Structural Determinants of *BRCA1* Translational Regulation*

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The *BRCA1* gene is involved in sporadic breast and ovarian cancer mainly through reduced expression. *BRCA1* mRNAs containing different leader sequences show different patterns of expression. In a normal mammary gland mRNA with a shorter leader sequence, 5'-UTRa is expressed only, whereas in breast cancer tissue mRNA with a longer leader, 5'-UTRb is expressed also. We show that the translation efficiency of transcripts containing 5'-UTRb is 10 times lower than those containing 5'-UTRa. The structures of 5'-UTRa and 5'-UTRb were determined by chemical and enzymatic probing aided by a new method developed for monitoring the number of co-existing stable conformers. Specific factors responsible for reduced translation of mRNA containing 5'-UTRb were determined using a variety of transcripts with mutations in the leader sequence. These factors include a stable secondary structure formed by truncated Alu element and upstream AUG codons. The novel mechanism by which *BRCA1* may be involved in sporadic breast and ovarian cancer is proposed. It is based on the expression patterns of *BRCA1* mRNAs and differences in their translatability. According to this mechanism the deregulation of the *BRCA1* transcription in cancer, resulting in a higher proportion of translationally inhibited transcripts containing 5'-UTRb, contributes to the decrease in the *BRCA1* protein observed in sporadic breast and ovarian cancers.

Breast cancer is one of the leading causes of death among women, and the lifetime risk of developing this malignancy has reached 10% in many countries in the Western world. The great majority of breast cancer is sporadic, and only a small proportion has been attributed to germ line mutations in predisposing genes (1). *BRCA1* was the first major breast/ovarian cancer susceptibility gene identified (2). Interestingly, three additional transcripts with different 5'-UTRb containing exon 1b. These uORFs may lower the efficiency of the *BRCA1* protein synthesis and contribute to putative translational regulation of the *BRCA1* expression (21).

In this study, we have shown that *BRCA1* is indeed translationally regulated, and we provide evidence that stable structures present in a longer mRNA leader, and to a lesser extent the uORFs, are factors involved in this regulation. This has led us to the hypothesis that the deregulation of the *BRCA1* transcription in cancer, resulting in a higher proportion of translationally inhibited transcripts containing exon 1b, contributes to
### EXPERIMENTAL PROCEDURES

**RT-PCR Analysis of BRCA1 mRNA Expression**—Total RNA was extracted from human tissues using standard procedures (23). To prepare cDNA from total RNA, random hexamers and avian myeloblastosis virus-reverse transcriptase (Promega) were used according to the manufacturer’s recommendations. RT-PCR was performed in a 20-μl reaction containing a cDNA template, 1 μM of each primers F and R, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 200 μM of each of the dNTPs, and 0.5 units of Taq DNA polymerase (Promega) under the following conditions: 95 °C for 3 min; 35 cycles, 94 °C for 1 s, 55–62 °C for 1 s, and 72 °C for 20 s (see Table I for primer sequences and fragment-specific annealing temperatures). Expression studies were performed using the Quick Screen cDNA from human testis and three cDNA libraries (all from CLONTECH), one from a normal human mammary gland and two from breast cancer tissue from different individuals. They confirmed the results obtained for cDNAs prepared from the corresponding tissues that were described earlier (21).

**Preparation of DNA Templates for in Vitro Transcription and Translation Experiments**—Two mRNAs were prepared that contained either exon 1a or exon 1b sequences in their 5'-UTRs fused with the entire luciferase coding sequence. The corresponding DNA templates were synthesized by ligating two phosphorylated PCR products: either luc and exon 1a or luc and exon 1b (Table I). The pGEM-luc vector and luciferase-specific primers Fluc and Rluc (Table I) were used to amplify the luciferase cDNA fragment. The 5'-terminal phosphates were introduced during PCR with phosphorylated primers (Fluc, Rex1a, or Rex1b). Ligation substrates were purified in polyacrylamide gel under nondenaturing conditions. DNA ligation reaction performed for 16 h at 4 °C contained 0.5 pmol of each PCR product, 10 units of T4 DNA ligase (Promega), 30 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 10 mM diethiothreitol, and 1 mM ATP. Ligation products served as templates to amplify two cDNA constructs ex1a-luc (primers Fex1a and Rluc) and ex1b-luc (primers Fex1b and Rluc) in the following PCR cycling conditions: 25 cycles, 94 °C for 1 s, 58 °C for 1 s, and 72 °C for 2 min. The PCR products that were used to prepare templates for in vitro transcription were probed by electrophoresis in 10% polyacrylamide-urea gels and were reamplified with DNA oligomers containing a T7 RNA polymerase promoter sequence (T7Fex1α or T7Fex1b) as forward primers and Rluc as a reverse primer.

Four **BRCA1** transcripts described in the legend to Fig. 7B were also prepared. The two gel-purified PCR products described earlier, ex1a-2 and ex1b-2, were used as templates in the amplification of T7ex2stop, T7ex1b-2stop, and T7ex1b(M4)-2stop DNA constructs, respectively. Two other purified amplification products ex1a-11 and ex1b-11 were used for synthesis of longer templates for in vitro transcription, T7ex1a-11 and T7ex1b-11. The PCR conditions used were as described under "RT-PCR Analysis of BRCA1 mRNA Expression" and are shown in Table I.

Next four **BRCA1**-specific transcripts were prepared from DNA templates T7ex1b-11-mut1AUG, T7ex1b-11-mut2AUG, T7ex1b-11-mut3AUG, and T7ex1b-11-mut1–3AUG, which contained mutations either in the individual or in all three AUG codons of uORFs (Fig. 7E). These templates were generated in two steps from a purified amplification product ex1b-11, by a modification of the overlap extension PCR mutagenesis protocol (24). In the first step two different types of products, with partially overlapping sequences, were obtained. The first type of PCR product was amplified using Fex1b primer and one of the mutagenic primers as follows: Rmut1AUG, 5'-CAGAGGGATACGCTCGAGGAGCAGAAGCTTGCTGAGGTAGGACACAGAGTCTTCTGCTGAGGAGCTCGAGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTCTTCTGCTGAGGAGCTGAGGACACAGAGTCTTCTGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTCTTCTGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTCTTCTGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTCTTCTGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGCTGAGGACACAGAGTGCCGAGCTGAGGAGC...
the further selection of pure mutants. These two types of PAGE-purified PCR products (0.1 pmol each) served as both a template and as a primer in the second PCR step carried out under the following conditions: 5-min initial denaturation at 94 °C, and 10 cycles, 20 s at 94 °C and 2 min at 70 °C. 2 μl of this crude PCR product was used to prepare templates for in vitro transcription. The 20-μl amplification reaction was performed with primers T7Fex1b and R11.1 in the conditions described above.

The control transcript EMCV RNA was synthesized by in vitro transcription of a template generated by PCR from the pIRES-EGFP vector (CLONTECH). This transcript contains the 5'-UTR region of the EMCV virus and the enhanced GFP ORF encoding the 27-kDa polypeptide. The DNA sequences of all constructs and structures of their transcripts were determined.

In Vitro Translation—Twelve DNA constructs that are described above served as templates for the synthesis of 10 BRCA1 mRNA fragments as follows: Ex2-2top, Ex1a-2sttop, Ex1b-2sttop, Ex1b(M4)-2stop, Ex1a-11, Ex1b-11, Ex1b-11-mut1AUG, Ex1b-11-mut2AUG, Ex1b-11-mut3AUG, Ex1b-11-mut1-3AUG and two chimeric BRCA1-luciferase transcripts, Ex1a-luc and Ex1b-luc. A transcription reaction carried out into a 50-μl volume contained 1–5 pmol of DNA template, 400 units of T7 RNA polymerase, 0.05 mM GTP, 0.5 mM ATP, CTP, UTP, 0.5 mM m7GpppG cap analog and 50 units of RNasin, in a 1× T7 polymerase buffer (Promega) supplemented with 10 mM dithiothreitol. Uncapped transcripts were synthesized using a 0.5 mM concentration of GTP instead of cap analog. Incubation was at 37 °C for 1 h. The RNA products were purified in denaturing 6% polyacrylamide gels. Transcripts encoding short BRCA1 peptides (5 pmol), the longer BRCA1 transcripts Ex1a-11, Ex1b-11 (2 pmol), and chimeric BRCA1-luciferase mRNAs (2 pmol) were used as templates for an in vitro translation reaction carried out in a 50-μl volume, containing 35 μl of rabbit reticulocyte lysate (RLR) (Promega), 2 μCi of [35S]methionine (1200 Ci/mmol; ICN), and other amino acids at a 20 μM concentration. Translation reactions were performed at 30 °C for 1 h. The luciferase-encoding chimeric mRNAs were also used for in vitro translation in wheat germ extract (Promega) according to the manufacturer's recommendations. All reactions were stopped by adding an SDS sample buffer and heating at 100 °C for 10 min. Translation products were analyzed in 10–18% Tris/glycine/SDS-polyacrylamide gels, and visualized by PhosphorImaging (Typhoon, Molecular Dynamics). Translation efficiencies were determined from measurements of [35S]methionine incorporated into a polypeptide.

Preparation of DNA Templates for the Synthesis of RNAs Used for Structure Probing—DNA templates for in vitro transcription were synthesized by PCR from purified amplification products, ex1a-2, ex1a-11, ex1b-2, and ex1b-11 (see “RT-PCR Analysis of BRCA1 mRNA Expression”), by using forward primers containing a T7 RNA polymerase promoter. PCR conditions used were as described above and shown in Table I. The in vitro transcription was performed as described above, with an exception that the cap analog was replaced by a 3 μM guanosine, and GTP was used at a concentration of 0.5 mM. The RNA products were purified and 5'-end-labeled with T4 polynucleotide kinase and [γ-32P]ATP (5000 Ci/mmol; ICN). All labeled transcripts were controlled for the number of stable conformers in which they exist by 6–10% polyacrylamide gel electrophoresis (acrylamide/ bisacrylamide, 29:1), performed under nondenaturing conditions (25).

Fluorescent Labeling of RNA at the 3'-End and Capillary Electrophoresis—The fluorescent labeling of in vitro transcripts at their 3'-end was achieved with 6-carboxyfluorescein derivative R110 of dUTP and terminal deoxynucleotidyltransferase. Transcripts were extended by a single [R110]dUTP residue only. The reaction mixture contained 50 pmol of transcript, 10 pmol of [R110]dUTP, 25 units of terminal deoxynucleotidyltransferase (Promega), 20 units of RNasin, 100 mM cacydlate buffer (pH 6.8), 0.5 mM CoCl2, and 0.1 mM dithiothreitol. The reaction was performed at 37 °C for 30 min. The labeled transcripts were purified in polyacrylamide gel, dissolved in a 50 μl buffer contain-
and under nondenaturing conditions (5% GeneScan polymer, 45 mM Tris borate buffer), using a standard capillary 42 cm long by 50 μm. The RNA samples were electroinjected, together with either the ROX-500 or TAMRA-500 internal standard, at 15 kV for 10 s, and electrophoresis was performed at 15 kV.

Lead Cleavages, Nuclease Digestions, and the Analysis of Reaction Products—Prior to structure probing reactions, the 32P-labeled transcripts were supplemented with an unlabeled RNA carrier to the final concentration 8 μM and were subjected to a denaturation and renaturation procedures in a solution containing 20 mM Tris-HCl (pH 7.2), 80 mM NaCl, 20 mM MgCl2, by heating the sample at 75 °C for 1 min and slowly cooling it to room temperature. Limited RNA hydrolysis was initiated by mixing 5 μl of the RNA sample described above with 5 μl of a probe sample containing lead ions, S1, T1, V1, or C3 nuclease in water at different concentrations, as described in the legends to figures. All reactions were performed at 20 °C for 20 min and stopped by adding an equal volume of stop solution (7.5 mM urea, 20 mM EDTA, and dyes) and sample freezing. The products of the RNA cleavages were separated in 6–12% polyacrylamide gels containing 7 M urea, 90 mM Tris borate buffer, and 2 mM EDTA, along with products of limited digestion of the same RNA with ribonuclease T1 in semidenaturing conditions and with an alkaline hydrolysis ladder prepared by incubating the labeled RNA in hot formamide (26). Electrophoresis performed at 1500 V was followed by autoradiography at ~80 °C with an intensifying screen.

RESULTS

BRCA1 mRNA Containing 5'-UTRb Is Expressed in Breast Cancer Tissue—PCR primers were designed to amplify sequences corresponding to BRCA1 exon 1a, exon 1b, exons 1a-2, and exons 1b-2 from cDNA (Fig. 1A). BRCA1 mRNA with both types of first exon fused to exon 2 was detected in total cDNA from normal human testis tissue (Fig. 1B). All of the PCR products that were obtained were of the length expected. Nucleotide sequencing (Fig. 1C) confirmed their identity with the corresponding fragment of the BRCA1 sequence from GenBank™ (accession number L78833). The BRCA1 transcripts expressed in a normal mammary gland were compared with those expressed in breast cancer tissue (Fig. 1D). It is apparent that BRCA1 mRNA containing the exon 1b sequence is expressed in breast cancer tissue but not in a normal mammary gland. On the other hand, BRCA1 mRNA containing exon 1a was present in both normal and cancer tissue. The corroborated results that have been reported by other authors (21) provided a rationale for the analysis of the translational abilities of these mRNAs.

Exon 1b Transcript Strongly Inhibits the Translation of a Downstream Sequence—In order to evaluate the influence of transcripts from BRCA1 exons 1a and 1b on translation efficiency, two constructs were prepared by fusing these exons with the coding sequence of the luciferase gene (Fig. 2A). The translatability of transcripts from these constructs was investigated in RRL and in wheat germ extract (WGE) (Fig. 2B). The expected 62-kDa translation product obtained from the transcript containing BRCA1 exon 1b was much less abundant than from the transcript with exon 1a. The difference was about 9 times in RRL and 10 times in WGE (Fig. 2C). Our further experiments were designed to determine the molecular basis of this large difference in translation efficiency. We began with the structure analysis of two different 5'-UTRb of the BRCA1 mRNA.

Structural Features of Ex1a Transcript Are Revealed by the Analysis of Truncated and Expanded Transcripts—The 121-nt exon 1a contributes 87% to the total length of BRCA1 5'-UTRb. To determine its secondary structure limited chemical cleavages and enzymic digestions of the transcript were performed under conditions preserving the natural folding of the RNA. The 5'-end-labeled transcript Ex1a was treated with lead ions that cleave the flexible single-stranded regions (27, 28) and with nucleases T1 and C3 showing the specificity for G and C residues, respectively, within the single-stranded regions. Two
other nuclease were also used as follows: S1 and V1, digesting single- and double-stranded regions, respectively, but having no base specificity (29). Attempts to correlate the experimental data with any secondary structure predicted by the Mfold program (30) failed, suggesting the co-existence of two or more stable conformers. Thus, further structure analysis was aided by a newly developed method allowing for an assessment of the number of co-existing stable conformers. This method, which is based on capillary electrophoresis of transcripts fluorescently labeled at their 3'-end with terminal deoxynucleotidyltransferase, was found superior to traditional PAGE of RNA under denaturing conditions (Fig. 3A). A capillary electrophoresis was performed in a 6% polyacrylamide gel in denaturing conditions. RNA structure modules present in two conformers of Ex1a-2 are indicated. C, patterns of cleavages generated by S1 nuclease in the 5'-end-labeled: lane 1, Ex1a-11 and, lane 2, Ex1a-2. One extra concentration of S1 nuclease was used, S4, 4 units/μl, and other conditions are as described in the legend to Fig. 3D, except for electrophoresis in 6% gel. D, proposed secondary structures of two co-existing stable conformers of Ex1a-2. Structures distinguishing conformers I and II are shown in frames. Cleavages observed in this fragment of the structures, which is common to both conformers, are shown. A shaded circle shows the localization of the translation START codon. RNA structure motifs and modules present in Ex1a-2 are also indicated. Note that both ends of the Ex1a-2 form 7 bp, separated by the 3-nt internal loop. This interaction has no biological relevance, because three guanine residues involved were artificially introduced at the 5'-end of the transcript to facilitate efficient in vitro transcription.

focused on establishing the secondary structures of these conformers. A majority of lead cleavage and nuclelease digestion sites found in the Ex1a-102 nt transcript (Fig. 3D) were also present in the Ex1a (data not shown) and Ex1a-2 (Fig. 4B and C) transcripts. This suggested that the structure of the 5'-end domain of one of the stable conformers of Ex1a-2 (conformer I) (Fig. 4D) was identical to the structure of the Ex1a-102 nt transcript. On the other hand, there were also differences in the cleavage patterns of Ex1a-102nt and the corresponding part in the longer transcripts. These differences allowed us to propose the structure for conformer II (Fig. 4D) which accommodates well the reactivity of all the sites that are unreactive in conformer I.

The structure of 5'-domain spanning nucleotides C14 to A122 is different in conformers I and II. Three secondary structure modules are present in this part of conformer I. The most stable is module M2-Ia (ΔG = −41.5 kcal/mol), composed of 70 nucleotides, and which contains two relatively long helical regions (Fig. 4D). The secondary structure of the corresponding fragment of conformer II contains only two such modules. The most stable module M2-IIa, composed of 80 nucleotides, has a calculated ΔG = −43.5 kcal/mol. Both stable conformers of Ex1a-2 have the same secondary structure within its 3'-half, which is absent in the two shorter molecules Ex1a-102nt and Ex1a. The structure includes a short hairpin M4a (ΔG = −4 kcal/mol) and a more stable structure module M5a (ΔG = −25 kcal/mol). The M5a module consists of 61 nucleotides, G146 to U207. G146, which is part of the BRCA1 AUG initiation codon,
The translation efficiency of capped Ex1a-11 and capped Ex1b-11 was much higher than that of the uncapped RNA in the whole transcript concentration range studied. This result left very little room for other mechanisms for initiating translation of the EMCV transcript. As shown in Fig. 6A, the translation efficiency of the uncapped RNA in the whole transcript concentration range studied. This result left very little room for other than cap-dependent mechanism of initiation to occur. On the contrary, the in vitro translation of the EMCV transcript was indeed cap-independent, which indicated that its initiation could take place at the IRES.

The Translation of BRCA1 mRNAs Is Cap-dependent—Two transcripts Ex1a-11 and Ex1b-11 were prepared and contained the entire BRCA1 5'-UTRa and 5'-UTRb, respectively, and a large fragment of the BRCA1 ORF ending with part of exon 11. They were used in two types of experiments. The first type was designed to compare the translation efficiency of transcripts having or not having the cap structure at their 5'-end (31). The EMCV transcript known to be translated in a cap-independent but IRES-dependent process (32, 33) was used as a negative control. As shown in Fig. 6A, the translation efficiency of the uncapped RNA in the whole transcript concentration range studied. This result left very little room for other than cap-dependent mechanism of initiation to occur. On the contrary, the in vitro translation of the EMCV transcript was indeed cap-independent, which indicated that its initiation could take place at the IRES.

The Secondary Structure Present in Ex1b Is More Stable—Exon 1b contributes 95% to the nucleotide sequence of BRCA1 5'-UTRb (Fig. 1A), and its transcript as revealed by both PAGE and CE has a homogeneous structure (data not shown). Structure probing was performed for the 379-nt-long Ex1b transcript (data not shown) as well as for the Ex1b-2 transcript containing the exon 1b sequence plus 99 nt of exon 2 (Fig. 5A). The results revealed that the structure of Ex1b and the corresponding portion of Ex1b-2 were identical and consisted of four modules (Fig. 5B). The M1b module, composed of 96 nucleotides, from the 5'-part of both transcripts contains six helices, two internal loops, a five-nucleotide bulge, and a three-way junction from which helices h4, h5, and h6 diverge. The free energy calculated for the entire M1b module is ΔG = −96 kcal/mol. The M2b module spanning nucleotides G88-C186 is slightly more stable (ΔG = −94.5 kcal/mol). The fragment of Ex1b-2 transcript corresponding to exon 2 forms an autonomous structure composed of three modules, M5b, M6b, and M7b (Fig. 5B). The structure of the first module is similar, and the structure of the second module is identical to the structure modules M4a and M5a of Ex1a-2 (Fig. 4D). The results of these structural studies allowed us to address the question of what specific features of 5'-UTRb were responsible for the strongly reduced translation efficiency of its corresponding transcript (Fig. 2). The candidates included stable elements of the secondary structure, short uORFs, or both. However, before answering this question we gathered some experimental data to prove that BRCA1 translation is initiated as expected by the standard scanning mechanism.

The Secondary Structure Present in Ex1b and Ex1b-2 transcripts. A, cleavages induced in Ex1b-2 by three structure probes. In lanes P4, S4, and T4, the lead ions, S1 nuclease, and T1 ribonuclease were at the following concentrations: 2 mM, 4 units/µl, and 2 units/µl, respectively. Other probe concentrations are as described in the legend to Fig. 3D. Electrophoresis was performed in a 6% polyacrylamide-urea gel. Positions of selected G residues and AUG codons of main BRCA1 ORF are also indicated. Vertical lines span sequence fragments corresponding to the secondary structure modules present. B, secondary structure of Ex1b-2. The AUG codons of three short uORFs, BRCA1-specific START codon, and Alu sequence ends are indicated. Structure of exon 2 sequence is shown in a frame.
In the second type of experiments the level of the translation initiation factor 4E (eIF-4E) was lowered gradually by the titration of RRL with an increasing concentration (0–1 mM) of the m'GpppG cap analog, as described earlier (34). eIF-4E is a component of the cap-binding protein complex eIF-4F that is required for 40 S ribosomal subunit binding to 5'-end of mRNA (35). Again, the efficiency of the in vitro translation decreased significantly with the increase of the cap analog concentration for both BRCA1 transcripts to a similar extent, whereas it remained unchanged for EMCV RNA (Fig. 6B). At 0.25 and 1 mM concentration of the cap analog the in vitro translation of BRCA1 transcripts exhibited only about 50 and 10%, respectively, of the efficiency shown in the absence of an extra amount of m'GpppG in the reaction mixture (Fig. 6C). The results of these experiments indicate that the translation of the BRCA1 transcripts containing either 5'-UTRs or 5'-UTRb is mostly if not entirely cap-dependent and occurs via ribosome scanning mechanism (34).

**Stable Secondary Structure Is Primarily Responsible for the Translational Inhibition of BRCA1 mRNA Containing 5’-UTRb**—Our further in vitro translation experiments with 5'-UTRb- and 5'-UTRb-containing transcripts Ex1a-2stop and Ex1b-2stop, coding for BRCA1 N-terminal polypeptide, were performed to confirm the large difference in their translatability. The third transcript Ex1b(M4)-2stop with truncated 5’-UTRb was used to separate the potential translation-inhibiting effects of uORFs from those caused by the strong secondary structure. In this transcript the secondary structure modules M3b, M4b, and M5b were present, whereas AUG codons of the uORFs were absent (Fig. 7A). The fourth transcript (Ex2stop) contained a short 27-nt fragment of 5’-UTR, corresponding to exon 2, devoid of all uORFs and stable secondary structures (Fig. 7, A and B). In vitro translation from all four transcripts was conducted in RRL (Fig. 7C), and translation efficiencies relative to that of Ex1a-2stop are shown in Fig. 7D. The translation efficiency of Ex1b-2stop was only about 10% of that of Ex1a-2stop, which closely resembled the results obtained with luciferase reporter (Fig. 2, B and C). This result reinforced our earlier conclusion that different BRCA1 5’-UTRs were responsible for the observed difference in translation efficiency. The comparisons between the 5’-UTRb-containing transcript and its truncated variants revealed that the efficiency of the translation of Ex1b-2stop is 14 times lower than that of the Ex2stop. The efficiency of the translation of the Ex1b(M4)-2stop is nearly 6 times lower than that of the Ex2stop which suggests the significant role of the strong secondary structure module M4b in translation inhibition. However, the 2.5-fold higher translation efficiency of Ex1b(M4)-2stop compared with Ex1b-2stop raised the question whether the deletion of uORFs or the absence of the two secondary structure modules M1b and M2b was responsible for this effect.

**uORFs Contribute to the Translational Inhibition of BRCA1 mRNA Containing 5’-UTRb**—To address specifically the question of the involvement of uORFs in translational regulation of BRCA1 mRNA with 5'-UTRb, a new set of four transcripts was prepared (Fig. 7E). These transcripts contained point mutations either in each individual AUG or in all three AUG codons of the uORFs. It turned out after their in vitro translation that the elimination of all three uORFs raised the translation efficiency from the main BRCA1 ORF 2.8 times (Fig. 7, F and G). This result corresponds well to the 2.5-fold difference in the translation efficiency of Ex1b(M4)-2stop and Ex1b-2stop transcripts and reflects the contribution of uORFs to the decreased efficiency of translation of 5’-UTRb-containing transcripts. Mutations of the individual AUGs showed that uORF2 and uORF3 may have the strongest contribution to this effect (Fig. 7G). Taking into account these results and the results obtained with the 5’-UTRb deletion mutants, we conclude that the decreased efficiency of translation from BRCA1 mRNA containing 5’-UTRb should be attributed to the predominant part to the presence of the strong secondary...
structure module M4b and to a lesser extent, about 30%, to the presence of uORFs in this leader sequence.

**DISCUSSION**

The Dereegulation of BRCA1 Transcription in Breast Cancer—The occurrence of two or more transcripts differing in their 5′ termini is thought to represent an evolutionary gain of refined transcriptional and translational control (36). In the BRCA1 gene, two promoters, α and β, produce transcripts beginning with either the exon 1a or exon 1b sequence, respectively (22). We have shown here that BRCA1 mRNA with a shorter 5′-UTRa is the sole transcript in blood leukocytes and in normal mammary glands, whereas both mRNAs are expressed in testis and in breast cancer tissue. Both BRCA1 mRNAs were found at variable levels and ratios in breast and ovarian cancer cell lines and primary tumors (21). A significant difference between the BRCA1 transcript pattern observed in a normal mammary gland and in breast cancer tissue (Fig. 1D) suggests the deregulation of BRCA1 transcription in cancer, resulting in the activation of the promoter β. Disturbances in tissue-specific transcription factors, repressors, and methylation of the CpG island in which the α promoter is located are among the effects (37, 38) possibly involved in the switch from promoter α to β.

BRCA1 Is Down-regulated at the Translation Level by the Stable Secondary Structure of 5′-UTRb—The translation efficiency of eukaryotic mRNAs may vary considerably depending on the properties of their 5′-UTRs. Statistically, 5′-UTRs of low expression mRNAs are longer; their GC content is higher, and they have a less optimal context of the AUG codon of the main ORF (39). Furthermore, they more frequently contain upstream AUG than 5′-UTRs of high expression mRNAs, and their leader sequences often form strong secondary structures (39–41). Most of these features, lowering the efficiency of translation initiation, are present in BRCA1 5′-UTRb, which strongly inhibits the cap-dependent translation in comparison to 5′-UTRa (Figs. 2, 6, and 7). The observed 1 order of magnitude difference in translatability (Figs. 2C and 7D) prompted us to search for the specific determinants of translation efficiency within the BRCA1 mRNA 5′-UTRs. We have analyzed the secondary structures of the BRCA1 leaders using experimental methods for the RNA structure probing in solution (42–44). The characteristics of the stable structures present in 5′-regions of BRCA1 mRNAs containing different 5′-UTRs, in terms of their potential to inhibit translation initiation, is shown in Fig. 7A. The environment of the initiation codon of the BRCA1 ORF has the same structure in both mRNAs. The AUG codon is located at the base of a quasistable module ($\Delta G = -25.5$ kcal/mol) named $M5a$ and $M6b$ in transcripts Ex1a-2 and Ex1b-2, respectively (Fig. 7A). Previously, structures with a similar location and free energy, about $-30$ kcal/mol, were shown to have little effect on translation efficiency, whereas more stable structures, with $\Delta G$ below $-50$ kcal/mol, located in 5′-UTR, inhibited translation efficiently (45, 46). Thus, the structure of the $M5a/M6b$ module is not expected to

Positions of the N-terminal BRCA1 30AA polypeptide, the 42-kDa internal marker protein, and the unincorporated methionine are indicated. **D**, the [35S]methionine incorporated was counted, and the results averaged ± S.E. from six independent experiments are shown in the graph. Translation efficiencies are expressed relative to that of an Ex1a-2stop transcript taken as 100%. E, transcripts used to analyze the influence of upstream AUG codons of 5′-UTRb on the translation efficiency of the BRCA1 N-terminal sequence (281AA). These transcripts include the wild type Ex1b-11 and its mutants Ex1b-11-mut1AUG, Ex1b-11-mut2AUG, Ex1b-11-mut3AUG, and Ex1b-11-mut1-3AUG with base substitution in the upstream AUG codons. **F**, 12% SDS-PAGE of translation products obtained from five transcripts described in E, G, translation efficiencies of four mutant transcripts, described in E, are shown relative to that of a wild type Ex1b-11 transcript taken as 100%.

The results are averages ± S.E. from three independent experiments.
The Predominant Part of BRCA1 5′-UTRb Derives from the Alu Sequence—The central part of 5′-UTRb contains the 232-nt long sequence (nucleotides 126–357), which belongs to the Alu-Sx subfamily of repetitive sequences (2, 50). The Alu insert present in BRCA1 leader is 60 nt shorter than the full size Alu dimer. It lacks the first 50 nt of the left monomer and 10 nt of the right monomer. The entire uORF3 is located in the BRCA1 Alu sequence. Part of the secondary structure characteristic for Alu dimer (51) is preserved in the BRCA1 leader (Fig. 5B). A fragment of the left monomer is engaged in base pairing with the non-Alu section of the 5′-UTRb and forms the module M2b. The sequence corresponding to the Alu right monomer lacks the hairpin designated “domain I” in the Alu secondary structure (51). The most stable element of the Alu secondary structure, “domain III,” is preserved in the BRCA1 5′-UTRb. It forms the M4b module that contributes most significantly to the translational inhibition of BRCA1 mRNA containing 5′-UTRb (Fig. 7D). Our study shows for the first time that the Alu sequence located in 5′-UTR strongly inhibits the initiation of translation by its stable secondary structure and uORF. As more than 1 million copies of Alu elements account for 10% of the human genome, they affect both genome organization and gene regulation (52–55). Our search revealed that about 4% of fully spliced human mRNAs contain complete or truncated Alu sequences, which are often located in 5′-UTRs. Alu-mediated gene regulation at the mRNA level is therefore a more common phenomenon, and its biological importance needs to be more fully revealed.

Coupled Transcriptional Deregulation and the Translational Inhibition of BRCA1 May Occur in Breast Cancer—In order to improve our understanding of the role of BRCA1 in cell homeostasis and in breast and ovarian carcinogenesis, two fundamental questions have to be answered. How is the expression of BRCA1 regulated in normal cells? How is it down-regulated in breast and ovarian cancer? Many proteins interacting with BRCA1 have been identified (56), and two major cellular functions have been assigned to the BRCA1 protein, DNA repair and transcriptional regulation (57). Several mechanisms of BRCA1 down-regulation in sporadic cancer have been proposed. These include monoallelic or biallelic deletion of the BRCA1 locus and transcriptional silencing by promoter methylation (56). The latter effect may also be achieved either by loss of proteins positively regulating BRCA1 expression (58) or by an increase in proteins playing a role of its negative regu-
Translational Regulation of the BRCA1 Gene

In the model shown in Fig. 8, we propose that BRCA1 may be down-regulated in breast cancer by the promoter switching coupled with translational inhibition. According to this model, the amount of BRCA1 protein may decrease significantly. Increased contribution from BRCA1 factors responsible for switching mRNAs with different leader sequences may serve as an in spicatory marker in sporadic breast and ovarian cancer. We have revealed the molecular basis underlying this mechanism. This involved in sporadic breast and ovarian cancer. We have revealed the molecular basis underlying this mechanism. This

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