Mammary Tumor Induction in Transgenic Mice Expressing an RNA-Binding Protein

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ABSTRACT
(10,12),(990,990)

We have analyzed mammary tumors arising in transgenic mice expressing a novel, multifunctional RNA-binding protein. The protein, which we call the c-myc mRNA coding region instability determinant binding protein (CRD-BP), binds to c-myc, insulin-like growth factor II, and β-actin mRNAs, and to H19 RNA. Depending on the RNA substrate, the CRD-BP affects RNA localization, translation, or stability. CRD-BP levels are high during fetal development but low or undetectable in normal adult tissues. The CRD-BP is linked to tumorigenesis, because its expression is reactivated in some adult human breast, colon, and lung tumors. These data suggest the CRD-BP is a proto-oncogene. To test this idea, the CRD-BP was expressed from the whey acidic protein (WAP) promoter in mammary epithelial cells of adult transgenic mice. The incidence of mammary tumors was 95% and 60% in two lines of WAP-CRD-BP mice with high and low relative CRD-BP expression, respectively. Some of the tumors metastasized. Nontransgenic mice did not develop mammary tumors. H19 RNA and insulin-like growth factor II mRNA were up-regulated significantly in non-neoplastic WAP-CRD-BP mammary tissue. WAP-CRD-BP mice are a novel model for mammary neoplasia and might provide insights into human breast cancer biology.

INTRODUCTION

RNA-binding proteins can affect gene transcription and mRNA translation, localization, and stability (1, 2). Diseases ranging from familial mental retardation to Alzheimer’s to cancer have been associated with the overexpression of RNA-binding proteins or with mutations in RNA-binding protein genes (3–5). Here, we describe transgenic animals in which overexpression of an RNA-binding protein induces mammary adenocarcinomas.

The protein binds to at least four different RNAs, is found primarily in the cytoplasm, and has multiple functions. It has received various names in the literature, depending on the laboratory in which it was discovered (Table 1). We call it the c-myc mRNA coding region instability determinant binding protein (CRD-BP), because it binds to the c-myc mRNA coding region and stabilizes c-myc mRNA (6–9). It is also called the zipcode-binding protein and the insulin-like growth factor II (IGF-II) mRNA-binding protein. Zipcode-binding protein binds to the “zipcode” segment of β-actin mRNA and localizes the mRNA to the leading edge of fibroblast lamellipodia (10). IGF-II mRNA-binding protein binds to the 5′-untranslated region of isoform 3 of IGF-II mRNA and affects the mRNA in two ways, by localizing it to fibroblast lamellipodia and by repressing its translation (11, 12). IGF-II mRNA-binding protein also binds to and localizes H19 RNA in fibroblasts (13). Other members of the CRD-BP family include human KH domain protein overexpressed in pancreatic cancer, which is overexpressed in pancreatic and other gastrointestinal cancers, p62, a human hepatocellular carcinoma autoantigen, several Xenopus and Drosophila proteins involved in egg maturation, and a mouse protein implicated in neuronal cell differentiation (5, 14–20). The properties of various structurally related CRD-BP family members highlight their evolutionary significance and their multifunctional nature.

The CRD-BP appears to be an oncogene. The CRD-BP is expressed abundantly during embryonic development but is silenced shortly after birth (7, 21). It is undetectable in many normal adult tissues, but its expression is reactivated in some human breast, colon, lung, and mesenchymal tumors (21–24). In contrast, CRD-BP expression is not reactivated in regenerating liver of adult rats after partial hepatectomy (7). This finding suggests that tumor-specific CRD-BP reactivation plays a role in the tumorigenesis process and is not simply a consequence of tumor cell replication.

Two observations link the CRD-BP to human breast tumors. First, the CRD-BP gene is amplified in ~30% of human breast cancer cases (25). Second, the CRD-BP is undetectable in non-neoplastic human breast tissue but is expressed in ~60% of human breast tumors (22). To address the potential role of CRD-BP gene reactivation in breast carcinogenesis, we expressed the CRD-BP from the mouse whey acidic protein (WAP) promoter in transgenic mice. The WAP promoter targets transgene expression to mammary epithelial cells, and is induced in pregnant and lactating females (26, 27). Because normal adult tissues contain little or no CRD-BP, the transgenic mice provide an excellent model for studying CRD-BP function in the absence of background CRD-BP expression. We report that CRD-BP-expressing mice develop mammary adenocarcinomas that are capable of metastasizing. Two known CRD-BP targets, IGF-II mRNA and H19 RNA, are up-regulated in CRD-BP-expressing mammary tissue.

MATERIALS AND METHODS

Transgene Construction and Transgenic Mice. Oligomers comprising the Xenopus β-globin 5′-untranslated region were annealed, kinased, and ligated to a mouse CRD-BP segment containing exon 1, intron 1, and exons 2–15 to generate plasmid pMiniCRDBP. This plasmid includes all of the CRD-BP coding sequences plus 266 bp of the CRD-BP 3′-untranslated region. An NcoI site was added at the start of CRD-BP exon 1, changing the second amino acid from asparagine to aspartic acid. The bovine growth hormone polyadenylation site was amplified by PCR from pcDNA3 (Invitrogen, Carlsbad, CA), digested with SflI and Kpnl, and ligated to pMiniCRDBP that had been digested with Xhol and Kpnl to generate pMiniCRDBP-BGH. A Bluescript construct containing the mouse WAP promoter and the 3′-flanking region of the WAP gene, kindly provided by Dr. Eric Sandgren (University of Wisconsin School of Veterinary Medicine, Madison, WI), was digested with Kpnl. The WAP-containing vector sequence was then ligated to Kpnl-digested pMiniCRDBP-BGH. The resulting plasmid was flanked by 2.42 kb of WAP gene 5′-flanking sequence and by 4.6 kb of WAP gene 3′-flanking sequence (Fig. 1).

The complete transgene construct was excised from the plasmid backbone by EcoRI digestion and was injected into pronuclei, which were then implanted into FVB/J hosts (The Jackson Laboratory, Bar Harbor, ME) by the Transgenic Animal Facility (University of Wisconsin, Madison, WI). WAP-CRD-BP transgenic founders were identified by PCR genotyping of tail DNA in a 20-μl reaction containing 10 μl of 2× PCR Master Mix (Promega, Madison, WI) and 1 μM CRD-BP primers (forward primer 5′-TTGCGATCC-CACCTGACCCTC-3′; reverse primer 5′-TGTGGTAGGCTGCG-CCTT-GGC-3′) in the following conditions: 96°C, 2 min; [94°C, 45 s; 60°C, 45 s; and 72°C, 1 min] × 30 cycles; and 72°C, 5 min. Amplification of the
endogenous Crd-bp gene generated a 527-bp band, and amplification of the WAP-CRD-BP transgene generated a 212-bp band.

Founders were mated to FVB/J mice to establish the colony. Two lines, designated 1100 and 900, were maintained for the experiments reported here. Some animals were the result of transgenic brother/sister mating. To maximize WAP gene transcription, female transgenics were mated continuously by keeping males in the cages. All of the animals were housed and treated in accordance with the Guide for the Care and Use of Laboratory Animals.

**Northern Blotting.** Mammary tissue was dissected and immediately frozen in liquid nitrogen. Total RNA was extracted using an RNA Wiz kit (Ambion, Austin, TX) as per the manufacturer’s directions. Ten μg of RNA were mixed with an equal volume of 2× loading dye (50% deionized formamide, 17.8% formaldehyde, 1× morpholinoethanesulfonic acid buffer (40 mM morpholinoethanesulfonic acid, 10 mM sodium acetate, 2 mM EDTA, and 24 mM NaOH), 0.04% bromophenol blue, 0.04% xylene cyanol, and 4.2% glycerol) and electrophoresed in a 1× morpholinoethanesulfonic acid/formaldehyde gel at 85 V for 4 h. The gel was soaked twice in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate) for 20 min each. RNA was transferred to a Hybond N membrane (Amersham, Piscataway, NJ) for 12 to 16 h in 10× SSC. RNA was cross-linked to the membrane with a Stratalinker (Stratagene, La Jolla, CA) set on Auto Crosslink. The membrane was prehybridized in nonradioactive hybridization buffer [50% deionized formamide, 7% SDS, 250 mM NaCl, 1 mM EDTA, 0.04% xylene cyanol, and 4.2% glycerol] and electrophoresed in an Amersham banding system (Amersham). The RNA was first DNase-treated using a DNA-Free kit (Ambion), and 1 μg of RNA was then used to synthesize cDNA, as described above.

**Quantitative PCR.** Quantitative PCR was carried out using Sybr Green PCR Mix (Applied Biosystems, Foster City, CA) as per the manufacturer’s instructions with the following primer sets: IGF-II, forward: 5′-TCAGAGAGCCTACAGGTGCCAT-3′; reverse: 5′-GGTTCTTGGTGTGTACCATG-3′; and 18S RNA forward: 5′-CGCCGGTAGGTTGAATTTCTC-3′; reverse: 5′-CGA- CCTCGGACTTGGTTGC-3′. 18S RNA was used as an internal quantitation standard. Analysis was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems).

**ELISA.** Protein was extracted from mouse mammary glands by homogenization in lysis buffer [25 mM ethylene glycol-bis-(β-aminooxy) ether] 1/5, N,N,N′,N′-tetraacetic acid, 10 mM KCl, 1.5 mM MgCl2, 20 μM protease inhibitor mixture (Sigma, St. Louis, MO), and 10 mM Tris-Cl (pH 7.4) followed by centrifugation at 4°C for 5 min. One μg of protein was diluted to 100 μl with 1× PBS, distributed in duplicate wells of a high-binding ELISA plate (CoStar), and incubated overnight at 4°C. The protein was then removed, and wells were incubated with blocking solution (5% BSA in PBS) for 2 h at room temperature. Blocking solution was removed, and primary antibodies that had been diluted in blocking solution were added to appropriate wells for 2 h at room temperature. The antibodies were as follows: c-Myc: C-19 rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) 1:1000; IGF-II: mouse monoclone (Upstate, Waltham, MA) 1:5000; and cytokeratin 18: mouse monoclonal clone Ks 18.04 (Progen, Heidelberg, Germany) 1:1000. Antibody solutions were removed. The plate was then washed eight times with wash solution (0.1% Tween 20 in PBS). Mouse IgG horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) was diluted 1:5000 in blocking solution and added for 1 h at room temperature. After removal of the secondary antibody, the wells were washed eight times with wash solution. Peroxidase reagent (Sigma) was added at 100 μl/well, and the plates were incubated at room temperature for 30 min. Reactions were stopped by adding 50 μl of 12% H2SO4. Absorbance was measured on a Spectra SLT ELISA plate reader, and c-Myc and IGF-II levels were normalized to cytokeratin 18 levels.

**Histology.** Mammary glands and tumors were dissected and immediately stored in 10% neutral-buffered formalin at 4°C overnight. They were then embedded in paraffin, sectioned, and stained with H&E.

**Statistical Analysis.** Tumor incidence was assessed using Fisher’s exact test. RNA levels in transgenic and nontransgenic mice were assessed with the Wilcoxon rank sum test. Both tests were performed using the Mstat software package (Norman Drinkwater, University of Wisconsin, McArdle Laboratory). Experiment-wise error rates (α′ = 0.05) were calculated by the Dunn-Šidák method \( \alpha' = 1-(1-\alpha)^{1/n} \).

**RESULTS**

**Generation of Mice Expressing the CRD-BP in the Mammary Gland.** The CRD-BP is a multifunctional RNA-binding protein, which is reactivated in some human breast cancers (Table 1; Ref. 22). To determine whether the CRD-BP plays a role in mammary carcinogenesis, we engineered female mice that express the CRD-BP in mammary tissue and monitored the animals for mammary tumors. Two CRD-BP-expressing founder lines, designated 1100 and 900.
CRD-BP INDUCED MAMMARY TUMORIGENESIS

were generated using a WAP-CRD-BP construct (Fig. 1). Transgenic females were then mated continuously to maximize WAP gene promoter activity. Nontransgenic control animals were also continuously mated. To quantify transgene expression, mammary glands were harvested at day 12 of pregnancy from two females of each transgenic line. Total cell RNA was extracted and analyzed by Northern blotting with a CRD-BP-specific probe. The blot was then stripped and reprobed for the epithelial cell marker cytokeratin 18. CRD-BP mRNA abundance was quantitated by normalization to cytokeratin. The CRD-BP was expressed in both transgenic lines, with 7–8-fold higher expression in line 1100 than in line 900 (Fig. 2). These lines are subsequently referred to as high and low CRD-BP expressers, respectively. Nontransgenic pregnant females contained no detectable CRD-BP mRNA (Fig. 2, NT). This result is consistent with previous findings indicating that the CRD-BP is expressed minimally, if at all, in normal adult tissues (7, 21). Therefore, pathological changes arising in WAP-CRD-BP mice should result from transgene-driven CRD-BP.

Mammary Tumor Induction in WAP-CRD-BP Mice. Animals were monitored for mammary tumors by palpation weekly. Only animals that were at least 40 weeks of age and had produced at least two litters were included in the analysis. Palpable tumors developed in both transgenic lines, but more so in the high-expressing 1100 line than in the low-expressing 900 line (Table 2). There was a strong statistical correlation between CRD-BP expression and mammary tumor formation (Table 2). Ninety-five percent of high-expresser animals developed palpable mammary tumors by an average age of 53 weeks, whereas 60% of low-expresser mice developed mammary tumors by 60 weeks (Fig. 3). No palpable tumors were detected in nontransgenic animals, consistent with previous reports on the rarity of mammary tumors in FVB mice (28, 29). To our knowledge, the CRD-BP is the first RNA-binding protein shown to be capable of inducing mammary tumors in vivo.

Histological Analysis of CRD-BP-Induced Mammary Tumors. All of the animals with palpable mammary gland masses were sacrificed, and their mammary glands and tumors were analyzed histologically (Fig. 4; Table 3). The majority of tumor-bearing animals had only a single palpable tumor in one mammary gland, although 2 animals each developed two palpable tumors. Microscopic analysis revealed multiple areas of carcinoma in multiple glands in many mice (Table 3). It is unclear if these neoplasms were micrometastases of the primary tumor or independent tumors. Primary tumors ranged from well-differentiated adenocarcinomas and papillary adenocarcinomas to adenocarcinomas and anaplastic carcinomas (Figs. 3 and 4). Ductal carcinoma in situ and metastases to lymph nodes, lung, and ovary were also observed. Nearly all of the nonneoplastic mammary glands from multiparous WAP-CRD-BP transgenics showed areas of hyperplasia (data not shown). Whole body autopsies were not routinely performed on these animals. Therefore, we cannot exclude the possibility of metastases to other tissues.

CRD-BP Expression in WAP-CRD-BP Mammary Tumors. CRD-BP mRNA was detected in <50% of WAP-CRD-BP mammary tumors by Northern blotting (data not shown). However, 14 of 17 tumors expressed transgene-derived CRD-BP mRNA by reverse transcription-PCR analysis (Fig. 5). (The CRD-BP band in the tumor of mouse number 15 is present but faint.) The WAP promoter is active only in pregnant and lactating females, but the majority of CRD-BP-expressing, tumor-bearing animals had not been pregnant for many weeks. This observation implies that the CRD-BP is required for the maintenance and/or continued growth of most tumors. There was no correlation between CRD-BP expression and tumor type.

Because the CRD-BP gene is reactivated in some human breast cancers, reverse transcription-PCR was performed on WAP-CRD-BP tumor tissue using primers specific for endogenous mouse CRD-BP. Approximately 50% of the high-expressing tumors also expressed endogenous CRD-BP (data not shown). There was no correlation between endogenous CRD-BP expression and tumor type. The mechanism for reactivation of the endogenous Crd-bp gene is not obvious and requires additional studies.

Expression of Known CRD-BP Target RNAs in Non-Neoplastic Mammary Tissue. The CRD-BP and its orthologues bind to at least four RNA targets (Table 1). To determine whether CRD-BP expression affected the abundance of these targets, Northern blotting was performed with mammary tissue RNA from high-expresser females who were in day 12 of their first pregnancy. The goal of this experiment was to assess CRD-BP function in mammary tissue that had not undergone significant

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**Table 2 Mammary tumor incidence in multiparous female WAP-CRD-BP+ mice**

Female mice from each line were mated continuously to maximize CRD-BP expression, and tumor development was monitored by palpation. Control animals were either nontransgenic litter mates or wild-type FVB/J mice with ages ranging from 40 to 85 weeks. A two-sided Fisher’s exact test was used to obtain P values.

<table>
<thead>
<tr>
<th>Line</th>
<th>Animals with tumors/total</th>
<th>Incidence (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>High CRD-BP</td>
<td>18/19</td>
<td>95</td>
<td>&lt;10⁻⁴</td>
</tr>
<tr>
<td>Low CRD-BP</td>
<td>15/25</td>
<td>60</td>
<td>&lt;10⁻⁴</td>
</tr>
<tr>
<td>Nontransgenic</td>
<td>0/19</td>
<td>0</td>
<td>N/A</td>
</tr>
</tbody>
</table>

WAP, whey acidic protein; CRD-BP, coding region instability determinant binding protein; N/A, not applicable.
bars are primers designed to amplify all of the IGF-II mRNA isoforms. By this mRNA was measured using quantitative reverse transcription-PCR with genic mice was below the detection level of Northern blotting, IGF-II RNA was 8-fold higher in transgenic mammary tissue than in nontransgenic mammary gland tested (Fig. 6, pregnancy were indistinguishable histologically. In this context, it is important to note that mammary glands of secondary effects related to neoplasia or to other mammary gland pathol-

Table 3 Histologic analysis of CRD-BP<sup>a</sup> induced mammary tumors

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Low CRD-BP</th>
<th>High CRD-BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple adenocarcinomas</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Papillary adenocarcinoma</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Anaplastic carcinoma</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ductal carcinoma &lt;i&gt;in situ&lt;/i&gt;</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Other metastases</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
</tbody>
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<sup>a</sup> CRD-BP, coding region instability determinant binding protein.

<sup>b</sup> Ovary and lung.

proneoplastic or neoplastic changes. At the time point chosen, CRD-BP-mediated regulation of its RNA targets should reflect primary effects, not secondary effects related to neoplasia or to other mammary gland pathology. In this context, it is important to note that mammary glands of nontransgenic and WAP-CRD-BP females at day 12 of the first pregnancy were indistinguishable histologically.

H19 RNA and IGF-II mRNA were significantly up-regulated in every WAP-CRD-BP mammary gland tested (Fig. 6, a and b). On average, H19 RNA was 8-fold higher in transgenic mammary tissue than in nontransgenic tissue (Table 4). Because IGF-II mRNA abundance in nontransgenic mice was below the detection level of Northern blotting, IGF-II mRNA was measured using quantitative reverse transcription-PCR with primers designed to amplify all of the IGF-II mRNA isoforms. By this assay, IGF-II mRNA was increased 100-fold in WAP-CRD-BP mammary glands as compared with nontransgenic glands (data not shown). Neither β-actin nor c-myc mRNA abundance was affected by CRD-BP expression in WAP-CRD-BP animals (Table 4).

Fig. 4. Histology of mammary tumors arising in multiparous whey acidic protein-coding region instability determinant binding protein (WAP-CRD-BP) mice. Tumor sections were stained with H&E. A, adenocarcinoma. B, papillary adenocarcinoma. C, adenocarcinoma. D, anaplastic carcinoma. E, ductal carcinoma <i>in situ</i>. F, metastatic adenocarcinoma to lymph node. A–E, ×100 magnification. F, ×200 magnification. Scale bars are ~100 μm.

Effect of CRD-BP Expression on c-Myc and Intracellular IGF-II Protein. To determine whether CRD-BP expression affected c-Myc protein abundance, total protein was extracted from the same mammary tissue homogenates used for Fig. 6, and c-Myc protein was quantitated by ELISA. There was no statistically significant difference between the WAP-CRD-BP and the nontransgenic samples (Fig. 7A). Therefore, CRD-BP expression did not appear to affect either c-myc mRNA or c-Myc protein abundance.

Because the CRD-BP binds to the 5'-untranslated region of IGF-II leader 3 mRNA and represses translation of this one isoform in cultured cells (11), we also compared intracellular IGF-II protein by ELISA. There was no difference between nontransgenic and WAP-CRD-BP mammary tissues (Fig. 7B). WAP-CRD-BP mammary tissue contains 100-fold more total IGF-II mRNA (all of the isoforms) than does nontransgenic mammary tissue, but intracellular IGF-II protein levels are comparable. Possible mechanisms accounting for these observations are discussed below.

DISCUSSION

The CRD-BP is an RNA-binding protein that was identified in different laboratories by virtue of its affinity for β-actin, c-myc, and IGF-II mRNAs, and for H19 RNA (Table 1). CRD-BP expression is reactivated in some human cancers, including ~60% of human breast cancers (21–24). These and other observations prompted us to ask if CRD-BP expression induces tumors in adult murine mammary tissue. Mammary tumors were observed in up to 95% of WAP-CRD-BP transgenic animals (Table 2). Nontransgenic adult mice did not express the CRD-BP and did not develop mammary tumors. On the basis of data from our two transgenic lines (high and low expressers), we tentatively conclude that tumor incidence correlates with CRD-BP abundance.

A spectrum of pathological entities was observed in tumor-bearing transgenic mice and included mammary hyperplasia, ductal carcinoma <i>in situ</i>, well-differentiated papillary adenocarcinomas and adenocarcinomas, as well as adenocarcinomas and anaplastic carcinomas (Fig. 4; Table 3). Some of the tumors metastasized. We do not understand the basis for the different mammary tumor types in mice expressing the same WAP-CRD-BP transgene. Presumably, additional factors such as the

Fig. 5. Expression of transgene-derived coding region instability determinant binding protein (CRD-BP) mRNA in mammary tissues from high-CRD-BP expressing mice. Reverse transcription-PCR was performed on RNA extracted from normal mammary tissue or mammary tumors arising in high-expressing whey acidic protein-CRD-BP mice. The transgene PCR product indicated by the arrow is 212 bp. Numbers indicate individual animals. N, tissue in which no palpable tumor was detectable. T, tissue from a palpable tumor. E, mouse embryo tissue. L, 100-bp DNA ladder. W, water control (no RNA added).

<table>
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<td>2</td>
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<td>6</td>
</tr>
<tr>
<td>Other metastases</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
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</tbody>
</table>

<sup>a</sup> CRD-BP, coding region instability determinant binding protein.

<sup>b</sup> Ovary and lung.
number of pregnancies, hormonal status, secondary mutations, and epigenetic changes influence mammary tumor type in these animals.

There are some similarities between human breast cancer and CRD-BP-induced mouse mammary tumors. Tumor latency is relatively long in humans and WAP-CRD-BP mice. Ductal carcinoma in situ is common in human breast cancer and is also observed in WAP-CRD-BP mammary tissue. Adenocarcinomas are common forms of breast tumors in humans and mice, and the human and mouse tumors can metastasize. Whole animal dissections of tumor-bearing mice were not routinely performed. Therefore, additional examples of metastases to other tissues such as bone and liver might have gone undetected.

The quantitation of CRD-BP target RNAs (H19, IGF-II, c-myc, and β-actin) was carried out with mammary tissues collected at day 12 of the first pregnancy. This time point was chosen for two reasons. First, the WAP-CRD-BP transgene is active at day 12 of pregnancy. Second, and more importantly, mammary tissues of transgenic and nontransgenic animals are indistinguishable histologically on day 12 of the first pregnancy (data not shown). Therefore, any differences in the gene expression profile of these tissues should reflect primary effects of CRD-BP expression, not secondary effects related to CRD-BP-induced hyperplasia or neoplasia. The levels of β-actin and c-myc mRNA and of c-Myc protein were unaffected by CRD-BP expression (Fig. 6, c and d; Fig. 7A). The β-actin result was not unexpected, because the CRD-BP (zipcode-binding protein) affects the location but not the abundance of β-actin mRNA (10). The c-myc result was unexpected, because the CRD-BP stabilizes c-myc mRNA in cells and in vitro (30). Therefore, we predicted that c-myc mRNA would be up-regulated in WAP-CRD-BP mice. One explanation for the c-myc result is that the mRNA was stabilized by the CRD-BP, but other transcriptional and post-transcriptional regulatory systems functioned to maintain the mRNA at a constant level. This hypothesis is consistent with the complex, multilevel regulation of the c-Myc gene (31, 32). An alternative explanation arises from the observation that c-myc mRNA stability and CRD-BP function are linked to ribosomal pausing under conditions of amino acid deprivation (33). Perhaps the mammary epithelial cells of pregnant mice are rich in amino acids and tRNAs that abrogate translational pausing in the c-myc CRD. As a result, the CRD-BP would not be required to stabilize the mRNA. Additional studies are required to distinguish between these and other hypotheses.

IGF-II mRNA is up-regulated by CRD-BP expression, whereas IGF-II protein levels are unaffected (Fig. 6b; Fig. 7B). These results are interesting, because multiple IGF-II mRNA isoforms are up-regulated in WAP-CRD-BP mice, but the CRD-BP (IGF-II mRNA-binding protein) is reported to bind only to the leader 3 IGF-II mRNA isoform (11). Therefore, the pathway for CRD-BP-mediated up-regulation of many IGF-II mRNA isoforms is unclear. Moreover, the CRD-BP represses IGF-II leader 3 mRNA translation in cultured cells but is not reported to affect translation of the other isoforms (11). Because these other isoforms were so abundant in transgenic tissue, we predicted that IGF-II protein would be up-regulated, which is not the case (Fig. 7B). Perhaps translation of the leader 3 mRNA isoform is blunted by the CRD-BP, whereas the other IGF-II mRNA isoforms are, for unrelated reasons, poorly translated in transgenic mammary tissue. Alternatively, CRD-BP expression might accelerate the export of newly synthesized IGF-II protein from mammary cells. Intracellu-

Table 4 Quantitation of RNA in 12-day pregnant mice

<table>
<thead>
<tr>
<th>RNA</th>
<th>Nontransgenic</th>
<th>WAP-CRD-BP</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>H19</td>
<td>0.48</td>
<td>4.0</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>IGF-II</td>
<td>ND</td>
<td>0.93</td>
<td>N/A</td>
</tr>
<tr>
<td>c-myc</td>
<td>0.76</td>
<td>0.82</td>
<td>&gt;0.3</td>
</tr>
<tr>
<td>β-actin</td>
<td>2.5</td>
<td>2.8</td>
<td>&gt;0.3</td>
</tr>
</tbody>
</table>

A, WAP, whey acidic protein; CRD-BP, coding region instability determinant binding protein; ND, not detected; N/A, not applicable.

Fig. 6. Expression of known coding region instability determinant binding protein target RNAs in mammary tissue of pregnant females. Total RNA was extracted from mammary tissue of mice at day 12 of their first pregnancy. Ten μg of RNA were electrophoresed, transferred, and hybridized with the indicated 32p-labeled DNA probe. Each lane represents RNA from a different mouse.

Fig. 7. ELISA analyses on mammary tissue of pregnant females. Total cell protein was extracted from the mammary tissue of 10 nontransgenic and 8 whey acidic protein-coding region instability determinant binding protein females at day 12 of their first pregnancy. One μg of protein was bound to duplicate wells of an ELISA plate and was probed with the appropriate antibody: (A) rabbit polyclonal c-Myc antibody and a mouse monoclonal cytokeratin 18 antibody; (B) mouse monoclonal IGF-II antibody and mouse monoclonal cytokeratin 18 antibody. c-Myc and insulin-like growth factor-II values were normalized to cytokeratin 18 levels. Average values for each set of animals are shown; bars, ±SDs.
lar IGF-II protein abundance would thereby remain steady, although IGF-II mRNA translation increased. It is also possible that CRD-BP expression enhances the degradation of newly synthesized IGF-II protein. Accelerated transport or rapid degradation of IGF-II protein could be explained by the CRD-BP-mediated localization of IGF-II mRNA (11, 12).

H19 RNA is also up-regulated in WAP-CRD-BP mammary tissue, as compared with nontransgenic glands (Fig. 6A). This observation is of particular interest for several reasons. First, the CRD-BP is reported to bind to and localize H19 RNA in cultured cells but not to up-regulate H19 RNA (13). Our data suggest the CRD-BP up-regulates H19 RNA in vivo. Perhaps CRD-BP binding both localizes and stabilizes H19 RNA in mammary tissue. Second, WAP-CRD-BP mice might provide a novel tool for investigating H19 RNA, which is not translated, has no known function, and is imprinted in conjunction with IGF-II (34–36). Contra- dictory roles for this RNA have been reported in the literature, indicating that H19 RNA might have tumor-suppressing or tumor-promoting properties (37–39). Understanding the basis for our in vivo observations might uncover novel interactions between the H19 and IGF-II genes, and might reveal some of the functions of H19 RNA. Lastly, H19 RNA is overexpressed in 72% of human breast cancers (40). In view of the results reported here, it might be worthwhile to investigate if CRD-BP activation reported here, it might be worthwhile to investigate if CRD-BP-induced mammary tumorigenesis is H19-dependent.

**Note Added In Proof**

We have performed preliminary immunohistochemical staining for estrogen receptor expression in adenocarcinomas from six randomly chosen WAP-CRD-BP mice (MC-20 antibody, Santa Cruz Biotechnology, 1:100). Positive background, to determine whether CRD-BP-induced mammary tumorigenesis is H19-null background, to determine whether CRD-BP-induced mammary tumorigenesis is H19-dependent.

**ACKNOWLEDGMENTS**

We thank Dr. Eric Sandgren for providing the WAP construct backbone, and for advice and suggestions on creating transgenic animals, Dr. Amy Moser for advice and suggestions on creating transgenic animals, Dr. Jennifer Schmidt for critical reading of the manuscript.

**REFERENCES**


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