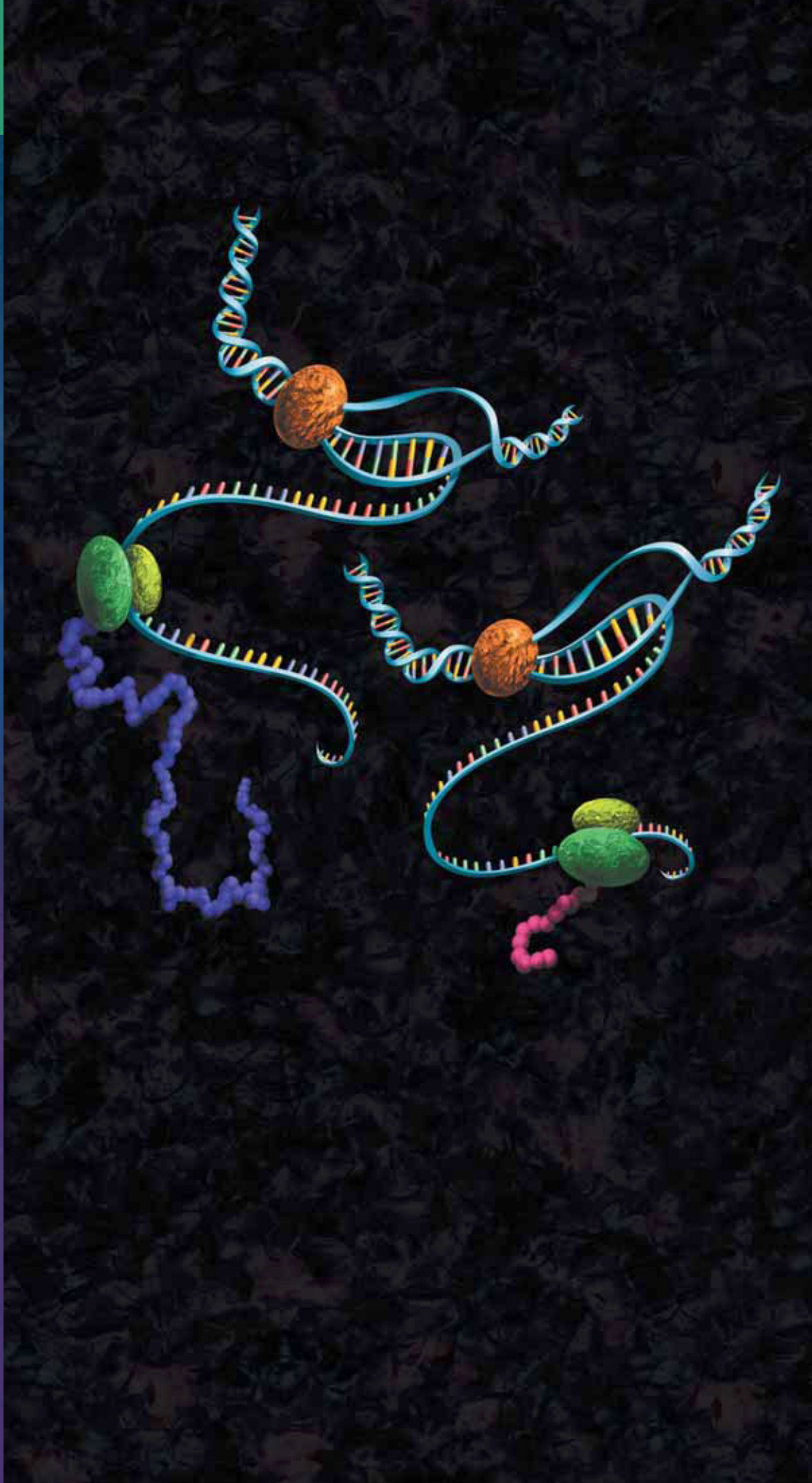
CHAPTER

4

About the Image:
This diagram depicts production of a truncated protein (right) as compared to a normal length protein (left) using the Protein Truncation Test. This test has been used, in vitro, to determine whether a gene mutation results in a shortened translation product that may lead to a cancerous cell.



Chapter Four: The Protein Truncation Test

Contents

Introduction

Mutations in a gene can range from large deletions to single point mutations. Many of the large deletions or translocations can be readily detected. For example, 95% of the cases of chronic myelogenous leukemia contain the Philadelphia chromosome, which is a translocation of part of chromosome 22 to chromosome 9. The abnormality can be detected by Southern blotting as aberrant or additional reactive bands when compared to normal samples (1). In this translocation, the *abl* proto-oncogene is translocated into the *bcr* gene resulting in the expression of a bcr-abl fusion protein. The chimeric transcript can be readily detected by RT-PCR(f) (2). Point mutations or small deletions, however, are much more difficult to detect. In Duchenne muscular dystrophy (DMD), for example, one third of the reported mutations in the gene *DMD* are not detectable as intragenic deletions or duplications (3–5). Techniques such as single strand confirmation polymorphism (6) can detect sequence differences but cannot distinguish between a polymorphism that may result in no phenotype (e.g., conservative amino acid change) and a polymorphism with a definite effect on the protein produced (e.g., premature termination of sequence).

A rapid solution to these problems can be achieved through a procedure known as PTT (protein truncation test).

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*For Laboratory Use.

PTT Principle

A simple way to judge whether a mutation results in a truncation or not, is to translate the protein in vitro. Roest *et al.* (7) developed the protein truncation test (PTT) to rapidly screen for these mutations. PTT is composed of four steps: i) isolation of nucleic acid, either genomic DNA, total RNA or poly (A)+ RNA; ii) amplification of a specific region of the gene of interest; iii) in vitro transcription and translation of the product of the amplification reaction; and iv) detection of the translation products. The shorter protein products of the mutant alleles are easily distinguished from the full-length protein product of the normal allele (Figure 1). PTT has been used to analyze many genes in addition to *DMD* (Table 1).

Amplified sequences for PTT can be generated across the entire protein coding sequence or they can be generated to specific exons. The key feature of PTT is a specifically designed PCR primer to allow coupled in vitro transcription/translation of the amplified sequence. The primer contains a T7 bacteriophage promoter sequence at the 5′-end that directs transcription. Usually, additional nucleotides are present upstream of the T7 promoter. Even the addition of a single G nucleotide upstream of the promoter increases the transcriptional efficiency (8). While T7 is the most commonly used promoter, T3 RNA polymerase promoter can be used as well. SP6 promoters are not well-suited for coupled transcription/translation of linear DNA (9). Promega offers a system specifically for the expression of PCR products, the TNT® T7 Quick for PCR DNA[c,d,e] (Cat.# L5540)*. A 3–6bp spacer separates the promoter sequence from an optimal eukaryotic translation initiation sequence, which includes the initiation codon ATG. The optimal eukaryotic translation initiation sequence is referred to as a Kozak consensus sequence (10). The bacteriophage promoter, spacer and Kozak sequence are followed by sequences specific to the target (Table 2). At the 3′-end of the target, the primer can include a stop codon if the amplified sequence does not contain the native stop codon (9). Restriction enzyme recognition sites can also be engineered into both primers to aid

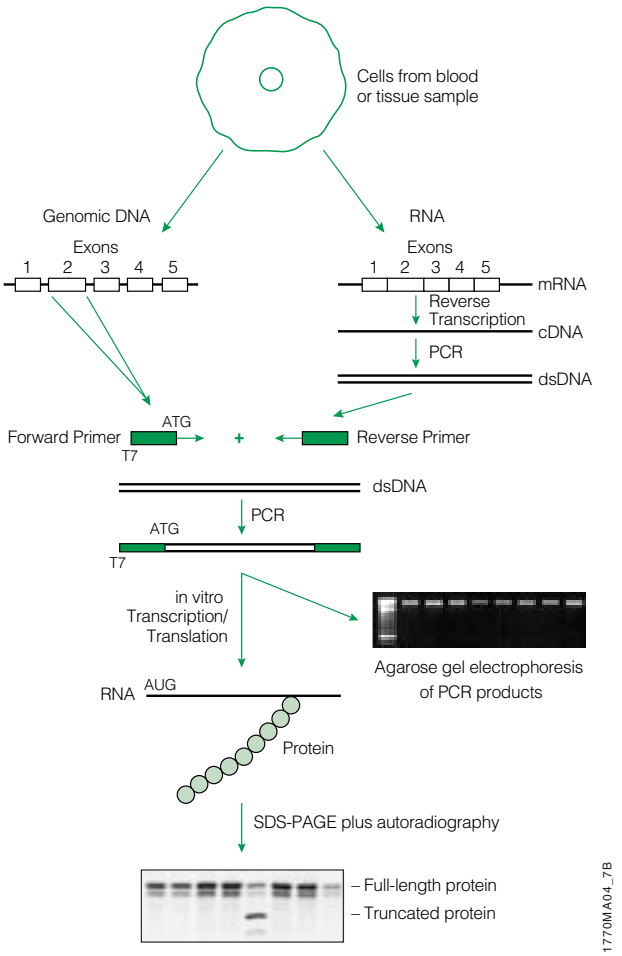

Figure 1. Schematic diagram of the Protein Truncation Test.

in subcloning of the PCR product if verification of a mutation is needed. The advent of PCR product cloning vectors has abrogated the need for inclusion of the restriction sites into PCR primers (11).

Source Considerations

The PTT test can be applied to individual exons of a gene via amplification of genomic DNA. Hogervorst *et al.* (12) analyzed genomic DNA of stored heparinized blood for mutations in the breast and ovarian cancer gene, *BRCA1*. Greater than 75% of the reported mutations in *BRCA1* result in truncated proteins. Primers were designed to amplify exon 11, which encodes 61% of the *BRCA1* gene product. Members of 35 families were analyzed, and all produced the correct size of PCR(f) product from the exon. The PCR product was transcribed and translated in vitro with [^{35}S]methionine and analyzed by SDS-PAGE and autoradiography. Six mutations resulting in truncated proteins were identified. The mutant PCR products were directly sequenced and were found to be the result of either insertions or deletions yielding frameshift mutations and premature stop codons. Genomic DNA has also been used to analyze the genes *BRCA2* (13), *APC* (14,15) and *PLEC1* (16) by PTT.

Use of genomic DNA as the source of nucleic acid for PTT has some drawbacks in that individual exons must be analyzed. To analyze the entire coding sequence of a gene like *BRCA1*, 24 individual exons would need to be amplified and analyzed. Besides requiring a large number of amplifications, assuming all the exons are large enough to translate, analysis of the individual exons could miss truncation mutations that could result in aberrant exon splicing. In the same study that amplified exon 11 of the *BRCA1* gene from genomic DNA for PTT analysis, Hogervorst *et al.* (12) isolated total RNA from freshly isolated peripheral blood lymphocytes. The sequences corresponding to exons 2–10 were amplified by RT-PCR(f) and analyzed by PTT. One subject had a mutation in one allele that resulted, first, in a smaller RT-PCR product and, second, in a truncated protein by PTT. The mutation was directly sequenced and resulted from aberrant splicing of exons 9 and 10. Thus, using RT-PCR and PTT, larger portions of a gene can be amplified and analyzed, picking up aberrant splicing mutations not identified by analysis of the exons via amplification of genomic DNA. In most cases, when RT-PCR is used as the method to generate targets, the entire coding region is broken into several smaller fragments. For example, three amplifications were used to test the entire coding region of the *TSC2* gene by PTT (17). When using multiple targets to span an entire coding region, the amplimers should overlap so that a mutation at the 3′-end of one target (that does not cause a

Table 1. Genes Analyzed with the Protein Truncation Test[a].

Condition	Gene	Ref.
Familial Adenomatous Polyposis	*APC*	14,15
Hereditary Desmoid Disease	*APC*	22
Ataxia Telangiectasia	*ATM*	23
Hereditary Breast and Ovarian Cancer	*BRCA1*	12
	BRCA2	13
Familial Hypocalciuric Hypercalcemia	*CASR*	24
Cystic Fibrosis	*CFTR*	25
Chorioderemia	*CHM*	26
Duchenne Muscular Dystrophy	*DMD*	7,27
Fanconi Anaemia	*FAA*	28
Congenital Muscular Dystrophy	*laminin-α2*	29
Hereditary Non-Polyposis Colorectal Cancer	*hMSH2*	30
	hMLH1	31
Neurofibromatosis Type 1	*NF1*	32
Neurofibromatosis Type 2	*NF2*	33
Aniridia	*PAX6*	34
Paroxysmal Nocturnal Haemoglobinuria	*PIG-A*	35
Polycystic Kidney Disease	*PKD1*	36,37
Epidermolysis Bullosa with Muscular Dystrophy	*PLEC1*	16,44–46
Dystrophic Epidermolysis Bullosa	*COL7A1*	43
Breast Cancers, Gliomas, Melanomas	*PTEN/MMAC1*	38,39,40
Rubenstein-Taybi Syndrome	*RTS*	41
Familial Tuberous Sclerosis	*TSC2*	17

[a]*More references available in The Protein Truncation Test Bibliography (BL002) and Mutation Detection (BR043) also available on the Internet at www.promega.com*

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significant change in molecular weight) will be detected in another target having the same codons near the 5′-end.

Detection and Primer Design

The detection method for PTT products must be considered when designing primers for amplification (18). Typically, [^{35}S]methionine is the label of choice but other labels such as [^{35}S]cysteine and [^{3}H]leucine could be used as well. Thus, the amplified segments should contain one or more of these amino acids. The reactions are resolved on an SDS-PAGE gel and either directly dried or fluorographically enhanced and exposed to X-ray film (9). The dried gels can also be analyzed by phosphorimaging. When radioactive incorporation is not an option, non-radioactive techniques are available. Proteins can be tagged with biotin by inclusion of biotinylated lysine tRNA in the translation reaction (9,19). The biotin moiety is then detected with a streptavidin-enzyme conjugate and developed via either a colorimetric or chemiluminescent reaction (19). For example, PTT has been applied to the *APC* gene using translation with a biotinylated lysine tRNA (20). Other methods for non-radioactive detection include the inclusion of an epitope tag in the 5′-primer so that the translation products can be analyzed by Western blotting with an antibody that binds the epitope (21). When dealing with a heterozygous condition, both the normal and mutant targets will be amplified and both the truncated and full-length protein will be detected, unless the allelle is on the X or Y chromosome of male subjects, no matter which detection method is chosen.

PTT offers a quick and easy method for analyzing a protein coding sequence for truncation mutations. However, the method has some limitations. If the truncated sequence does not translate well or does not contain the appropriate amino acid for labeling, the mutation could be overlooked. Also, if the truncation is very near the 3′-end of the target, truncation could be missed due to the inability of SDS-PAGE to resolve such differences. If the mutations are very near the 5′-end of the coding sequence, the mutation could be missed as well. Refinements of PTT detection, such as the incorporation of an epitope tag into the 5′ PCR primer (21), could allow detection of these mutations, since incorporation of a specific amino acid is not needed for detection. Finally, incorporation of fluorescence-tagged amino acids may simplify the detection of proteins by PTT and can possibly be used for quantitation of the mutant protein (18).

Table 2. Sequences of Different T7-Modified Oligonucleotide Primers for In Vitro Transcription and Translation[a].

Restriction Site Sequence	T7 Bacteriophage Sequence	Spacer	Eukaryotic Translation Initiation Sequence	Ref.
GGATCC	TAATACGACTCACTATAGGG	AG	CCACC ATG	13,42
GGATCC	TAATACGACTCACTATAGGG	AG	CCACC ATG G	30,31
GGATCC	TAATACGACTCACTATAGG	AACAG	CCACC ATG	7,15
nnn[b]	TAATACGACTCACTATAGG	AACAG	CCACC ATG G	12,28

[a]Sequences provided are for only the upstream portion of the 5′ primer that is not gene specific. For gene-specific use, the eukaryotic translation initiation sequence would be followed by 17–20 bases exactly complementary to the sequence of interest.
[b]n = any nucleotide