PD partially blocked R5020-induced Luciferase activity (Fig. R.19A, upper panel). As a control, we checked also in transient tranfections, the effect of PD on the hormone-dependent MMTV-Luc reporter activation (Fig. R.19A, middle panel), as well as on a constitutively active promoter, the cytomegalovirus (CMV) promoter, fused with luciferase reporter gene (Fig. R.19B, lower panel). Our results confirmed that Erk pathway inhibition totally blocks hormone-induced MMTV promoter activation (Fig. R.19A, middle panel). Moreover, the inhibition was hormone-specific since the levels of constitutive expression of CMV promoter remained unaffected in the cells treated with PD (Fig. R.19B, lower panel).

To complete the results obtained we next checked the possible role of Erk pathway and other PR signalling-mediated effects on endogenous 11β-HSD2 expression in T47D cells, using PD inhibitor, ICI 182,780 (ICI), a specific ER antagonist, and Wortmannin (WM), an inhibitor of phosphoinositol 3-kinase (PI3K) pathway (Fig. R.19B). Comparable with transient transfections experiments, R5020-induced 11β-HSD2 mRNA expression after 6 h of treatment was partially inhibited by PD inhibitor (Fig. R.19B, red bars). Surprisingly, no significant differences were observed after 2 and 12 h of treatment (Fig. R.19B, red bars).

Previous works determined that interaction of ligand-activated PR with ER is necessary for Erk pathway activation and hormone induction of the MMTV promoter (Ballare et al. 2003, Vicent et al. 2006). In our experiments, cells treated with ICI showed normal levels of progesterone-induced 11β-HSD2 expression after 2, 6 and 12 h of treatment (Fig. R.19B, yellow bars).

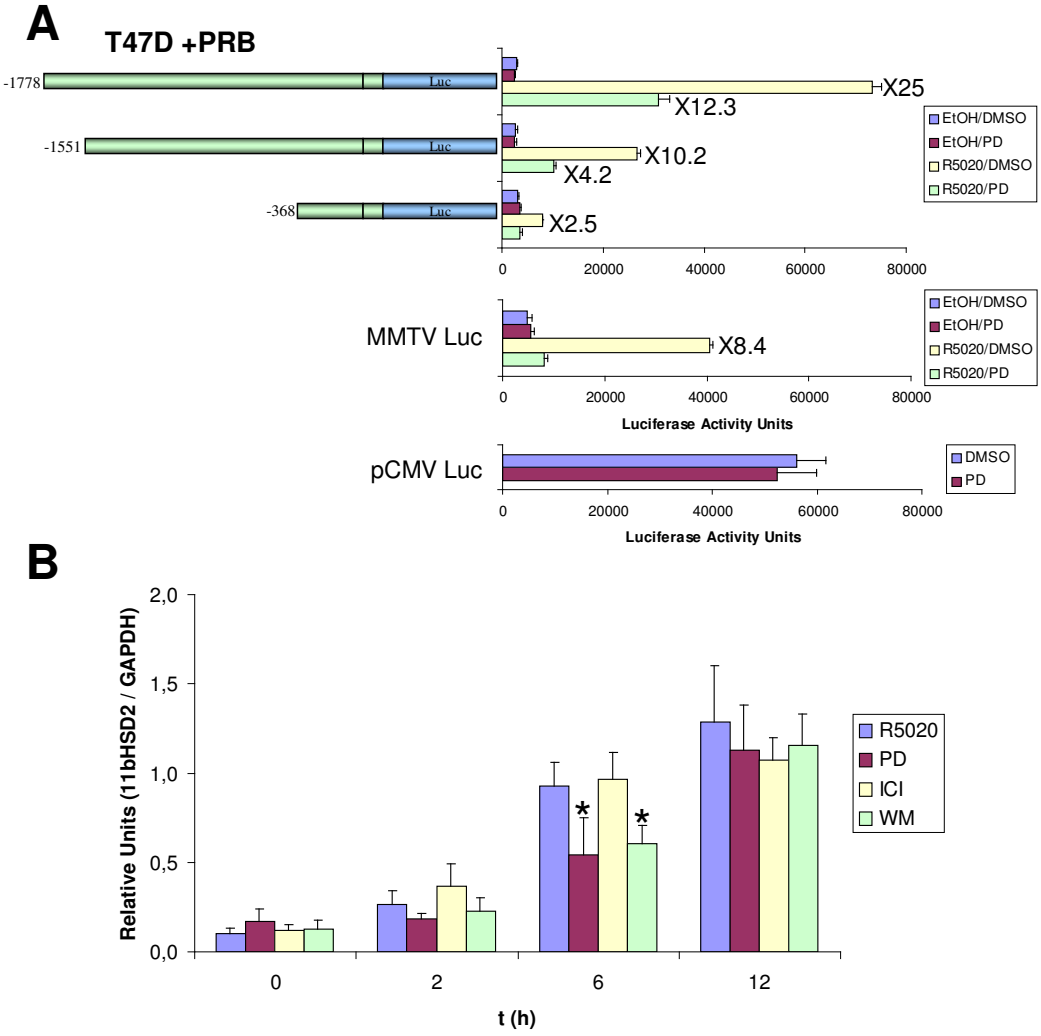

Figure R.19: Involvement of MAPK and PI3K / Akt pathways in hormone activation of 11β–HSD2 promoter expression

(A) ***Effect of MAPK inhibition on the expression of the 11β–HSD2 reporter constructs***

T47D cells co-transfected with 1.5 µg of pSG5-PRB wt and 1.5 µg of indicated 11β–HSD2 promoter constructs (upper panel); MMTV-Luc reporter vector (middle panel); or pCMV-Luc reporter vector (lower panel) were treated with R5020 10 nM (upper and middle panels) or vehicle (lower panel) for 16 h and luciferase activity was determined. When indicated, cells were pre-treated with PD 98059 50 µM (PD) or vehicle for 1 h. For normalization equal amounts of cellular extract of each sample were used. The values represent the mean luciferase arbitrary units ±SD of two experiments performed in duplicate. Fold inductions in response to hormone are indicated.

(B) ***Effect of different signalling pathway inhibitors on hormone-induced endogenous 11β–HSD2 gene expression***

T47D cells cultured as in Figure 1B were untreated (0) or treated with R5020 10 nM for 2, 6 or 12 h. When indicated, PD98059 50 µM (PD, red bars); Wortmannin 0.1 µM (WM, green bars); or ICI182,780 10 µM (ICI, yellow bars), was added 1 h before hormone induction. Then cells were harvested, total RNA was prepared and cDNA was generated by RT. 11β–HSD2 expression was analysed by Real Time PCR with specific primers. To normalize the data GAPDH expression was used as a control.

Finally, as in the case of PD effects, WM affected hormone-induced 11β-HSD2 mRNA expression after 6 h of treatment, although no significant differences were observed after 2 and 12 h of treatment (Fig. R.19B, green bars).

With these results we concluded that 11β-HSD2 response to hormone is partially affected by MEK and PI3K / Akt pathways inhibition. Together with the previous results, we conclude that JAK / STAT pathway is the major signalling pathway player induced by hormone in the regulation of 11β-HSD2. This differs from the MMTV promoter model, that is only affected by the ER / Src / Ras / Erk pathway.

R.10. Characterization of post-translational histone modification marks and transcription factors recruitment to the 11β-HSD2 promoter regulatory regions

R.10.1. RNA polymerase II recruitment to 11β-HSD2 promoter regions in response to hormone treatment

Promoter regions are known to regulate gene transcription through the recruitment of complexes including transcription factors and cofactors, as well as the core transcriptional machinery including RNA polymerase II (Pol II) (Ogata, Sato & Tahirov 2003). To characterize transcriptional activation of 11β-HSD2 promoter in a chromatin context, we checked Pol II recruitment to the different promoter regions by ChIP (Fig. R.20A, lanes 7, 8 and 9). Pol II was recruited in response to hormone to the proximal and the coding promoter regions as expected, but, importantly, was also recruited at distal and middle promoter regions (Fig. R.20A, lanes 7,8 and 9).

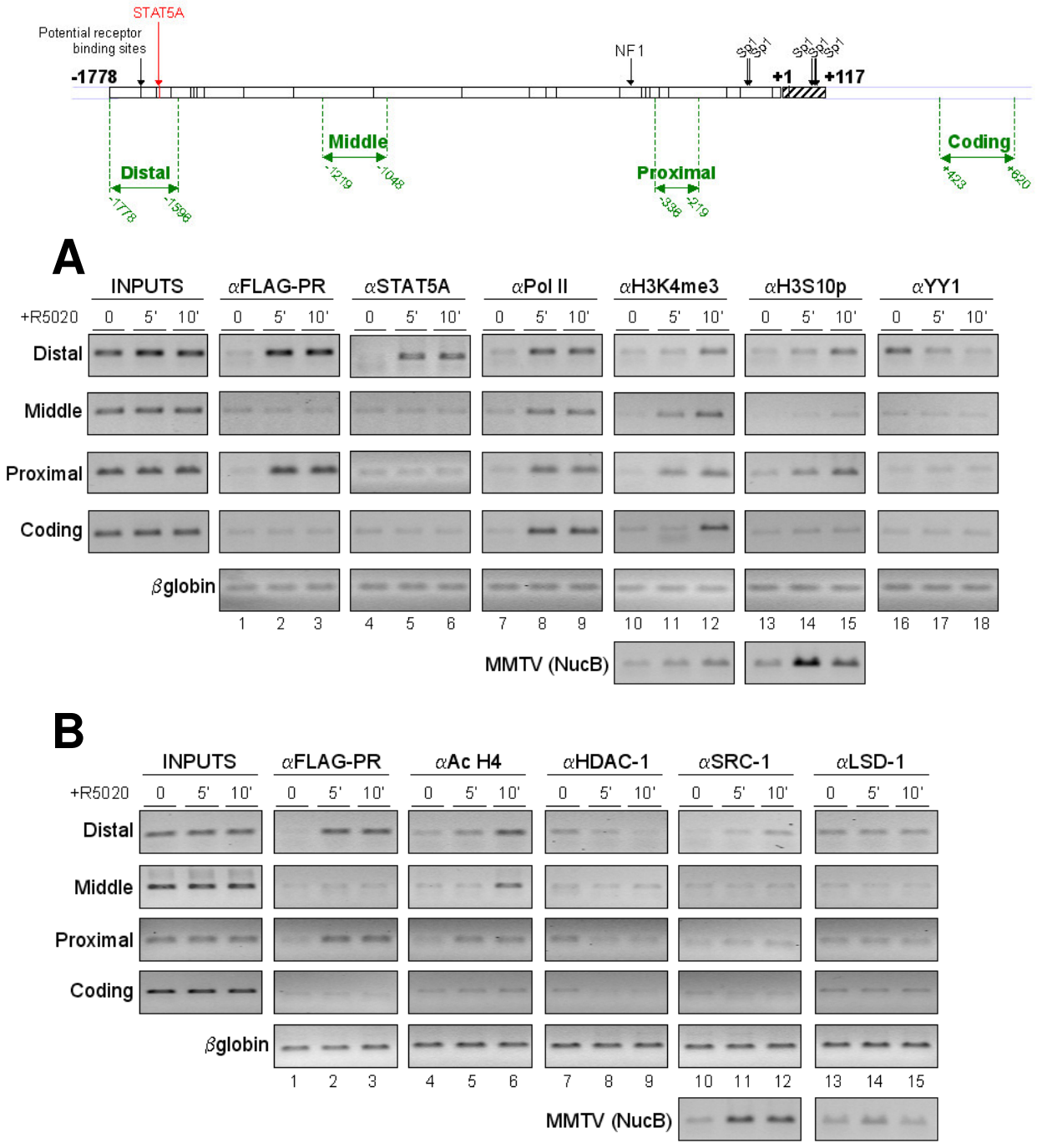

Figure R.20: Characterization of histone post-translational modifications marks and transcription factors recruitment to the 11β–HSD2 promoter regulatory regions by chromatin immunoprecipitation

TYML cells expressing PRB wt tagged with FLAG cultured as in Figure R.1B, were untreated (0) or treated with R5020 10 nM for 5 or 10 min, harvested, and used for ChIP experiments with antibodies against FLAG-tag; STAT5A; Pol II; H3K4Me3; H3S10p; and YY-1 (A) or FLAG-tag; AcH4; HDAC-1; SRC-1; and LSD-1 (B). The precipitated DNA fragments were subjected to PCR analysis with specific primers corresponding to the indicated 11β–HSD2 promoter regions or MMTV nucleosome B, and the β–globin gene as a control.

R.10.2. Histone code changes upon hormone induction

To better characterize the functional role of the different 11β-HSD2 promoter regions in R5020-induced promoter activation, we assessed by ChIP the enrichment of three important histone modifications: trimethylation of lysine 4 at histone H3 (H3K4me3); H4 acetylation (AcH4) and phosphorylation of histone H3 at serine 10 (H3S10p) (Fig. R.20 A and B). H3K4me3 is enhanced in the 5' transcribed region of genes and is linked to active transcription (Schneider et al. 2004). AcH4 is a general mark of transcriptional activation of many genes (Peterson, Laniel 2004). Finally, H3S10p occurs during gene activation by diverse stimuli and is required for transcriptional activation (Thomson et al. 1999). In response to progesterone, H3S10 is rapidly phosphorylated in the MMTV promoter in a region limited to the regulatory nucleosome B, by Msk1, recruited by PR in a complex PR-Erk-Msk (Vicent et al. 2006).

Correlating with Pol II recruitment results, progestin treatment induced an increase of H3K4me3 all along 11β-HSD2 promoter (Fig. R.20A, lanes 10, 11 and 12). In the same way, the levels of AcH4 were enriched at the distal, middle and proximal promoter regions upon hormone treatment, but the levels did not change at the coding region (Fig. R.20B, lanes 4, 5 and 6). Hormone treatment clearly increased H3S10p at the distal and proximal promoter regions (Fig. R.20 A, lanes 13, 14 and 15), correlating with PR recruitment (Fig. R.20 A, lanes 1, 2 and 3). A weaker increase was observed at the middle region, while no changes in H3S10p were observed at the coding region (Fig. R.20 A, lanes 13, 14 and 15).

Taken together these results support the idea that the different regions along 11β-HSD2 promoter are participating in the hormone-dependent transcriptional regulation of 11β-HSD2 gene.

R.10.3. *In vivo* binding of transcription regulatory factors and cofactors to the different 11β-HSD2 promoter regions

Sequence analysis of the 11β-HSD2 promoter revealed several binding sites for YY-1 factor at the distal promoter region. YY-1 is a Zinc finger protein

that acts as an activator or a repressor depending of promoter context (Thomas, Seto 1999). In tumour cell lines, YY-1 has been mostly related with repression of transcription because its ability to form complexes with histone deacetylases, as HDAC-1 (Yao, Yang & Seto 2001). ChIP was used to assay the binding of YY-1 and HDAC-1 factors to the 11β-HSD2 promoter regions (Fig. R.20 A, lanes 16, 17 and 18; and R.20B, lanes 7, 8 and 9). The results showed that in both cases, YY-1 and HDAC-1 are bound to the promoter at the distal region only in hormone-deprived conditions. YY-1 factor binding is only observed at distal promoter, in which the predicted binding sites were located (Fig. R.20 A, lanes 16, 17 and 18). On the contrary, HDAC-1 binding was also observed to the proximal and coding regions in the absence of hormone treatment (Fig. R.20B, lanes 7, 8 and 9). YY-1 and HDAC-1 were released from their binding regions upon hormone treatment.

These results suggested that YY-1 protein together with HDAC-1 could be repressing 11β-HSD2 gene expression in hormone-starved cells.

To test the recruitment of another factor that could be acting as PR, Pol II and STAT5A collaborators, we checked SRC-1 binding (Fig. R.20B, lanes 10, 11 and 12). SRC-1 acts as a transcriptional co-activator for steroid hormone receptors (Onate et al. 1995, Shim et al. 1999). Remarkably, it has been reported that SRC-1 collaborates together with STAT5A and GR to regulate β–casein gene expression (Litterst et al. 2003). In the same way, our results showed that hormone treatment increased SRC-1 recruitment to the distal 11β-HSD2 promoter region, while no change was observed at the rest of the checked regions (Fig. R.20B, lanes 10, 11 and 12).

This result suggested that SRC-1 could be collaborating with STAT5A and PR in the progesterone-dependent 11β-HSD2 promoter regulation.

Finally, we checked the binding of a transcription repressor, LSD-1, that specifically demethylates H3K4 (Shi et al. 2004), which, as we have seen, is linked to active transcription (Fig. R.20A, lanes 13, 14 and 15). No changes in the binding of LSD-1 to 11β-HSD2 promoter regions were observed neither in

the absence of hormone nor following hormone treatment (Fig. R.20A, lanes 13, 14 and 15).

This result indicated that LSD-1 could not be involved in the demethylation of H3K4me3 at 11β-HSD2 promoter regions in hormone deprived conditions.

R.11. Progesterone activates transcription upstream of the characterized 11β-HSD2 transcription start site

R.11.1. Active RNA polymerase II is present along the entire 11β-HSD2 promoter upon hormone induction

We have previously showed that Pol II was recruited along the 11β-HSD2 promoter in response to hormone (Fig. R.20A, lanes 7, 8 and 9). In the same way, we checked the hormone-dependent presence of the phosphorylated form of RNA polymerase (p-Pol II) over these regions (Fig. R.21). ChIP experiments showed an increase of active RNA Pol II amount along the 11β-HSD2 promoter, from the distal to the coding region, when the cells were treated with R5020 for 5, 10 and 30 min (Fig. R.21, right panel). As a control we checked phospho-RNA Pol II recruitment to MMTV nucleosome B (Fig. R.21, right panel). Active RNA Pol II amount was increased in the nucleosome B upon hormone treatment for 10 and 30 min.

Our results suggested that hormone-dependent activation of 11β-HSD2 transcription starts upstream the described transcriptional start site.

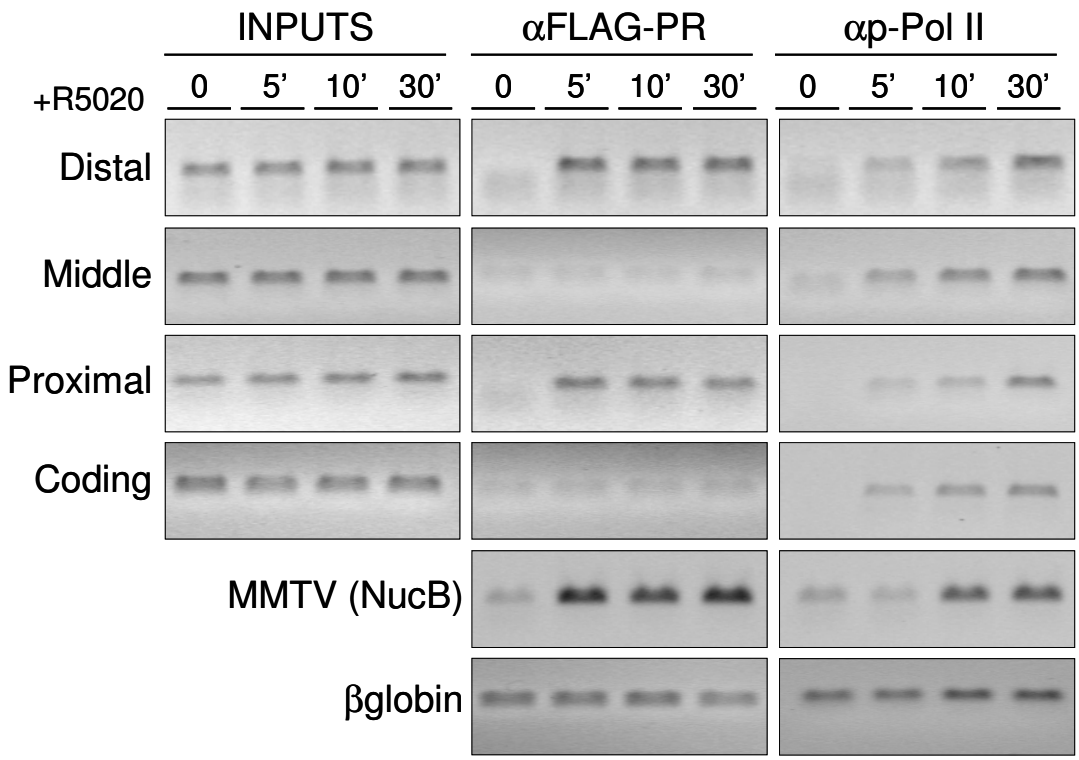

Figure R.21: Active RNA polymerase II is present along the entire 11β–HSD2 promoter upon hormone induction
TYML cells expressing PRB wt tagged with FLAG cultured as in Figure R.1B, were untreated (0) or treated with R5020 10 nM for 5 or 10 min, harvested, and used for ChIP experiments with antibodies against FLAG-tag and phospho-RNA Pol II (p-Pol II). The precipitated DNA fragments were subjected to PCR analysis with specific primers corresponding to the indicated 11β–HSD2 promoter regions or MMTV nucleosome B, and the β–globin gene as a control.

R.11.2. Transcription is detected upstream the described transcription start site

To study the transcriptional relevance of the distal 11β-HSD2 promoter region, we analysed 11β-HSD2 mRNA expression in T47D cells treated with R5020 by Oligo-dT RT-PCR cDNA amplification, and using primers that specifically amplified the distal region between nt –1778 and –1596 (Fig. R.22). In the same experiment we also analysed 11β-HSD2 mRNA expression using primers that specifically amplified the proximal region between nt –336 and –219 (Fig. R.22). The results indicated that in both cases, the specific distal and proximal primers were able to amplify the 11β-HSD2 cDNA, showing an increase of 11β-HSD2 mRNA expression after R5020 treatment for 6 h (Fig. R.22, middle panels, lanes 2 and 3). As control, we checked hormone-dependent 11β-HSD2 mRNA expression in the same samples using specific

forward and reverse primers located in exon 3 and exon 4, respectively (Fig. R.22, upper panel, lanes 2 and 3).

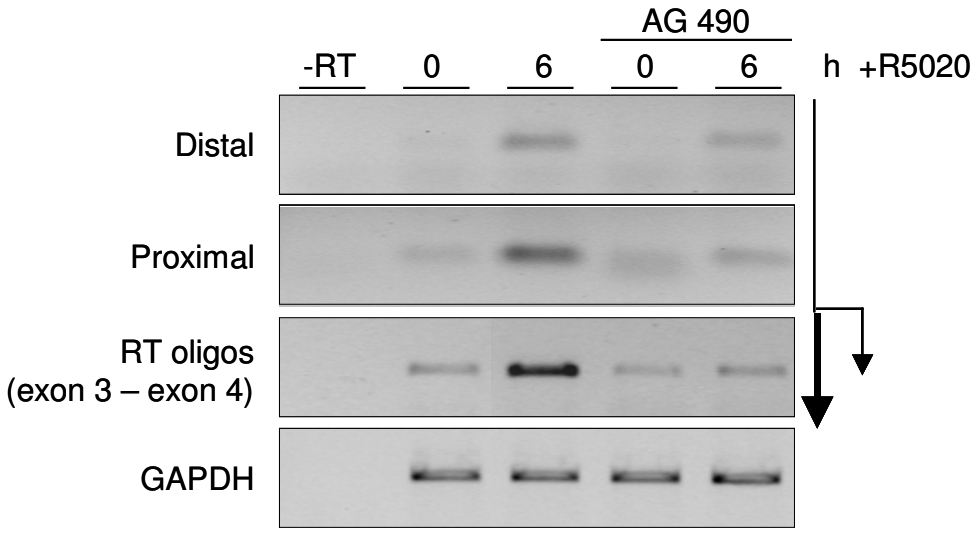

Figure R.22: The presence of hormone-induced transcripts is detected upstream the described 11β–HSD2 transcription start site
TYML cells expressing PRB wt tagged with FLAG cultured as in Figure R.1B were treated with EtOH or R5020 10 nM for 6 h. Then cells were harvested, total RNA was prepared and cDNA was generated by RT. When indicated the cells were pre-treated with AG 490 50 μM (AG). 11β–HSD2 gene expression was analysed using primers that specifically amplified the promoter distal region, the proximal region and primers located in exon 3 and exon 4 (RT oligos). GAPDH specific primers were used as a control (lower panel).

These results indicated the existence of cDNA covering the region upstream of the previously described 11β-HSD2 gene transcription start site. This may suggest that 11β-HSD2 mRNA expression in response to progesterone was initiated upstream of the previously reported transcription start site.

To describe the hormone-dependent transcription activation with more detail, we decided to check 11β-HSD2 mRNA expression using specific distal and proximal primers, in cells treated with progesterone and in the presence of the JAK / STAT pathway inhibitor (Fig. R.22, middle panels, lanes 4 and 5). As in the control PCR using exon 3 and exon 4 primers (Fig. R.22, upper panel, lanes 4 and 5), progestin-induced 11β-HSD2 mRNA expression was decreased in cells treated with AG inhibitor (Fig. R.22, middle panels, lanes 4 and 5).

Therefore, JAK / STAT pathway seemed to be involved in the progesterone-dependent 11β-HSD2 mRNA expression initiated upstream the characterized transcription start site.

R.11.3. The distal region has hormone-responsive promoter activity by itself

To explore whether the hormone-dependent transcription activation upstream the previously described 11β-HSD2 transcription start site was exclusively dependent of the distal region, we cloned two new promoter deletion constructs (Fig. R.23). These two new constructs consisted of the deletion of the region between nt –1345 and –368; and the cloning of the region between –1778 and –1345 bp in front of the luciferase reporter gene (Fig. R.23). The resultant reporter constructs co-transfected with PRB isoform in T47D-YV cells and in T47D cells were able to induce luciferase activity in response to R5020 at the same level that entire 11β-HSD2 promoter reporter vector (Fig. R.23).

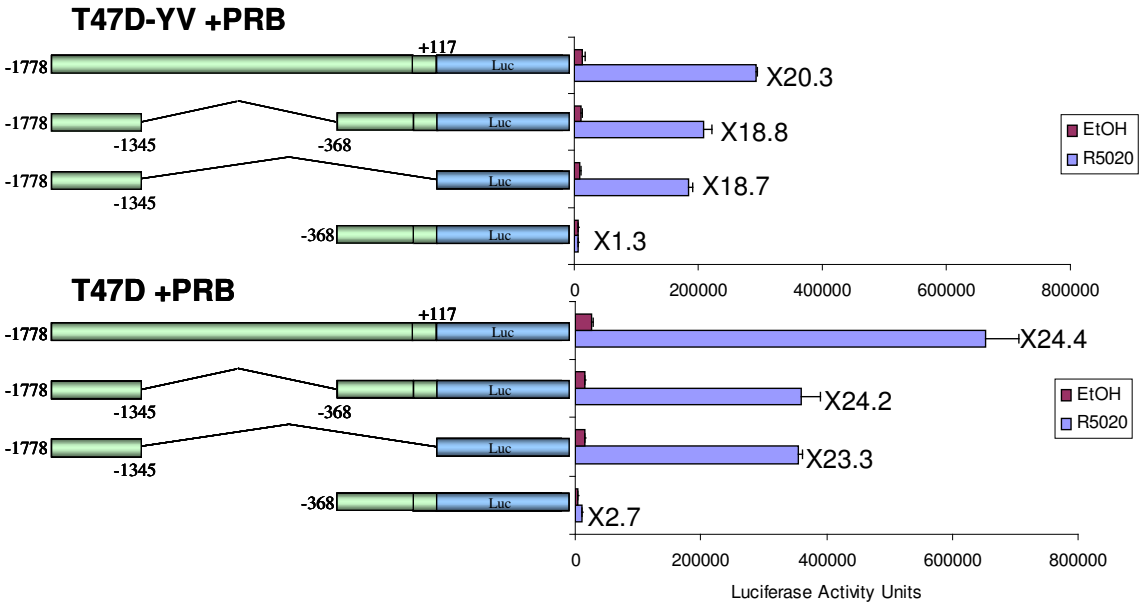

Figure R.23: The distal region has hormone-responsive promoter activity by itself in the context of a luciferase reporter construct

T47D-YV (upper panel) or T47D (lower panel) cell co-transfected with 1.5 µg of pSG5-PRB wt expression vector and 1.5 µg of the indicated 11β–HSD2 deletion constructs, were treated with ethanol or R5020 10 nM for 16 h and luciferase activity was measured. For normalization equal amounts of cellular extract of each sample were used. The values represent the mean luciferase arbitrary units ±SD of two experiments performed in duplicate. Fold inductions in response to hormone are indicated.

These results along with the deletion analysis experiments (Fig. R.3–R.5) demonstrated that distal 11β-HSD2 promoter region between –1778 and –1345 bp not only was indispensable for R5020-induced 11β-HSD2 promoter activation but also had hormone-responsive promoter activity by itself.

DISCUSSION

D.1. Hormone-regulation of the 11β-HSD2 promoter activation in breast cancer cells

D.1.1. 11β-HSD2 promoter expression in T47D cells is induced by progestins and depends on the classical PR activation.

The *HSD11B2* gene was principally described in kidney and placenta due to the activity of the enzyme codified by this gene, involved in the metabolism of cortisol (Agarwal et al. 1995, Agarwal, White 1996). However, Northern blot analysis using multiple human tissues had shown also high levels of the 11β-HSD2 gene expression in other mineralocorticoid target tissues as colon, and in ovary, prostate and testis (Albiston et al. 1994). Importantly, it has been described the up-regulation of 11β-HSD2 in neoplastic tissue and cell lines in those that normally express 11β-HSD1 (Rabbitt et al. 2002). This is the case of the breast tumours specimens and breast cancer cell lines as T47D, MCF7 and ZR-75-1 cell lines. As it has been explained in the Introduction (see chapter I.3.1.2), 11β-HSD2 isozyme has a high oxidative activity that inactivates glucocorticoids and abrogates their anti-proliferative effect. Thus, 11β-HSD2 up-regulation would have a profound effect on tumour cell proliferation. T47D cell mRNA expression analysed by RT-PCR had shown a strong expression of 11β-HSD2 whereas 11β-HSD1 is not expressed (Arcuri et al. 2000). Moreover, recent studies, also in T47D cells, have identified that *HSD11B2* is the most robustly regulated gene by glucocorticoids and progestins (Wan, Nordeen 2002).

We have confirmed by immunofluorescence and mRNA expression analysis by RT-PCR that 11β-HSD2 is expressed in T47D cells in response to progesterone and that the gene expression is specifically induced by progestins through the classical activation of PR because antiprogestin RU 486 abolished the hormone-induced 11β-HSD2 expression. This result indicates that *HSD11B2* gene is not only expressed in T47D cells but also its activation is regulated by hormones. Thus, hormone-dependent 11β-HSD2 promoter induction provided an excellent endogenous model to study progesterone gene regulation in T47D cells.

D.1.2. The distal region (-1778 to –1345) of the 11β-HSD2 promoter is necessary for hormone-regulated promoter expression.

In silico analysis of the 11β-HSD2 promoter using bioinformatic tools have shown the presence of different potential hormone responsive elements (HREs) along the promoter (Fig. R.2B). However, none of these potential HREs coincides with a canonical HRE (GGTACA nnn TGTTCT) defined as an inverted sequence or palindrome. Almost all the potential HREs located along the 11β-HSD2 promoter are half-sites. Therefore, from sequence comparison it was very difficult to focus our attention in a specific potential region within the 11β-HSD2 promoter that could be responsible of the hormone-dependent 11β-HSD2 promoter regulation. Consequently, we decided to perform a promoter deletion analysis to identify the minimal region involved in the 11β-HSD2 promoter induction by progestins in T47D cells (Figs. R.3-6).

Reporter gene constructs that were under the control of 11β-HSD2 promoter fragments terminating at nts –1778 or –1345 were induced by progestins and glucocorticoids in T47D cells and T47D-YV clonal derivative cells co-transfected with PR-expression vectors. When the promoter was shortened, the induction was abolished indicating the loss of the region necessary for the 11β-HSD2 promoter hormone regulation. Previous work in JEG-3 choriocarcinoma cells identified the presence of negative and positive regulatory regions responsible of the basal 11β-HSD2 promoter transcription regulation (Agarwal, White 1996) (Fig. I.20). Remarkably, the behaviour of the 11β-HSD2 deletion constructs analysed here confirmed the possible involvement of these regulatory regions in the promoter basal transcription also in T47D cells (Fig. R.3, red bars).

When the promoter fragments were tested in T47D cells overexpressing PRB or PRA isoforms, progestin-induction was increased (Fig. R.4), although T47D cells are known to contain high levels of PR (Horwitz et al. 1978). One possible explanation could be that the 11β-HSD2 promoter is negatively regulated by some abundant factor, and the balance between this and PR is displaced more efficiently when PR is overexpressed. We have shown that HDAC-1 and YY-1 proteins are recruited to the 11β-HSD2 promoter in progesterone-untreated serum-starved cells (Fig. R.20A and B). YY-1 / HDAC-1

complex involvement in the 11β-HSD2 promoter activation will be discussed extensively below.

A different explanation could be that endogenous PR and overexpressed PR are functionality different, either in their cell localization or post-translational modification (Abdel-Hafiz et al. 2002, Boonyaratanakornkit et al. 2007). It has been previously described that PRs are post-translationally modified by sumoylation and by phosphorylation in a time-dependent manner after protein synthesis (Abdel-Hafiz et al. 2002, Sheridan, Francis & Horwitz 1989). PR transient transfected and overexpressed would be post-translational modified differentially, and could show a different transcriptional behaviour compared with endogenous PR, since post-translational modification are involved in PR stabilization, targeting and / or trafficking (Seeler, Dejean 2001). In the same way, recently it has been described that PR intracellular localization is important in its transcriptional activity (Boonyaratanakornkit et al. 2007). At equilibrium, the majority of PR is in the nucleus, but in breast cancer cells, it has been suggested that between 3 and 5 % of PR is located in the cytoplasm (Ballare et al. 2003). PR post-translational modification and / or other factors still unknown could be mediating the subcellular localization and the transcriptional effects.

D.1.3. Involvement of PRA and PRB isoforms on hormone-regulated 11β-HSD2 promoter activation.

In order to investigate the possible role of PRA and PRB in the hormone-regulation of the 11β-HSD2 promoter, we performed transient co-transfections, co-expressing PRA or PRB isoforms and the different 11β-HSD2 promoter deletion constructs (Figs. R.4 and 5). We used T47D cells (Fig. R.4) and T47D-YV cells (Fig. R.5), that is a PR-negative clonal derivative cell line of T47D cells. Our results showed that in both cases PRB induction of 11β-HSD2 expression is higher than PRA induction. This effect was previously reported and in breast cancer cells has remarkable consequences. PRB and PRA differential transcriptional activity is cell specific and promoter specific. With reporter constructs containing a single PRE, PRA displays similar transactivation activity to PRB. However, this activity is reduced or inactive when more complex response elements are used, i.e. the MMTV promoter (Vegeto et al. 1993).

The mechanisms by which PRA and PRB exert such apparently different transcriptional activities in various cell and promoter systems remain unknown. The physical differences at the N-terminal end of the two receptors are clearly responsible for some transcriptional differences (Fig. I.2). In addition, to the fact that AF-3 is unique to PRB, the PRB–specific region has a distinct conformation in solution (Bain et al. 2001) and is likely to mask an inhibitory domain that is active in the N-terminus of the PRA protein (Huse et al. 1998). Motifs contained in AF-3 have been involved in the interaction between PRB and coactivators. This suggests that coactivators may bind differently to the two PRs or that the two receptors bind to different subgroups of coactivators (Giangrande et al. 2000). Furthermore, differential cofactor requirements between gene promoters may lead to differences in the transcriptional efficacy of the two PRs on the same promoter (McKenna, O'Malley 2002).

On the other hand, it would be expected that changes in the relative amounts of PRA and PRB would result in altered target gene expression patterns if the two isoforms are transcriptionally distinct. When patterns of gene regulation were examined in T47D cells expressing exclusively PRA or PRB, a remarkably small overlap was seen between the sets of genes regulated by the two receptors, with the subset of genes regulated by PRB exceeding in number those regulated by PRA (Richer et al. 2002). Moreover, when the data in human tissues and null animals are taken together, they suggest that the two isoforms either work cooperatively to mediate progesterone action or that each isoform has distinct physiological roles that are probably cell specific and promoter specific.

D.1.4. PR is recruited in response to hormone to two different regions of the 11β-HSD2 promoter, involving two different mechanisms.

ChIP assays using TYML cells expressing PRB wt tagged with FLAG, revealed that PR is recruited to the 11β-HSD2 promoter in response to hormone to the distal and the proximal 11β-HSD2 promoter regions (Fig. R.7). Hormone-regulated PR recruitment to the distal promoter correlates with the deletion analysis results discussed before (Fig. R.3-6). Surprisingly, the PR recruitment in response to hormone to the proximal promoter was as strong as that to the

distal promoter (Fig. R.7, panels A-B and G-H). Although the deletion promoter constructs that correspond with the proximal promoter (still present in deletion –368) showed a clear abolishment of hormone induction, remarkably, in the optimal experimental circumstances (T47D cells overexpressing PRB), these constructs retained a residual promoter activity and hormone response (Fig. R.4A). Hormone-regulated PR recruitment to the proximal promoter explains this progestin activation of 2-3-fold induction observed with the deletion constructs –839, -571 and –368 co-transfected with PRB in T47D cells and suggested that PR exerts its role in the 11β-HSD2 promoter through more than one region and maybe through different mechanisms.

ChIP assays using another TYML-derived cell line, expressing a FLAG-tagged version of PRB isoform with point mutations in the DNA binding domain (PRB DBDm) revealed that hormone-regulated direct PR binding to DNA only occurs in the proximal 11β-HSD2 promoter region, whereas mutations in the PRB DBD do not affect the hormone-dependent PR recruitment to the distal 11β-HSD2 promoter regions (Fig. R.8A). This result defined that direct PR binding to DNA is the mechanism that regulates the hormone-induced PR recruitment to the proximal promoter region. Well-known MMTV recruitment also depends completely on DBD. Conversely, the result also suggested that hormone-dependent PR recruitment to the distal promoter region is governed by a mechanism in which PR DBD is not involved, and as a consequence, direct PR DNA binding does not occur in the distal 11β-HSD2 promoter region.

Computer predictions suggested the presence of a putative STAT5A binding site in the distal 11β-HSD2 promoter region (Fig. R.10A). ChIP experiments using the TYML-derived cell line expressing FLAG-tagged PRB wt revealed that STAT5A protein is recruited to the distal 11β-HSD2 promoter region in response to hormone (Fig. R.10B). Moreover, the JAK / STAT pathway inhibitor AG 490 affected not only hormone-regulated STAT5A recruitment to the distal 11β-HSD2 promoter region but also hormone-dependent PR recruitment to the same region is almost totally affected by the JAK / STAT pathway inhibition (Fig. R.11A). These results showed that STAT5A recruitment to the distal 11β-HSD2 promoter region is involved in the mechanism of hormone-regulated PR recruitment to this region.

There are different PR regulated promoters in which this regulation is mediated by different mechanisms, including PR direct and indirect binding to the DNA. As it has been reviewed in the Introduction (see chapter I.1.5), PR direct binding is involved in the hormone-regulated MMTV promoter, investigated not only *in vitro* (Cato, Henderson & Ponta 1987, Chalepakis et al. 1988) but also *in vivo* in T47D cells (Truss et al. 1995, Vicent, Melia & Beato 2002). However, there are other SHR-regulated promoters in which this regulation is mediated by the combination of several mechanisms. p21 promoter PR regulation occurs by the binding to Sp1 proteins in the proximal promoter (Owen et al. 1998). In the p21 regulation PR binding to the promoter is connected with transcriptional machinery complex recruitment and activation by the linking role of other factors and complexes, i.e. p300/CBP, to activate the promoter in response to progestins (Owen et al. 1998).

Another example is the estrogens-regulated *CCND1* gene promoter, in which ERα recruitment is accompanied by AP-1 and Oct-1 recruitment (Cicatiello et al. 2004). ERα does not bind directly to the cyclin D1 promoter but is required as an AP-1 corregulator. Moreover, in a cell-type specific manner ERα is recruited to the cyclin D1 promoter region and to another enhancer region downstream from the coding region (Eeckhoute et al. 2006). In the second recruitment region, ERα acts collaborating with FOXA1 and other transcription factors.

Therefore, the complexity in the SHR regulation correlates with the complexity of the promoter itself. In the case of the 11β-HSD2 promoter, that spans ~1.8 kb, the results showed that hormone-regulated PR recruitment to the distal and the proximal promoter regions would be controlled by two different mechanisms, in which PR is directly recruited to the DNA in response to hormone and progestins regulate PR binding to the distal 11β-HSD2 promoter region through the STAT5A recruitment to the same region. However, ChIP experiments using the JAK / STAT pathway inhibitor AG showed a minimal residual hormone-dependent PR recruitment to the distal promoter region (Fig. R.11A), suggesting that another mechanisms could be involved in the PR recruitment in response to hormones to this region.

On the other hand, although hormone-regulated STAT activation and recruitment to the distal 11β-HSD2 promoter regions is involved in the hormone-dependent PR recruitment to the same region, by the moment, we are not able to demonstrate neither that PR and STAT directly interact, nor that cytoplasm phospho-STAT activation in response to progestins and STAT translocation to the nucleus is accompanied by PR, forming a complex. In spite of this, there is a previous work that describes STAT phosphorylation by JAK in response to progestins in T47D cells (Proietti et al. 2005). In addition, STAT and GR interaction as well as STAT-ERα interaction, have been reported *in vitro* by GST pull-down (Faulds et al. 2001, Stoecklin et al. 1997). More remarkably, STAT3 and PR associate in response to medroxyprogesterone acetate (MPA, synthetic progesterone) in C4HD cells (murine mammary tumour cell line) (Proietti et al. 2005).

The complexity in hormone-dependent PR regulation of 11β-HSD2 promoter is also revealed by the differential PRB DBDm effect in the promoter activation in transient transfections and endogenous 11β-HSD mRNA expression (Fig. R.9). The results showed that PRB DBDm transient co-transfection with 11β-Luc reporter vector partially affects hormone dependent luciferase expression under the control of the 11β-HSD2 promoter (Fig. R.9A), whereas PRB DBDm stably expressed in T47D cells does not affect the normal hormone-dependent 11β-HSD2 mRNA expression (Fig. R.9B). Again, these results could be explained by the post-translational modifications that PR undergoes or, more likely, by the chromatin structure of the endogenous 11β-HSD2 promoter, that cannot be adopted by transiently transfected 11β-HSD2 promoter codified by the 11β-Luc reporter vector. Hormone-regulated recruitment of chromatin remodelling complexes leading changes in the chromatin structure, would be involved in the progestins-dependent 11β-HSD2 promoter activation and hormone-regulated PR recruitment mechanisms.

D.2. JAK / STAT pathway is functionally important for hormone dependent 11β-HSD2 promoter activation.

To test the functional relevance of STAT5A on hormone-dependent 11β-HSD2 promoter regulation, the effect of the JAK / STAT pathway inhibitor, AG

490, was checked in transient transfection experiments using the reporter vector expressing luciferase gene under the control of the 11β-HSD2 promoter, and in the hormone-regulated endogenous 11β-HSD2 mRNA expression (Fig. R.12A and B). In both cases, the JAK / STAT pathway inhibition abolished the 11β-HSD2 promoter activation in response to progestins, suggesting the functional relevance of this pathway in the regulation of the 11β-HSD2 gene expression mediated by hormones.

STAT5A is an important transcription factor involved in the regulation of many target genes in response to cytokines and growth factors. In breast cancer cells, R5020 induces JAK / STAT pathway activation through phosphorylation of JAK2 and STAT3 and 5 (Proietti et al. 2005). Because the prediction by *in silico* analysis of one STAT5A binding site at the distal promoter region and the *in vivo* confirmation of its relevance by ChIP experiments (Figs. R.2B and R.10B), we tested in transient transfections the effect of a constitutive active or a dominant negative STAT5A forms (CA-STAT5A and DN-STAT5A) on the hormone-regulated 11β-HSD2 promoter activation (Fig. R.13). CA-STAT5A form is characterized by its elevated DNA binding and transactivation activities with stable Tyr phosphorylation and nuclear accumulation, whereas the DN-STAT5A has a deletion in its C-terminal transactivation domain so, as a consequence, still binds to DNA following activation but is unable to induce the transcription of target genes (Ariyoshi et al. 2000, Moriggl et al. 1996). The overexpression of wt-STAT5A or the expression of the CA-STAT5A robustly increased the progestins-dependent 11β-HSD2 promoter activation, while the expression of the DN-STAT5A form abolished the hormone-regulated 11β-HSD2 promoter induction. In another set of experiments, we checked the effect of DN-STAT5A form in combination with the different promoter deletion constructs (Fig. R.14). Remarkably, DN-STAT5A has a strong effect on the hormone-activation of the longest deletion constructs (–1778 and –1551), while the remaining hormone induction obtained with the shortest promoter constructs (-1345, -839 and –368) is not abolished by the DN-STAT5A co-expression (Fig. R.14B).

These results together with those obtained by ChIP using antibodies against FLAG-PR and STAT5A (Figs. R.7 and R.10B) suggest that, although

the predicted putative STAT5A binding site is located upstream nt –1551 (nts –1654 to –1646), the functional involvement of STAT5A in the progesterone-dependent 11β-HSD2 promoter regulation requires a more extended region upstream nt –1345. Since DN-STAT5A is characterized by a deletion in the C-terminal STAT5A transactivation domain, this mutated STAT5A form is unable to bind to many STAT5A corregulators (Moriggl et al. 1996). CBP, ERα, SRC-1 and Oct-1 interact with STAT5 via its transactivation domain (Magne et al. 2003, Pfitzner et al. 1998, Xu et al. 1998), showing that, although is not the only way of interaction with STAT5, mutations on the STAT transactivation domain can disrupt its cooperative action with transcription factors and corregulators.

shRNA mediated knock-down of STAT5A was not sufficient to affect neither the hormone-regulated 11β-HSD2 promoter expression nor the progestin-dependent STAT5A and PR recruitment to the distal promoter region (Fig. R.15). In spite of the high levels (~70%) of STAT5A expression inhibition reached by the cells expressing the shRNA against STAT5A (Fig. R.15A), the remaining STAT5A expression was sufficient to full-occupancy of the 11β-HSD2 promoter in response to hormone (Fig. R.15C). Thus, PR is also recruited to the same region after the hormone treatment and, more relevant, the levels of hormone-regulated STAT5A and PR recruitment were comparable with those reached in the control cells expressing an irrelevant shRNA. Therefore, higher levels of STAT5A expression inhibition, by the combination of several shRNAs against STAT5A in the same cell, are probably needed to observe an effect on the hormone-dependent 11β-HSD2 promoter regulation.

Microarray experiments were used to analyse the effect of the JAK / STAT pathway inhibition by AG 490 in the hormone-mediated induction of gene expression (see chapter R.8). Using a customized human cDNA microarray containing 750 genes of interest in breast cancer and hormone regulation, we obtained a set of 35 genes significantly induced by progesterone. A subset of these progesterone target genes were affected by the incubation with the JAK / STAT inhibitor (Fig. R.17). This result revealed that although only a small subset of genes induced by hormone are affected by JAK / STAT pathway

inhibition, this is not unique to the 11β-HSD2 promoter and would be explaining the progesterone-regulated expression of other target genes.

Remarkably, mRNA analyses in the presence of AG inhibitor revealed that the JAK / STAT pathway inhibition affected the basal expression of different progesterone target genes, as *CCND1, DUSP1, MYC* and *JUN (*Fig. R.18). This basal expression effect was also observed in the case of the 11β-HSD2 gene, not only in mRNA expression analyses (Figs. R.12B and 18A) but also in the experiments with the DN-STAT5A form (Fig. R.13), in which basal 11β-HSD2 promoter activation is decreased when JAK / STAT pathway and STAT5A phosphorylation are inhibited. These results talks in favour of a general role of JAK / STAT in those genes, independent of hormones.

D.3. MAPK and PI3K / Akt pathways are partially involved in hormone activation of the 11β-HSD2 promoter expression.

As it was commented before (see chapter I.1.3.2.), SHs induce rapid responses of cytoplasmic kinase pathways. In the well-known MMTV promoter activation by progestins, the Src / Ras / Erk pathway is activated by ER through the interaction between PR and ER (Ballare et al. 2003, Migliaccio et al. 1998). Hormone-dependent MMTV promoter activation was not affected by Wortmannin (WM), specific inhibitor that blocks the PI3K / Akt pathway (Vicent et al. 2006). In the present work, we checked the effect of Erk pathway inhibitor, PD 98059 (PD), in the hormone-regulated 11β-HSD2 promoter activation in transient transfections and in the endogenous 11β-HSD2 mRNA expression (Fig. R.19). In both cases, PD partially blocked progesterone-induced 11β-HSD2 promoter activation. The same result was obtained by the inhibition of PI3K / Akt pathway, showing that this cascade is also partially involved in the hormone-regulated 11β-HSD2 expression (Fig. R.19). Surprisingly, ICI 182,780 (ICI), a specific ER antagonist, did not block the normal hormone-activated 11β-HSD2 promoter induction.

In breast cancer cells, cytoplasmic ER and PR interact through the ERID I and II domains located in the central region of PR (Fig. I.4). This interaction seems to be necessary for the hormone dependent activation of the signalling cascades (Ballare et al. 2003, Migliaccio et al. 1998), and for the progesterone-

induction of the MMTV promoter (Vicent et al. 2006). However, progesterone can also crosstalk to kinase pathways through an interaction of PR with SH3 domain of c-Src (Boonyaratanakornkit et al. 2001). Therefore, Src / Ras / Erk progesterone-dependent activation could be directly mediated by PR, and activated Src could not be only activating Erk pathway but also PI3K / Akt pathway, to partially regulate hormone-induced 11β-HSD2 activation.

Our results showed that, although JAK / STAT pathway functional involvement is necessary for the progesterone-regulated 11β-HSD2 promoter activation, MAPK and PI3K / Akt kinase pathways are also partially involved in this regulation. Further studies will be necessary to determine the contribution of MAPK and PI3K / Akt pathways in the hormone-regulated 11β-HSD2 gene expression.

D.4. Characterization of post-translational histone modifications marks and transcription factors recruitment to the 11β-HSD2 promoter regulatory regions.

Using ChIP experiments we determined the recruitment pattern of several cooperating transcription factors along the 11β-HSD2 promoter and the enrichment of different post-translational histone modifications related with transcription. Recruitment of transcription factors to regulatory sequences can be mediated by direct recognition of the DNA or indirectly through protein-protein interactions. Promoter DNA regions contain several transcription factors binding sites where recruitment of multiprotein complexes occurs to transmit signals to the basal transcription machinery (Ogata, Sato & Tahirov 2003). Although numerous potential binding sites exist in the regulatory regions, the exact *in vivo* recruitment mechanisms remain to be established.

Especially interesting is the presence of several putative binding sites for YY-1 factor at the distal 11β-HSD2 promoter region. ChIP experiments showed that YY-1 factor is present at the distal region in progesterone- and serum-starved cells (Fig. R.20A). The results correlates with the HDAC-1 recruitment observed in the same cellular conditions (Fig. R.20B). HDAC-1 is present in serum-deprived conditions at all the checked regions along the 11β-HSD2 promoter, suggesting that could have an important role in the transcriptional

regulation of this promoter. YY-1 / HDAC-1 complex has been extensively related with repression of transcription, in which histone deacetylase enzymes reverse the modifications carried out by acetyltransferases. The deacetylases may be targeted to the transcriptionally repressed regions by specific repressor proteins (Yao, Yang & Seto 2001). Moreover, YY-1 can be deacetylated by HDAC-1 resulting in stable HDAC activity associated with YY-1 protein. The presence of HDAC-1 and YY-1 simultaneously in the distal 11β-HSD2 promoter region, the same region in which PR and STAT5A are recruited in response to hormone, could be revealing a regulatory mechanism to control hormone-dependent 11β-HSD2 activation. In this mechanism, YY-1 / HDAC-1 complex would have a repressive role in serum-deprived cells, whereas PR and STAT5A hormone-dependent recruitment would be part of the transcription activation complex that triggers the transcription machinery recruitment and activation.

In addition to STAT5A and PR, many other cofactors and corregulators contribute to transcription. SRC-1 is an extensively studied coactivator that binds nuclear hormone receptors directly and stimulate their transcriptional activity. Members of the SRC-1 family have been shown to act as coactivators for steroid, retinoid and thyroid hormone receptors, by modifying transcriptionally repressed chromatin or by enhancing stabilization of transcription preinitiation complexes (Beato, Herrlich & Schutz 1995). SRC-1 has HAT activity, but also interacts (and may recruit) other HATs. Its coactivation function is mediated through direct ligand-dependent interaction with the receptors but also with other transcription factors, such as STAT5A. SRC-1 enhances the cooperative action of GR and STAT5A and directly interacts with STAT5A (Litterst et al. 2005). Given the ability of SRC-1 to directly interact with SHRs and STAT5 to enhance transcriptional activation, we expected that SRC-1 would be recruited to the 11β-HSD2 promoter in response to hormone. ChIP experiments revealed that SRC-1 is hormone-dependent recruited to the distal 11β-HSD2 promoter region (Fig. R.20B). This result suggested that SRC-1 would be probably taking part in the transcriptional activation complex together with PR and STAT5A, although we can not precise whether interacts with PR, STAT5A or both at the 11β-HSD2 promoter.

Distinct histone amino-terminal modifications have synergistic or antagonistic interaction affinities for chromatin-associated proteins, which in turn activate or silence transcription (Jenuwein, Allis 2001). The combination of histone amino-terminal modifications, also named “histone code” represents a fundamental regulatory mechanism. We checked here the enrichment of three important histone modifications related with activation of transcription: trimethylation of lysine 4 at histone H3 (H3K4me3), H4 acetylation (AcH4), and phosphorylation of serine 10 at histone H3 (H3S10p) (Fig. R.20). ChIP experiments showed an enrichment of H3K4me3 along the 11β-HSD2 promoter in response to hormone. AcH4 was also enriched in response to progestins at the distal, middle and proximal 11β-HSD2 promoter regions, but not at the coding region. These results correlated with the role in activation of transcription of these histone modifications. Interestingly, AcH4 enrichment in response to hormone inversely correlates with HDAC-1 recruitment to the 11β-HSD2 promoter. HDAC-1 bound to the 11β-HSD2 in serum-deprived cells is released after hormone treatment, coinciding with an AcH4 enrichment at the same regions (Fig. R.20B).

Progesterone-treatment also increased H3S10p at the distal and the proximal 11β-HSD2 promoter regions. Recently, it has been determined an important role of H3S10p in transcriptional activation by progestins (Vicent et al. 2006). In response to the hormone, rapid phosphorylation of H3S10 is detected at the MMTV nucleosome B. H3S10p triggered by progestins is mediated by MsK1, activated by PR-signalling-MAPK-Erk and recruited by PR in a complex PR – Erk – Msk. Given the partial involvement of MAPK pathway in the hormone-dependent 11β-HSD2 promoter activations (Fig. R.19), Erk / Msk could be also involved in the 11β-HSD2 gene transcriptional regulation by progestins, although further analyses are needed to characterize their involvement with detail.

As we have seen, H3K4me3 is enriched in response to hormone along the 11β-HSD2 promoter (Fig. R.20A). LSD-1 is a transcriptional repressor associated with specific demethylation of H3K4 (Shi et al. 2004). Recently, some reports have related LSD-1 not only with H3K4 specific demethylation, but also with H3K9 demethylation (Garcia-Bassets et al. 2007). Surprisingly, LSD-1

would be involved *in vivo* in simultaneously modulate distinct developmental gene activation and repression programmes (Wang et al. 2007). LSD-1 would be basally recruited to the target promoters, and gene activation or repression seems to be regulated by the recruitment of distinct LSD-1 containing coactivators or correpressors complexes. Our results suggest that LSD-1 is constitutively bound to the 11β-HSD2, remarkably in the case of the distal promoter region (Fig. R.20B). LSD-1 binding is not displaced by the hormone treatment, indicating that its possible role could be mediated by coactivators or correpressor recruitment to the same region, forming a complex with LSD-1, instead of regulated recruitment/displacement of LSD-1.

D.5. Progesterone activates transcription upstream of the characterized 11β-HSD2 transcription start site.

ChIP experiments revealed that RNA Polymerase II (RNA Pol II) and phosphorylated form of RNA Pol II (p-RNA Pol II) were recruited to the 11β-HSD2 promoter in response to hormones (Figs. R.20A and 21). Surprisingly, hormone-regulated pRNA Pol II is recruited along the 11β-HSD2 promoter, from the distal to the coding region.

The presence of activated form of RNA pol II at the distal and the proximal promoter regions, correlating with hormone-dependent PR recruitment to the same regions, could be suggesting the presence of a “loop” within the promoter chromatin structure connecting both regions, where PR and RNA Pol II are taking part of a multiprotein complex. Genes in higher eukaryotes often require distant enhancer sequences for high-level expression. Enhancers act at a distance to create a favorable environment for transcription and to act as entry sites for factors. The communication occurs through direct interaction between the distant enhancer and the promoter by various mechanisms that “loop” the intervening sequences (Bulger, Groudine 1999, Mueller-Storm, Sogo & Schaffner 1989). However, this mechanisms have been related with long-range enhancer action, in which distant enhancer elements function in close proximity to clusters of genes that they regulate *in vivo*, suggesting direct chromatin regulatory interactions that normally involved wide regions of several Kbs (Carter et al. 2002). The presence of hormone-recruited RNA Pol II in the

middle 11β-HSD2 promoter region together with the differential recruitment pattern of transcriptional factors and repressors at the distal and proximal 11β-HSD2 promoter regions (i.e. STAT5A and YY-1) discarded the role of a possible loop linking the distal and the proximal regions.

On the other hand, another possible explanation for the RNA Pol II presence along the 11β-HSD2 promoter would be that the transcriptional machinery complex could be "scanning" through the entire promoter in search of the transcriptional start site. According with this model, RNA Pol II would be loaded at the beginning of the promoter in response to hormones, but the transcription would not be started up to the transcription start site. However, the results revealed that phosphorylated RNA Pol II form is also loaded from the distal promoter region, indicating that transcription is initiated at that region.

Correlating with these results, transient co-transfection in T47D and T47D-YV cells of PRB and a 11β-HSD2 promoter deletion construct only containing the distal promoter region between nts –1778 and –1345, revealed that the distal region has hormone-responsiveness and promoter activity by itself (Fig. R.23). In the same way, the analysis of the endogenous 11β-HSD2 mRNA expression in response to hormones also showed that cDNA generated by Oligo-dT RT-PCR covered a region upstream of the previously described 11β-HSD2 gene transcription start site (Fig. R.22). The amount of this cDNA is increased in response to hormone and affected by the JAK / STAT pathway inhibition. Together, these results suggest the existence of a transcription start site upstream of the previously described for the 11β-HSD2 gene.

HSD11B2 gene structure was described from kidney and placenta tissues and cell lines. Using bacteriophage clones and human genomic libraries by hybridization with HSD11β2 cDNA from these tissues, gene was characterized and transcriptional start site was determined (Agarwal et al. 1995). By Northern blot hybridization of human kidney, placenta, testis and lung tissues, the major transcript found was of about 2 Kb, coinciding with the cDNA description found in the literature and the principal genome browsers (USCS, Emsembl). However, the evidence of minor transcripts with higher length was reported in the same Northern blot experiments, suggesting the existence of other transcription start sites in the *HSD11β2* gene. Our results revealed the

presence of transcripts that start upstream the described transcriptional start site in breast cancer cells and, more importantly, the relevance of the upstream transcription initiation in response to hormone.

D.6. A model for the 11β-HSD2 promoter activation by progestins

Hormone-dependent PR recruitment to the 11β-HSD2 promoter to two different regions has revealed two differential mechanism of regulation by which PR binds directly to DNA at the proximal promoter regions, while PR binding to the distal promoter is mediated by progesterone-regulated transcription factor STAT5A recruitment to the same region. In the process of 11β-HSD2 promoter regulation by hormones there are not only involved PR and STAT5A, but also another transcriptional corregulator, SRC-1, that binds in response to progesterone to the distal promoter region.

The JAK / STAT pathway activation is functionally relevant for the hormone-activated 11β-HSD2 gene expression. Another two cytoplasmic kinase pathways are partially involved in the 11β-HSD2 regulation by progesterone: MAPK and PI3K / Akt pathways, although we do not know by the moment the detailed involvement of these pathways or whether another signalling pathways are also involved.

YY-1 / HDAC-1 complex seems to be involved in the repression of the 11β-HSD2 promoter in serum-deprived conditions, since the two factors are bound to the distal promoter region in the absence of hormone. YY-1 and HDAC-1 are both released from the promoter when the cells are treated with progesterone, and, concomitantly, this displacement leads to an enrichment in AcH4 levels along the promoter, indicating the transcriptional activation.

Hormone-induced transcription is also indicated by the enrichment in H3K4me3 and H3S10p along the promoter from the distal region. These events activated by hormones are accompanied by progesterone-regulated RNA Pol II and phosphorylated RNA Pol II recruitment to the 11β-HSD2 promoter. Importantly, since the RNA Pol II and the activated form of RNA Pol II are detected from the distal 11β-HSD2 promoter region, and hormone-induced mRNA expression analysis revealed cDNAs covering the distal and the proximal promoter regions, we suggested that transcription initiates upstream of

the previously described transcriptional start site. We do not know where would be located the upstream transcriptional start site or whether we are detecting some short regulator transcript located in this particular region. However, this observation seems to be particular of breast cancer cells, since previous works performed with kidney and placenta tissues and cell lines did not revealed the existence of another transcriptional start site.

Although future investigations will explain with more detail the 11β-HSD2 promoter regulation, taken together the results presented here have shown that the 11β-HSD2 promoter provides an excellent endogenous model to study progesterone gene regulation in breast cancer cells.

CONCLUSIONS

1. The 11β-HSD2 gene is expressed in breast cancer cells and its promoter activation is activated by progestins through the activation of PR in T47D cells.

2. *In silico* analyses of 11β-HSD2 promoter reveals several potential hormone responsive elements along the promoter, as well as the presence of putative binding sites for other relevant transcription factor and co-regulators, such as STAT5A and YY-1, in addition to previously described NF-1, NFkB and Sp1 binding sites.

3. Experiments of promoter deletion analysis in the context of luciferase reporter constructs have determined that the distal promoter of the 11β-HSD2 gene, between nts -1778 and –1345, as necessary for strong response to progestins and glucocorticoids. Nonetheless, the proximal promoter region maintains residual responsiveness to progestins.

4. Progesterone receptor binds to the distal (-1778 to –1586 aproximately) and the proximal (-336 to –219 aproximately) regions of the 11β-HSD2 promoter 5 min after hormone addition.

5. Hormone-regulated PR binding to the proximal 11β-HSD2 promoter region is regulated by a mechanism in which the DBD of PR is required, indicating that hormone-activated PR binds directly to DNA in the proximal promoter region.

6. STAT5A binds in response to hormone to the distal 11β-HSD2 promoter region.

7. PR and STAT5A binding is affected by an inhibitor of JAK / STAT pathway, suggesting the involvement (direct or indirect) of STAT5A in the recruitment of PR to the promoter, and the participation of this signalling pathway upon hormone induction.

8. A minimal progestins-regulated residual PR binding to the distal promoter region is observed when the STAT5A activation is inhibited, indicating that another mechanism may be involved in PR recruitment to this region in addition to STAT5A recruitment.

9. In addition, interfering with JAK / STAT pathway (by inhibitor or dominant negative STAT5A) abolishes 11β-HSD2 hormone-response. JAK / STAT may have a more general role on 11β-HSD2 regulation, because also basal expression is diminished when the pathway is blocked.

10. A small subset of cellular genes showing progesterone regulation are affected by JAK / STAT pathway inhibition, indicating that JAK / STAT pathway involvement in progestins-dependent gene expression is not unique to the 11β-HSD2 gene.

11. Inhibition of MAPK and PI3K / Akt pathways partially affects 11β-HSD2 promoter activation by progesterone, indicating that the influence of the non-genomic actions of PR on the 11β-HSD2 gene differs from the previously observed for the MMTV promoter.

12. Progesterone-induced enrichment of trimethylation of lysine 4 at histone H3, phosphorylation of serine 10 at histone H3 and acetylation of H4 are detected along the 11β-HSD2 promoter, as a result of the rapid transcriptional activation of the promoter after hormone treatment.

13. YY-1 transcriptional repressor factor together with HDAC-1, is recruited under serum-deprived cell-growth conditions to the distal 11β-HSD2 promoter region, where it was predicted the presence of a putative YY-1 binding site. These proteins could be part of a repressive complex that is displaced upon hormone activation, concomitant with acetylation of histones.

14. SRC-1 coactivator is recruited in response to progesterone to the distal 11β-HSD2 promoter region, together with STAT5A and PR, suggesting

that the three proteins could be collaborating in the in the hormonal activation of 11β-HSD2 promoter.

15. Activated RNA Polymerase II (Pho Ser 5) was found at all 11β-HSD2 promoter regions tested, including the distal and middle regions, early in response to progestins. This indicated and was indeed confirmed by RT-PCR, that transcripts encompassing the promoter region were synthesized in response to hormone and depending on JAK / STAT pathway.

16. Moreover, luciferase reporter assays indicated that the distal 11β-HSD2 promoter between nts. –1778 and –1345 has hormone responsiveness by itself. All together, these results suggested that hormone-induced 11β-HSD2 mRNA expression starts upstream the previously described transcriptional start site.

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