Chromatin Immunoprecipitation-mediated Target Identification Proved Aquaporin 5 is Regulated Directly by Estrogen in the Mouse Uterus

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Contents

I. Summary

Estrogens play a central role in the reproduction of vertebrates and affect a variety of biological processes. The major target molecules of estrogens are nuclear estrogen receptors (ERs), which have been studied extensively at the molecular level. However, it remains unclear which genes are regulated directly by ERs, especially at the level of the whole organism rather than cultured cells. To identify the genes that are regulated directly by ERs *in vivo*, I subjected estrogen-treated mouse uteri to a modified chromatin immunoprecipitation (ChIP)-mediated target cloning strategy (Chapter 1).

Sequence analysis of one of the anti-ERα antibody-precipitated DNA fragments and alignment with the mouse genomic sequence revealed that the promoter region of the gene encoding aquaporin 5 (*AQP5*) contains an estrogen response element (ERE) (Chapter 2). Quantitative polymerase chain reaction (PCR) and DNA microarray analyses confirmed that *AQP5* is activated soon after the administration of estrogen. Reporter assays also revealed that the

ERE in the promoter region of *AQP5* is functional and is activated directly by estrogen. In contrast, while *AQP8* is also expressed in the estrogen-treated uterus, reporter assays failed to detect a functional ERE. None of the other *AQP* genes responded to estrogen. The ChIP-mediated target identification strategy employed in this study may be useful for the study of other transcription factor networks.

II. Introduction

Estrogens play crucial roles in reproduction and other biological processes. Their functions in mammals are mediated by their binding to two ERs, namely, ERα and ERβ. The estrogen/ER complexes appear to act in both a genomic and non-genomic manner. First, the ERs function as ligand-dependent transcription factors that bind DNA at EREs (Fig. 1). The consensus ERE is a 13 bp palindromic inverted repeat separated by 3 bp composed of variable bases. Second, estrogen/ER complexes also rapidly activate membrane-initiated kinase cascades such as the mitogen-activated protein kinase (MAPK) (Migliaccio, 1996) and phosphatidylinositol-3-OH (PIP3) kinase (Simoncini *et al.* 2000) signaling pathways (Fig. 2). Since the net effect of estrogen involves both genomic and non-genomic activities (Kahlert *et al.* 2000; Wong *et al.* 2002), it is important to distinguish between these mechanisms when seeking to understand the functions of estrogen.

The uterus is a major target organ for estrogen and its condition both physiologically and morphologically depends upon

estrogen levels. In the mouse uterus, the major function of estrogen is to induce hypertrophy by mediating water imbibition and cell proliferation (Fig. 3). ERα is the most important ER in uterus physiology since it is expressed at high levels in the uterus and studies with ERα and ERβ null mice (Lubahn *et al.* 1993; Krege *et al.* 1998) have revealed that ERα, not ERβ, plays an essential role in reproduction.

Although the influence of estrogen on the uterus is quite well understood, our understanding of the estrogen-activated transcriptional networks in the uterus remains limited. While several groups have examined the uterine gene expression profile (Watanabe *et al.* 2002; Hewitt *et al.* 2003; Moggs *et al.* 2004), it remains unclear which of these genes are directly activated by estrogen/ERs, and which are activated by the non-genomic effects of estrogen.

A direct genomic interaction occurs between ER and specific sequences of DNA known as ERE. A consensus ERE has been derived from highly estrogen-responsive sequences from the *Xenopus laevis* genes encoding vitellogenin A1, A2, B1, B2 (Klein-

Hitpass *et al.* 1988). It is a 13 bp palindromic inverted repeat with a 3 bp spacing of variable bases. Although there are many reports of EREs on various species, few genes have complete ERE sequence. Almost all genes have only ERE-half site or similar sequence (Table 1). In this reason, it is too difficult to identify the target genes from genomic sequences.

In the work described in this thesis, I have sought to comprehensively identify the uterine genes that are regulated directly by estrogen. To do so, I developed the ChIP-mediated target cloning method described in Chapter 1. This method generated a number of anti-ERα antibody-immunoprecipitated, fragments that were subjected to ligation-mediated PCR (LMPCR), amplified and sequenced. Candidate target genes located in the vicinity of these putative ERE-bearing fragments were then identified by examination of the whole mouse genome sequence. When the estrogen-induced uterine transcription of these candidate target genes was determined by reviewing previously acquired microarray data, a number of uterine genes that are clearly directly regulated by the DNA-binding of ERα

were identified. One of these is the gene encoding aquaporin 5 (*AQP5*). In chapter 2, we analyzed the function of *AQP5* and examined its responsiveness to estrogen. The estrogen responsiveness of the other *AQP* genes was also determined.

Fig. 1. Genomic effect of the estrogen/ER complex

Estrogens act by binding the estrogen receptors ERα and ERβ. These receptors can function as ligand-dependent transcription factors that bind genomic DNA at EREs, thereby directly mediating the genomic effects of estrogen. E2, 17b-estradiol; RNA pol, RNA polymerase; ER, estrogen receptor; ERE, estrogen response element.

Fig. 1.

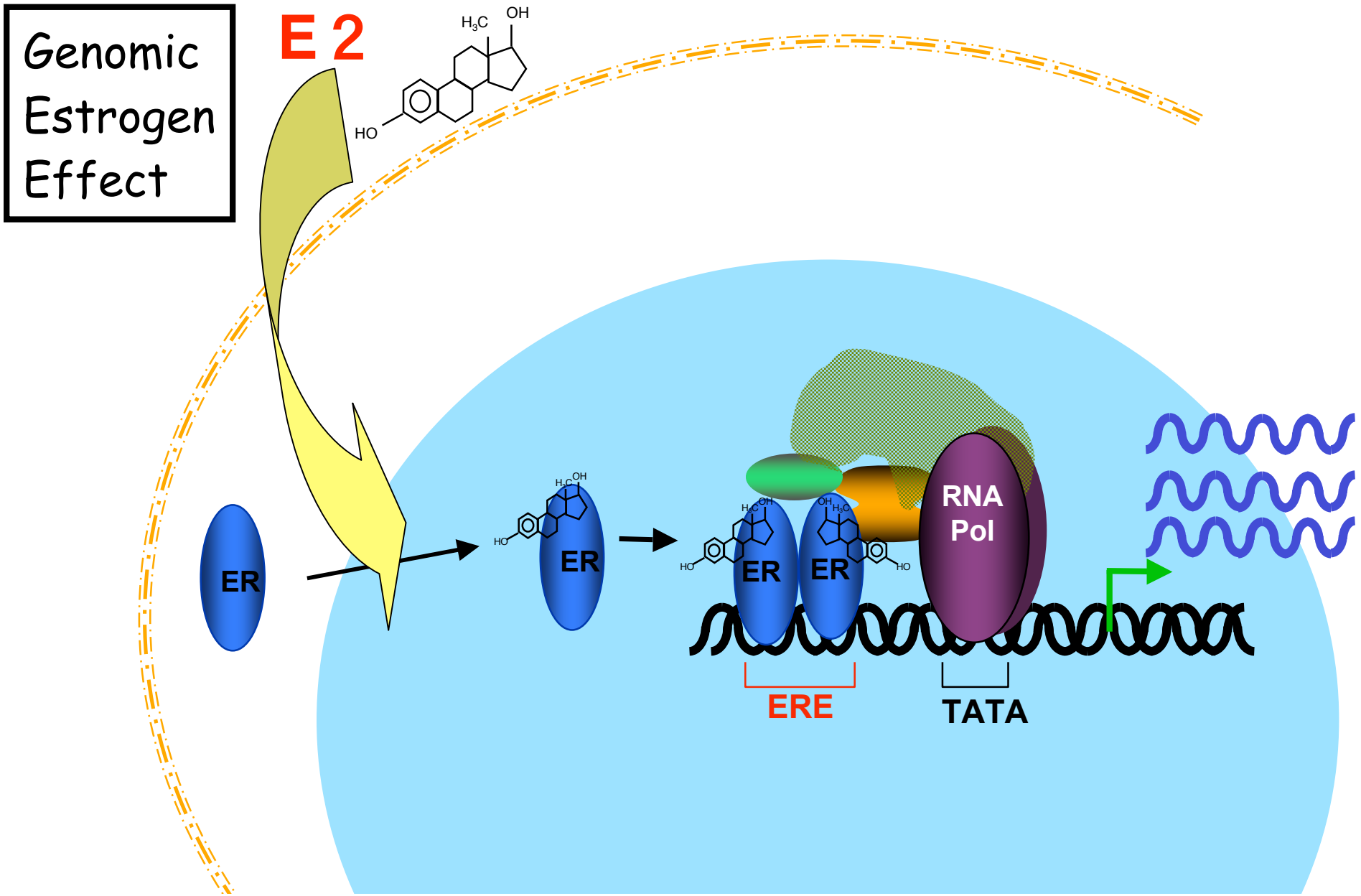

Genomic
Estrogen
Effect
E 2
H3C
OH
HO
ER
ER
ER
ER
RNA
Pol
ERE
TATA

Fig.2. Non-genomic effect of the estrogen/ER complex

Estrogen/ER complexes can rapidly activate intracellular signal transduction cascades such as the MAPK (Migliaccio, 1996) and PI3K (Simoncini *et al.* 2000) signaling pathways. E2, 17b-estradiol; IGF-1, insulin-like growth factor-1; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate ; ERK1/2, extracellular signal-regulated kinase 1/2.

Fig. 2.

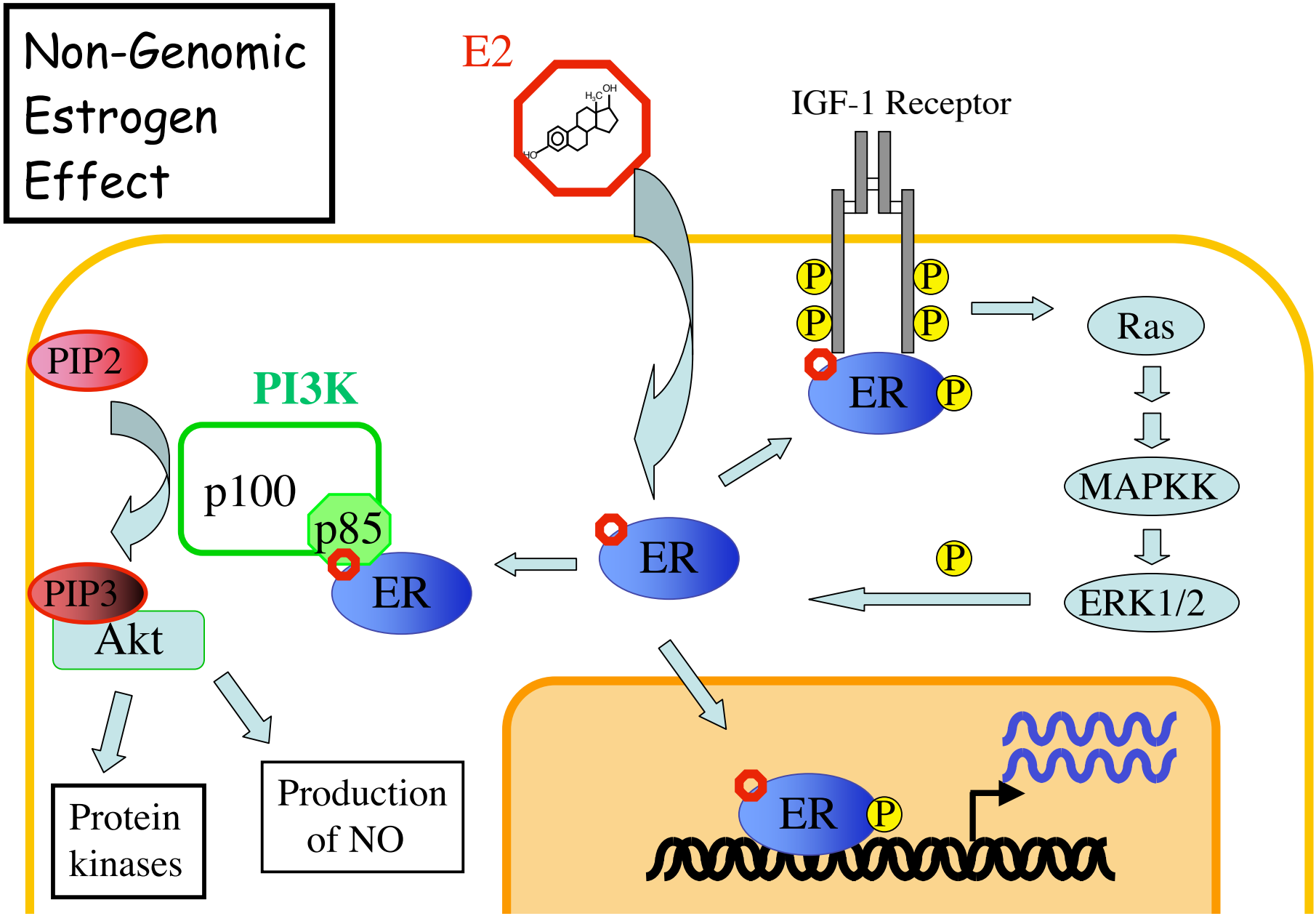

Non-Genomic
Estrogen
Effect
E2
IGF-1 Receptor
P
P
P
P
ER
P
Ras
MAPKK
ERK1/2
P
PIP2
PI3K
p100
p85
ER
PIP3
Akt
ER
Protein
kinases
Production
of NO
ER
P

Fig. 3. Major effects of estrogen on mouse uterus

In the mouse uterus, the major functions of estrogen are to first induce water imbibition 6 h after estrogen exposure and then cell proliferation at 16-24 h. These functions of estrogen together cause the uterus to become hypertrophic.

Fig. 3.

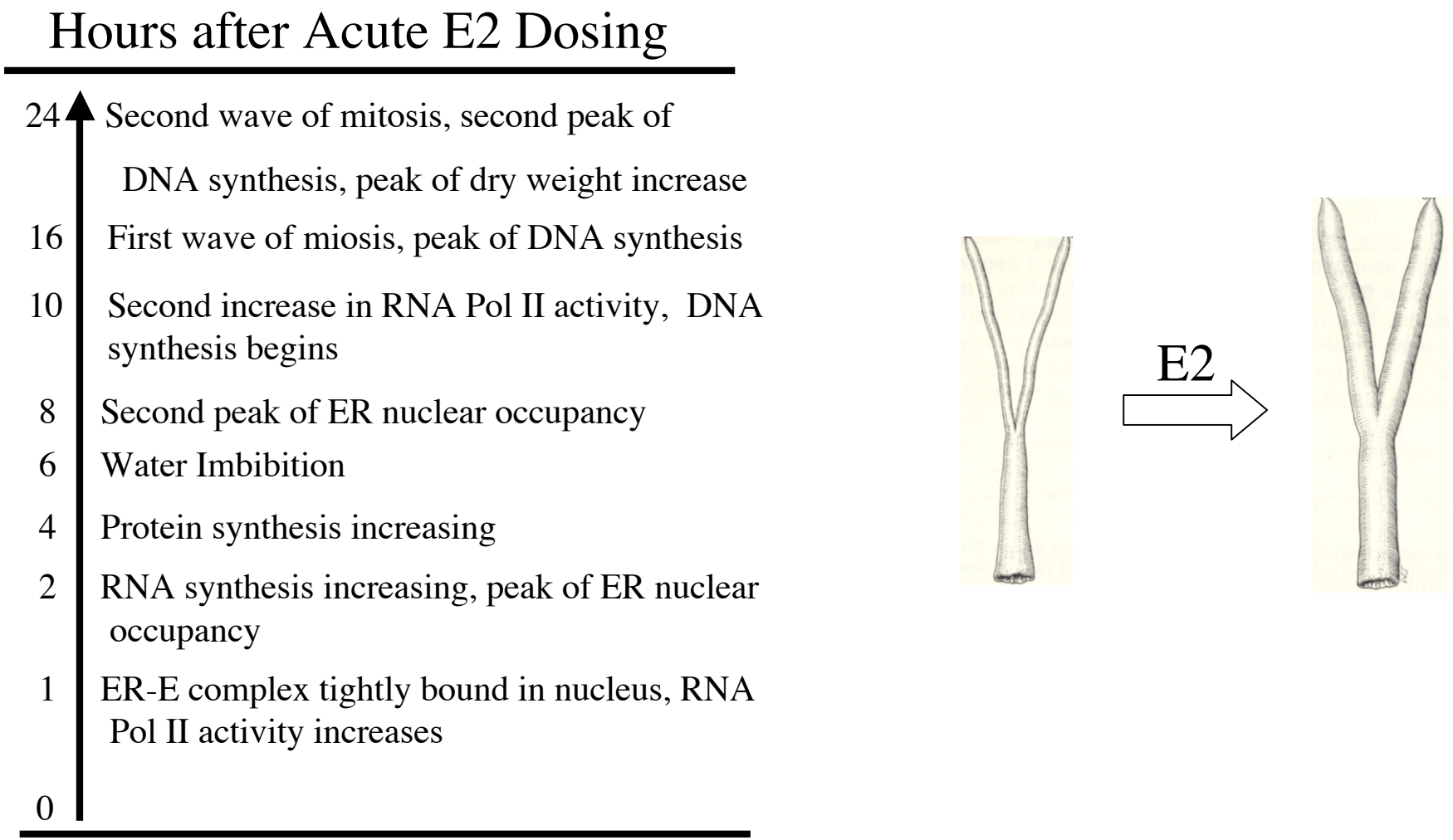

Hours after Acute E2 Dosing
24
Second wave of mitosis, second peak of DNA synthesis, peak of dry weight increase
16
First wave of miosis, peak of DNA synthesis
10
Second increase in RNA Pol II activity, DNA synthesis begins
8
Second peak of ER nuclear occupancy
6
Water Imbibition
4
Protein synthesis increasing
2
RNA synthesis increasing, peak of ER nuclear occupancy
1
ER-E complex tightly bound in nucleus, RNA Pol II activity increases
0
E2

Table 1. Reported EREs of various species

ERs interact directly with EREs in DNA. The consensus ERE is a 13 bp palindromic inverted repeat separated by 3 bp of variable bases (GGTCAnnnTGACC). While many EREs have been identified in various species, few genes carry the complete consensus ERE sequence. Instead, most genes only have the ERE half-site or a similar sequence.

Table 1.

	Consensus EREs	GGTCAnnnTGACC	
Human	**angiotensinogen**	**AGGGCATCGTGACCC**	**-63**
	bcl-2	**GGTCGCCAGGACC**	**+195**
		GGTCCCCATGACC	**+276**
	BRCA1	**GGTCAGGCTGGTCTGGAACTCCTGACC**	***+2023***
	cathepsin D	**GGCCGGGCTGACC**	**-270**
	complement C3	**AGGTGGCCCTGACC**	**-236**
	cytochrome c oxidase 7related protein	**GGTCAAGGTGACC**	**+443**
	ERbeta	**GGTCAGGCTGGTC**	**-1510**
	Ha-ras	**GCGCTGACC**	***+1713***
	hepatic alpha 2u golobulin	**GGTCATTTCCTGTGACT**	**-606**
	lactoferrin	**AGGTCAAGGCGATCT**	**-374**
	progesterone receptor	**GCAGGAGCTGACC**	**+744**
	quinone reductase	**AGTCACAGTGACTC**	**-476**
	pS2	**AGGTCAGCGTGGCCA**	**-405**
	VEGF	**AATCAGACTGACT**	**-1560**
Rat	**creatine kinase B**	**GGTCAGAACACCC**	**-569**
	lutenizing hormone B	**GGACAGATGGTGTCC**	**-1126**
	oxytocin	**GGAACAGTTTGACCC**	**-115**
	prolactin	**ACCTCACCAGCTGCTT**	**-1713**
	vasopressin	**GGCCAGCCTGACC**	***-4324***
	VEGF	**GGGCAAAGTGACT**	**-10**
Mouse	**c-fos**	**GGTCTAGGAGACC**	**-278**
	oviduct specific glycoprotein	**GGTCACCACAGCC**	**-115**
	Hepatocyte Growth Factor	GGTCAGAAAGACC	-872
Rabbit	**uteroglobin**	**AGGTCACCATGCCCT**	**-275**
Guinea pig	**estrogen sulfotransferase gene 2**	**AGGTCATGTTGTTC**	**-982**

III. Chapter 1

Methodology of ChIP–mediated Target Cloning

Introduction

Although estrogen regulates the expression of many genes, it remains unclear which of these are regulated directly by estrogen/ER binding to EREs. Membrane-binding assays (Inoue *et al.* 1993), computational analyses (Bourdeau *et al.* 2004; Kamalakaran *et al.* 2005) and ChIP-on-chip analyses (Carroll *et al.* 2005; Laganiere *et al.* 2005) have been used previously to identify ER target genes in a unbiased manner. The membrane-binding assays involved mixing restriction enzyme-digested total genomic DNA with the DNA-binding domain of ER; after passing this mixture through a nitrocellulose filter, the ER protein-bound fragments were retained and cloned into plasmids (Fig. 4). The computational analyses involved building a database of putative promoter regions and systematically analyzing these regions for the presence of motifs recognized by ER (Fig. 5).

The recently developed ChIP-on-chip technique, unlike the other two methods, enables the whole genome in living cells to be investigated for the presence of transcription factor-bound genes.

This method is based on the standard ChIP method, which detects the specific association of a transcription factor with a known DNA region in living cells (Fig. 6). In standard ChIP, the cells are usually fixed by formaldehyde to crosslink the proteins that are closely associated with DNA and then sonicated to fragment the DNA. The fragments are subsequently incubated with antibodies specific for the transcription factor in question. The immunoprecipitated protein–DNA complexes are then purified and the DNA sequences are determined by PCR, generally by using specific primers. This method is useful for confirming the binding of a protein to a gene that was previously identified by other methods but cannot be used to identify unknown target genes. This limitation was overcome by the ChIP-on-chip method. In this method, the immunoprecipitated transcription factor-bound DNA fragments are used to probe microarrays consisting of promoter regions. This method has been used successfully to identify previously unknown ER-binding genes in the uterus but it is hampered by the large number of arrays needed. Moreover, these studies used cultured cell lines such as MCF-7 as their genomic

cycle at 23-25^{o}C, fed laboratory chow (CE-2; CLEA, Tokyo, Japan) and given tap water *ad libitum*. To assess the effect of estrogen on uterine gene expression, 8-week-old mice were ovariectomized and injected intraperitoneally two weeks later with 50 mg kg^{-1} body weight of 17β-estradiol (E2) (Sigma-Aldrich Japan, Tokyo, Japan) or with sesame oil (Nakarai Tesque, Kyoto, Japan) as a vehicle control. The whole uterus was collected immediately or 1 and 2 h after treatment. All animal experiments were approved by the institutional Animal Care Committee.

ChIP-mediated target cloning

Sampling

The uteri were subjected to perfusion fixation with 30 ml of 1% formaldehyde in saline, then homogenized in phosphate-buffered saline (PBS) containing 0.125 M glycine by using a Physcotron (NS-310E, Microtec, Chiba, Japan). The homogenates were centrifuged at 700 x*g* for 5 min at 4ºC and the pellets were kept in a –80ºC freezer until use in the following 6-day ChIP procedure.

Day 1: uterine homogenate lysis, sonication and CsCl gradient

material. Consequently, the estrogen target genes of the whole uterus remain poorly understood.

To overcome these limitations, I have established the ChIP-mediated target cloning method. This method can be used to identify previously unknown transcription factor-binding genes in whole organs and tissues (Fig. 7). It differs from the ChIP-on-chip method in several way. First, because whole tissues are used as the DNA material, fixation occurs by perfusion. Second, to reduce the background, the homgenized tissues are fractionated through a density gradient. Third, the purified chromatin fragments are ligated with oligonucleotide linkers before sequencing.

In this chapter, the methodology of ChIP-mediated target cloning and the ER-bound uterine clones that were obtained with this method are discussed.

Methodology

Animals

Female C57BL/6J mice were housed under a 12 h light/dark

centrifugation

The pellets were resuspended in lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], 0.5 mM EGTA and 0.25% Triton X-100), incubated for 10 min on ice, and subjected to microcentrifugation at 700 x*g* for 5 min at 4ºC. The pellets were then washed by wash buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], 0.5 mM EGTA, 200 mM NaCl), resuspended in sonication buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA [pH 8.0], 0.5 mM EGTA), and sonicated with 15 10-sec pulses at the maximum setting by using a Bioruptor sonicator (Cosmo Bio, Tokyo, Japan). Between the pulses, the samples were cooled for 20 sec. SDS was then added to the samples to a final concentration of 2%. After 1 h rotation at room temperature, the samples were microcentrifuged at 15,000 r.p.m. for 5 min and the supernatants were collected into 2 ml tubes. The supernatants were sonicated as described above, thus yielding DNA with an average length of ~500 bp, and then centrifuged at 15,000 r.p.m. to remove debris. The supernatants were subsequently loaded onto a cesium chloride (CsCl) step gradient

consisting of 1.5 ml each of 1.75 g/ml and 1.5 g/ml CsCl, and 1 ml of 1.3 g/ml CsCl supplemented with 0.5% (vol/vol) N-lauroylsarcosine. The samples were then centrifuged at 44,000 r.p.m. in a SW55Ti rotor for 24 h at 4ºC.

Day 2: dialysis of uterine chromatin-bearing fractions

0.1 ml fractions were collected from the bottom of the gradient and the fractions containing the crosslinked chromatin were combined and dialyzed twice for over 6 hours against 1 L of Tris-EDTA (TE) buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) containing 10% glycerin.

Day 3: incubation of cross-linked uterine chromatin with anti-ERα antibody

The samples were collected into 2 ml tubes, after which 5 M NaCl, 0.2 M EGTA, 10% Triton X –100, 10% SDS and 10% sodium deoxycholate were added to yield the final concentrations of these chemicals in RIPA buffer (i.e. 10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1 mM EDTA [pH 8.0], 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate). The samples were then

pre-cleared with 300 ml of 50% slurry protein G sepharose for 1 h at 4ºC and microcentrifuged at 3,000 r.p.m. for 3 min. The supernatants were placed into new 2 ml tubes and incubated with 10 mg anti-ERα polyclonal rabbit antibody (H-184; Santa Cruz, CA) overnight at 4ºC. Alternatively, the 0 h uterus samples were incubated with an anti-AcH3 antibody, which recognizes acetylated histone 3.

Day 4: immunoprecipitation of anti-ERα antibody-bound chromatin and cross-link reversal

The samples were precipitated by incubation with protein G sepharose for 2 h at 4ºC and then microcentrifuged at 3,000 r.p.m. for 2 min. The pellets were washed 3 times with 1 ml of RIPA buffer, 3 times with 1 ml of RIPA buffer containing 1 M Nacl, twice with 1 ml of LiCl wash buffer (0.25 M LiCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate), and twice with 1 ml of TE buffer. The samples were then incubated twice for 10 min with 200 ml elution buffer (0.1 M sodium bicarbonate, 1% SDS). The cross-linking was reversed by incubation for 6 h at 65ºC.

Day 5: cloning of immunoprecipitated uterine DNA fragments and ligation with oligonucleotide linkers

The immunoprecipitated samples were incubated with proteinase K for 4 h at 45ºC, then extracted with phenol/chloroform and ethanol precipitated. The precipitated DNA fragments were cloned into pGEM-T vector (Promega, Tokyo, Japan) by ligation-mediated PCR (LMPCR). For this, the DNA fragments were first phosphorylated with T4 polynucleotide kinase (Takara Bio Inc., Shiga, Japan) as follows: the DNA pellets were resuspended in 75 ml of denaturation buffer and preincubated for 2 min at 90ºC, after which 10 ml of blunt end kinase buffer, 1 ml of 100 mM ATP, 2 ml of T4 polynucleotide kinase (10 unit/ ml), and 12 ml of sterilized water were added. The samples were incubated for 1 h at 37ºC and then for 2 min at 90ºC, after which they were extracted with phenol/chloroform and ethanol precipitated. The phosphorylated samples were then blunt-ended with T4 DNA polymerase (Takara., Shiga, Japan) as follows: the DNA pellets were resuspended in 5 ml of sterilized water and then 1 ml of 10x T4 polymerase buffer, 1 ml of

0.1% BSA, and 1 ml of 1.7 mM dNTP mix was added. The samples were preincubated for 5 min at 70ºC, after which they were transferred to 15ºC. One ml of T4 polymerase was then added and the samples were incubated for 2 h at 15ºC, after which the samples were extracted with phenol/chloroform, ethanol precipitated, and resuspended with 4 ml of sterilized water. Subsequently, 1ml of 100 pmol oligonucleotide linkers and 5 ml solution I from the Takara ligation kit Ver. II (Takara) were added and the samples were incubated overnight at 16ºC. The oligonucleotide sequences of the linkers were F 5'-GCGGTGACCCGGGAGATCTGAATTC-3'. and R 5'- GAATTCAGATC-3'.

Day 6: PCR, cloning and sequence analysis of ChIP-selected uterine chromatin fragments

After ligating the oligonucleotide linkers, the DNA fragments were amplified by PCR in the presence of 0.1 nM F primer, 0.2 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 1 x PCR buffer and 1 U LA Taq (Takara) in 20 mL of reaction buffer. After 30 amplification cycles, the PCR products were purified by

using the QIAquick PCR purification kit (QIAGEN, Tokyo, Japan) and cloned into the pGEM-T easy vector. Transformed colonies were then picked randomly and their inserts were sequenced.

Results

In this study, the ChIP assay was modified so that the ERα-bound DNA sequences in estrogen-treated murine uteri could be cloned and identifed. As described below, this procedure successfully identified novel ERα-regulated genes in the murine uterus, thereby expanding our understanding of the direct genomic effects of estrogen treatment on the mammalian uterus. This procedure can thus be generally used to identify unknown target promoters of known transcriptional factors that function in whole organisms or their organs and tissues.

To modify the ChIP procedure so that unknown targets could be identified, it was necessary to lower the background. This was achieved by employing cesium chloride step gradient centrifugation. While unfixed sample DNA would normally accumulate in the lowest

cesium chloride fraction, the DNA of fixed samples localize in the middle layers (Fig. 8). Moreover, to isolate the protein–DNA complexes from whole tissues and organs, fixation was achieved by perfusion fixation. Finally, to identify unknown target genes, the immunoprecipitated chromatin fragments were purified and ligated with oligonucleotide linkers before being amplified, cloned and sequenced.

Ovariectomized mice were treated with E2 or the sesame oil vehicle and their uteri were harvested immediately (0 h) or 1 or 2 h later. Analysis of these samples by ChIP-mediated target cloning assays generated a number of clones. When the inserts of these clones were sequenced and mapped to the whole mouse genome sequence (Ensemble sequence, Fig. 9), mouse-specific fragments were not isolated from the sesame oil-treated mice, regardless of when they were harvested and which immunoprecipitating antibody was used (data not shown). With regard to the E2-treated mice, when the anti-ERα antibody was used for immunoprecipitation, 52, 72 and 67 clones were obtained from the 0, 1 and 2 h uteri, respectively (Fig.

10). Sequence analysis revealed these clones together contained 5, 71 and 63 unique sequences, respectively. Thus, many more uterine DNA sequences are bound by ERα 1 or 2 h after E2 treatment than at the 0 h timepoint. When the anti-AcH3 antibody was used to immunoprecipitate the 0 h-harvested uterus DNAs, 46 clones were obtained. As expected, most of these contained unique murine sequences.

Putative target genes that had transcription start sites within 100 kbp of the precipitated fragments were then identified. As shown in Figure 9, this criterion means that each ChIP fragment could have (at least) two putative target genes with start sites within 100 kbp. These genes were classified into three groups according to whether their start sites were 0~5 kbp, 5~50 kbp, or 50~100 kbp away from the ChIP fragments. To help identify which of these genes are most likely to be actual target genes, we examined previously acquired microarray data that show the genome-wide transcription patterns of estrogen-treated murine uteri**.** These microarray data were generated by treating mice with E2, harvesting their uteri at 0, 1, 2, 6,

12, and 24 h later, and then subjecting the RNAs from these samples to microarray analysis. Many of the genes whose start sites were 0-5 kbp away from the precipitated DNA fragment were upregulated by estrogen (Fig. 11A), while only a few of those with start sites 5-50 kbp away were similarly upregulated (Fig. 11B). In contrast, none of the genes with the most distant start sites (50-100 kbp away) were upregulated by estrogen (Fig. 11C). These observations suggest that the ERα–bound EREs in the uterus are most likely to be within 50 kbp of the transcription start site of their target genes.

I then analyzed the distances between the ChIP fragments and transcriptional start sites of their putative target genes. When the frequency of ChIP fragments per kbp of the transcription start sites was calculated and plotted in Figure 12, this analysis revealed that the vast majority of the ChIp fragments were clustered around the transcriptional start sites. Only a few fragments were located far from the transcriptional start sites. This confirms that most ERα–bound EREs in the uterus are most likely to be within 50 kbp of the transcription start site of their target gene.

Re-evaluation of the microarray analysis revealed that several of the putative target genes located close to the ChIP fragments were upregulated by estrogen (Fig. 11A). Of particular interest were *AQP5* (NM_009701), *Adrenomedullin (Adm)* (NM_009627), *Chemokine orphan receptor (COR)* (NM_007722), *serum inducible kinase* (M96163), *HMG-CoA reductase* (M62766). To confirm that ERα directly induces the transcription of these genes by binding to the EREs identified by the modified ChIP assay, murine uteri harvested 0, 1, 2 or 6 h after estrogen treatment were subjected to the standard ChIP assay depicted in Figure 6**.** This revealed that ERα does bind to the promoter region of these genes in an estrogen-dependent manner (Fig. 13A and data not shown). Moreover, the ERα-binding patterns corresponded to the estrogen-stimulated expression patterns of these genes that were revealed by the microarray analysis (Fig. 13B and data not shown). Thus, most of these seven genes were upregulated 1 or 2 h after injecting estrogen, after which their expression levels decreased.

We recently published a report showing that the *Adm*

gene is upregulated by estrogen (Watanabe *et al.*, 2006). This paper revealed that the *Adm* gene is upregulated 1 h after injecting estrogen, after which the expression levels decreased (Fig. 14). Two ERE-half sites were identified about 700 bp upstream of the *Adm* gene (Fig. 14B). My standard ChIP analysis reveals here that ERα indeed binds to this region 1 h after estrogen injection but that its binding decreases thereafter (Fig. 13B and 14C). This binding pattern corresponds with the expression pattern of this gene (Fig. 14A) and confirms that the ChIP-mediated target cloning assay can be used to identify unknown target genes of known transcription factors.

Discussion

Previously, we used DNA microarray analysis to identify the genes in the mouse uterus that are activated by exposure to estrogen (Watanabe *et al.* 2002). However, while we identified hundreds of genes, it was not clear which of those genes were activated directly by estrogen through the binding of estrogen/ERα to EREs in the gene. In the study described here, I identified a number

of potential ERα target genes by ChIP-mediated target cloning. Comparison of these genes with those identified by the DNA microarray analysis revealed a number of genes that are directly targeted by ERα. Thus, the ChIP-mediated target cloning method can be successfully used to identify the direct target genes of known transcription factors, including nuclear receptors.

The ChiP-mediated target cloning method has a number of advantages over other methods that have been used previously to identify the direct target genes of ERα in the uterus. In particular, while filter binding, computational, and ChIP on Chip approaches have yielded useful information, these studies focused primarily on cultured cells. I have found previously that estrogen response genes are differently expressed depending on the organ or tissue, and that these patterns also vary throughout various maturation (Watanabe *et al.* 2004). Consequently, to gain a greater understanding of the function of estrogen during the development and maturation of the whole organism, it will be necessary to identify the direct target genes of ERα in whole organs. While it is potentially possible to achieve this

goal with the computational approach and the ChIP-on-chip method, both methods have a number of limitations. The computational approach is hampered by the diversity of ERE sequences (Table 1), while the ChIP-on-Chip technique requires a large set of arrays. A recent report describing the use of ChIP with a paired-end ditag sequencing strategy (Wei *et al.* 2006) could overcome the latter problem. Nevertheless, the techniques available to date for obtaining a genome-wide and organ-wide perspective of the target genes of a particular transcription factor have a number of deficiencies.

The ChIP-mediated target cloning method described here differs from other ChIP-based methods in a number of ways. In particular, I introduced a density gradient step that would to remove uncoupled DNA fragments prior to immunoprecipitation to reduce background. Other steps also helped to reduce background, namely, perfusing the mouse tissue, sonicating, and washing the bound samples. Indeed, I could not calculate the non-specific contamination of DNA fragments during my ChIP-mediated target cloning procedure since none of the fragments that were precipitated from the sesame

oil-treated or the 0 hour E2-treated uterus could be mapped to the mouse genome (Fig. 10). In contrast, when the sequences of the precipitated DNA clones obtained from 1 or 2 hour-treated uteri were compared to the mouse genome and mapped, most were unique sequences. These observations suggest that my protocol has a very low background, and indicate that the DNA-binding of ERα depends on the presence of estrogen.

That the ChIP-mediated target cloning method was successful in identifying the direct target genes of ERα in the uterus is indicated by my analysis of the microarray expression patterns of the candidate genes I identified. Many of those in the 0-5 and 0-50 kbp groups were indeed upregulated by estrogen (Fig. 11A). Moreover, I confirmed by conventional ChIP analysis that ERα binds to the promoter region of these genes in the presence of estrogen (Fig. 13). Two of those genes are the *Adm* and *COR* genes. The *Adm* gene encodes a secretory protein whose receptor is called *COR*. I showed that the expression and ERα-binding of both *Adm* and *COR* were both upregulated 1 h after injecting estrogen, after which they

dropped in parallel (Fig. 13B and 14A). Thus, ERα directly regulates the transcription of *Adm* (NM_009627) and *COR* (NM_007722). Other interesting genes identified by my study included *AQP5* (NM_009701), *serum inducible kinase* (M96163), and *HMG-CoA reductase* (M62766). Also of interest are the genes that are downregulated by estrogen, which are not discussed further here.

When I analyzed the distance between the EREs identified by my ChIP-mediated target cloning assays and their putative target genes, I found that they could be divided into three groups depending on whether they were 0-5 kpb, 5-50 kbp, or 50-100 kbp away from the transcription start sites of their target genes. When the microarray data for these genes was reviewed, it was found that more genes in the 0-5 kbp group were upregulated by estrogen than those in the 5-50 kbp group, while none of the 50-100 kbp group were upregulated by estrogen (Fig. 11). Moreover, when I analyzed the distances between the ChIP fragments and the transcriptional start sites of their putative target genes, I found most of the fragments were clustered around the transcriptional start site (Fig. 12). These results

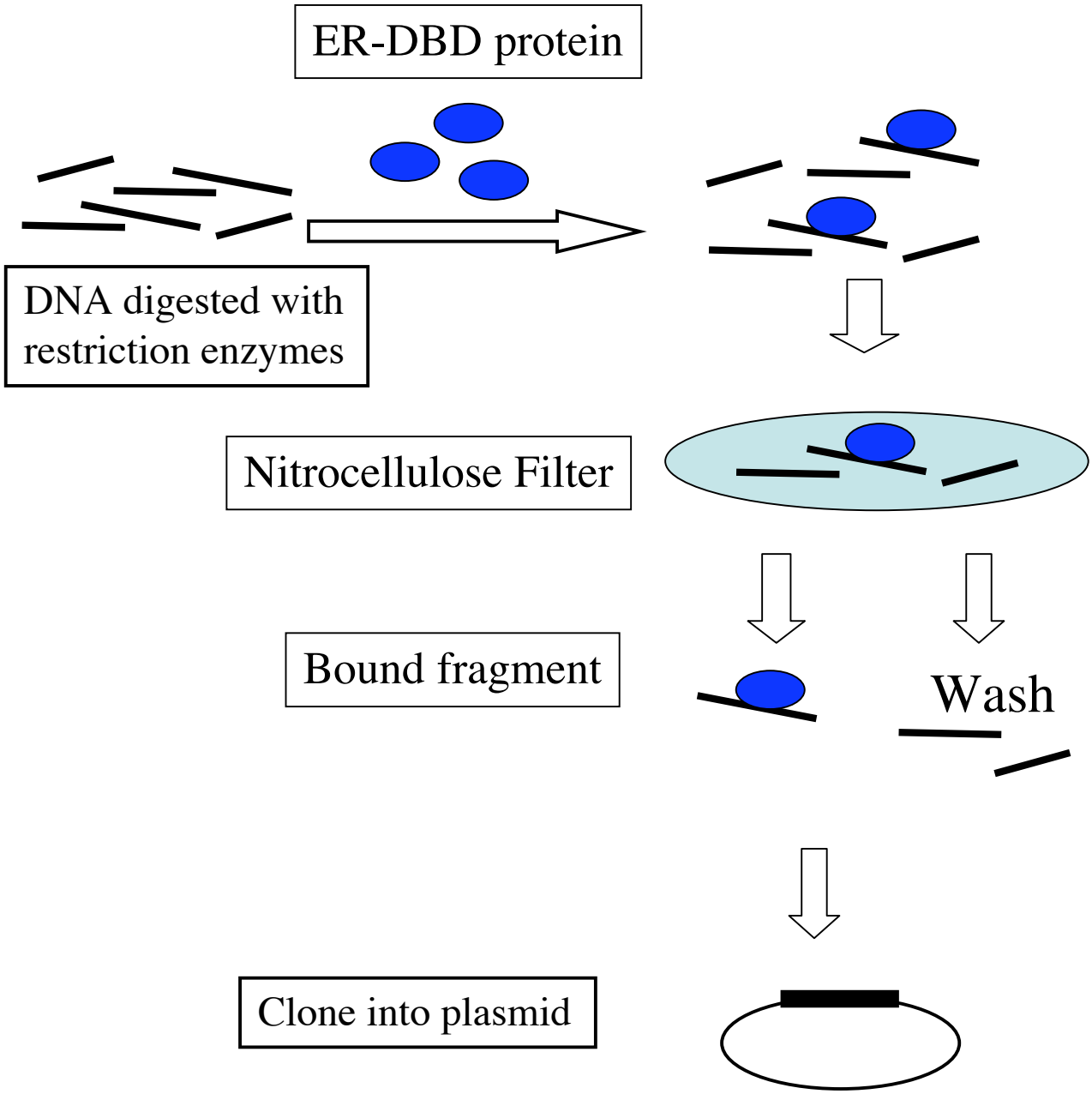
suggest that ERα tends to bind near the transcriptional start site.

The ChIP-mediated target cloning method described in this chapter can also be used to identify the direct target genes of other transcription factors. For example, it could be used to understand the function of ERβ in the brain and prostate if a functional anti-ERβ antibody is available. Indeed, the only limiting factor in ChIP-mediated target cloning is the requirement of functional antibodies. Moreover, the ChIP-mediated target cloning data could also be useful in improving our understanding of other microarray data, thus increasing our understanding of how estrogen works.

Fig. 4. Membrane-binding assay

Total genomic DNA is digested by restriction enzymes, mixed with the DNA-binding domain of ER and passed through a nitrocellulose filter. The ER protein-bound fragments are retained, purified, and cloned into vectors.

Fig. 4.

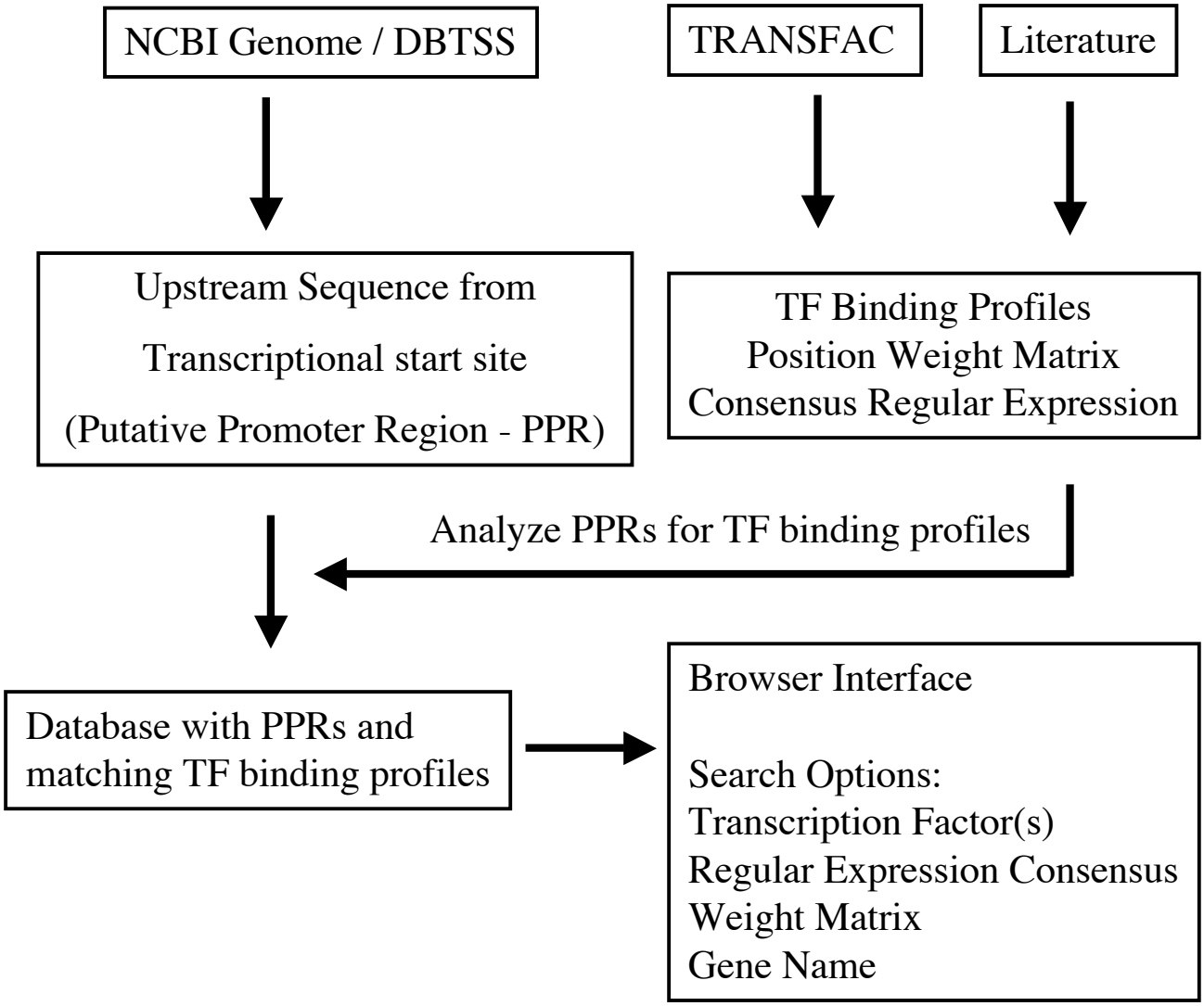


Nucleic Acids Research, 19 : 4093, 1991.

Fig. 5. Computational analysis

A database of putative promoter regions is built and then systematically analyzed for motifs recognized by the transcription factor (TF) in question (Kamalakaran *et al.* 2005).

Fig. 5.



Journal of Biological Chemistry,280 : 21491, 2005.

Fig. 6. The standard ChIP assay

Cells are fixed with formaldehyde to crosslink proteins that are closely associated with the DNA, after which they are sonicated to fragment the DNA. The samples are then incubated with antibodies specific for the transcription factor in question. Subsequently, the cross-links of the immunoprecipitated protein–DNA complexes are reversed and the freed DNA fragments are sequenced by PCR (usually by using gene-specific primers).

Fig. 6.

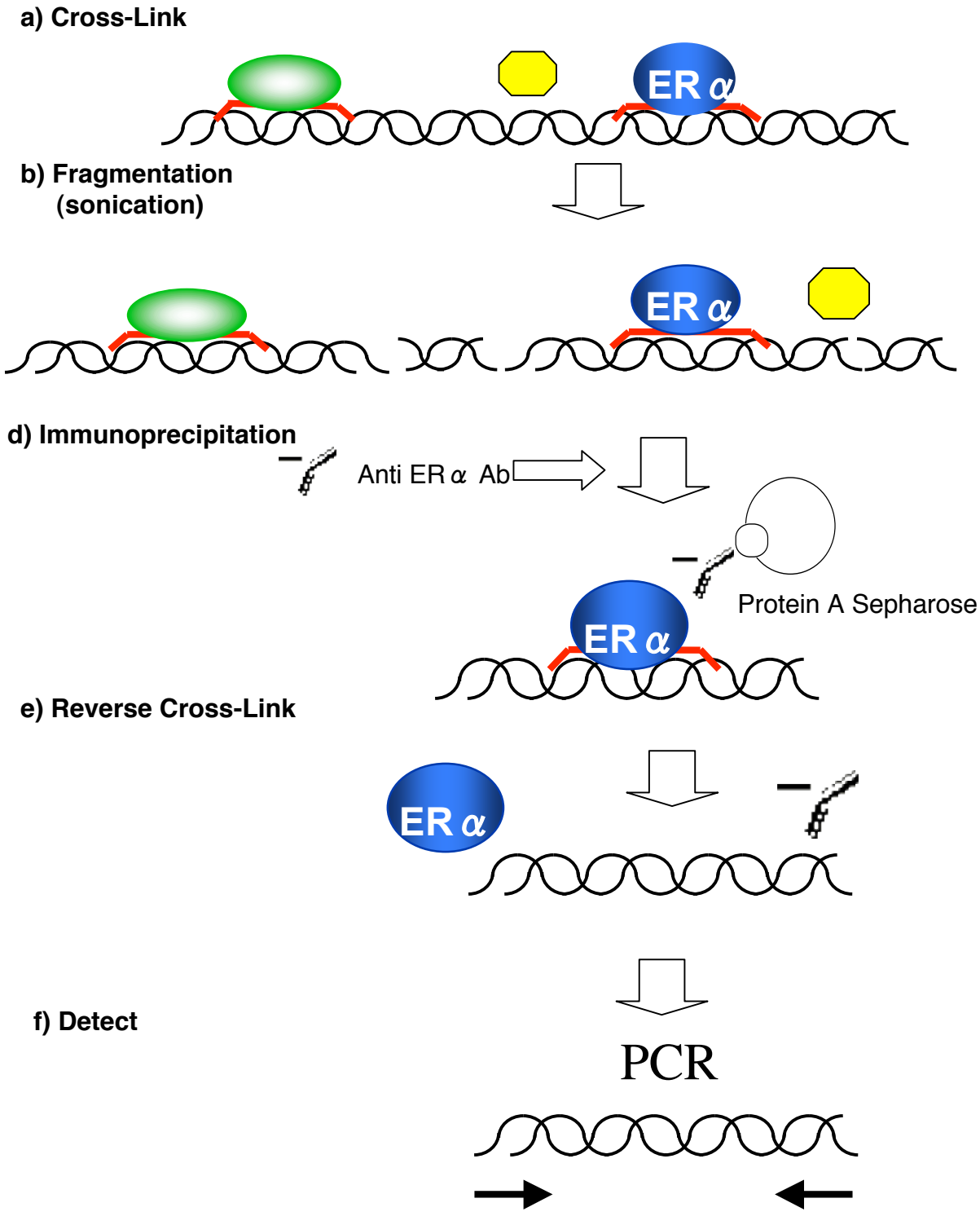

a) Cross-Link
ER α
b) Fragmentation
(sonication)
ER α
d) Immunoprecipitation
Anti ER α Ab
Protein A Sepharose
ER α
e) Reverse Cross-Link
ER α
f) Detect
PCR

Fig. 7. The ChIP-mediated target cloning assay

This modified ChIP procedure can be used to clone unknown target genes of particular transcription factors in animal organs. The procedure differs from the standard ChIP assay described in Figure 6 as follows. First, cross-linking was performed by perfusion fixation, which allows whole organs to be sampled by this technique. Second, to reduce the background, density gradient fractionation of the organ homogenates was introduced. Third, the purified chromatin-fragments were ligated with oligonucleotide linkers by LMPCR before cloning, amplification and sequencing.

Fig. 7.

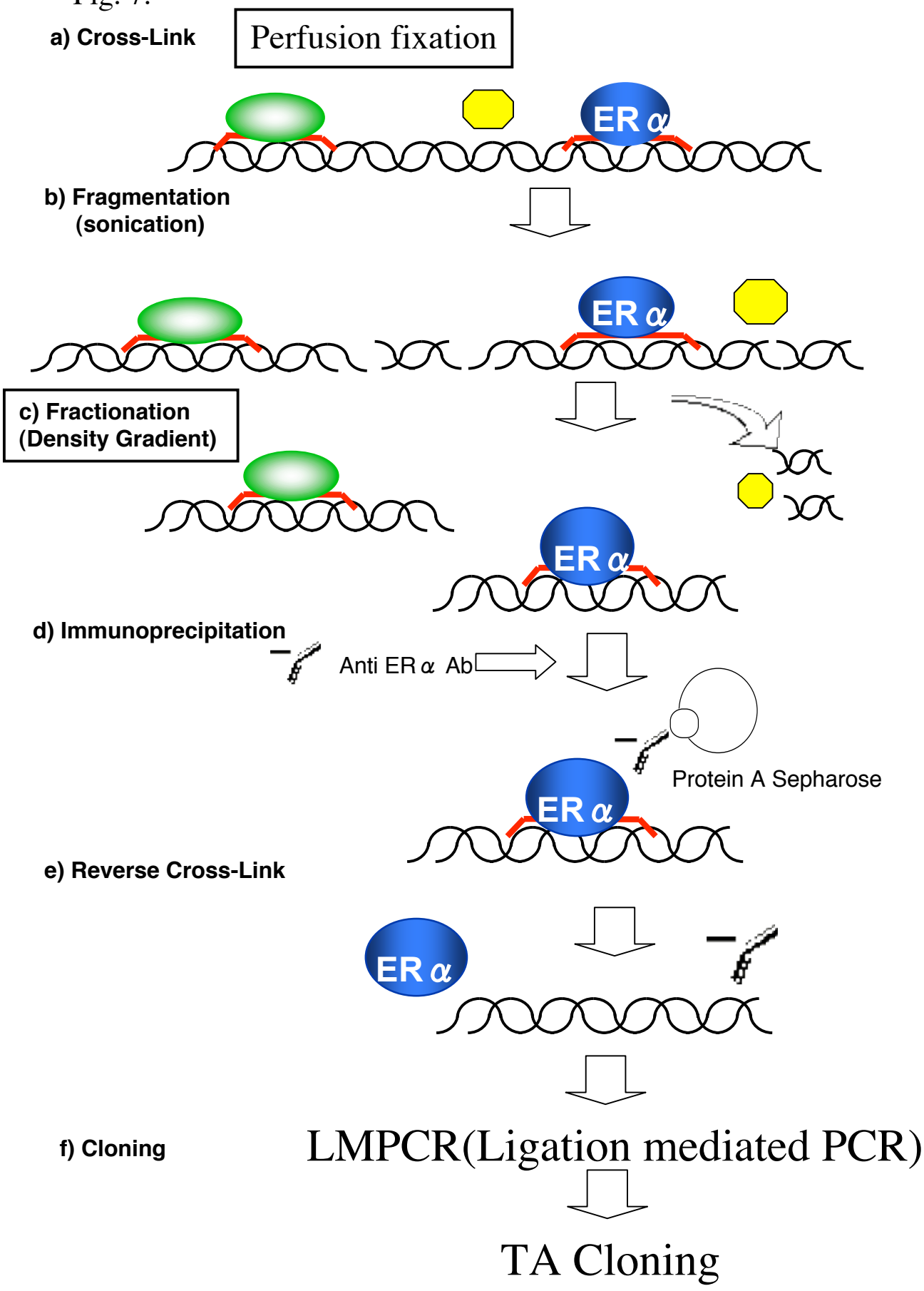
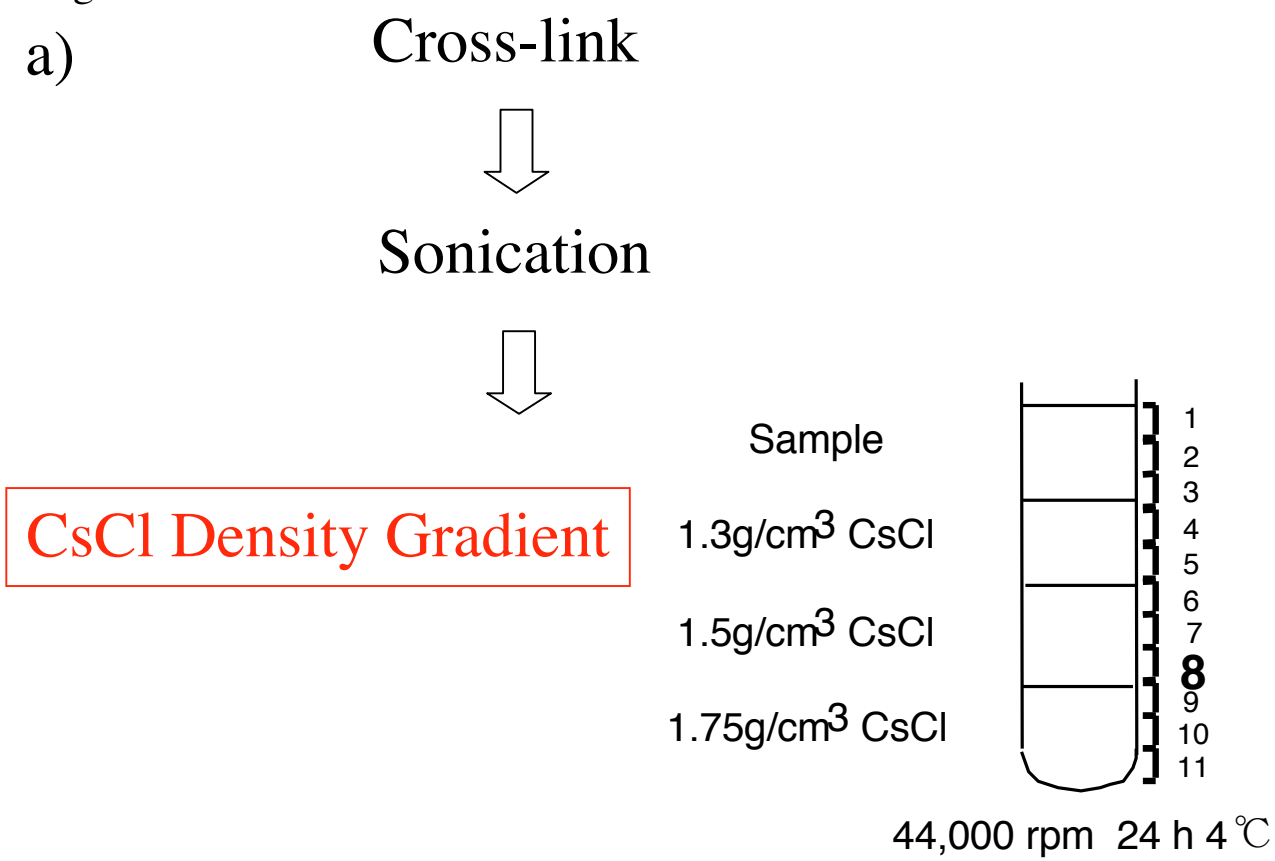

a) Cross-Link
Perfusion fixation
ER α
b) Fragmentation
(sonication)
ER α
c) Fractionation
(Density Gradient)
ER α
d) Immunoprecipitation
Anti ER α Ab
Protein A Sepharose
ER α
e) Reverse Cross-Link
ER α
f) Cloning
LMPCR(Ligation mediated PCR)
TA Cloning

Fig. 8. CsCl density gradient fractionation

To reduce the background of the ChIP-mediated target cloning assay, the cross-linked and sonicated organ homogenates were subjected to cesium chloride step gradient centrigugation (a). When unfixed samples were used, the DNA accumulated in the lowest two fractions (fractions 10 and 11), while the DNA from fixed samples were concentrated in the middle layers (particularly fraction 8) (b). FA, formaldehyde.

Fig. 8.


a)
Cross-link
Sonication
CsCl Density Gradient
Sample
1.3g/cm3 CsCl
1.5g/cm3 CsCl
1.75g/cm3 CsCl
1
2
3
4
5
6
7
8
9
10
11
44,000 rpm 24 h 4℃

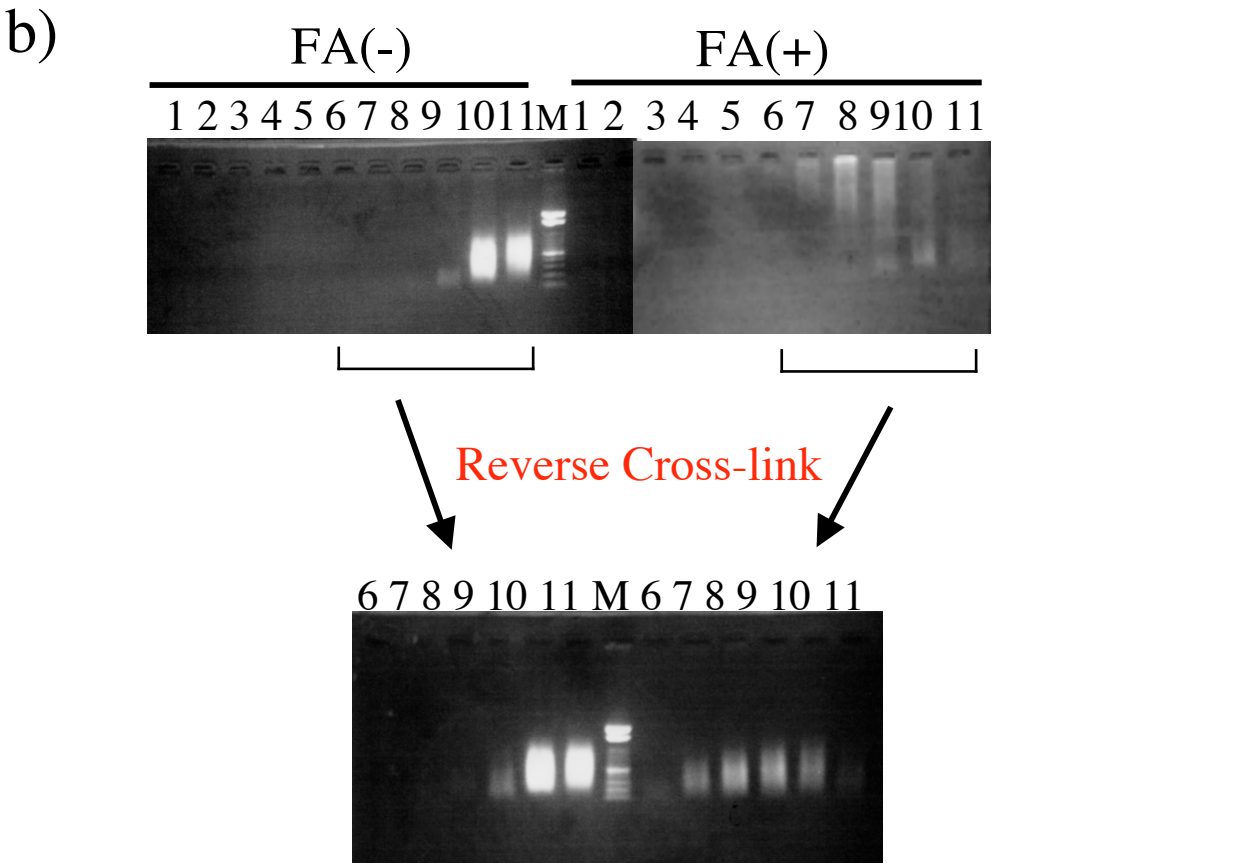

b)
FA(-)
FA(+)
1 2 3 4 5 6 7 8 9 10 11 M 1 2 3 4 5 6 7 8 9 10 11
Reverse Cross-link
6 7 8 9 10 11 M 6 7 8 9 10 11

Fig. 9. Comparison of the precipitated DNA sequences with the murine whole genome sequence.

The sequences of the precipitated DNAs were compared to the Ensemble mouse genome sequence. Potential target genes were identified on the basis of the proximity of their transcription start sites to the fragments.

Fig. 9.

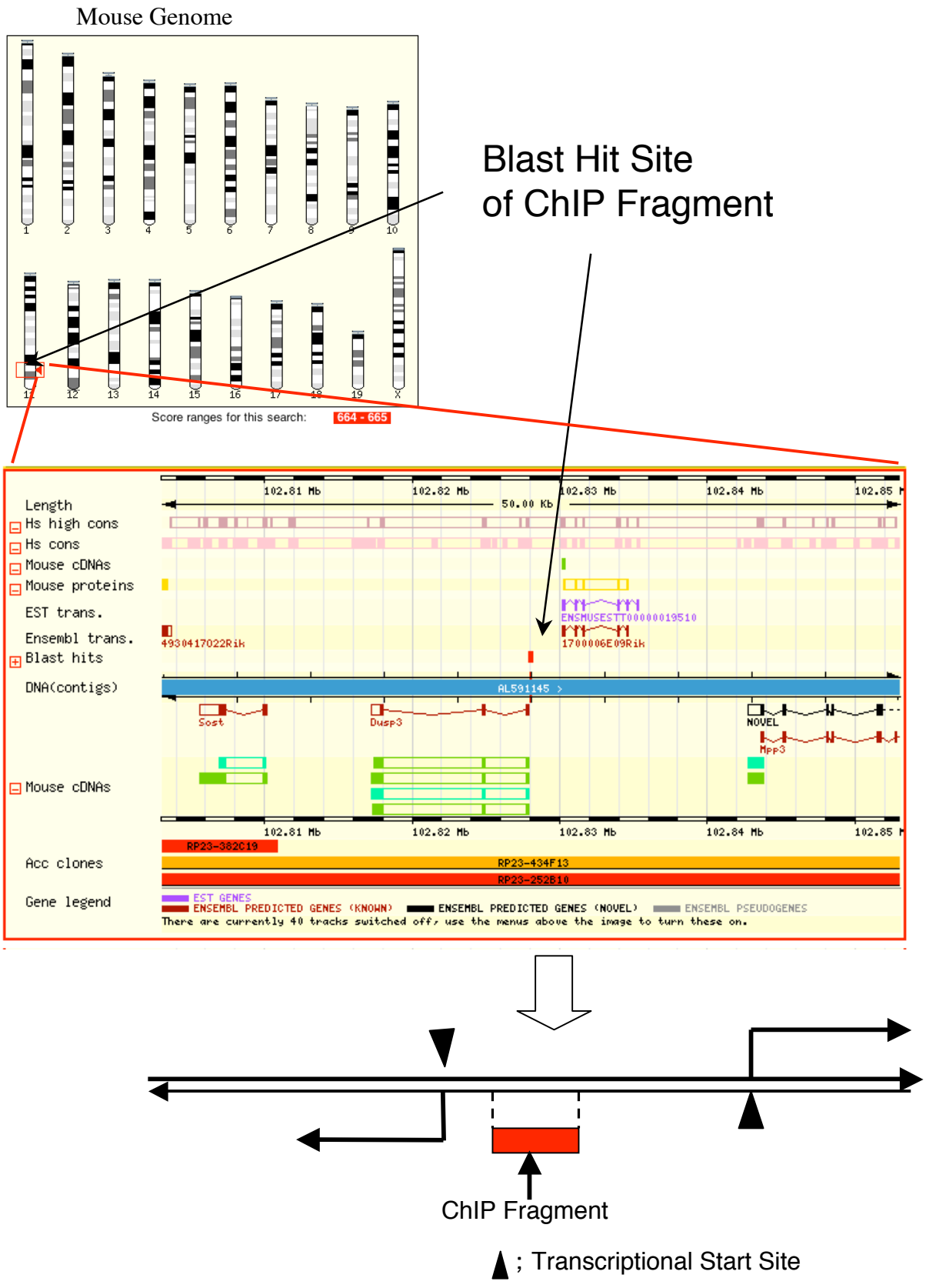
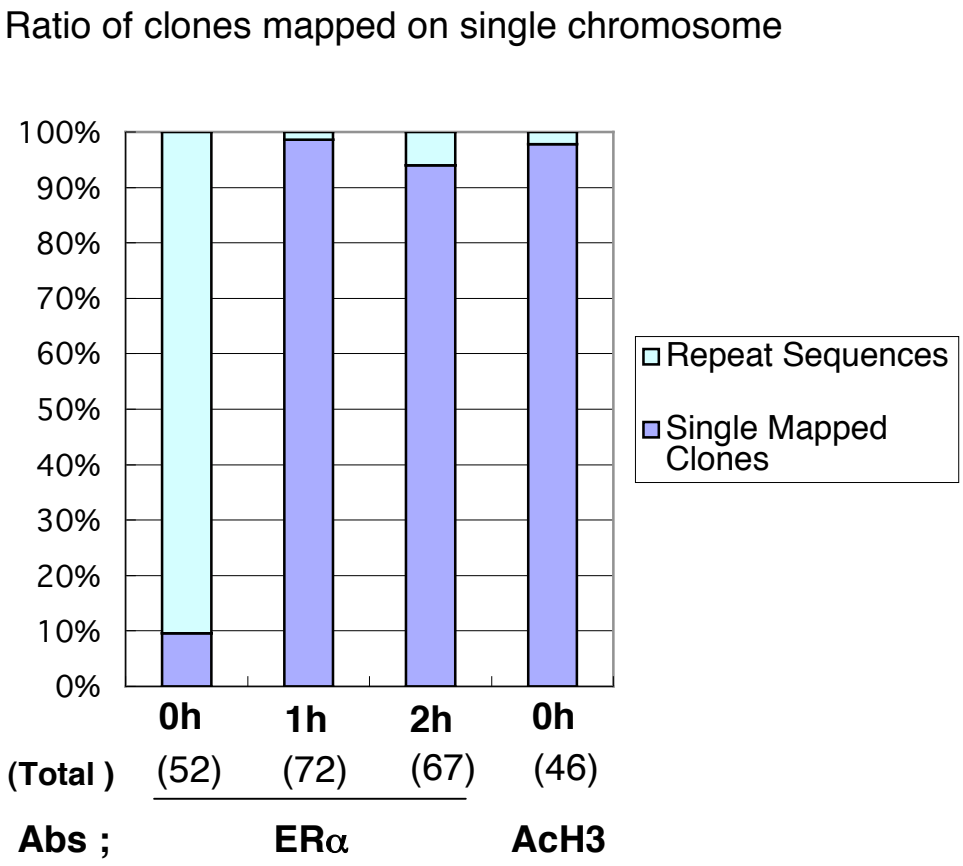

Mouse Genome
Blast Hit Site
of ChIP Fragment
Score ranges for this search: 664 - 665
Length
Hs high cons
Hs cons
Mouse cDNAs
Mouse proteins
EST trans.
Ensembl trans.
Blast hits
DNA(contigs)
Mouse cDNAs
Acc clones
Gene legend
102.81 Mb
102.82 Mb
102.83 Mb
102.84 Mb
102.85 Mb
50.00 Kb
ENSMUSESTT00000019510
4930417022Rik
1700006E09Rik
AL591145 >
Sost
Dusp3
NOVEL
Mpp3
RP23-382C19
RP23-434F13
RP23-252B10
EST GENES
ENSEMBL PREDICTED GENES (KNOWN)
ENSEMBL PREDICTED GENES (NOVEL)
ENSEMBL PSEUDOGENES
There are currently 40 tracks switched off; use the menus above the image to turn these on.
ChIP Fragment
; Transcriptional Start Site

Fig. 10. Proportion of clones that contain a unique murine sequence.

Sequence analysis of the anti-ERα antibody-precipitated sequences generated by ChIP-mediated target cloning revealed most (94-99%) of the fragments from 1 and 2 h-treated uteri were unique sequences. In contrast, there were only 5 unique sequences from the immediately harvested E2-treated uteri (90% of these clones carried repeat sequences). As expected, nearly all of the anti-AcH3 antibody-generated clones obtained from 0 h uteri were unique sequences (98%). AcH3, acetylated histone 3; Abs, antibodies.

Fig. 10.



	0h		1h		2h		AcH3 0h	
	clones	%	clones	%	clones	%	clones	%
Repeat sequence	47	90.4	1	1.4	4	6	1	2.2
Identified	5	9.6	71	98.6	63	94	45	97.8
Total	52	100	72	100	67	100	46	100

Fig. 11. Estrogen-induced expression patterns of genes with transcription start sites within 100 kbp of the ChIP fragments

Putative target genes with start sites within 100 kbp of the precipitated fragments were determined by analyzing the Ensemble murine whole genome sequence. These candidate genes were classified into three groups depending on whether their start sites were 0~5 kbp (A), 5~50 kbp (B), or 50~100 kbp (C) away from the ChIP fragments. The estrogen-stimulated uterine expression patterns of these genes were then determined by examining previously acquired microarray data. These data were generated by treating mice with E2, harvesting their uteri at various timepoints over 24 h, and then subjecting the RNAs from these samples to microarray analysis. The intensity of transcription relative to the 0 h standard (set at 1) is indicated.

Fig. 11.

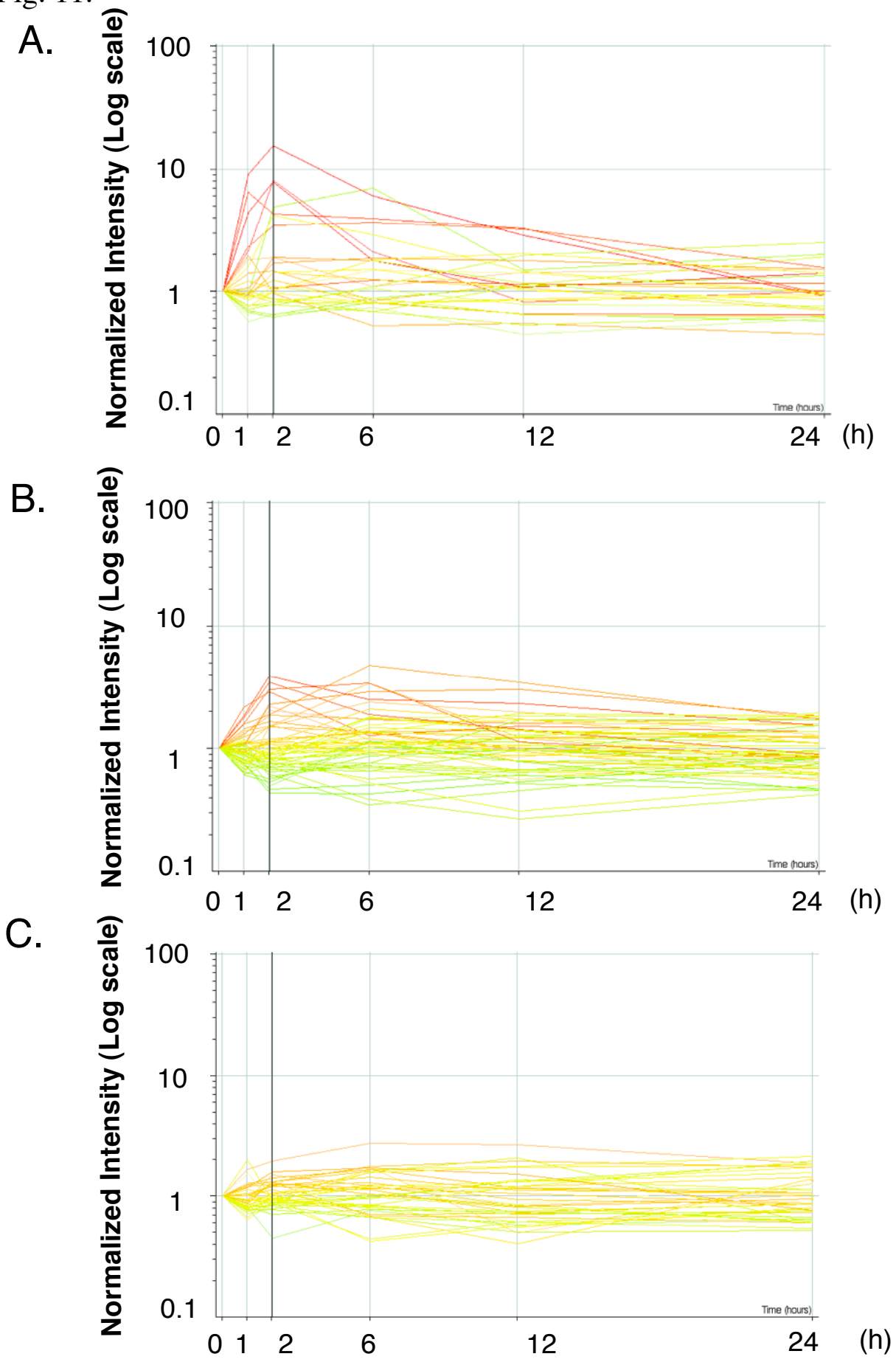

A.
Normalized Intensity (Log scale)
100
10
1
0.1
Time (hours)
0 1 2 6 12 24 (h)
B.
Normalized Intensity (Log scale)
100
10
1
0.1
Time (hours)
0 1 2 6 12 24 (h)
C.
Normalized Intensity (Log scale)
100
10
1
0.1
Time (hours)
0 1 2 6 12 24 (h)

Fig. 12. Distribution of the ChIP-DNA fragments relative to the transcriptional start sites of their putative target genes

The ChIP-DNA fragments were classified according to where they were located in their putative target genes relative to the transcription start site (A) and the frequency of fragments at particular distances from the transcription start site was plotted (B). Most of the fragments clustered around the transcriptional start sites.

Fig. 12.

A.

	fragments	length	per 1k
exon	26	0.5	52
intron	28	0.5	56
initiation	66	0.5	132
1k	39	1	39
5k	13	4	3.25
10k	19	5	3.8
50k	38	40	0.95
100k	21	50	0.42
3' 50k	29	50	0.58
3' 100k	8	100	0.08
	287		

B.

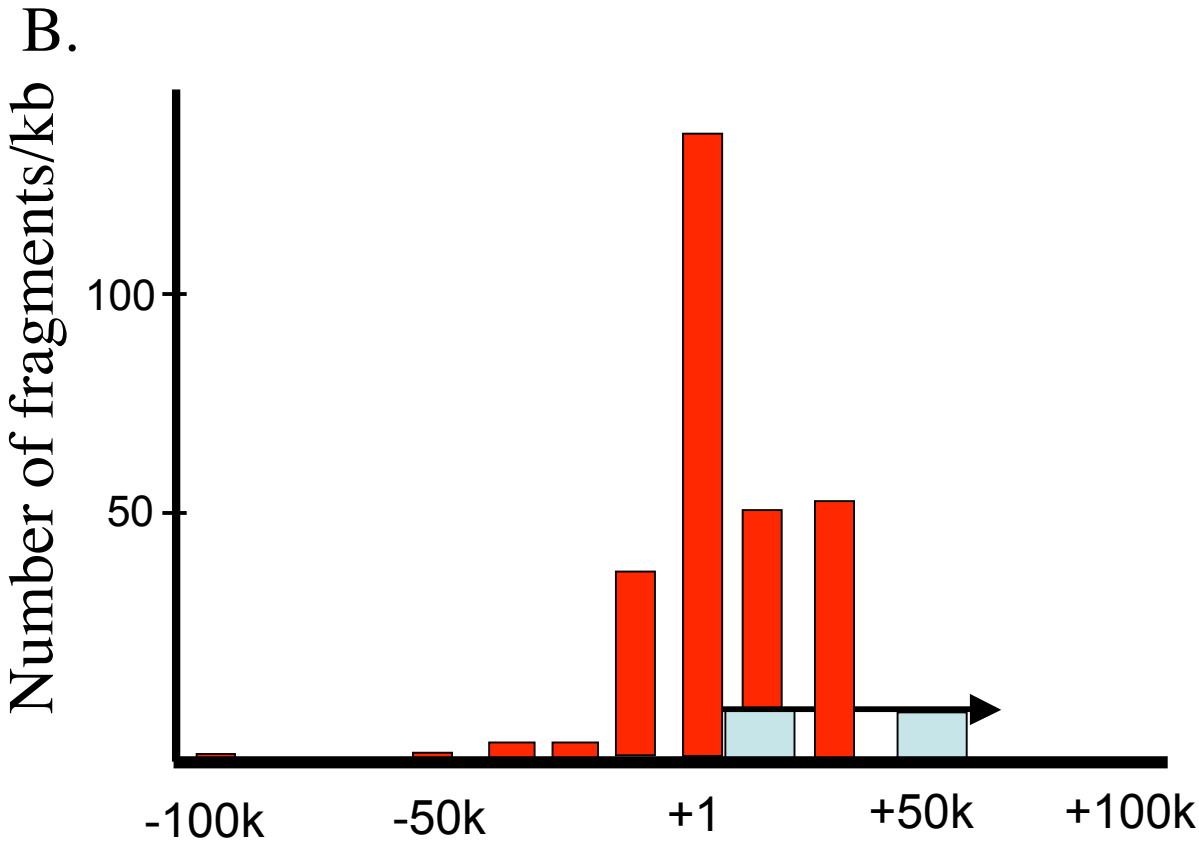


Fig. 13. Correlation between the kinetics of ERα-binding to estrogen response genes and their expression patterns

When the estrogen-induced uterine expression patterns of the genes identified by ChIP-mediated target cloning were examined by reviewing previously acquired microarray data, a number with apparent EREs within 5 kbp of the transcription start site were found to be upregulated by estrogen. Six of these were examined by conventional ChIP analysis to confirm that the EREs putatively identified by ChIP-mediated target cloning were indeed bound by ERα in the estrogen-treated uterus (A). The microarray data showing the temporal estrogen-induced expression patterns of these genes in the uterus are shown in (B). The kinetics of ERα binding to the EREs and gene expression matched for all of the genes examined: both were upregulated 1 h after estrogen injection, and then declined.

Fig. 13.

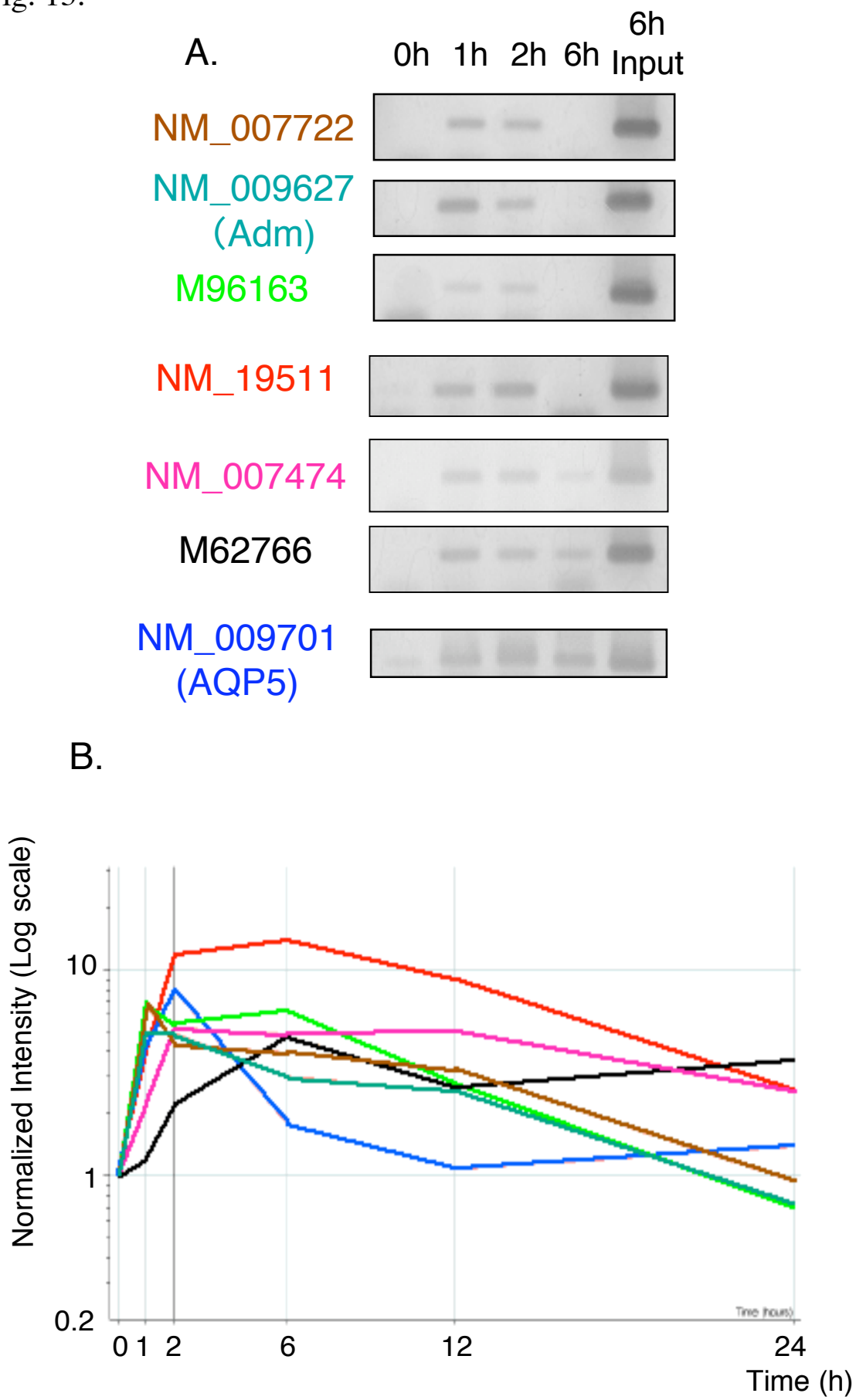
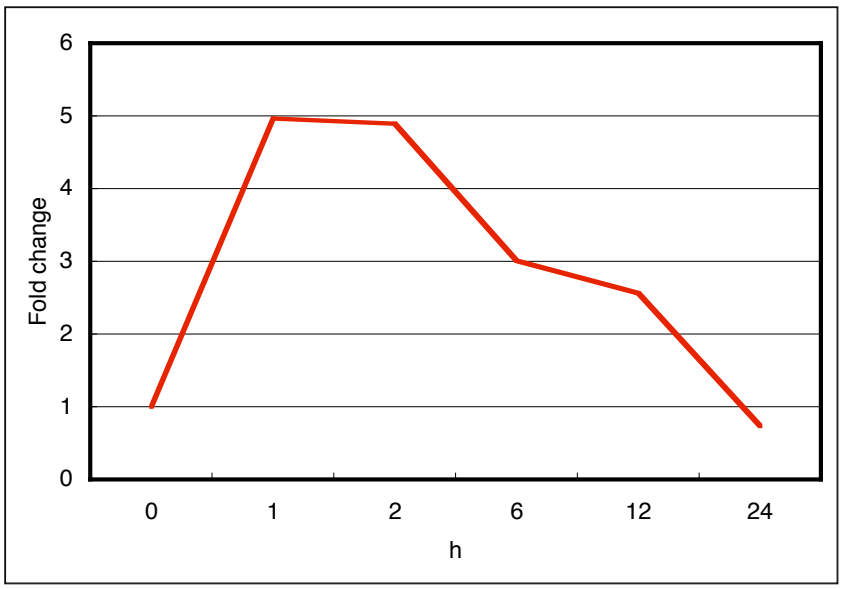

A.
0h
1h
2h
6h
6h Input
NM_007722
NM_009627 (Adm)
M96163
NM_19511
NM_007474
M62766
NM_009701 (AQP5)
B.
Normalized Intensity (Log scale)
10
1
0.2
0 1 2
6
12
24
Time (h)

Fig. 14. The *Adm* gene is directly regulated by ERα

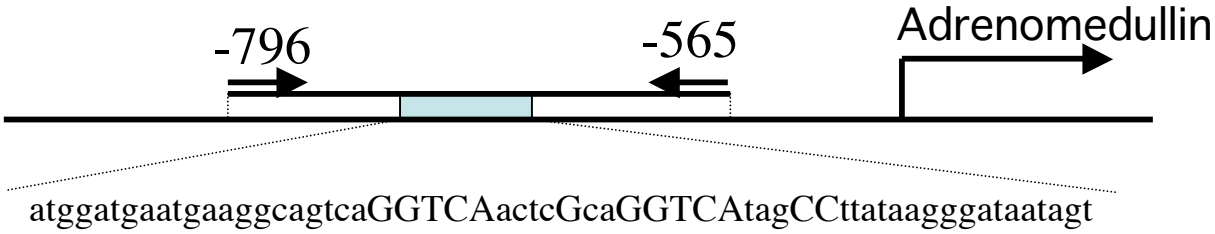
The *Adm* gene is upregulated in the uterus 1 h after injecting estrogen, after which ist expression levels dropped (A). Two ERE-half sites were detected about 700 bp upstream of the *Adm* gene (B) and conventional ChIP analysis revealed that ERα does bind to this region of the *Adm* promoter (C). Abs; antibodies.

Fig. 14.

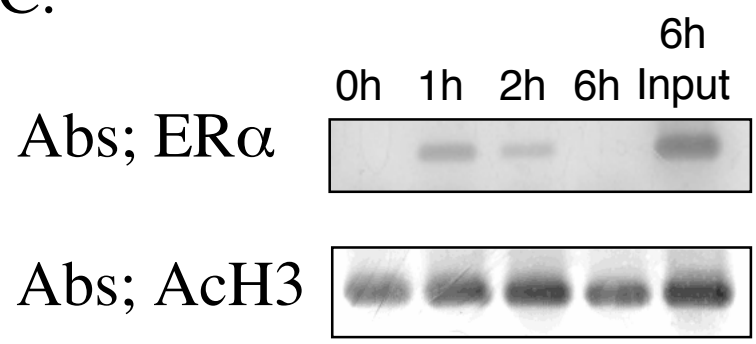
A.


6
5
4
3
2
1
0
Fold change
0
1
2
6
12
24
h

B.


-796
-565
Adrenomedullin
atggatgaatgaaggcagtcaGGTCAactcGcaGGTCAtagCCttataagggataatagt

C.


0h
1h
2h
6h
6h
Input
Abs; ERα
Abs; AcH3

IV. Chapter 2

Aquaporin 5 is Regulated Directly by Estrogen in the Mouse Uterus

Introduction

A prominent effect of estrogen is water imbibition in the uterine endometrium (Fig. 1). This changes the uterine environment in the peri-implantation period, thus making it hospitable for implantation. The water transport required for water imbibition is regulated in particular by water channels termed aquaporins (AQPs). The AQPs are a family of transmembrane channel proteins that have six transmembrane domains. Thirteen AQPs (AQP0-AQP12) have been identified in mammals (Table 2).

Chapter 1 describes the ChIP-mediated target cloning method that I developed and applied to identify the genes in the uterus that are directly regulated by estrogen/ERα binding. One of these genes was *Aquaporin5 (AQP5)* (Fig. 13), which is located in mouse chromosome 15. AQP5 is an apical plasma membrane water channel that has been found in the salivary glands, lacrimal glands, and airway epithelium and that plays an important role in the fluid secretion by these tissues (Table 2). The translocation of AQP5 to the apical plasma membrane in parotid glands is induced by calcium and

NO (Ishikawa et al,2006) (Fig. 15).

AQP5 expression in the mouse uterus has been detected previously in the glandular epithelium (Richard et al, 2003). AQP1, 2, 3, 4, and 8 have been found to be expressed in the uterus as well, as determined by protein and RNA expression studies (Table 2) (Offenberg *et al.* 2000; Jablonski *et al.* 2003; Richard *et al.* 2003). These observations together suggest that the AQP family is involved in the morphological transformations of the uterus that take place during the reproductive cycle.

Estrogen treatment of ovariectomized mice has been found to upregulate AQP5 expression in the uterus (Richard et al, 2003), and similar observations have been made for AQP2 (Jablonski *et al.* 2003). However, it remains unclear whether the *AQP* family genes are directly regulated by ERα.

In this Chapter, I confirm that the *AQP5* gene is directly regulated by ERα and that its activity in the uterus depends on E2 stimulation. In contrast, none of the other *AQP* family genes are directly regulated by ERα.

Materials and Methods

Animals

Female C57BL/6J mice were housed, ovariectomized and injected with E2 or sesame oil as described in Chapter 1. The whole uterus was collected immediately (0 h) or 1, 2, 6, 12 or 24 h later. All animal experiments were approved by the institutional Animal Care Committee.

ChIP-mediated target cloning

The method was described in Chapter 1.

ChIP-PCR analysis

Mouse uterine genomic DNA was prepared and sonicated as described above. The sonicated samples were pre-cleared with protein G Sepharose for 1 h at 4 ºC, then incubated with either 10 mg anti-ERa polyclonal rabbit antibody (Santa Cruz, CA) or 10 mg anti-acetylated histone H3 (Lys9) antibody (Cell Signaling, MA) overnight

at 4 ºC. The samples were precipitated with protein G Sepharose, washed 3 times with RIPA buffer (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1 mM EDTA [pH 8.0], 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate) and 3 times with 1 M NaCl in RIPA buffer and twice with LiCl wash buffer (0.25 M LiCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate) and twice with TE buffer. Then samples were recovered by incubation in elution buffer (0.1 M sodium bicarbonate, 1% SDS). Cross-linking was reversed by incubation for 6 h at 65 ºC, followed by incubation with proteinase K for 4 h at 45 ºC. Samples were extracted with phenol/chloroform and ethanol precipitated. In general, 1/30th of the precipitated DNA was used in a PCR amplification reaction. PCR amplification was performed in the presence of 0.1 nM primers, 0.2 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 1 x PCR buffer and 1 U of AmpliTaq Gold (PerkinElmer Japan, Tokyo, Japan) in 20 mL of reaction buffer. After 35 amplification cycles, the PCR products were analyzed by agarose electrophoresis. As a negative control, the same experiments were

performed with IgG. The primer sequences used to amplify the precipitated DNA are listed in Tables 3 and 4.

Preparation of labeled cRNA and microarray analysis

Total uterine RNA was extracted by using TRIzol reagent (Invitrogen, Tokyo, Japan) and the RNeasy total RNA purification kit (Qiagen, Tokyo, Japan). cRNA probes were prepared from purified RNA by using a CodeLink Expression assay reagent kit (Amersham Bioscience K.K., Tokyo, Japan). The amplified cRNA (10 mg) was hybridized to oligonucleotide DNA microarrays (CodeLink Uniset Mouse I Bioarray, Amersham Bioscience K.K., Tokyo, Japan), which were scanned and processed using a GenePix 4000B scanner and GenePix Pro software (Axon Instruments, Union City, CA), respectively. To confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, the experiment was repeated independently at least twice. The expression data were analyzed by using GeneSpring software (Agilent Tech. Japan, Tokyo, Japan).

Quantitative real time-PCR

cDNA was synthesized from total RNA purified using Superscript II RT(-) (Invitrogen, Tokyo, Japan) and random primers at 42ºC for 60 min. Quantitative PCR reactions were performed by using primers designed for amplifying short (<100 bp) PCR products (Tables 3 and 5) and the PE Prism 7000 sequence detector (Applied Biosystems, Tokyo, Japan), SYBR-Green PCR Core Reagents (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. The PCR conditions were as follows: 50ºC for 2 min, 95ºC for 10 min, and 40 cycles of 95ºC for 15 sec and 60ºC for 1 min in 15 ml volumes. RNA equivalents for each sample were obtained by standardizing the ribosomal protein L8 (L8) levels.

Plasmid construction

To generate pERαcDNA3.1, a DNA fragment encoding mouse ERα was amplified from uterine cDNA by using the primers 5'-GGCGAATTCATGACCATGACCCTTCACAC-3' and 5'-

GCAGTCGACTCAGATCGTGTTGGGGAAGC-3'. This fragment was digested with *EcoR*I and *Sal*I and then ligated into pcDNA3.1(+) (Invitrogen, Tokyo, Japan).

To generate reporters bearing the 5' flanking regions of AQP genes, these regions were amplified by using the primers listed in Table 5. The fragments were then digested with *Mlu*I and *Bgl*II and ligated into pGL2-basic (Promega, Tokyo, Japan). To generate the shorter *AQP5* reporters p103AQP5-luc and p13AQP5-luc, their inserts (-103 to +74 and -13 to +74 of the *AQ5* 5' region, respectively), were amplified by using the forward primers 5'-cgacgcgtTGGGTGAGACCGACCGGGTCAAGATG-3' and 5'-cgacgcgtAAAGGCCGGCCGGAGAGGGA-3', respectively. To generate the mutated *AQP5* ERE reporter pm103AQP5-luc, the primer 5'-cgacgcgtTGGGTGAGACCGACCGaaTCAAGATGCTCC-3' was used for amplification.

Luciferase reporter assay

HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. For ligand-dependent transcription assays, the cells were seeded in 24-well tissue culture plates and grown in phenol red-free DMEM supplemented with 5% charcoal/dextran-treated fetal calf serum (JRH Biosciences, Inc. Lenexa, KA). HEK293T cells were transfected by using FuGene6 (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer's instructions and treated the following day with ethanol or 10 nM E2. Typically, the cells were transfected with 0.3 μg luciferase reporter plasmid, 50 ng pERαcDNA3.1 (ERα expression plasmid) and 50 ng pRL-TK reporter plasmid (cDNA encoding *Renilla* luciferase downstream of the thymidine kinase promoter) (Promega, Tokyo, Japan). Luciferase values were normalized to the *Renilla* luciferase activity.

Results

AQP5 is a target of ERα

As described in Chapter 1, ChIP-mediated target cloning

identified a number of ERα-binding DNA fragments in the mouse uterus that had been treated for 1 h with estrogen. One of these mapped to Chr:15 99647562-99647991, which is only 6 bases upstream of *AQP5* (Chr:15 99,647,997 - 99,652,041; Fig. 16). While this fragment did not contain a canonical ERE, a highly-conserved motif was found upstream between -85 and -73 (Fig. 16). To confirm that ERα binds to this putative ERE, we performed a conventional ChIP using primers that amplify each of the seven regions of the coding and 5' flanking and coding areas of *AQP5* shown in Figure 17A (Table 3). Only the regions that contained the putative ERE and its adjacent sequences (regions 4 and 5) could be precipitated from estrogen-treated mouse uteri (Fig. 17B). Sesame oil-treated uteri and 0 h estrogen-treated uteri did not show ERα binding to this ERE (data not shown and Fig. 17C). Similar results were obtained by using monoclonal anti-ERα antibodies (data not shown). Temporal analysis revealed that ERα binding to this ERE was detected 1 h after estrogen administration and continued for at least 6 h (Fig. 17C). Thus, the putative ERE at -85/-73 upstream of the *AQP5* transcription

start site appears to participate in the estrogen response of the uterus.

In contrast, all DNA fragments could be precipitated with anti-acetylated histone H3 antibody (anti-AcH3). Modification of the N-terminal tails of histones H3 and H4 plays an important role in chromatin formation (Richards & Elgin 2002). That Lys9 of H3 was acetylated indicates that the chromatin of the 5' flanking region of *AQP5* is active. In contrast, no PCR product was detected from samples precipitated with IgG (data not shown).

The ERE in the 5' flanking region of *AQP5* is functional

To determine whether the putative *AQP5* ERE plays a functional role in estrogen-dependent transcriptional activation, we constructed three luciferase reporter constructs that lack the -611/+74, -103/+74, or -13/+74 5' flanking regions of *AQP5* (p611AQP5-luc, p103AQP5-luc and p13AQP5-luc, respectively) (Fig. 18A). HEK293T cells were transformed with these reporters together with the pERαcDNA3.1 plasmid and cultured with or without estrogen.

The positive control reporter that contains 3 canonical EREs was activated by estrogen (Fig. 18B). The two constructs containing the putative ERE (p611AQP5-luc and p103AQP5-luc) were also activated by estrogen, unlike the construct lacking the putative ERE (p13AQP5-luc) (Fig. 18B). The p103AQP5-luc construct that had a mutation in the ERE (pm103AQP5-luc; GGTCA->aaTCA) was also not activated by estrogen (Fig. 18B). Thus, the ERE element in the 5' region of *AQP5* that bound by ERα is functional.

Effect of estrogen treatment on the transcription of other *AQP* genes

DNA microarray analysis of mouse uteri 1 h after treatment revealed a >2-fold up- or down-regulation in 216 and 204 genes, respectively. As *AQP5* is located between *AQP2* and *AQP6* (Fig. 16), we examined whether the other *AQP* genes were also affected by estrogen. At 1 h post-treatment, expression of *AQP8* was activated as well as *AQP5* (Fig. 19). However, *AQP2* and *AQP6,* which flank *AQP5,* were not activated by estrogen; nor were *AQP1*, *3*, *4*, *7* or *9* (Fig. 19). These observations support those of Richard *et al*.

(Richard *et al.* 2003), who found the *AQP5* and *AQP8* genes are expressed in uterus and are spatiotemporally regulated during the peri-immplantation period.

To confirm these patterns, we examined the temporal expression changes of all *AQP* genes by semi-quantitative PCR. Indeed, *AQP5* and *AQP8* were activated within 1 h of estrogen administration and continued to be activated for at least 2 h (Fig. 20). Although *AQP5* activation declined after 6 h, substantial amounts of its transcript were still present at 24 h (Fig. 20). The other *AQP* genes, including *AQP2* and *AQP6*, were not activated by estrogen.

AQP genes other than *AQP5* are not directly activated by ERα

We found weak ERE motifs in the <1 kb proximal promoter regions upstream of *AQP8* and similar motifs were identified upstream of the other *AQP* genes (Table 4). To determine whether these motifs are functional EREs, we constructed reporters containing 1 kb of the sequences upstream of the *AQPs* (Table 5). HEK293T cells were transfected with these reporters along with pERαcDNA3.1

and treated with or without estrogen. Apart from *AQP5*, none of the *AQP* genes were activated by estrogen (Fig. 21), including *AQP8*.

To determine whether the weak EREs could be recognized by ERα in the uterus, we performed ChIP analysis using anti-ERα and PCR primers were designed to amplify putative ERE sequences in the 5' flanking region of each *AQP* gene (Table 5). The only DNA fragment that could be amplified by PCR contained the ERE of *AQP5* (Fig. 22). Thus, it is unlikely that ERα binds to the flanking region of other *AQP* genes despite their presence of half EREs.

Discussion

Aquaporin expression in the uterus

Estrogen has marked effects on the uterus. Within 1-2 h of estrogen administration, uterine wet weight begins to increase *via* water imbibition. While vascular endothelial growth factors are believed to play crucial roles in this process (Cullinan-Bove & Koos

1993), how the AQP water channels that transport the water contribute to this process remains poorly understood. However, AQPs have been found to play an important role in the peri-implantation stage and it is known that *AQP1,4* and *5* are expressed at that stage (Li *et al.* 1994; Jablonski *et al.* 2003; Richard *et al.* 2003; Lindsay & Murphy 2004).

In this study, we demonstrated that ERα regulates *AQP5* directly. However, by using *in situ* hybridization, Richard *et al*. identified *AQP1*, not *AQP5*, as the estrogen response gene(Richard *et al.* 2003). This discrepancy may be due to differences in the methods of detection used; in general, quantitative PCR is more accurate for evaluating changes in mRNA than *in situ* hybridization. However, Jablonski *et al*. also did not detect expression of *AQP5* at the protein level in the uterus (Jablonski *et al.* 2003), although a recent study of the mouse cervix did detect the expression of *AQP5* by northern hybridization (Anderson *et al.* 2006). Again, these discrepancies may be due to differences in experimental design. Nevertheless, my study clearly shows by both DNA microarray and

quantitative PCR analyses that *AQP5* is an estrogen target gene in the uterus. Since water imbibition is a prominent and early effect of estrogen, *AQP5* may play a critical role in this process.

Estrogen responsiveness of the other aquaporins

As indicated in Table 5, half EREs could be found in the 5' flanking regions of most *AQP* genes. However, reporter assays and standard ChIP analysis of the estrogen-treated uterus indicates that all of these half EREs apart from that in the *AQP5* promoter are not functional. The possibility that this is because the EREs cannot be accessed by ERα is unlikely since the ERE-containing regions of the promoters were all acetylated on Lys 9 of histone H3.

Although requirement of cis element necessary for ERα binding is still not clear, accumulation and analysis of functional EREs *in vivo* such as this study may be important. It was particularly notable that a functional ERE in the 5' region of *AQP8* could not be identified, since this gene, like *AQP5*, is an estrogen response gene. This suggests either that there is a functional ERE in another region of this

gene or that this gene is activated by a secondary effect of estrogen. Supporting the latter possibility is that another direct target of estrogen, the *Adm* gene, is activated 1-2 h after estrogen administration and then shows a decline in expression that almost reaches baseline at 6 h (Watanabe *et al*., 2006) (Fig. 13 and 14); this is very similar to the expression profile of *AQP5* (Fig. 20). In contrast, while *AQP8* expression also peaks at 1 h, its expression thereafter is sustained until 12 h (Fig. 20).

AQP5 is expressed at high levels in the salivary and lachrymal glands (Raina *et al.* 1995), which are target tissues of Sjogren's syndrome, an autoimmune disorder that occurs primarily in females. A relationship between *AQP5* and this syndrome has been reported (Steinfeld *et al.* 2001; Tsubota *et al.* 2001). In addition, a recent study has suggested that estrogen may affect lymphopoiesis (Shim *et al.* 2004). Our discovery of a functional ERE in the promoter region of *AQP5* suggests that aberrant estrogen responses of AQP5 in the salivary and lachrymal glands may be participating in the immunopathology of Sjogren's syndrome.

Table 2. Function and expression patterns of the AQPs

The AQPs belong to a family of small, hydrophobic, integral membrane proteins. Thirteen members have been identified in the mouse. They are expressed in many epithelia and endothelia involved in fluid transport, as well as in cell types that are thought not to perform fluid transport, such as skin, fat and urinary bladder cells.

Table 2.

AQP	Permeability	Tissue expression	Cell types
AQP0	?	Eye lens fiber cells	fiber cells
AQP1	Water	Kidney tubules, endothelia, erythrocytes	Endothelial Myoepithelial
AQP2	Water	Kidney collecting duct	Ductal
AQP3	Water, glycerol	Kidney collecting duct, airway epithelium	Acinar
AQP4	Water	Astroglia, Kidney collecting duct, airways	Ductal
AQP5	Water	Glandular epithelia, corneal epithelium. Alveolar epithelium, gastrointestinal tract	Ductal Acinar
AQP6	Chloride?	Kidney collecting duct intercalated cells	?
AQP7	Water, glycerol	Adipose tissue, testis, kidney proximal tubules	?
AQP8	Water	Liver, pancreas, intestine, salivary gland,testis	Myoepithelial
AQP9	Water, small solt.	Liver, white blood cells, testis, brain	?
AQP11	?	Liver, Kidney	?
AQP12	?	Pancreatic acinar cells	?

Fig. 15. Function of AQP*5* in the rat parotid gland

AQP5 is an apical plasma membrane water channel in salivary glands, lacrimal gland, and airway epithelium and plays an important role in the fluid secretion characterizing these tissues. The translocation of AQP5 protein to the apical plasma membrane in parotid glands is induced by calcium (Ca^{2+}) and nitric oxide (NO). RyR, Ryanodine receptor; PIP2, phosphatidylinositol (4,5)-bisphosphate; IP3, inositol (1,4,5)-triphosphate; IP3R, IP3 receptor; CaMK II, calmodulin-dependent kinase II; PKG, cGMP-dependent protein kinase; PLC, phospholipase C; MLCK, myosin light chain kinase.

Fig. 15.

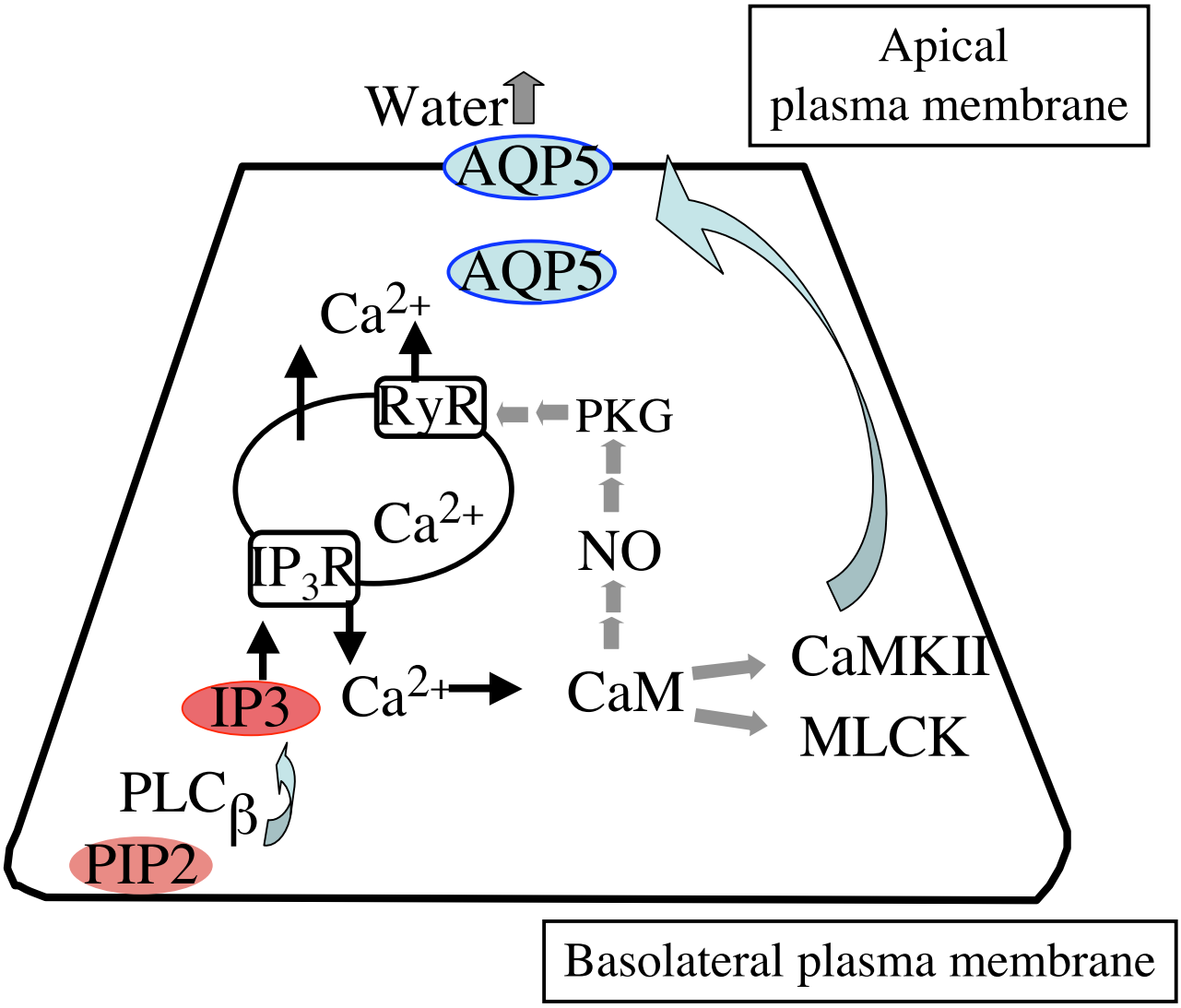


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Fig. 16. Genome map around *AQP5*

The locations of *AQP2*, *AQP5* and *AQP6* on chromosome 15 are mapped. The ERE scores were calculated by using the PATSER program (Hertz and Stormo, 1999). The putative ERE sequence in the 5' flanking region of *AQP5* detected by ChIP-mediated target cloning is indicated.

Fig. 16.

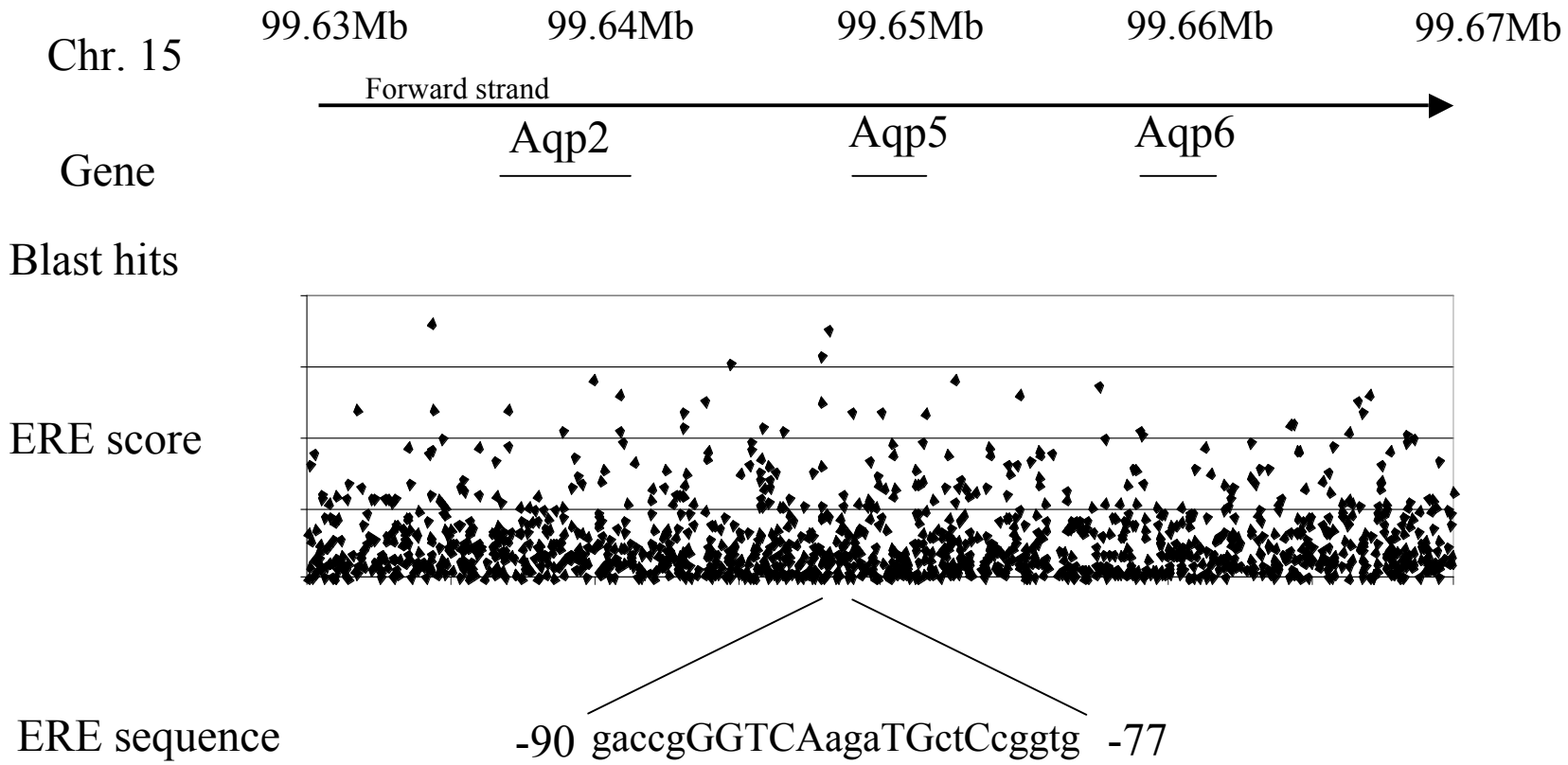

Chr. 15
99.63Mb
99.64Mb
99.65Mb
99.66Mb
99.67Mb
Forward strand
Gene
Aqp2
Aqp5
Aqp6
Blast hits
ERE score
ERE sequence
-90 gaccgGGTCAagaTGctCcggtg -77

Fig. 17. Standard ChIP of the 5' flanking region of *AQP5*

A. The 5' region of *AQP5* is schematically depicted to show which regions were examined by PCR following ChIP (regions numbered 1-7). The transcriptional initiation site and exons are indicated by an arrowhead and solid black boxes, respectively. Note that the putative ERE detected by ChIP-mediated target cloning is located 6 bp up from the transcription start site.

B. Standard ChIP analysis of the 5' regions of *AQP5* numbered from 1-7. ChIP was performed by using anti-ERα or anti-AcH3 antibodies on samples of uterine tissue that were prepared 1 h after injecting estrogen.

C. Analysis of ERα-binding to the *AQP5* promoter region over time after estrogen injection. Standard ChIP was performed on uterine tissue samples harvested 0, 1, 2 and 6 h after estrogen injection. The DNA fragments were precipitated with anti-ERα antibody and then amplified by PCR using primers for region 5 shown in (A), which flank the ERE region.

Fig. 17.

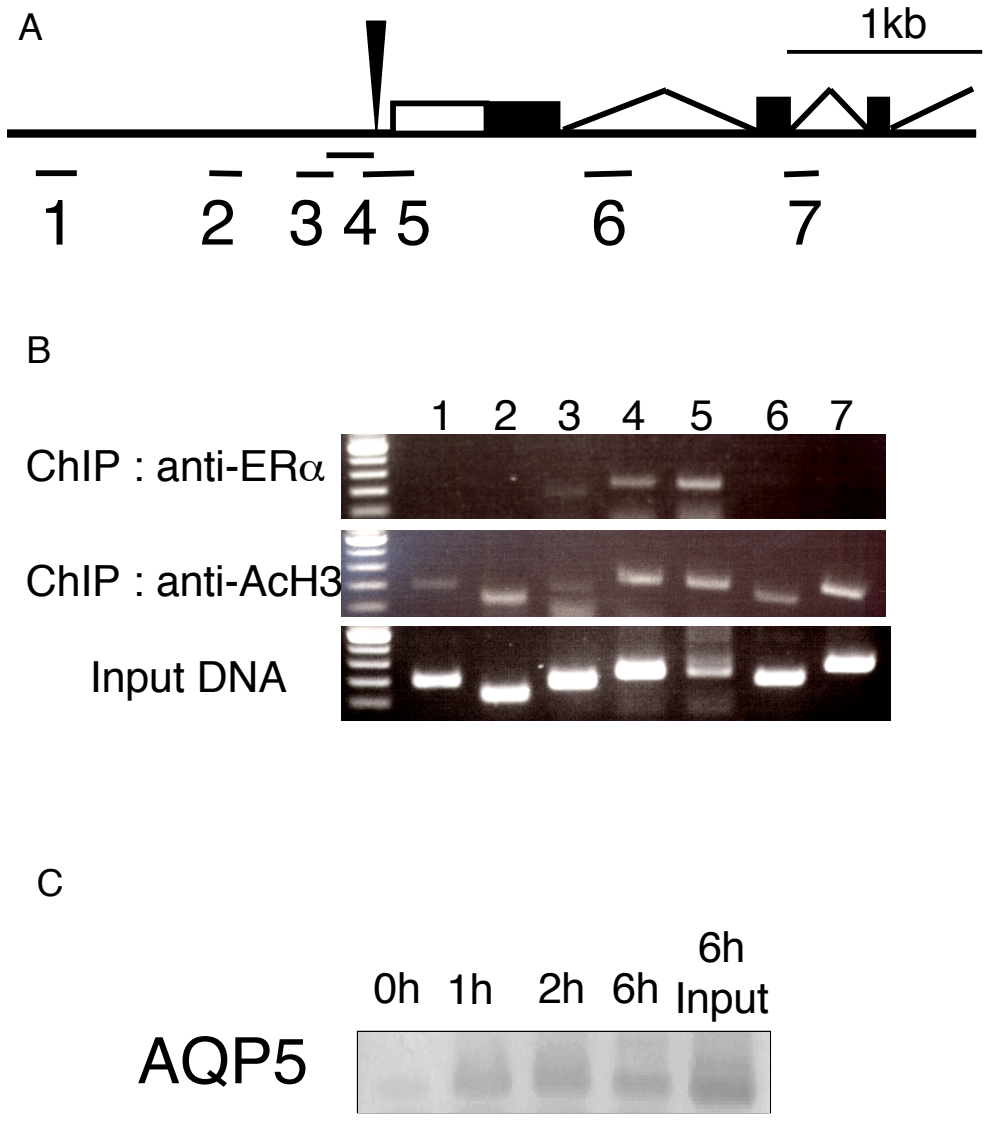
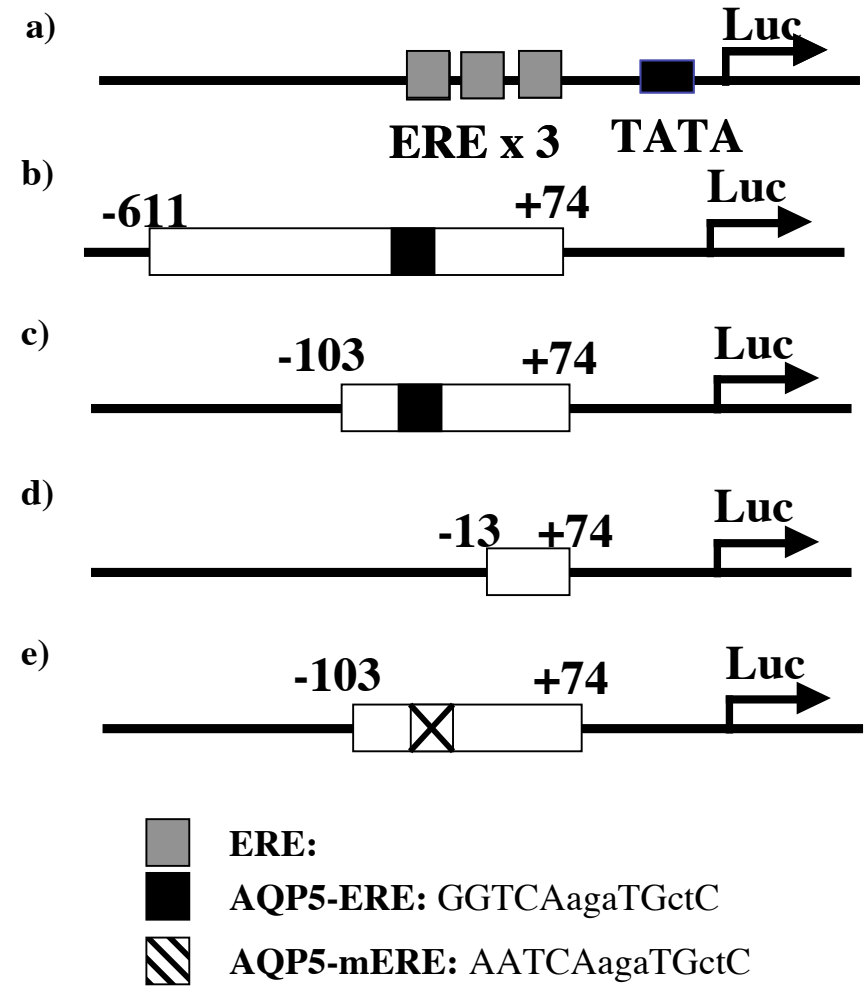

A
1kb
1
2
3
4
5
6
7
B
1 2 3 4 5 6 7
ChIP : anti-ERα
ChIP : anti-AcH3
Input DNA
C
0h 1h 2h 6h 6h Input
AQP5

Fig. 18. Presence of a functional ERE in the 5' flanking region of *AQP5*

A. Luciferase reporter constructs. The positive control (a) contains 3 canonical ERE elements (gray boxes). One construct has the -103/+74 region of the 5' flanking region of *AQP5* (b, p611AQT5-luc; c) while the other two carry deletions of this region (p103AQT5-luc; and d, p13AQP5-luc). The fifth construct contains a mutation of the putative ERE sequence (e, pm103AQP5-luc). The black boxes indicate the putative ERE motif in the *AQP5* promoter region (-86GGTCAagaTGctC-74). The crossed box indicates a mutation in the putative ERE motif (AATCAagaTGctC).

B. The response of 5' flanking regions of *AQP5* to estrogen stimulation. HEK293 cells were transfected with the luciferase reporter constructs shown in (A) along with an ERα expression vector and a control plasmid and then treated with or without estrogen. The luciferase intensities detected in the presence of estrogen were divided by those in the absence of estrogen and the relative differences are shown.

Fig. 18. A.


a)
Luc
ERE x 3
TATA
b)
-611
+74
Luc
c)
-103
+74
Luc
d)
-13
+74
Luc
e)
-103
+74
Luc
ERE:
AQP5-ERE: GGTCAagaTGctC
AQP5-mERE: AATCAagaTGctC

B.

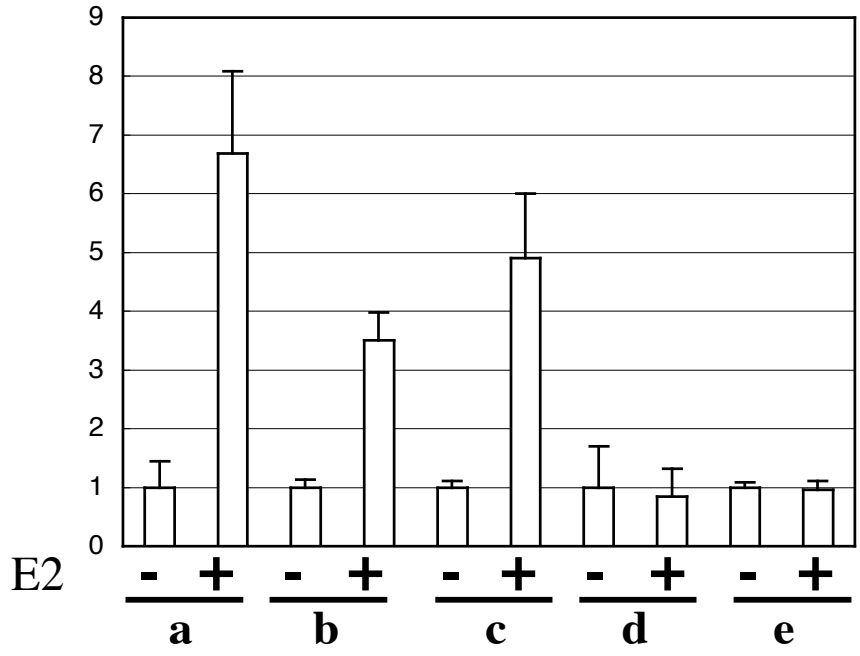

9
8
7
6
5
4
3
2
1
0
E2
- + - + - + - + - +
a
b
c
d
e

Fig. 19. DNA microarray analysis of the expression profiles of the *AQP* genes

Gene expression levels (intensity of fluorescence) of the *AQP* genes determined 1 h after the administration of estrogen (X-axis) compared with those at 0 h (Y-axis). The *AQP* genes on the DNA microarray are indicated.

Fig. 19.

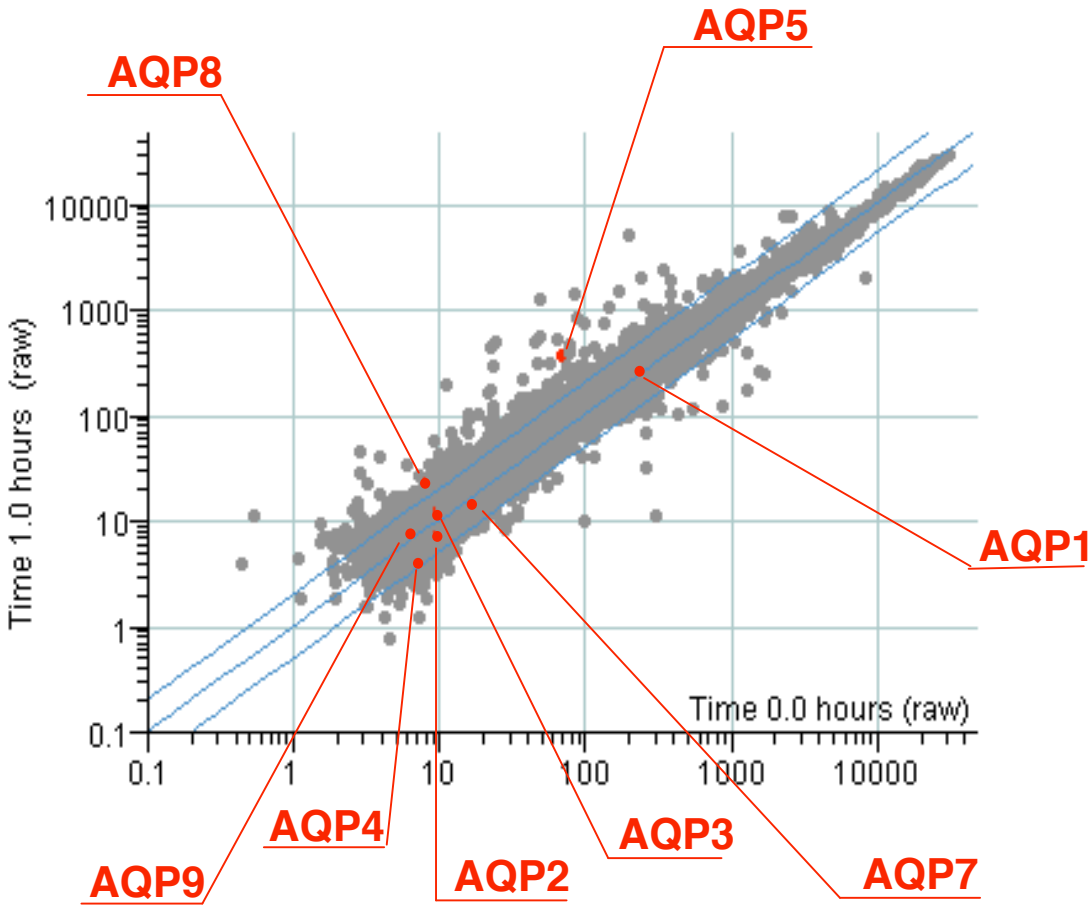

AQP8
AQP5
AQP1
AQP4
AQP9
AQP2
AQP3
AQP7
Time 1.0 hours (raw)
Time 0.0 hours (raw)
10000
1000
100
10
1
0.1
0.1
1
10
100
1000
10000

Fig. 20. Temporal changes in *AQP* gene expression after estrogen treatment

Total RNAs were prepared from mouse uteri at 0, 1, 2, 6, 12 and 24 h after estrogen administration and the expression levels of *AQP* genes were estimated by using quantitative PCR. The changes relative to expression at 0 h are indicated using a log scale.

Fig. 20.

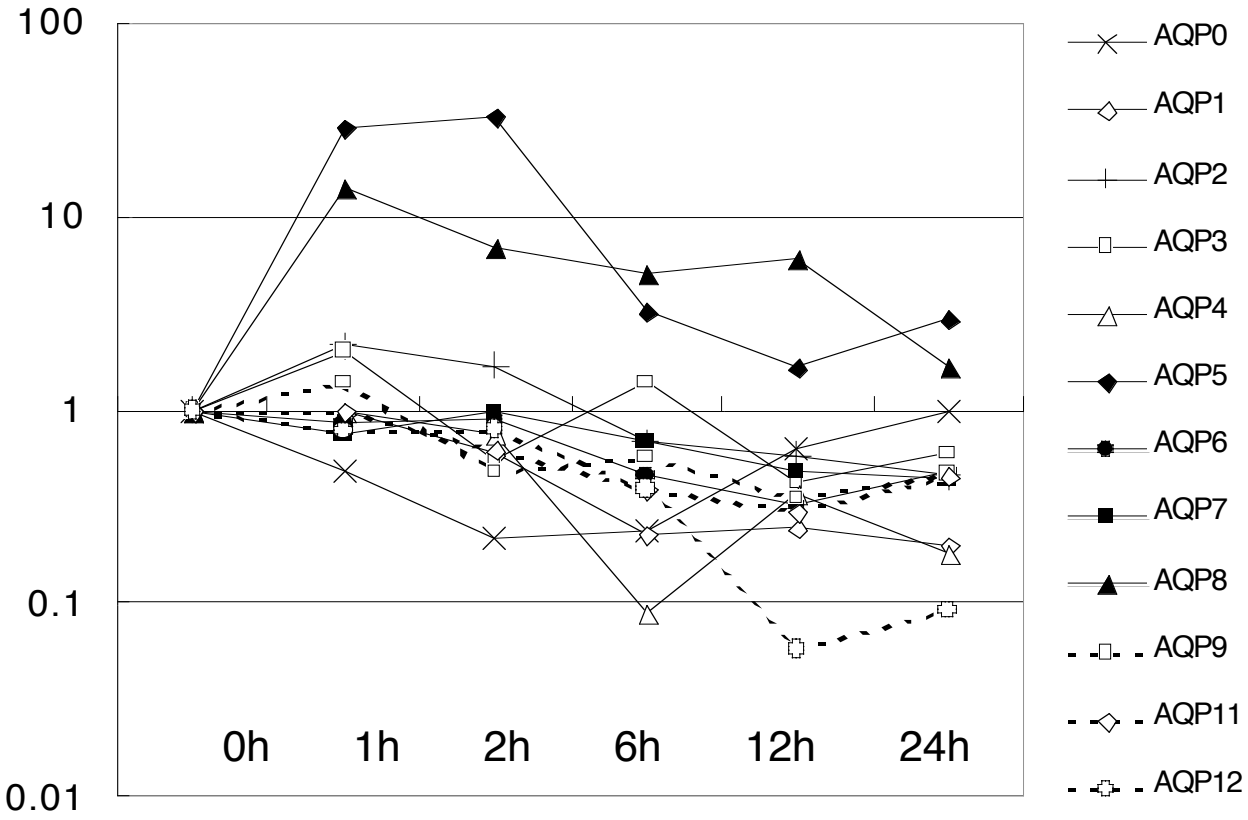

100
10
1
0.1
0.01
0h
1h
2h
6h
12h
24h
AQP0
AQP1
AQP2
AQP3
AQP4
AQP5
AQP6
AQP7
AQP8
AQP9
AQP11
AQP12

Fig. 21. Luciferase reporter assay of *AQP* genes

Estrogen activation of *AQP* genes was examined by using luciferase reporter constructs containing the 5' flanking region of each gene (Table 5). The luciferase activities of the constructs in the presence (open) or absence (gray) of estrogen are indicated relative to a control reporter. ERE indicates the positive control containing three repeats of the canonical ERE motif.

Fig. 21.

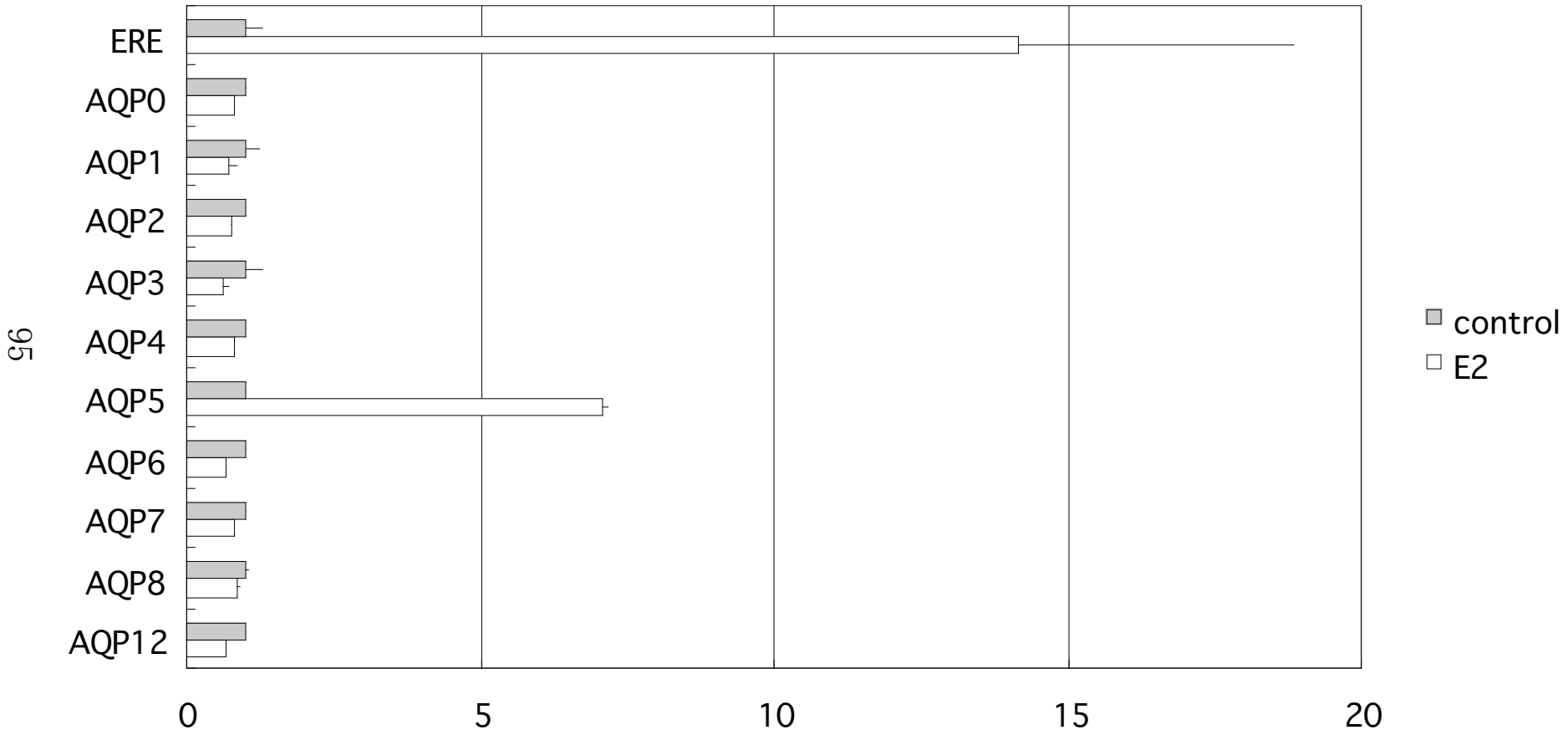

ERE
AQP0
AQP1
AQP2
AQP3
AQP4
AQP5
AQP6
AQP7
AQP8
AQP12
0
5
10
15
20
control
E2
95

Fig. 22. Standard ChIP analysis of the 5' flanking regions of *AQP* genes in the estrogen-treated uterus

Standard ChIP was performed by using anti-ERα or anti-AcH3 antibodies on samples of uterine tissue that were prepared 1 h after estrogen injection. The precipitated DNA fragments were amplified by PCR using primers (Table 4) designed to amplify the putative EREs (Table 4) of each *AQP* gene.

Fig. 22.

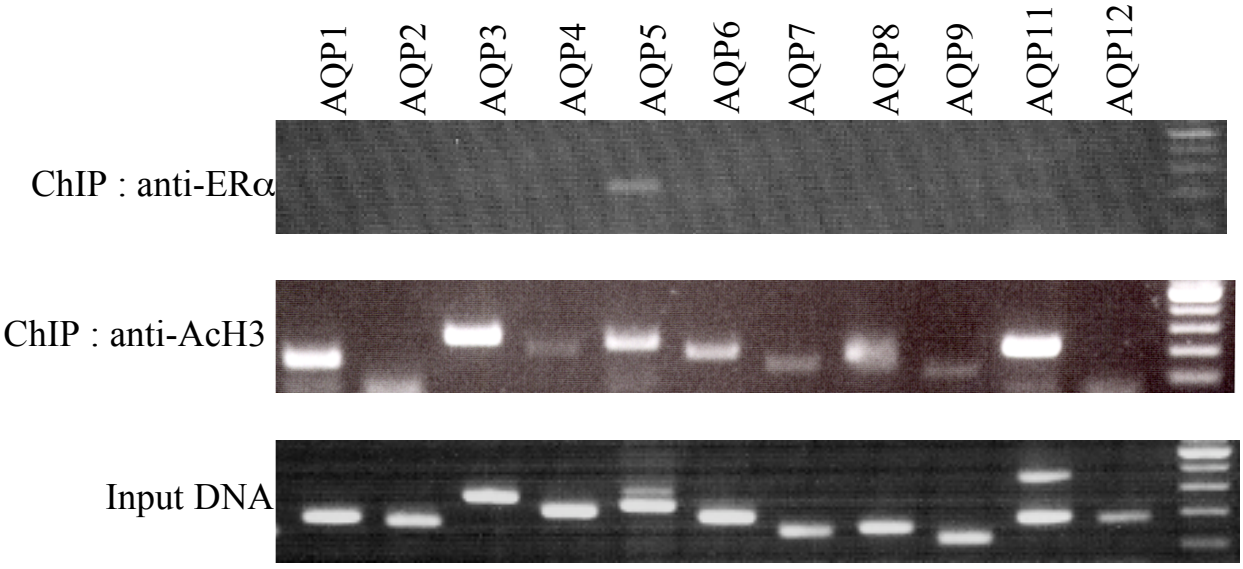

AQP1
AQP2
AQP3
AQP4
AQP5
AQP6
AQP7
AQP8
AQP9
AQP11
AQP12
ChIP : anti-ERα
ChIP : anti-AcH3
Input DNA

Table 3. Primer sequences used for ChIP analysis of *AQP5*

Primers used for the ChIP-PCR assay of AQP5 (Fig. 17).

Table 3. Primer sequences for cloning, quantitative PCR and ChIP analysis

Primer Set	Position	Direction	Primer sequence
1	-1885	F	AATCTGCTTGTCTCTGCCTC
	-1691	R	CCTTCTCTTTCCCAGCTAAC
2	-946	F	GACAAGAGGAAGCTGGGAAC
	-818	R	CCGGCCTATCTCACTTTCTA
3	-506	F	GAAAGACCAACAGGGACAAG
	-318	R	TTCTCAGTGGTAGCCCTTGG
4	-338	F	CCCAAGGGCTACCACTGAGA
	-101	R	ACGGACGGGTCAGAGTGATG
5	-161	F	GGGCGGATAAGGAGCTAAGA
	76	R	GTGCGTGCTGGGCTCTCCTG
6	1026	F	CCTGGCTTCTCTTCACATTC
	1228	R	GTTGCTCCAGACCTCCATCC
7	1996	F	CTCCCCAGCCTTATCCATTG
	2269	R	GTCTCTGTGCTCGCCCTCCC

Table 4. Putative ERE sequences in the 5' region of *AQP* genes and primer sequences used for ChIP analysis

Shown are the highest-scoring putative ERE-like motifs that were identified in the 5' flanking regions (within 1 kb) of the *AQP* genes (Heinemeyer *et al*., 1999).

Table 4. Primer sequences used for ChIP analysis of *AQP5*

Gene	Position	Putative ERE Sequence	Score	Position	Direction	ChIP Primer Sequence
AQP1	-348/-330	TCAGGCAcCCTTGAgCTTT	72	-467	F	TCAGAGTGGGATGGGACAGG
	-810/-828	GAAtGACAGCTgGACCTGA	72	-296	R	GGCCAGAGAAATCCAGGTGT
AQP2	-327/-355	GCtGtGTcCAGTGACCTTC	72	-430	F	CAGAAGAAAGACCATCCAGT
				-268	R	CCAGCCCCCACAATGACCAC
AQP3	-581/-563	TCcAGACgGTGTGACCaGG	76	-617	F	AAGGAGAAAGCCCAGGTATC
				-355	R	TGCGATGACTGGATAGAACA
AQP4	-			-174	F	CTGAAATGCCCTGTGTCTAT
				37	R	AGCTCTGTCACTCATGCCTT
AQP5	-123/-105	TCtcaTCACTCTGACCCGT	78	-163	F	GGGCGGATAAGGAGCTAAGA
	-70/-88	CCgGaGCATCTTGACCCGG	78	74	R	GTGCGTGCTGGGCTCTCCTG
AQP6	-843/-825	AAgAGGCACGGTGACaCTT	70	-945	F	CCAGGTGCAGCCAGGGTTAG
				-748	R	CTTCGGGACCTTGTTCTCAG
AQP7	-714/-696	TCAGcTCcCTCTGACCTCA	83	-751	F	CTGAACCCAGACAAACCATT
				-602	R	TAGGAAAAGTATGCCCAAGG
AQP8	-490/-472	TGcAGGCAGTGTGAgCaGA	71	-569	F	TGCCGATGAAACAGTGAAAG
	-571/-553	AAcAGTGAAAGTGACtCGA	71	-419	R	GACCTACGGGCTTACCTACC
AQP9	+22/+4	ATtAGGAACTGTGACtTAA	72	-57	F	GCAAACAAATAGCAATGAGC
				74	R	ATCTCTGGAGGCGACTAAAG
AQP11	-717/-735	ATtGGGCACCTTtACCTGC	74	-199	F	CGGAGTGTGCAAAGATCAAG
				17	R	AGGGGACAACGGTCTGTAGA
AQP12	-92/-74	ACttGGCtCTGTGACCaAT	76	-105	F	TCCTCTGTGGGTGTTCCTTG
				79	R	TCAGTCTGGGTTCTACAAGG
consensus		NNARGNNANNNTGACCYNN				

Table 5. Primer sequences used for quantitative PCR and reporter construct tpreparation

Shown are the highest-scoring putative ERE-like motifs that were identified in the 5' flanking regions (within 1 kb) of the *AQP* genes (Heinemeyer *et al*., 1999).

Table 5. ERE-like motifs identified in the promoter regions of *AQP* genes

Gene	RefSeq Number	Direction	Position	Q-PCR Sequence	Position	Reporter Sequence
AQP1	NM_007472	F	2246	TCACAGCTGCACACTCGTCTC	-932	cgacgcgtCCACTTTCCACCATCACTCG
		R	2319	TGGGTCCTCAGTGCCCTTAT	28	gaagatctCTCGACTTAACCGCTGGATG
AQP2	NM_009699	F	1237	GGGTGTAAAAGTGCTCGTCCAT	-917	cgacgcgtCTGTGGTAAGGGTGGCTCTG
		R	1287	TCTGCACGTGAGGAAAGAAACA	30	gaagatctGCTCTTCCTCCCTCCCTCTC
AQP3	NM_016689	F	1535	GGCTGAAGTCCAGGTCGTAAGT	-868	cgacgcgtGCACTGCTATGAACGTGTGG
		R	1634	GGAGTTTCCCACCCCTATTCC	66	gaagatctCAACTCCTTCTGTCGACCCA
AQP4	NM_009700	F	1030	GAGGACAGCACTGAAGGCAGA	-939	cgacgcgtTTAAGAGCCAGAGAACCTAC
		R	1097	TCCTTAATGGGTGGCAGGAA	74	gaagatctGTGCTGAGCATTGTTTCCTG
AQP5	NM_009701	F	1527	TTGTGAAGGCAGTGCAAGCT	-611	cgacgcgtCGCAGAAACGCAGGAACACA
		R	1579	CACCCCTTTCTGGGATGGT	74	gaagatctGTGCGTGCTGGGCTCTCCTG
AQP6	NM_175087	F	1436	TGGATCCCTGTCTTGGAGAAA	-998	cgacgcgtAGACAGGGAGGGTGGCATTT
		R	1517	TGGGCTCTGAAGCTCCTTCAT	70	gaagatctGCCAGGAACTCAGCAAAAAG
AQP7	NM_007473	F	2206	TCAAGACAGGGTTTCTCGGTG	-988	cgacgcgtCATGGGTGCAGGCTACAGAC
		R	2294	AGGCAGGCGGATTTCTGAG	63	gaagatctTGTCTTTCAGCCTCCGTCTC
AQP8	NM_007474	F	1220	GGGATTAGAAGGGCTGAGAAGG	-943	cgacgcgtTCAGACAAGAAGCGGCAGAG
		R	1311	GAATTGGGTTCCAAACCCAAC	47	gaagatctTCTAGGTCACAGACTGGAGG
AQP9	NM_022026	F	2061	TGACCTGAGCAAGTTGCCCT	NT	
		R	2140	CAGTCGGCTAGCAAGCTTCTG		
AQP11	NM_175105	F	1110	CAGTCAAGCTGGATGCGACA	NT	
		R	1172	AAGCTGAAGCAGGAGGCGT		
AQP12	NM_177587	F	924	CCGGCAGAAAAGCAAATACC	-981	cgacgcgtTTTGCTTAGCCTGTCCTGTG
		R	1019	ACGGCCCTTTGCCACTACT	54	gaagatctTGGGTTCTACAAGGAGCAAG

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