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Characterization of the Rat and Mouse Homologues of the *BRCA2* Breast Cancer Susceptibility Gene

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Abstract

Inherited *BRCA2* mutations confer profound susceptibility to human breast and ovarian cancer. The rat and mouse *Brca2* homologues share 58% and 59% identity (72% similarity), respectively, with the human BRCA2 protein. The Brca2 proteins also share a potential nuclear localization signal (human codons 3263–3269) and a highly conserved large carboxyl region (77% identity, 86% similarity between human and rodents) that may represent important functional domains. At least six of eight previously described BRC repeats have been highly conserved in rats and mice. Expression studies demonstrate an 11–12 Kb transcript with rodent tissue-specific patterns of expression consistent with human *BRCA2*. These results will facilitate studies of *Brca2* function during normal and neoplastic development.

Introduction

Inherited alterations in the human *BRCA1* or *BRCA2* genes confer a profound predisposition to breast and ovarian cancer development (reviewed in Ref. 1). Although men who inherit *BRCA1* mutations do not have an increased breast cancer risk, male *BRCA2* mutation carriers exhibit a significantly increased risk for this rare disease. *BRCA2* carriers also appear to be predisposed to a broad spectrum of cancers including elevated cancer risks in pancreas, larynx, prostate, and ocular melanoma (1). The human *BRCA2* gene was mapped to chromosome 13q12–13 (2) and positional cloning efforts recently lead to its identification (3, 4). *BRCA2* contains 27 exons that encode a predicted protein of 3418 amino acids. *BRCA1* and *BRCA2* appear to function as tumor suppressors because both alleles are inactivated during neoplastic development in familial tumors (1). Unlike BRCA1, which contains a RING finger zinc-binding domain (5), the BRCA2 gene product lacks obvious homology to proteins described previously. Potential granin motifs, related to secretory function, have been proposed for the human BRCA1 and BRCA2 proteins (6), but the significance of this putative motif has been questioned. Eight copies of a repeated motif (known as BRC[2] repeats) have been noted within exon 11 of the human *BRCA2* gene (7, 8). We have determined sequences for the complete coding regions of both the mouse and rat homologues as an initial step toward better understanding of *BRCA2* function. Several new domains of interest have been identified based on evolutionary conservation although overall sequence homology between human and rodent BRCA2 proteins is relatively low, as reported previously for BRCA1 (9).

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[2] The abbreviations used are: BRC, 25-amino acid repeat in BRCA2 proteins; NLS, nuclear localization signal.

Materials and Methods

Isolation of the Murine and Rat Brca2 Homologues. A bacterial artificial chromosome clone (BAC593D1) containing the *Brca2* gene was obtained by screening the CJ7 embryonic stem cell library (Research Genetics), which was derived from 129Sv mice. High density filters were hybridized in 40% formamide at 42°C with a ^{32}P-labeled, 1.3-kb PCR product containing exon 10 of the human *BRCA2* gene (5'-TTTCTATGAGAAAGGTTGTGAG and 5'-AGCAGAAAAAAACACAGAAGGA). Filters were then washed twice in 2× SSC (1× SSC: 1.5 M NaCl/0.15 M sodium citrate, pH 7.0) for 5 min at room temperature, twice in 2× SSC/1% SDS for 40 min at 60°C, and twice in 0.1× SSC/0.1% SDS for 30 min at room temperature. A 7-kb *Pst*I fragment from BAC593D1 was subcloned into pSK (Stratagene) and sequenced to determine the mouse *Brca2* genomic region extending from exons 9–11. Overlapping cDNA clones corresponding to the remaining exons were obtained by screening a BALB/c lambda gt11 mouse testes cDNA library (Clontech) with a series of mouse-specific PCR products. The extreme 5' end of the coding region, including the first 10 amino acids (and the untranslated portion of exon 2), was obtained using the mouse Genome Walker kit (Clontech) with genomic DNA from ICR Swiss mice. *Brca2*-specific nested primers used to generate this PCR product were 5'-TGTGCTGCATCTCGCCTT and 5'-CGCCTTAAAAATTTCCCAAA.

The initial rat sequence in exon 11 was obtained using various combinations of mouse primers with rat genomic DNA as a template. The 3' portion of the cDNA was isolated by screening a Sprague Dawley Lambda ZAP II testes cDNA library (Stratagene). A single large cDNA clone (6-1A) containing exons 11–27 was isolated and sequenced. Sequences for exons 1–10 were obtained through 5' rapid amplification of cDNA ends using a Marathon-ready Sprague Dawley rat testis cDNA amplification kit (Clontech) and a series of nested gene-specific reverse rat primers.

PCR products and isolated plasmid subclones were sequenced using an ABI PRISM dye terminator kit with an ABI 373A fluorescent sequencer (Applied Biosystems). Sequence comparisons of the human and rodent *Brca2* sequences were accomplished using the GAP, BLAST, and PILEUP alignment software (Program Manual for the Wisconsin Package, version 9.0; Genetics Computer Group). Motif searches were performed with GCG and the PSORT protein localization prediction program (10).

Expression. A mouse multiple-tissue Northern filter (Clontech) containing 2 μg of poly(A)$^+$ RNA from adult BALB/c mice was hybridized to a ^{32}P-labeled mouse cDNA PCR product probe spanning exons 3–9 (5'-CAGAAGCCCCCCCATACA and 5'-CTTCTTGCTGGTTTTTGTTTTC). Hybridizations were carried out in Hybrisol I (Oncor) at 42°C overnight, and the filter was washed as described by the manufacturer. Autoradiography was performed for 2 weeks with X-OMAT AR film (Kodak).

RNase protection assays were performed using total RNA from adult Sprague Dawley rat tissues extracted with Tri Reagent (Molecular Research Center). An antisense RNA probe corresponding to nucleotides 3449–3583 of the rat sequence was synthesized with T3 RNA polymerase using the Riboprobe *in vitro* transcription system (Promega Corp.). Probes were prepared as described by the manufacturer. RNase protection assays were performed using a RPA II assay kit (Ambion). Forty μg of total RNA were hybridized overnight at 43°C with 2.7×10^5 cpm of high specific activity *Brca2* antisense probe and 3×10^4 cpm of low specific activity rat cyclophilin probe (Ambion) as an internal control. Hybridized RNAs were digested with 0.25 units of RNase A and 10 units of RNase T1 for 30 min at 37°C and then precipitated. Samples were resuspended and denatured in 8 μl of 95% formamide, 0.025% xylene

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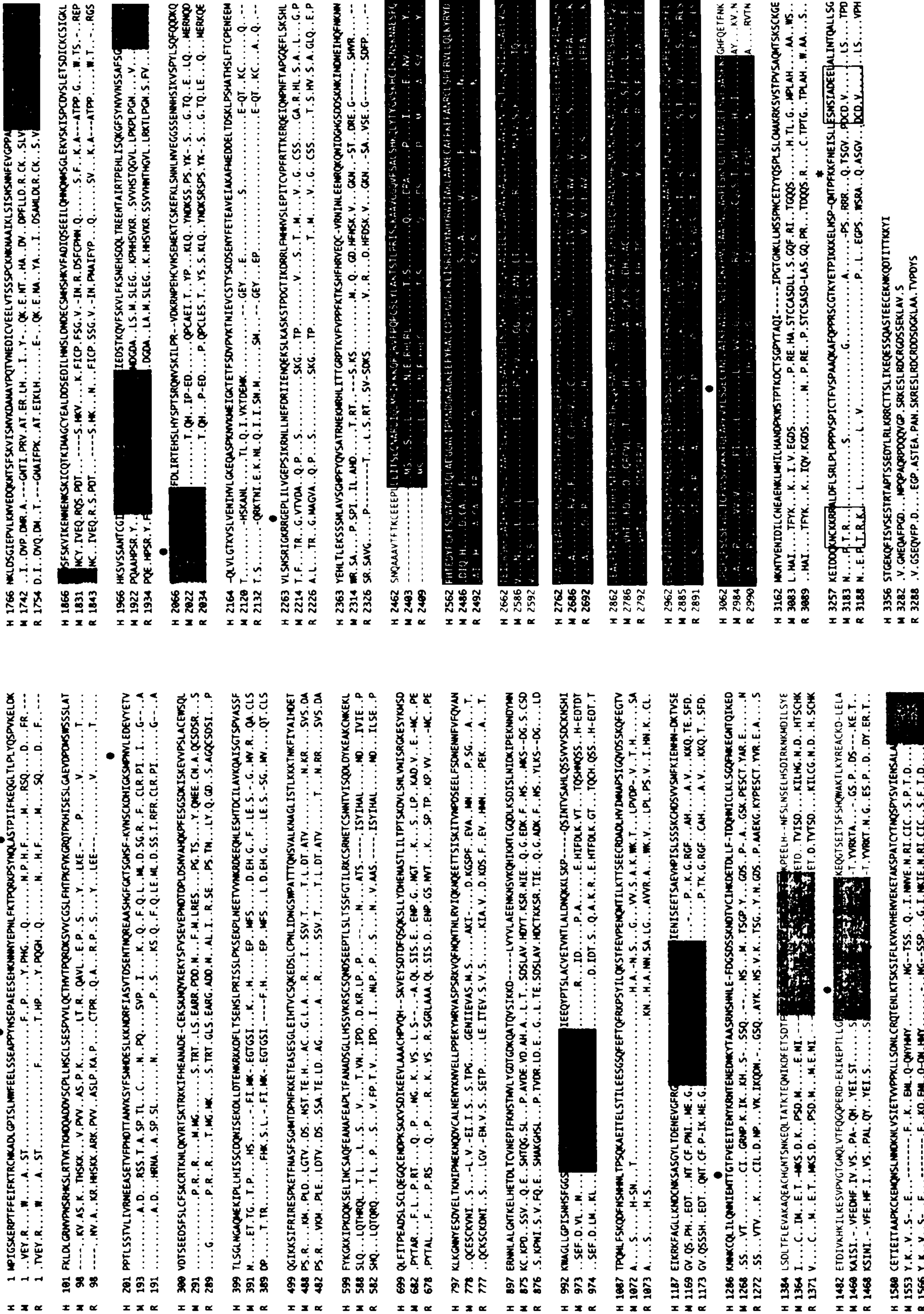

Fig. 1. Alignment of the predicted human (U43746), mouse (U89652), and rat (U89653) Brca2 amino acid sequences using the GCG PILEUP program. Only rodent differences from human BRCA2 are shown. *Dots,* identity of human and rodent gene products; *dashes,* gaps introduced in the alignment. Core sequences for the BRC repeats in exon 11 are highlighted in *light gray.* A large COOH terminal domain (represented by human codons 2479–3152), which has a 77% amino acid identity between the human and both rodent homologues and a 93% identity between rat and mouse, is highlighted in *black.* A putative NLS (human codons 3263–3269) and the previously proposed granin motif (human codons 3335–3344) are *boxed.* The *asterisk* over human codon 3326 shows the location of a polymorphic stop codon in human BRCA2. *Filled circles,* sites of missense alterations that have been conserved between the human and rodent Brca2 proteins.

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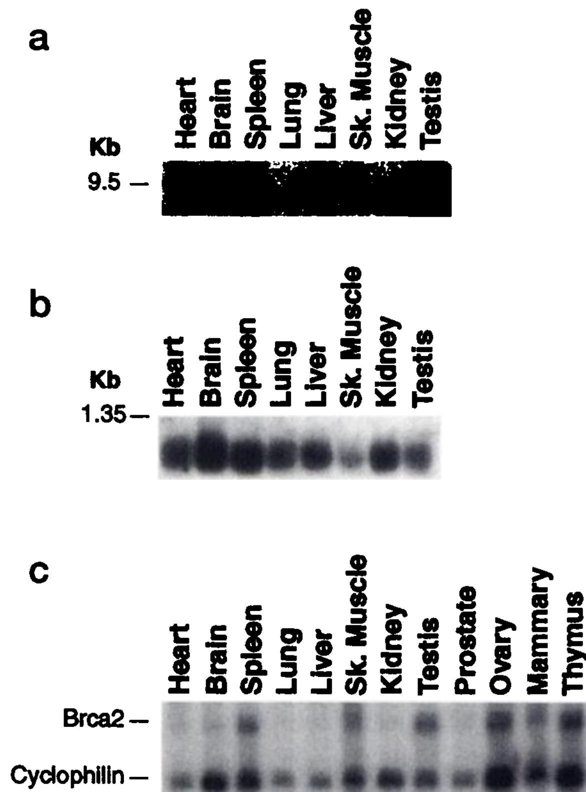

Fig. 2. Expression of *Brca2* in adult rodent tissues. *a,* Northern analysis of the 11–12-kb *Brca2* transcript in the indicated mouse tissues using a probe containing mouse exons 3–9. *b,* Northern analysis of the 0.7-kb *cyclophilin* transcript used as a control for loading of poly(A)$^+$ RNA. *c,* RNase protection analysis of *Brca2* transcripts from the indicated Sprague Dawley rat tissues. Protected fragment sizes are 135 and 103 nucleotides for *Brca2* and *cyclophilin* RNAs, respectively. *Sk.,* skeletal.

cyanol, 0.025% bromphenol blue, 0.5 mM EDTA, and 0.025% SDS prior to electrophoresis on a 5% acrylamide, 8 M urea gel at 250 V for 2 h. Gels were quantitated using a PhosphorImager (Molecular Dynamics).

Results and Discussion

We have determined the sequence of the coding regions of the rat (U89653) and mouse (U89652) *Brca2* homologues to identify potential functional domains based on evolutionary conservation. Fig. 1 shows the alignment of the human, mouse, and rat Brca2 proteins. The human BRCA2 protein consists of 3418 amino acids with a predicted molecular weight of M_r 384,000, whereas the mouse and rat Brca2 proteins encode products of 3329 and 3343 amino acid residues with predicted molecular weights of M_r 370,000 and 372,000, respectively. The largest gap between the alignment of the human BRCA2 protein with the rodent gene products exists at the 3′ end of exon 13. The human protein has an additional 19 residues that are absent in both rodent homologues at this exon/intron junction. An additional in-frame methionine is present nine amino acids upstream of the start codon designated for the rat protein (data not shown). Although this rat methionine has a favorable Kozak consensus sequence for translation, the mouse and human homologues lack an analogous start codon. Additional studies will be required to determine the actual translation start site for the rat Brca2 protein. The mouse and rat coding regions are 73 and 72% identical with the human *BRCA2* gene at the nucleotide level, respectively. The human BRCA2 protein exhibits 59 and 58% identity (72% similarity), respectively, with the mouse and rat predicted gene products. As expected, comparisons between the mouse and rat *Brca2* genes reveal a much higher overall conservation with 90% nucleotide identity and 84% amino acid identity (90% similarity). Although most human cancer susceptibility genes are more highly conserved with their rodent homologues (11), previous work has shown that the human *BRCA1* gene has undergone a similar degree of divergence with other mammalian species (9, 12, 13). This relatively high level of divergence suggests that large portions of the Brca1 and Brca2 proteins may not be subject to strong evolutionary constraints. Although the overall conservation is low, a large COOH terminal domain (codons 2479–3152 of human BRCA2) shows 77% amino acid identity (86% similarity) between the human and rodent gene products (Fig. 1). This 674-amino acid domain has 93% identity (94% similarity) between the mouse and rat proteins. In addition, at least seven regions of 22 or more amino acids are conserved throughout the BRCA2 proteins, where identity between these three species is 95% or greater (human codons 22–43, 1006–1042, 1102–1130, 2220–2243, 2581–2616, 2797–2819, and 3287–3308).

To demonstrate that the mouse consensus sequence represents the *Brca2* homologue rather than a related gene, we have mapped its location in the mouse genome (14). A $(TTTGG)_8$ simple sequence repeat was identified in intron 10 by sequencing BAC593D1. Segregation analysis of this marker with DNAs from a mouse backcross panel localized the *Brca2* gene to the telomeric region of mouse chromosome five, which is syntenic with human 13q12-q13. In addition to *Brca2,* the murine homologues of at least three other human genes from 13q12 (*FLT1, FLT3,* and *ATRC1*) have been mapped to this distal region of mouse chromosome five.

Further confirmation that these rodent genes are truly *BRCA2* homologues was obtained by Northern analysis and RNase protection assays. Northern analysis of multiple mouse tissues with a mouse exon 3–9 probe revealed transcripts of the expected size (approximately 11–12 kb) that exhibited a tissue-specific pattern of expression comparable to human *BRCA2* (4). The 11–12-kb transcript was most abundant in testis and was also detectable at lower levels in heart, spleen, liver, and skeletal muscle (Fig. 2*a*). The Northern filter shown in Fig. 2*a* was stripped and rehy-

Repeat	Species	Codon	Sequence
BRC1	Human	1009	FRTASNKEIKLSEHNIKKSKMFFKD
	Mouse	988	...............V.........
	Rat	991	.............D.V.........
BRC2	Human	1219	FYSAHGTKLNVSTEALQKAVKLFSD
	Mouse	1199	.C..L....S..N...R..M.....
	Rat	1204	.C..L....S..N...R..M.....
BRC3	Human	1428	FQTASGKNISVAKELFNKIVNFFDQ
	Mouse	1401	........TR.S..SL..S..I.NR
	Rat	1412	.........R.S..SL..S..IL..
BRC4	Human	1524	FHTASGKKVKIAKESLDKVKNLFDE
	Mouse	1498	...........MQ............
	Rat	1510	...........MQ............
BRC5	Human	1671	FYTSCSRKTSVSQTSLLEAKKWLRE
	Mouse	1630	Y..ED....C.RES..SKGR.....
	Rat	1645	C..GD....C.GES..SKG......
BRC6	Human	1844	FRIASGKIRLCSHETIKKVKDIFTD
	Mouse	1813	.IT.H------.Q..E-RT.E.V..
	Rat	1828	.ITTH------.Q..V-RM.E....
BRC7	Human	1978	FSTASGKSVQVSDASLQNARQVFSE
	Mouse	1931	.......AI.......EK.......
	Rat	1946	.......A........EK.......
BRC8	Human	2058	FSTASGKQVSILESSLHKVKGVLEE
	Mouse	2011	....G..L.TVS..A......M...
	Rat	2026	....G..L.TVS..A......M...

BRC Consensus FxTASGKx$^{I}_{V}$x$^{I}_{V}$SxxxLxKxxxx$^{L}_{F}$x$^{D}_{E}$

Fig. 3. Conservation of the core BRC repeats in the human, mouse, and rat Brca2 proteins. *Dots,* identity of human and rodent gene products; *dashes,* gaps introduced in the alignment. The BRC repeat consensus as defined by Bignell *et al.* (8) is shown; *x,* nonconsensus residues.

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Table 1 *Conservation of BRCA2 sequence variants and neutral polymorphisms*

Codon	Human *BRCA2*		Mouse *Brca2*	Rat *Brca2*	Control frequency[a]	Origin[b]	Ref.
	Normal	Variant					
Neutral polymorphisms							
372	His	Asn	Ser	Asn	0.30		22
991	Asn	Asp	Asp	Asp	0.02		22
1147	Asn	Ser	Asn	Asn	0.19		22
1915	Met	Thr	Ile	Ile	0.04		22
2034	Arg	Cys	His	His	0.01		22
3326	Lys	Stop	Gln	Gln	0.02		17
BRCA2 sequence variants							
42	Tyr	Cys	Tyr	Tyr		U	21
75	Ala	Pro	Ala	Ala		U	23
289	Asn	His	Asn	Asn		U	23
355	Val	Leu	Val	Ala		U	23
630	Thr	Ile	Thr	Pro		U	23
728	Asp	Ala	Asn	Asp		G	23
1283	Val	Gly	Phe	Phe		U	21
1302	Thr	Asn	Thr	Thr		U	21
1529	Gly	Arg	Gly	Gly		U	23
1880	Asn	Lys	Ser	Ser		U	23
2071	Ser	Ile	Ser	Ser		U	21
2274	Gly	Val	Gly	Gly		G	23
2415	His	Asn	Gln	Arg		S	24
2421	Gln	His	His	His		G	23
2466	Ala	Val				G	25
2787	Arg	His	Cys	Arg		S	25
3095	Asp	Glu	Asp	Asp		S	26
3098	Tyr	His	Leu	Leu		U	21
3103	Ile	Met	Val	Val		U	23
3357	Thr	Arg	Val	Val		U	23
3412	Ile	Val				G	23

[a] The frequency of the less common allele in normal populations is presented for neutral polymorphisms.
[b] The origin of the variation is designated as G (germ-line), S (somatic), and U (unknown).

bridized with a 165-nucleotide mouse *cyclophilin* antisense RNA probe (Ambion; Fig. 2*b*). Comparable loading was observed for the multiple mouse tissues in this experiment with the exception of skeletal muscle. RNase protection assays for *Brca2* were also performed using total RNA from a panel of 12 Sprague Dawley rat tissues (Fig. 2*c*). Quantitative analysis of rat *Brca2* expression normalized to *cyclophilin* showed that expression in testis was 2–3-fold higher than in spleen, skeletal muscle, thymus, mammary gland, heart, ovary, and prostate. *Brca2* expression in liver and lung was 4-fold less than in the testis, whereas levels in the kidney and brain were reduced 12-fold compared to testis when normalized to *cyclophilin*. These observations of *Brca2* expression are consistent with previous results for *BRCA1* expression in humans and rodents (5, 9, 12). *BRCA2* and *BRCA1* expression in normal human and tumor-derived breast epithelial cells is regulated by the cell cycle in a similar manner (15). Likewise, coordinate regulation of *Brca2* and *Brca1* expression during mouse mammary cell proliferation and differentiation *in vitro* has been described (16). These observations have led to the proposal that these breast cancer susceptibility genes may function in a common biochemical pathway (15, 16).

Jensen *et al.* (6) have reported that the human BRCA1 and BRCA2 proteins contain a potential granin motif, which has been defined by the following consensus motif: (E,D)(N,S)L(S,A,N)*XX*(D,E)*X*(E,D)L. This granin motif is poorly conserved in the rodent Brca2 homologues (Fig. 1), as was observed with BRCA1 (6, 12). Although the human BRCA2 protein contains six of the seven constrained amino acids characteristic of this motif (codons 3335–3344), the rodent Brca2 protein sequences have retained only five of these seven critical amino acids. The serine or asparagine at the second position has been replaced with a cysteine, and the leucine at the third position has been replaced with an aspartic acid in both rodent proteins. Interestingly, Mazoyer *et al.* (17) have found a polymorphic stop codon that removes the final 93 amino acids of the human BRCA2 protein. This stop codon would delete the putative granin domain (Fig. 1), but an increased risk for cancer has not been noted between normal individuals and breast cancer patients carrying this polymorphism (17). Given the apparent lack of phenotypic differences related to this polymorphic stop codon and the extreme divergence between humans and rodents at the extreme 3′ end of the BRCA2 protein sequence (Fig. 1), the presence of an important functional domain in this region seems unlikely.

The BRC motif (Fig. 1) was initially described as an internal repeat in the human BRCA2 protein (7). The sequences of BRC repeats have recently been determined for the monkey, pig, dog, hamster, and mouse Brca2 proteins, and this work demonstrated that the BRC repeats have been more highly conserved throughout mammalian evolution than other portions of exon 11 (8). A comparison of the human BRC repeats with mouse and rat sequences is shown in Fig. 3. We found six of the eight rat BRC repeats to have 9 or more of the 13 consensus residues defined by Bignell *et al.* (8). The BRC3, 4, 7, and 8 repeats exhibited the strongest conservation in the rat Brca2 protein relative to the consensus (85–92%), whereas the BRC1 and 2 repeats were 77 and 70% conserved, respectively. As noted previously (8), the fifth and sixth BRC repeats have diverged significantly from the BRC consensus; their conservation is less than the overall homology between the entire rat and human BRCA2 proteins.

Several potential new domains of importance were identified by comparison of the human and rodent Brca2 homologues. The large homologous COOH terminal domain (Fig. 1) is suggestive of a similar 3′ region of the BRCA1 protein that is relatively highly conserved between mammals (13). Chapman and Verma (18) have shown that the COOH domain of the human BRCA1 protein has transcriptional transactivation activity. By analogy, this BRCA2 region may function in a similar manner. In addition, we observed a potential NLS in the COOH terminus of human BRCA2 (codons 3263–3269; Fig. 1) and its rodent homologues. This putative motif consists of a positively charged core peptide unit of KKRR in the human BRCA2 protein, whereas the mouse and rat contain RKRR and RKKR, respectively. This motif was predicted for all three species by the PSORT program (10). Most core NLSs consist of a hexapeptide with at least four arginines or lysines; acidic residues and prolines are found frequently in the flanking regions of this hexapeptide stretch (19). A number of variations of the standard basic hexapeptide exist in many

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eukaryotic species, and it is not uncommon for the exact amino acid positions of a NLS to be slightly altered in the homologous gene of different species (19). Core NLSs are present in nearly all nuclear proteins and absent from nearly all cytoplasmic proteins. The BRCA1 protein also contains a pair of NLSs and appears to be localized to the nucleus of normal human cells (20). Recent work suggests that BRCA1 plays a role in both mitotic and meiotic cell cycle control through its interaction with the RAD51 protein (20).

The majority of *BRCA2* mutations are nonsense or frameshift alterations resulting in the premature termination of protein synthesis (1). However, multiple missense alterations have been reported (21), and these may be either disease related or simply represent polymorphic variations. In the absence of a functional assay, interspecies comparisons may help to clarify the biological relevance of such missense alterations. Table 1 shows the conservation of residues between humans and rodents for the reported polymorphisms resulting in an amino acid change and for unclassified human *BRCA2* sequence variants. It is difficult to classify most *BRCA2* missense alterations as true mutations *versus* rare polymorphisms because many of these changes have not been shown to segregate with disease in families, and variant allele frequencies are unknown for appropriate control populations. Functionally significant, disease-related missense mutations should be highly conserved across species, whereas polymorphic variants are more likely to occur at nonconserved residues. Consistent with this hypothesis, only one of the polymorphic variants (17%) reported in the literature was conserved across all three species (Table 1). In contrast, 8 of 21 (38%) of the sequence variants with unknown consequences have been conserved among all three species (Fig. 1), whereas four others were only conserved between human and one of the two rodent species. These observations are similar to previous results reported for the conservation of human *BRCA1* variants among species. Seven of 18 (39%) *BRCA1* alterations were maintained among humans, mice, and dogs (13). Three of these conserved missense variants (A1708E, P1749R, and M1775R) disrupt the transcriptional transactivation activity of BRCA1 (18). By analogy, a subset of these conserved *BRCA2* variants may also prove to be functionally significant and may be useful in assays designed to test proposed BRCA2 functions.

In conclusion, we have identified several potentially important domains of the *BRCA2* gene product based on evolutionary conservation. The functional significance of the BRC repeats, the putative NLS motif, and the large COOH terminal domain merit experimental verification. In addition, we have developed the tools to study the control of *Brca2* expression during normal growth control and differentiation as well as neoplastic development in rats and mice. The ability to manipulate the murine genome through gene targeting and transgenic techniques makes the mouse a powerful model for disease studies. Our isolation and characterization of the rodent *Brca2* homologues provide the necessary reagents for the creation of mice with specific *Brca2* alterations on defined genetic backgrounds.

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