

# The estrogen-responsive adrenomedullin and receptor-modifying protein 3 gene identified by DNA microarray analysis are directly regulated by estrogen receptor

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## Abstract

Recent studies have revealed that hundreds of genes in the uterus are activated by estrogen. Their expression profiles differ over time and doses and it is not clear whether all these genes are directly regulated by estrogen via the estrogen receptor. To select the genes that may be regulated by estrogen, we treated mice with several doses of estrogen and searched for those genes whose dose–response expression pattern mirrored the uterine growth pattern. Among those genes, we found that the dose-dependent expression of the adrenomedullin (ADM) gene correlated well with the uterotrophic effect of estrogen. ADM expression is induced early after estrogen administration and is restricted to the endometrial stroma. The spatiotemporal gene expression pattern of ADM was similar to that of receptor-modifying protein 3 (RAMP3). RAMP3 is known to modify calcitonin gene-related receptor (CRLR) so that it can then serve as an ADM receptor. Chromatin immunoprecipitation assays indicated that the estrogen receptor binds directly to the ADM promoter region and RAMP3 intron after estrogen administration. It was also shown that neither the ADM nor RAMP3 gene could be activated in estrogen receptor- $\alpha$ -null mouse. Although uterine ADM expression has been reported to occur in the myometrium, our observations indicate that estrogen-induced ADM is also expressed in the uterine stroma and that such variable, spatiotemporally regulated ADM expression contributes to a wider range of biological effects than previously expected.

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## Introduction

The uterus is a major target organ of estrogen and undergoes drastic changes after estrogen administration. Early uterine responses to estrogen include many physiological biochemical changes such as hyperemia, calcium influx, histamine release, eosinophil infiltration, cAMP level changes, enhanced glucose oxidation, and increased RNA and protein synthesis. After DNA synthesis and mitosis, later stage effects include cellular hypertrophy and hyperplasia and the result is the growth of the uterus. This uterotrophic effect of estrogen is a demonstrable phenotype that can be used to estimate the potency of estrogens. Notably, not all the physiological and biochemical changes associated with estrogen treatment are necessary for the uterotrophic effect. For example, while cAMP is elevated after estrogen administration (Szego & Davis 1967), uterine growth is independent of cAMP (Zor *et al.* 1973). This is also true for ornithine decarboxylation and prostaglandins. Thus, it remains unclear which processes are essential for the effects of estrogen in target tissues.

In the level of gene expression, it is also not clear whether estrogen-response genes are directly regulated by estrogen receptor (ER) or not. Recently, the effect of estrogen on the uterus has been studied at the level of gene expression by DNA microarray analysis (Watanabe *et al.* 2002). As a result, hundreds of genes have been listed as being estrogen-responsive and the temporal changes in their expression after estrogen exposure have been analyzed (Watanabe *et al.* 2003). This has vastly aided our understanding of the effect of estrogen on the uterus at the genetic level. However, this new methodology, like the classical methodology, cannot determine whether a particular gene-expression change is the direct effect of estrogen or not.

In our previous study we found that not all estrogen-responsive genes are uniformly activated by estrogen and that their estrogen-dose-dependent, gene-expression patterns are not necessarily identical with the uterotrophic effect of estrogen (Watanabe *et al.* 2003). For example, while the uterotrophic effect of estrogen is directly proportional to the estrogen doses used, some

genes are activated by a low dose of estrogen but this activation is saturated or diminished by higher doses of estrogen. These various estrogen-response patterns are generally attributed to the differences of promoter context but the contribution of ER is not clear. In this study, we selected the adrenomedullin (ADM) and receptor-modifying protein 3 (RAMP3) genes, whose expression patterns correlate closely with the uterotrophic effect, and studied the direct contribution of ER to their expression.

## Experimental procedures

### Animals

Female C57BL/6J mice and ER $\alpha$ - and - $\beta$ -null mice (Dupont *et al.* 2000) were housed under a 12 h:12 h light/darkness cycle. To assess the effect of estrogen on uterine gene expression, mice were ovariectomized at 8 weeks of age and 2 weeks later injected intraperitoneally with estrogen (or sesame oil as a vehicle control; Nakarai Tesque, Kyoto, Japan). The whole uterus ( $n=4$ ) were collected 6 h later. The estrogen used was 17 $\beta$ -estradiol (Sigma-Aldrich Japan, Tokyo, Japan) and it was injected at 0.05, 0.5, 5 or 50  $\mu\text{g}/\text{kg}$  body weight (b.w.). To assess the effects of the different estrogen doses on uterine growth, mice were ovariectomized and 2 weeks later injected intraperitoneally every 24 h for 7 days with the different 17 $\beta$ -estradiol doses or sesame oil. The whole uterus ( $n=5$ ) were then collected and weighed. All animal experiments were approved by the institutional animal care committee.

### Preparation of labeled cRNA and microarray analysis

Total uterine RNA was extracted using TRIzol reagent (Invitrogen, Tokyo, Japan) and cRNA probes were prepared from the purified RNA by using an Affymetrix cRNA probe kit according to the manufacturer's protocol (Affymetrix Japan, Tokyo, Japan). All preparations met the recommended criteria of Affymetrix for use on their expression arrays. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74 A; Affymetrix Japan), and the scanned data were analyzed with GeneChip software (Affymetrix Japan) and processed as described previously (Watanabe *et al.* 2002). To confirm the estrogen-related changes in gene expression revealed by the DNA microarray analysis, we independently repeated the same experiment at least twice. The expression data were analyzed with GeneSpring software (Agilent Tech. Japan, Tokyo, Japan).

### Quantitative real-time PCR

cDNA was synthesized from total RNA purified as described above by using Superscript II reverse transcriptase (Invitrogen) with random primers at 42 °C for 60 min. Quantitative PCRs were performed by using

the PE Prism 7000 sequence detector (Applied Biosystems, Tokyo, Japan), SYBR-Green PCR core reagents (Applied Biosystems) and the appropriate primers according to the manufacturer's instructions. The primers were chosen to amplify short PCR products (<100 bp), and their sequences were as follows: RAMP1 (NM\_016894), 5'-TTTCATTGCGCTCCCCATT-3' and 5'-CCAGACCACCAGTGCAGTCAT-3'; RAMP2 (NM\_019444), 5'-AGTTGCATGGACTCTGTCAAGGA-3' and 5'-TGCCTGCTAATCAAAGTCCAGTT-3'; RAMP3 (NM\_019511), 5'-CCGGATGAAGTACTCATCCCA-3' and 5'-CCACCAGGCCAGCCATAG-3'; ADM (U77630) 5'-ATAAGCCTCATTACTACTTGA-3' and 5'-TTGCACGTTTCCTCGTAGGT-3', and CRLR (calcitonin gene-related receptor; NM\_018782) 5'-CACTCTGATGCTCTCCGCAGT-3' and 5'-GGCTGTACCCTTGCATGTCAC-3'. Gene-expression levels were normalized to the expression levels of NM\_012053 (ribosome L8, Rpl8), whose primer sequences were 5'-ACAGAGCCGTTGTTGGTGTG-3' and 5'-CAGCAGTTCTCTTTGCCTTGT-3'. Gel electrophoresis and melting-curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands.

### In situ hybridization

*In situ* hybridization was performed using PCR fragments containing a portion of the ADM gene (nucleotides 58–586; U77630), the RAMP2 gene (nucleotides 160–706; NM\_019444) or the RAMP3 gene (nucleotides 681–1059; NM\_019511). [ $\alpha$ -<sup>35</sup>S]UTP (Amersham Biosciences, Tokyo, Japan)-labeled antisense and sense RNAs were obtained by using T7 and T3 RNA polymerases and an *in vitro* transcription kit (Stratagene, Funakoshi, Tokyo, Japan). After DNase digestion, the probes were fragmented by alkali hydrolysis. The tissues were embedded in compound (Sakura Fintech Co., Ltd., Tokyo, Japan) and sectioned (10  $\mu\text{m}$ ) using a cryostat. Sections were fixed on slides with 4% paraformaldehyde for 10 min and rinsed in PBS. The slides were then acetylated in 0.1 M triethanolamine with a 1/400 vol. acetic anhydride, rinsed again in PBS, and dehydrated in graded ethanol. After air drying, the hybridization mixture was added. Hybridization was performed at 50 °C overnight with  $5 \times 10^7$  d.p.m./ml probe in 100  $\mu\text{l}$  hybridization solution under coverslips. After the hybridization, the slides were washed and incubated in RNase A solution (20  $\mu\text{g}/\text{ml}$ ) at 37 °C for 30 min, and then dipped in NTB-2 nuclear track emulsion (Kodak, Rochester, NY, USA). After 14 days exposure, the slides were developed and counterstained with hematoxylin. The sections were evaluated and photographed under dark-field illumination using a Zeiss microscope. The dark-field (changed to red) and bright-field images were merged.

## Chromatin immunoprecipitation

Mouse uteri were fixed with 1% formaldehyde and homogenized using phycotron (NS-310E; Microtec, Chiba, Japan) in PBS containing 0.125 M glycine. The samples were then centrifuged at 700 g for 5 min at 4 °C and the pellets were incubated with lysis buffer (10 mM Tris/HCl, pH 8.0, 10 mM EDTA, 0.5 mM EGTA and 0.25% Triton X-100) for 10 min. The samples were collected by microcentrifugation, suspended in sonication buffer (10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5 mM EGTA), and sonicated with a Bioruptor sonicator (Cosmo Bio, Tokyo, Japan) to an average length of approximately 500 bp. The samples were precleared by treatment with Protein G–Sepharose for 1 h at 4 °C and then incubated with 10 µg anti-ERα polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-acetylated histone H3 antibody (Cell Signaling, Beverly, MA, USA) overnight at 4 °C. After precipitation by the addition of Protein G–Sepharose the samples were washed five times with RIPA buffer (10 mM Tris/HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS and 0.1% sodium deoxycholate). The precipitated samples were recovered by incubation with elution buffer (0.1 M sodium bicarbonate and 1% SDS). Crosslinks were reversed by incubation at 65 °C for 6 h followed by incubation with proteinase K at 45 °C for 4 h. Thereafter, the samples were extracted with phenol/chloroform and the DNA fragments were precipitated with a 1/10 vol. of NaCl and 2.5 vol. of ethanol. Generally, 1/30 of the precipitated DNA was used for PCR amplification. PCR amplification was performed in the presence of 0.1 nmol primers, 0.2 mM each nucleotide (dATP, dCTP, dGTP and dTTP), 1 × PCR buffer and 1 U AmpliTaq Gold (PerkinElmer Japan, Tokyo, Japan) in 20 µl of reaction buffer. After 35 cycles of amplification the amplified DNA was analyzed by agarose electrophoresis. As a negative control the same experiments were performed with IgG: no amplified DNA was obtained. The primer sequences used to amplify the putative estrogen-response element (ERE) of the ADM gene were (−796) 5′-ATCCTCA CGTTTATGATGGA-3′ (−777) and (−565) 5′-CGG ATTTCGTAATAAGGGCA-3′ (−584). The primer sequences used to amplify the putative ERE of the RAMP3 gene were (13 531 bp from the 5′ end of the first intron) 5′-AGAGTGTACGTGTGGACAGG-3′ (13 550 bp) and (13 735 bp from the 5′ end of the first intron) 5′-CTGTGACAGCAGGAGGACAG-3′ (13 716).

## Results

### The ADM gene expression pattern correlates well with the uterotrophic effect of estrogen

To select genes that may be involved in the estrogen-induced uterine response, we injected ovariectomized

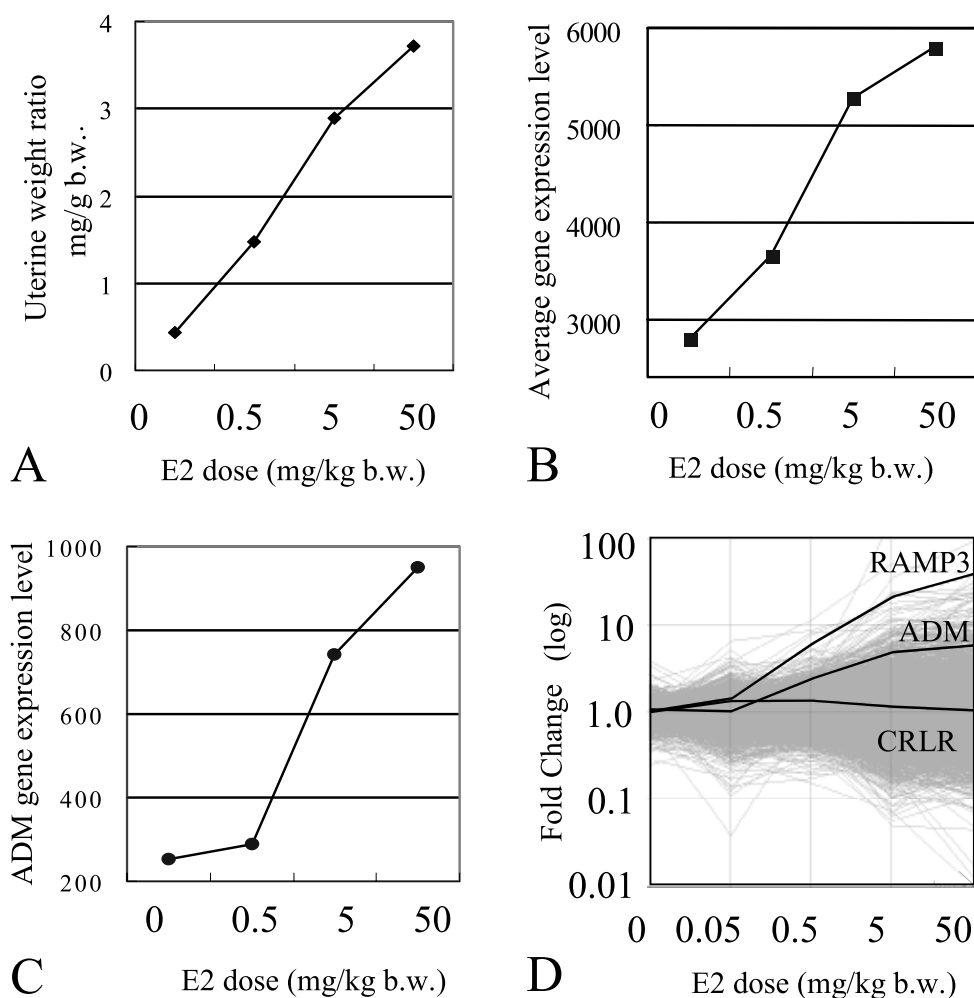
mice with four different doses of estradiol and harvested the uteri 6 h later. The total uterine RNAs were then subjected to DNA microarray analysis, which determined the expression levels of 10 000 genes. The average gene-expression levels were compared with the estrogen dose response of uterine growth (determined by daily injecting ovariectomized mice for a week with the four different estrogen doses and then weighing the whole uteri; Fig. 1A). The genes whose expression levels correlated well with the uterotrophic effect of estrogen were selected.

Of the 10 000 genes examined, 338 had a correlation coefficient of greater than 0.95. The average expression levels of these genes, as determined by the microarray analysis, are shown in Fig. 1B. The 338 selected genes included the ADM gene (Fig. 1C). In addition, the RAMP3 gene displays a similar estrogen-induced expression pattern, as shown by Fig. 1D. This is of interest because RAMP3 modifies the calcitonin gene-related receptor (CRLR), after which CRLR can serve as a receptor for ADM (McLatchie *et al.* 1998). However, CRLR gene expression was unaffected by estrogen treatment (Fig. 1D). The good correlation between ADM and RAMP3 gene expression prompted us to examine the gene-expression regulation of ADM and RAMP3 by estrogen.

### ADM and RAMP3 gene expression are activated early after estrogen administration

To examine the temporal changes in ADM gene expression by estrogen exposure, we injected ovariectomized mice with 5 µg/kg b.w. estradiol, harvested the uteri 1, 2, 4 or 6 h later, and subjected the whole uterine RNA to quantitative PCR to evaluate ADM expression. The experiment was performed three times. While ADM gene expression in the uteri varied quite widely 2–4 h after estrogen administration, it was elevated 1 h after estrogen administration and continued to increase thereafter until around 4 h after estrogen administration, after which ADM expression started to decrease (Fig. 2A). Thus ADM gene expression was induced early after estrogen stimulation. RAMP3 gene expression was also induced immediately after estrogen administration and continued until 4 h after estrogen administration (Fig. 2B).

There is also another modifying protein that confers ADM receptor function onto CRLR, namely RAMP2 (McLatchie *et al.* 1998). However, we found RAMP2 expression was not effectively activated by estrogen (Fig. 2C). Moreover, RAMP1, a third CRLR-modifying protein that modifies CRLR to act as a receptor for calcitonin-gene-related peptide (CGRP; McLatchie *et al.* 1998), was substantially repressed by estrogen (Fig. 2C). Furthermore, CGRP, which binds to receptors bearing RAMP3 or RAMP1 (McLatchie *et al.* 1998), was

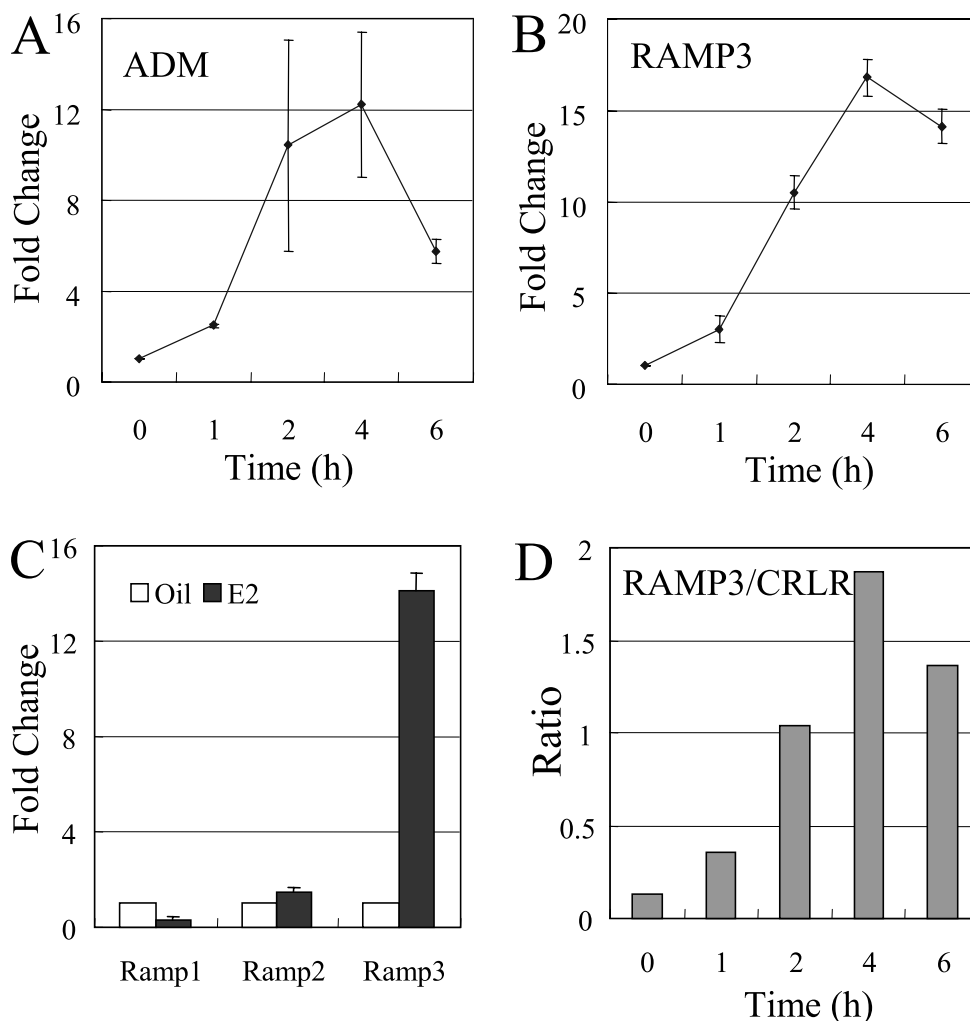


**Figure 1** Correlation between estrogen dose-dependent uterine ADM gene expression and the uterotrophic effect of estrogen. (A) Uterotrophic effect of estrogen. Estrogen dose-dependent uterine growth is indicated by a ratio to body weight. (B) Average gene-expression levels of the selected genes; 338 genes whose expression correlated well with the uterotrophic effect were selected and the average gene-expression levels of the 338 genes at the different estrogen doses (calculated from the fluorescent signal intensity of each gene in the DNA microarray analysis) are indicated. (C) Estrogen-dependent gene activation of ADM gene. The gene-expression level of ADM (one of the 338 genes) is indicated. The x axis indicates the estradiol dose administered while the y axis indicated the gene-expression levels. (D) Estrogen dose-dependent changes in ADM, RAMP3 and CRLR gene expression (solid lines) relative to their expression levels at 0  $\mu\text{g}/\text{kg}$  b.w. estradiol (fold expression). Gene expression of all the 10 000 genes examined is shown in gray. While ADM and RAMP3 gene expression were activated by estrogen, CRLR gene expression was not changed. The x axis indicates the estradiol dose administered while the y axis indicates fold changes in gene expression on a log scale. Note that because of the normalization algorithm, the gray gene expression is not exactly 1.0 but spread around 1.0 at the 0 mg/kg b.w. dose. E2, 17 $\beta$ -estradiol.

expressed in the absence of estrogen at much lower levels than ADM and was unaltered by estrogen administration (data not shown).

To examine how the expression levels of RAMP3 relate to CRLR expression upon estrogen exposure, the ratio of RAMP3 mRNA to CRLR mRNA was calculated from data obtained by quantitative PCR. As

indicated in Fig. 1D, CRLR expression is unaltered by estrogen. Although RAMP3 mRNA levels were one-tenth of those of CRLR before estrogen administration, estrogen induced RAMP3 gene transcription, with the result that RAMP3 and CRLR were expressed at equivalent levels 2 h after estrogen administration, shown by mRNA levels. Although protein-level confirmation is

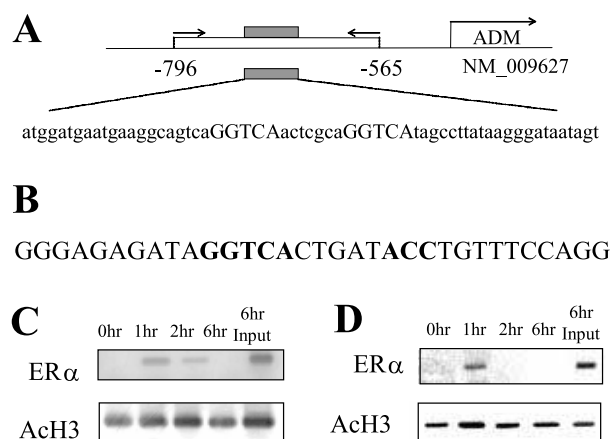


**Figure 2** Temporal changes in ADM and RAMP3 gene expression. Ovariectomized mice were injected with 5  $\mu\text{g}/\text{kg}$  b.w. estradiol and total uterine RNA was isolated 1, 2, 4 and 6 h later. ADM (A) and RAMP3 (B) gene-expression levels were estimated by quantitative PCR. Shown is the change in expression over time relative to the gene-expression level at 0 h (fold change). Uterine RNA was prepared from three independent experiments and average fold changes and errors are indicated. The x axes indicate the time after estrogen administration while the y axes indicate fold changes in gene expression. (C) Of the different RAMP genes, only RAMP3 was strongly activated by estradiol. Total uterine RNA was isolated 6 h after injecting 5  $\mu\text{g}/\text{kg}$  b.w. estradiol and the change in RAMP gene expression relative to expression in the sesame-oil-injected control uteri was estimated by quantitative PCR. Uterine RNA was prepared from three independent experiments and the average fold changes and errors are indicated. (D) Gene expression of RAMP3 relative to CRLR expression mRNA after estradiol administration was determined by quantitative PCR. The ratio of RAMP3 mRNA to CRLR mRNA was calculated on the basis of the copy numbers of RAMP3 and CRLR mRNA. Approximately equal amounts of mRNA were detected 2 h after estrogen administration. The x axis indicates time after estrogen administration while the y axis indicates the ratio of RAMP3 mRNA to CRLR mRNA. E2, 17 $\beta$ -estradiol.

essential, the rapid increase of RAMP3 mRNA may affect the properties of the ADM receptor and it appears that the net effect of estrogen exposure is to convert existing CRLR protein into an ADM receptor.

#### The ER binds to the promoter region of the ADM and RAMP3 genes

Since ADM expression is induced early after estrogen administration, we examined whether the ADM gene



**Figure 3** Chromatin immunoprecipitation of the ADM promoter region. (A) Schematic diagram of the ADM promoter region. The ADM gene is indicated on the right. The putative ERE is indicated by a black box. The nucleotide sequence containing the two direct repeats is indicated (the repeats are indicated by capital letters). (B) The open box indicates the region amplified by PCR. The nucleotide sequence of RAMP3 intron containing the putative ERE is indicated (the motif is highlighted in bold). (C) The ADM promoter region was precipitated by anti-ER $\alpha$  antibody. Chromatin immunoprecipitation was performed on mouse uteri obtained 0, 1, 2 and 6 h after treatment with 5  $\mu$ g/kg b.w. estradiol. At 1 and 2 h after estrogen administration, the ADM promoter region was precipitated by anti-ER $\alpha$  (upper panel). When chromatin immunoprecipitation using anti-acetylated histone H3 (AcH3) antibody was performed, the ADM promoter region was precipitated both before and after estrogen administration (lower panel). Without specific antibodies, no DNA was amplified (data not shown). (D) The RAMP3 intron was precipitated by anti-ER $\alpha$  antibody. Chromatin immunoprecipitation was performed on mouse uteri obtained 0, 1, 2 and 6 h after treatment with 5  $\mu$ g/kg b.w. estradiol. At 1 h after estrogen administration, the RAMP3 intron was precipitated by anti-ER $\alpha$  (upper panel). When chromatin immunoprecipitation using anti-acetylated histone H3 antibody was performed, the same region was precipitated both before and after estrogen administration (lower panel). Without specific antibodies, no DNA was amplified (data not shown).

can be directly activated by the ER. Analysis of the sequence upstream of the ADM gene revealed direct repeats of the canonical ERE motif between positions -744 and -748 and between positions -756 and -760 from the 5' end of the ADM gene (NM\_009627; Fig. 3A). [The actual distance from the transcriptional start site may differ since the transcriptional start site database (<http://dbtss/hgc/jp/>) showed that the ADM gene has multiple transcriptional start sites located between positions -9 and -14.] To examine whether the ER can bind to the element *in vivo*, we performed chromatin-immunoprecipitation assays with uterine DNA obtained 0, 1, 2 and 6 h after treatment with 5  $\mu$ g/kg b.w. estradiol. A DNA fragment containing the direct repeats was precipitated by the anti-ER $\alpha$  antibody only after estrogen administration (Fig. 3C), suggesting

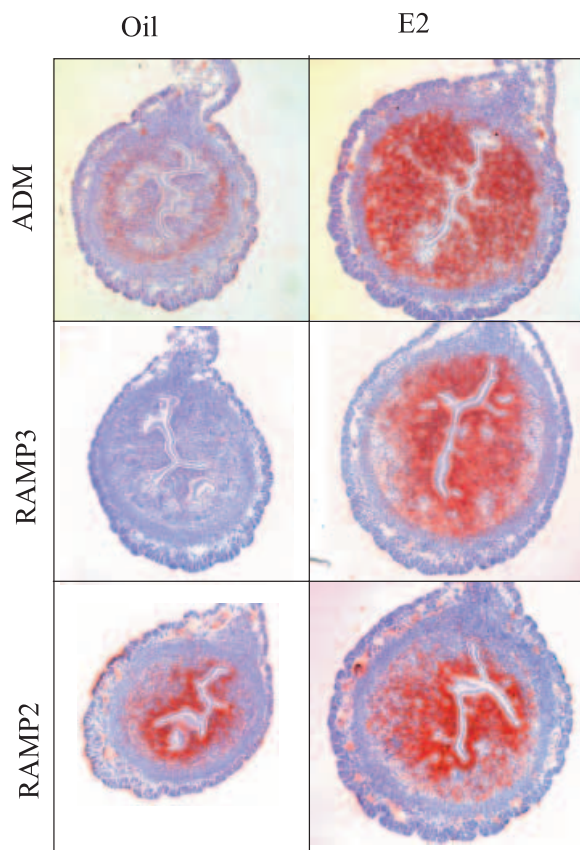
that the ER binds to the promoter region of the ADM gene and activates its transcription. Unlike the ligand-dependent binding of the ER to the ADM promoter, anti-acetylated histone H3 antibody precipitated the direct repeat-bearing DNA fragment even before estrogen was added (0 h). Thus the chromatin structure of the ADM gene is open before the addition of ligand and is accessible to the ER when the ligand is administered. Similarly RMAP3 expression was also activated by estrogen. Although ERE could not be found near the transcription start site, a putative ERE was found in the first intron of RAMP3 gene (13 620 bp from the 5' end of the first intron; Fig. 3B). Chromatin immunoprecipitation confirmed that the DNA fragment containing the putative ERE could be precipitated by anti-ER $\alpha$  only when estrogen was administered (Fig. 3D). Similar to ADM gene, anti-acetylated histone H3 antibody could precipitate the DNA fragment of RAMP3 even before estrogen administration.

### The ADM gene is expressed in uterine stroma cells

To examine the location of ADM gene expression in the uterus, we used *in situ* hybridization to detect ADM mRNA in uteri obtained 6 h after stimulation with 5  $\mu$ g/kg b.w. estrogen. ADM mRNA was mainly detected in the endometrial stroma (Fig. 4). In contrast to the weak signal in unstimulated ovariectomized uteri, the ADM gene was strongly expressed in the stroma of the estrogen-stimulated uteri. Thus estrogen induces ADM gene expression in the stroma only. Interestingly, the ADM gene was not expressed in the myometrium or epithelial cells. A similar expression pattern was observed for RAMP3, namely weak expression in unstimulated uteri and strong expression in the stroma of estrogen-stimulated uteri. The distribution of RAMP3 mRNA in the stroma was similar to that of CRLR mRNA (data not shown). In contrast, RAMP2 expression did not change after estrogen administration, although its expression was limited to the stroma. Thus the temporal (Fig. 2) and spatial (Fig. 4) expression of the ADM and RAMP3 genes correlated closely.

### ADM gene is not activated in ER $\alpha$ -null mice

To examine whether ADM gene activation is dependent on ER, we examined the gene-expression profile of ER $\alpha$  and - $\beta$ -null mice using DNA microarray. As shown in Fig. 5, ADM was activated in wild-type and ER $\beta$ -null mice but not ER $\alpha$ -null mice. This result confirmed that ER $\alpha$  is responsible for the gene activation of ADM. Similarly, RAMP3 was also activated in wild-type and ER $\beta$ -null mice but not ER $\alpha$ -null mice. These results suggest that both ADM and RAMP3 genes are regulated by ER $\alpha$  but not ER $\beta$ .

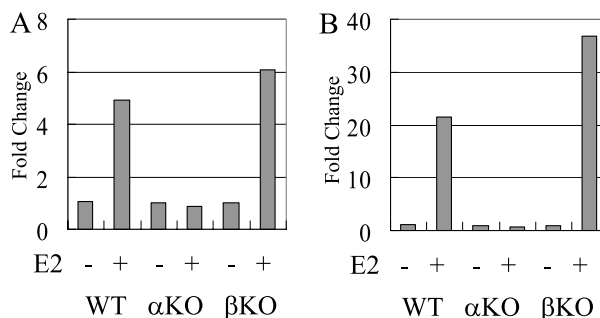


**Figure 4** Distribution of ADM, RAMP3 and CRLR mRNAs in estrogen-treated uteri. Ovariectomized mice were given 5 µg/kg b.w. estradiol and their uteri were isolated 6 h later. ADM, RAMP3 and CRLR gene expression was examined by *in situ* hybridization. As a control, mice were injected with sesame oil. The dark-field images were converted to red and merged with bright-field images. E2, 17β-estradiol.

## Discussion

Since the development of DNA microarray technology, many genes have been listed as being responsive to specific stimuli. However, despite knowing which genes may be activated or repressed by a particular stimulus, the biological significance of this information is still largely lacking. Moreover, many genes identified by DNA microarray analysis have not been validated to respond to the stimulus in question by other methods. In this study, we focused on ADM and RAMP3 genes that were shown by DNA microarray analysis to be activated by estrogen and showed that the ADM and RAMP3 genes are directly regulated by the ER.

ADM was originally identified as a potent vasorelaxant peptide that is produced by pheochromocytoma cells (Kitamura *et al.* 1993b). The human form consists of 52 amino acid residues and the mouse and rat forms consist of 50 amino acid residues (Sakata *et al.* 1993).



**Figure 5** ADM and RAMP3 genes are not activated in ER $\alpha$ -null mice. Female C57BL/6J mice and ER $\alpha$ - and - $\beta$ -null mice were ovariectomized at 8 weeks of age and injected with either 17β-estradiol (E2, +; 50 µg/kg body weight) or vehicle (-) at 10 weeks of age. The whole uteri were collected 6 h later, and the gene-expression profile was analyzed by DNA microarray. Calculated fold changes are indicated. (A) ADM, (B) RAMP3. WT, wild type;  $\alpha$ KO, ER $\alpha$ -knockout;  $\beta$ KO, ER $\beta$ -knockout.

Subsequent studies revealed that ADM is expressed not only in vascular endothelial cells but also in the adrenal gland (Kitamura *et al.* 1993a), kidney (Kitamura *et al.* 1993a), heart (Perret *et al.* 1993), bone (Cornish *et al.* 1997) and other tissues. Thus ADM is active in the cardiovascular system (Ishiyama *et al.* 1993), the endocrine system (Yamaguchi *et al.* 1995) and the central nervous system (Wang *et al.* 1995). It is also known to be expressed in the reproductive system as its expression in the uterus has been observed; moreover, uterine ADM expression increases during pregnancy (Di Iorio *et al.* 1999, Michishita *et al.* 1999, Thota *et al.* 2003, Upton *et al.* 1997). Furthermore, studies of the relationship between estrogen and ADM expression in the uterus have revealed a positive correlation between estrogen levels and ADM gene expression (Cameron *et al.* 2002, Ikeda *et al.* 2004, Jerat & Kaufman 1998). Thus ADM appears to participate in physiological processes affecting the uterus.

Many studies on ADM are mainly concerned with its long-lasting activity in physiological processes such as hypotension (Kitamura *et al.* 1995), bone development (Cornish *et al.* 2003) and gestation (Thota *et al.* 2003, Upton *et al.* 1997). With regard to the reproductive system, ADM has been reported to generally act as a relaxant or vasodilator (Makino *et al.* 1999, Yanagita *et al.* 2000). However, the data presented in this paper show that locally expressed ADM can also function in the uterus in a more transient manner, since estrogen-induced ADM gene expression in the uterus only occurs in the stroma over a short period. Interestingly, although ADM, RAMP3 and CRLR are only expressed in the stromal cells, this kind of local effect of ADM has been observed in many tissues (Kato *et al.* 1997, Martinez *et al.* 1997, Nishimura *et al.* 1997, Seguchi *et al.* 1995, Takahashi *et al.* 1997), including the

uterus (Nikitenko *et al.* 2000). Further analysis of ADM signaling is needed to clarify the effects of estrogen on uterine growth.

In the present study, we also demonstrated by chromatin-immunoprecipitation assays that the ADM promoter is recognized by the ER in a ligand (estrogen)-dependent manner. Although we used whole uteri for this analysis, these results appear to reflect the effects of ADM in the stroma, which is abundant in the uterus. Our data show that rapid binding of the ER directly to the ADM promoter may be important for ADM gene activation. A putative ERE was also detected in the RAMP3 gene, though it was found in the first intron of the gene. Chromatin immunoprecipitation confirmed that this region could be recognized by ER $\alpha$  in a ligand-dependent manner.

In summary we have shown that ADM and RAMP3 are genes that are directly activated by the ER, and that ADM and RAMP3 are directly recognized by the ER $\alpha$ . Similarities between the gene-activation patterns of ADM and RAMP3 suggest that a combination of RAMP3 and CRLR functions as an ADM receptor after estrogen administration. Although functional analysis of these genes is essential, the early activation of ADM after estrogen administration suggests that ADM plays important roles in the reproductive system in different ways: first, in processes such as pregnancy, where its involvement and expression are prolonged (Di Iorio *et al.* 1999, Michishita *et al.* 1999, Thota *et al.* 2003, Upton *et al.* 1997), and second, in processes such as estrogen-induced uterine growth, where its involvement and expression are transient.

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