Recently, we have described a high level of homology between *BRCA1/NBR2* and *pseudoBRCA1/NBR1(1A)* promoters with highly conserved putative regulatory elements (Dimitrov et al., 2001). Since the intergenic sequence between *BRCA1* and *NBR2* is known to act as a bi-directional promoter (Xu et al., 1997b; Suen and Goss, 1999), and our previous observation indicated coordinated expression of *Brca1* and *Nbr(1a)* during mouse spermatogenesis, we decided to ask about a possible relationship between the expression of both genes in the cell lines and primary epithelial cell cultures derived from the breast cancer and from the normal breast tissue. Furthermore, we report here *in silico* identification of two novel protein domains in the NBR1 protein sequence, implying a role of the gene in the cellular ubiquitin system. The possible involvement of both *NBR1* and *BRCA1* in the ubiquitin pathway suggests that the genes might be functionally connected.

Material and Methods

Cells

HBL-100, MCF7 and T47D cell lines were purchased from Interlab Cell Lines Collection (ICLC, Genova, Italy), and MDA-MB-231 and SK-BR-3 cells were a kind gift from Dr. Jan Kovář. MCF7, T47D and MDA-MB-231 cells were grown in DMEM medium and HBL-100, and SK-BR-3 cells were grown in McCoy's 5A medium, all supplemented with 10% foetal bovine serum (GibcoBRL-Life Technologies Ltd., Paisley, UK), 2mM L-glutamine (GibcoBRL-Life Technologies) and antibiotics in 5% CO_2 at 37°C.

Primary mammary epithelial cells from normal (NME cells) and tumour tissue (BT15, BT18, BT19, BT27, BT101, BT117, MBC1 and MBC3 cells) were cultured on the feeder layer of irradiated 3T3 cells as described previously (Matoušková et al., 1998; Matoušková et al., 2000). All malignant cell cultures were derived from ductal infiltrating carcinomas, except BT18 cells, which were obtained from an atypical medullar infiltrating carcinoma, and MBC1 and MBC3 cells, derived from cutaneous metastases of ductal infiltrating carcinomas. The details concerning the origin and characteristics of the primary cell cultures will be described elsewhere (Krásná and co-workers, in preparation).

Isolation of RNA and semi-quantitative RT-PCR

Total RNA was isolated using the guanidine-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) from exponentially growing cells. The semi-quantitative RT-PCR assay was carried out as described by Gause and Adamovicz (Gause and Adamovicz, 1995) with modifications. Three μg of total RNA were reverse transcribed in the presence of 1x reverse-transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) (GibcoBRL-Life Technologies), 1 mM each deoxynucleotide triphosphate (MBI, Fermentas GmbH, St. Leon-Rot, Germany), 50 pmol of Oligo$(dT)_{22}$ primer and 200 U of M-MLV reverse transcriptase (GibcoBRL-Life Technologies) in a 25-μl final volume. The reaction mixture was incubated 10 min at 37°C, 40 min at 42°C, heated to 75°C for 15 min, cooled on ice for 10 min and stored at –20°C until PCR amplification. The following pairs of primers for PCR were used: HBR16F (5′ GCAATGGAAGAAAGTGTGAGC) and HBR22R (5′ GCCAAGGGTGAATGATGAAAG) for *BRCA1*, HNB187F (5′ CCCATTACCCCACTCTTTCC) and HNB5R (5′ ATGGTGCCCTTCGTGGACTTG) for *NBR1(1A)*, mNb1bF (5′ CGCGAACGGTCCTTGCACT) and HNB8R (5′ TCAGAGGAGCCCACAACAGGTC) for *NBR1(1B)*, HNB187F and HNB23R (5′ TCTACCTCCTTTCCACATTCCA) for *NBR2*, ACTF 5′ (CCTCGCCTTTGCCGATCC) and ACTR (5′ GGATCTTCATGAGGTAGTCTGTC) for *β-ACTIN*, and GF (5′ ACCACAGTCCATGCCATCAC) with GR (5′ TCCACCACCCTGTTGCTGTA) for *GAPDH*. The number of cycles required for linearity of amplification was individually determined for each pair of primers in order to determine the exponential phase of each particular reaction. PCR was performed in the PTC-225 system (MJ Research, Inc., Waltham, MA) in the presence of 1x PCR buffer (75 mM Tris-HCl, pH 8.8, at 25°C, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20) (MBI Fermentas), 1.5 mM $MgCl_2$ (MBI Fermentas), 0.1 μM primers, 200 μM dNTPs, and 1 U of Taq DNA Polymerase (MBI Fermentas) in a 50-μl final volume with incubation times as follows: 1 min initial denaturation at 95°C, 30 s denaturation at 94°C, 45 s annealing and 30 s extension at 72°C. The annealing temperatures and the numbers of cycles for the particular genes were: 57°/27 cycles for *BRCA1*, 55°/35 cycles for *NBR1(1A)*, 50°/33 cycles for *NBR1(1B)*, 60°/29 cycles for *NBR2*, 60°/25 cycles for *β-ACTIN* and 60°/21 cycles for *GAPDH*. The PCR products were separated by electrophoresis on 2% agarose gel. Quantification was performed densitometrically using the GeneSnap and GeneTools software (Syngene, Synoptics Ltd., Frederick, MD). Data were normalized to the levels of *GAPDH* and *β-ACTIN* and analyzed by the GraphPad PRISM software (GraphPad Software, Inc., San Diego, CA).

Results

Domain organization of the NBR1 protein

The search for conserved protein domains in the predicted protein sequence of NBR1, using the recent version of the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de) (Schultz et al., 2000), resulted in the identification of two new motifs. The previously reported zinc finger positioned between amino acids 211 and 256, determined as a

member of the B-box family of zinc fingers, showed highest similarity with the ZZ class of zinc fingers. This domain was followed by the described coiled-coil domain between amino acids 288–329. A well conserved octicosapeptide repeat (OPR) was identified between amino acids 43–72 at the amino-terminus of the protein. Both ZZ zinc finger and the OPR motif are thought to be involved in protein-protein interactions (Chang et al., 1994; Ponting et al., 1996). A less conserved ubiquitin-associated domain (UBA) was predicted at the carboxy-terminus of NBR1 between amino acids 917 and 956. This motif is present in a wide variety of different proteins involved in the cellular ubiquitin pathway (Hofmann and Bucher, 1996). The expected location of the UBA domain within the Nbr1 polypeptide strongly suggests that it could be functional. Moreover, two additional proteins were identified with the same domain structure as the Nbr1: the *Drosophila* ref(2)P protein, involved in spermatogenesis and in the multiplication of sigma rhabdovirus in flies, and the mouse, rat and human homologues of p62 protein, participating in multiple signal-transduction pathways and in the ubiquitin system in the cell. All three proteins possess OPR, ZZ zinc finger and UBA domains in the same conserved spatial organization (Fig. 1b). These findings indicate that Nbr1, p62 and ref(2)P might be the founder members of a novel protein family, conserved between different species.

NBR1(1A) splice variants are expressed in normal and breast cancer cell lines

The *Nbr1/NBR1(1A)* splicing variant has been observed so far only in the mouse and human testis (Dimitrov et al., 2001) and in a myeloblast cell line

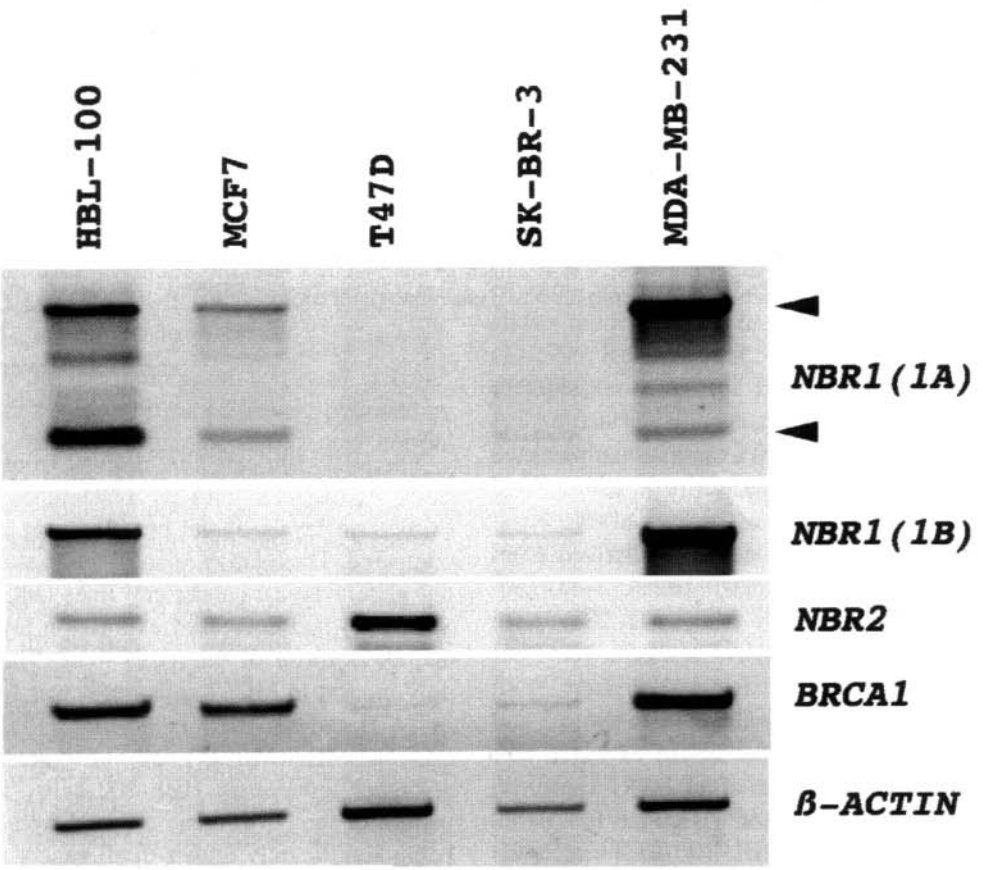

Fig. 2. mRNA levels of *BRCA1, NBR1(1A), NBR1(1B), NBR2* and *β-ACTIN* genes evaluated by semi-quantitative RT-PCR. Three μg of total RNA were used from the permanent human breast cancer cell lines MCF7, T47D, SK-BR-3 and MDA-MB-231 and the normal breast-derived HBL-100 cell line. The arrowheads indicate two variants of exon 1A of *NBR1* transcripts – the 131 bp long and the 463 bp full-length variant. The PCR amplification products were separated on 2% agarose gel.