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 synten 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]

Received August 10, 1995; revised version accepted September 14, 1995.

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
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 Brcal. We find that Brcal 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. Brcal is located on Chromosome 11, in a region of conserved synteny with human chromosome 17q21. Interestingly, Brcal is expressed during the development of the neuroepithelium and of several epithelial organs, including the kidney, skin, and mammary gland. Significantly, Brcal 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 Brcal structure

A 752-bp fragment of Brcal (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 Brcal clones from a mouse embryonic day 17 (E17) cDNA library. The longest cDNA clones terminated $\sim$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 Brcal 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.)

The mouse cDNA predicts an 1812-amino-acid protein similar in size and structure to the human BRCA1 gene product (Fig. 2A). Brcal 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, Brcal 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 Brcal gene

Sequence analysis of genomic clones resulted in the characterization of the complete structure of exon 11
Expression of Brcal mRNA in cell lines and embryos

When probed with a 5' cDNA fragment (Fig. 1, probe B), a single major Brcal transcript was detected on Northern blots of poly(A)+ RNA prepared from a 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 untranscribed 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-
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, Fi][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
whisker pads, with only moderate expression in the overlying epidermis (Fig. 6E). The reason for the locally modulated levels of Brcal 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 Brcal in adult tissues
and differentiation of the mammary gland

Expression of Brcal 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. Brcal 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 Brcal 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 Brcal 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, Brcal mRNA levels again increase [lanes 6–8]. By day 5 of regression, Brcal 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 Brcal 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]. Brcal transcripts were localized specifically in the mammary epithelial 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-
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 -.

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, BRCAl, 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...
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 1676–1712 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 Brcal 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 Brcal mRNA in tumors might indicate an inhibition, or disconnection, of a signaling system that regulates Brcal activity.

Materials and methods

Cloning and sequencing

Oligonucleotides that contain sequence from exon 11 of human Brcal (forward primer, 5’-TCATGCCAGCTCATTACAGC; reverse primer 5’-TCCGGTTGTAGTTCCCTG) 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 AflIII genomic library (Stratagene, La Jolla, CA). Positive clones were mapped (Fig. 1), and the complete 3317-nucleotide sequence of Brcal activity.

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Genetic mapping

The mouse Brcal gene was mapped by analysis of the progeny of the multilocus crosses: [NFS/N or C58/1 × Mus musculus musculus] × M. m. musculus [Kozak et al. 1990] and [NFS/N × M. spreitus] × Mus spreitus or C58/1 [Adamson et al. 1991]. Progeny of these crosses have been typed for >850 markers, including chromosome 11 markers ecotropic viral integration site 2 (Ev2l), small inducible cytokine a2 (Soya2), 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 Brcal cDNA probe [Fig. 1, probe B] identified BgIII 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 C58/1, and 11.8, and 7.0 in M. spreitus. 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/FCGF3 transgenic mice (Muller et al. 1990). AC204, AC236, and AC816 are mammary adenocarcinoma cell lines from γ-globulin-+H-αras 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 μg/ml of streptomycin.

Northern blot analysis

Northern blots were prepared from 2 μg 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 Brcal 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× SSC, 20 mM Tris–HCl at pH 7.5, 10% dextran sulfate, 1× Denhardt’s solution, 1% SDS, 20 μg/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 μm sections were mounted on microscope slides prior to in situ hybridization as described by Irura-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 Brcal mRNA [probe B]. Riboprophes 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 immunossay with an alkaline phosphatase–labeled anti-DIG antiserum, and hy-brid 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).

Acknowledgments

We thank Dr. Luisa Iruela-Arispe for stimulating discussions and help with in situ hybridizations, Dr. Karen A. Jones for...
reading the manuscript, Ann Kuo for assistance with the library screening, and the Biopolymer Facility, HHMI, Harvard Medical School, for cycle sequencing. T.F.L., C.D., and P.L. were supported by the Howard Hughes Medical Institute. A.E. is the recipient of the Dorot Foundation postdoctoral fellowship.

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*Genes Dev.* 1995, 9:
Access the most recent version at doi:10.1101/gad.9.21.2712