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hAG-2, the Human Homologue of the *Xenopus laevis* Cement Gland Gene XAG-2, Is Coexpressed with Estrogen Receptor in Breast Cancer Cell Lines[1]

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hAG-2, the human homologue of the *Xenopus laevis* cement gland gene *Xenopus* Anterior Gradient-2 (XAG-2), has been found to be coexpressed with estrogen receptor (ER) in breast cancer cell lines by using suppression subtractive hybridization. Sequence analysis of clones obtained from a human breast adenocarcinoma MCF7 cDNA library suggests that at least three mRNA transcripts, differing in their 3′-untranslated regions, are transcribed from the hAG-2 gene. Northern blot analyses identified two principal hAG-2 signals in all ER-positive breast carcinoma cell lines that were absent in cell lines not expressing, or minimally expressing, ER. Coexpression of hAG-2 with ER suggests that hAG-2 may be involved in the tumor biology specific to the well-differentiated phenotype of hormonally-responsive breast cancers.

Key Words: **estrogen receptor; breast cancer; differentiation; Northern analysis.**

Hormone-responsive breast cancer is associated with the expression of the estrogen receptor (ER) transcription factor. Breast carcinomas that express ER (ER-positive) have significant biological and histopathological differences when compared to breast carcinomas that fail to express ER (ER-negative) [1, 2]. Generally ER-negative breast carcinomas are less well-differentiated and tend to be more aggressive clinically than ER-positive breast tumors [3-7]. Although the expression of ER is intimately associated with the biology of breast carcinoma, the mechanisms regulating transcription of ER and the role of ER in tumorigenesis have not been elucidated. In addition, the molecular basis for the differences between ER-positive and ER-negative tumors and the relationship of ER to the hormone-responsive phenotype remain unclear.

The tumor biology underlying differences between hormone-responsive and hormone-unresponsive breast cancers may be elucidated by the identification of genes that are differentially expressed between these two sub-groups. Recent studies from this laboratory and others have identified several proteins that may contribute to the differing phenotypes including the transcription factors AP-2γ/ERF-1 [8] and GATA-3 [9], proteins involved in proliferation and differentiation that include progesterone receptor [10], pS2 [11, 12] and heat shock protein 27,000 [13, 14], and the intermediate filament proteins cytokeratin 8 and 18 [15-17], and a G protein-coupled receptor (GPR30) [18].

By using the technique of suppression subtractive hybridization (SSH) [19], this laboratory recently reported the identification of 29 gene fragments that were expressed in the ER-positive MCF7 breast carcinoma cell line that were absent or minimally expressed in the ER-negative MDA-MB-231 breast carcinoma cell line [17]. One of these gene fragments, DEME-2 (GenBank EST accession AA506763), exhibited an expression pattern that correlated with the expression of ER in a panel of eight breast carcinoma cell lines [17]. The full-length message containing the DEME-2 fragment has now been isolated and found to represent the human homologue of the *Xenopus laevis* cement gland gene Xenopus Anterior Gradient-2 or XAG-2 [20] (GenBank accession AF025474) (previously referred to as np77 [21] GenBank accession U82110), named according to its pattern of expression during cement gland development. DEME-2 has therefore been renamed hAG-2. Co-expression of hAG-2 with ER suggests that hAG-2 may be involved in the tumor biology specific to the well-differentiated phenotype of hormonally-responsive breast cancers.

[1] The nucleotide sequences described in this paper have been submitted to the GenBank database at NCBI and assigned the following accession numbers: hAG-2/C, AF007791; hAG-2/I, AF038452; hAG-2/R, AF038451; mAG-2, AF044262.

Abbreviations: ER, estrogen receptor; SSH, suppression subtractive hybridization, CNS, central nervous system; NCBI, National Center for Biotechnology Information; bp, base pair(s); kb, kilobase(s).

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MATERIALS AND METHODS

Cell lines. All cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD). Cell lines MCF7, T-47D, BT-20, MDA-MB-231, HBL-100 were maintained in Minimal Essential Medium (Life Technologies), ZR-75-1 and BT-474 were maintained in RPMI 1640 (Life Technologies, Inc.) and MDA-MB-361 was maintained in Leibovitz's L-15 medium (Life Technologies, Inc.). Media were supplemented with 10% fetal calf serum (Hyclone), 10 U/mL penicillin G (Life Technologies, Inc.), 10 μg/mL streptomycin (Life Technologies, Inc.), and 6 ng/mL bovine insulin (Sigma). All cell lines were incubated at 37°C in 5% CO_2 except MDA-MB-361 which was maintained in a CO_2-free environment.

Poly(A)$^+$ RNA isolation and SSH. Poly(A)$^+$ RNA was isolated from cell lines using the Fast Track Kit 2.0 (Invitrogen) according to the manufacturer's instructions. SSH was performed with the CLONTECH PCR-Select cDNA Subtraction Kit (Clontech) as described previously [17].

Northern blot analysis. Northern blots containing poly(A)$^+$ RNA from human breast carcinoma cell lines were prepared, hybridized and washed as described previously [22]. The commercial human and mouse Multiple Tissue Northern (MTN) blots (Clontech) and the Human RNA Master Blot (Clontech) were hybridized [in ExpressHyb Hybridization Solution (Clontech) with probes labelled by random priming (Boehringer Mannheim)] and washed according to the conditions recommended by the manufacturers.

Screening of cDNA libraries. The human breast adenocarcinoma MCF7 cell line cDNA library described previously (23) was screened to isolate hAG-2. Approximately 1×10^6 λ phage plaques were probed with the ^{32}P-labelled 376 bp hAG-2 (previously called DEME-2) fragment isolated previously by SSH [17], using the conditions described in Thompson and Weigel [22]. Isolated plaques were processed to excise the cDNA inserts from the λ vector to yield recombinant pBK-CMV phagemids (Stratagene) in *Escherichia coli* XL1-Blue MRF′ cells (Stratagene).

The cDNA encoding mAG-2, the mouse homologue of hAG-2, was isolated from a 17-day mouse embryo cDNA library (5′-STRETCH PLUS, oligo(dT) + random primed, λgt10; Clontech) by screening with hAG-2/R (see Results section for terminology). Approximately 1×10^6 λ phage plaques were screened with the ^{32}P-labelled 1077 bp hAG-2/R clone, using conditions as described in Thompson and Weigel [22]. The cDNA inserts were released from the λ vector by *Eco*R I digestion, and the fragments cloned into pBluescript KS+ (Stratagene) by transformation of *E. coli* XL1-Blue MRF′ cells.

DNA sequence analysis. DNA sequencing reactions were carried out on double-stranded templates using the dideoxynucleotide chain-termination method [24] with [α-^{35}S]dATP (Amersham Pharmacia Biotech) and the Sequenase version 2.0 DNA Sequencing Kit (Amersham Pharmacia Biotech) with SP6 (pCR II) or T3 (pBK-CMV and pBluescript KS+) and T7 promoter primers, and custom primers obtained from Operon Technologies. Template DNA for all sequencing reactions was prepared using the Wizard Plus Minipreps DNA Purification System (Promega). All sequence data reported was determined on both DNA strands.

The nucleotide sequences of the hAG-2 and mAG-2 cDNA clones and the 175 amino acid protein sequences of hAG-2 and mAG-2 were compared against the NCBI databases (http://www.ncbi.nlm.nih.gov/) using BLASTn and BLASTp, respectively. In addition, the amino acid sequence of hAG-2 and mAG-2 were analyzed using the PROSITE Motif dictionary [http://www.genome.ad.jp/SIT/MOTIF.html] [25], SOSUI (Secondary Structure Prediction of Membrane Proteins) [http://www.tuat.ac.jp/~mitaku/sosui/], PSORT II [http://psort.nibb.ac.jp:8800/] [26], and the programs at the ExPASy server [http://expasy.hcuge.ch].

RESULTS

Identification and Sequence of hAG-2

A 376 bp cDNA fragment, DEME-2, was isolated previously from ER-positive MCF7 cells (tester) using SSH with ER-negative MDA-MB-231 cells as driver [17]. By using the 376 bp ^{32}P-labelled SSH fragment as probe, approximately 76 positive λ phage plaques were identified in a MCF7 cDNA library. Following phage purification and insert excision to phagemids, six cDNA clones were sequenced and revealed three different, though overlapping, transcripts that were designated hAG-2/C, hAG-2/I and hAG-2/R. Comparisons of these cloned cDNA sequences with the GenBank/EMBL databases using BLASTn and BLASTp indicated a high similarity with the *Xenopus laevis* cement gland gene XAG-2 (Xenopus Anterior Gradient-2) [20] (GenBank accession AF025474) (previously known as np77; [21] GenBank accession U82110), with a lower similarity to XAG-1 [20] (GenBank accession U76752). In the likelihood that these clones represent the human homologues of XAG-2, they were named hAG-2 (human Anterior Gradient-2). The three different hAG-2 transcripts have been submitted to GenBank with the accession numbers AF007791 (hAG-2/C), AF038452 (hAG-2/I) and AF038451 (hAG-2/R).

The combined 1,702 nucleotide sequence of the hAG-2 cDNAs obtained from the sequence data derived for the individual hAG-2/C and hAG-2/I and hAG-2/R transcripts is depicted in Figure 1A, in addition to the deduced 175 amino acid sequence of the hAG-2 protein. The sequence of the 376 bp hAG-2 (previously DEME-2) fragment isolated by SSH lies within the 3′ noncoding region and is underlined in Figure 1A. The individual hAG-2 transcripts isolated from the MCF7 cDNA library are also indicated. The hAG-2/R cDNA clone was 1,077 bp in length (including a polyA-tail with 14 'A's), the 5′ end beginning at nucleotide -58 and terminating at base +1,006 (denoted with the symbol (*) in Fig. 1A). The hAG-2/C cDNA clone was 838 bp in length (including a polyA-tail with 32 'A's) with the 5′ end at nucleotide -10 and the 3′ end at +796 (denoted with the symbol (^) in Figure 1A). The hAG-2/I cDNA clone was 859 bp in length that began at nucleotide +786 and terminated at base +1,644 (denoted with the symbol (°) in Figure 1A). It is emphasized that the full length sequence depicted in Figure 2 does not represent an isolated biological transcript, but combines the sequences of the individual transcripts (hAG-2/C, hAG-2/I and hAG-2/R) obtained from the library screening.

Isolation and Sequence of mAG-2

The 1,077 bp hAG-2/R cDNA clone was used to probe a fetal mouse Multiple Tissue Northern (MTN) blot containing 2 μg/lane of poly(A)$^+$ RNA from 7-, 11-, 15-

A

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  -58 ACCGCATCCTAGCCGCCGACTCACACAAGGCAGGTGGGTGAGGAAATCCAGAGTTGCC   -1
      *                                              ^

    1 ATG GAG AAA ATT CCA GTG TCA GCA TTC TTG CTC CTT GTG GCC CTC TCC TAC ACT CTG GCC AGA GAT ACC ACA GTC   75
    1 M   E   K   I   P   V   S   A   F   L   L   L   V   A   L   S   Y   T   L   A   R   D   T   T   V     25

   76 AAA CCT GGA GCC AAA AAG GAC ACA AAG GAC TCT CGA CCC AAA CTG CCC CAG ACC CTC TCC AGA GGT TGG GGT GAC  150
   26 K   P   G   A   K   K   D   T   K   D   S   R   P   K   L   P   Q   T   L   S   R   G   W   G   D     50

  151 CAA CTC ATC TGG ACT CAG ACA TAT GAA GAA GCT CTA TAT AAA TCC AAG ACA AGC AAC AAA CCC TTG ATG ATT ATT  225
   51 Q   L   I   W   T   Q   T   Y   E   E   A   L   Y   K   S   K   T   S   N   K   P   L   M   I   I     75

  226 CAT CAC TTG GAT GAG TGC CCA CAC AGT CAA GCT TTA AAG AAA GTG TTT GCT GAA AAT AAA GAA ATC CAG AAA TTG  300
   76 H   H   L   D   E   C   P   H   S   Q   A   L   K   K   V   F   A   E   N   K   E   I   Q   K   L    100

  301 GCA GAG CAG TTT GTC CTC CTC AAT CTG GTT TAT GAA ACA ACT GAC AAA CAC CTT TCT CCT GAT GGC CAG TAT GTC  375
  101 A   E   Q   F   V   L   L   N   L   V   Y   E   T   T   D   K   H   L   S   P   D   G   Q   Y   V    125

  376 CCC AGG ATT ATG TTT GTT GAC CCA TCT CTG ACA GTT AGA GCC GAT ATC ACT GGA AGA TAT TCA AAT CGT CTC TAT  450
  126 P   R   I   M   F   V   D   P   S   L   T   V   R   A   D   I   T   G   R   Y   S   N   R   L   Y    150

  451 GCT TAC GAA CCT GCA GAT ACA GCT CTG TTG CTT GAC AAC ATG AAG AAA GCT CTC AAG TTG CTG AAG ACT GAA TTG  525
  151 A   Y   E   P   A   D   T   A   L   L   L   D   N   M   K   K   A   L   K   L   L   K   T   E   L    175

  526 TAAAGAAAAAAAAATCTCCAAGCCCTTCTGTCTGTCAGGCCTTGAGACTTGAAACCAGAAGAAGTGTGAGAAGACTGGCTAGTGTGGAAGCATAGTGAACA  625

  626 CACTGATTAGGTTATGGTTTAATGTTACAACAACTATTTTTAAGAAAAACATGTTTTAGAAATTTGGTTTCAAGTGTACATGTGTGAAAACAATATTGT  725

  726 ATACTACCATAGTGAGCCATGATTTTCTAAAAAAAAAAATAAATGTTTTGGGGGTGTTCTGTTTTCTCCAACTTGGTCTTTCACAGTGGTTCGTTTACCA  825
                                           ------                 °         ^

  826 AATAGGATTAAACACACACAAAATGCTCAAGGAAGGGACAAGACAAAACCAAAACTAGTTCAAATGATGAAGACCAAAGACCAAGTTATCATCTCACCAC  925

  926 ACCACAGGTTCTCACTAGATGACTGTAAGTAGACACGAGCTTAATCAACAGAAGTATCAAGCCATGTGCTTTAGCATAAAAGAATATTTAGAAAAACATC  1025
                                                                                      *

 1026 CCAAGAAAATCACATCACTACCTAGAGTCAACTCTGGCCAGGAACTCTAAGGTACACACTTTCATTTAGTAATTAAATTTTAGTCAGATTTTGCCCAACC  1125

 1126 TAATGCTCTCAGGGAAAGCCTCTGGCAAGTAGCTTTCTCCTTCAGAGGTCTAATTTAGTAGAAAGGTCATCCAAAGAACATCTGCACTCCTGAACACACC  1225

 1226 CTGAAGAAATCCTGGGAATTGACCTTGTAATCGATTTGTCTGTCAAGGTCCTAAAGTACTGGAGTGAAATAAATTCAGCCAACATGTGACTAATTGGAAG  1325
                                                                         ------

 1326 AAGAGCAAAGGGTGGTGACGTGTTGATGAGGCAGATGGAGATCAGAGGTTACTAGGGTTTAGGAAACGTGAAAGGCTGTGGCATCAGGGTAGGGGAGCAT  1425

 1426 TCTGCCTAACAGAAATTAGAATTGTGTGTTAATGTCTTCACTCTATACTTAATCTCACATTCATTAATATATGGAATTCCTCTACTGCCCAGCCCCTCCT  1525

 1526 GATTTCTTTGGCCCCTGGACTATGGTGCTGTATATAATGCTTTGCAGTATCTGTTGCTTGTCTTGATTAACTTTTTTGGATAAAACCTTTTTTGAACAGA  1625

 1626 AAAAAAAAAAAAAAAAAAA                                                                                   1644
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B

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XAG-2   MQTGLSLACLVLLCSVLGEAALRKPKRQAGATDTNGAAKSEPAPVKTKGLKTLDRGWGEDIEWAQTYEEGLAKARENNKPLMVIHH
mAG-2   MEK-FSVSAILLLVAI--SGTLAK------DTTVKSGAKKDPKDSRPKLPQTLSRGWGDQLIWTQTYEEALYRSKTSNRPLMVIHH
hAG-2   MEK-IPVSAFLLLVAL--SYTLAR------DTTVKPGAKKDTKDSRPKLPQTLSRGWGDQLIWTQTYEEALYKSKTSNKPLMIIHH
        *.  .... ..** ..    .* .       *  . .** .    . *  .** ****... *.*****.* ... .*.***.***

XAG-2   LEDCPYSIALKKAFVADKMAQKLAQEDFIMLNLVHPVADENQSPDGHYVPRVIFIDPSLTVRSDLKGRYGNKLYAYDADDIPELIT
mAG-2   LDECPHSQALKKVFAEHKEIQKLA-EQFVLLNLVYETTDKHLSPDGQYVPRIVFVDPSLTVRADITGRYSNRLYAYEPSDTALLYD
hAG-2   LDECPHSQALKKVFAENKEIQKLA-EQFVLLNLVYETTDKHLSPDGQYVPRIMFVDPSLTVRADITGRYSNRLYAYEPADTALLLD
        *..** * **** *  .*  **** *.*..****   .* . ****.**** .*.*******.*. ***.*.****.. * . *

XAG-2   NMKKAKSFLKTEL
mAG-2   NMKKALKLLKTEL
hAG-2   NMKKALKLLKTEL
        *****   *****
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FIG. 1. Nucleotide and deduced amino acid sequences of hAG-2 cDNA and alignment with other species. (A) The 1,702 bp nucleotide sequence of hAG-2 determined by DNA sequence analysis is shown with the first nucleotide of the start codon designated as +1. The nucleotide sequence of hAG-2 contains an open reading frame which encodes a protein of 175 amino acids as indicated. The region underlined indicates the sequence of the 376 bp DEME-2/hAG-2 fragment isolated by SSH. The 5′ and 3′ ends of the hAG-2/R, hAG-2/C and hAG-2/I cDNA clones are denoted beneath the nucleotide sequence with the symbols (*), (^) and (°), respectively. The occurrences of the potential polyadenylation signal motif, AATAAA, are underlined with a dashed line. Sequence analyses utilized the computer program DNA Strider 1.2 [30]. (B) The deduced protein sequences of human hAG-2, mouse mAG-2 and *Xenopus laevis* XAG-2 were aligned using Clustal V [31]. Identical amino acids are indicated with an asterisk and conserved amino acids are indicated with a period.

and 17-day old whole mouse embryos. A strong hybridization signal was obtained from the 17-day whole mouse embryo (data not shown). Subsequently, a 17-day whole mouse embryo cDNA library screened with the hAG-2/R cDNA clone yielded a 781 bp cDNA clone encoding the mouse homologue of hAG-2, named mAG-2. The nucleotide sequence for mAG-2 has been submitted to the GenBank database at NCBI and assigned accession number AF044262. DNA sequence analysis revealed that mAG-2 was 87% similar to hAG-2/R at the nucleotide level and also encoded 175 amino acid protein.

An alignment of the protein sequences of hAG-2, mAG-2 and XAG-2 is shown in Figure 1B. hAG-2 and mAG-2 have a similarity of 98.3% with 91.4% identical residues, whereas each of the mammalian proteins share 73.3% similarity with XAG-2 and with 47.4 % identical residues. Analysis of the protein sequences of hAG-2 and mAG-2 with PSORT II [26] predicts that both proteins have cleavable 20 amino acid signal peptides, a feature that is similar to the 18 amino acid signal peptide predicted for XAG-2 [21]. However, the predicted isoelectric points for the mature portions of both the hAG-2 and mAG-2 proteins is 9.06, whereas that predicted for the mature XAG-2 protein is substantially lower at 7.17. An analysis of the hAG-2 and mAG-2 proteins using the PROSITE Motif dictionary [25] did not reveal any features that might allow a functional role to be ascribed to either protein. The results from SOSUI did indicate that hAG-2 and mAG-2 would be soluble proteins based on the hydrophobicity index.

Expression Patterns of hAG-2 and mAG-2

As described above, sequence analyses of the hAG-2 clones suggests that at least three separate mRNA transcripts, hAG-2/R, hAG-2/C, and hAG-2/I, that differ in their polyA-tails are encoded by the hAG-2 gene. Northern blot analysis using the hAG-2/R transcript as probe identified two mRNA messages of approximately 0.9 kb and 1.6 kb in all ER-positive breast carcinoma cell lines, as shown in Figure 2. Interestingly, the same blot probed with the 376 bp hAG-2 SSH fragment detected only the 1.6 kb message as shown in Figure 2. The use of a cDNA probe encoding ER confirmed that all breast cancer cell lines described as ER-positive (MCF7, T-47D, MDA-MB-361, ZR-75-1 and BT-474) did indeed express ER, and the use of a β-actin probe demonstrated that similar amounts of RNA from each cell line were present (Figure 2).

The analysis of hAG-2 expression in a human Multiple Tissue Northern (MTN) showed a strong signal in lung with two bands of approximately 0.9 kb and 1.6 kb in size, and a weaker signal in pancreas, as shown in Figure 3A. When a human RNA Master Blot was analyzed for hAG-2 expression, as shown in Figure 3B, strong signals were detected in trachea (F3), lung (F2), stomach (C8), colon (C4), prostate (C7) and small intestine (E3), with weaker signals (listed in decreasing order) in pituitary gland (D4), salivary gland (D7), mammary gland (D8), bladder (C5), appendix (F1), ovary (D2), fetal lung (G7), uterus (C6), pancreas (D3), kidney (E1), fetal kidney (G3), testis (D1), placenta (F4) and thyroid gland (D6) (the latter two are seen on a longer exposure of the blot). Expression of hAG-2 was absent in all parts of the human central nervous system (A1-A8, B1-B7), heart (C1), aorta (C2), skeletal muscle (C3), adrenal gland (D5), liver (E2), spleen (E4), thymus (E5), peripheral leukocyte (E6), lymph node (E7), bone marrow (E8), fetal brain (G1), fetal heart (G2), fetal liver (G4), fetal spleen (G5) and fetal thymus (G6) (Fig. 3B). (F5-F8 and G8 do not contain RNA, H1-H8 are controls).

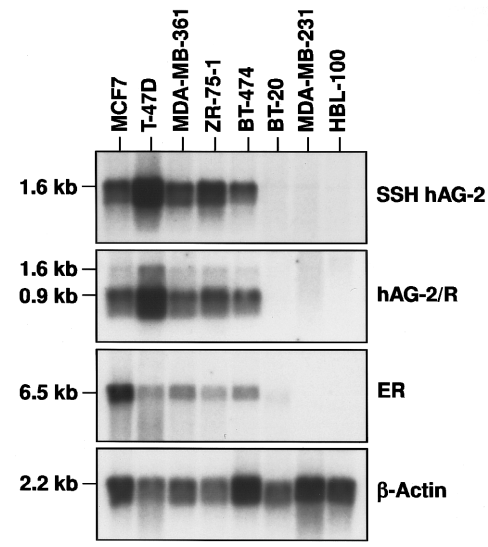

FIG. 2. Northern analysis of poly(A)$^+$RNA from a panel of human breast carcinoma cell lines. A Northern blot comprised of 1 μg of poly(A)$^+$ RNA per lane from the ER-positive breast carcinoma cell lines MCF7, T-47D, MDA-MB-361, ZR-75-1, BT-474, and BT-20 [the latter makes low levels of a mutated ER mRNA] and the ER-negative breast carcinoma cell lines MDA-MB-231 and HBL-100 was hybridized sequentially with the following ^{32}P-labelled cDNA probes as indicated: the 376 bp DEME-2/hAG-2 fragment isolated by SSH, the 1,077 bp hAG-2/R transcript, ER and β-actin.

Similarly, Northern analysis of mAG-2 expression, shown in Figure 4, displayed strong signals in adult mouse lung tissue and 17-day old mouse embryos, with weaker signals in 15-day old mouse embryos and adult mouse skeletal muscle, testis and liver. mAG-2 expression was absent from 7- and 11-day old mouse embryos, and adult mouse tissues including brain, heart, spleen and kidney. Figure 4 illustrates that only one message of approximately 0.9 kb was observed in all the mouse tissues examined.

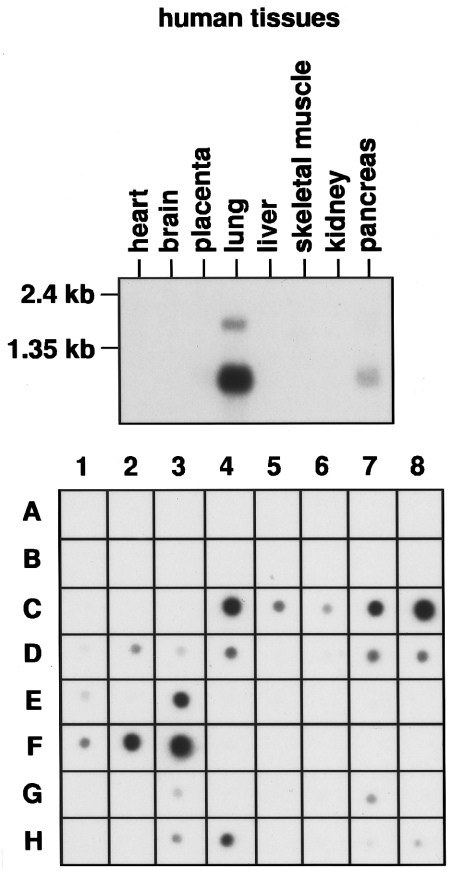

FIG. 3. hAG-2 expression in various tissues. A human Multiple Tissue Northern (MTN) blot (Clontech) containing 2 μg of poly(A)$^+$ RNA per lane from the tissues indicated (A) and a Human RNA Master Blot (Clontech) (B) were probed with the hAG-2/R cDNA that had been labelled with ^{32}P as described in the Materials and Methods section.

DISCUSSION

Suppression subtractive hybridization has allowed the identification of hAG-2, the human homologue of the *Xenopus laevis* cement gland gene XAG-2, as being co-expressed with ER in ER-positive breast cancer cell lines. Although considerable data is emerging regarding the role of XAG-2 during development of the frog, it is important to establish the role of hAG-2 in a mammalian system in order to understand its potential involvement in hormone-responsive breast carcinomas. The limitations of working with a human system lead us to isolate mAG-2, the mouse homologue of hAG-2. Mouse genetics has provided a useful model system with which to study mammalian gene expression and control. Due to the similarity between hAG-2/mAG-2 with the XAG proteins, we speculate that hAG-2 and mAG-2 will be expressed in a gradient in ectodermal tissues and is likely involved in the proliferation of mammalian tissues, including hormone-responsive breast carcinomas.

Although the biological roles of hAG-2 and mAG-2 are unknown, some hints as to their possible functions may be derived from the literature pertaining to the *Xenopus* homologues XAG-1 and XAG-2. In *Xenopus laevis*, the XAG family of genes are expressed in a gradient in the ectoderm during early development of the cement gland, and appear to be important factors during differentiation of this organ [20, 21, 27]. The cement gland is a mucus-secreting organ that lies at the extreme anterior of the frog embryo and is involved in the attachment of the embryo to a solid support prior to swimming and feeding [20, 27]. Interestingly, the tissues hAG-2 has been found to be expressed in contain mucus secreting cells and/or function as endocrine organs. The cement gland arises from the outer layer of embryonic ectoderm and forms a cone of columnar epithelium. The *Xenopus* anterior gradient genes, XAG-1, XAG-2 and XAG-3 are expressed at high levels in the cement gland and disappear during involution [20, 27]. Similar to hAG-2 and mAG-2, the XAG-1 and XAG-2 genes encode secreted proteins [21, 27] that are later expressed in the pharynx and lung primordium [27]

Evidence suggests that XAG-2 is activated by many of the Spemann organizer-secreted signals involved in neural induction, including noggin, chordin, follistatin and cerberus [21]. It has been suggested that homeobox gene, *Xenopus* otx2, acts as transcriptional activator of cement gland differentiation genes including XAG-1 [28]. Recently overexpression of XAG-2 was shown to induce cement gland differentiation and the expression of the anterior neural markers *Xenopus* otx2 and XIF3 [21]. Furthermore XGSK3 (*Xenopus* glycogen synthase kinase 3 beta) has been shown to activate both *Xenopus* otx2 and XAG-1 [29]. It is anticipated that additional information regarding the regulation and function of the XAG-2 will contribute significantly to defining these parameters for hAG-2 and mAG-2.

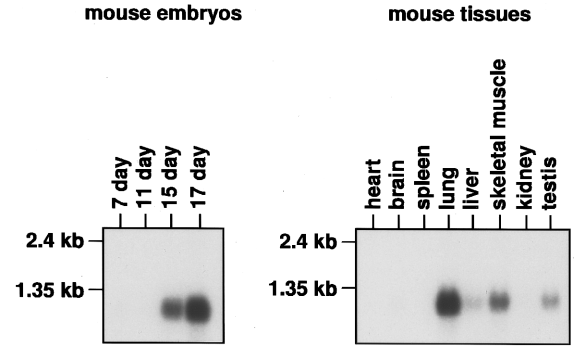

FIG. 4. mAG-2 expression in various tissues. Mouse Multiple Tissue Northern (MTN) blots (Clontech) containing 2 μg of poly(A)$^+$ RNA per lane from whole mouse embryos at different stages of development (A) and from adult mouse tissues (B) were probed with the mAG-2 cDNA that had been labelled with ^{32}P as described in the Materials and Methods section.

The original premise of this study was based on the hypothesis that the enhanced response to endocrine therapy and improved prognosis of women with ER-positive breast cancer may be attributed to a pattern of gene expression characteristic of the hormone-responsive phenotype. The identification of the novel gene hAG-2, co-expressed with ER in ER-positive breast cancer cell lines, is one such candidate gene that may contribute to the well-differentiated phenotype associated with hormone-responsive tumors. It will be of interest to analyze the 5′-flanking region of the hAG-2 gene in order to identify potential regulatory elements within the hAG-2 promoter. It will be particularly interesting to determine if hAG-2 gene expression is regulated in any way by the transcription factors ER, AP-2γ/ERF-1 or GATA-3 that have been implicated as potential contributors to the phenotypic differences of breast cancer tumors.

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REFERENCES

1. Fisher, E. R., Redmond, C. K., Liu, H., Reckette, H., and Fisher, B. (1980) *Cancer* **45,** 349–353.
2. Fisher, E. R., Sass, R., and Fisher, B. (1987) *Cancer* **59,** 1554–1559.
3. Knight, W. A., III, Livingston, R. B., Gregory, E. J., and McGuire, W. L. (1977) *Cancer Res.* **37,** 4669–4671.
4. Osborne, C. K., Yochmowitz, M. G., Knight, W. A., and McGuire, W. L. (1980).*Cancer* **46,** 2884–2888.
5. Mason, B. H., Holdaway, T. M., Mullins, P. R., Yee, L. H., and Kay, R. G. (1983) *Cancer Res.* **43,** 2985–2990.
6. Chevallier, B., Heintzmann, F., Mosseri, V., Daunce, J. P., Bastit, P., Graic, Y., Brunelle, P., Basuyau, J. P., Comoz, M., and Asselain,B. (1988) *Cancer* **62,** 2517–2524.
7. Henry, J. A., Nicholson, S., Farndon, J. R., Westley, B. R., and May, F. E. B. (1988) *Br. J. Cancer* **58,** 600–605.
8. McPherson, L. A., Baichwal, V. R., and Weigel, R. J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4342–4347.
9. Hoch, R. V., Thompson, D. A., Baker, R. J., and Weigel, R. J. (1998) Manuscript submitted.
10. May, F. E. B., Johnson, M. D., Wiseman, L. R., Wakeling, A. E., Kastner, P., and Westley, B. R. (1989) *J. Steroid Biochem.* **33,** 1035–1041.
11. Masiakowski, P., Breathnach, R., Bloch, J., Gannon, F., Krust, A., and Chambon, P. (1982) *Nucleic Acids Res.* **10,** 7895–7902.
12. Brown, A. M. C., Jeltsch, J.-M., Roberts, M., and Chambon, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6344–6348.
13. Moretti-Rojas, I., Fuqua, S. A. W., Montgomery, R. A., III, and McGuire, W. L. (1988) *Breast Cancer Res. Treat.* **11,** 155–163.
14. Ciocca, D. R., Oesterreich, S., Chamness, G. C., McGuire, W. L., and Fuqua, S. A. W. (1993) *J. Natl. Cancer Inst.* **85,** 1558–1570.
15. Trask, D. K., Band, V., Zajchowski, D. A., Yaswen, P., Suh, T., and Sager, R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2319–2323.
16. Taylor-Papadimitriou, J., Stampfer, M., Bartek, J., Lewis, A., Boshell, M., Lane, E. B., and Leigh, I. M. (1989) *J. Cell Sci.* **94,** 403–413.
17. Kuang, W. W., Thompson, D. A., Hoch, R. V., and Weigel, R. J. (1997) *Nucleic Acis Res.* **26,** 1116–1123.
18. Carmeci, C., Thompson, D. A., Ring, H. Z., Francke, U., and Weigel, R. J. (1997) *Genomics* **45,** 607–617.
19. Diatchenko, L., Lau, Y. C., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., and Siebert, P. D. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6025–6030.
20. Sive, H. L., Hattori, K., and Weintraub, H. (1989) *Cell* **58,** 171–180.
21. Aberger, F., Weidinger, G., Grunz, H., Richter, K. (1998) *Mech. Dev.* **72,** 115–130.
22. Thompson, D. A., and Weigel, R. J. (1998) *Eur. J. Biochem.* **252,** 169–177.
23. Thompson, D. A., McPherson, L. A., Carmeci, C., deConinck, E. C., and Weigel, R. J. (1997) *J. Steroid Biochem. Mol. Biol.* **62,** 143–153.
24. Sanger, F., and Coulson, A. R. (1975) *J. Mol. Biol.* **94,** 441–448.
25. Bairoch, A., Bucher, P., and Hofmann, K. (1997) *Nucleic Acids Res.* **25,** 217–221.
26. Nakai, K., and Kanehisa, M. (1992) *Genomics* **14,** 897–911.
27. Sive, H. L., and Bradley, L. (1996) *Dev. Dyn.* **205,** 265–280.
28. Gammill, L. S., and Sive, H. (1997) *Development* **124,** 471–481.
29. Itoh, K., Tang, T. L., Neel, B. G., and Sokol, S. Y. (1995) *Development* **121,** 3979–3988.
30. Marck, C. (1988) *Nucleic Acids Res.* **16,** 1829–1836.
31. Higgins, D. G., Bleasby, A. J., and Fuchs, R. (1992) *Comput. Applic. Biosci.* **8,** 189–191.