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Localization of BRCA1 and a Splice Variant Identifies the Nuclear Localization Signal

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Inherited mutations in *BRCA1* confer susceptibility to breast and ovarian neoplasms. However, the function of BRCA1 and the role of BRCA1 in noninherited cancer remain unknown. Characterization of alternately spliced forms of *BRCA1* may identify functional regions; thus, we constructed expression vectors of *BRCA1* and a splice variant lacking exon 11, designated BRCA1Δ672-4095. Immunofluorescence studies indicate nuclear localization of BRCA1 but cytoplasmic localization of BRCA1Δ672-4095. Two putative nuclear localization signals (designated NLS1 and NLS2) were identified in exon 11; immunofluorescence studies indicate that only NLS1 is required for nuclear localization. RNA analysis indicates the expression of multiple, tissue-specific forms of *BRCA1* RNAs; protein analysis with multiple antibodies suggests that at least three BRCA1 isoforms are expressed, including those lacking exon 11. The results suggest that BRCA1 is a nuclear protein and raise the possibility that splicing is one form of regulation of BRCA1 function by alteration of the subcellular localization of expressed proteins.

BRCA1 was isolated by positional cloning methods as a gene linked to breast cancer in families with a pattern of autosomal dominant inheritance of the disease (21). Single dominant susceptibility alleles are thought to account for 5 to 10% of all breast cancers, and *BRCA1* germ line mutations are widely held to be responsible for approximately 50% of all inherited breast cancers. Inherited *BRCA1* mutations are also thought to be responsible for the disease in 80 to 90% of all families with breast-ovarian cancer syndrome (9). Women inheriting mutations in *BRCA1*, most of which are truncating mutations that result in nonfunctional or unstable proteins (8), have an 85% chance of developing breast cancer in their lifetime (9). An on-line database now provides a listing of known *BRCA1* mutations (http:// www. nchgr. nih. gov/ Intramural_research/ Lab_transfer/Bic/index.html), but little is known about the regulation of this gene or the function of its protein product. Unfortunately, mutations in *BRCA1* are distributed evenly over the gene, providing little in the way of clues for localizing critical functional regions.

BRCA1 is a large gene, with a coding region of 5.5 kb and a total mRNA of approximately 8.0 kb. There is little identifiable homology to known genes, with the exception of a short region in the 5′ end (spanning exons 2 to 5) that encodes a RING finger with a typical Cys_3-His-Cys_4 structure. This is a zinc-binding motif that is found in a family of transcription factors and may be a protein-protein interaction site. Initial reports also provided evidence for a complex pattern of alternate splicing and the potential for translation of a number of BRCA1 protein isoforms (21). *BRCA1* fits the model of a classic tumor suppressor gene, a hypothesis supported by recent work demonstrating that expression of BRCA1 inhibits growth of breast and ovarian cancer cell lines and MCF7-based tumor development in nude mice (16). Additional data supporting *BRCA1* as a tumor suppressor gene are provided by Rao et al. (23), who demonstrated that down-regulation of *BRCA1* expression by introduction of *BRCA1* antisense cDNA transforms NIH 3T3 cells. There is some evidence that *BRCA1* is directly or indirectly regulated by ovarian hormones (14, 20), but again, a detailed analysis of the molecular pathways has not been performed. In fact, BRCA1 has yet to be directly linked to any known cellular pathway despite efforts by many investigators to identify protein partners and regulatory mechanisms.

Based on the high incidence of breast cancer in *BRCA1* germ line mutation carriers, there has been widespread speculation that BRCA1 would be implicated in the pathogenesis of a significant number of sporadic tumors, not just those that arise in women with inherited mutations. However, to date, *BRCA1* mutations have been detected in very few sporadic breast cancers, suggesting that BRCA1 may not play a significant role in the development of these cancers (12). Given the absence of somatic *BRCA1* mutations in the face of the dramatic effect of *BRCA1* germ line mutations, several groups have investigated mechanisms other than protein truncation or loss that could tie BRCA1 to the pathogenesis of noninherited tumors. Thompson et al. (28) examined *BRCA1* transcripts in a series of normal and malignant breast tissues, including a number of in situ carcinomas, and demonstrated a decrease in *BRCA1* mRNA levels in sporadic breast cancers compared to normal breast cells. Furthermore, by utilizing an RNase protection assay with RNA samples obtained by microdissection, a stepwise decrease in the *BRCA1* transcript levels as a function of tumor progression was demonstrated (28). Additional data supporting the ostensible function of BRCA1 in growth regulation was provided by accelerated growth of breast cancer cell lines when they were treated with antisense oligonucleotides of *BRCA1* (28). Chen et al. provided other evidence of a role for BRCA1 in sporadic cancers by demonstrating an accumulation of BRCA1 in the cytoplasm of 80% of cells derived from sporadic breast cancers (6, 7), while BRCA1 (p220) was located in the nuclei of HBL-100 cells (a normal breast epithelial cell line immortalized with simian virus 40). The nuclear ex-

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clusion and/or cytoplasmic sequestration of BRCA1 in malignant cells suggests an indirect suppression of protein function in malignant cells if the target of action of BRCA1 resides in the nucleus. Finally, the most recent data provide conflicting evidence that BRCA1 is a 190-kDa protein localized to the cytoplasm and cell membrane and present as a secreted protein in cell culture supernatants (17). The source of this controversy is likely to lie in the nonspecificity of many antisera raised to BRCA1 peptides, and a consensus opinion has not arisen.

Our data strongly support the hypothesis that BRCA1 is a nuclear protein with a molecular mass of 220 kDa. The present study describes the isolation and expression of two cDNAs of *BRCA1*, including a splice variant designated BRCA1Δ672-4095. BRCA1Δ672-4095 is generated by exclusion of exon 11 by in-frame splicing and produces a 97-kDa protein. In contrast to BRCA1, BRCA1Δ672-4095 localizes to the cytoplasm. This observation led to the identification of a nuclear localization signal (NLS) in *BRCA1* exon 11 as determined by immunofluorescence studies of deletion mutants. Finally, we describe a complex tissue-specific pattern of multiple splice forms of *BRCA1* and suggest that splicing may play a role in the regulation of BRCA1 function by altering the subcellular localization of various isoforms.

MATERIALS AND METHODS

Construction of *BRCA1* and BRCA1Δ672-4095 expression vectors. Four separate cDNA fragments of *BRCA1* with overlapping endogenous restriction enzyme sites were amplified from total RNA of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines, utilizing reverse transcription PCR (RT-PCR). The internal *Eco*RI, *Kpn*I, and *Bam*HI sites present within each fragment were utilized for ligation and assembly of the full-length *BRCA1* cDNA (Fig. 1a). *Hin*dIII and *Not*I linkers were added to the proximal and distal ends of the primers of fragments 1 and 4, respectively, to facilitate subsequent subcloning. The primer pairs used for each of the fragments and their positions relative to the coding sequence are as follows: fragment 1, F(−6-21) (5′-GCAAGCTTGC CACCATGGATTTATCTGCTCTTCGC-3′) and R(1003-982) (5′-GGGAGTC CGCCTATCATTACAT-3′); fragment 2, F(765-788) (5′-AGAGGCATCCAGA AAAGTATCAGG-3′) and R(3170-3152) (5′-GGAGCCCACTTCATTAGTAC -3′); fragment 3, F(2135-2157) (5′-GTGTTCAAATACCAGTGAACTTA-3′) and R(4096-4073) (5′-AAGTTTGAATCCATGCTTTGCTCT-3′); and fragment 4, F(3879-3902) (5′-GCTAGCTTGTTTTCTTCACAGTGC-3′) and R(5676-5707) (5′-GCGCGGCCGCGTAGCCAGGACAGTAGAAGGA-3′). Fragments were amplified by using PWO polymerase (Boehringer Mannheim Biochemicals). The *Hin*dIII and *Not*I sites are underlined. Each of the four fragments was subcloned separately into the pCR3 vector (Invitrogen) by TA cloning as suggested by the manufacturer, with one additional step: the amplified products were tagged with a single deoxyadenine by incubating them with 500 mM dATP and 10 U of *Taq* polymerase at 70°C for 1 h. BRCA1Δ672-4095 was amplified by performing RT-PCR with the proximal primers (nucleotides [nt] −6 to 21) and distal primers (nt 5707 to 5676) (Fig. 1b) and RNAs of four separate EBV-transformed lymphoblastoid lines. The amplified product was inserted into the pCR3 vector as described for *BRCA1*. The sequences of both clones were verified by the dideoxy chain termination method and automated sequencing. Two sequence variants in pCR3-BRCA1 were repaired by site-directed mutagenesis with the Altered Sites II *In Vitro* Mutagenesis Systems (Promega). Three sequence variants in BRCA1Δ672-4095 were repaired by substituting restriction fragments from wild-type *BRCA1*. K38R was repaired by using a *Hin*dIII/*Acc*I (nt 1 to 533) restriction fragment; Y1563C and N1745D were repaired by using a *Bgl*II fragment (nt 4668 to 5276). All repairs were confirmed by sequencing.

In vitro translations. *BRCA1* and BRCA1Δ672-4095 in the pCR3 vector were inserted 3′ to a heterologous T7 phage promoter. One microgram of each plasmid was translated in a rabbit reticulocyte system (Promega) according to the instructions of the manufacturer.

Immunoprecipitations and Western blots. Immunoprecipitations were performed in radioimmunoprecipitation assay buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 1% aprotinin, and 250 μM phenylmethylsulfonyl fluoride) with a 1:200 dilution of the C-20 or D-20 polyclonal antibody (Santa Cruz Biotechnology Inc.) for 1 h at 4°C, and 40 μl of protein A-agarose beads (GIBCO/BRL) was added and incubated for another hour. C-20 recognizes a C-terminal epitope corresponding to amino acids 1843 to 1862; D-20 recognizes an N-terminal epitope corresponding to amino acids 2 to 21. Three washes were performed with the radioimmunoprecipitation assay buffer. For Western blotting, peptide competitions involved addition of equal volumes of the peptide, C-20P (Santa Cruz Biotechnology), and C-20 antibody in the buffer. The translated and immunoprecipitated proteins were electrophoresed through a 9% polyacrylamide gel. Western blotting of in vitro-translated BRCA1Δ672-4095 was performed by the ECL method (Amersham). The antibody (C-20 or D-20) was applied at a dilution of 1:200, and competitions were performed with an equal volume of the peptide (C-20 only).

Combination immunoprecipitation-Western blotting was performed by incubating cellular extracts with a mixture of three N-terminal antibodies (D-20 with MS13 and MS110 [gifts from David Livingston]) for 1 h in 10 mM Tris-HCl (pH 7.5)–1% Nonidet P-40–150, mM NaCl–0.1% SDS (IP buffer) with freshly added 250 μM phenylmethylsulfonyl fluoride, 1% aprotinin, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, and 40 μl of protein A-agarose beads (GIBCO/BRL). A 1:200 dilution was used for each of the antibodies. The immunoprecipitated beads were washed three times with the IP buffer, boiled for 10 min in dissociation buffer (625 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.1% bromophenol blue), and electrophoresed in a 9% polyacrylamide gel. The separated proteins were electroblotted onto a nitrocellulose membrane (6 h) and immunoblotted with the C-20 C-terminal antibody (1:200 dilution) by utilizing the ECL detection system.

Transfections and immunofluorescence analysis. Ten micrograms of pCR3, pCR3-BRCA1, and pCR3-BRCA1Δ672-4095 was transfected into NIH 3T3 or COS-7 cells by calcium phosphate precipitation (13) with modifications as outlined by Beckman et al. (2) and Kiledjian et al. (19) or by using Lipofectin (GIBCO/BRL) according to the instructions provided by the manufacturer. Cells were maintained in Dulbecco modified Eagle medium (high glucose)–10% fetal bovine serum–100 U of penicillin per ml–100 mg of streptomycin per ml–150 mg of glutamine per ml. After 24 h, the cells were trypsinized, plated at 60% confluence in chamber slides (Nunc), and incubated overnight. Microtransfections by both methods were also performed on chamber slides, obviating the need for trypsinization. After a phosphate-buffered saline (PBS) wash, the cells were treated with cold acetone (2 min) or 3% paraformaldehyde (10 min) and incubated for 1 h in PBS with 1% Triton X-100 and 10% goat serum. The primary antibody was applied to the cells at a dilution of 1:200 (C-20 and D-20), 1:100 (MS110 and MS13), or 1:10 (Ap16 and SG11) in a solution of PBS and 0.3% Triton X-100. MS110 and MS13 are mouse monoclonal antibodies raised against a glutathione *S*-transferase fusion protein containing BRCA1 amino acids 1 to 304, Ap16 was similarly raised to recognize amino acids 1313 to 1863, and SG11 is a mouse monoclonal antibody raised by using a peptide corresponding to amino acids 1843 to 1863; all monoclonal antibodies (used as cell culture supernatants) were gifts of D. Livingston and R. Scully (25). A 1:200 dilution of secondary goat anti-rabbit-fluorescein isothiocyanate antibody (Boehringer Mannheim Biochemicals) was added. Prior to and after each step of antibody applications, cells were washed three times (10 min each) with PBS containing 0.3% Triton X-100. After the washes, the cells were mounted in AF1, an antiquenching reagent (Citifluor). The cells were photographed with immunofluorescence microscopy.

Construction of deletion mutants. Sequence overlap extension amplification was used in generating the deletion nucleotides encoding the two potential NLSs. For the NLS1 deletion, two PCR products were generated by using four sets of primers. The sequences of the primers and their nucleotide positions with respect to the start codon are as follows: 883(F) (5′-AGAGGCATCCAGAAAA GTATCAGG-3′) and 1620(R) (5′-TGATGTAGGATTTGTGAGGGGACG-3′); 1620(F) (5′-TCACAAATCCTACATCAGGCCTT-3) and 2504(R) (5′-TTT TGCCTTCCCTAGAGTGCTAAC-3′). The underlined nucleotides denote the overlap with sequences which extend beyond the NLS1 coding sequence but exclude the NLS1 coding sequences. Products of 737 and 874 bp were amplified, gel purified, mixed, and amplified again with primers 1620 (sense) and 2504 (antisense), generating a product of 1621 bp. The amplified product was digested by utilizing internal *Eco*RI (nt 1025) and *Kpn*I (nt 2431) sites and ligated into pCR3-BRCA1. For the NLS2 coding sequence deletion, exactly the same conditions were utilized, except the overlap extension primers used were as follows: 1937(F) (5′-AAGCACCCTTCTTCTACCAGGCAT-3′) and 1937(R) (5′-GTA GAAGAAGGTGCTTTGAATT-3′).

RNA analysis. MTN Northern blots (Clontech) containing poly(A)$^+$ RNA from human tissues were hybridized with the BRCA1Δ672-4095 probe according to the recommendations of the manufacturer. The probe was labeled by the random hexamer method (10) and purified with a Sephadex G-50 bead column. RT-PCR on total RNAs of various tissues was performed with primers in exon 6 (nt 215 to 239) (5′-GCCTACAAGAAAGTACGAGATTTAG-3′) and exon 12 (nt 4152 to 4132) (5′-GCTTGTTTCACTCTCACACCC-3′). One microgram of total RNA was reverse transcribed by utilizing the Superscript preamplification system (GIBCO/BRL), with conditions recommended by the manufacturer, and amplified under the following conditions: preheating at 94°C for 3 min and 32 cycles of amplification (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s).

RESULTS

In vitro expression of BRCA1 and BRCA1Δ672-4095. In order to define potentially important functional regions of BRCA1, we began characterizing alternately spliced variants of the complex *BRCA1* gene. Splice variants were isolated by

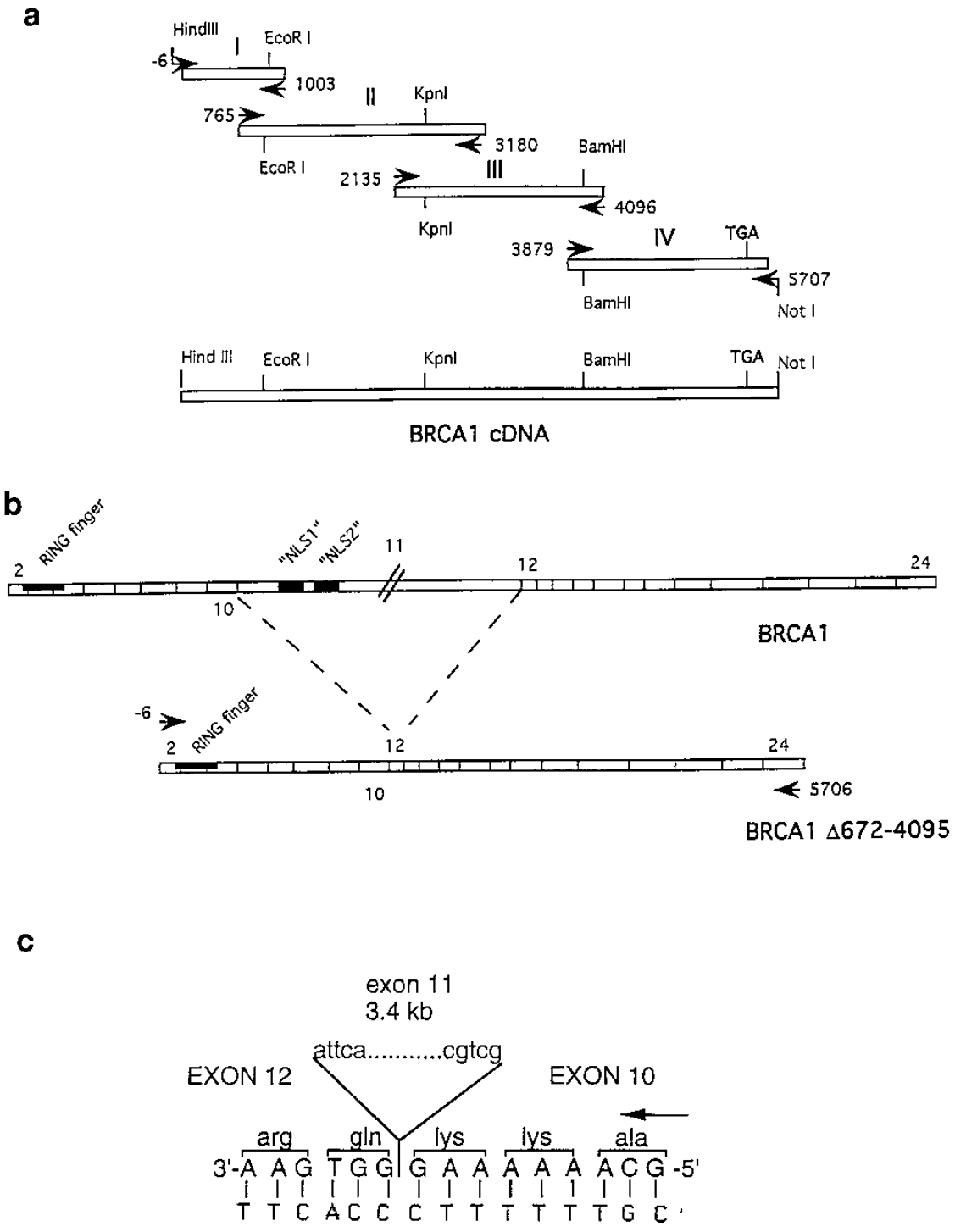

FIG. 1. pCR3-BRCA1 and pCR3-BRCA1Δ672-4095 (a) Construction of expression constructs. Four overlapping fragments (I to IV) were amplified by RT-PCR. The positions of the primers (arrows) in relation to the translational start site are indicated. The four fragments were ligated together by using the overlapping restriction enzyme sites denoted. The position of the stop codon (TGA) is also indicated. (b) Schematic representation of BRCA1 and BRCA1Δ672-4095. The RING finger motif, NLS1, and NLS2 are indicated. BRCA1Δ672-4095, which has an in-frame splice deletion of exon 11, lacks both NLS1 and NLS2. Numbers denote exons. (c) Sequence analysis of the BRCA1Δ672-4095 exon 10-12 splice junction.

PCR amplification of cDNA derived from EBV-transformed human lymphoblastoid cell lines known to contain *BRCA1* alleles without disease-associated sequence variants (5) by using primers from the extreme 5′ and 3′ ends of the coding region of *BRCA1*. We constructed an expression vector containing the full-length coding region of *BRCA1* (pCR3-BRCA1) and another containing the alternate splice BRCA1Δ672-4095, from which exon 11 is removed by in-frame splicing (pCR3-BRCA1Δ672-4095) (Fig. 1b and c). pCR3-BRCA1 was constructed in four segments as illustrated

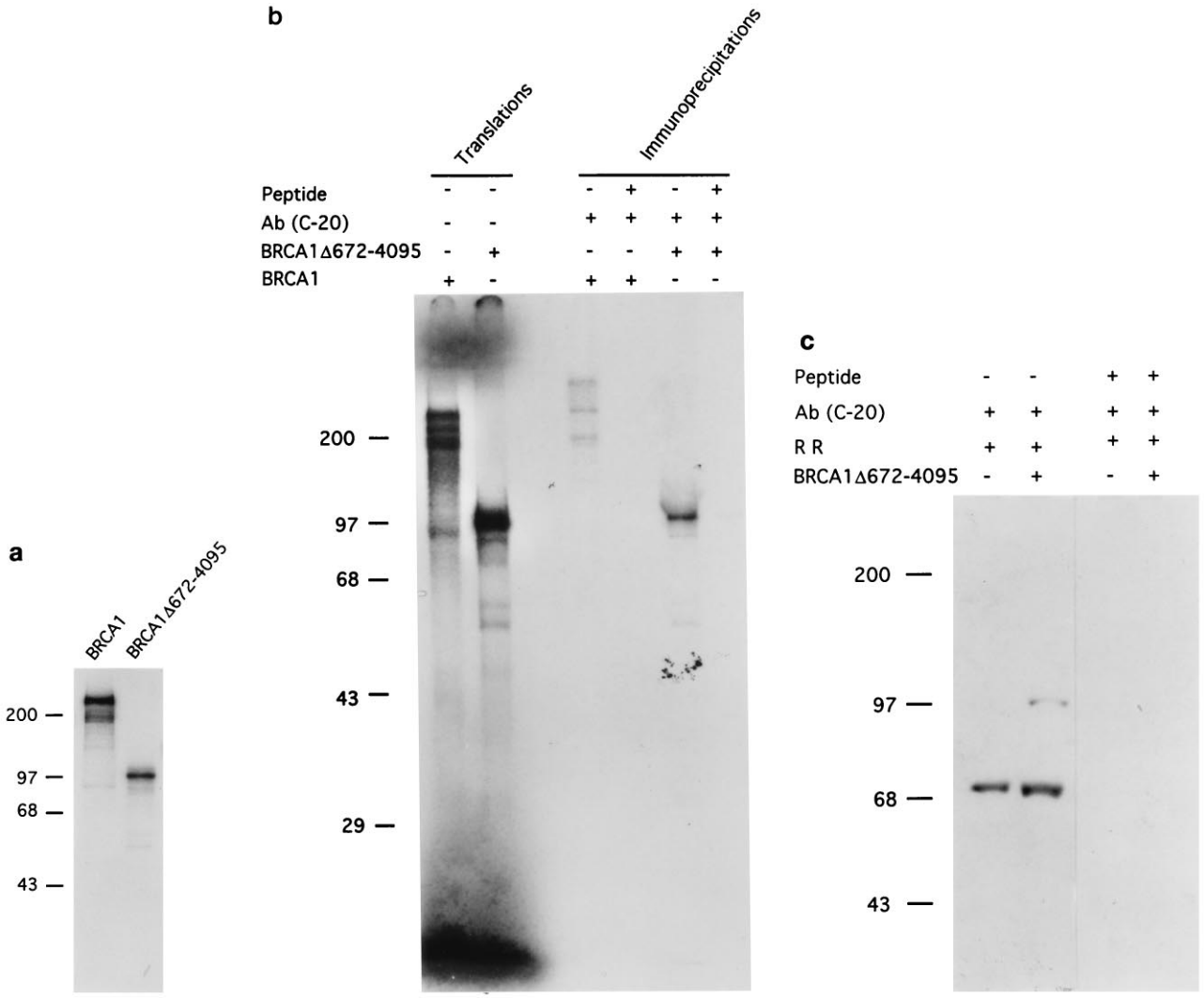

FIG. 2. In vitro expression of BRCA1 proteins. (a) In vitro-translated proteins of BRCA1 and BRCA1Δ672-4095. Molecular mass standards (in kilodaltons) are indicated on the left. (b) Immunoprecipitation of the translated products and peptide competition with C-20 antibody (Ab) and C-20P peptide (Santa Cruz Biotechnology Inc.). (c) Western blot (with C-20) of translated BRCA1Δ672-4095 (97 kDa) and competitions with C-20P peptide. RR, rabbit reticulocytes. Of note, while the appearance of a 68-kDa protein is inhibited by the presence of the C-20P peptide, this protein is present in rabbit reticulocyte lysate in the absence of an expression construct.

in Fig. 1a; BRCA1Δ672-4095 was amplified in its entirety by utilizing the proximal and distal primers (Fig. 1b). The *BRCA1* cDNA is 5,592 bp in size, and BRCA1Δ672-4095 is 2,163 bp in size. Both pCR3-BRCA1 and pCR3-BRCA1Δ672-4095 were sequenced fully in both directions. Two missense mutations in the coding region of pCR3-BRCA1 (presumably generated as PCR artifacts) were repaired by site-directed mutagenesis with the Altered Sites II *In Vitro* Mutagenesis Systems (Promega). The sequence of pCR3-BRCA1Δ672-4095 contained three base substitutions as compared to the reported wild-type *BRCA1* (GenBank accession number U14680), also probably representing PCR-generated artifacts, as none of these changes have been reported as disease-associated mutations or polymorphisms (Breast Cancer Information Core on-line database). The sequence variants in BRCA1Δ672-4095 were repaired by substituting appropriate restriction fragments from wild-type *BRCA1* as noted in Materials and Methods. The deduced amino acid sequences of BRCA1 and BRCA1Δ672-4095 predict the expression of 208- and 81-kDa proteins, respectively, and the in vitro-translated proteins were electrophoresed with mobilities of 220 and 97 kDa (Fig. 2a). Translated BRCA1 products were immunoprecipitated by the C-20 antibody (Santa Cruz Biotechnology Inc.) (Fig. 2b). The C-20 antibody also recognized the in vitro-translated BRCA1Δ672-4095 in a Western blot assay (Fig. 2c), and the 97-kDa protein band was abrogated by specific peptide competition. A 68-kDa band also abrogated by peptide competition was present in rabbit reticulocyte lysate in the absence of pCR3-BRCA1Δ672-4095.

Subcellular localization studies. To study the subcellular localization of BRCA1 and BRCA1Δ672-4095, fibroblast transfections and immunofluorescence studies were performed. NIH 3T3 and COS-7 cells transfected with pCR3-BRCA1 showed nuclear staining (Fig. 3a). In contrast, cells transfected with pCR3-BRCA1Δ672-4095 showed cytoplasmic staining (Fig. 3b). The cytoplasmic localization of BRCA1Δ672-4095 suggested the presence of an NLS within exon 11.

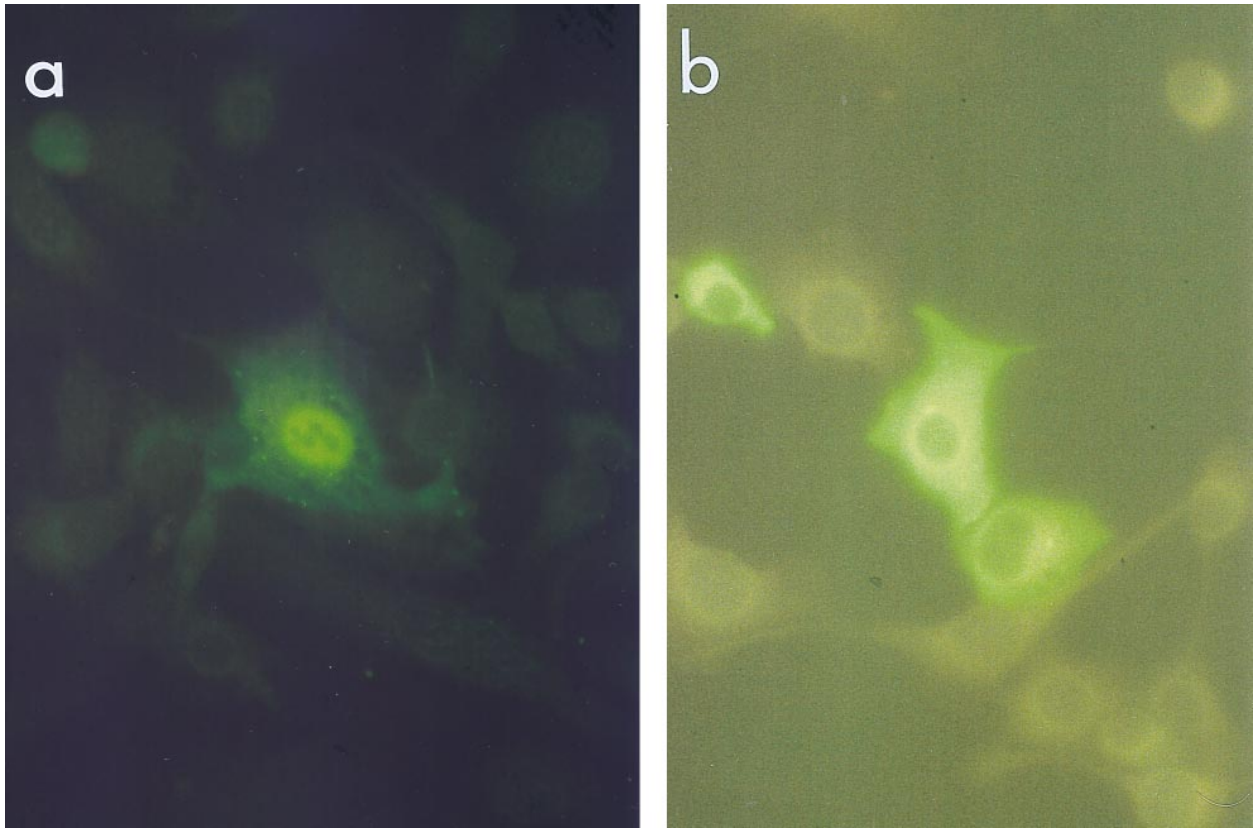

FIG. 3. Subcellular localization studies of NIH 3T3 cells transfected with pCR3-BRCA1 and pCR3-BRCA1Δ672-4095. (a) BRCA1 localizes in the nuclei of NIH 3T3 cells following transient transfection, as detected by immunofluoresence with C-20 antibody. (b) BRCA1Δ672-4095 is localized in the cytoplasm of NIH 3T3 cells following transient transfection, as detected by immunofluoresence with C-20 antibody. The data shown are representative of 10 transient transfections of each construct, in both NIH 3T3 and COS-7 cells, and with C-20, D-20, MS110, MS13, Ap13, and SG11. A minimum of 100 cells were scored in total. Magnification, ×187.5.

Identification of the NLS. Analysis of the amino acid sequence of BRCA1 indicated two potential NLSs: amino acids 501 to 507 (NLS1; KCKRKRR) and 607 to 614 (NLS2; KKN RLRRK) (4). In order to determine whether either of these sequences was functional, two deletion mutants that lacked either NLS1 or NLS2 were constructed. Transfection of fibroblasts with expression vectors containing these deletion mutants demonstrated that NLS1, but not NLS2, was essential for nuclear localization, as the encoded protein missing NLS1 was identified in the cytoplasm (Fig. 4a). In contrast, deletion of NLS2 did not disrupt the nuclear localization of the encoded protein (Fig. 4b).

Expression of multiple, tissue-specific *BRCA1* transcripts. To determine the expression pattern of BRCA1Δ672-4095 mRNA in human tissues, Northern analysis of tissue mRNAs was performed with the BRCA1Δ672-4095 probe, and multiple mRNA species were detected (Fig. 5a). An 8-kb mRNA, presumed to be the full-length *BRCA1* transcript, was detected at highest levels in testis. Low levels of this transcript also were detected in small intestine, ovary, thymus, spleen, skeletal muscle, placenta, and heart when the full-length *BRCA1* probe was used, but they were undetectable with the BRCA1Δ672-4095 probe, probably because of the absence of exon 11 (3.5 kb) in that probe and the low level of transcript present in these tissues (21). A 4.6-kb transcript was detected with the BRCA1Δ672-4095 probe in every tissue examined except colon and peripheral blood lymphocytes. Additional splice variants were also detected by the Northern analysis. A 7-kb RNA was present in skeletal muscle, as were smaller transcripts varying from 1.5 to 2.2 kb. In particular, a single species of about 2 kb in small intestine and a doublet in liver at 1.8 kb are more highly expressed than other transcripts. In heart, signals were detected at 4.6, 3, 2, and 1 kb. These transcripts may lack part or all of exon 11, as they were not detected by previously published Northern analyses, which were done primarily with probes derived from exon 11 (21). The 7-kb transcript is likely to retain exon 11 and may have other exons spliced out.

Based on the sizes of the BRCA1Δ672-4095 coding region (2.1 kb) and the *BRCA1* 3′ untranslated region (2.5 kb), we hypothesize that the 4.6-kb band corresponds to BRCA1Δ672-4095. RT-PCR assays confirmed the presence of BRCA1Δ672-4095 RNA in a number of tissues (Fig. 5b). The primers utilized in this amplification were derived from exon 6 (nt 215 to 239) and exon 12 (nt 4132 to 4152) and were expected to amplify a product of 490 bp derived from BRCA1Δ672-4095. A product of this size was amplified in most tissues, including breast tissue, but was not observed in pancreas and liver. A 607-bp band also was detected in all tissues except ovary and thyroid tissues. Sequence analysis of the amplified fragments indicated that the 490-bp fragment is derived from BRCA1Δ672-4095. The 607-bp fragment, designated BRCA1Δ789-4098, contains the first 120 bp of exon 11, lacks the remainder of exon 11, and lacks the first codon of exon 12.

In order to determine whether BRCA1 isoforms derived from BRCA1Δ672-4095 and/or BRCA1Δ789-4098 were present in tissue, a combination immunoprecipitation-Western blotting was performed. A mixture of three antibodies that

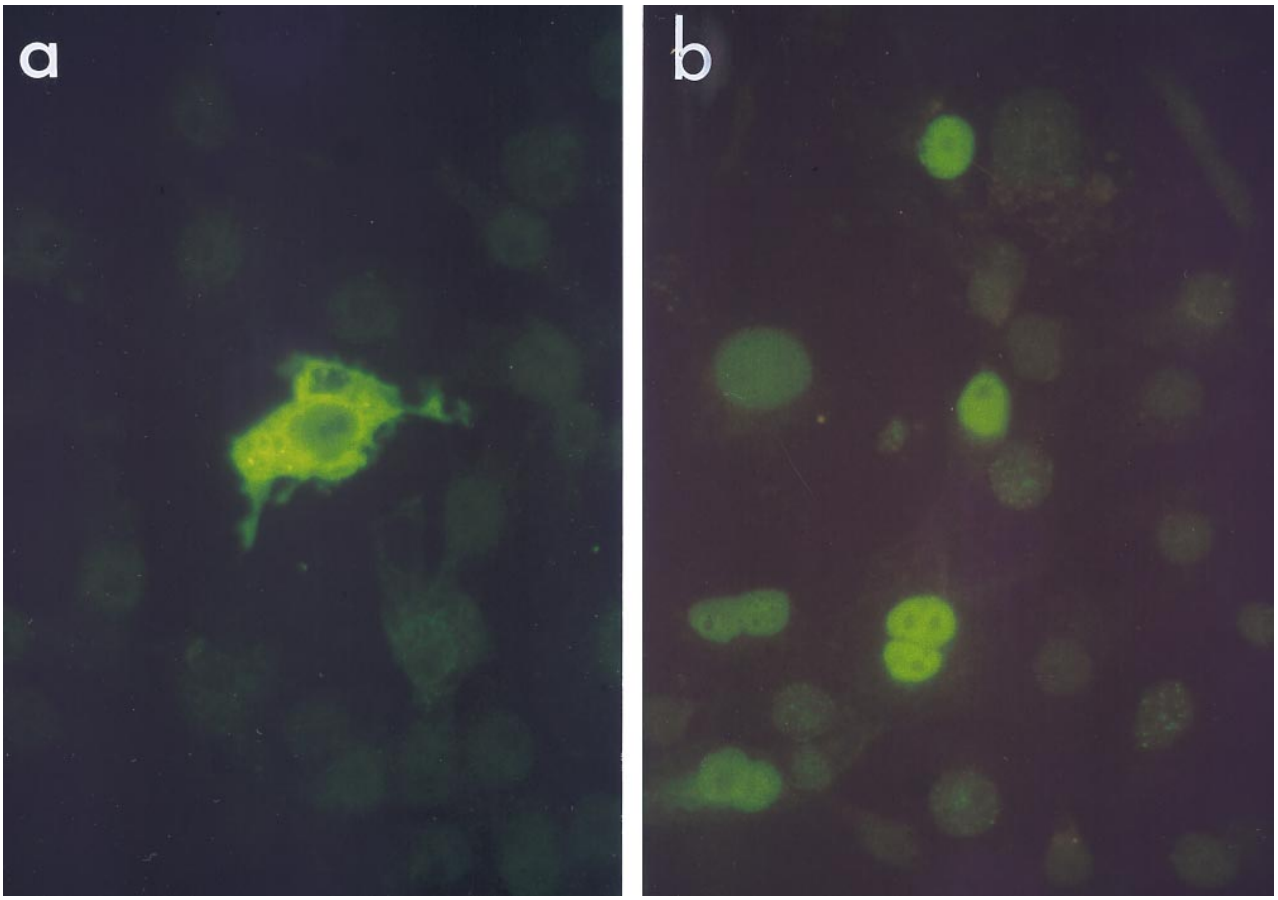

FIG. 4. Identification of the NLS. Transient transfection and immunofluorescence, with C-20 antibody, of pCR3-BRCA1 deletion mutants lacking NLS1 (a) and NLS2 (b) in NIH 3T3 cells are shown. The data shown are representative of five transient transfections of each construct, in both NIH 3T3 and COS-7 cells, and with C-20 (NLS1 and NLS2) and D-20 (NLS1). A minimum of 100 cells were scored in total. Magnification, ×62.5.

recognize the N terminus of BRCA1 was used for immunoprecipitation of cell extracts prepared from the breast epithelial cell line HBL-100. The immunoprecipitates were transferred to nitrocellulose and probed with the C-terminal BRCA1 antibody C-20 (Fig. 6). By using this approach, three protein species were detected with apparent molecular masses of 220, 100, and 97 kDa (p220, p100, and p97, respectively). These isoforms are consistent with the full-length BRCA1 protein (p220), BRCA1Δ672-4095 (p97), and BRCA1Δ789-4098 (p100). This combination of antibodies would be expected to recognize only proteins with both the N and C termini intact, excluding cleavage products of the full-length protein and any variant BRCA1 isoforms generated by splicing which deletes either end of the protein, adding further weight to the hypothesis that the proteins detected by this approach represent the predicted BRCA1 isoforms lacking exon 11 and therefore lacking the NLS.

DISCUSSION

BRCA1 is a gene which is clearly important in the pathogenesis of at least a portion of breast cancers; however, nothing is known about the regulation of *BRCA1* expression or the function of its product. Because (i) the production of multiple alternately spliced RNAs from a single gene is a frequently utilized means of increasing regulatory versatility and allows for cell-type-specific gene regulation and (ii) BRCA1 appears to produce multiple splice variants, we began characterizing several of the alternately spliced *BRCA1* RNAs that either were abundant as detected by Northern blotting or exhibited a tissue-specific pattern of expression. We have isolated and sequenced two such variants. The first of these variants, BRCA1Δ672-4095, is generated by splice removal of exon 11, a large, 3.5-kb exon that makes up approximately 60% of the coding region of *BRCA1*. As an initial step in characterizing BRCA1Δ672-4095, we determined the subcellular location of BRCA1Δ672-4095 and found that in contrast to the full-length BRCA1, which is found primarily in the nucleus, BRCA1Δ672-4095 is found in the cytoplasm. On examination of the *BRCA1* sequence, two putative NLSs were identified, both in exon 11. We sought to determine whether either or both of these sequences were necessary for nuclear localization, using deletion mutants lacking one or the other of these signals and subsequent transfection of these constructs into fibroblasts. The results of the experiments indicate that only one of the two putative NLSs appears to be required for nuclear transport of BRCA1 and that the splicing which removes exon 11 from BRCA1Δ672-4095 renders this isoform cytoplasmic. There is widespread speculation that BRCA1 may be a transcription factor, based on the presence of the RING finger motif at the 5′ end found in a family of zinc-binding proteins with direct or indirect DNA interactions: this family includes the product of the *RET* proto-oncogene, which is responsible for familial medullary thyroid carcinoma; the product of the recombinase-

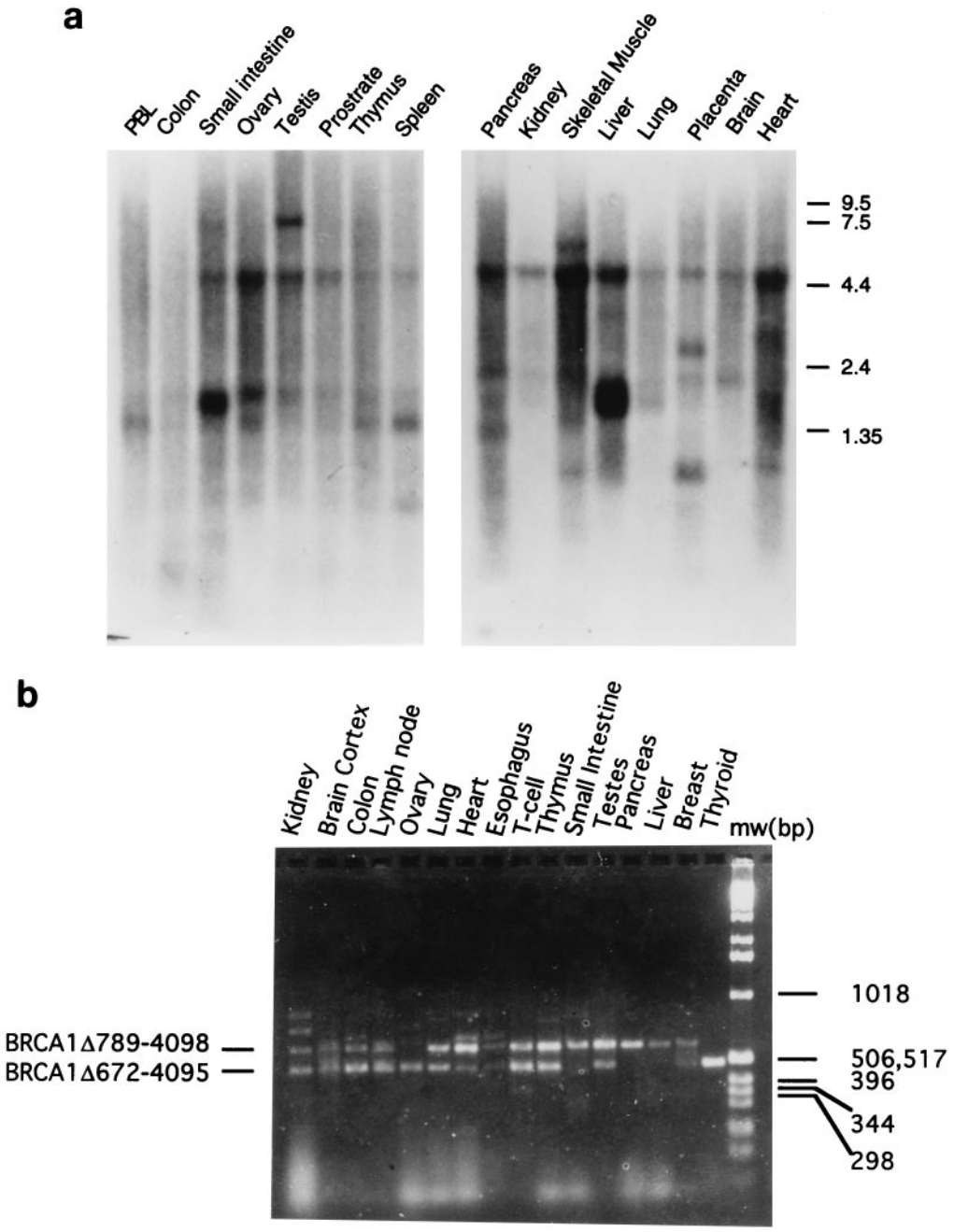

FIG. 5. Tissue-specific expression of multiple forms of BRCA1 mRNA. (a) Northern analysis of tissue mRNAs hybridized to BRCA1Δ672-4095 probe. Molecular size standards (in kilobase pairs) and tissues of origin are indicated. PBL, peripheral blood lymphocytes. (b) RT-PCR of total RNAs from indicated the tissues with primers from exons 6 and 12. These primers are expected to produce a product of 490 bp from BRCA1Δ672-4095. The expected PCR product derived from BRCA1Δ672-4095 at 490 bp is marked. A PCR product of the expected size (607 bp) for BRCA1Δ789-4098 also is marked. BRCA1Δ789-4098 also lacks most of exon 11 but is produced by utilization of a cryptic splice junction 120 bp into exon 11. Molecular size standards are indicated.

activating gene *RAG1*, which is critical for immunoglobulin gene rearrangement; and *Xenopus* nuclear factor 7 (Xnf7), a putative transcription factor expressed during development at the midblastula transition (11). If BRCA1 does have a critical function in the nucleus, splicing which alters the subcellular localization may represent an important regulatory mechanism for separating BRCA1 from its substrate at appropriate times during development and/or phases of the cell cycle.

As noted above, splicing is an important regulatory mechanism, providing, for example, a means of limiting gene expression to a phase of the cell cycle, a tightly defined period of embryonic development, or a spatially defined location in an organism. In addition, splicing may represent a functional on-off switch analogous to direct transcriptional regulation by producing an unstable transcript or one that does not contain an open reading frame. Alternate splicing has been shown to play an important role in the regulation of genes important in development and differentiation, and examples of cell- and

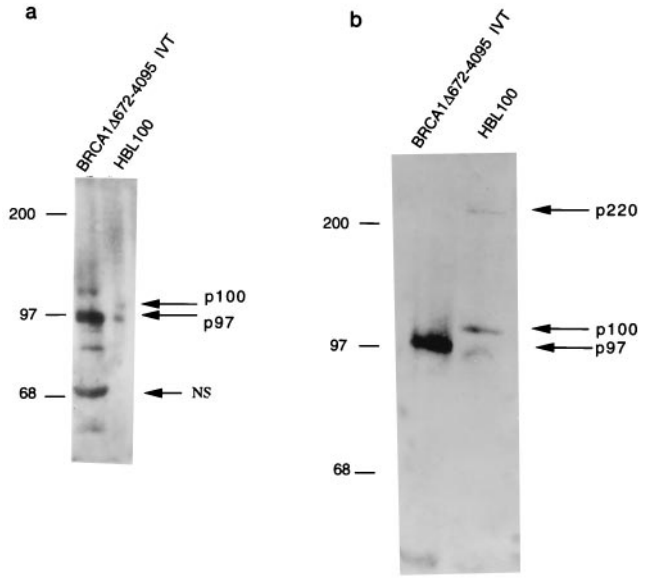

FIG. 6. In vivo expression of BRCA1 isoforms. Combination immunoprecipitation-Western blotting was used to detect BRCA1 isoforms in the breast epithelial cell line HBL-100. Numbers on the left of each panel are molecular masses in kilodaltons. (a) Three N-terminal antibodies (D-20, MS13, and MS110) were used for immunoprecipitation of 100 μg of cellular extract, and protein subsequently was detected by Western blotting with a C-terminal antibody (C-20). The in vitro-translated product (IVT) of pCR3-BRCA1Δ672-4095 is as indicated in the adjacent lane for size comparison. NS, protein species present in rabbit reticulocyte lysate mock translations (not thought to be related to BRCA1). With this concentration of cellular extract, only p97 (BRCA1Δ672-4095) and p100 (BRCA1Δ789-4098) are detectable. (b) A fourfold larger amount of HBL-100 cellular extract (400 μg) was subjected to the same conditions with the exception of the deletion of one of the three N-terminal antibodies (D-20) used for immunoprecipitation. The in vitro-translated product (IVT) of pCR3-BRCA1Δ672-4095 again is included for size comparison and also was subjected to immunoprecipitation in a manner identical to the cellular extract. p220 (the full-length product of *BRCA1*) is now clearly visible. The band designated NS in panel a is no longer present in the in vitro translation after immunoprecipitation and blotting with BRCA1 antibodies.

tissue-specific splicing abound; previous studies have suggested a role for BRCA1 function in both of these pathways (20).

As with splicing, there are examples of subcellular compartmentalization as a regulatory mechanism. Rexach and Blobel have defined a complex of proteins in the cytosol that recognize a core NLS and allow binding of the relevant protein to the transporter complex, followed by dissociation of the complex on the interior periphery of the nucleus (24). Presumably, an NLS may occur anywhere in a polypeptide but must be accessible to the nuclear transporter complex. Comparisons of the NLSs of a majority of proteins demonstrate that they consist of four or five arginine and lysine residues within a hexapeptide motif (4). BRCA1 NLS1 (KLKRKRR) and NLS2 (KKNRLRRK) both have six basic residues. As noted above, only NLS1 appears to be necessary for nuclear transport. The strongest core NLS appears to be a contiguous stretch of five to nine arginines and lysines, for example, in simian virus 40 large T antigen (18), adenovirus 5 DBP (22), and the c-abl (IV) gene product in mouse (29). The NLS1 of BRCA1, which contains five arginines and lysine in tandem, could be classified as a strong core signal. In our hands, the deletion of NLS2 does not disrupt the nuclear localization of BRCA1. As noted, NLS2 has a leucine residue and an asparagine residue interrupting the contiguous stretch of arginines and lysines. Previous studies also have identified a nonfunctional putative NLS with four arginines and lysines (15). Nevertheless, the region encompassing the BRCA1 putative NLS2 is evolutionarily conserved at all positions in murine BRCA1 (1, 3, 26), suggesting a functional role for this domain. We have not tested the hypothesis that NLS2 enhances nuclear transport.

Given the current controversy surrounding the subcellular localization of BRCA1, the importance of the cell types used in the study of cellular localization cannot be discounted. Slavicek and coworkers (27) demonstrated that the karyophilic sequence from adenovirus E1A proteins used for nuclear localization experiments functioned in *Xenopus* oocytes but not in somatic cells. We provide data only for subcellular localization in fibroblasts.

While much of the discussion of the role of BRCA1 regulation by splicing and/or nuclear localization is speculative, examples where these mechanisms are functionally important for genes with potentially similar functions support the idea that further study of the mechanisms may yield important clues to the factors that influence BRCA1 expression and, by extension, the repression of the malignant phenotype in the breast epithelium. In addition, the significant number of "regulatory" mutations in *BRCA1*-linked families that result in allele-specific loss of *BRCA1* transcript as the germ line defect (believed to account for 10 to 15% of all *BRCA1* mutations) may be explained by exploring mechanisms of aberrant splicing that results in unstable transcripts. Finally, study of the regulation of splicing of BRCA1 may yield data that are applicable to other tumor suppressor genes.

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