Biochemical and Biophysical Research Communications **275,** 108–114 (2000)
doi:10.1006/bbrc.2000.3276, available online at http://www.idealibrary.com on IDEAL®

WISP-2 as a Novel Estrogen-Responsive Gene in Human Breast Cancer Cells

Hidekuni Inadera,*,†,[1] Shin-ichi Hashimoto,*,[1] Hong-Yan Dong,* Takuji Suzuki,* Shigenori Nagai,* Taro Yamashita,* Nobuaki Toyoda,* and Kouji Matsushima*,[2]

**Department of Molecular Preventive Medicine and CREST, and †School of Medicine and Environmental Science Center, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan*

Received July 17, 2000

In order to search for novel estrogen-responsive genes, we performed serial analysis of gene expression (SAGE) for estrogen-treated MCF-7 human breast cancer cells. SAGE analysis of 31,000 and 30,856 tags from non-treated and 17 β-estradiol (E2)-treated cells for 24 h, respectively, facilitated the identification of 15,037 different transcripts. Comparison of these two SAGE libraries indicated a remarkable similarity in expression profiles. Among the identified transcripts, four genes were found to be markedly increased for E2-treated cells compared with control cells. Three of the transcripts were cathepsin D, pS2 and high mobility group 1 protein, which have been described as estrogen-inducible genes. The fourth gene was WISP-2 (Wnt-1 inducible signaling pathway protein 2) which has recently been reported as an up-regulated gene in the mammary epithelial cell line C57 MG transformed by the Wnt-1 oncogene. The increase in WISP-2 mRNA was completely prevented by co-incubation with a pure anti-estrogen ICI 182,780, but not by coincubation with cycloheximide, indicating that WISP-2 is directly regulated by the estrogen receptor. The WISP-2 gene was also induced by treating with environmental estrogens, such as bisphenol-A or nonylphenol. This study represents the first comprehensive gene expression analysis of estrogen-treated human breast cancer cells.

Key Words: **breast cancer cells; estrogen; serial analysis of gene expression; WISP-2.**

The activation of transcription by steroid hormones is mediated by their specific nuclear receptors acting as ligand-activated transcription factors interacting specifically with cognate responsive elements. Estrogens interact with the estrogen receptor (ER) with high affinity. The ER is a member of the steroid hormone receptor superfamily, and the mechanism of action of estrogens is similar to that of other steroid hormones (1, 2). Upon binding to estrogens, the ER becomes activated and binds to specific estrogen-response element in the enhancer region of target genes (1, 2).

Estrogen plays a crucial role in cell growth and proliferation of breast cancer cells (3, 4). This process includes DNA synthesis and cell division. Several studies have shown that this E2-induced response is accompanied by modulation of the expression of a set of genes that regulate cell cycle progression (5, 6). For example, treatment of MCF-7 cells with 17 β estradiol (E2) is accompanied by increased c-fos, cyclin D1 mRNA and protein, cdk7-, cdk2-, and cdk4-dependent kinase activities, cdc25A phosphatase protein, and increased phosphorylation of retinoblastoma (Rb) protein (5–8). Many other genes such as c-myc (9), E2F1 (10), bcl-2 (11), insulin receptor substrate-1 (12), insulin-like growth factor-binding protein-4 (13), c-Ha-ras (14) were also induced by E2 in breast cancer cells. After addition of E2, transcripts of these genes were induced rapidly and transiently in human breast cancer cells (5–9, 11, 14).

Recently, scientific attention has been drawn to a number of environmentally-dispersed chemicals that may bind to estrogen receptors and mimic estrogenic activity, such chemicals being termed environmental estrogens (xenoestrogens) (15, 16). These include a number of pesticides and herbicides, some polychlorinated biphenyls, and some alkylphenolic compounds (16, 17). These compounds are suspected of playing a pivotal role in alterations to the normal sexual development of wildlife species (16–18). Thus, novel mark-

Abbreviations used: ActD, actinomycin D; BPA, bisphenol-A; CHX, cycloheximide; E2, 17 β estradiol; ER, estrogen receptor; GAPDH, glyceraldehyde phosphate dehydrogenase; FCS, fetal calf serum; NP, nonylphenol; SAGE, serial analysis of gene expression; WISP-2: Wnt-1 inducible signaling pathway protein 2; XE, xenoestrogen.

[1] These two authors contributed equally to this work.

[2] To whom correspondence should be addressed. Fax: +81-3-5684-2297. E-mail: koujim@m.u-tokyo.ac.jp.

0006-291X/00 $35.00

ers to assess the extent of chronic exposure to such chemicals are clearly warranted.

To search novel estrogen-responsive genes and possibly markers for the chronic exposure to xenoestrogens (XEs), we conducted serial analysis of gene expression (SAGE) to establish a comprehensive gene expression profile in estrogen-treated and -untreated human breast cancer cell line, MCF-7. SAGE generates a 14- or 15-bp "tag" sequence from a defined position within the transcript, which is sufficient to uniquely identify an individual transcript (19–23). By using SAGE, we tried to identify a set of estrogen responsive genes in MCF-7 cells treated with E2 for 24 h.

MATERIALS AND METHODS

Materials. The human breast cancer cell line MCF-7 (24) was obtained from American Type Culture Collection (Manassas, VA). Charcoal, 17 β-estradiol (E2), and bisphenol-A (BPA), were purchased from Sigma Chemical Co. (St. Louis, MO). Nonylphenol (NP) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). The estrogen antagonist ICI 182,780 was purchased from Tocris (Bristol, UK). All other chemicals were of the highest purity available from commercial sources.

Cell culture and treatment. MCF-7 cells were treated as described previously (25). Cells were grown in minimum essential medium (MEM: Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS). For induction experiments, cells were cultured in phenol red-free medium containing 10% FCS treated with dextran-coated charcoal for three days, then added the indicated concentrations of estrogens. Stock solutions of E2, BPA, and NP were prepared in ethanol and the final concentration of ethanol was 0.1% or less.

SAGE protocol. The SAGE protocol has been described in detail elsewhere (19–23). MCF-7 cells were treated, for 24 h, with 10 nM E2 in the presence of 10% serum treated with dextran-coated charcoal in phenol-red free MEM. Control cells were treated with 0.1% ethanol (vehicle). Total RNA from the cells was isolated by direct lysis in RNAzolB (TEL-TEST, INC., Friendswood, TX). Polyadenylated RNA was isolated using the FastTrac mRNA purification kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Polyadenylated RNA was converted to double-stranded cDNA by the SuperScript Choice System (GIBCO-BRL, Gaithersburg, MD) with a 5′-biotinylated oligo dT primer. Double-stranded cDNA was digested with *NlaIII* and most 3′ primed fragments were bound to DynabeadsM-280 Streptavidin (Dynal A.S., Oslo, Norway). Subsequent to ligation of the oligonucleotides containing recognition sites for *Bsm F1*, the linkered cDNA was released from the beads by digestion with *BsmF1*. Released tags were ligated to one another, blunt-ended, concatemerized, and cloned into the *SphI* site of the pZero-1 vector (Invitrogen, Carlsbad, CA). Samples were sequenced by an ABI PRISM 377 DNA Sequencer with a BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA). Sequenced files were analyzed by SAGE software (19), NCBI's SAGE database (http://www.ncbi.nlm.nih.gov/SAGE/) and NCBI's sequence search tool (Advanced BLAST search, http://www.ncbi.nlm.nih.gov//BLAST/).

Northern blot analysis. Northern blot analyses were performed as described (25). Human WISP-2, cathepsin D, pS2 and high mobility group 1 protein cDNA probes were generated by PCR from MCF-7 cDNA using specific primers and amplified products were confirmed by DNA sequencing.

Statistical analysis. The statistical significance of difference between samples was calculated as described previously by Madden, S.L. *et al.* (23) using the equation $(N_1 - kN_1^{1/2}) - (N_2 + kN_2^{1/2})$, where N_1 and N_2 represent the larger and smaller of the two numbers, respectively, and k is the degree of confidence.

RESULTS

SAGE Tag Abundance in MCF-7 Cells

A total of 31,000 tags from nontreated (ethanol vehicle only) MCF-7 cells facilitated the identification of more than 15,000 different transcripts. Table 1 lists the top 50 transcripts from MCF-7 cells. The most frequently expressed gene in MCF-7 cells was identified as keratin 8, with an expression frequency of 1.72% (Table 1). Keratin 8 is the major component of the intermediate-filament cytoskeleton of simple epithelia (26, 27). A high expression rate for many genes encoding ribosomal proteins was observed.

Table 2 lists the top 50 transcripts of 30,856 tags in E2-treated MCF-7 cells. Comparison of the two SAGE libraries indicated a remarkable similarity in expression profiles.

Comparison of Expression Patterns in E2-Untreated and Treated MCF-7 Cells

Among the identified transcripts, several genes were found to be significantly increased for E2-treated cells as compared with control MCF-7 cells (Table 3). To validate this observation, we performed Northern blotting using specific probes. We observed that the expression of four genes was confirmed to be increased in E2-treated cells compared with nontreated cells (Fig. 1). Among these four genes, three have already been reported as estrogen-responsive genes, cathepsin D (28), pS2 (29) and high mobility group 1 protein (30). The remaining gene is identified here as a novel estrogen-responsive gene, WISP-2 (Wnt-1 inducible signaling pathway protein 2). WISP-2 has been recently cloned as an up-regulated gene in a mouse mammary epithelial cell line, C57MG transformed by Wnt-1, an oncogene activated by the insertion of mouse mammary tumor virus in virus-induced mammary adenocarcinoma (31). We failed to confirm the other genes to be E2-inducible by Northern blotting (data not shown).

Time Course of WISP-2 Expression and Dose-Dependent Induction by E2

Analyses of WISP-2 mRNA revealed that E2 upregulated WISP-2 mRNA in a time-dependent manner (Fig. 2). In the absence of E2, expression of WISP-2 was observed. This is probably due to low amount of estrogen still present in the charcoal-treated serum. The expression of WISP-2 mRNA was enhanced, and this increase continued for up to 72 h subsequent to the addition of E2. In contrast, the expression of WISP-2

TABLE 1

Transcripts Profile in MCF-7 Cells

Abundance (%)	Tag sequence	UnGene cluster	Description
1.72	CCTCCAGCTA	73742	keratin 8
1.62	CCCATCGTCC		Multiple match
1.35	TTGGTCCTCT	108124	homologue to yeast ribosomal protein L41
1.24	CACCTAATTG		Multiple match
1.23	TGATTTCACT		Multiple match
1.19	TTCATACACC	mito	tag matches mitochondrial sequence
0.97	CAAGCATCCC	223214	EST
0.81	CCCGTCCGGA	180842	ribosomal protein L13
0.75	CTAAGACTTC		Multiple match
0.75	AAAAAAAAAA		Multiple match
0.71	AGCCCTACAA		Multiple match
0.70	ATAATTCTTT	539	ribosomal protein S29
0.69	CCAGAACAGA		Multiple match
0.63	CAAACCATCC	65114	keratin 18
0.60	TGCACGTTTT	169793	ribosomal protein L32
0.59	CACTACTCAC		No match
0.50	CCTCAGGATA		Multiple match
0.49	AAAACATTCT	mito	tag matches mitochondrial sequence
0.48	TAGGTTGTCT	119252	tumor protein, translationally-controlled 1
0.48	CTGGGTTAAT	126701	ribosomal protein S19
0.47	ACCCTTGGCC	mito	tag matches mitochondrial sequence
0.46	ACTTTTTCAA	149587	DKFZp 564E1616
0.43	ATTTGAGAAG	mito	tag matches mitochondrial sequence
0.41	CTCATAAGGA	mito	tag matches mitochondrial sequence
0.39	GTGAAACCCC		Multiple match
0.38	AAGGTGGAGG	163593	ribosomal protein L18a
0.37	TTGGGGTTTC	62954	ferritin, heavy polypeptide 1
0.37	CCTGTAATCC		Multiple match
0.36	CGCCGCCGGC	182825	ribosomal protein L35
0.36	GCAGCCATCC	4437	ribosomal protein L28
0.35	CCCCAGCCAG	75459	ribosomal protein S3
0.34	GGCCGCGTTC	5174	ribosomal protein S17
0.34	ACTAACACCC		Multiple match
0.34	ATGGCTGGTA	182426	ribosomal protein S2
0.33	AAGACAGTGG	184109	ribosomal protein L37a
0.32	TGGTGTTGAG	75362	clone 1033B10 on chromosome 6p21.2–21.31
0.31	TGTGTTGAGA	181165	eukaryotic translation elongation factor 1 alpha 1
0.31	CACAACGGT	19453	ribosomal protein S27
0.31	AGCACCTCCA	75309	eukaryotic translation elongation factor 2
0.30	CGCCGACGAT	21205	interferon, alpha-inducible protein
0.29	GGGAAGCAGA		No match
0.28	CCTAGCTGGA	182973	peptidylprolyl isomerase A
0.28	GAAGCAGGAC	180370	cofilin 1
0.28	ACCGCCTGTG	214455	EST
0.28	TAAAAAAAAA		Multiple match
0.28	GGGCTGGGGT		Multiple match
0.27	GAAAAATGGT	181357	laminin receptor 1 (67 kD)
0.27	AGGCTACGGA	119122	23 kD highly basic protein
0.26	CATTTGTAAT		Multiple match
0.26	CCACTACTGC		No match

Note. Top 50 transcripts expressed in MCF-7 cells are listed. The tag sequence represents the 10-bp SAGE tag. UnGene cluster are listed. More information on this table is available at our web site (http://www.prevent.m.u-tpkyp.ac.jp/SAGE.html).

mRNA remained low levels for no E2-treated cells (Fig. 2).

Dose–response experiments indicated that the induction of WISP-2 mRNA by E2 is dose-dependent and that the estimated EC_{50} levels lay between 10 and 100 pM (Figs. 3A and B).

The Induction of the WISP-2 Gene by E2 Is Mediated through an ER in MCF-7 Cells

To clarify whether the effect of E2 on WISP-2 gene expression was mediated by an ER, a pure anti-estrogen ICI182,780 was added, contemporaneously,

TABLE 2

Transcript Profile in E2-Treated MCF-7 Cells

Abundance (%)	Tag sequence	UnGene cluster	Description
1.62	CCCATCGTCC		Multiple match
1.35	TTGGTCCTCT	108124	homologue to yeast ribosomal protein L41
1.35	CACCTAATTG		Multiple match
1.14	TTCATACACC	mito	tag matches mitochondrial sequence
1.11	TGATTTCACT		Multiple match
0.96	CCTCCAGCTA	73742	keratin 8
0.86	ATGGCTGGTA	182426	ribosomal protein S2
0.83	CCAGAACAGA		Multiple match
0.82	CCCGTCCGGA	180842	ribosomal protein L13
0.79	CAAGCATCCC		Multiple match
0.78	CTGGCCCTCG		Multiple match
0.76	CTAAGACTTC		Multiple match
0.75	AAAAAAAAA		Multiple match
0.58	ATAATTCTTT	539	ribosomal protein S29
0.57	AGCCCTACAA		Multiple match
0.56	TGCACGTTTT	169793	ribosomal protein L32
0.53	CACTACTCAC		No match
0.51	TGTGTTGAGA	181165	eukaryotic translation elongation factor 1 alpha 1
0.46	AAGACAGTGG	184109	ribosomal protein L37a
0.43	TGGTGTTGAG	75362	clone 1033B10 on chromosome 6p21.2–21.31
0.42	GAAAAATGGT	181357	laminin receptor 1 (67 kD)
0.41	CTCATAAGGA		Multiple match
0.41	TAGGTTGTCT	119252	tumor protein, translationally-controlled 1
0.40	CTGGGTTAAT	126701	ribosomal protein S19
0.40	ATTTTGAGAAG		Multiple match
0.38	CCTAGCTGGA	182937	peptidylprolyl isomerase A
0.38	GTGACCACGG	36451	glutamate receptor, ionotropic
0.37	TTCAATAAAA		Multiple match
0.36	CGCCGCCGGC	182825	ribosomal protein L35
0.35	CCACTACTGC		No match
0.34	AAGGTGGAGG	163593	ribosomal protein L18a
0.34	AGGCTACGGA	119122	23 kD highly basic protein
0.34	ACCCTTGGCC		Multiple match
0.34	CCTGTAATCC		Multiple match
0.33	CCTCAGGATA		Multiple match
0.32	CACAAACGGT	195453	ribosomal protein S27
0.32	CAAACCATCC	65114	keratin 18
0.32	CCCCAGCCAG	75459	ribosomal protein S3
0.32	CCCAAGCTAG		Multiple match
0.31	CTCAACATCT	73742	keratin 8
0.29	TTGGGGTTTC	62954	ferritin, heavy polypeptide 1
0.29	AGGGCTTCCA	29797	DNA segment on chromosome X 648 expressed sequence
0.29	GTGAAACCCC		Multiple match
0.29	GGCCGCGTTC	5174	ribosomal protein S17
0.29	CATTTGTAAA		Multiple match
0.29	AATCCTGTGG	178551	ribosomal protein L8
0.28	AAAACATTCT	mito	tag matches mitochondrial sequence
0.27	ACTAACACCC		Multiple match
0.27	GAAGCAGGAC	180370	confilin 1
0.27	GCAGCCATCC	4437	ribosomal protein L28

Note. Top 50 transcripts expressed in MCF-7 cells are listed. The tag sequence represents the 10-bp SAGE tag. UnGene cluster are listed. More information on this table is available at our web site (http://www.prevent.m.u-tokyo.ac.jp/SAGE.html).

with E2 (Fig. 4). The presence of ICI182,780 completely precluded the induction of the WISP-2 gene by E2, indicating that the induction of WISP2 mRNA by E2 is ER-dependent (Fig. 4, lane 3).

In order to determine whether the induction of WISP-2 mRNA requires protein synthesis, MCF-7 cells were treated with E2 in combination with a protein-synthesis inhibitor, cycloheximide at 10 μg/ml. The induction of WISP-2 was not abolished by cycloheximide (lane 4), suggesting that the increase in WISP 2 mRNA elicited by E2 is independent from new protein synthesis. Next, we investigated as to whether the E2 effect on WISP-2 mRNA expression occurred at the transcriptional or the post-transcriptional level, by

TABLE 3

Increased Transcripts in E2-Treated MCF-7 Cells

Tag number					
Untreated	Treated	Fold	Tag sequence	UnGene cluster	Description
41	241	5.9	CTGGCCCTCG	1406	pS2 protein
104	266	2.6	ATGGCTGGTA	182426	ribosomal protein S2
20	76	3.8	GAAATACAGT	79572	cathepsin D
8	51	6.4	AGTAGGTGGC	mito	tag matches mitochondrial sequence
1	16	16.0	CACACGGGCG	194679	WNT1 inducible signaling pathway protein 2
1	16	16.0	GAAATTTAAA	189509	high mobility group 1 protein
3	27	9.0	GGCAGAGGAC	118639	non-metastatic cells 1, protein (NM23A) expressed in

Note. Table represents the increased tags showing differentials with confidence $P < 0.001$. Statistical significance between samples was calculated as described under Materials and Methods. The tag sequence represents the 10-bp SAGE tag. Tag number indicates the number of times the tag was identified.

treating cells with 10 nM E2 in the presence of 5 μg/ml of an RNA-synthesis inhibitor actinomycin D. In contrast to cycloheximide, the RNA-synthesis inhibitor actinomycin D prevented the induction of WISP2 mRNA for E2-treated MCF-7 cells (lane 5).

WISP-2 Gene Is also Regulated by Xenoestrogens

In order to clarify as to whether the expression of the WISP-2 gene is also regulated by xenoestrogens (XEs), we treated cells with the XEs, bisphenol-A (BPA) (32) and nonylphenol (NP) (33). Our results indicate that treatment of MCF-7 cells with BPA or NP induced the expression of WISP-2 (Fig. 5, lanes 2 and 3), and the treatment of MCF-7 cells with a mixture of BPA and NP resulted in an additive effects (lane 4). These effects of XEs were antagonized by a pure antiestrogen, ICI 182,780, implying that the effects of these compounds are also mediated through an ER (lanes 5 and 6).

DISCUSSION

One approach to understanding the molecular basis of estrogen action is to identify differences in gene expression profile between estrogen treated and non estrogen-treated cells. Several methods, such as Northern blotting, RT-PCR, differential display, and subtraction, are useful for such studies. However, these technologies can analyze only a limited numbers of genes, and the quantitative analysis of the transcription of individual genes is difficult. Although microarray technology can potentially examine the expression patterns of a relatively large number of genes, the method can only examine expressed sequences that have already been identified. The SAGE method allows for a quantitative and simultaneous analysis of a large numbers of transcripts in any particular cell system, without prior knowledge of the genes (19–23). Thus, we chose to use SAGE to identify the novel estrogen responsive genes.

MCF-7 human breast cancer cells express the ER and represent a prototypical estrogen-responsive cell line which has been widely used for studies on estrogen- and antiestrogen-induced responses (3, 4). The present study identified WISP-2 as a novel estrogen-responsive gene in MCF-7 cells. This gene has

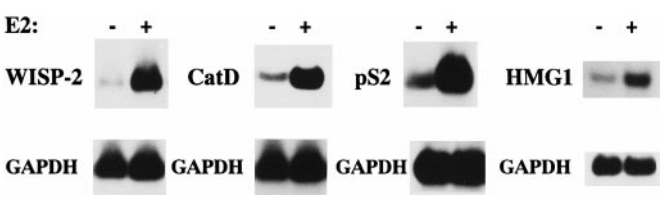

FIG. 1. Northern blot analysis of estrogen-regulated mRNAs. Differential expression patterns were confirmed by Northern blot analyses. Total RNA was isolated at 24 h from MCF-7 cells in the presence (+) or absence (−) of 17 β estradiol (E2) (10 nM). The blotted membrane was hybridized with ^{32}P-labeled WISP-2 or cathepsin D (Cat D) or pS2 or high mobility group (HMG) 1 protein cDNA probe. GAPDH mRNA is shown as a loading control.

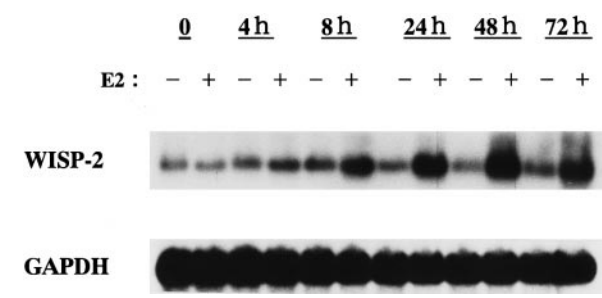

FIG. 2. Time course of WISP-2 induction by E2 in MCF-7 cells. Total RNA was isolated at indicated times from MCF-7 cells in the presence (+) or absence (−) of 17β-estradiol (E2) (10 nM). The blotted membrane was probed for WISP-2 mRNA, and GAPDH mRNA is shown as a loading control. The WISP-2 signal under E2 treatment was determined by densitometric analysis and was normalized to the GAPDH signal from each lane. The data expressed as percent of the time 0 were as follows: 0, 100; 4 h, 152; 8 h, 260; 24 h, 479; 48 h, 520; 72 h, 403.

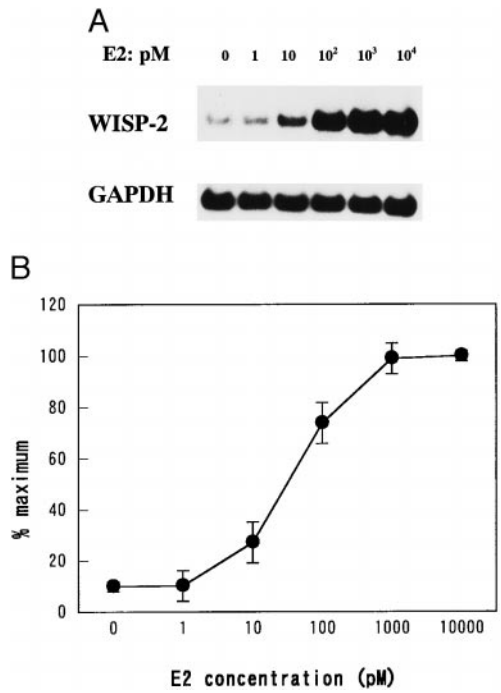

FIG. 3. Dose dependence of WISP-2 induction by E2. (A) MCF-7 cells were treated for 48 h with the indicated concentrations of E2. Total RNAs were analyzed on a Northern blot using ^{32}P-labeled WISP-2 or GAPDH cDNA probe. (B) The levels of RNA were determined by densitometric analysis. Values were corrected for variations with GAPDH levels. Results are expressed as percentages of the maximum. Results are expressed as the mean ± SD of three independent experiments.

recently been identified by Pennica *et al.* (31) as being located downstream from Wnt-1 signaling. To identify downstream genes in the Wnt signaling pathway that are relevant to the transformed cell phenotype, these

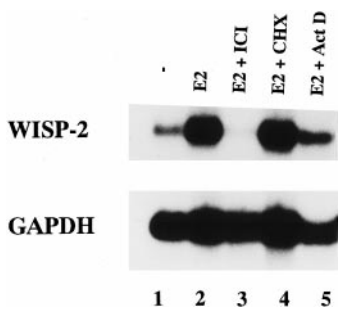

FIG. 4. Effect of ICI182,780 (ICI), cycloheximide (CHX), actinomycin D (Act D) on E2-mediated increase in WISP-2 mRNA levels in MCF-7 cells. Cells were treated for 24 h with E2 (10 nM), ICI 182,780 (1 μM), cycloheximide (10 μg/ml), actinomycin D (5 μg/ml), individually or in various combinations, as indicated. The blotted membrane was hybridized with ^{32}P-labeled WISP-2, or GAPDH probe. The WISP-2 signal was determined by densitometric analysis and was normalized to the GAPDH signal from each lane. The data expressed as percent of the lane 1 were as follows: lane 1, 100; lane 2, 420; lane 3, 12; lane 4, 410; lane 5, 120.

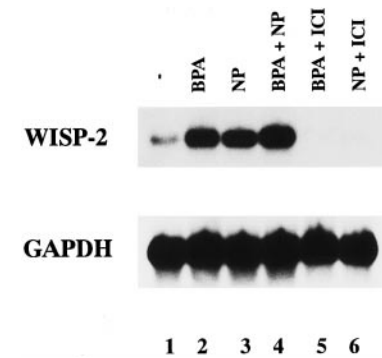

FIG. 5. Northern blot analysis of WISP-2 mRNA levels after treatment with xenoestrogens. Cells were grown in medium containing 1 μM bisphenol-A (BPA), 1 μM nonylphenol (NP) or both for 48 h. The antiestrogen ICI 182,780 was added contemporaneously. Total RNA was then extracted, and 20 μg total RNA were loaded into each lane. The blotted membrane was hybridized with ^{32}P-labeled WISP-2 or GAPDH cDNA probe. The WISP-2 signal was determined by densitometric analysis and was normalized to the GAPDH signal from each lane. The data expressed as percent of the lane 1 were as follows: lane 1, 100; lane 2, 612; lane 3, 628; lane 4, 888; lane 5, 0; lane 6, 0.

authors performed a PCR-based cDNA subtraction strategy, using RNA isolated from C57MG mouse mammary epithelial cells and C57MG cells stably transformed by a Wnt-1 retrovirus. The WISP-2 gene is up-regulated in C57MG cells transformed by the Wnt-1 retrovirus. WISP-2 is a member of the CCN family of growth factors which includes connective tissue growth factor (31, 34, 35). Members of the Wnt signaling pathway have been implicated in the pathogenesis of colon cancer and breast cancer (36). The rat ortholog, rCop-1, was identified as a gene whose expression became lost after cell transformation (37). Transfection of rCop-1 into transformed cells suppressed their growth and this was attributed to cell death rather than growth arrest (37). Potential tumor-suppressive properties are further suggested by the finding that WISP-2 is underexpressed in human colon tumors (31). Thus, the expressed levels of WISP-2 in mammary tumors may be a novel marker to predict the disease's prognosis.

The exposure of MCF-7 cells to estrogens results in the induction of certain mRNAs and proteins. Previous studies have demonstrated that the induction of cathepsin D and pS2 in human mammary cells takes place at the transcriptional level and occurs in the absence of de novo protein synthesis (28, 29, 38). Like these two genes, WISP-2 mRNA was induced in the absence of de novo protein synthesis. Although nuclear run-on experiments have not been performed, the RNA synthesis inhibitor actinomycin D abolished the induction of WISP-2 mRNA for E2-treated cells, suggesting a direct stimulation of transcription by E2. The identification of structural elements which are involved in mediating the estrogen-response will be necessary to

fully understand the molecular mechanism of WISP-2 gene induction by estrogens.

XEs are non-steroidal, human-produced chemicals that enter the body by ingestion, inhalation or adsorption (15–18). These compounds are suspected to play a causative role in alterations of sexual development in wildlife species (16–18). Thus, novel markers to assess the extent of individual exposure to such chemicals are clearly warranted. Due to the existence of a putative signal sequence and the absence of a transmembrane domain, WISP-2 is considered to be secreted protein (31). For humans, WISP-2 mRNA is expressed in adult skeletal muscle, colon, ovary, and fetal lung (31). Although the expression of the WISP-2 in these organs is regulated by estrogens yet to be determined, the expressed level of WISP-2 may be a novel marker to evaluate the exposure to estrogenic compounds. Further study is now underway in order to identify the WISP-2 protein utilizing a specific antibody derived from culture supernatants or from the blood stream of individuals exposed chronically to low concentrations of xenoestrogens.

In summary, we show that WISP-2 is a novel estrogen responsive gene in the MCF-7 human breast cancer cell line, and this effect is directly regulated by an ER. The role of the WISP-2 in the progression of breast cancer, and whether or not the WISP-2 could be a novel marker for breast cancer prognosis and exposure to xenoestrogens remain to be established.

ACKNOWLEDGMENTS

We are very grateful to Drs. V. Velculescu, L. Zhang, W. Zhou, B. Vogelstein, and K. Kinzler for their help in SAGE analysis. We acknowledge Dr. A. Kutlaca for the review of the manuscript. This study was supported by Core Research for Evolutional Science and Technology and the Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of the Japanese Government.

REFERENCES

1. Tsai, M. J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486.
2. Muramatsu, M., and Inoue, S. (2000) *Biochem. Biophys. Res. Commun.* **270,** 1–10.
3. Dickson, R. B., and Lippman, M. E. (1987) *Endocrinol. Rev.* **8,** 29–43.
4. Dickson, R. B., and Lippman, M. E. (1986) *Cancer Surv.* **5,** 617–624.
5. Foster, J. S., and Wimalasena, J. (1996) *Mol. Endocrinol.* **10,** 488–498.
6. Prall, O. W. J., Sarcevic, B., Musgrove, E. A., Watts, C. K. W., and Sutherland, R. L. (1997) *J. Biol. Chem.* **272,** 10882–10894.
7. Duan, R., Porter, W., and Safe, S. (1998) *Endocrinology* **139,** 1981–1990.
8. Foster, J. S., and Wimalasena, J. (1996) *Mol. Endocrinol.* **10,** 488–498.
9. Dubik, K., Dembinske, T. C., and Shiu, R. P. (1987) *Cancer Res.* **47,** 6517–6521.
10. Wang, W., Dong, L., Saville, B., and Safe, S. (1999) *Mol. Endocrinol.* **13,** 1373–1387.
11. Dong, L., Wang, W., Wang, F., Stoner, M., Reed, J. C., Harigai, M., Samudio, I., Kladde, M. P., Vyhlidal, C., and Safe, S. (1999) *J. Biol. Chem.* **274,** 32099–32107.
12. Molloy, C. A., May, F. E. B., and Westley, B. R. (2000) *J. Biol. Chem.* **275,** 12565–12571.
13. Qin, C., Singh, P., and Safe, S. (1999) *Endocrinology* **140,** 2501–2508.
14. Pethe, V., and Shekhar, P. V. M. (1999) *J. Biol. Chem.* **43,** 30969–30978.
15. Safe, S. H. (1995) *Environ. Health Perspect.* **103,** 346–351.
16. Colborn, T. (1995) *Environ. Health Perspect.* **103,** 135–136.
17. Ashby, J., Houthoff, E., Kennedy, S. J., Stevens, J., Bars, R., Jekat, F. W., Campbell, P., Miller, J. V., Carpanini, F. M., and Randall, G. L. P. (1997) *Environ. Health Perspect.* **105,** 164–169.
18. Colborn, T., vom Saal, F. S., and Soto, A. M. (1993) *Environ. Health Perspect.* **101,** 378–385.
19. Velculescu, V. E., Zhang, L., Vogelstein, B., and Kinzler, K. W. (1995) *Science* **270,** 484–487.
20. Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. (1997) *Nature* **389,** 300–305.
21. Hashimoto, S., Suzuki, T., Dong, H.-Y., Yamazaki, N., and Matsushima, K. (1999) *Blood* **94,** 837–844.
22. Hashimoto, S., Suzuki, T., Dong, H.-Y., Nagai, S., Yamazaki, N., and Matsushima, K. (1999) *Blood* **94,** 845–852.
23. Madden, S. L., Galella, E. A., Zhu, J., Bertelsen, A. H., and Beaudry, G. A. (1997) *Oncogene* **15,** 1079–1085.
24. Soule, H. D., Vazquez, J., Long, A., Albert, S., and Brennan, M. (1973) *J. Natl. Cancer Inst.* **51,** 1409–1415.
25. Inadera, H., Sekiya, T., Yoshimura, T., and Matsushima, K. (2000) *Endocrinology* **141,** 50–59.
26. Kulesh, D. A., Cecena, G., Darmon, Y. M., Vasseur, M., and Oshima, R. G. (1989) *Mol. Cell. Biol.* **9,** 1553–1565.
27. Liao, J., Ku, N. O., and Omary, M. B. (1997) *J. Biol. Chem.* **272,** 17565–17573.
28. Cavailles, V., Augereau, P., and Rochefort, H. (1991) *Biochem. Biophys. Res. Commun.* **174,** 816–824.
29. Berry, M., Nunez, A. M., and Chambon, P. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1218–1222.
30. Chau, K. Y., Lam, H. Y. P., and Lee, K. L. D. (1998) *Exp. Cell Res.* **241,** 269–272.
31. Pennica, D., Swanson, T. A., Welsh, J. W., Roy, M. A., Lawrence, D. A., Lee, J., Brush, J., Taneyhill, L. A., Deuel, B., Lew, M., Watanabe, C., Cohen, R. L., Melhem, M. F., Finley, G. G., Quirke, P., Goddard, A. D., Hillan, K. J., Gurney, A. L., Botstein, D., and Levine, A. J. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14717–14722.
32. Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L., and Feldman, D. (1993) *Endocrinology* **132,** 2279–2286.
33. Soto, A. M., Justicia, H., Wray, J. W., and Sonnenschein, C. (1991) *Environ. Health Perspect.* **92,** 167–173.
34. Lau, L. F., and Lam, S. C. T. (1999) *Exp. Cell Res.* **248,** 44–57.
35. Brigtock, D. R. (1999) *Endocr. Rev.* **20,** 189–206.
36. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) *Science* **275,** 1787–1790.
37. Zhang, R., Averboukh, L., Zhu, W., Zhang, H., Jo, H., Dempsey, P. J., Coffey, R. J., Pardee, A. B., and Liang, P. (1998) *Mol. Cell Biol.* **18,** 3131–3141.
38. Cavailles, V., Augereau, P., Garcia, M., and Rochefort, H. (1988) *Nucleic Acids Res.* **16,** 1903–1919.