

Rapid detection of *BRCA1* mutations by the protein truncation test

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More than 75% of the reported mutations in the hereditary breast and ovarian cancer gene, *BRCA1*, result in truncated proteins. We have used the protein truncation test (PTT) to screen for mutations in exon 11, which encodes 61% of *BRCA1*. In 45 patients from breast and/or ovarian cancer families we found six novel mutations: two single nucleotide insertions, three small deletions (1-5 bp) and a nonsense mutation identified two unrelated families. Furthermore, we were able to amplify the remaining coding region by RT-PCR using lymphocyte RNA. Combined with PTT, we detected aberrantly spliced products affecting exons 5 and 6 in one of two *BRCA1*-linked families examined. The protein truncation test promises to become a valuable technique in detecting *BRCA1* mutations.

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Hereditary breast cancer is a genetically heterogeneous condition. Two loci have been mapped by linkage analysis, *BRCA1* to chromosome 17q (ref. 1) and *BRCA2* to chromosome 13q (ref. 2). At least 45% of families with site-specific breast cancer and more than 80% of families with breast and ovarian cancer are linked to *BRCA1* (ref. 3). The population frequency of *BRCA1* mutations has been estimated to be one in 800 women, and the risk for breast cancer conferred by mutations in this gene is 85% by age 70 (ref. 4). With the isolation of the *BRCA1* gene⁵, DNA-based genetic testing of high-risk individuals has become feasible. Some 5–8% of all breast cancers are thought to be hereditary, while a further 10% show familial clustering but without a clear mode of inheritance⁶. Hence, many women self-referring to a cancer family clinic for counselling will not belong to a high-risk family. Also, many high-risk families will not have defects in *BRCA1* (ref. 3). However, given the potential benefits mutation testing may offer women, it will be difficult to restrain the demand for widespread testing for *BRCA1*.

The genomic structure of *BRCA1* is complex, and the rapid detection of mutations is a technical challenge^{7,8}. Logistic problems are to be expected if the DNA test is going to be provided routinely by health care institutions, such as cancer family clinics or clinical genetic centres. However, of the 38 distinct gene alterations published so far, 86% result in a truncated protein due to nonsense or frameshift mutations^{5,8–12}. These mutations might be rapidly screened by the protein truncation test (PTT)^{13–15}, which detects mutations leading to premature termination of protein synthesis and which has been successfully applied to the *APC* and *NFI* tumour suppressor genes, among others. Briefly, PCR is performed using forward

primers containing a T7-promoter sequence and a eukaryotic translation initiation sequence. Protein products are synthesized in a coupled *in vitro* transcription/translation reaction and analysed by gel electrophoresis. Truncated proteins are easily discriminated from full size, wild-type products and their size directly pinpoints the site of the mutation.

We have previously developed this test for the diagnosis of Duchenne muscular dystrophy (DMD)¹³ and familial adenomatous polyposis (FAP)¹⁴. Here, we investigated the efficiency of PTT to identify truncating mutations in *BRCA1*.

Detection of mutations in exon 11

For mutation screening by PTT, we examined 45 patients. Twenty-nine of them belonged to research families in which at least three patients shared the same haplotype at polymorphic markers closely flanking *BRCA1*: cen-D17S250–*THRA1*–D17S855–D17S579–tel (ref. 3). An additional six patients belonged to research families for which no linkage data were available but which contained either at least one case of ovarian cancer or had a high incidence of breast cancer. Finally, ten patients referring themselves to our clinical genetic centre were tested upon request. Table 1 summarizes the estimated probabilities for each family that they harbour a mutation in *BRCA1*.

As RNA samples were not available from most families, we initially focused on exon 11 which is the largest *BRCA1* exon and encodes 61% of the protein. This allowed us to analyse previously collected DNA samples from our families. We designed three primer pairs (A, B and C) of which the PCR products partially overlap and together span exon 11 almost completely (see Methods). Fragments

Table 1 Estimated probabilities of the investigated families and patients*

a, Families analysed for linkage (29)				
Posterior prob. %	Total number	Number HBC ^b	Number HBOC ^b	
>90	13	3	10	
75–90	6	3	3	
25–75	8	8	0	
0–25	2	0	2	
Total	29	14	15	

b, Families analysed without linkage evidence (6)				
Family	Prior prob.	Affected members	Breast cancer	Ovarian cancer
RUL26 ^c	0.67	6	6	0
RUL27	0.45	7	7	0
RUL36	0.45	5	5	0
RUL78	0.81	4	3	2
RUL85	0.92	9	7	3
RUL102	0.92	3	1	2

c, Index cases analysed (10)				
	Prior prob.	Affected members	Breast cancer	Ovarian cancer
MOC18	0.35	3	3	0
MOC22	0.45	4	4	0
MOC23	0.81	3	2	1
MOC29	0.61	2	0	2
MOC31	0.45	5	5	0
MOC46	0.30	2	1	1
MOC47	0.92	6	4	3
MOC48	0.81	4	4	1
MOC50	0.46	2	1	1
RUL93	0.92	4	0	4

*Prior probabilities were estimated using Easton *et al.*³, Narod *et al.*¹⁷ and Shattuck-Eidens *et al.*¹².

^bHBC, Hereditary breast cancer; HBOC, hereditary breast and ovarian cancer.

^cMean age of onset <45 years.

of expected sizes were obtained after amplification of genomic DNA with each of the primer pairs (Fig. 1a). After amplification of the fragments from patients of the 35 selected families, we observed only normal, wild-type sized products. However, PTT analysis showed that five cases contained a mutation in one of the *BRCA1* alleles as, in addition to the normal protein product, a truncated protein was observed after *in vitro* transcription and translation of the fragments. Truncated proteins were generated from fragment A of family EUR17, from fragment B of families RUL47, RUL49 and RUL21 and from fragment C of family RUL77. In patients from families RUL47 and RUL49 the truncated proteins appeared to be of similar size, suggesting that both families might contain identical mutations.

Among the ten patients who requested genetic counselling, two (MOC46 and MOC47) were found to carry mutations in fragment A of exon 11 (not shown). Patient MOC47 derived from a family with multiple cases of ovarian and early-onset breast cancer, while MOC46 was treated for ovarian cancer at age 47 and had a paternal grandmother who died from breast cancer at age 50. She was investigated because her sister was considering prophylactic ovariectomy.

Segregation of the truncated *BRCA1* protein

The cosegregation of the truncated protein with the disease phenotype and the reconstructed disease haplotype, was

verified by performing PTT on individual family members from three different families (Fig. 2). In all cases the haplotype carrier status, in affected or non-affected members, concurred with the presence of the truncated protein. Note that in one patient in family RUL47, who was diagnosed with breast cancer at age 41, truncated protein was absent. Thus, PTT showed that this patient, despite belonging to a true *BRCA1*-family, did not inherit the disease allele. Another early-onset case of sporadic breast cancer was observed in family RUL49 (not shown). Interestingly, this family also contained a male breast cancer patient, but we were unable to retrieve pathological material from this deceased patient to establish whether or not this patient was a carrier of the *BRCA1* mutation.

PTT combined with RT-PCR

Two families, one site-specific breast cancer family (RUL5) and one breast-ovarian cancer family (RUL19), were negative for the PTT analysis of exon 11, although their lod scores (1.31 and 1.0, respectively) suggested *BRCA1* involvement. To examine the remaining coding region of *BRCA1*, we performed RT-PCR on total RNA isolated from fresh peripheral blood lymphocytes obtained from two patients and used these fragments for PTT analysis. Figure 3a shows the RT-PCR fragments (lane 1, exons 2–10, and lane 5, exons 12–24) together with the genomic PCR fragments of exon 11 (lanes 2–4). The RT-PCR of exons 2–10 resulted in a doublet, the largest band having the expected size while the lower was approximately 100 bp smaller. Sequence analysis of this smaller band revealed the absence of exons 9 and 10 (not shown). This doublet was observed in various intensity ratios in 10 out of 12 different samples analysed. Figure 3b shows the PTT analysis of all five protein encoding fragments from a control and the RUL19 patient. We observed a doublet at about 32 kD in lane 1 of the control. This probably reflects the alternative splicing of exons 9 and 10. In addition to the full-length product, a prominent extra band of 15 kD (or less) was observed in the RUL19 patient (lane 1). Its size suggested a mutation early in the 5'-region of *BRCA1*. No other aberrant bands were detected in the samples analysed of this patient nor of the RUL5 patient.

Analysis of *BRCA1* mutations

Direct sequencing validated our results obtained by PTT (Table 2). Most translation terminating mutations identified are frameshift mutations, due to either insertion or deletion of one or more nucleotides. As suggested by our PTT results, an identical nonsense mutation, leading to a stop at codon 780, was found both in family RUL47 and family RUL49. As both families also share a common disease haplotype at four markers tested, it is very likely that both families have a common origin.

With regard to RUL19, sequence analysis of the RT-PCR products revealed the presence of two aberrantly spliced products, one having a deletion of 22 nucleotides (bp 310–331) affecting exon 5 and one having a deletion of 92 nucleotides (bp 310–401) affecting exons 5 and 6. Indeed, after RT-PCR of exons 3–7, we clearly observed two smaller products in addition to the wild type product. Both deletions cause premature truncation at codons, 64 and 65 respectively, and affect the zinc finger sequence located in this region of *BRCA1* (ref. 5). Sequence analysis of the intron/exon boundaries of exon 5 using primers described by Friedman *et al.*¹¹ did

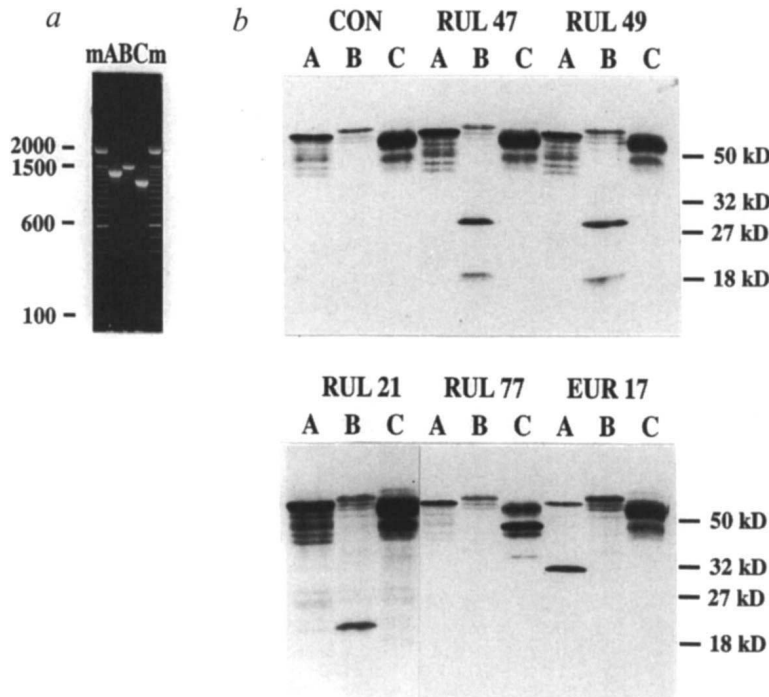


Fig. 1 PTT analysis of *BRCA1* exon 11 reveals premature termination. *a*, PCR products of fragments A, B and C obtained from control DNA have the correct size. Fragments were analysed on a 1.5% agarose gel and stained with ethidium bromide. *b*, PTT analysis of the exon 11 PCR fragments. PTT revealed truncated proteins of 27 kD using fragment B of RUL47 and RUL49 and of 20 kD when using fragment B of RUL21. A truncated protein of 46 kD was seen after using fragment C of RUL77 and of 29 kD using fragment A of EUR17. Translated products were analysed by 12% SDS-PAGE and made visible using 2,5-Diphenyloxazole and autoradiography.

not reveal any abnormalities compared to the wild type sequence.

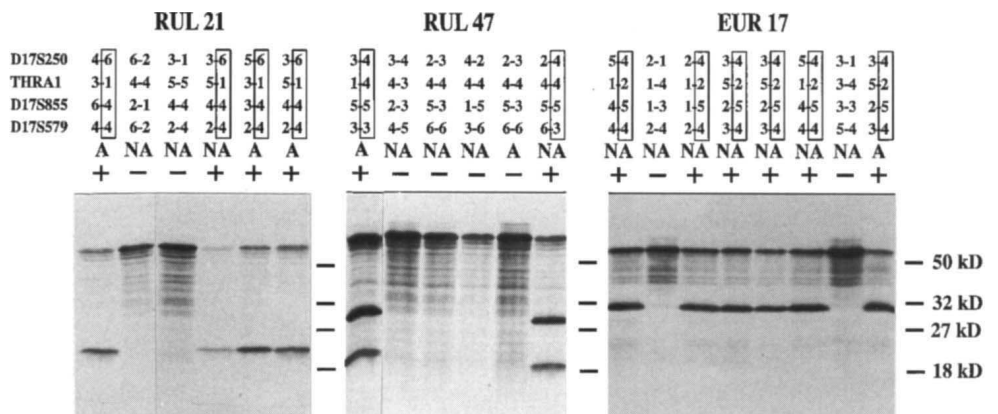
Discussion

A candidate gene for *BRCA1* was recently identified⁵, and confirmed by mutation analysis in high risk breast cancer families⁸⁻¹¹. The scattering of the mutations across the coding sequence of *BRCA1*, with no apparent clustering as yet¹², as well as the complex genomic organization of *BRCA1*, and the genetic heterogeneity of hereditary breast cancer, imply that rapid screening in a routine diagnostic setting will be a major technical challenge^{7,8}. We have evaluated the protein truncation test to detect *BRCA1* chain-terminating mutations. PTT, with its comprehensive screening of segments of up to 2,000 bases, poses an attractive alternative to single-stranded conformation polymorphism (SSCP) analysis. Furthermore, mutations identified by PTT will have an immediate clinical relevance, whereas amino acid substitutions, additionally detected by SSCP and direct sequencing, might still turn out to be false positives due to

rare polymorphisms with no causal relation to disease outcome. PTT therefore has a clear clinical advantage over SSCP when screening isolated index cases.

PTT analysis of exon 11 revealed six truncating mutations in 45 patients tested (13%), one of which was found in two unrelated families sharing the same disease haplotype. In a large collaborative study¹², 21 distinct truncating exon 11 mutations were found among 372 examined patients (6%). As in our study, the patients were mainly selected from high-risk families. This indicates that PTT appears to be at least as efficient as SSCP analysis in screening for these mutations. In contrast to SSCP and direct sequencing, PTT can screen the complete coding region of *BRCA1* in only five fragments. This is a major reduction in laborious work, although it would also require an RNA based approach, which is not routine in most diagnostic laboratories. Furthermore, the possibility remains that truncating mutations decrease mRNA stability and thus might decrease the detection of the mutated allele derived mRNA after RT-PCR/PTT. However, the detection of a truncated band, migrating at

Fig. 2 PTT analysis of members of *BRCA1* affected families RUL21, RUL47 and EUR17. The appropriate fragments (RUL21 and RUL47 fragment B, EUR17 fragment A) were amplified and subjected to PTT. Products were analysed on a 12% SDS-PAGE. Haplotype analysis of the individual family members are depicted at the top. Family members having a tumour are marked by an A (affected) otherwise by NA (non-affected), members containing the truncated protein are marked by +, if not by -.



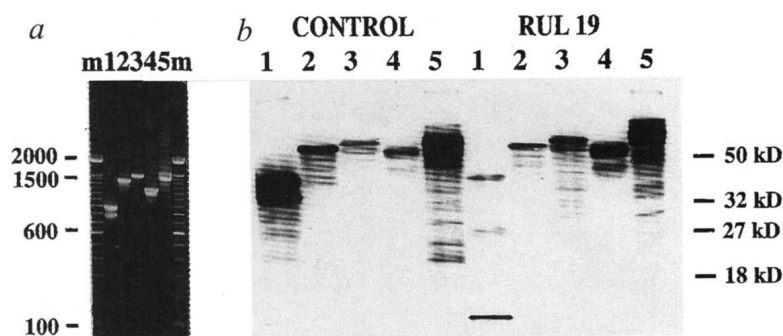


Fig. 3 PTT analysis of the complete coding region of *BRCA1* using fragments obtained by RT-PCR and genomic amplification of a control and RUL19, a 17q linked family. *a*, lanes 1 and 5 represent the amplified RT-PCR products (lane 1, exons 2–10, lane 5, exons 12–24). Lanes 2–4 represent the genomic amplification of exon 11 fragments A, B and C respectively. *b*, PTT analysis of these fragments. Compared to the control, a band of 15 kD or less can be detected in lane 1 of RUL19. The protein doublet in lane 1 of the control is probably derived from alternatively splicing. Products were analysed by 12% SDS-PAGE.

a different and patient specific position is a qualitative test which is sensitive to a level of 10–20% of the normal mRNA. Moreover, successful detection of carriers using RNA has been reported for DMD^{13,16} and FAP¹⁵. A more direct comparison of the sensitivities and efficiencies of the techniques on identical data sets is necessary to assess the relative values of each technique.

Both here and in Shattuck-Eidens *et al.*¹² the proportion of identified mutations is low ($\leq 20\%$), despite the fact that we concentrated on exon 11 and PTT does not identify missense mutations. Several factors may account for this low proportion. First, the fraction of families truly caused by *BRCA1* might be lower than expected. Only seven out of 29 haplotyped families had lod scores >0.60 . Furthermore, 23 out of 45 patients examined belonged to families in which, in addition to breast cancer, there was

at least one verified case of ovarian cancer, implying a greater prior probability of *BRCA1* involvement¹⁷. This prior probability may vary in populations: a linkage study¹⁸ of 16 Dutch breast/ovarian cancer families suggested a lower proportion being linked to *BRCA1*. Second, an unknown portion of the *BRCA1* mutations could be gross chromosomal aberrations (deletions, duplications, inversions), or located outside the regions covered by PCR, or regulatory silencing mutations. Such mutations would escape detection by PTT or SSCP since only wild-type alleles would be amplified. Finally, it is still possible that another gene, closely linked to *BRCA1*, might be responsible for an unknown portion of breast and breast/ovarian cancers. On the other hand, in four out of five families with strong evidence of linkage to *BRCA1* (lod scores >1), mutations were detected by PTT indicating that indeed most mutations are truncations. Only exhaustive analysis of *BRCA1* in families with conclusive high lod scores at flanking markers can determine whether the high negative test rate, as observed in a number of studies^{8,10,12} and here, is real or not.

Of the eight predisposing mutations we identified, seven were distinct and novel, occurring in codons not previously reported to be mutated. The exception was a 1-bp deletion from an (A)₇ string at position 1129–1135, where Simard *et al.*¹⁰ found an insertion of an A. Most of the small frameshift deletions and insertions reported so far occur at repetitive mononucleotide strings^{5,10}. In addition, more complex repeats are also affected, such as the deletion of AGAAG at codon 733, which itself is imperfectly repeated five times in the nucleotide string 2300–2321. The exact nature of the mutation in the RUL19 family still has to be established. Although we detected two aberrantly spliced mRNAs affecting exons 5 and 6, we found no changes in the exon 5 sequence. (However, a mutation interfering with mRNA processing or the PCR primer binding site could have resulted in amplification of only the normal allele.)

Table 2 Predisposing mutations in the *BRCA1* gene

Family	Codon	Mutation	Nucleotide change	Effect on coding seq.	Exon	Prior prob.	Lod score	Post. prob.
EUR17	431	1409insT	ins. of T after 1409	Frameshift	11	92%	1.11	$>99\%$
RUL21	731	2312del5	del. 5 bp from 2312	Frameshift	11	67%	1.18	$>95\%$
RUL47 ^a	780	Q780X	C to T at 2457	Gln780Stop	11	92%	-0.59	11%
RUL49 ^{a,b}	780	Q780X	C to T at 2457	Gln780Stop	11	0%	-1.1	0%
RUL77	1273	3937insG	ins. of G after 3937	Frameshift	11	81%	1.41	$>97\%$
MOC47	337	1129delA	del. of A at 1129	Frameshift	11	92%	na	na
MOC46	439	1436delT	del. of T at 1436	Frameshift	11	30%	na	na
RUL19 ^c	64	Aberrant splicing	del. 22bp after 309	Frameshift	5	92%	1.0	$>99\%$
	64	Aberrant splicing	del. 92 bp after 309	Frameshift	5+6			

^aBoth families have a case of sporadic breast cancer and thus a negative lod score.

^bAccording to Narod *et al.*¹⁷, the prior probability in families with male breast cancer is 0% (95% confidence interval). In the absence of male breast cancer, the prior probability in the RUL49 family would be 92% (Menko *et al.* manuscript in preparation).

^cAberration observed at cDNA level only.

Table 3 BRCA1 primers

Name	Position 5'-3'cDNA ^c	Exon	Purpose	Fragment length ^d PCR products
Genomic PCR				
BR11F1 ^a	793-813	11	Fragment A	1333
BR11R1	2125-2103	11	Fragment A	
BR11F2 ^a	1921-1943	11	Fragment B	1463
BR11R2	3383-3359	11	Fragment B	
BR11F3 ^a	3061-3082	11	Fragment C	1123
BR11R3	4183-4161	11	Fragment C	
RT-PCR with nested sets				
BR1F1	36-57	1	1 st PCR exons 2-10	
BR11R1	2125-2103	11	1 st PCR exons 2-10	
BR2F2 ^a	100-123	2	2 nd PCR exons 2-10	880
BR11R4	979-958	11	2 nd PCR exons 2-10	
BR11F4	4011-4032	11	1 st PCR exons 12-24	
BR24R1 ^b		24	1 st PCR exons 12-24	
BR11F5 ^a	4153-4173	11	2 nd PCR exons 12-24	1541
BR24R2	5693-5672	24	2 nd PCR exons 12-24	

^aT7-primer with promoter and sequence for initiation of translation: GCTAATACGACTCACTATAGGAACAGACCACCAITGG. *BRCA1* primer sequence at 3'-position. The open reading frame starts with the ATG codon.

^bPrimer sequence GTAGCCAGGACAGTAGAAGGA: exon 24 reverse primer¹¹.

^cPosition according to sequence BRCA1 by Miki *et al.*⁵, accession number U14680.

^dLength without T7 primer sequence as described above.

So far, the majority of the breast and breast/ovarian cancer patients cannot be traced back to *BRCA1* mutations. Simple, rapid and efficient mutation screening techniques will be necessary to stepwise reduce the diagnostic workload, especially since it is reasonable to expect a large increase in the number of requests for predictive DNA-testing. As expected from the finding that 85% of the *BRCA1* mutations lead to truncated proteins, our study shows that PTT is an attractive method to apply in an early phase of *BRCA1* mutation screening.

Methods

DNA and RNA isolation. Genomic DNA was prepared from heparinized blood samples as described¹⁹. For the isolation of total RNA, peripheral blood lymphocytes were purified using Histopaque 1077 (Sigma) followed by rinsing in cold PBS. The resulting cell pellets were subjected to extraction with RNazol B as described by the manufacturer (Cinna, Biotecx Laboratories Inc.).

PCR on genomic DNA. For PTT analysis, 3 overlapping fragments

covering exon 11 were amplified using 100 ng of genomic DNA and primer sets (Table 3). 1× PCR reaction of 50 µl contained 5 µl 10× PCR buffer (166 mM (NH₄)₂SO₄, 670 mM Tris-HCl pH 8.8, 67 mM MgCl₂, 100 mM beta-mercaptoethanol), 1,500 mM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, 10% v/v DMSO, 50 µg BSA and 2 U Amplitaq *Taq* polymerase (Perkin-Elmer-Cetus). 32 cycles of PCR (at 93 °C for 1 min, at 58 °C for 1 min, and at 72 °C for 4 min on a Perkin-Elmer-Cetus DNA thermal cycler) were followed by an incubation at 72 °C for 5 min. The PCR products were subsequently analysed on a 1.5% agarose gel and used for PTT.

Reverse transcription and nested PCR (RT-PCR). Samples of total RNA (1-3 µg) were incubated in 32 µl TE (10 mM Tris pH 7.5, 0.1 mM EDTA) containing 100 ng of random primer (Promega) at 65 °C for 10 min. The sample was then snap-chilled on ice and made to total 60 µl with a premix containing 12 µl 5× RT (BRL), 6 µl 100 mM DTT (BRL), 6 µl 10 mM dNTPs (Pharmacia), 1 µl RNasin (Promega) and 600 U MMLV reverse transcriptase (BRL). The reaction was incubated at 42 °C for 1 h. For the first PCR, a 20 µl mixture containing 2.5 µl 10× PCR buffer (described above), 1,500 mM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, 12.5% v/v DMSO, 25 µg BSA, 1 U Amplitaq *Taq* polymerase (Perkin-Elmer-Cetus) was added to 5 µl of reverse transcription reaction and covered with mineral oil. PCR was performed as above. For the nested (2nd) PCR, samples of the previously obtained product (3 µl) were added to a 50 µl mixture containing 5 µl 10× Supertaq PCR buffer, 5 µl 2 mM dNTPs, 20 pmol forward primer, 20 pmol reverse primer and 0.2 U Supertaq polymerase (HT Biotechnology, Cambridge, England). Nested PCR was carried out similarly to the first PCR. For primers, see Table 3.

Protein truncation test. Modified primers, containing a T7 promoter and an eukaryotic translation initiation sequence were used to generate PCR products suitable for PTT analysis. PTT analysis was carried out by adding 200-400 ng T7-PCR product to the TnT/T7 coupled reticulocyte lysate system (Promega). The synthesized protein products were separated on a 12% SDS-polyacrylamide minigel system. Fluorography was obtained by washing the gels in DMSO/PPO. Dried gels were exposed for 16-40 h for autoradiography.

Direct sequence analysis. Appropriate PCR fragments were synthesized and direct sequence analysis was performed with the USB PCR product sequencing kit, using ³⁵S-dATP as isotope. The samples were analysed on a 6% acrylamide gel at 60 W for various times. Gels were fixed, dried and exposed to X-ray film.

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