Expression of *Brca1* is associated with terminal differentiation of ectodermally and mesodermally derived tissues in mice

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We have isolated genomic and cDNA clones of *Brca1*, a mouse homolog of the recently cloned breast cancer-associated gene, *BRCA1*. *Brca1* encodes an 1812-amino-acid protein with a conserved zinc finger domain and significant homology to the human protein. *Brca1* maps to Chromosome 11 within a region of conserved synteny with human chromosome 17, consistent with the mapping of the human gene to 17q21. *Brca1* transcripts are expressed in a variety of cultured cells but reveal a specific and dynamic expression pattern during embryonic development. For example, expression is observed first in the otic vesicle of embryonic day 9.5 (E9.5) embryos. This expression diminishes and is replaced by expression in the neuroectoderm at E10.5. By E11–12.5, higher levels are observed in differentiating keratinocytes and in whisker pad primordia. Transcripts also become evident in epithelial cells of the E14–17 kidney. *Brca1* expression occurs in differentiating epithelial cells of several adult organs as well, suggesting a general role in the functional maturation of these tissues. Consistent with this, *Brca1* transcripts are expressed in both alveolar and ductal epithelial cells of the mammary gland. During pregnancy, there is a large increase in *Brca1* mRNA in mammary epithelial cells, an increase that parallels their functional differentiation. Because high rates of breast cancer are associated with loss of *BRCA1* in humans, it is possible that this gene provides an important growth regulatory function in mammary epithelial cells. In addition, increased transcription of mammary *Brca1* during pregnancy might contribute, in part, to the reduced cancer risk associated with exposure to pregnancy and lactation.

[Key Words: *Brca1* expression; mouse homolog; embryonic development; terminal differentiation; transcription; mammary gland; Ring finger]

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Roughly 5% of the breast cancers diagnosed in North America and Europe are attributed to family history.¹ One candidate gene, referred to as *BRCA1*, is thought responsible for 45% of these hereditary cancers, and mutant alleles confer a lifetime risk approaching 85% (Easton et al. 1993, 1995). A variable risk of ovarian cancer is also present and approaches 60% in some kindreds. Positional cloning led to the identification of the human *BRCA1* gene in 1994 (Miki et al. 1994). Conceptual translation of cDNAs from this locus revealed a large C3HC4-type zinc finger protein expressed in the breast and ovary, as well as in a variety of adult tissues that appear unaffected by disease. Germ-line mutations have been identified in >80 afflicted families (Castilla et al. 1994; Friedman et al. 1994; Futreal et al. 1994). Disease

follows the loss of the wild-type allele, a result that classifies *BRCA1* as a tumor suppressor gene. Whereas *BRCA1* mutations do not contribute greatly to nonfamilial late-onset breast cancers, somatic mutations have been characterized in a number of sporadic cases of ovarian cancer (Hosking et al. 1995; Merajver et al. 1995). Both inherited and somatic mutations result in truncation of the gene product, alterations of the amino acid sequence, or reduced transcription of the *BRCA1* mRNA.

Mutations in tumor suppressor genes typically act recessively, and result, by diverse mechanisms, in loss of cellular growth control (p53, Rb protein, APC, DCC, NF-1), or in reduced fidelity of DNA repair (p53, MSH2). The p53 gene product is involved in both cell cycle and DNA-repair activities. C3HC4-type zinc finger proteins (also known as Ring finger or A box proteins) show *trans*-activation activity for a number of viral and cellular genes (Patarca et al. 1988) and have been implicated in

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DNA repair [Jones et al. 1988]. However, unlike other zinc finger motifs, C3HC4-finger proteins might not have direct DNA-binding activity and their mode of action is unknown [Freemont 1993]. Recent antisense experiments indicate that inhibition of *BRCA1* transcription leads to elevated proliferation in certain breast cell lines [Thompson et al. 1995]. Thus, it seems reasonable to predict that *BRCA1* will be involved, at least in part, in cell cycle control.

Because little is known about the role of *BRCA1* in development and cellular regulation, we set out to define its role in mice, a species where the contribution of specific genes to aspects of development and disease are studied more easily. To establish its structure and pattern of expression, and to test the hypothesis that loss of expression might play a role in mouse breast cancer, we have cloned a mouse homologue that we term *Brca1*. We find that *Brca1* encodes an 1812-amino-acid protein that has 58% amino acid identity with the human protein. The amino-terminal zinc finger domain is 100% identical, as are smaller domains of unknown function. *Brca1* is located on Chromosome 11, in a region of conserved synteny with human chromosome 17q21. Interestingly, *Brca1* is expressed during the development of the neuroepithelium and of several epithelial organs, including the kidney, skin, and mammary gland. Significantly, *Brca1* expression increased sharply during pregnancy-induced mammary gland development in a pattern consistent with its regulation by pregnancy-associated hormonal changes. Because sequence conservation is an indication of functional conservation, it would appear that study of conserved features between *BRCA1* genes in diverse species will provide considerable insight into functional domains of this large tumor suppressor locus.

Results

Characterization of *Brca1* structure

A 752-bp fragment of *Brca1* (Fig. 1, probe A) was amplified from mouse genomic DNA with primers derived from exon 11 of the human gene [Miki et al. 1994] and used to isolate clones from a mouse genomic library. One genomic clone was found to contain the complete sequence of an exon homologous to human exon 11. Fragments from this sequence were used to isolate *Brca1* clones from a mouse embryonic day 17 (E17) cDNA library. The longest cDNA clones terminated ~15 bp short of the expected ATG initiation site, and oligonucleotides from this region were used to isolate additional genomic clones that overlapped this region (Fig. 1; data not shown). Sequence analysis of the overlapping clones shown in Figure 1 revealed that 6515 bp of *Brca1* cDNA had been identified. An ATG triplet at the 5' end of this sequence was followed by a 5437-bp open reading frame. A 1002-bp 3'-untranslated region contained an -AATAAA- polyadenylation signal that was followed by a long tract of adenosine residues. (The nucleotide sequence is available from GenBank under accession no. U36475.)

Figure 1. Map of *Brca1* clones. Predicted structure of *Brca1* mRNA based on the complete characterization of four independent cDNA clones (T.1-5) and partial characterization of two genomic fragments (CD.1-2 and T.8-12). The arrow indicates the location of the ATG codon predicted to be the start of translation. The location of exon 11 is indicated as an open rectangle, and the 5436-bp open reading frame by a thick line. Noncoding sequence are indicated by a thin line. The T.8-12 genomic fragment contained the sequence for exon 2, which contains the start of translation. Exon 1, a noncoding exon identified in human cDNAs, has not been identified. The location of probes used for genomic mapping and mRNA expression studies are also included.

The mouse cDNA predicts an 1812-amino-acid protein similar in size and structure to the human *BRCA1* gene product (Fig. 2A). *Brca1* is 100% identical to the human protein over a 49-amino-acid region that includes the amino-terminal C3HC4-zinc-binding domain. The amino-terminal location is similar to the placement of homologous domains in rpt-1, a regulator of the IL-2 receptor [Patarca et al. 1988], RAD18, a DNA repair gene from yeast [Jones et al. 1988], ring1, a zinc-binding protein [Lovering et al. 1993], the human oncogenes *ret*, *MEL18*, and *BMI-1* [Takahashi et al. 1988; Tagawa et al. 1990; Haupt et al. 1991; van Lohuizen et al. 1991], and others [Freemont 1993; Lovering et al. 1993].

Overall, *Brca1* shares 58% amino acid identity with the predicted human protein. When conservative substitutions were included, homology increased to 73%. Mouse and human *BRCA1* proteins also share similar charge and hydrophilic character. In particular, the amino terminus has several clusters of basic residues, whereas the middle and carboxyl terminus of the protein are generally acidic in character and contain a proportionally high number of serines (Fig. 2B). Whereas nothing is known about the subcellular distribution or phosphorylation state of *BRCA1*, two conserved clusters of basic residues appear homologous to nuclear localization signals found in SV40 [Kalderon et al. 1984] (Fig. 2A). Several conserved serines are found in acidic environments similar to known sites of phosphorylation by casein kinase II and glycogen-synthase kinase 3 [Litchfield et al. 1990; Park et al. 1994].

Organization and location of the *Brca1* gene

Sequence analysis of genomic clones resulted in the characterization of the complete structure of exon 11

Figure 2. Mouse *Brca1* encodes an 1812-amino-acid protein with an amino-terminal C3HC4-type zinc finger. (A) Deduced amino acid sequence of *Brca1* is given in one-letter symbols. The amino acids predicted to comprise a C3HC4-type zinc finger are underlined. (▼) The boundaries of exon 11. Two conserved basic motifs similar to the SV40 nuclear localization signal (Kalderon et al. 1984) are shown with open underlines. The location of several conserved serines that lie within acidic environments are indicated by a star. The complete sequence of the derived *Brca1* cDNA has been deposited in GenBank (accession no. U36475). (B) A bar diagram of the predicted *Brca1* protein is shown with the location of the amino terminus (NH₂), the zinc-finger (ZF), two potential nuclear localization signals (NLS), and a relatively acid- and serine-rich domain. Vertical lines above indicate the locations of gaps between the human (Miki et al. 1994) and mouse proteins. Gaps result in a net loss of 53 amino acids in the mouse sequence.

(clone CD.1-2) and the intron 1–exon 2 boundary (clone T.8–12) (Fig. 1; data not shown). The extensive similarity between the 5' end of the mouse coding region with human exon 2 and the conserved structure of exon 11 indicate extensive similarity between human and mouse in the organization of the *Brca1* locus.

The mouse *Brca1* gene was mapped by Southern blot analysis of progeny from two multilocus crosses as described (Kozak et al. 1995) (Fig. 3). Linkage was detected with several markers on Chromosome 11, including *Wnt3* and *Neu/Erbb2*, which are also linked to the human *BRCA1* locus (Lossie et al. 1994). This map location places *Brca1* within a region of conserved synteny with human chromosome 17q21–22 and rat chromosome 10.

Expression of *Brca1* mRNA in cell lines and embryos

When probed with a 5' cDNA fragment (Fig. 1; probe B), a single major *Brca1* transcript was detected on Northern blots of poly(A)⁺ RNA prepared from a number of normal and transformed mouse cell lines (Fig. 4A). This major transcript migrated at ~7.2 kb on 1% agarose–formaldehyde gels. This size is in reasonable agreement with the 6.5-kb mouse cDNA sequence that has been characterized thus far, the difference accounted for by the presence of one or more untranslated exons lying 5' of the start of translation and by the poly(A) tract at the 3' end. Minor transcripts were also evident at ~4.6 and 5 kb (Fig. 4A,B, arrowheads).

Similar transcripts were identified in both normal and transformed mammary epithelial cells, as well as in primary tumors derived from the mammary and salivary glands of transgenic mice (Fig. 4A,B). In addition, a large number of nonepithelial tumors and cell lines also ex-

Figure 3. *Brca1* is located on Chromosome 11. A diagram of Chromosome 11 is given with the relative positions of eight genes mapped previously to this region. (●) The centromere. Minimization of recombinant frequencies between *Brca1* and these markers placed it centromeric to *Wnt3* and *Hmg14-1* but distal to *Erbb2* and *Fkbp-rs*. Recombination fractions for adjacent markers are given at right and were derived from analysis of the progeny of two multilocus crosses: The first fraction was derived from crosses carried out with *M. spretus*; the second fraction was from *M. m. musculus*. Numbers in parentheses represent the recombinational distances ±S.E. and were calculated according to Green (1981). No double recombinants were identified within this interval in either cross. To the left of the map are human map locations for the underlined genes. The *M. m. musculus* cross was not typed for *Hoxb* and *Erbb2*.

Figure 4. Expression of *Brca1* mRNA in cell lines, primary tumors, and embryonic brain. (A,B) A 7.2-kb transcript is detected in a variety of cultured cells when probed with a 900-bp fragment from the 5' end of the *Brca1*-coding region (Fig. 1, probe B). (A) Mammary tumor cell lines: AC816, *ras*-transformed (lane 1); M158, *myc*-transformed (lane 2); NK417, *neu*-transformed (lane 3). Nontransformed mammary cell lines: CL-S1 (lane 4); NMuMG (lane 5). (B) Primary mammary tumors from transgenic mice over-expressing *neu* (lane 6), *ras* (lane 7), and *myc* (lanes 9 and 10). (Lane 8) Salivary gland tumor from a *ras*-transgenic mouse; (lane 11) NIH-3T3 cell line. Smaller transcripts are also identified (arrowheads).

(C) Additional bands (arrows) are visible in blots probed with a 4-kb fragment from the middle and 3' end of the *Brca1*-coding region (Fig. 1, probe C). Mammary cell lines: SMF, *neu*-transformed (lane 12); 16MB9a, *myc*-transformed (lane 13); AC204, *ras*-transformed (lane 14); NMuMG, non-transformed (lane 15). Solid arrows indicate smaller transcripts identified in lanes 12, 14, and 15. Open arrows indicate those in lane 13. (D) *Brca1* mRNA expression peaks in E12.5 head and is expressed in brain throughout development: E10.5 head (lane 16), E12.5 head (lane 17), E14.5 brain (lane 18), E16.5 brain (lane 19), E18.5 brain (lane 20), day 2 postpartum brain (lane 21), adult brain (lane 22). Timed Swiss-Webster embryos were prepared as described in the text. The adult brain tissue was from an 8- to 10-week-old male FVB mouse. Transformed cell lines and tumors were derived from transgenic mouse lines overexpressing *ras*, *myc*, *neu*, or *int2*, as described in the text. All lanes contain 2 μ g of poly(A)⁺ RNA analyzed on 1% agarose/formaldehyde gels. Blots were exposed to x-ray film for 5 days. RNA loading was verified by reprobing blots with a fragment of the 28S rRNA and exposed for 2–8 hr.

pressed *Brca1* mRNA (Table 1). Amplification of the 5' 250 bp from several normal and transformed cell lines by RT-PCR failed to identify any major differences between *Brca1* transcripts (not shown). Whereas many tumors and cell lines appeared to express relatively high levels of *Brca1* transcript, one *myc*-based mammary tumor had significantly reduced levels (Fig. 4, lane 10). Whereas it has been suggested that reduced expression of *Brca1* contributes to human breast disease (Thompson et al. 1995), it remains to be determined whether the reduction in *BRCA* expression seen in this mammary tumor is linked to transformation.

Several fibroblast- and lymphoid-derived tumors also expressed *Brca1*, (Table 1), as did NIH-3T3 cells (Fig. 4, lane 11). Expression in these cells often seemed lower than in epithelial cell lines, but this was not always the case (Table 1).

When a probe corresponding to the middle and 3' ends of the *Brca1* cDNA was used (Fig. 1, probe C), smaller transcripts were observed (Fig. 4C). These smaller transcripts appear to vary between cell lines. One pattern of transcripts was observed in *ras*, *neu*, and normal mammary epithelial cell lines, whereas a fourth line derived from a *myc* overexpressing mouse gave two smaller transcripts (cf. transcripts identified by arrows in lanes 12, 14, and 15 with those identified by open arrows in lane 13). These transcripts appear to lack exons 5' of exon 11.

Northern blots of RNA from embryonic head [E10.5 post coitus (p.c.) and 12.5 p.c.] and brain (E14 p.c. through day 2 postnatal) show expression of *Brca1* (Fig. 4D). Low levels of *Brca1* transcripts were detected in embryonic heads of E10.5 embryos and increased dramatically in E12.5 embryonic head tissue (Fig. 4, lanes 16 and 17, respectively). *Brca1* mRNA was evident in isolated brain

tissue of mice between E14.5 and postnatal day 2 (Fig. 4, lanes 18–21), very low levels of message were detected in adult brain (lane 22).

In situ hybridization for Brca1 expression in embryos

To identify more precisely the cells in which *Brca1* was expressed, we prepared staged embryos for *in situ* hybridization. Whole-mounted embryos displayed *Brca1* mRNA in their otic vesicles at E9.5 (Fig. 5A). In E10.5 embryos, otic vesicle staining was diminished and tissues in the forebrain became distinctly positive (Fig. 5B). This expression was specific as neither sense nor irrelevant probes stained this region (Fig. 5C; and data not shown). Although epithelial staining was eliminated by proteinase treatment of whole-mounted embryos, clear expression was evident in the neuroepithelium of sectioned embryos between E10.5 and E15.5 (Fig. 5D). Later in development, expression in the mesonephros (a precursor of the kidney) became evident (Fig. 5E). Sectioned embryos from E14.5 through E17.5 show that the expression was localized to tubular epithelial cells of the developing kidney [Fig. 5F,F(i)].

Localized expression in the skin of sectioned embryos was evident beginning at E10.5 (Fig. 6A). Intense hybridization in keratinocytes continued throughout embryonic development and was associated with both basal and suprabasal cells (Fig. 6B). Expression was reduced, but present, in adult skin (not shown). Expression in keratinocytes was most evident in dorsal skin and was weaker in the face and ventral body wall (Fig. 6C,E). Transcripts in facial skin of E13.5 embryos show a periodic pattern reminiscent of whisker follicle development (Fig. 6D). At E15.5, strong reactivity was identified in

Table 1. Expression of *Brca1* mRNA in cell lines and tumors

	Status ^a	Source ^a	Genotype ^b	Expression
Cell lines				
NIH-3T3	N	E	wt	+
1615	N	M	wt	++
1636	N	M	wt	++
AC204	T	MA	<i>ras</i>	+++
AC236	T	MA	<i>ras</i>	+++
AC816	T	MA	<i>ras</i>	+++
SMF	T	MA	<i>neu</i>	+++
16mb9a	T	MA	<i>myc</i>	+++
NX1128	T	MA	<i>int2</i>	+++
HPI-2	H	M	<i>int2</i>	++
AC101	T	F	<i>ras</i>	+++
AC280	T	F	<i>ras</i>	++
AC139	T	UF	<i>ras</i>	++
Primary tumors				
adenocarcinoma		MA	<i>neu</i>	++
adenocarcinoma		MA	<i>myc</i>	++
adenocarcinoma		MA	<i>myc</i>	++
adenocarcinoma		MA	<i>ras</i>	++
adenocarcinoma		S	<i>ras</i>	++
lymphoma ^c		Th	<i>myc/p53</i> ^{-/-}	++
lymphoma ^c		LN	<i>myc/p53</i> ^{-/-}	++

Northern analysis of 2 µg of poly (A)⁺ mRNA from subconfluent cultures with ³²P-labeled probe B. Samples with a visible 7.2-kb band after 1-day exposure were considered + + +, those with a band after 5 days were considered + +, and those with a faint band after 5 days were considered +.

^a(N) Normal; (H) hyperplasia; (T) transformed; (wt) wild type; (E) embryo; (M) mammary gland; (MA) mammary adenocarcinoma; (F) fibrosarcoma; (UF) uterine fibrosarcoma; (S) salivary gland; (Th) thymus; (LN) lymph node.

^bCell lines were derived from mice either wild type (wt) or overexpressing *ras*, *myc*, *neu*, or *int2*, as described in the text.

^cLymphomas arose in mice overexpressing *c-myc* and lacking functional *p53*.

whisker pads, with only moderate expression in the overlying epidermis (Fig. 6E). The reason for the locally modulated levels of *Brca1* in the skin is not known but could result from well-known differences in the timing of epidermal differentiation or in local differences in the expression of particular keratinocyte markers (Vassar and Fuchs 1991).

Expression of *Brca1* in adult tissues and differentiation of the mammary gland

Expression of *Brca1* mRNA was evident in several adult tissues (Table 2). Highest levels were evident in spleen, thymus, and lymph nodes, and in epithelial organs like the Harderian gland, an intraorbital lachrymal gland found in mice. Very low expression was detected in adult brain, kidney, and skin, tissues with the highest levels of embryonic expression. No expression was detected in heart, liver, or lung. *Brca1* mRNA also was expressed in the breast. The pattern of expression in adult tissues is thus similar to that reported for human *BRCA1* (Miki et al. 1994).

Because of the association of *BRCA1* with human cancers of the breast, we characterized the expression in mammary tissue at several stages of maturation. Interestingly, the steady-state level of *Brca1* mRNA expression increased dramatically during pregnancy (Fig. 7A;

Table 2). Expression was elevated ~10-fold by day 10 of pregnancy and remained high through day 17 (Fig. 7A, lanes 3,4). This increase in expression parallels the well-characterized increase in α -casein and transferrin mRNAs, both of which continue to rise as constitutive milk proteins during pregnancy and lactation (Fig. 7A, lanes 3–5). During lactation, steady-state levels of *Brca1* mRNA appear to fall (Fig. 7A, lane 5). This apparent decrease is characteristic of genes expressed by mammary cells and can be attributable either to its down-regulation or to dilution resulting for the large accumulation of milk protein mRNAs. During postlactational regression of the mammary gland, *Brca1* mRNA levels again increase (lanes 6–8). By day 5 of regression, *Brca1* levels appear slightly elevated over those seen in the virgin gland (cf. lanes 2 and 8). Again, it is not clear whether this difference is because of an increase in transcription or an increase in the number of mammary epithelial cells relative to adipocytes in the postlactational versus virgin mammary gland.

To identify cells expressing *Brca1* mRNA in the mammary gland and to test whether transcription is down-regulated during lactation, we carried out in situ hybridization on sectioned glands at different stages of development. Expression was low but detectable in virgin mammary glands (Fig. 7B). *Brca1* transcripts were localized specifically in the mammary epithelial cells,

Figure 5. *Brca1* is expressed in the embryonic nervous system and kidney. *Brca1* mRNA expression was analyzed in mouse embryos by in situ hybridization. (A) Day 9.5 p.c., a dorsal view of a de-epithelialized Swiss-Webster embryo showing positive reaction product in the otic vesicles (OV) hybridized with an antisense *Brca1* riboprobe (AS); inset (i), a lateral view of a similar embryo. (B) De-epithelialized E10.5 embryo AS showing diminished otic staining and beginnings of forebrain (FB) staining. (C) Similarly prepared E10.5 control embryo hybridized with a sense riboprobe (S). (D) Section of E11.5 embryo, mesencephalon AS. (E) Day 15.5 embryos, S or AS, arrows identify staining in mesonephros (M) (note high background associated with older embryos). (F) Section of day 17.5 kidney AS; arrows identify nephric tubules. Inset (i), higher magnification of section in F. Asterisks identify tubule lumens, and lines identify the basal lamina of one tubule; cytoplasmic *Brca1* mRNA is identified by the brown reaction product. Staged mouse embryos were fixed in 4% paraformaldehyde and prepared for whole mount, or sectioned after imbedding in paraffin. Embryos were stained with antisense or sense riboprobes to the 5' end of *Brca1* (probe B) labeled with D.G.-UTP. Hybridization was detected by an enzyme-linked immunoassay that produces a dark brown reaction in positive cells.

whereas adipocytes appeared negative. After day 17 of pregnancy, *Brca1* mRNA was increased dramatically in both tubular and alveolar epithelial cells but not in the surrounding fat pad (Fig. 7C). Epithelial expression was not elevated appreciably by day 5.5 of pregnancy, a fairly early stage in which epithelial hyperplasia has yet to

begin, but was seen by day 13.5 (not shown), consistent with the up-regulation of *Brca1* mRNA on RNA blots (Fig. 7A, lane 3). During lactation, *Brca1* transcripts were still evident in mammary epithelial cells surrounding ducts and in alveoli. This expression does appear lower than that seen during pregnancy. However, signal inten-

Figure 6. *Brca1* is expressed in embryonic skin and whisker pads. *Brca1* transcripts were identified by in situ hybridization in sections of embryonic mice. (A) Antisense (AS) *Brca1* hybridization to dorsal skin at day 12.5 p.c.; (B) AS of dorsal skin at day 16.5; (C) AS of ventral skin at day 12.5. (D) AS of facial skin at day 13.5; (E) facial skin at day 15.5. Open arrows in A–C and E identify hybridization in basal and suprabasal keratinocytes; solid arrows in D–F identify developing whisker pads. Swiss-Webster embryos were hybridized with antisense (A,B,D–F) or sense (C) riboprobes as described previously (probe B).

Table 2. Expression of *Brcal* mRNA in adult tissues

Tissue	Expression
Heart	—
Liver	—
Lung	—
Brain	±
Kidney	±
Skin	±
Thymus	+
Axillary lymph node	+
Harderian gland	+
Testis	+
Spleen	++
Ovary	+
Mammary gland	
virgin	+
day 10 pregnancy	++
day 10 lactation	—
day 5 regression	+

Northern analysis of 2 µg of poly (A)⁺ mRNA from 8 to 10-week-old FVB male mice or 12-week-old female mice. Probe = ³²P-labeled probe B (Fig. 1). Tissues with a 7.2-kb band visible after 5-day exposure were considered ++, those with a band after a 14-day exposure were considered +, those with very faint bands at 14 days were considered ±, tissues without mRNA detected by this method are labeled —.

sity cannot be considered an unbiased measure of RNA molecules per cell. Because considerable hyperplasia occurs during pregnancy and lactation, similar-sized sections show different percentages of a cell's cytoplasm, making direct comparisons of signal intensity hard to interpret. However, it is clear that *Brcal* mRNA continues to be present during lactation. We also have found that staining is still present, but much reduced, in ducts after 7 days of regression (not shown). These results indicate that elevated expression of *Brcal* is a consequence of pregnancy and might contribute to mammary cell differentiation or withdrawal from the cell cycle during this period.

Discussion

Genetic analysis of familial breast cancer pedigrees and tumors has suggested that loss of function in one or more growth inhibitory genes (*p53*, *Rb*, *BRCA1*, and *BRCA2*) play causative roles in the development of neoplastic diseases of the breast (Hall et al. 1990; Duffy 1993). Such recessive tumor suppressor alleles might act alone or in concert with dominantly acting oncogenes (such as *c-myc* and *neu/erbB2/HER2*) that are often amplified in tumor tissue (Bieche et al. 1994; Tavassoli et al. 1995). Tumor suppressor genes appear to comprise multiple classes but typically act to limit cell-cycle progression or to regulate DNA repair. In addition to their obvious role as antioncogenes, identification of these genes has provided new insights into normal pathways of cellular growth control. In particular, *p53* and *Rb* gene products are major regulators of cell-cycle control in mammalian

cells and are deleted or mutated in a large percentage of human tumors.

In cloning a mouse homolog of the *BRCA1* gene, we have shown that the coding sequence is conserved moderately and that both genomic structure and organization are well conserved. Complete conservation is notable in the zinc-binding domain and in short regions of unknown function. Both human and mouse proteins are fairly acidic. This is particularly evident in the carboxyl terminus that has been proposed to comprise an acidic blob-type interaction domain (Miki et al. 1994). In addition, we have identified clusters of basic amino acids that might comprise nuclear localization sites at codons 495–501 and 598–608 (Kalderon et al. 1984). Other clusters of basic amino acids occur throughout the amino-terminal half of the protein but are not, in general, as

Figure 7. *Brcal* is increased by pregnancy in mammary gland epithelial cells. *Brcal* transcripts were identified in mammary epithelial cells at all stages of maturation but increase dramatically during pregnancy and lactation. (A) Blot of poly(A)⁺ RNA. (Lane 1) 5-week-old virgin; (lane 2) 8-week-old virgin; (lane 3) day 10 pregnancy; (lane 4) day 17 pregnancy; (lane 5) day 2 lactation; (lane 6)

well conserved. Nuclear localization sites have been observed in other zinc-binding proteins, and their presence in a region encoded by exon 11 might suggest a subcellular targeting function for this unusually large exon (Patarca et al. 1988). Several potential serine phosphorylation sites are conserved between mouse and human sequences. Two of these sites (Ser-582/Ser-586 and Ser-1174/Ser-1178 in the mouse sequence) have the potential for sequential phosphorylation by casein kinase II followed by glycogen synthase kinase 3 (Fiol et al. 1990).

Because of the relatively large size of the *Brca1* gene product, the identification of specifically conserved amino acids likely will prove useful in identification of functional domains. In this regard, it is interesting that a number of families with *BRCA1*-associated pedigrees have mutations that fall within the last 200 amino acids of the coding domain (Futreal et al. 1994; Miki et al. 1994; Merajver et al. 1995). Regions of amino acid identity in this region are relatively short (64% identity overall), with the longest corresponding to amino acids 1680–1712 of human *BRCA1* and amino acids 1623–1654 of mouse, which are identical in 30/32 amino acids (94%). The remainder of the carboxyl terminus has smaller stretches of identity but shows considerable conservation in the pattern of hydrophobic and charged residues (81% similarity) that might comprise an interaction domain. Whereas the role of the carboxyl terminus is not clear, the location of several human mutations in this region make functional characterization a priority.

The adult expression pattern of *Brca1* is very similar to that described for the human gene (Miki et al. 1994). However, the pattern of expression in embryonic tissues and during mammary development has not been shown previously. We find that *Brca1* mRNA is highly expressed during specific stages in the development of a variety of ectodermally derived tissues, including brain, skin, whisker pads, and mammary epithelial cells. In addition, transcripts are seen in mesodermally derived kidney epithelial cells, suggesting additional roles in epithelial differentiation. *Brca1* transcripts were also abundant in lymphoid organs, reproductive organs, and cultured fibroblasts, suggesting a more generalized role in these tissues. Because it has been reported that reduced *BRCA1* transcription is associated with increased rates of cell proliferation (Thompson et al. 1995), it appears curious that high levels of mRNA expression are associated with so many proliferating cells, both during development and in culture. Particularly intriguing are the elevated levels associated with transformed tissues, especially in mammary tumors and lymphomas. In preliminary experiments, we have detected no rearrangements or gross mutations within *Brca1* transcripts in transformed tissues but have noted reduced transcription in one mammary tumor and a novel size class or splice variant in another. Because somatic mutations in the *BRCA1* locus appear rare in spontaneous human breast cancers (Futreal et al. 1994), such mutations might also be rare in mouse models of the disease.

Although *Brca1* expression in the mammary epithelium was expected from the data on human disease (Hall

et al. 1990), the pregnancy-dependent increase in expression was not predicted. Expression clearly was elevated in mid-pregnancy (day 10) and paralleled the expression of milk protein mRNAs (this study) as well as that reported for *c-myc* and *p53* (Elson et al. 1995). *Brca1* expression continued during lactation, as assessed by in situ hybridization, and decreased between 5–7 days after weaning. These results give support to the idea that *Brca1* might act as a regulator of cellular maturation or transcription because little proliferation is seen by late pregnancy. It is also unlikely that *BRCA1* plays a direct role in apoptosis because its expression is not elevated in glands following weaning, a period of extensive cell death in the mammary gland (Lefebvre et al. 1992).

Differentiation is a common theme in cells that express *Brca1*. It is interesting that *Brca1* is expressed in several epithelial cell types during developmental transitions (day 12 neuroepithelium, keratinocyte differentiation, and mammary epithelial maturation). This association does not appear to hold for transformed cells or for cells in culture, and it remains to be seen how *Brca1* function is regulated in these cases. Because *Brca1* transcripts were identified in epidermal basal cells of E12.5 embryos, it is clear that *Brca1* transcription occurs in proliferating cells in vivo.

Because reductions in *Brca1* transcripts have been reported in spontaneous human breast tumors (Thompson et al. 1995), we tested RNAs from a variety of mouse tumors and tumor cell lines for the size and abundance of their *Brca1* message. We observed high levels of the 7.2-kD *Brca1* mRNA in all cell lines and primary tumors tested and saw few obvious differences between normal and transformed cell lines. Tumor cell lines included those from mammary adenocarcinomas, as well as from dermal fibrosarcomas and two different *p53*-null lymphoid tumors (Elson et al. 1995). In addition, cell lines derived from fibroblasts (NIH-3T3) displayed high levels of *Brca1* mRNA. We note that cell lines expressed 5- to 10-fold more *Brca1* than samples from normal tissues. Thus, we found little evidence for loss or rearrangement of *Brca1* transcripts in mouse tumors and found that high levels of expression were present in a variety of cells established in culture. As with the *p53* tumor suppressor gene, mutations in the coding region of *Brca1* might be present in these cells and therefore high levels of mRNA expression might not correlate with functional protein.

We show that *Brca1* encodes a zinc finger protein expressed during the differentiation of a variety of epithelial tissues. Genes of this type have been shown to regulate transcriptional control in some systems. Taken together, these results support a model in which *Brca1* functions as a regulator of differentiation, a process that often includes transcriptional control and the inhibition of cell-cycle progression. Because we observe *Brca1* transcription in proliferating cells in vitro, it is probable that activity of its gene product is regulated in a complex manner, similar to other tumor-suppressor genes, notably the *Rb* gene product (Weintraub et al. 1995). Our observation that *Brca1* is elevated during pregnancy is particularly interesting in light of data that the breast

becomes incrementally more resistant to transformation following pregnancy and lactation (Russo and Russo 1995). Genes like *Brca1* thus might function to alter irreversibly cells in which they are expressed, making them less likely to reenter a proliferative state, a function that would be consistent with sites of expression identified in this study. If this is the case, elevated expression of *Brca1* mRNA in tumors might indicate an inhibition, or disconnection, of a signaling system that regulates *Brca1* activity.

Materials and methods

Cloning and sequencing

Oligonucleotides that contain sequence from exon 11 of human *BRCA1* (forward primer, 5'-TCATGCCAGCTCATTACAGC; reverse primer 5'-TCCGTTTGGTTAGTTCCTG) were used to amplify a 752-bp fragment from human DNA by PCR, and the fragment was subcloned into a TA cloning vector (Invitrogen, San Diego, CA). A similar fragment was amplified from mouse DNA with the same primers and subcloned (Fig. 1, probe A). The inserts were sequenced to confirm their identities, and the mouse sequence was used to screen a 129/SvJ mouse λ fixII genomic library (Stratagene, La Jolla, CA). Positive clones were mapped (Fig. 1), and the complete 3317-nucleotide sequence of mouse exon 11 was determined in both directions by a combination of manual dideoxy sequencing (Sequenase, U.S. Biochemical, Cleveland, OH) and automated cycle sequencing on an ABI-373A sequencer (Applied Biosystems, Inc., Foster City, CA). Probes from the 5' and 3' ends of exon 11 were then used to screen an E17 embryo λ gt10 cDNA library (Clonetek, Palo Alto, CA). Positive clones were sequenced in both directions using internal primers. Sequences were analyzed with sequence analysis programs from DNASTAR (Madison, WI), the Genetics Computer Group (Madison, WI), and the NCBI Blast server (Altschul et al. 1990).

Genetic mapping

The mouse *Brca1* gene was mapped by analysis of the progeny of the multilocus crosses: (NFS/N or C58/J $\times$ *Mus musculus musculus*) $\times$ *M. m. musculus* (Kozak et al. 1990) and (NFS/N $\times$ *M. spretus*) $\times$ *Mus spretus* or C58/J (Adamson et al. 1991). Progeny of these crosses have been typed for >850 markers, including chromosome 11 markers ecotropic viral integration site 2 (*Evi2*), small inducible cytokine $\alpha 2$ (*Scya2*), homeo box 2 cluster (*Hoxb*), avian erythroblastosis oncogene B2 (*ErbB2*), FK506 binding protein-related protein (*Fkbp-rs*), wingless-related mouse mammary tumor virus (MMTV) integration site *int-4* (*Wnt-3*), high mobility group 14 related sequence 1, (*Hmg14-1*) and protein kinase C α (*Pkca*) as described previously (Kozak et al. 1995). The mouse *Brca1* cDNA probe (Fig. 1, probe B) identified BglII fragments of 12.4, 3.5, and 2.8 kb in parental *M. m. musculus* DNA, 8.9, 3.5, and 2.8 in NFS/N and C58/J, and 11.8, and 7.0 in *M. spretus*. The data were stored and analyzed using the program LOCUS developed by C.E. Buckler [National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD]. Recombinational distances and standard errors were calculated according to Green (1981). Genes were ordered by minimizing the number of double recombinants.

Cell lines

Mammary epithelial cell lines CL-S1 and NMuMG, and NIH3T3 fibroblasts were obtained from the American Type

Culture Collection. Mammary epithelial tumor cell lines HPI-2 and NX1128 were from MMTV-*int-2/FGF3* transgenic mice (Muller et al. 1990). AC204, AC236, and AC816 are mammary adenocarcinoma cell lines from ζ -globulin-v-Ha-*ras* transgenic mice (Leder et al. 1990). AC101 and AC280 are fibrosarcomas, and AC139 is a uterine fibrosarcoma from the same line. M158 and 16MB9a are mammary adenocarcinoma cell lines from MMTV-*c-myc* transgenic mice (Stewart et al. 1984). NK417 and SMF are mammary adenocarcinoma cell lines from MMTV-*neu/NT* (*erbB2*) transgenic mice (Muller et al. 1988). Cell lines were grown to ~85% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin.

Northern blot analysis

Northern blots were prepared from 2 μ g of poly(A)⁺ mRNA isolated from various mouse tissues, and tumor cell lines derived from FVB or Swiss-Webster mice (Chomczynski and Sacchi 1987). Blots were probed with probe B, or with probe B in combination with a 4-kb *EcoRI* fragment corresponding to the 3' end of the *Brca1* cDNA (probe C, Fig. 1). Probes were prepared by random-labeling of the insert with [α -³²P]CTP. Hybridization was carried out in S-buffer for 4–16 hr at 42°C [S-buffer = 48% formamide, 4.8 $\times$ SSC, 20 mM Tris-HCl at pH 7.5, 10% dextran sulfate, 1 $\times$ Denhardt's solution, 1% SDS, 20 μ g/ml of denatured herring sperm DNA (Sambrook et al. 1989)]. Blots then were washed extensively in 0.1% SSC at 60°C prior to exposure to autoradiographic film. The amount and quality of RNA present on each blot was assessed by reprobing with a cloned fragment of the 28S rRNA (Rich and Steitz 1987). Quantitation of autoradiographic signals was carried out on a Computing Densitometer equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Tissue preparation and in situ hybridization

Staged mouse embryos were obtained by natural mating of Swiss-Webster or C57BL/6 mice. Noon of the day that followed successful mating was considered to be day 0.5 p.c. Gestation was terminated between day 8.5 p.c. and day 18.5 p.c. and embryos were fixed with fresh 4% paraformaldehyde in phosphate-buffered saline (pH 7.6). Embryos, staged by morphological criteria (Rafferty 1970), were processed for whole-mount in situ hybridization as described (Chan et al. 1995), or were embedded in paraffin, and 5 μ m sections were mounted on microscope slides prior to in situ hybridization as described by Iruela-Arispe et al. (1993).

Riboprobe templates were generated by linearization of pBS-KSII plasmids (Stratagene, La Jolla, CA) that contained a subclone of the 5' 900 bp of *Brca1* mRNA (probe B). Riboprobes were synthesized with digoxigenin(DIG)-UTP in in vitro transcription reactions using T3 or T7 RNA polymerase as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Hybridization was detected by enzyme-linked immunoassay with an alkaline phosphatase-labeled anti-DIG antisera, and hybrids were visualized with an enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) as described by the manufacturer (Boehringer Mannheim).

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