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Molecular Cloning and Characterization of Mouse EBAG9, Homolog of a Human Cancer Associated Surface Antigen: Expression and Regulation by Estrogen

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We previously identified a human estrogen-responsive gene, EBAG9 (ER-binding fragment-associated antigen9) (Watanabe, T. *et al., Mol. Cell. Biol.* 18, 442–449, 1998). It was later reported as RCAS1 (receptor-binding cancer antigen expressed on SiSo cells) that induced apoptosis and suppressed the growth of several cells such as activated T cells (Nakashima, M. *et al., Nat. Med.* 5, 938–942, 1999). Here, we have isolated both cDNA and genomic DNA of mouse EBAG9/RCAS1. Mouse EBAG9 gene spans about 30 kb in genomic DNA and consists of 7 exons. Mouse EBAG9 cDNA encodes a protein that contains the transmenbrane segment and coiled-coil domain. An alignment between the predicted mouse and human EBAG9 shows a high degree of homology at the amino acid level (98%). Northern and Western blot analyses demonstrate that EBAG9 is expressed in several tissues including the heart, brain, spleen, liver, kidney, and testis, and also in developing embryo. In the uterus, a target organ for estrogen, the EBAG9 was shown to be upregulated *in vivo* by 17β-estradiol. To determine the biological action of mouse EBAG9, NIH3T3 fibroblastic cells were incubated with recombinant EBAG9 protein, resulting in suppression of cell growth. These findings suggest that EBAG9 is an *in vivo* estrogen-responsive gene that inhibits the cell growth.

Key Words: **estrogen; estrogen receptor; cell growth; estrogen responsive gene; gene structure; EBAG9.**

Abbreviations used: ER, estrogen receptor; ERE, estrogen responsive element; bp, base pair(s); EBAG9, ER-binding fragment-associated gene 9; RCAS1, receptor-binding cancer antigen expressed on SiSo cells; SSC, standard saline citrate; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; DIG, digoxigenin; dpc, days of *post coitus;* BrdU, five-bromo-2′-deoxyuridine; ORF, open reading frame.

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Estrogen plays important roles in the reproductive system as a sex steroid hormone (1). It also functions in the central nervous system (2, 3), skeletal system (4, 5), and cardiovascular system (6, 7). Moreover, estrogen plays critical roles in carcinogenesis including growth control of breast cancers (8), immunological function (9–11) and induction of apoptosis (12–14). These various actions are mediated by the estrogen receptor (ER) which is a member of the steroid/thyroid hormone receptor superfamily (15–17). ER binds to the estrogen responsive element (ERE) located in the regulatory region of target genes in the ligand dependent manner and regulates their transcription directly. However, relatively few genes that are directly regulated through the ERE by this hormone have been identified so far (18, 19). To obtain more estrogen-responsive genes, we have developed a method designated "genomic binding site cloning" and isolated several ERE-containing genomic fragments from human CpG island library (20). Using one of these fragments named EB9 as a probe, we have cloned a novel estrogen-responsive gene, EBAG9 (ER-binding fragment-associated antigen 9) from MCF-7 cDNA library (20). The cDNA of human EBAG9 encodes an open reading frame (ORF) of 213 amino acids. The EBAG9 mRNA is upregulated by estrogen in MCF-7 breast cancer cells (20) and its promoter responds to estrogen through the complete palindromic ERE that was located in the 5′-upstream region of the gene (21).

Recently, RCAS1 (receptor-binding cancer antigen expressed on SiSo cells), which had been isolated as the antigen recognized by 22-1-1 antibody (22) against a human uterine adenocarcinoma cell line SiSo (23) was isolated as a type II transmenbrane protein (24), having identical amino acid sequence with EBAG9 (25). Type II transmenbrane protein is a protein of which

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the N-terminal exists in the cytoplasm and the C-terminal region exists outside of the cell (24). EBAG9/RCAS1 protein detected using 22-1-1 antibody was found in various cell lines, and also in uterine and ovarian carcinomas (22, 23). The frequency of EBAG9/RCAS1 expression was correlated with poor prognosis in uterine cancer (22). Moreover, it was suggested that putative receptor for EBAG9/RCAS1 was present on T and NK cells through which EBAG9/RCAS1 inhibited proliferation of these cells stimulated by IL-2 and anti-CD3 antibody (25). These observations support the idea that EBAG9/RCAS1 expressed in tumor cells may be involved in escape from immune system action of the tumor cells (25).

Thus, EBAG9/RCAS1 is assumed to have a role in proliferation and invasion of cancer as an estrogen-responsive gene. So far, the expression, regulation and function of EBAG9 gene especially in normal tissues have been unknown. Here, we have cloned the mouse homolog of human EBAG9 cDNA. The mouse EBAG9 cDNA contained a consensus sequence for eukaryotic translation initiation site (CANCATG) (26, 27) surrounding the first ATG codon. We also isolated the mouse EBAG9 genomic clones and analyzed the genomic organization. Northern and Western blot analyses demonstrated that mouse EBAG9 was expressed in several tissues. Recombinant EBAG9 protein was shown to affect cell proliferation of NIH3T3 cells. We then demonstrated regulation of EBAG9 *in vivo* by estrogen in mouse uterus.

MATERIALS AND METHODS

Screening of mouse cDNA and genomic libraries and DNA sequencing and analysis. To obtain mouse EBAG9 clones, partial EBAG9 cDNA fragments were obtained by RT-PCR using mouse uterus RNA which was extracted as described previously (28). On the basis of the information obtained from the putative mouse EBAG9 EST sequence (AA657187), two primers (5′-TATTCGTCTGTCCCTAAGCAG and 5′-TTAGGATAGCTTCACACCG) which corresponded to the amino acid sequence 48-213 were used in the RT-PCR. The PCR products were subcloned into pCR2.1 (Invitrogen, Carlsbad, CA) and the insert was used for the probe. A Lamda ZAP II cDNA library (5.0×10^5 plaques) (Stratagene, La Jolla, CA) prepared from poly(A)$^+$ RNA of mouse brain was screened (18) with the ^{32}P-labeled fragment of the partial EBAG9 cDNA described above. The plaque-transferred filters were hybridized with the probe at 65°C for 18 h in 5× standard saline citrate (SSC). The filters were washed twice at room temperature for 15 min in 2× SSC, 0.1% SDS, and then exposed to X-ray film at −80°C for 24–48 h. Further screening was repeated, until a single positive signal was obtained. To isolate the gemonic clones from mouse Lamda FIX II library (Stratagene), a mouse EBAG9 open reading frame (ORF) cDNA fragment was used as a probe.

The nucleotide sequences were determined by sequencing both strands of the plasmid using ABI Prism BigDye Terminators and analyzed on an ABI PRISM 310 automated DNA sequencer (PE Applied Biosystems Division, Foster City, CA).

Northern blot analysis of various mouse tissues. A mouse multiple tissue Northern blot of poly(A)$^+$ RNA, purchased from Clonthech (Palo Alto, CA), was probed with a ^{32}P-labeled EBAG9 ORF cDNA fragment according to the manufacturer's instruction.

Expression and preparation of proteins. For the production of GST-EBAG9 fusion protein, the EBAG9 ORF cDNA fragments were amplified by PCR using 5′-ATACGGAATTCCGATGGCCATCACTCAGTTTCGG and 5′-GTCCCTCGAGGTTAGGAAAGCTTCACACCGATTTTG primers and ligated into the EcoRI-XhoI site of a prokaryotic expression vector pGEX4T-2 (Amersham Pharmacia Biotech, Arlington Heights, IL). The fusion protein was expressed in *Eschericha coli* strain BLR (DE3) pLysS, by incubation with 0.1 mM isopropyl β-D-thiogalactoside for 3 h. The cells were sonicated in cell lysis buffer (25% sucrose, 50 mM Tris–HCl (pH 7.4), 0.5% NP-40, 5 mM $MgCl_2$). After intact cells and debris were removed by centrifugation, GST fusion protein was purified by Glutathione Sepharose 4B (Amersham Pharmacia Biotech) by elution with reduced glutathione (29). The purified fusion protein was desalted with Hi Trap Desalting column (Amersham Pharmacia Biotech) to remove remaining detergent, and was sterilized by passing through a filter unit with 0.22-μm pores, divided into aliquots and stored at −80°C. For GST-ΔEBAG9 fusion protein, partial EBAG (48-213 amino acids) generated by PCR was ligated in frame into the EcoRI-XhoI site of pGEX4T-2. Recombinant GST protein was also synthesized as a control.

Preparation of anti-EBAG9 polyclonal antibody. Rabbit polyclonal anti-EBAG9 antisera were generated by subcutaneous injection of GST-EBAG9 fusion protein emulsified in complete Freund's adjuvant (Difco LABORATORIES, Detroit, MI). Expression and preparation of GST-EBAG9 fusion protein was described under expression and preparation of proteins. To remove antibody to GST and purify the antiserum specific for EBAG9, CNBr-actived Sepharose 4B (Amersham Pharmacia Biotech) coupled with recombinant GST protein or GST-EBAG9 fusion protein were used. One and a half grams of each CNBr-activated Sepharose 4B coupled with 20 mg of recombinant GST protein or GST-EBAG9 fusion protein was prepared as depletion matrix or affinity matrix, respectively. Four millilers of antiserum was added to the depletion matrix and incubated rotating end-over-end at room temperature for 1.5 h. After the matrix was centrifuged at 1000*g* for 2 min, the supernatant was removed and added on the affinity matrix, and then incubated in the same way as for depletion matrix. The affinity matrix with the antibody was transferred to the empty column and eluted with 0.15 M glycine, pH 2.5 into the tube containing 2 M Tris–HCl, pH 8.0 and mixed briefly.

Characterization of anti-EBAG9 polyclonal antibody and Western blot analysis. For characterization of EBAG9 antibody, Western blot analysis was performed using recombinant EBAG9 protein. Full lengths of mouse and human EBAG9 ORF cDNA were ligated into eukaryotic expression vector pcDNA3 (Invitrogen), and then mouse and human EBAG9 proteins were produced using TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI). Western blot analysis was performed as described (18). Briefly, 10 μg of each sample was fractionated on SDS-12.5% polyacrylamide gels, and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore Corp., Bedford, MA). The membranes were blocked at room temperature for 1 h in phosphate-buffered saline (PBS) with 3% skim milk, then incubated at room temperature for 1 h with 5 ml each of 1:2000 diluted purified anti EBAG9 antibody. Each membrane was washed in PBS with 0.1% Tween 20 and incubated with 1:5000 diluted horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech) at room temperature for 1 h. Bands were visualized with the chemiluminescence-based ECL or ECL plus detection system (Amersham Pharmacia Biotech). The membranes were exposed to X-ray film or placed on the STORM system (Molecular Dynamics, Sunnyvale, CA). To detect the distribution of EBAG9 at the protein level, tissues of 8-week-old ICR mice purchased from SLC (Shizuoka, Japan) were collected and homogenized in lysis buffer (5 mM phosphate buffer, pH 7.4, with 0.1% Triton X-100), then 10 mg of each sample was used for Western blot analysis. To analyze estrogen regulation, 8-week-old ICR mice were ovariectomized. Two weeks after surgery, the mice were treated with 17β-estradiol (20 μg/kg) or

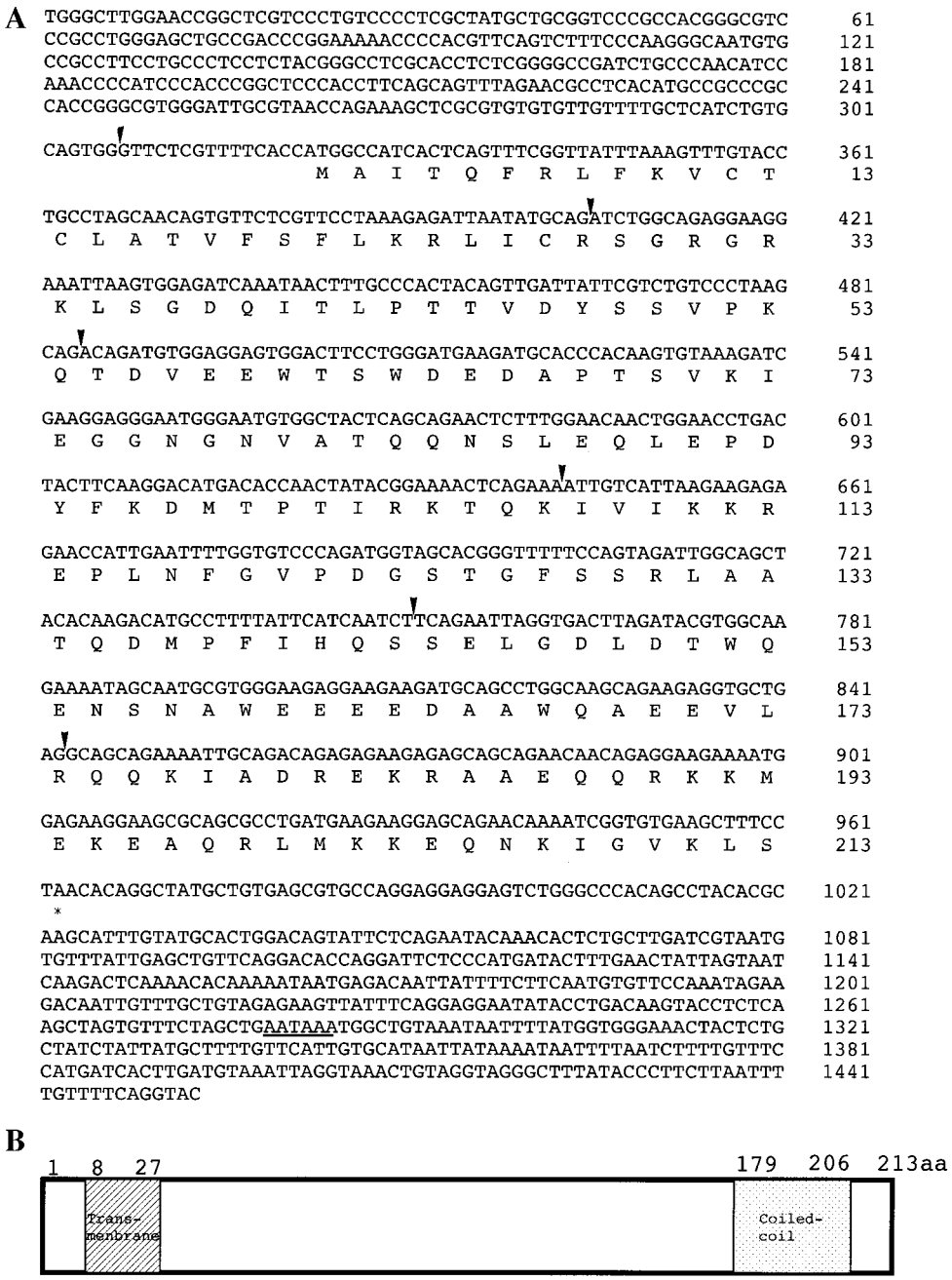

FIG. 1. Molecular structure of mouse EBAG9. (A) Nucleotide and deduced amino acid sequences of mouse EBAG9. The deduced amino acids are shown below their respective codons. The TAA stop codon (*) and the polyadenylation signal (underline) are also shown. The positions of intron–exon junctons are indicated by arrowheads. (B) Diagrammatic representation of mouse EBAG9 protein. Trans-membrane segment (hatched box) and coiled-coil domain (stippled box) are shown. aa, amino acid.

olive oil (solvent control) and were sacrificed at the indicated time. The uteri of these mice were collected for Western blot analysis.

In situ hybridization. For mouse EBAG9 RNA probe, mouse EBAG9 cDNA fragment (nucleotide 1-1454) was subcloned into the EcoRI/NotI site of eucaryotic exprssion vector pcDNA3 (Invitrogen) and amplified. In situ hybridization was performed essentially as described (30). Briefly, digoxigenin (DIG)-labeled antisence RNA probes were synthesized by RNA Labeling kit (Boehringer Mannheim Corp., Indianapolis, IN) and hybridization was performed at 55°C for 12 h and slides were washed. Hybridized probes were detected with anti-DIG-alkaline phosphate antibody (Boehringer Mannheim Corporation) and finally visualized with DAB kit (Funakosi, Tokyo, Japan).

Assay for cell proliferation. NIH3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% calf serum (GIBCO BRL, Rockville, MD). 3×10^4 cells in 5 cm petri dishes were cultured with 1 μg/ml of GST-EBAG9, GST-ΔEBAG9 or GST as described. At 24–72 h culture, cells were collected and counted with Coulter Counter (BECKMAN COULTER, Fullerton, CA). 1×10^3 cells/well were cultured in 96 well-plates with the same concentra-

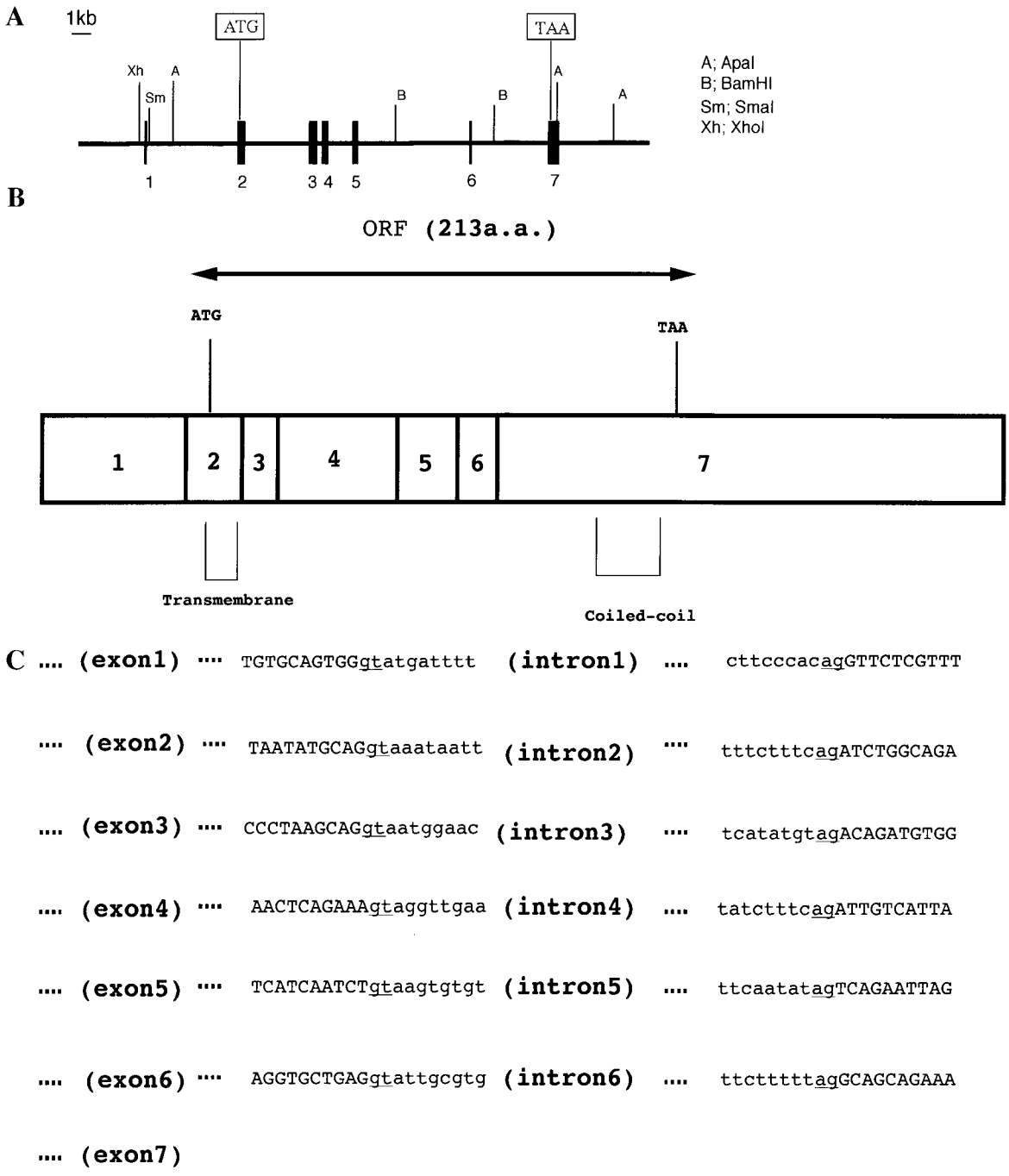

FIG. 2. Genomic organization of mouse EBAG9. (A) Genomic clones containing mouse EBAG9 coding sequences were isolated and mapped with restriction enzymes. Exons are shown as boxes and numbered. The first methionine codon of the open reading frame is located in exon 2, whereas the stop codon and poly $(A)^+$ addition signal is located in the last exon 7. kb, kilobase pairs. (B) Schematic representation of mouse EBAG9 cDNA. Exons 1–7 are shown by boxes. The open reading frame is indicated by arrow. aa, amino acid. (C) Nucleotide sequences of each exon–intron junction. The donor sites (GT) and acceptor sites (AG) of introns are underlined.

tion of each protein. At 24–72 h culture, DNA synthesis was detected by Cell Proliferation ELISA System (Amersham Pharmacia Biotech).

RESULTS

Isolation of mouse EBAG9 cDNA. Two EBAG9 clones were isolated by the screening of a mouse brain cDNA library. Both clones were found to be derived from the same RNA by sequencing. The longest assembled cDNA was 1454 bp which encodes an ORF of 213 amino acids (Fig. 1A), containing a consensus sequence for eukaryotic translation initiation site (CANCATG) (26, 27) surrounding the first ATG codon. The 3′-

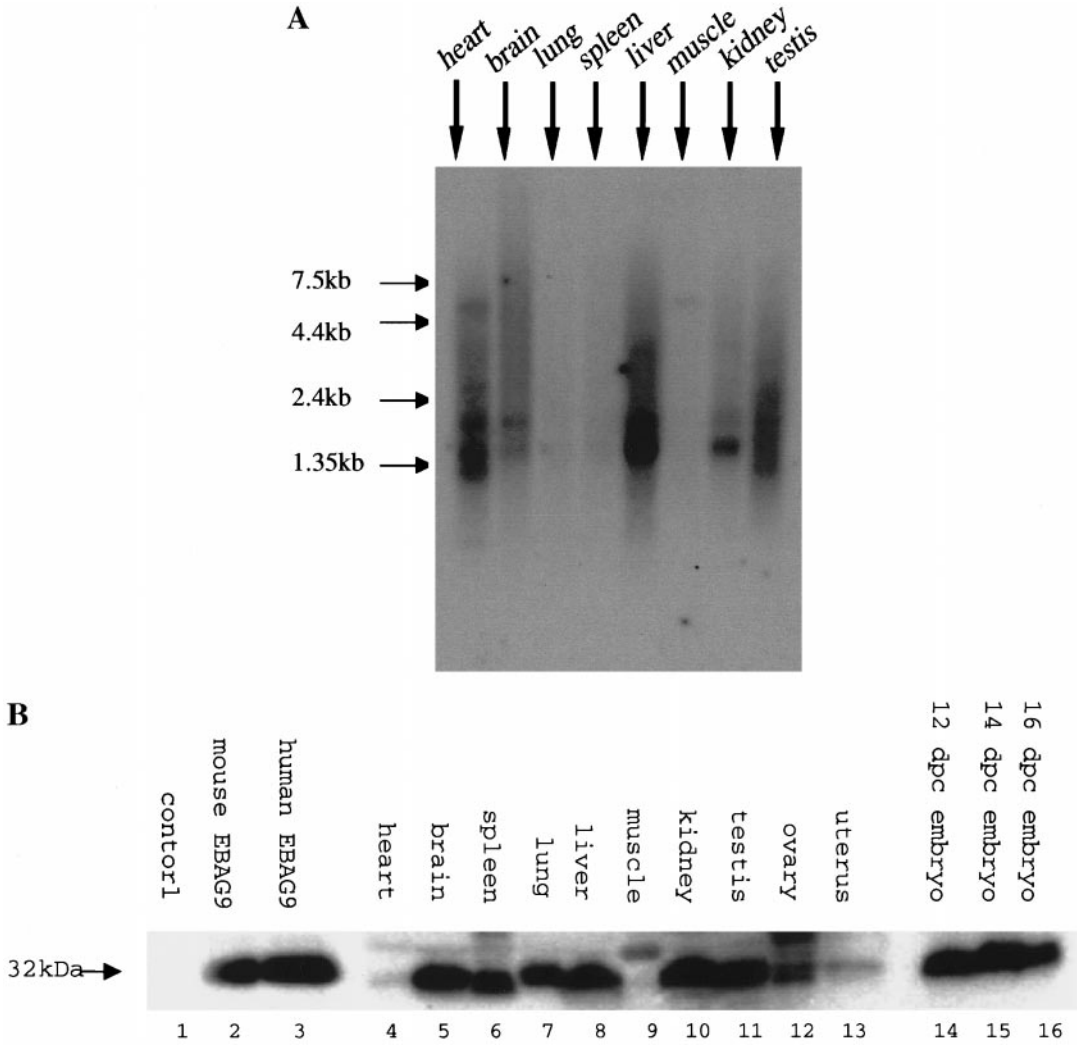

FIG. 3. Tissue distribution of mouse EBAG9 mRNA and protein. (A) Northern blot analysis of various mouse tissues using ^{32}P-labeled mouse EBAG9 ORF cDNA. kb, kilobase pairs. (B) Immunoblot analysis of mouse EBAG9. The cell extracts from various mouse tissues and embryos were resolved by SDS–PAGE and transferred to PVDF membrane. Blot was probed with the EBAG9-specific polyclonal antibody (1:2000) and a 32-kDa native protein was detected. Lane 1, control (premix of TNT T7 Quick Coupled Transcription/Translation System); lane 2, *in vitro* translated mouse EBAG9; lane 3, *in vitro* translated human EBAG9; lanes 4–13, heart, brain, spleen, lung, liver, muscle, kidney, testis, ovary, uterus; lanes 14–16, embryo of 12, 14, and 16 dpc, respectively. dpc days of *post coitus.*

terminus of the cDNA ended with a poly $(A)^+$ tail preceded by a polyadenylation signal at nt 1280-1285 (underlined in Fig. 1A).

Structure of mouse EBAG9 cDNA. Mouse EBAG9 cDNA encodes a protein of calculated relative molecular mass of 24,376 Da. The putative mouse EBAG9 protein contains a transmenbrane segment (24) near the N-terminal position and a coiled-coil domain (31–33) in the C-terminal region (Fig. 1B), indicating that mouse EBAG9 is a type II membrane protein (24).

Isolation and structural analysis of mouse EBAG9 gene. We screened a mouse genomic library with mouse EBAG9 cDNA as a probe and obtained overlapping genomic clones. The mouse EBAG9 gene spans about 30 kb of genomic DNA and contains 7 exons (Fig. 2A). Exon 1 is 308 bp in length and contains only 5′ untranslated segment of EBAG9 cDNA. The exon 2, 98 nucleotides long, specifies the remaining 15 bp untranslated nucleotides and the first 28 amino acids of the protein, of which 21 represent the transmenbrane segment. Exon 3 to 6 encode amino acid residues 29-174, which comprise the spacing region between transmenbrane segment and coiled-coil domain. Exon 7 encodes amino acid residues 175-213, of which 28 represent the coiled-coil domain (Fig. 2B). The 5′ donor and 3′ acceptor splice sites in all of the introns conform to the consensus sequences for exon–intron boundaries (34) (Fig. 2C).

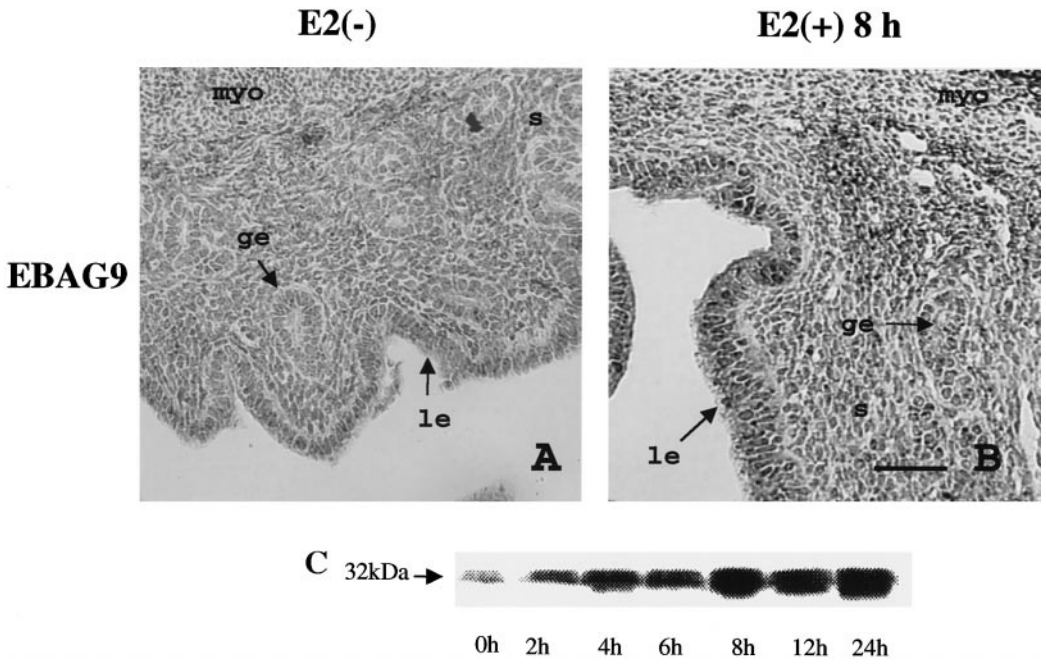

FIG. 4. EBAG9 was upregulated by estrogen in mouse uterus. *In situ* hybridization of 17β-estradiol treated ovariectomized mouse uterus using EBAG9 RNA probe at 0 h (A) and 8 h (B). To facilitate orientation, the luminal epithelium (le), the glandular epithelium (ge), the stroma (s), and myometrium (myo) of uterus are indicated. The scale bar indicates 100 μm. (C) Western blot analysis using anti-EBAG9 anti-serum show that the level of EBAG9 signals is elevated after subcutaneous injection of 17β-estradiol. The experiment was carried out three times and a representative pattern is shown.

Expression of mouse EBAG9. Northern blot analysis was performed using the DNA fragment of EBAG9 ORF cDNA fragment as a probe. Six different sizes of transcripts were detected. Two transcripts, 1.5 and 1.8 kb in size, were detected in the heart, brain, liver, kidney, and testis. In the spleen and lung, 1.5 kb transcripts were weakly detected. A 1.2 kb transcript was detected in the heart and testis. A 3 kb transcript was detected in the heart, liver, and testis. A 4 kb transcript was detected in the liver, and a 6.5 kb was detected in the heart and muscle (Fig. 3A). Although the EBAG9 mRNA was detected in almost all tissues examined, the levels of the expression were various among tissues. Higher levels of expression were observed in the heart, liver, and testis, whereas the mRNA was hardly detectable in the spleen, lung, and muscle.

Using the generated antibody (for details see Materials and Methods), Western blot analysis detected 32-kDa bands, which corresponded to the predicted molecular weight of EBAG9, in various mouse tissues and also in various days of post coitus (dpc) of mouse embryo (Fig. 3B). The signals detected by the anti-EBAG9 antibody are shown in the brain, spleen, lung, liver, kidney, testis, and embryo. In the heart, muscle, ovary, and uterus, the bands are detected at a lower intensity compared with that of other organs.

Regulation of EBAG9 protein by estrogen in mouse uterus. To examine estrogen responsiveness of the mouse EBAG9 gene *in vivo,* the effects of estrogen administration on the amount of EBAG9 transcript and protein were studied in the ovariectomized mouse uterus. To show the distribution of EBAG9 mRNA, we performed *in situ* hybridization using EBAG9 antisence RNA probes. In overiectomized mouse uterus without 17β-estradiol, EBAG9 transcripts were detected in grandular luminal and epithelial cells, and also in stromal cells at a lower level of signals (Fig. 4A). In the uterus after treatment of 17β-estradiol (8 h), the EBAG9 transcripts were significantly increased in epithelial cells, stromal cells, as well as in myometrium (Fig. 4B). By Western blot analysis, the level of signals detected by anti-EBAG9 antibody was increased gradually, and reached about 3 times at 8 h after subcutaneous injection of 17β-estradiol (Fig. 4C).

Inhibition of cell growth by EBAG9. To analyze the involvement of EBAG9 in the regulation of cell growth, NIH3T3 mouse fibroblastic cells were treated with 1 μg/ml of GST-EBAG9, GST-ΔEBAG9, or GST proteins. Each of their viability was evaluated for 72 h. The growth of NIH3T3 cells was decreased when the cells were treated with GST-EBAG9 compared with GST at 48 h (Fig. 5A). To analyze the effect of EBAG9 on DNA synthesis, NIH3T3 cells were treated with 1 μg/ml of GST-EBAG9, GST-ΔEBAG9, or GST proteins. Five-

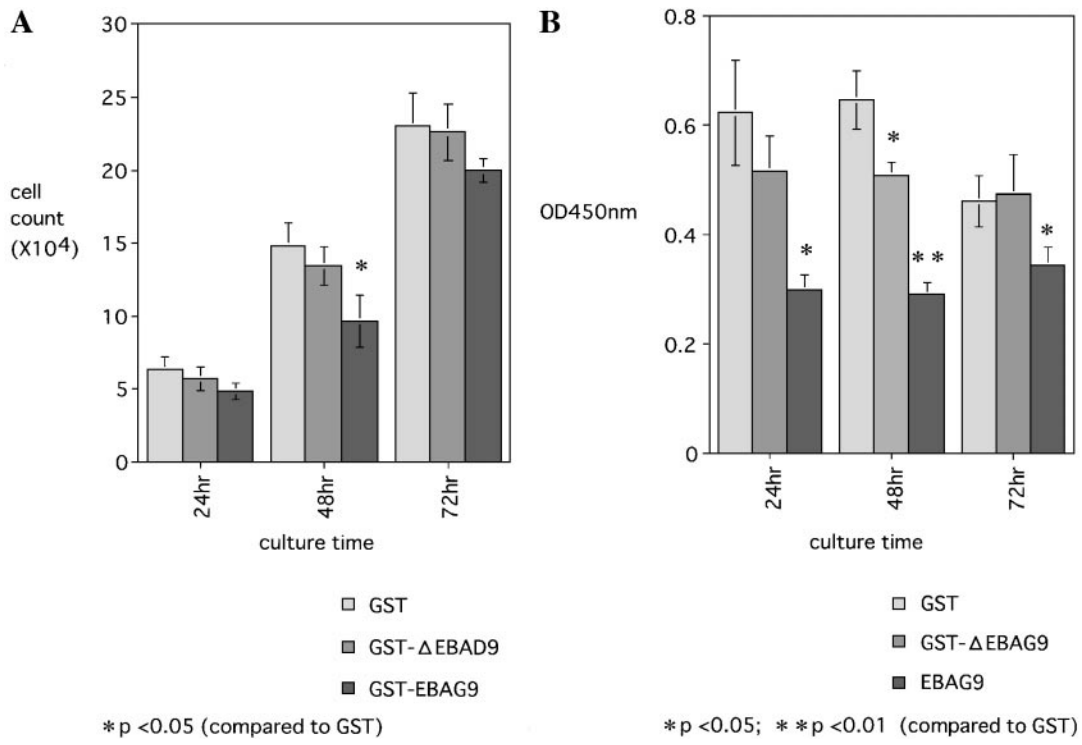

FIG. 5. Inhibition of growth of NIH3T3 cells by EBAG9. (A) Suppression of cell growth by recombinant EBAG9 protein. NIH3T3 cells were cultured with GST, GST-ΔEBAG9, or GST-EBAG9 and cell numbers were counted every 24 h. The cell growth was inhibited at 48 h by GST-EBAG9. (B) Supression of DNA synthesis by EBAG9. NIH3T3 cells were cultured with GST, GST-ΔEBAG9, or GST-EBAG9 and BrdU uptake was measured every 24 h. DNA synthesis was suppressed by both GST-EBAG9 and GST-ΔEBAG9 at 48 h. Values shown represent mean ± SE from three separate experiments. n = 3, * $P < 0.05$, ** $P < 0.01$.

bromo-2′-deoxyuridine (BrdU) was added to the cells and the DNA synthesis of the cells was measured at 24, 48, and 72 h. BrdU-incorporation to the cells was decreased when the cells were treated with GST-EBAG9 at 24, 48, and 72 h and with GST-ΔEBAG9 at 48 h, compared with GST (Fig. 5B).

DISCUSSION

In the present study, the mouse homolog of the human EBAG9/RCAS1 was cloned and characterized. Human EBAG9 has been isolated as an estrogen-responsive gene by genomic binding site cloning using a recombinant ER protein. EBAG9 is highly conserved between mouse and human (98% homology) in the amino acid level compared with other type II transmembrane proteins such as FasL (76.9%) (35) and TNF-α (75%) (36), suggesting that EBAG9 has biologically essential function beyond the species. Especially, the coiled-coil domains of mouse and human EBAG9 are identical, and these domains are supposed to have important function. In fact, some of coiled-coil domains present in proteins such as cFos-cJun (37), Gal4 (38), G protein β/γ heterodimer (39, 40), DNA polymerase I (41) were known to have important functions of dimer formation and protein–protein interaction. As mouse EBAG9 is a membrane protein of which receptor-like molecule is suggested to be present (25), we assume that the coiled-coil domain has functions of dimerization or binding to its receptor.

The EBAG9 mRNA was expressed at a relatively high level in the heart, liver, and testis. In other organs, such as the brain, lung, kidney, and skeletal muscle, the expression of EBAG9 mRNA was detected, but the intensity was different among the organs. The sizes of EBAG9 transcripts were variable as shown by multiple bands ranging from 1.2–6.5 kb. It was mainly expressed as 1.5 and 1.8 kb products, but other sizes of the products were observed such as 1.2, 3, 4, and 6.5 kb, suggesting the existence of alternative splicing or alternative end. In order to analyze the expression of EBAG9 at the protein level, we performed Western blot analysis in various mouse tissues. EBAG9 protein detected as a 32 kDa band is present in the brain, spleen, lung, liver, kidney, and testis as well as in the embryo. The relatively low levels of expression were observed in the heart, uterus, and ovary, and it is hardly detectable in the skeletal muscle. Comparing with the expression pattern of EBAG9 at the mRNA level, we detected some disparity between the expression at the protein level in some tissues. In the lung and spleen, for instance, the expression of mRNA was hardly seen, al-

though the expression at the protein level was relatively high. In the case of the heart, the EBAG9 protein was hardly detectable while the mRNA levels was relatively high. Thus, we have detected the expression of EBAG9 protein at least in normal mouse organs including embryo. It was suggested that the EBAG9 protein was expressed in the tumor cells and involved in immune escape from the attack by the lymphocytes (25). Here, we also demonstrated that EBAG9 was expressed in normal tissues and suppressed the growth of fibroblastic NIH3T3 cells. Deletion mutant analysis suggested that N terminal portion of EBAG9 was indispensable for full activity of cell growth inhibition. We speculate that EBAG9 controls morphogenesis or organogenesis considering its relatively robust expression in embryonal stages.

Upregulation of human EBAG9 protein was detected at 8 h after estrogen treatment in MCF-7 cells (20). In the present study, the regulation of mouse EBAG9 protein by estrogen was demonstrated in mouse uterus. We performed Western blot analysis using estrogen treated ovariectomized mice. The level of EBAG9 protein was increased about three times at 8 h by estrogen treatment, and its higher level was maintained until 24 h. Therefore, EBAG9 is suggested to estrogen responsive *in vivo.*

It is reported that the human EBAG9 bound to its putative receptor and induced the apoptosis or cell growth inhibition of its receptor positive cells (25). In the present study, we demonstrated that mouse EBAG9 had the function of cell growth inhibition on NIH3T3 cells. Although we studied the induction of apoptosis by mouse EBAG9 with terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling method, the apoptosis could not be detected by this method (data not shown). These differential observations may be due to the difference of experimental conditions, cells, or species.

Taken together, we demonstrated in this study that mouse EBAG9 was expressed in normal tissues and embryo, and functions to inhibit the cell growth. Moreover, we demonstrated that EBAG9 protein was upregulated by estrogen in mouse uterus. In mouse uterus, the endometrial cells proliferate by estrogen via estrogen receptor (42). The factors driving the cell growth such as c-fos (43), c-jun (44), TGF-β (45), VEGF (46), and cyclin D1 (47, 48) are known to be regulated by estrogen, and contribute to the cell growth in the uterus. For the cells of vascular smooth muscle (49–51) and certain kinds of tumor cells (52), on the other hand, estrogen inhibits the cell growth. Thus, estrogen has opposite dual functions to the cell growth. Besides the cloning of EBAG9, identification of EBAG9 receptor and the elucidation of regulatory machinery of EBAG9 will contribute to the explanation of these differential functions of estrogen to cell proliferation, differentiation, and cell cycle.

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REFERENCES

1. George, F. W. a. W., J. D. (1988) The Physiology of Reproducion, Raven Press, New York, NY.
2. Simerly, R. B., Chang, C., Muramatsu, M., and Swanson, L. W. (1990) *J. Comp. Neurol.* 76–95.
3. Toran-Allerand, C. D., Miranda, R. C., Hochberg, R. B., and MacLusky, N. J. (1992) *Brain Res.* 25–41.
4. Komm, B. S., Terpening, C. M., Benz, D. J., Graeme, K. A., Gallegos, A., Korc, M., Greene, G. L., O'Malley, B. W., and Haussler, M. R. (1988) *Science* 81–84.
5. Eriksen, E. F., Colvard, D. S., Berg, N. J., Graham, M. L., Mann, K. G., Spelsberg, T. C., and Riggs, B. L. (1988) *Science* 84–86.
6. Orimo, A., Inoue, S., Ikegami, A., Hosoi, T., Akishita, M., Ouchi, Y., Muramatsu, M., and Orimo, H. (1993) *Biochem. Biophys. Res. Commun.* 730–736.
7. Stumpf, W. E., Sar, M., and Aumuller, G. (1977) *Science* 319–321.
8. Henderson, B. E., Ross, R. K., and Pike, M. C. (1991) *Science* 1131–1138.
9. Marbaix, E., Kokorine, I., Moulin, P., Donnez, J., Eeckhout, Y., and Courtoy, P. J. (1996) *Proc. Natl. Acad. Sci. USA* 9120–9125.
10. Polan, M. L., Loukides, J., Nelson, P., Carding, S., Diamond, M., Walsh, A., and Bottomly, K. (1989) *J. Clin. Endocrinol. Metab.* 1200–1206.
11. Galien, R., and Garcia, T. (1997) *Nucleic Acids Res.* 2424–2429.
12. Davis, E. C., Popper, P., and Gorski, R. A. (1996) *Brain. Res.* 10–18.
13. Gutierrez, L. S., Eliza, M., Niven-Fairchild, T., Naftolin, F., and Mor, G. (1999) *Breast. Cancer Res. Treat.* 245–253.
14. Mor, G., Amir-Zaltsman, Y., Barnard, G., and Kohen, F. (1992) *Endocrinology* 3633–3640.
15. Jensen, E. V. a. J., h. I. (1962) *Recent Prog. Hormone Res.* 387–414.
16. Evans, R. M. (1988) *Science* 889–895.
17. Green, S., and Chambon, P. (1988) *Trends Genet.* 309–314.
18. Inoue, S., Orimo, A., Hosoi, T., Kondo, S., Toyoshima, H., Kondo, T., Ikegami, A., Ouchi, Y., Orimo, H., and Muramatsu, M. (1993) *Proc. Natl. Acad. Sci. USA* 11117–11121.
19. Muramatsu, M., and Inoue, S. (2000) *Biochem. Biophys. Res. Commun.* 1–10.
20. Watanabe, T., Inoue, S., Hiroi, H., Orimo, A., Kawashima, H., and Muramatsu, M. (1998) *Mol. Cell. Biol.* 442–449.
21. Ikeda, K., Sato, M., Tsutsumi, O., Tsuchiya, F., Tsuneizumi, M., Emi, M., Imoto, I., Inazawa, J., Muramatsu, M., and Inoue, S. (2000) *Biochem. Biophys. Res. Commun.* 654–660.
22. Sonoda, K., Nakashima, M., Kaku, T., Kamura, T., Nakano, H., and Watanabe, T. (1996) *Cancer* 1501–1509.
23. Sonoda, K. (1995) *Int. J. Oncol.* 1099–1104.
24. Stoffel., K. H. a. W. (1993) *Biol. Chem. Hoppe-Seyler* 166.
25. Nakashima, M., Sonoda, K., and Watanabe, T. (1999) *Nat. Med.* 938–942.
26. Cavener, D. R. (1987) *Nucleic Acids Res.* 1353–1361.
27. Kozak, M. (1987) *Nucleic Acids Res.* 8125–8148.
28. Sambrook, J., Fritsch, F., and Maniatis, T. (1989) Molecular

Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

29. Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., and Hirai, H. (1994) *EMBO J.* 3748–3756.
30. Ishikawa, K., Fujigasaki, H., Saegusa, H., Ohwada, K., Fujita, T., Iwamoto, H., Komatsuzaki, Y., Toru, S., Toriyama, H., Watanabe, M., Ohkoshi, N., Shoji, S., Kanazawa, I., Tanabe, T., and Mizusawa, H. (1999) *Hum. Mol. Genet.* 1185–1193.
31. Berger, B., Wilson, D. B., Wolf, E., Tonchev, T., Milla, M., and Kim, P. S. (1995) *Proc. Natl. Acad. Sci. USA* 8259–8263.
32. Lupas, A. (1996) *Trends Biochem. Sci.* 375–382.
33. Lupas, A. (1996) *Methods Enzymol.* 513–525.
34. Shapiro, M. B., and Senapathy, P. (1987) *Nucleic Acids Res.* 7155–7174.
35. Takahashi, T., Tanaka, M., Inazawa, J., Abe, T., Suda, T., and Nagata, S. (1994) *Int. Immunol.* 1567–1574.
36. Iraqi, F., and Teale, A. (1997) *Immunogenetics* 459–461.
37. Glover, J. N., and Harrison, S. C. (1995) *Nature* 257–261.
38. Marmorstein, R., Carey, M., Ptashne, M., and Harrison, S. C. (1992) *Nature* 408–414.
39. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell* 1047–1058.
40. Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) *Nature* 311–319.
41. Beese, L. S., Derbyshire, V., and Steitz, T. A. (1993) *Science* 352–355.
42. Korach, K. S. (1994) *Science* 1524–1527.
43. Weisz, A., and Bresciani, F. (1988) *Mol. Endocrinol.* 816–824.
44. Weisz, A., Cicatiello, L., Persico, E., Scalona, M., and Bresciani, F. (1990) *Mol. Endocrinol.***4,** 1041–1050.
45. Nelson, K. G., Takahashi, T., Lee, D. C., Luetteke, N. C., Bossert, N. L., Ross, K., Eitzman, B. E., and McLachlan, J. A. (1992) *Endocrinology* 1657–1664.
46. Cullinan-Bove, K., and Koos, R. D. (1993) *Endocrinology* 829–837.
47. Zhang, Z., Laping, J., Glasser, S., Day, P., and Mulholland, J. (1998) *Endocrinology* 961–966.
48. Altucci, L., Addeo, R., Cicatiello, L., Germano, D., Pacilio, C., Battista, T., Cancemi, M., Petrizzi, V. B., Bresciani, F., and Weisz, A. (1997) *Endocrinology* 978–984.
49. Dubey, R. K., Gillespie, D. G., Mi, Z., Rosselli, M., Keller, P. J., and Jackson, E. K. (2000) *Hypertension* 262–266.
50. Dai-Do, D., Espinosa, E., Liu, G., Rabelink, T. J., Julmy, F., Yang, Z., Mahler, F., and Luscher, T. F. (1996) *Cardiovasc. Res.* 980–985.
51. Farhat, M. Y., Lavigne, M. C., and Ramwell, P. W. (1996) *FASEB J.* 615–624.
52. Messa, C., Pricci, M., Linsalata, M., Russo, F., and Di Leo, A. (1999) *Scand. J. Gastroenterol.* 79–84.