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A complete protein truncation test for *BRCA1* and *BRCA2*

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The protein truncation test (PTT) is currently the fastest method in general use for detecting previously unidentified mutations in tumor suppressor genes. Greater than three kilobases of coding sequence can be screened by one PCR reaction, one coupled *in vitro* transcription/translation reaction, and one lane on an SDS-PAGE gel. The 16 kb of *BRCA1/2* coding sequence can be screened with nine overlapping segments. Since 90% of *BRCA1/2* mutations result in a truncated protein product, the theoretical false negative rate for a *BRCA1/2* PTT screen should be 10%. In practice the false negative rate is much higher, especially when cDNA is used as template. However, the actual false negative rate for a given screen will depend on the details of how the test is performed. Design of the overlapping segments, gel parameters, and nonsense mediated mRNA decay can all influence the effectiveness of the screen. *BRCA1/2* screening by PTT can be optimised by considering these variables. Furthermore, nonsense-mediated mRNA decay can be inhibited by blocking protein synthesis with cycloheximide.

Keywords: Protein truncation test; mutation detection; *BRCA1*; *BRCA2*

Introduction

The protein truncation test (PTT), also referred to as the *in vitro* synthesised protein assay (IVSP), was first described by Roest *et al*¹ and Powell *et al*² as a means of detecting mutations in the dystrophin gene and the *APC* gene, respectively. Using PTT, one can identify the subset of genetic mutations which result in a truncated protein product by synthesising the protein encoded by a test sequence *in vitro* and determining its size. Although PTT is an indirect approach to identifying alterations in DNA and is unable to detect missense

mutations, it has become a popular screening method because it is such an efficient means of screening coding sequence. Alternate approaches such as SSCP and direct sequencing require much more effort per megabase screened than PTT, and generate excess data that are irrelevant to mutation detection. For example, sequencing will determine the nucleotide sequence of a gene (virtually all of which will be wild type) in addition to detecting alterations that predispose to disease. Similarly, SSCP detects benign polymorphisms as well as disease-causing mutations. PTT, on the other hand, only detects alterations in a coding sequence that lead to a truncated protein product. Fortunately, many genetic diseases are caused by inheritance of loss of function mutations, and premature truncation of the protein product is a common mechanism leading to loss of function. Thus a protein truncation assay can efficiently detect mutations predisposing to disease.

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Protein truncating mutations are commonly found in genes predisposing to cancer. For example, 95% of the *APC* gene mutations associated with familial adenomatous polyposis³ and 71% of the mutations in the mismatch repair genes predisposing to hereditary non-polyposis colorectal cancer⁴ are translation terminating mutations. The breast cancer susceptibility genes *BRCA1* and *BRCA2* follow the same pattern. Eighty-six percent of *BRCA1* mutations detected by sequencing and SSCP generate a truncated protein,⁵ as do 20 of the first 21 (95%) reported *BRCA2* mutations.^{6,7,8} More recently detected mutations described in the BIC online data base confirm this trend.⁹ These truncating mutations are dispersed throughout the 48 exons containing the 16 kb of *BRCA1/2* coding sequence, implying that a screen of the entire coding sequence is appropriate. In certain human subpopulations, such as the Ashkenazim, founder effects will allow for efficient screening of specific mutations.¹⁰ However, in most clinical settings the heterogeneity of the patient population and the large number of still unidentified *BRCA1/2* mutations will result in an unacceptably high false negative rate for allele specific testing. The recently described DNA chip-based assay promises to offer high throughput detection of novel *BRCA1/2* mutations,¹¹ although this technology is not yet available for routine diagnostics.

PTT has been used by at least three groups to screen the entire *BRCA1* coding sequence.¹²⁻¹⁴ In each case, the general strategy is the same: cDNA is used as PCR template for the 21 smaller exons containing 40% of the coding sequence, while the 3427 basepairs (60% of the coding sequence) of exon 11 are amplified from genomic DNA. *BRCA1* is PCR-amplified in five or six overlapping segments of convenient size, and the entire gene is screened for truncating mutations by analysing the five or six PCR products by *in vitro* transcription/translation and SDS-PAGE. In the three publications cited, 138 patients were screened by PTT for *BRCA1* exon 11 mutations using gDNA as template, and 10 novel mutations were detected; 95 patients were screened by PTT for mutations in exons 2-10 and 12-24 using cDNA as template, yet not a single mutation that could be verified by an alteration in the DNA sequence was identified. Thus the ability of PTT to detect *BRCA1* mutations appears to be below its theoretical expectations, especially when cDNA is used as starting material.

The disappointing detection frequency for PTT is most likely due to a number of technical factors that

must be addressed. Firstly, the overlap between segments must take into account the inability of tris/glycine buffered SDS-PAGE to resolve proteins smaller than 12 kilodaltons. Secondly, means must be found to minimize and cope with the background signals caused by PCR artifacts that can arise when cDNA is used as template. Thirdly, the problem of nonsense mediated mRNA instability must be solved.

Materials and Methods

Template Preparation

Genomic DNA was isolated by the salting-out method.¹⁵ For cDNA, PBLs from 20 ml of fresh heparin-treated blood purified by Ficoll hypac, or 10⁷ cells grown in tissue culture are used to make total RNA isolated with the Triazol reagent (Gibco, Basel, Switzerland). The RNA pellet is used directly to make cDNA by random priming MoMuLV RT. Final volume of the RT rxn is 50 µl. The cDNA is heat treated for 5 min at 95°C and is used directly for PCR.

PCR

Hot start is done for all PCR reactions. All PCR reactions using genomic DNA as template are performed in a final volume of 20 µl, 100 ng of template, with primer concentration at 200 nM. Amplification is achieved by 50 cycles of PCR with 2 mM MgCl₂ and 1 U thermostable polymerase consisting of a mixture of 40 parts Taq to 1 part Pwo, a thermostable polymerase with proofreading activity (GIBCO BRL, Basel). All cycling is done at 94°C for 30 s, 55°C for 30 s, 68°C for 2 min, except for segment 5 which works best with a hybridisation temperature of 50°C. Segments requiring cDNA template are amplified in two steps using 1 µl of cDNA. In the first step, external primers at 200 nM in a final volume of 10 µl are used for 20 cycles of amplification. Internal primers are then added to the 10 µl of PCR products to a final concentration of 400 nM in 30 µl and 30 cycles are performed with the same temperature/time profile and enzyme concentration as above. There are three exceptions to these primer concentrations – external segment 4 primers are at 100 nM, and internal segment 4 and internal segment 8 primers are at 250 nM final concentration.

PTT

The PCR products are transcribed into *BRCA1/2* mRNA and translated into protein simultaneously using a minimum amount of PTT reagent similar to that provided in the TNT coupled transcription/translation kit from Promega (Madison, WI, USA), but having one-fourth the concentration of labelled methionine and a homemade buffer. The PTT reagent is made up in bulk and frozen in aliquots once at -70°C. 1.2 ml of PTT reagent is made as follows: 1 ml rabbit reticulocyte lysate and 40 µl 1 mM amino acids minus methionine from Promega (Madison, WI, USA), 40 µl high concentration (90 units/µl) T7 polymerase, 40 µl translational grade S35-methionine, and 20 µl RNase inhibitor (90 U/µl) from Amersham (Buckinghamshire, UK). To these five components is added 80 µl of a homemade buffer that consists of 10 mM rNTPs and 25 mM MgCl₂.

2 μ l of PCR product (at 2 mM $MgCl_2$) are added to 3 μ l of PTT reagent and incubated at 30°C for 1 h. After the reaction is complete, 10 μ l of loading buffer containing SDS and BME are added to each sample, the samples are heated to 95°C for 2 min, and 5 μ l of each is subjected to tris/glycine discontinuous SDS-PAGE on a 5–18% gradient gel at 35 ampere constant current for 120 minutes. When the bromophenol blue dye has reached the bottom of the gel, the gel is fixed in methanol/acetic acid for 30 min, dried and used to expose Kodak Biomax film for 18 h at room temperature. No fluorographic agents are used.

Cell Lines and Cycloheximide Treatment

BRCA1 mutant cell lines GM13713 (Glu1250ter), GM13705(1251del4bp), GM13709 (2135delA), GM13712(2279insA), and GM13707(390del140bp), were purchased from the Coriell Institute (Camden, New Jersey, USA). 2×10^6 1611delC EBV transformed cells were grown in RPMI + 10% FCS with or without cycloheximide (Sigma, Saint Louis, MO, USA) at 300 μ g/ml for 12 h before harvesting total RNA.

gDNA and cDNA Sequencing

Cycle sequencing was done on PCR products using Thermo-sequenase (Amersham, Bucks, UK). The extension products were analysed on a Licor automated sequencer (Lincoln, Ne., USA). gDNA from the 1611delC mutant was amplified by 35 cycles of amplification using primers from positions 1461–1480 and 1864–1883 of *BRCA1*. cDNA was used as template for nested PCR in which the 5' external primer was in exon 1 (the 5' external primer for segment 1, see Table 1). The internal primers were the same as those used to amplify gDNA.

Results

The entire 15 849 basepairs of coding sequence for *BRCA1/2* were divided into 9 overlapping segments ranging in size from 473 to 1148 codons and having overlaps of 250 codons (Figure 1a). Nested PCR using cDNA as template was used to amplify segments 1, 3, 4, 5, 8, and 9, while standard PCR was used to amplify segments 2, 6, and 7 from genomic DNA (Figure 1b). The sequences of the primers used for PCR are listed in Table 1. PCR products were transcribed and translated into radio-labelled protein using an inexpensive home-made lysate-RNA polymerase mixture and analysed by SDS-PAGE (Figure 1c, and methods).

Sufficient Resolution With Gradient Gels

All 1143 codons of *BRCA1* exon 11 are contained in segment 2. In order to determine the limits of mutation detection using tris/glycine SDS-PAGE, known truncations spanning exon 11 were analysed on a 5–18% gradient gel (Figure 2a, lanes 1–11). The smallest difference detected between the full length protein and a mutant is seen with the 1031 residue product of the 3875del4 mutation,¹⁶ lane 1. The 10% difference in size of these relatively large proteins is easily resolved in the upper range of the gradient gel. Lane 2 contains a 1019 residue mutant protein that is indistinguishable from that of lane 1, since they differ in size by only 1.2%. Importantly, however, the difference between the

Table 1 *BRCA1/2* Primers

Segment	5'	3'	Size (BP)
1 external	TCTGCTCTGGGTAAAGTTCA (85–104)	GTTGGGGAGGCTTGCCTTCT (1538–1519)	1453
1 internal	T7-GATTTATCTGCTCTTCGCGT (123–142)	GTTGGGGAGGCTTGCCTTCT (1538–1519)	
2	T7-GCTTGTGAATTTTCTGAGAC (792–811)	TAAGTTTGAATCCATGCTTTGC (4214–4193)	3460
3 external	ATTCAAGCAGAACTAGGTAG (3321–3340)	TAGCCAGGACAGTAGAAGGA	2287
3 internal	T7-GAAGTAGTTCAGACTGTAA (3462–3481)	TCAGTAGTGGCTGTGGGGGATC (5711–5690)	
4 external	GCCGGGAGAAGCGTGAGGGG (101–120)	TGGTGGTGGCTGGCCAGCTT (1930–1911)	1736
4 internal	T7-CCTATTGGATCCAAAGAGA (232–250)	TGGTGGTGGCTGGCCAGCTT (1930–1911)	
5 external	GAAGATAGTTTTTCATTATG (1141–1160)	CCTTTCATTAGCTACTTGAAG (2922–2901)	1737
5 internal	T7-AAAAATCTACAAAAGTAAGA (1180–1200)	GAGAAAAGTTCTTCAGAGTCTGG (2879–2857)	
6	T7-GGTTTATTGCATTCTTCTGTG (2137–2157)	TTCTTTAATCTGAGTGTTC (4374–4355)	2275
7	T7-CCAAGCTACATATTGCAGAAG (3625–3645)	CCCACTAAGATAAGGGGCTC (7070–7051)	3483
8 external	TCTGTCCAGGTATCAGATGC (6181–6200)	CTCTTTTGTGGGCTCCAC (8810–8791)	2527
8 internal	T7-ACCAAGCAAGTCTTTTCCAAAG (6247–6268)	AGATGATGTCTTCTCCATCC (8736–8717)	
9 external	GAAGATTATTTTGGTAAGGA (7921–7940)	CGCTGAGGTAAATTGAAAC (10620–10600)	2611
9 internal	T7-CTCATACCCTCCAATGATGG (7987–8006)	GGTTTGAAATTATATTCCAG (10560–10541)	

The numbers in parentheses for primers for segments 1–3 refer to the positions according to Genbank accession number U14680, except for the segment 3 external 3' primer, which is the reverse primer for *BRCA1* exon 24 from Friedman *et al* 1995. For segments 4–9, the numbers refer to positions according to Genbank accession number U43746. T7 refers to the T7 polymerase and Kozak consensus sequence TAATACGACTCACTATAGGGAGACCACCATG.

mutant and wild-type signals in lane 2 is sufficient to detect the mutation. This holds true for truncations spanning exon 11 and generating products as small as 138 amino acids (lanes 3–11). An exon 11 mutant of 123

residues can be detected as well (see lane 4, Figure 2c), although this band was not seen on some occasions, suggesting that reproducibility becomes a problem with such small fragments (see Discussion). Segment 2 has a

Figure 1 a) Nine segments span the entire *BRCA1/2* coding sequence. Overlaps are 250 codons each. b) The nine PCR products for each of the nine segments. c) Protein products synthesized in vitro for the nine *BRCA1/2* segments are analysed on 5–18% gradient PAGE.

distinct and highly reproducible artifactual doublet of approximately 500 amino acids in length. This artefact

does not interfere with mutation detection, however, since the doublet intensity is significantly less than the

Figure 2 a) Truncations spanning segment 2. Five patients that have a wild-type pattern are shown as controls (C), followed by 10 patients harbouring mutations in exon 11 of *BRCA1*. lane 1: Coriell GM13705, lane 2: Coriell GM3713, lane 3: patient 1540, lane 4: patient 1668, lane 5: patient 1564, lane 6: Coriell GM13712, lane 7: Coriell GM13709, lane 8: patient 1560, lane 9: patient 1604, lane 10, Coriell GM13707. Lane 11 used as template a PCR product amplified from gDNA using the 5' primer for segment 2 and a 3' primer complimentary to positions 1181–1200 of *BRCA1*. b) Truncations in segment 3 derived from cDNA. C indicates the normal pattern seen in controls. In lanes 1 and 2 cDNA from Coriell GM13713 cells was used in 2 independent PCR amplifications, while lanes 3 and 4 used cDNA from Coriell GM13714A cells. c) *BRCA1* mutations in overlap seen in two segments. Lanes 1 and 2 are segment 1, lanes 3 and 4 are segment 2. Lanes 1 and 3 are controls, while lanes 2 and 4 show the pattern for patient 1686. Mutations in the patients have been described in Garvin et al 1996, except patient 1564 which has the mutation 2804 del 2bp.

truncation products. A mutation induced truncation product co-migrating with the doublet is easily seen in lane 7 of Figure 2a.

PTT is generally more difficult and less reliable when cDNA is used as template, and yet 48% of the coding sequence analysed by PTT in the present scheme is derived from cDNA. Figure 2b demonstrates that mutations at either end of segment 3, which contains exons 12–24 of *BRCA1* amplified from cDNA, can be reproducibly detected. Lanes 1 and 2 show the segment 3 pattern for a nonsense mutation at codon 1250 in *BRCA1*.¹⁶ PCR products from two independent PCR amplifications were used as template for each PTT. The expected truncation product of 137 residues is consistently detected, as well as additional unreproducible truncation products. Similarly, lanes 3 and 4 demonstrate the clear and reproducible signal from the 5438insC mutant. TEXTREF ID = "rf16" > 16 The 714 residue truncated protein is only 35 residues shorter than the wild-type protein, and yet this 5% size difference is easily resolved.

When the overlap between PTT segments is sufficient, some mutations occurring in the overlap will be detected in both segments. Figure 2c demonstrates that this is the case for *BRCA1* 1129insA, which generates a truncated segment 1 of 344 residues (72% of full length) and a 123 residue segment 2.

Mutations in cDNA can be Distinguished from Artefacts

PTT can often generate unreproducible truncation products when cDNA is used as template for the PCR amplification. Sequencing of the PCR product often reveals an unusual splice variant or a deletion, thus explaining the PTT result. However, confirmatory sequencing of genomic DNA fails to detect the expected splice site alteration or the expected deletion, indicating that PCR products derived from cDNA template are prone to artefacts. The over-representation of an unusual splice variant can be explained by fortuitous PCR amplification of that particular species, whilst a deletion can be explained by internal priming by partially extended PCR products, and subsequent preferential amplification of the smaller amplicon. In any case, these are PCR artefacts that occur randomly during the amplification process and should not be reproducible. Figure 3 demonstrates this to be the case for segment 8 of patient 4. The cDNA from patient 4 was of low quality and was difficult to amplify, however PCR products of the correct size were eventually generated. Unlike the control pattern, the PTT pattern

Figure 3 Non-reproducibility of artifacts but reproducibility of truncations caused by mutations. Segment 8 for three patients lacking mutations and having the normal pattern are each indicated as C (control). Template from three independent PCR amplifications from patient 4 were used for PTT in lanes marked 4. Similarly, three independent PCR products from patient 25, who has a 2 bp deletion at position 7297 of *BRCA2* generating a 373 residue segment 8, were used in lanes marked 25.

for patient 4 was inconsistent. For comparison, patient 25 has a reproducible truncation product of 373 residues due to a two base-pair deletion at position 7297 in exon 14 of *BRCA2*. For each cDNA template, independent PCR amplifications were performed, and each PCR product was used as template for PTT.

Nonsense-mediated mRNA Decay is Inhibited by Cycloheximide

Premature termination of protein synthesis in eukaryotes can result in message instability, a process termed nonsense-mediated mRNA decay (recently reviewed by Jacobson and Pelz.¹⁷ Importantly, transcript instability for a number of *BRCA1* mutants has been reported.^{18,19} Although truncating *BRCA1* and *BRCA2* mutations can be identified using cDNA as starting material (see Figures 2b, 2c and 3), nonsense mediated decay in a subset of *BRCA1/2* mutants will increase the false negative rate for any test that uses cDNA as starting material and is a cause for concern. The mechanism responsible for mRNA instability in certain *BRCA1* mutants is unclear; however, it is reasonable to propose that protein synthesis is required for the cell to discriminate between mutant and wild-type messages. In such a scenario, global inhibition of protein synthesis should equalise the steady state levels of mutant and wild-type mRNA. Figure 4 demonstrates this is the case

for the previously characterised *BRCA1* 1611delC mutant.¹⁴ EBV transformed cells harbouring the mutation were grown in the presence or absence of cycloheximide, an inhibitor of protein synthesis. Genomic DNA or cDNA was used as template to amplify codons 447–581 of *BRCA1* and the PCR products were sequenced. Genomic DNA has the expected diminished signal for the T residue at 1612 and an equivalent T signal at 1611 due to the 1 base-pair deletion in the mutant allele. Mutant cDNA from untreated cells gives a barely detectable T signal at position 1611, while the cDNA from cycloheximide treated cells generates a signal intensity equal to that obtained with mutant genomic DNA, indicating that nonsense-mediated mRNA decay has been inhibited. Similar inhibition of nonsense mediated decay was obtained when protein synthesis was inhibited in a cell line containing the gln526ter mutation (data not shown).

Discussion

Mutation detection in the *BRCA1* and *BRCA2* genes poses a formidable challenge. Many unidentified *BRCA1/2* mutations still exist in the human population, implying that the rapid and simple methods which detect known mutations will have an unacceptably high false negative rate. Furthermore, mutations are spread throughout the genes, thus a complete scan of the 5283 codons contained in *BRCA1/2* is required for an accurate screen. DNA sequencing is one method for detecting mutations in *BRCA1/2* and involves generat-

ing, collecting, and analysing 32 000 data points, since both strands of the 16 kb of coding sequence must be sequenced. This approach can detect the presence of mutations, but it will also provide an enormous amount of unneeded information, namely the complete *BRCA1/2* gene sequence of the patient. The cost of this unnecessary information is reflected in the cost of the assay, which is often prohibitive. Fortunately, other methods are available which provide less information than sequencing, but are effective in detecting mutations.

PTT provides very limited information on the test sequence in that it only detects mutations resulting in premature termination of protein synthesis. The limited information content of PTT is reflected in its rapidity and low cost. A theoretical PTT of *BRCA1* and *BRCA2* relying on nine overlapping segments will generate nine wild-type data points representing the full length protein products, and the presence or absence of one (or at most two) additional data points indicating the presence of a mutation.

Limitations of SDS-PAGE

All examples of PTT published to date use tris/glycine SDS-PAGE to determine protein size. It is important when using this technique to keep in mind that the relation of logMW to relative mobility is linear over only a limited range of molecular weight. On a 12% gel, for example, proteins with a molecular mass below 16 000 run with the buffer front, and thus with unincorporated radio-labelled methionine,²⁰ and higher acrylamide concentrations do little to improve the

Figure 4 Cycloheximide blocks nonsense-mediated mRNA decay. Genomic DNA, cDNA from untreated cells, and cDNA from cells treated with 300 mgr:g/ml cycloheximide were used as template to amplify codons 447–581 of the *BRCA1* gene from cells harbouring the 1611delC mutation. The T ladder profile for gDNA between 1607 and 1612 shows the expected signal at 1611 due to the 1 base-pair deletion in the mutant allele, as well as the wild-type signal at 1612. cDNA template from untreated cells lacks the signal at 1611, indicating the absence of the mutant message. Cycloheximide treatment restores the signal at 1611.

lower limit of detection. The resolution of small proteins can be improved by using tricine/urea gels, although some proteins are still not detected because they are eluted from the gel during the washing step.²¹ Since a washing step to remove unincorporated S-35 methionine is required when doing PTT, and since tricine is relatively expensive and not commonly used for SDS-PAGE, 130 residues can be considered a conservative lower limit of detection for PTT when 5–18% gradient gels are used with a tris/glycine buffer. The largest segments used for screening *BRCA1/2* are the 1143 and 1148 residue segments 2 and 7, respectively. As shown in Figure 2a, a 10% reduction (115 residues) in size for these large segments is enough to detect a mutation. Therefore, the overlap between segments should be at least 245 (130 + 115) residues to insure proper coverage at the segment junctions. Overlaps of this size are much larger than the 14–22 codon overlaps used in DMD screening,²² and the 9–107 codon overlaps used for the first *BRCA1* PTT.¹²

In the present study all overlaps are 250 residues, leaving only the 5' and 3' ends of *BRCA1/2* without adequate coverage. The 5' ends of each gene lack coverage for the first 130 codons. Mutations truncating segments 3 and 9 by less than 10% (75 and 83 residues, respectively) at the 3' end of each gene might also be scored as false negatives, although this is a conservative figure, since a 5% shift in segment 3 was detected in lanes 3 and 4 of Figure 2b. Thus truncations occurring within a total of 418 codons, or 8% of the *BRCA1/2* coding sequence, might be missed due to the limitations of tris/glycine SDS-PAGE. Assuming that truncating mutations are spread evenly throughout the *BRCA1/2* coding sequence, 92% of the truncated proteins generated in the coupled transcription/translation reaction can be detected. Since 90% of *BRCA1/2* mutations result in truncations, the theoretical false negative rate for this PTT screen is 17%, assuming that the problem of nonsense mediated mRNA decay is adequately resolved.

PCR Artefacts when Using cDNA Template

The low abundance of *BRCA1* and *BRCA2* mRNA in peripheral blood leukocytes necessitates nested PCR amplification of cDNA. Under the PCR conditions used, a significant and apparently unavoidable number of PCR amplifications from cDNA template generates artefacts that produce truncated protein products in the PTT. Much time and effort can be wasted attempting to find the alterations in gDNA responsible for these false

positive signals. Fortunately these artefacts are distinguished from true mutations by their irreproducibility. Thus any putative mutation detected by PTT when cDNA is the starting material should first be verified by an independent nested PCR-PTT before proceeding with sequencing.

Nonsense Mediated mRNA Decay

cDNA is used as starting material for 48% of the *BRCA1/2* PTT screen. In such an approach mutant message must be present at detectable levels in the patient's cells that serve as a source of mRNA. For at least six *BRCA1* mutations this is not the case and premature truncation of the *BRCA1* protein is accompanied by loss of transcript.^{18,19} Clearly then, nonsense mediated decay of *BRCA1/2* mRNA can increase the false negative rate of a PTT screen.

The 1611de1C mutation in *BRCA1* results in loss of transcript^{18,19} (see Figure 4), and yet many other truncating mutations both 5' and 3' to 1611 do not. The 185de1AG mutation of *BRCA1*, for example, can be easily detected by sequencing cDNA (data not shown), indicating that truncating mutations at the extreme 5' end of the coding sequence do not necessarily lead to loss of transcript. Similarly the nonsense mutation at codon 1828 at the extreme 3' end of *BRCA1* is easily detectable from cDNA (see Figure 2b, lanes 3 and 4). In yeast, *cis*-acting sequences 3' to the premature stop codons are required for mRNA destabilisation.²³ Perhaps similar elements are adjacent to the *BRCA1* mutations which cause nonsense mediated mRNA decay, but are not adjacent to those mutations that have no effect on message stability.

Regardless of the mechanism of nonsense mediated mRNA decay, any means to stabilise mutant message should increase the accuracy of PTT and any other mutation detection scheme that relies on cDNA. Thus the finding that inhibition of protein synthesis by cycloheximide treatment can stabilise mutant *BRCA1* mRNA may have practical applications. Perhaps routine cycloheximide treatment of blood samples prior to RNA isolation will reduce that component of the false negative rate caused by nonsense mediated mRNA decay.

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