PRODUCTION
OF RECOMBINANT HUMAN BRCA1 ENCODED PROTEINS
in
E.coli

A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE
OF BİLKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

BY
BERNA S. ÖZÇELİK
AUGUST 1997

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BERNA S. ÖZÇELİK Berna S. Özçelik.

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TO THE MEMORY OF MY FATHER,

ENVER ÖZCELİK

WITH ENDLESS LOVE AND YEARNING

AND

FOR MY MOTHER AND SISTER

KERİMAN and AYSİN

I certify that I read this thesis and in my opinion it is fully adequate, in scope and in quality, as a dissertation for the degree of Master of Science.

Prof. Dr. Mehmet Öztürk

I certify that I read this thesis and in my opinion it is fully adequate, in scope and in quality, as a dissertation for the degree of Master of Science.

Assis. Prof. Dr. Işık Yulug

I certify that I read this thesis and in my opinion it is fully adequate, in scope and in quality, as a dissertation for the degree of Master of Science.

Prof. Dr. Semra Kocabıyık

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Prof. Dr. Mehmet Baray

Director of Institute of Engineering and Science

ABSTRACT

Production of Recombinant Human BRCA1 Encoded Proteins in E.coli.

Berna S. Özçelik

M.S. in Molecular Biology and Genetics

Supervisor: Prof. Dr. Mehmet Öztürk

August 1997, 137 pages

Among the risk factors that cause breast cancer, heredity emerges as the major determinant. BRCA1 is the first gene which was found to be associated with inherited early-onset breast cancer. BRCA1 is spread over a 100 kb region on human chromosome 17q21 and consists of 22 coding exons which are transcribed into a 7.8 kb long mRNA. This mRNA is abundant in breast and ovarian tissues and encodes a polypeptide of 1863 amino acids. The exact cellular function of BRCA1 remains to be elucidated. As there are many gaps of knowledge and many conflicts about the cellular functions of BRCA1, new points of views and technical approaches should be generated. As such studies require the presence of purified protein products, we aimed to express and purify BRCA1 encoded proteins. In this study we cloned the BRCA1 gene in four overlapping fragments into the pCR-Script Amp, sk (+) cloning vector and subcloned the carboxyl terminal into the pQE expression vector. The 73 kD gene product was purified by affinity chromatography.

ÖZET

İnsan BRCA1 Rekombinant Proteininin E.*coli*'de Üretilmesi

Berna S. Özçelik

Yüksek Lisans Tezi, Moleküler Biyoloji ve Genetik Bölümü

Tez yöneticisi: Prof. Dr. Mehmet Öztürk

Ağustos 1997, 137 sayfa

BRCA1 geni, ailesel, genç yaşta baş gösteren meme kanseri ile ilişkisi olduğu saptanan ilk gendir ve onyedinci kromozomda yer almaktadır. BRCA1 geni 17. kromozomun q kolunda 100 kilobazlık bir alana yayılmıştır. 24 ekzonu vardır ve bunlardan 22 tanesi transkripsiyon ile 7.8 kilobaz uzunluğunda bir mRNA kodlar. Bu mRNA'nın en bol memede ve overde bulunduğu ve translasyonu sonucu 1863 amino asit uzunluğunda bir polipeptid oluştuğu saptanmıştır. BRCA1 proteininin hücredeki rolü henüz tam olarak bilinmemektedir. BRCA1'in hücredeki işlevleri hakkında pek çok bilgi eksikliği ve çelişkiler mevcut olduğundan, yeni bakış açılarının ve tekniklerin geliştirilmesi gereklidir. Bu tip çalışmalarda saflaştırılmış proteine ihtiyaç duyulduğundan, çalışmalarımızı bu yöne yönlendirdik. Bu çalışmada BRCA1 genini dört kesişen fragman halinde pCR-Script Amp, SK (+) vektörüne ve karboksil terminalini bir ekspresyon vektörüne klonladık. 73 kilodaltonluk bir ekspresyon ürününü afinite kromatografi ile saf bir şekilde izole ettik.

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ABBREVIATIONS

amp.	amplification
bisacrylamide	N, N, methylene bis-acrylamide
bp	base pairs
c-terminus	carboxyl terminus
cDNA	complementary deoxynucleic acid
kb	kilobasepairs
kD	kilo daltons
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	diaminoethane tetra-acetic acid
EtBr	ethidium bromide
HBC	Hereditary Breast Cancer
IPTG	isopropylthio-β-D-galactoside
kan	kanamycin
LFS	Li-Fraumeni syndrome
LB	Luria-Bertani media
LOH	Loss Of Heterozygosity
ml	mililiter
mg	miligram
MQ	MilliQ water
nm	nanometer (1/109 of a meter)

N-terminus	amino terminus
MW	molecular weight
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
TAE	tris-acetic acid-EDTA
TBE	tris-boric acid-EDTA
TE	tris-EDTA
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
Tris	tris (hydroxymethyl)-methylamine
TSG	Tumor Supressor Gene(s)
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER I

INTRODUCTION

1-1 GENERAL INTRODUCTION

Breast cancer is one of the most common fatal malignancies affecting females in developed countries. It is estimated that one of eight women will develop breast cancer by age 95 (Miki *et al.*, 1994). In United States more than 180,000 new cases were observed in 1996, and each year about 45,000 women die of breast cancer in this country (Parker *et al.*, 1996). The etiology of breast cancer involves a complex interplay of genetic, endocrine, and dietary factors that are superimposed on the physiological status of the host. Among these risk factors family history emerges as the major determinant (Schildkraut *et al.*, 1989). Studies have shown that genetic factors contribute to approximately 5- to 10- % of all cases but 25% of cases diagnosed before age 30 (Claus *et. al.*, 1991). The two breast cancer susceptibility genes identified (BRCA1 and BRCA2) are believed to be responsible for more than 80% of familial cases diagnosed before age 80 (Ford *et al.*, 1994). Presence of a positive family history was observed even in sporadic cases and about one third of women with sporadic breast cancer have one or more first degree relatives having breast cancer (Marcus *et al.*, 1996). Breast cancer is 100 times less frequent in man than women and it is also rare in young women (<20 years of age). Its frequency increases progressively from 20 to 45 years of age, stabilizes around 55 years, then increases abruptly in older age. About 85% of breast cancers occur after 40 years of age. Life style and dietary factors also contribute to

occurrence of breast cancer as secondary risk factors, that daily exercise and dietary intake of phytoestrogens are thought to decrease the risk of developing breast cancer (Knekt *et al.*, 1996). Controversially daily uptake of alcohol increases the risk of breast cancer by 40% (Holmberg *et al.*, 1995). Treatment of advanced breast cancer is often pointless and disfiguring, making early detection very important by means of the medical management of the disease (Miki *et al.*, 1994).

Breast cancer is a tumor of the mammary gland. The anatomy of breast is shown in figure 1-1. The breast tissue is composed 10-15 galactophorous channels. The mammary gland is formed by a surface epithelium attached to myoepithelial cells and continuously exposed to hormonal factors. The 17β-estradiol (active estrogen) and progesterone play a critical role in the physiological regulation of mammary gland. Estrogen act mostly on the galactophours and induce mitotic activity of epithelial cells. The progesterone, which is secreted periodically, during the second phase of menstrual cycle, blocks mitotic activity induced by estrogens.

Breast tumors are classified as benign and malignant tumors. Among benign tumors, fibroadenomas are the most frequent forms of which develop before the age of 30 years. They are characterized by a glandular proliferation localized with a variable fibrous component. More than 98% of malignant breast tumors are carcinomas and are classified according their histological features as shown in table 1-1.

In situ carcinomas can either be ductal or lobular, arising from the ductal epithelium or from the epithelium of the lobules, respectively. Non-infiltrating carcinomas occur in the epithelium of galactophour channels or the lobules. They do not infiltrate the neighboring connective tissues. They represent about 7% of breast carcinomas. Infiltrating carcinomas are mostly ductal carcinomas (70%). Paget disease is a carcinoma composed of large tumor cells infiltrating the epidermis of the nipple. They represent about 2% of breast cancers and they are associated to another mammary carcinomas (Rubin E., Farber J. L.).

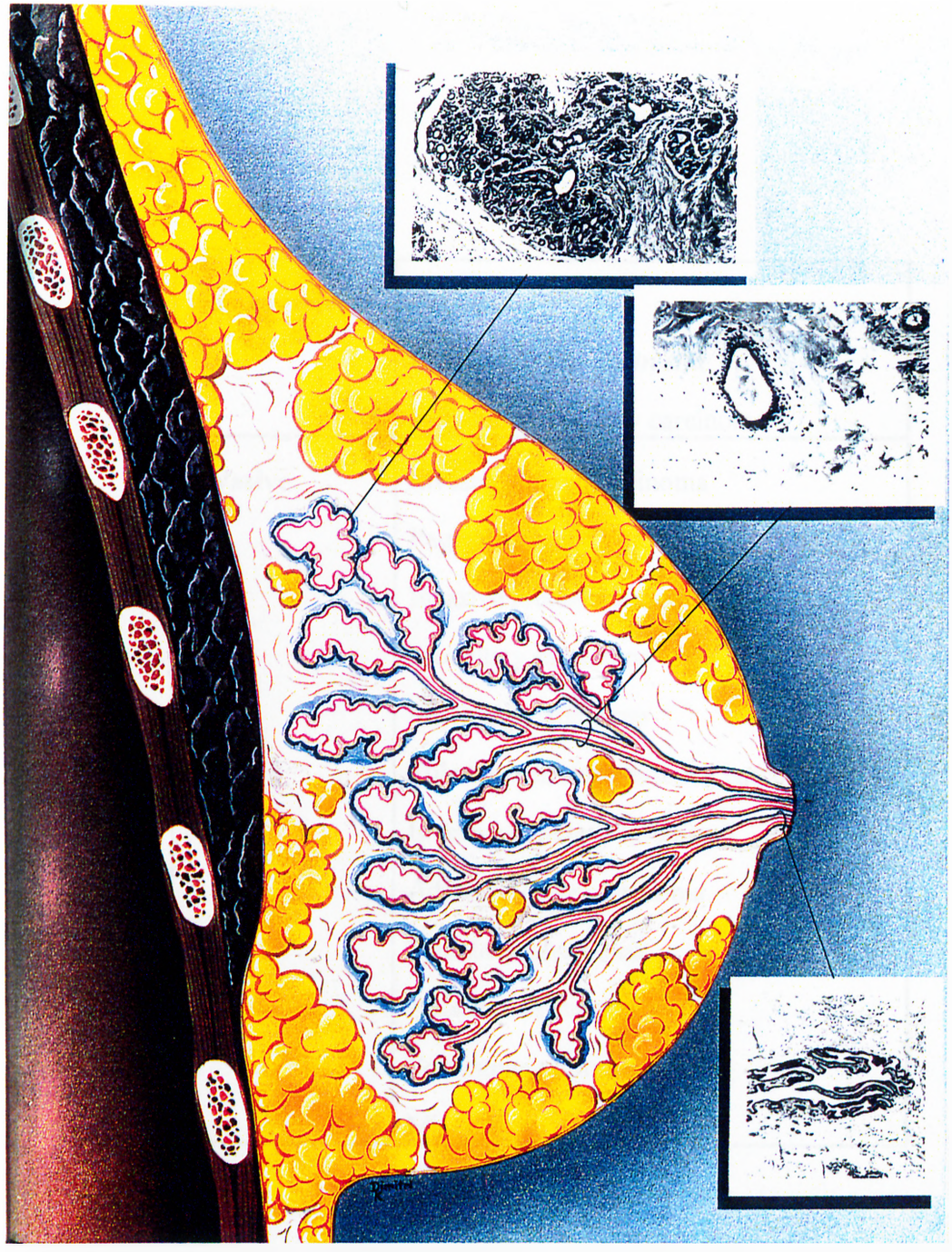


Figure 1-1: The anatomy of the breast

Table 1-1: World Health Organization Classification of carcinomas (Rubin E., Farber J. L.)

Non-invasive	Intraductal
	Lobular carcinoma *in situ*
	Intraductal papillary carcinoma
Invasive	Invasive ductal carcinoma
	Paget's disease
	Invasive lobular carcinoma
	Medullary carcinoma
	Mucinous carcinoma
	Tubular well-differentiated carcinoma
	Invasive papillary carcinoma
	Adenoid cystic carcinoma
	Secretory carcinoma
	Apocrine carcinoma
	Carcinoma with metaplasia
	Mixed type

1-2 MOLECULAR GENETICS OF BREAST CANCER

Development of cancer involves the activation of proto-oncogenes and the inactivation of tumor suppressor genes (Sager , 1989); (Weinberg, 1985). A schematic representation of the genetic alterations that take place in the progression from normal breast epithelium to metastatic carcinoma is shown in figure 1-2.

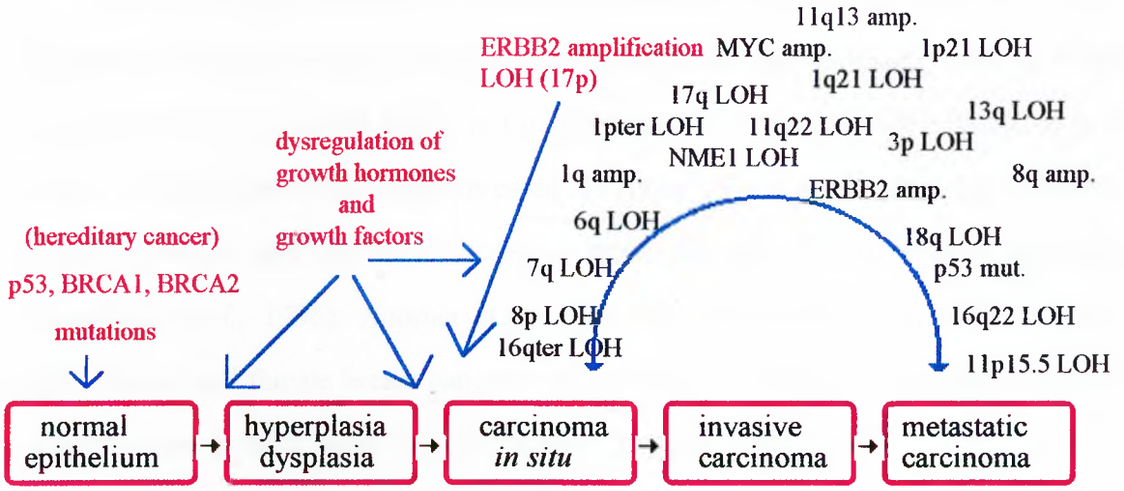


Figure 1-2: Genetic alterations that take place in the progression from normal breast epithelium to metastatic carcinoma

Although multiple cytogenetic abnormalities have been detected in breast cancer, few abnormalities have been noted to be consistent. Results of cytogenetic and direct DNA studies have showed that the most commonly affected chromosomes were 1, 6, 8, 13 and 17 (Wendy *et al.*, 1990). These abnormalities include, amplification of oncogenes, point mutations, and loss of tumor suppressor genes.

Gene amplification results in an increase in the number of copies of a gene, which in turn leads to increased mRNA synthesis and increased protein production.

The oncogene c-*erb*B-2 (HER-2 or *neu*) which is homologous to epidermal growth factor (EGF) is a cell surface growth factor receptor with receptor tyrosine kinase activity. Although it is not known whether the amplification of the *erb*B-2 gene plays a role in the initiation of mammary carcinogenesis, it was reported that it correlates with the aggressive behavior of breast cancer (Slamon *et al.*, 1989). This gene is amplified in adenocarcinomas of a variety of organs, including breast, where the gene is amplified in approximately 28% of cases (Berns *et al.*, 1995).

BCL1 (cyclin D) is another oncogene that was found to be amplified in breast carcinomas. Overexpression of BCL1 was observed at approximately 30% of breast cancers (Gillett *et al.*, 1994). BCL2 is a gene involved in apoptosis that it functions as an inhibitor of apoptosis when overexpressed. Recently it was shown that the expression pattern of BCL2 and p53 was altered in approximately 33% of breast carcinomas (Siziopikou *et al.*, 1996). Another study concerning the mutations in BCL2 and p53 genes in male and female breast cancers showed that $p53^{-}/BCL2^{+}$ phenotype was more frequently seen in male breast cancers (Weber-Chappuis *et al.*, 1996).

MDM2 is an oncogene which is transcriptionally transactivated by p53 tumor suppressor gene, and its elevated expression results in the downregulation of p53 activity (Momand *et al.*, 1992) (Wu X. *et al.*, 1993). Analysis of MDM2 and p53 expression levels in breast carcinoma cells showed that, at about 4 to 9% of breast tumors, MDM2 expression levels were elevated up to 8 folds resulting in decrease in p53 expression levels (McCann *et al.*, 1995); (Marchetti *et al.*, 1995).

Overexpression of c-myc by several mechanisms is found in a variety of different tumors (Vijer M. J., 1993). The myc nuclear proto-oncogene, located on chromosome 8q24, was found to be amplified in about 6% to 33% and was found to be overexpressed in 17% primary breast tumors (Berns *et al.*, 1995).

Some tumor suppressor genes involved in breast carcinomas were elucidated by loss of heterozygosity (LOH) assays. In breast cancers the most common deleted

regions include chromosome 5q21 (APC), 17p (p53), 17q21 (BRCA1), 13q12-14 (BRCA2 and Rb) and chromosome 10 (gene of Cowden Disease).

In a study of sporadic breast cancers (n=34), it was shown that 28% of the cases there was LOH in APC locus (5q21) (Thompson A. M., *et al.*, 1993). ApcMin (Min, multiple intestinal neoplasia) is a point mutation in the murine homolog of the APC gene. Mice that are heterozygous for Min mutation (Min-/+) mice develop multiple intestinal adenomas, just like humans carrying germ-line mutations in APC. Female mice carrying the Min mutation are also prone to develop mammary tumors (Moser *et al.*, 1993).

Tumor suppressor genes RB and p53 appear to be important in breast cancer (Stanbridge, 1990). The RB tumor suppressor gene codes for a nuclear DNA binding phosphoprotein which is phosphorylated and dephosphorylated in a cell cycle dependent manner and the phosphorylation is required for activation of a cell cycle dependent transcription activator, E2F (Wang *et al.*, 1994). Inactivation of RB is observed in all retinoblastomas and in lesser proportion in other types of tumors, including breast carcinomas (Lee E. *et al.*, 1988). In a study of 223 cases, loss of Rb expression was found at 21% of primary breast cancers (Anderson *et al.*, 1996).

Like the RB protein, p53 is involved in the regulation of normal cell cycle and, when inactivated, leads to uncontrolled cell proliferation, leading to carcinogenesis. The p53 tumor supressor gene is localized to 17p and encodes 53 kD phosphoprotein with multiple important functions concerning cell fate, including cell cycle arrest, apoptosis and induction of DNA repair (Vogelstein B. *et al.*, 1992); (Lee S. *et al.*, 1995); (Smith M. L., *et al.*, 1995). The p53 gene is the most commonly mutated gene in human tumors (Levine *et al.*, 1991). Results of several studies have shown that incidence of breast cancer was increased in Li-Fraumeni Syndrome families (LFS) (Ford D. and Easton 1995). LFS is a dominantly inherited syndrome in which family members are at high risk of developing a wide spectrum of tumors, including breast tumors, at an early age (Li and Fraumeni, 1969). It is also shown that expression of mutant p53 increases from 13%

to 50% as breast cancers progress from early *in situ* to advanced metastatic lesions. This suggests that p53 mutation is an early event in breast cancer and is more common in the advanced forms (Davidoff *et al.*, 1991). Lindblom *et al.*, studied the LOH at p53 locus in 82 familial breast carcinomas from 79 different families (Lindblom *et al.*, 1993). Their results indicated that LOH in the p53 region was seen in 25-30% of breast cancer families, 40% of early-onset breast cancers and 10-15% of late-onset breast cancers (Lindblom *et al.*, 1993).

Certain rare abnormalities of the androgen receptor located on the X chromosome appear to be associated with male breast cancer. Wooster *et al.*, identified a family in which two brothers with breast cancer had a constitutional mutation in the androgen receptor gene (Wooster *et al.*, 1992).

Breast cancer is also associated with autosomal dominantly inherited Cowden's disease and autosomal recessively inherited ataxia-telangiectasia (AT). AT is a rare disease in which homozygotes develop a progressive cerebellar ataxia, hypersensitivity to ionizing radiation, immune dysfunction and a striking predisposition to cancer (Swift *et al.*, 1990). Heterozygotes for the disease also have an increased cancer incidence, with solid tumors of the breast, ovary, pancreas, stomach, and the bladder being most common (Swift *et al.*, 1987). Epidemiological studies have suggested that heterozygotes for the ataxia telangiectasia gene, AT, on chromosome 11q are at elevated risk of breast cancer (Lynch *et al.*, 1994).

The overexpressed oncogenes/LOH at tumor suppressor genes and their participation to breast cancer are summarized in Table 1-2

Table 1-2: Oncogenes and TSGs involved in breast carcinomas

name of the gene	participation to breast cancer (%)	reference
ONCOGENES		
c-erbB-2	28	Berns *et al.*, 1995
BCL-1	30	Gillette *et al.*, 1994
BCL-2	33	Siziopikou *et al.*, 1996
MDM2	4-9	McCann *et al.*, 1995
c-myc	6-33 (amplification) 17 (overexpression)	Berns *et al.*, 1995
TUMOR SUPRESSOR GENES		
RB	21	Anderson *et al.*, 1996
p53	25-30	Lindblom *et al.*, 1993
APC	28	Thompson *et al.*, 1993
BRCA1	50 (hereditary)	Takahashi *et al.*,1995
BRCA2	35 (hereditary)	Wooster *et al.*, 1994

It is also reported that some tumor types co-segregate with breast cancer. Heterogeneity of breast cancer is illustrated in figure 1-2 (Lynch *et al.*, 1994).

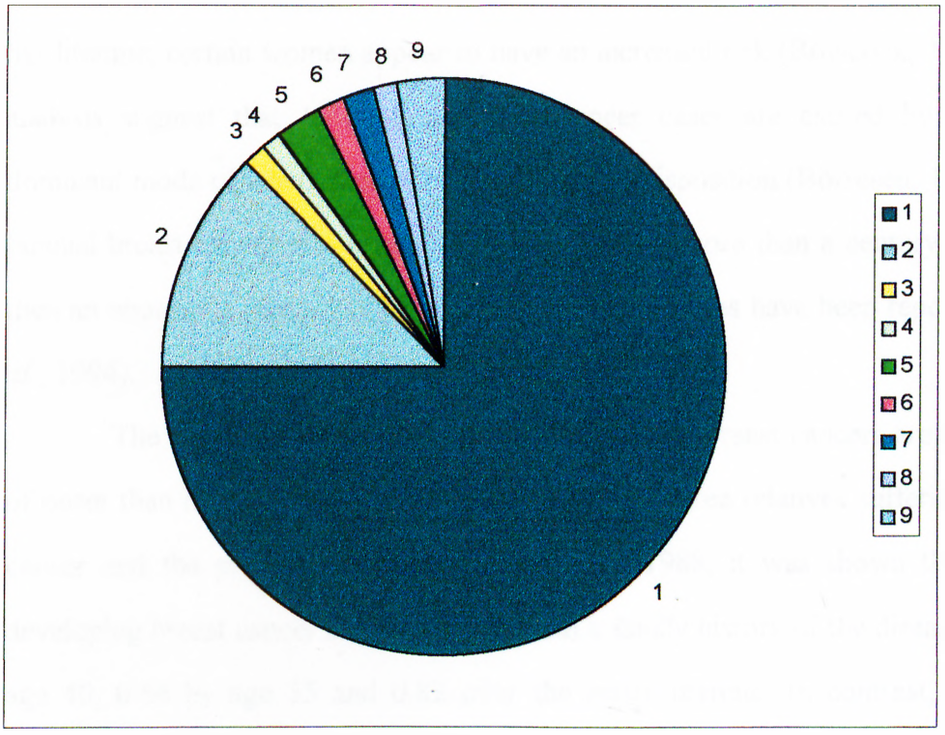


Figure 1-3: Heterogeneity of breast cancer
(1: sporadic breast cases, 2: polygenic breast cancers, 3: breast and miscellaneous tumors, 4: extraordinarily early onset breast cancer, 5: breast/gastrointestinal cancer, 6: breast/ovarian cancer, 7: site-specific breast cancer, 8: breast cancer and LFS, 9: breast cancer and Cowden's disease)

1-3 HEREDITARY FORMS OF BREAST CANCER

Although the incidence of breast cancer is estimated to be 1/9 for a woman over her lifetime, certain women appear to have an increased risk (Bowcock, 1993). Pedigree analysis suggest that 10% of all breast cancer cases are caused by an autosomal dominant mode of inheritance of breast cancer predisposition (Borresen, 1992). The first familial breast cancer was described by Paul Broca, more than a century ago and since then an enormous number of breast cancer-prone families have been reported (Lynch *et al.*, 1994).

The three important characteristics of familial breast cancers are the earlier age of onset than normal, presence of 3 or more first degree relatives suffering from breast cancer and the presenve of bilateral cancer. In 1988, it was shown that the risk of developing breast cancer for women who had a family history of the disease was 0.37 by age 40, 0.66 by age 55 and 0.82 over the entire lifetime. In contrast, risk of breast cancer in women without a family history was estimated to be 0.004 by age 40, 0.028 by age 55 and 0.081 over the entire lifetime. Females less than 15 years of age and males had a negligible risk (less than 0.001) (Newman *et al.*, 1988). These women who have increased risk harbor germ-line mutations that predispose to breast cancer susceptibility, and in general develop the disease at an earlier age. Such studies indicated the fact that 5-10% of breast cancer cases result from the inheritance of germline mutations in autosomal dominant susceptibility genes (Newman *et al.*, 1988).

Two genes have been identified that are believed to play an important role in the familial form of breast cancer. BRCA1 was the first locus associated with inherited early-onset breast cancer and was mapped to chromosome 17q21 by linkage analysis (Hall *et al.*, 1990). Subsequently it was shown that mutations in BRCA1 were responsible for almost all families with multiple cases of both breast and ovarian cancer, and approximately 50 % of the families with breast cancer only (Easton *et al.*, 1993).

There is also an increased risk for BRCA1 carriers to develop prostate cancer (3%) in males and colon cancer (4%) in both males and females (Ford *et al.*, 1994). Another breast cancer susceptibility gene BRCA2 was found to be located on chromosome 13q12-13 by linkage analysis in 1994 (Wooster *et al.*, 1994). Mutations at this locus were also involved in the development and progression of breast cancer roughly as much as BRCA1 but they do not appear to be associated with susceptibility to ovarian cancer although they are associated with cases of male breast cancer (Wooster *et al.*, 1994).

BRCA1, BRCA2 and p53 germ-line mutations do not explain all of the familial cases. Therefore it is believed that there must be other genes predisposing to breast cancer.

1-4 BREAST AND OVARIAN CANCER SUSCEPTIBILITY GENES

1-4.1 BRCA1

The first described breast and ovarian cancer susceptibility gene, BRCA1, was located to chromosome 17q-21 by linkage analysis (Hall *et al.*, 1990) and then isolated in 1994 by Miki *et al.* by positional cloning strategies (Miki *et al.*, 1994). The majority of breast cancer families with more than four affected members were linked to the BRCA1 region, suggesting a high penetrance of this gene (Easton *et al.*, 1993). BRCA1 is spread over a 100 kb region on chromosome 17q and consists of 24 exons, 22 of which are transcribed into a 7.8 Kb long mRNA which is abundant in breast and ovary (Miki *et al.*, 1994). Although alternatively spliced forms of the transcript have been identified the significance of the presence of these transcripts remain unknown (Lu *et al.*, 1996), (Xu *et al.*, 1995). The 7.8 Kb mRNA species encode a polypeptide of 1863 amino acids which contains a 123 amino acid long RING finger domain at the N-terminus (Miki *et al.*, 1994). Following the identification of the gene, mutation analysis

of breast cancer families showed that germline mutations of BRCA1 were responsible for approximately 50% of hereditary breast cancers (Friedman *et al.*, 1994). Somatic point mutations in BRCA1 in sporadic tumors were found to be very rare (Futreal *et al.*, 1994), but complete somatic deletion of one allele of BRCA1 was identified to occur approximately 50% of sporadic breast cancers and 70% of sporadic ovarian cancers (Takahashi *et al.*, 1995). Approximately 85% of mutations that have been identified throughout the coding region of BRCA1 give rise to truncated forms of protein that vary in length from 5% to 99% of full-length protein (Shattuck-Eidens *et al.*, 1995). Although any clustering of mutations had not been found, a founder effect had been observed in Ashkenazi Jewish families in which 185del AG mutation was frequently observed such that the carrier frequency of the BRCA1 185del AG mutation was approximately 1% among these individuals (Struewing *et al.*, 1995). In breast cancer patients who carry BRCA1 predisposing alleles, observations have shown that malignancy results from the loss of the wild-type allele suggesting that BRCA1 is a tumor suppressor (Neuhausen and Marshall, 1994). This hypothesis was supported by the identification of the fact that BRCA1 negatively regulates the proliferation of mammary epithelial cells (Thompson *et al.*, 1995) and that transfection of MCF-7 breast cancer cell lines with wild-type BRCA1 inhibits tumor progression (Holt *et al.*, 1996). In 1996 Chen *et al.* identified that BRCA1 is a 220 kD nuclear phosphoprotein and in 1997, BRCA1 was found to be located in perinuclear compartment of the endoplasmic reticulum-Golgi complex and in the tubes invaginating the nucleus (Coene *et al.*, 1997). Many line of evidence suggests that BRCA1 plays an important role during mammary epithelial cell proliferation and differentiation. As most of the breast cancer cases are associated with loss of BRCA1 in humans, it can be thought that this gene provides an important growth regulatory function in mammary epithelial cells (Lane *et al.*, 1995). Murine homologue of BRCA1 was found to be widely expressed in proliferating and differentiating cell types in the mouse during embryonic and mammary gland

development, and in adult tissues (Marquis *et al.*, 1995). Identification of the BRCT domain, which was found at the carboxyl terminal of BRCA1 and was conserved in 53BP1 (p53 binding protein) and the yeast DNA repair protein RAD9 in 1996, elucidated the fact that BRCA1 encoded proteins are likely to function in the cell nucleus and may be involved at cell-cycle checkpoints and DNA repair (Koonin and Altschul, 1996). Although BRCA1 was found to be related to the growth regulation of mammary epithelial cells, exact function of the gene and the proteins that it interact with were not elucidated till the identification of BARD1 (Wu *et al.*, 1996). Very recently BRCA1 was shown to be involved in DNA repair. In 1997, Scully *et al.*, found that BRCA1 was associated with RAD51 in mitotic and meiotic cells, suggesting that BRCA1 may participate in some nuclear processes such as recombination and DNA repair (Scully *et al.*, 1997(a)). Similarly, usage of hydrophobic cluster analysis in combination with linear methods of sequence analysis by Callebaut and Mornon , led to the identification of the presence of the repeated motif in the C-terminus of BRCA1 named BRCT, among several nuclear proteins closely related to cell-cycle regulation and DNA repair (Callebaut and Mornon, 1997). Very recently Scully *et al.*, hypothesized that BRCA1 is a component of RNA polymerase holoenzyme (Scully *et al.*, 1997(b)). BRCA1 will be discussed in more detail in chapter II.

1-4.2 BRCA2

The breast cancer susceptibility gene BRCA2 was located to chromosome 13q12.5 in 1994 (Wooster *et al.*, 1994). By performing genomic linkage analysis to 15 families that were not linked to BRCA1 but had multiple cases of early-onset breast cancer, Wooster *et al.*, localized the second breast cancer susceptibility gene roughly to a 6-cM interval on chromosome 13q12-q13, between the markers D13S1444 and D13S310, close to the marker D13S260. The further localization was done by Schutte *et*

al., (1995). Afterwards the gene was partially isolated by positional cloning in 1995 (Wooster *et al.*, 1995). The complete BRCA2 gene was then cloned in 1996 (Tavtigian *et al.*, 1996). The identified portion of BRCA2 cDNA consists of 11385 basepairs and lacks a poly adenylation signal and poly A tail. The BRCA2 gene encodes a 3418 amino acid long polypeptide that lacks significant homology to previously described proteins . However a very weak similarity to BRCA1 protein was observed, that was restricted to a 80 amino acid long region (aa.1394-1474 in BRCA1 and aa.1783-1863 in BRCA2); (Wooster *et al.*, 1995). Transcription of the gene results in a 11-12 kb transcript and high expression was detected at testis, breast and thymus with less amounts at spleen, lung and ovary. The gene is composed of 27 exons and distributed over roughly 70 kb of genomic DNA. The cDNA consists of >60% A/T unlike most of the human proteins. There is a CpG rich region at the 5' end of the gene suggesting a regulatory region. BRCA2 encoded protein was found to be highly charged, approximately one quarter of all amino acids were acidic or basic (Tavtigian *et al.*, 1996). Working with BRCA2 was hard because another very important TSG, the retinoblastoma gene, was located very close to the BRCA2 locus. In 52-63% of 78 sporadic primary breast tumors, allelic imbalance was observed at the 13q loci, 9 of which showed AI at BRCA2 locus but not Rb and 6 of which showed AI at Rb locus but not in BRCA2 (Hamann *et al.*, 1996). This data suggests that Rb and BRCA2 are distinct targets in sporadic breast cancer and the mutation incidence of BRCA2 in sporadic breast cancer is significantly higher than BRCA1 but still rare.

Germline mutations in the BRCA2 gene are believed to be associated with approximately 45% of breast cancer families and carriers have a moderately increased risk for developing ovarian cancer (Wooster *et al.*, 1994). As most breast tumors that occur in patients with germ-line BRCA2 mutations have been found to have a mutations that result in the loss of the wild-type BRCA2 allele, this gene is believed to be tumor suppressor (Collins *et al.*, 1995). Analysis of a family with multiple cases of male breast

cancer but no increase in female breast cancer identified the fact that BRCA2 confers high susceptibility to male breast cancer (Thorlacius *et al.*, 1995). As in the case of BRCA1 most of the mutations observed in BRCA2 result in the formation of truncated forms of the protein (Wooster *et al.*, 1995) (Phelan *et al.*, 1996).

Identification of the second breast cancer susceptibility gene BRCA2, allowed a comparison between the two breast cancer susceptibility genes, BRCA1 and BRCA2 (Tavtigian *et al.*, 1996). Both genes are rich in A/T content, contain a huge exon 11 (3426 bp for BRCA1 and 4932 bp for BRCA2) and are distributed over a 70-100 kb region in genomic DNA. Translation results in the production of highly charged proteins. Most of the mutations result in the formation of truncated forms of the protein products in both genes. Expression patterns of BRCA1 and BRCA2 are very similar, that it is highest in testis and breast.

CHAPTER II

BRCA1

2-1 LOCALIZATION OF EARLY-ONSET FAMILIAL BREAST CANCER TO CHROMOSOME 17q21

The first breast cancer susceptibility gene was identified by linkage analysis (Hall *et al.*, 1990). Linkage analysis can reveal the chromosomal location of the genes of interest by identifying polymorphic genetic markers of known location that are coinherited with the disease in families. These markers were originally protein polymorphisms, but have been replaced by DNA markers such as RFLPs (restriction fragment length polmorphisms) and VNTRs (variable number of tandem repeat polymorphisms) (Bowcock, 1993). As the family history of the disease was a significant risk factor in all populations, breast cancer was found suitable for this approach. Mapping the genes for familial breast cancer was important because alterations at the same loci may also be responsible for the sporadic cases. The main problems in the mapping of these genes were the unavoidable epidemiological realities and the molecular heterogeneity of the disease. The disease was common but only a small proportion of the disease was attributed to inherited susceptibility. Thus, members of a family may have multiple number of cases without inherited susceptibility and sporadic cases may occur in families with inherited disease.

Hall *et al.* made an extensive study of 23 families with 146 cases of breast cancer in 1990. These families shared the epidemiological features that were characteristics of

familial versus sporadic breast cancer: young age of diagnosis, frequent bilateral disease and frequent occurrence of disease among men. Genetic analysis of the 329 people in those families revealed a lod score of 5.98 for linkage of breast cancer susceptibility in early-onset families to D17S74, which is located in a 1 to 2 megabase region on chromosome 17q21. Negative lod scores were found in families with late-onset disease (Hall *et al.*, 1990). A map of human chromosome 17q12-23 is given in figure 2-1.

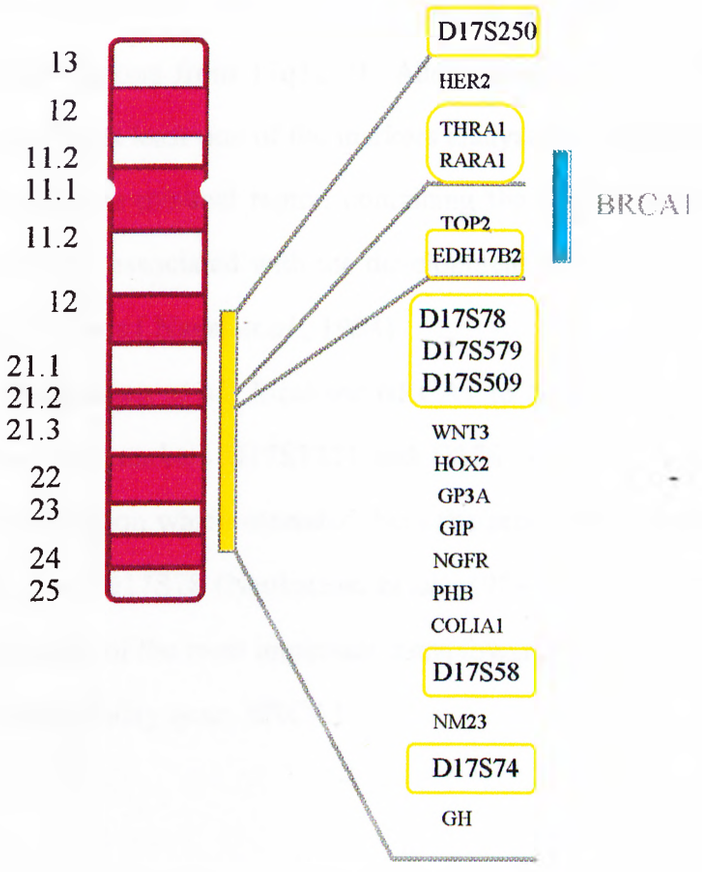


Figure 2-1: Map of human chromosome 17q12-23

Linkage of breast cancer susceptibility gene BRCA1 to chromosome 17q21 was then confirmed by a number of studies. In 1991 Narod *et al.* showed that the chromosomal region 17q12-q13 was associated with hereditary ovarian cancer as well as hereditary breast cancer (Narod *et al.*, 1991). Analysis of a single multi-affected breast-ovarian cancer pedigree showed the consistent inheritance of markers on chromosome 17q with the disease confirming that the disease in this family was due to the BRCA1 gene (Kelsell *et al.*, 1993). Involvement of BRCA1 in sporadic breast cancer was also identified in a study in which 100 cases were analyzed for the LOH by using 10 PCR-based polymorphic markers from 17q12-21. Allele losses were detected in 40 of 100 tumors informative for at least one of the markers analyzed. The most frequently deleted region overlaps with the minimal region containing the BRCA1 gene, suggesting that this gene might also be associated with the development or progression of a proportion of sporadic breast tumors (Nagai *et al.*, 1994).

In 1994 Neuhausen *et al.*, localized BRCA1 to a region of about 600 kilobase pairs (kb) between the markers D17S1321 and D17S1325 by constructing a physical map of the BRCA1 region which extended from the proximal boundary at D17S776 to the distal boundary at D17S78 (Neuhausen *et al.*, 1994). Localization of BRCA1 to a 600-kb region was one of the most important steps towards the cloning of the breast and ovarian cancer susceptibility gene, BRCA1.

2-2 MOLECULAR CLONING OF BRCA1

In October 1994, Miki *et al.*, identified a strong candidate for 17q-linked BRCA1 gene by positional cloning methods. For this purpose, first they found out sixty-five expressed sequences within the 600-kb region on 17q21. Characterization of these expressed sequences by DNA sequence, database comparison, transcript size, expression pattern, genomic structure and DNA sequence analysis of individuals from breast cancer kindreds identified three expressed sequences that merged into a small transcription unit. This transcription unit was located in the center of the 600-kb region.

Combination of sequences obtained from amplified polymerase chain reaction (PCR) products, complementary DNA (cDNA) clones and hybrid-selected sequences revealed the full-length BRCA1 cDNA. Translation of this cDNA showed that it has a single, long open reading frame encoding a protein of 1863 amino acids (Miki *et al.*, 1994).

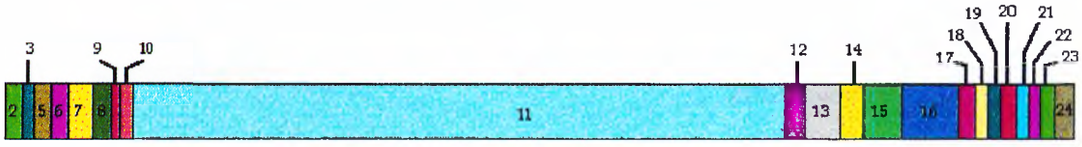
In order to prove that BRCA1 is responsible for susceptibility to breast and ovarian cancer it should be demonstrated that there are mutations in this locus in carrier individuals from kindreds that segregate 17q-linked susceptibility to breast and ovarian cancer. Analysis of different members from these kindreds have shown that there are frameshift, nonsense and regulatory mutations that cosegregate with predisposing BRCA1 alleles suggesting that this gene is BRCA1 (Miki *et al.*, 1994). Mutation analysis of BRCA1 gene of affected kindred members, performed by a number of laboratories after the cloning of BRCA1, further proved that this gene was responsible for the hereditary form of breast and ovarian cancer. Analysis of 50 probands with a family history of breast and/or ovarian cancer by Castilla *et al.* (1994), 63 breast cancer patients by Friedman *et al.*(1994), for germline mutations in the coding region of BRCA1 showed that there were germline mutations in the BRCA1 gene in more than 80% of affected families.

2-3 DNA, RNA AND PROTEIN STRUCTURE OF BRCA1

Miki *et al.*, found out that BRCA1 gene is composed of 24 exons 22 of which are transcribed into a 7.8 kb transcript and is distributed over roughly 100 kb of genomic DNA. The exon structure of BRCA1 is schematically represented in figure 2-2. The transcript was found to be most abundant in testis, thymus, breast and ovary. Probing genomic DNA samples from different species including humans, mice, rats, rabbits, sheeps, and pigs with BRCA1 sequences revealed the fact that BRCA1 is conserved among mammals (Miki *et al.*, 1994).

During the cloning of the gene, cDNA clones lacking one or more exons within the 5' portion of the gene were isolated indicating the presence of alternative splicing (Miki *et al.*, 1994). In 1996, Lu *et al.* identified the splice variants of BRCA1 which lacked most of the nucleotide sequences from exon 11 and were expressed abundantly in tumor-derived and normal breast cell lines (Lu *et al.*, 1996). These alternative BRCA1 transcripts were in frame and coding 80-85 kD BRCA1-derived proteins, lacking approximately 60% of the internal amino acids of full-length BRCA1. A schematic representation of the splice variant of BRCA1 are given in figure 2-3.

Generation of different mRNA species can be due to the presence of alternative splicing as indicated above, as well as the presence of distinct transcription sites. In 1995, Xu *et al.*, identified the presence of two different transcripts generated by the alternative use of dual promoters and alternative splicing, in primary tissues including that of placenta, mammary gland, testis and thymus, six normal or cancer cell lines, four primary breast tumor tissues and four primary ovarian tumor tissues (Xu *et al.*, 1995). In all of the samples studied, both transcripts were detected. The transcript named exon1a (which starts from the 5' 1st transcription start site was expressed abundantly in



5' UTR: 1-119

exon 1: 1-100
exon 2: 101-119
exon3: 200-253
exon5: 254-331
exon6: 332-420
exon7: 421-560
exon8: 561-665
exon9: 666-712
exon10: 713-788
exon11: 789-4215
exon12: 4216-4302
exon13: 4303-4476
exon14: 4477-4603
exon15: 4604-4794
exon16: 4795-5105
exon17: 5106-5193
exon18: 5194-5273
exon19: 5274-5310
exon20: 5311-5396
exon21: 5397-5451
exon22: 5452-5526
exon23: 5527-5586
exon24: 5587-5711

Figure 2-2: The exon structure of BRCA1

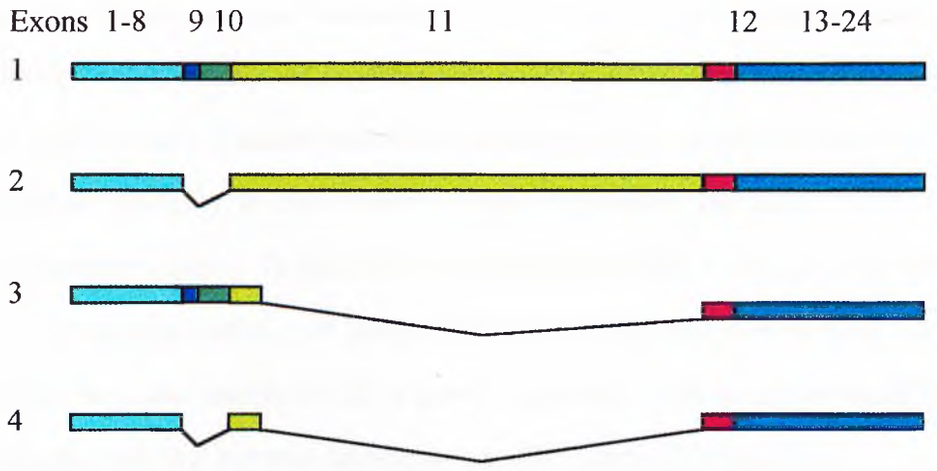


Figure 2-3: Splice variants of BRCA1
(1: full-length BRCA1; 2: BRCA1 lacking exons 9 and 10; 3: BRCA1 splice variant lacking 3309 nucleotides from exon 11; 4: BRCA1 splice variant lacking exons 9 and 10 as well as 3309 nucleotides from exon 11)

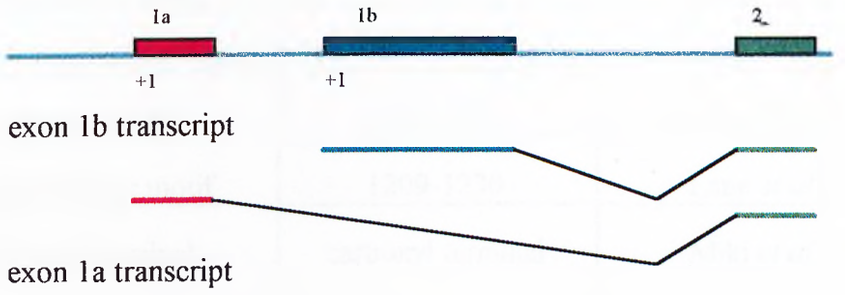


Figure 2-4: Exon 1a and exon 1b transcripts of BRCA1

mammary gland, and the transcript named exon 1b (which starts from the 5' 2nd transcription start site) was expressed abundantly in placenta, indicating a tissue-specific expression pattern. Transcription of a single gene from multiple promoters may provide additional flexibility in the control of gene expression (Kozak., 1991). A schematic representation of exon 1a and exon 1b transcripts of BRCA1 are given in figure 2-4.

Sequence analysis of human BRCA1 revealed the presence of a RING finger domain, two basic motifs (NLS), a granin sequence, a leucine zipper motif, and a highly conserved carboxyl terminal harboring the BRCT motif (Table 2-1).

Table 2-1: A summary of the identified domains and motifs on BRCA1

NAME OF THE MOTIF	LOCATION (aa, residues)	REFERENCE
RING finger motif	24-64	Miki *et al.*, 1994
Nuclear Localization Signal	500-506 and 604-611	Lane *et al.*, 1995
Granin motif	1214-1223	Jensen *et al.*, 1996
Leucine zipper motif	1209-1230	Lane *et al.*, 1995
Carboxyl terminal acidic loop	carboxyl terminal	Miki *et al.*, 1994
Carboxyl terminal minimal transactivation domain	1760-1863	Monteiro *et al.*, 1996
Carboxyl terminal BRCT motif	1671-1796	Holt *et al.*, 1996

Smith-Waterman and BLAST searches identified that the BRCA1-encoded protein contains the consensus Cys_3-His-Cys_4 (C3HC4) zinc-finger (ring finger) motif near the amino-terminus (amino acids 22-64) indicating the presence of a zinc-finger domain which suggests that BRCA1 might have a possible role in transcriptional regulation (Miki *et al.*, 1994). A schematic representation of the BRCA1 zinc finger domain is illustrated in figure 2-5 (Bienstock *et al.*, 1996). In 1996, Wu *et al.*, identified a novel protein, by using yeast two-hybrid system, named BRCA1-associated ring domain (BARD1), that was interacting with the N-terminal region (minimal region aa.s 1-100) of BRCA1 *in vivo*. The gene coding for BARD1 was shown to be located on chromosome 2q and just like BRCA1 contains a N-terminal ring motif and a C-terminal BRCT domain. These two proteins were shown to be co-expressed in the tested breast and ovarian carcinoma cell lines. It was also shown that BARD1-BRCA1 interactions were disrupted by tumorigenic amino acid substitutions in BRCA1 indicating that the formation of this complex was important for the BRCA1-mediated tumor suppression. Wu *et al.*, concluded that mutations in the BARD1 may also play an important role in breast carcinogenesis.

Smith-Waterman and BLAST searches also identified an acidic blob in the carboxyl region of the gene indicating a transactivation function. Examination of the globular domains of BRCA1 in order to define the sequence similarities, revealed a similarity between the 202 amino acid residue long C-terminal globular domain of BRCA1 and a human protein named p53BP1. Computer analysis showed that the same conserved region was also present in the yeast RAD9 protein, which is involved in the control of the DNA damage induced cell-cycle arrest. This domain was named as BRCT (BRCA1 C-terminus) and proposed to confer the ability to suppress breast cancer cell growth to BRCA1 (Koonin and Altschul, 1996) as the BRCA1 mutants lacking this region were incapable of performing their tumor suppressor functions (Holt *et al.*, 1996).

Another domain identified on the BRCA1 protein is the granin domain which is located at a close proximity of the N-terminal (amino acids 1214-1223) (Jensen *et al.*, 1996). By using SWISS PROT database, Jensen *et al.*, searched for protein motifs in BRCA1. Some biochemical features as well as the conserved granin sequence indicated that BRCA1 can be a granin. These features include the similarity between the isoelectric points (granins: between 4.9-5.6; BRCA1: 5.2) and the acidic amino acid composition of both BRCA1 and the granins.

Immunoprecipitation analysis of BRCA1 by anti-BRCA1 antibodies in synchronized T24 bladder carcinoma cells, identified the presence of two forms of 220 kD BRCA1 protein one hypo-phosphorylated and the other hyper-phosphorylated (Chen *et al.*, 1996). The phosphorylation status of specific domains of various proteins often correlates with many respects of cell growth and transformation. In this study it was also shown that phosphorylation of BRCA1 was cell cycle dependent, becoming evident in mid/late G1, rising to maximum in S phase and remain elevated through M phase. In order to define whether the phosphorylation of BRCA1 was carried out by cell-cycle dependent protein-kinases, cell lysates were immunoprecipitated with antibodies against various cyclin dependent kinases (cdk) and cyclins. Reimmunoprecipitation of the precipitates with anti-BRCA1 antibodies indicated that BRCA2 was phosphorylated by cdk-2 and kinases associated with cyclins D and A, consistent with the initiation of phosphorylation in mid-G1 and maximum phosphorylation during S phase.

Identity of the phosphorylated amino acid was assessed by immunoprecipitating BRCA1 with antibodies against the protein, and then performing a phosphoamino acid analysis (Ruffner and Verma, 1997). It was found out that, it was a serine phosphoprotein that undergoes hyperphosphorylation during late G1 and S phases of the cell cycle and was transiently dephosphorylated after M phase.

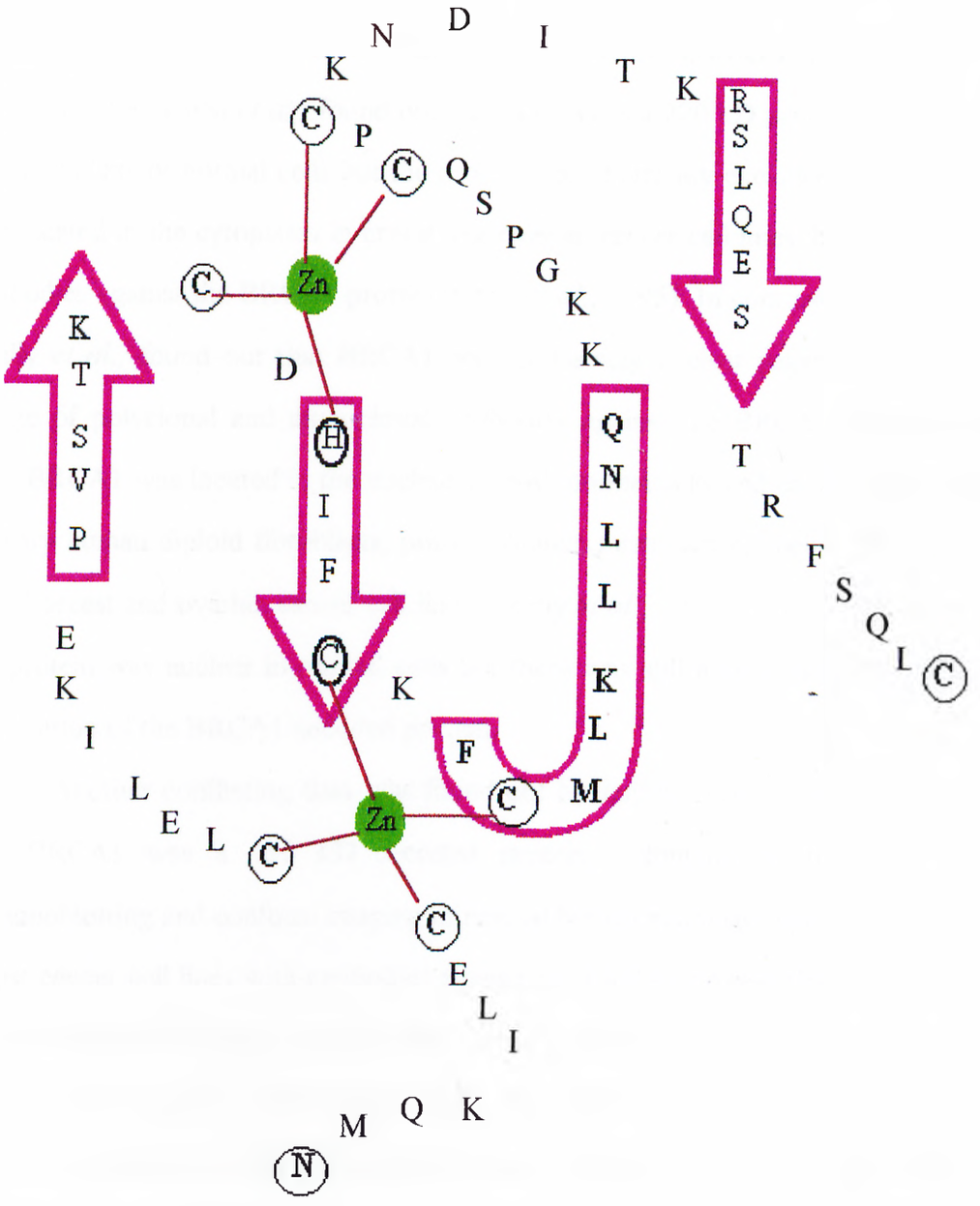


Figure 2-5: RING (zinc) finger domain of BRCA1

2-4 CELLULAR LOCALIZATION OF BRCA1 ENCODED PROTEINS

The localization of a protein may indicate some clues about its functions in a cell, therefore subcellular localization of the BRCA1 protein was extensively studied.

In 1995 Chen *et al.*, found out that BRCA1 is a 220 kD protein and is localized in the nucleus of normal cells but the protein was aberrantly excluded from the nucleus and located in the cytoplasm in breast and ovarian cancer cell lines, by using polyclonal antibodies against the BRCA1 protein (Chen *et al.*, 1995). In contrast to these findings, Scully *et al.*, found out that BRCA1 was exclusively nuclear regardless of cell type. Usage of polyclonal and monoclonal antibodies against the BRCA1 protein revealed that, BRCA1 was located in the nucleus of both normal cells and cancer cells including, primary human diploid fibroblasts, primary human mammary epithelial cells and all the tested breast and ovarian cancer cell lines (Scully *et al.*, 1996). Both groups agreed that the protein was nuclear in normal cells but there was still a confusion about the exact localization of the BRCA1 encoded protein.

Another conflicting data was found out by Jensen *et al.* (1996), who proposed that BRCA1 was a 190 kD secreted protein, belonging to the granin family. Immunoblotting and confocal imaging of normal human mammary epithelial cells and the breast cancer cell lines with antibodies against the BRCA1 protein showed that BRCA1 was localized to the Golgi network and secretory vesicles (Jensen *et al.*, 1996). But no other study except this provided evidence that BRCA1 is a 190 kD secreted protein. Later on, it was shown that the antibodies used by Jensen *et al.* were cross-reacting with the epithelial growth factor receptor (EGFR) which was a 190 kD protein and had a high expression pattern in the MDA-MB-468 cells used in the study (Wilson *et al.*, 1996).

In 1996, Chen *et al.*, characterized the BRCA1 as a 220 kD nuclear phosphoprotein by using highly specific antibodies. Generation of a baculovirus-based

expression system, allowed the purification of large amounts of full-length BRCA1. Co-migration of this *in-vitro* translated, recombinant, baculovirus derived BRCA1 with BRCA1 from human breast epithelial cell line, indicated that BRCA1 is a 220 kD protein. Immunostaining of bladder carcinoma cells with the two highly specific antibodies indicated the nuclear localization of the BRCA1 protein.

A study concerning the functional significance and cellular localization of BRCA1 splice variants showed that the alternatively spliced form of BRCA1 (BRCA1Δ 672-4095) which was lacking a large portion of exon 11 was located in the cytoplasm, in contrast to the full-length BRCA1 (Thakur *et al.*, 1997). This was explained by the presence of two nuclear localization signals (NLS) in exon 11 (amino acids; 500-508 and 609-615). The localization of the BRCA1 splice variant lacking exon 11 (BRCA1Δ11b) had also been shown to be cytoplasmic in another study, again pointing out the presence of NLSs in exon 11, which mediate the translocation of BRCA1 from cytoplasm to the nucleus (Wilson *et al.*, 1997). In this study it was also shown that overexpression of BRCA1 but not BRCA1Δ11b was toxic to the cells, indicating the accumulation of full length BRCA1 in the cytoplasm and the nucleus.

Very recently BRCA1 is localized in the perinuclear compartment of the endoplasmic reticulum-Golgi complex and in tubes invaginating the nucleus. Coene *et al.*, (1997) produced polyclonal antibodies against exon 11 and the C-terminal region of BRCA1 and then used these antibodies and two other monoclonal antibodies to stain human breast cancer cells, human T-leukemia cells known to express BRCA1, fibroblasts, normal breast epithelium and carcinoma cells. Confocal analysis indicated the presence of BRCA1 in the cytoplasm especially within the perinuclear region and as dot-like structures in the nucleus. Further confocal analysis showed that the dot-like structures in the nucleus in fact represent cross-sections through perinuclear originated threads or tubes and that number of these channels were increased in malignant cells compared to normal cells. Co-localization of these tubes with endoplasmic reticulum-

Golgi complex specific proteins indicated that these nuclear tubes may have been originated from the cytoplasmic structures. This study also indicated that the nuclear detection of BRCA1 was fixation dependent, that the cell-fixation procedures influence the outcome of the experiments. Therefore the previously described different subcellular localizations of BRCA1 may be due to the fixation techniques. Similarly, identification of different molecular weights for the BRCA1 may be due to the cross-reactivity of the antibodies used in the studies.

2-5 PUTATIVE FUNCTIONS OF BRCA1

2-5.1 BRCA1 IS A TUMOR SUPRESSOR GENE

Most of the tumors that occur in patients with germline BRCA1 mutations display heterozygosity at this locus which involves loss of the wild-type BRCA1 allele indicating that BRCA1 can be tumor suppressor acting as a negative regulator of tumor growth (Smith *et al.*, 1992). Cancer predisposing alleles typically carry mutations that result in loss or reduction of the gene function. A single inherited copy of the mutant allele results in predisposition to cancer and a mutation which results in the loss or inactivation of the gene in the wild-type allele completes one of the steps towards carcinogenesis (Miki *et al.*, 1994). The predisposing allele generally behaves as a recessive allele in somatic cells but the predisposition to cancer is inherited as a dominant genetic trait. In breast cancer patients who carry BRCA1 predisposing alleles, observations have shown that malignancy results from the loss of the wild-type allele suggesting that BRCA1 is a tumor suppressor (Kelsell *et al.*, 1993) (Neuhausen and Marshall, 1994). This hypothesis was supported by the identification of the fact that BRCA1 negatively regulates the proliferation of mammary epithelial cells (Thompson *et*

al., 1995), transfection of MCF-7 breast cancer cell lines with wild-type BRCA1 inhibits tumor progression (Holt *et al.*, 1996) and antisense RNA against BRCA1 transforms fibroblasts (Rao *et al.*, 1996).

Thompson *et al.* compared the expression of BRCA1 in normal mammary epithelium, carcinoma *in situ* and invasive breast cancer by the use of antisense oligonucleotides against BRCA1. Although the inhibition of BRCA1 expression by these oligonucleotides did not affect the non-mammary epithelial cells, accelerated growth of mammary epithelial cells was observed, suggesting the role of BRCA1 to be a negative regulator of mammary epithelial cell growth. Their results also indicated that BRCA1 mRNA expression was five-to-ten fold higher in normal mammary tissue than in invasive breast cancer samples and that BRCA1 mRNA levels were decreased during transition from carcinoma *in-situ* carcinoma to invasive cancer. Thus, BRCA1 is expressed at lower levels in breast cancer cells than in normal mammary cells and diminished expression of BRCA1 increases the proliferation rate of breast epithelial cells (Thompson *et al.*, 1995).

Analysis of the tumor inhibition effects of BRCA1 on breast cancer cell line MCF-7, showed that wild-type BRCA1 (but not mutant BRCA1) inhibits growth of breast cancer cells *in vitro* and rescues the cells from the tumorigenic phenotype (Holt *et al.*, 1996). MCF-7 breast cancer cell line has very low expression of BRCA1 mRNA and BRCA1 protein and contains a genomic loss of one copy of the 2 Mb region containing BRCA1. Transfection of wild-type BRCA1 into the breast and ovarian cancer cell lines resulted in the inhibition of growth whereas growth inhibition was not observed in transfected colon and lung cancer cell lines. This indicates the tissue specificity of BRCA1 protein in exerting its growth inhibitory effects. Transfection of a mutant BRCA1 which encodes a 340 amino-acid truncated form did not inhibit the growth of the cell lines. Interestingly, a truncated 1835 amino-acid long BRCA1 protein did not inhibit the growth of breast cell lines, while inhibiting the growth of ovarian cell lines.

This suggests that growth inhibition of breast cancer cells and ovarian cancer cells are mediated by different mechanisms and that this difference depends on the length of the truncated protein. Similarly when MCF-7 cells that were transduced with wild-type BRCA1 were injected in nude mice, no tumor development was observed, but when MCF-7 cells lacking wild-type BRCA1 were injected in nude mice, tumors developed in five of six mice indicating that BRCA1 can inhibit mouse breast carcinogenesis. Holt *et al.*, also demonstrated that wild-type BRCA1 can inhibit the growth of already established breast tumors in nude mice and improved the survival of these animals (Holt *et al.*, 1996).

Rao *et al.* (1996), postulated that if BRCA1 functions as a growth regulator in normal cells, inhibiting its function should result in transformation. Transfection of NIH3T3 cells that were expressing significant levels of BRCA1, with vectors containing antisense BRCA1 cDNA resulted in 3-5 fold decrease in the expression of BRCA1. There were no significant morphological changes in these cells, but increased proliferation rate, anchorage independence and the ability to grow in serum-free or low serum culture medium were observed which are the three important characteristics of transformed cells. Testing the tumorigenicity of the BRCA1-antisense cDNA harboring cells *in vivo*, showed that these cells were capable of generating tumors in nude mice.

All these data support the hypothesis that BRCA1 is a potent tumor suppressor gene and that the functional BRCA1 protein is present in normal breast and ovarian epithelium tissue and is altered, reduced, or absent in some breast and ovarian tumors (Miki *et al.*, 1994)

2-5.2 BRCA1, A SECRETED PROTEIN?

Identification of a granin consensus at BRCA1 amino acids 1214-1223, implicated the possibility that BRCA1 can be a secreted protein (Jensen *et al.*, 1996). Granins are secreted proteins, hence they are located in secretory granules. The expression of some granin family proteins is regulated by estrogen and their secretion is triggered by cyclic AMP (Thompson *et al.*, 1992). Granins contain a characteristic ten amino acid long motif and their overall amino acid content is highly acidic (Huttner *et al.*,1991). Intracellularly, granins are involved in regulated secretory pathway, and extracellularly they serve to regulate cellular secretion of other peptides in an endocrine or paracrine fashion.

As indicated in section 2-4, none of the other studies indicate that BRCA1 may function as a secreted protein. Rather, they indicated the exact localization of BRCA1 to the nucleus. Therefore now, it is unlikely that BRCA1 is a secreted protein.

2-5.3 BRCA1 AS A REGULATOR OF TRANSCRIPTION

The first identified conserved domain on BRCA1 was the ring finger domain near the amino-terminus (amino acids 22-64). The members of the RING finger family are putative DNA binding proteins, some of which are implicated in transcriptional regulation (Miki *et al.*, 1994). Similarly, presence of a highly negatively charged carboxyl terminal may indicate a transcriptional regulation function as in many eukaryotic transcription activators such a region is found (Monteiro *et al.*, 1996). Also the presence of the two nuclear localization signals in exon 11 and identification of the subcellular localization of BRCA1 to be nuclear supplied further evidence that BRCA1 acts as a regulator of transcription (Coene *et al.*, 1997). BRCA1 was also shown to induce apoptosis when overexpressed in several normal or malignant cell lines indicating

that BRCA1 products may act as a transcriptional regulator to activate or inhibit apoptosis inducing or repressing genes, respectively (Shao *et al.*, 1996).

The most convincing data supporting the transcriptional regulator role of BRCA1 was found out by Monteiro *et al.*, 1996. It was found out that, the C-terminal of the BRCA1 protein, comprising exons 16-24 (aa: 1560-1863) can act as a transactivation domain when fused to GAL4 DNA binding domain in both yeast and mammalian cells. Also the minimal transactivation domain was found to be involved by exons 21 to 24 (aa 1760-1863). Their results also indicated that mutations in the C-terminal region found in patients with breast or ovarian cancer had markedly impaired transcription activity.

Co-purification of the wild-type BRCA1 with the mammalian RNA polymerase II holoenzyme by Scully *et al.*, suggested that BRCA1 was a component of RNA polymerase II holoenzyme, and therefore involved in the regulation of transcription of at least some genes (Scully *et al.*, 1997). Purification of the holoenzyme from the HeLa whole cell extract, recovered the holoenzyme, and the components of the holoenzyme were separated by density gradient centrifugation. Results indicated the presence of BRCA1 in RNA polymerase II holoenzyme in association with pol II (core unit) and cdk8 (mammalian counterpart of the yeast SRB10). Similarly, BRCA1 was coimmunoprecipitated with the basal transcription factors TFIIF, TFIIE, and TFIIH. The effects of BRCA1 mutations that generate truncated protein products, on holoenzyme binding was also assessed. Results indicated that the truncated product was also able to bind holoenzyme but with a decreased efficiency, indicating the importance of the C-terminal of BRCA1 in this functional complex. Previously, it had been shown that hRAD51, which is shown to be associated with BRCA1 in mitotic and meiotic cells (Scully *et al.*, 1997), was also a component of the RNA polymerase II holoenzyme (Maldonato *et al.*, 1996). The inability to purify hRAD51 with BRCA1 in this study was

explained by the cell-cycle dependent association of the two proteins and the cells used in this study were asynchronous.

2-5.4 ROLE OF BRCA1 IN APOPTOSIS

In a wide variety of human malignancies decreased ability to undergo apoptosis is observed. In breast and ovarian carcinomas, this could be due to lack/decreased levels of functional BRCA1 proteins. In 1996, it was shown that BRCA1 plays a critical role in the regulation of programmed cell death, such that overexpression of BRCA1 results in apoptosis (Shao *et al.*, 1996). In order to define the role of BRCA1 in apoptosis, Shao *et al.*, developed mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1 by transfecting NIH3T3 and MCF7 cells with a vector harboring the BRCA1 cDNA. The morphological differences between the BRCA1 transfectants and the parental NIH3T3/MCF7 cells in prolonged periods of cell culture indicated that constitutive high level expression of BRCA1 may result in apoptosis. Serum withdrawal is known to induce apoptosis and by comparing the effects of serum deprivation on induction of apoptosis in BRCA1 transfectants and the parental cells, Shao *et al.*, showed that BRCA1 transfectants were more prone to apoptosis. Their results also indicated that exon 11 of BRCA1 was dispensable for the apoptotic function as the BRCA1 cDNA they used lacked the majority of exon 11. The mechanisms underlying the induction of apoptosis by BRCA1 are not clear but it is suggested that BRCA1 products may activate death inducing genes or repress death inhibiting genes either by direct protein-protein interactions or by acting as a transcriptional regulator.

2-5.5 BRCA1 AND DNA REPAIR

Very recently it was shown that BRCA1 and hRAD51 colocalize in S phase, interact physically and share a common space on the surfaces of zygotene and pachytene meiotic chromosomes, suggesting that BRCA1 participates in nuclear processes including recombination and genome integrity control (Scully *et al.*, 1997).

Usage of several different monoclonal and polyclonal antibodies indicated that endogenous BRCA1 forms discrete, dot-like structures in the nucleus in a S-phase of the cell-cycle dependent manner (Scully *et al.*, 1996). hRad51 is the human homologue of RecA and is a member of protein family which functions as a mediator of DNA strand-exchange functions, leading to recombination (Baumann *et al.*, 1996). Mutations in the RAD51 gene result in early embryonic lethality in mice (Tsuzuki *et al.*, 1996), and meiotic recombination and double-strand break-repair abnormalities in yeast (Shinohara *et al.*, 1992). Just like BRCA1, RAD51 forms S phase-specific nuclear dots (Tashiro *et al.*, 1996). This similar timing of appearance of nuclear dots suggests the colocalization of the protein products of these two genes in the same structure. Immunostaining of MCF7 cells with antibodies against BRCA1 and hRAD51 showed that these two proteins were colocalized in nuclear dot patterns (Scully *et al.*, 1996). Further analysis of this interaction revealed that this colocalization was mediated by sequences encoded by the 11^{th} exon of BRCA1.

Presence of such an interaction in mitosis and the background knowledge about the presence of Rad51 on unique, meiosis-specific DNA-protein complexes named synaptonemal complexes (Terasawa *et al.*, 1995), (Bishop, 1994), raised the probability that these two proteins may interact during meiosis. Analysis of the nuclei of zygotene spermatocytes obtained from human testis showed that both proteins were present on asynapsed elements of synaptonemal complexes.

Probable involvement of BRCA1 in DNA repair was also assessed by Callebaut *et al.*, (1997). Identification of the presence of the approximately 95 amino acid (aa) long repeated motif in the C-terminus of BRCA1 named BRCT (Koonin and Altschul, 1996), among several nuclear proteins closely related to cell-cycle regulation and DNA repair, including the 53BP1 (p53 binding protein 1), RAD9, XRCC1, RAD4, suggested that BRCA1 was involved in DNA repair (Callebaut *et al.*, 1997). Positions of the BRCT domains in BRCA1, 53BP1, RAD9, XRCC1, RAD4 are shown in figure 2-6.

The BRCT domain is predicted to consist of four β-strands and two α-helices, with the β-strands probably forming a core sheet structure. The size of the domain, its predicted secondary structure and the observed pattern of amino acid conservation indicate that this domain may be involved in protein-protein interactions. All proteins that contain the BRCT domain, the BRCT superfamily, are large multi-domain protein that are associated with DNA damage-responsive cell cycle checkpoints.

A model indicating the interaction of BRCA1, BRCA2 and p53 has just been proposed (Figure 2-7), (Brugarolas and Jacks, 1997). Very recently it was shown that, p21 and MDM2 levels were increased in Brca1 deficient mice, indicating a linkage between the cell cycle control and Brca1 (Hakem *et al.*, 1996). According to this model, RAD51, BRCA1 and BRCA2 form a complex and repair the damaged DNA. But when a mutation occurs in either BRCA1 or BRCA2, and repair is failed, p53 is activated.

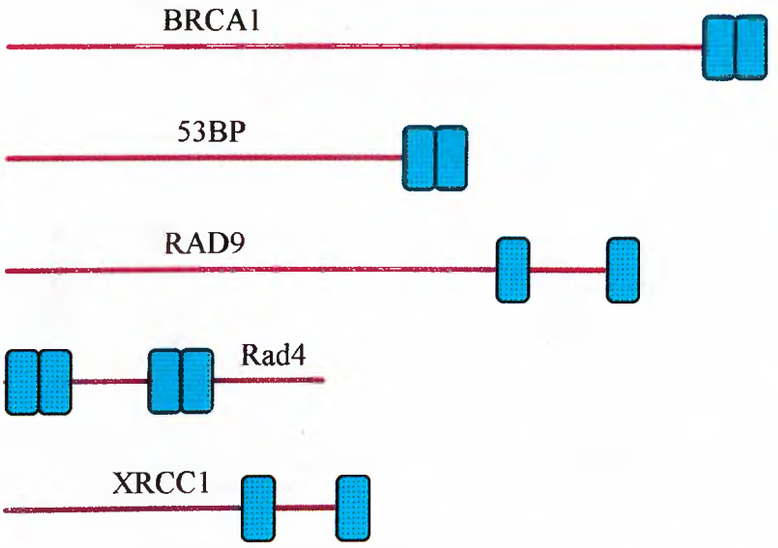


Figure 2-6 Positions of the BRCT domains in BRCA1, 53BP1, RAD9, XRCC1, RAD4 (blue boxes indicate the BRCT modules)

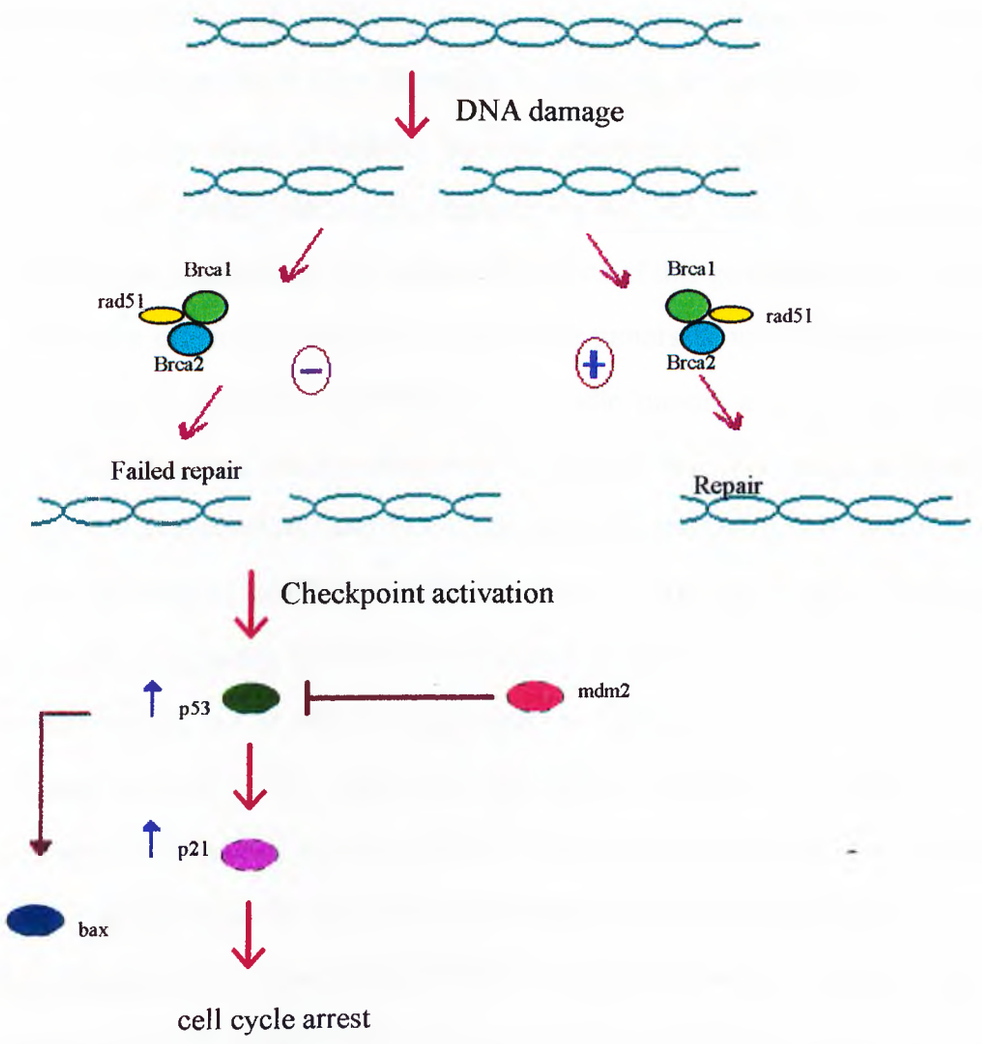


Figure 2-7: Interaction of BRCA1, BRCA2 and p53 in response to DNA damage

2-6 MAIN TYPES OF BRCA1 MUTATIONS IN BREAST CANCER

Germline mutations of the BRCA1 tumor suppressor gene are involved in a significant fraction of hereditary breast and ovarian cancers. So far, more than 100 distinct mutations have been identified in breast or breast/ovarian cancer families. Of these, a founder effect, 185delAG, has been observed in Ashkenazi Jewish families (Offit *et al.*, 1996). Allelic deletions that include the BRCA1 locus are common in breast and ovarian cancers, implying that somatic mutations of this gene may play an important role in the more common sporadic forms of these tumors as well (Takahashi *et al.*, 1995). Somatic point mutations in BRCA1 in sporadic tumors are very rare (Futreal *et al.*, 1994), but complete somatic deletion of one allele of BRCA1 occurs approximately 50% of sporadic breast cancers and 70% of sporadic ovarian cancers (Takahashi *et al.*, 1995). Expression analysis of BRCA1 mRNA in sporadic breast cancers indicated a decreased expression, suggesting that BRCA1 is altered at genomic level in hereditary breast and ovarian cancer, but at either the genomic or transcriptional levels in sporadic cancer (Thompson *et al.*, 1995). Approximately 85% of mutations that have been identified throughout the coding region of BRCA1 give rise to truncated forms of protein that vary in length from 5% to 99% of full-length protein (Shattuck-Eidens *et al.*, 1995). This suggests that C-terminal of BRCA1 is essential for the normal function of the protein in breast epithelial cells. Patients inheriting 1853Stop were shown to develop breast cancer at a very early age (Friedman *et al.*, 1994). Also truncated form of breast cancer was shown to be incapable of inhibiting cell growth *in vitro* (Holt *et al.*, 1995).

Recently, the severity of breast cancers classified according to the proliferative index were compared with the positions of mutations in BRCA1, in order to define a genotype-phenotype correlation. A significant correlation was found between truncations at the N and C termini of BRCA1 and the most highly proliferating cancers (Sobol *et al.*, 1996). Sobol *et al.*, compared two subgroups of BRCA1-associated breast cancer

families, the first with highly proliferating tumors, and the second composed of cases with a low proliferation rate, in order to define the relation between the proliferation rate of BRCA1-associated breast cancers and the site of the germ line mutation in the BRCA1 gene. Results showed that when the mutation was at the two terminal conserved domains of the BRCA1 protein, i.e., in the amino and carboxyl termini, the tumors showed a more aggressive pattern of proliferation. Similarly, analysis of BRCA1 mutations have suggested that the risk of breast and ovarian cancer are related to the position of the mutation, such that truncating mutations in the first two thirds of the coding region were found to be more associated with a higher ovarian cancer risk relative to breast cancer risk, than mutations in the last third.

A summary of the identified mutations of BRCA1 are summarized in figure 2-8.

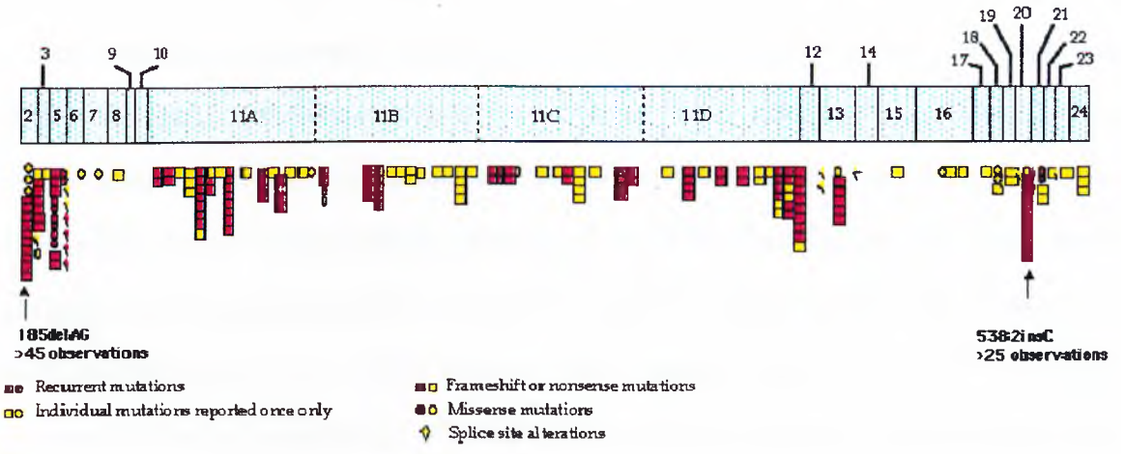


Figure 2-8: The identified mutations of BRCA1

Mutations of the BRCA1 gene were also shown to predispose to colon and prostate cancer as well as breast and ovary cancer. In 1994, Ford *et al.*, studied 33 families for linkage to BRCA1, in order to establish whether gene carriers were at an increased risk of any cancer other than breast and ovarian cancer. 87 cancers other than breast or ovarian cancer were observed in individuals with breast or ovarian cancer and their first-degree relatives compared with 69.3 expected, based on national incidence rates. Significant excesses were observed for colon cancer (P=0.002) and prostate cancer (P=0.006). No significant excesses were noted for cancers of other tissues.

2-7 EXPRESSION OF BRCA1 IN DIFFERENT STAGES OF DEVELOPMENT

Changes in the normal pathways of differentiation and development is one of the causes of carcinogenesis (Rajan *et al.*, 1997). The relationship between carcinogenesis and development is well exemplified in breast, that the hormonal changes taking place during menarche, menopause and age of the first full-time pregnancy are important factors by means of the occurrence of breast cancer. Thus, mammary gland development and mammary gland carcinogenesis are basically related (Rajan *et al.*, 1997). Because most of the breast cancer cases are associated with loss of BRCA1 in humans, it can be thought that this gene provides an important growth regulatory function in mammary epithelial cells (Lane *et al.*, 1995). Several line of evidence suggest that BRCA1 plays an important role in the regulation of mammary epithelial cell growth. Murine homologue of BRCA1 was found to be widely expressed in proliferating and differentiating cell types in the mouse during embryonic and mammary gland development, and in adult tissues (Marquis *et al.*, 1995). Understanding the role of hormones in the regulation of BRCA1 expression in breast and the role of BRCA1 in mammary gland development and differentiation is important because elucidation of these facts would lead to the identification of the relationship between carcinogenesis and the presence of mutant

BRCA1. For this reason, Marquis *et al.* analyzed the temporal and spatial pattern of Brca1 expression during normal mouse embryogenesis, in adult tissues, in the mammary gland during postnatal development, and in the mammary glands of animals whose levels of ovarian hormones have been experimentally manipulated. The embryonic expression observations indicated that Brca1 was expressed all over the embryo in the very beginning but a change occur in the expression pattern from this diffused pattern to tissue-specific pattern during later stages of the development. And these tissues were the ones in which rapidly growing cells were undergoing differentiation. Thus it was shown that Brca1 was expressed in rapidly proliferating cell types which are involved in differentiation. The same pattern was also observed during puberty and pregnancy. In the beginning of puberty Brca1 mRNA levels were found to be considerably higher in the differentiating mammary cells compared with males and the expression levels prior to puberty. In the same manner mammary epithelial cells proliferate rapidly and undergo differentiation during pregnancy. And Brca1 mRNA levels show a sharp increase early in pregnancy. In the same study tissue distribution of Brca1 was also analyzed in adult mice, and it was shown that Brca1 expression was high in breast (predominantly epithelial-specific), ovary (follicular-specific), uterus, testis, thymus, spleen, lymph nodes, and the liver. Spatial and temporal expression analysis of Brca1 during postnatal development of the mammary gland showed that Brca1 was expressed predominantly in an epithelial-specific pattern in the mammary gland of males and females at all stages of postnatal development. These results and the Brca1 expression analysis during puberty and pregnancy in adult rodents indicated that Brca1 plays an important role during development and that expression levels of the gene was modified directly or indirectly by hormones (Marquis *et al.*, 1995).

Another study concerning the Brca1 expression-cell proliferation/differentiation relationship was carried out by Lane *et al.*, (1995). In order to define the growth regulatory function of BRCA1 Lane *et al.*, studied the role of loss of expression of

BRCA1 in the development of breast cancer. Because mouse Brca1 shares 58% amino-acid identity with human BRCA1, and conserved sequence is an indicator of conserved function, and because identification of the role of mouse Brca1 in different stages of development would provide considerable insight into functional domains of BRCA1, as well as the role of human BRCA1 in development and carcinogenesis, mice were used as an animal model (Lane *et al.*, 1995). Expression of Brca1 in embryonic tissues was analyzed by *in situ* hybridization and revealed the fact that Brca1 mRNA expression was high in ectoderm derived tissues including brain, skin, and mammary epithelial cells as well as in mesoderm derived tissues such as kidney epithelial cells. Expression analysis of Brca1 in mammary tissue at different stages of maturation supported the results of Marquis *et al.*, that Brca1 mRNA expression levels increase dramatically during pregnancy when mammary epithelial cells start to proliferate and differentiate. Taken together, all these results support the model in which Brca1 functions as a regulator of differentiation (Lane *et al.*, 1995).

This hypothesis was further supported by the findings that homozygous mutations in Brca1 result in early embryonic lethality in mice (Gowen *et al.*, 1996). By creating a mouse line carrying a mutation in one Brca1 allele (Brca1 +/- line), Gowen *et al.*, examined the role of BRCA1 in normal tissue growth and differentiation and generated a potential model for cancer susceptibility associated with loss of BRCA1 function. Mice that were homozygous for the mutant allele (Brca1 -/-) died in utero between 10 and 13 days of gestation indicating that Brca1 is critical for normal development. Abnormalities in these mice were most evident in neural tube that their neuroepithelium was highly disorganized, with signs of both rapid proliferation and excessive cell death (Gowen *et al.*, 1996).

Necessity of the presence of wild type Brca1 in embryonic cellular proliferation was also analyzed by Hakem *et al.* (1996), by generating a null mutation of the Brca1 gene through deleting exons 5 and 6 ($Brca1^{5-6}$). The findings of this study indicated that

mice that were heterozygous for the mutant Brca1 ($Brca1^{+/-}$) were viable, phenotypically normal and fertile and did not develop breast and ovarian cancer by eleven months of age. However mice lacking a wild type allele of Brca1 ($Brca1^{-/-}$) were not viable and died early in embryogenesis (before day 7.5 of gestation) indicating that Brca1 is essential for postimplantation development at the time of initiation of gastrulation. In vivo, a dramatic increase in the expression of cyclin-dependent kinase inhibitor p21 due to increased p53, and decreased expression of cyclin E and MDM2, leading to reduced cell proliferation was observed in these embryos. Hakem *et al.* concluded that the death of Brca1(5-6) mutant embryos prior to gastrulation may be due to a failure of the proliferative burst required for the development of the different germ layers.

The increased expression of p21, which encodes a G1 cell cycle inhibitor and is a target for p53 transcriptional activation in the Brca1 mutants ($BRCA1^{5-6}$), suggested that the failure of the proliferative burst could be due to a G1 cell-cycle arrest, caused by increased p21 levels (Hakem *et al.*, 1997). Generation of a $Brca1^{5-6-/-}$, $p53^{-/-}$ mice and $Brca1^{5-6-/-}$, $p21^{-/-}$ showed that these mutations prolonged the survival of Brca1 mutant embryos from E7.5 to E9.5. This partial rescue indicates the presence of other genes, that take part in the developmental stages that act in association with the BRCA1 protein.

This relationship of BRCA1, with p53, mdm2, and p21 indicates that BRCA1 is directly or indirectly linked to cell-cycle.

2-8 EXPRESSION LEVELS OF BRCA1 DURING CELL CYCLE

Observations indicating the increased expression of BRCA1 during rapid cell proliferation and/or differentiation (Lane *et al.*, 1995); (Marquis *et al.*, 1995) and the presence of dot-like BRCA1 structures in the nucleus during S-phase (Coene *et al.*, 1997), suggested that BRCA1 expression levels were modified in relation with the cell cycle.

Analysis of BRCA1 expression levels in synchronized T24 bladder carcinoma cells, revealed that BRCA1 expression was very low in early G1, and increased as the cell cycle progress toward late G1, reached the maximum as the cells enter S phase and stayed at the maximum level throughout the S and the M phases (Chen *et al.*, 1996). The results of the immunostainings were consistent with the previous results, that when synchronized T24 cells were fixed at different stages of the cell cycle and stained with antibodies against the BRCA1 protein, maximum staining was obtained in the S-phase cells.

In contrast to all these data, Aprelikova *et al.*(1996), suggested that BRCA1 may not be directly involved in the regulation of the cell cycle in the breast cancer cells, based on the observations that the expression pattern of BRCA1 was not changed, when the breast cancer cell line MCF10A cells were arrested by growth factor deprivation or stimulation of cell proliferation by re-addition of growth factors.

2-9 HORMONAL REGULATION OF BRCA1 EXPRESSION

It had been reported that in addition to genetic factors, hormones of the pituitary and ovarian origin, including estrogens, progestins and prolactin, play a role in the control of normal and breast tumor cell proliferation (Nandi *et al.*, 1995). It had also been reported that, as cells progress from normal to tumorigenic state, response to the growth factors was lost (Clarke *et al.*, 1992).

In 1995, Marquis *et al.*, suggested that the expression pattern of Brca1 can be modulated by ovarian hormones, as the Brca1 mRNA levels were shown to be increased and stayed elevated throughout the pregnancy in mice. Treatment of ovariectomized mice with 17β-estradiol alone, progesterone alone, and with the combination of both hormones, showed that when these animals were treated with only any one of the hormones, there was no observable increase or only a slight increase in the levels of Brca1 mRNA expression, respectively, compared to intact mice. And when these animal were treated with the combination of the two hormones, there was a 4-to 5-fold increase in the expression level. These results suggest that, hormones play a key role in the regulation of BRCA1 expression.

Regulation of BRCA1 expression via hormones was also tested *in vitro*, by using estrogen-receptor positive MCF-7 cells (Gudas *et al.*, 1995). Gudas *et al.*, demonstrated that the depletion of estrogens resulted in a decreased expression of BRCA1 and treatment of the these cells with β-estradiol following the depletion, resulted in an increased BRCA1 expression. As it had been shown that BRCA1 transcription was regulated with the cell cycle, reaching to maximum levels in S phase (Chen *et al.*, 1996), Marks *et al.*(1997), investigated whether BRCA1 was directly induced by estrogen or the increased expression was due to the mitogenic activity of β-estradiol.

By using three estrogen receptor positive (ER^+) and one estrogen receptor negative (ER^-) cell lines, it was shown that following the depletion of estrogen,

treatment of the ER^+ cells resulted in induction of BRCA1, whereas there was no change in the expression pattern of BRCA1 in ER^- cells as expected. Usage of an antagonist of β-estradiol, tamoxifen citrate, in MCF7 cells showed that, although the expression of pS2 was not induced, BRCA1 expression was induced indicating that BRCA1 expression was not directly affected by β-estradiol. Furthermore, when β-estradiol was added to a previously depleted medium, an increase in the number of cells undergoing S phase was observed. Similarly use of two other mitogens, epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1), instead of β-estradiol induced the expression of BRCA1 in MCF7 cells. Their results indicated that, BRCA1 expression was linked to a promotion of DNA synthesis by these hormones and is not linked to estrogen directly.

2-10 BRCA1-BRCA2 INTERACTIONS

It is known that germline mutations in the putative tumor suppressor gene, BRCA1 predispose women to an elevated risk of breast cancer. Like BRCA1, germline mutations in the structurally unrelated gene BRCA2 predispose women to breast cancer. However germline mutations in BRCA2 also predispose male carriers to an increased risk of breast cancer (Thorlacius *et al.*, 1995). In addition BRCA2 mutations are also found in tumor types other than breast, including pancreas, prostate and colon (Thorlacius *et al.*, 1996). Recent studies have shown a relation between the expression of BRCA1 and BRCA2 (Rajan *et al.*, 1996). In 1996 Rajan *et al.*, analyzed the relationship between Brca2 expression and mammary epithelium cell proliferation and differentiation. Results indicated that Brca2 mRNA expression was down-regulated in response to serum deprivation, and up-regulated in rapidly proliferating and/or differentiating mammary epithelial cells, peaking at the G1/S boundary in a cell-cycle dependent manner during proliferation. The same pattern of expression was also

observed for Brca1 suggesting that regulation of Brca2 mRNA expression occurs coordinately with Brca1 and the expression is tightly regulated during mammary epithelium proliferation and differentiation. And, as these two genes are coordinately expressed during proliferation and/or differentiation of mammary epithelial cells, it is very possible that Brca1 and Brca2 may function in overlapping regulatory pathways. (Rajan *et al.*, 1997). Their results also indicated that the up-regulation of Brca1 and Brca2 expression during the differentiation of mammary epithelial cells occurs by a proliferation-independent pathway, because when the post confluent mammary epithelial cells were induced for differentiation, an increased expression of both Brca1 and Brca2 was observed (Rajan *et al.*, 1996).

2-11 BRCA1 HOMOLOGUES

Studies with the homologues of tumor suppressor genes from other species provide valuable informations about the biochemical features of the protein. Especially mouse, provides a good system for the characterization of the roles of these genes and other events take part during neoplastic transformation, as their genetic background can be controlled easily.

Comparison of human BRCA1 with the murine sequence revealed 72% identity at the nucleic acid level (Marquis *et al.*, 1995), and 73% similarity and 58% identity at the amino acid level. Mouse Brca1 is located on chromosome 11 and encodes an 1812 amino-acid protein (Abel *et al.*, 1995). The mouse homolog also contains a RING finger domain near the amino terminus, and a conserved highly acidic domain in the carboxyl terminus.

CHAPTER III

AIM AND STRATEGY

Identification of function of genes requires the presence of purified protein products. Therefore this study was directed towards the production and purification of BRCA1 encoded proteins. For this reason, we designed a project, in order to express and purify the BRCA1 encoded proteins. The first step of the project involves the cloning of the BRCA1 gene in four overlapping fragments into the cloning vector pCR-Script Amp SK(+). We planned to divide BRCA1 into four fragments for two reason. First in order to minimize mutations in the amplification step due to DNA polymerase mistakes, and second it is hard to produce large proteins in E. *coli*. The second part of the project involves the subcloning of these fragments into the expression vector pQE. As approximately 90% of mutations of BRCA1 result in the formation of truncated protein products lacking the carboxyl terminal and the presence of the BRCT domain in this region, we planned express the B4 fragment. The other three fragments of BRCA1 B1, B2, and B3 will also be subcloned to pQE and expressed in future.

A general outline of the experiments performed in this study is shown in figure 3-1.

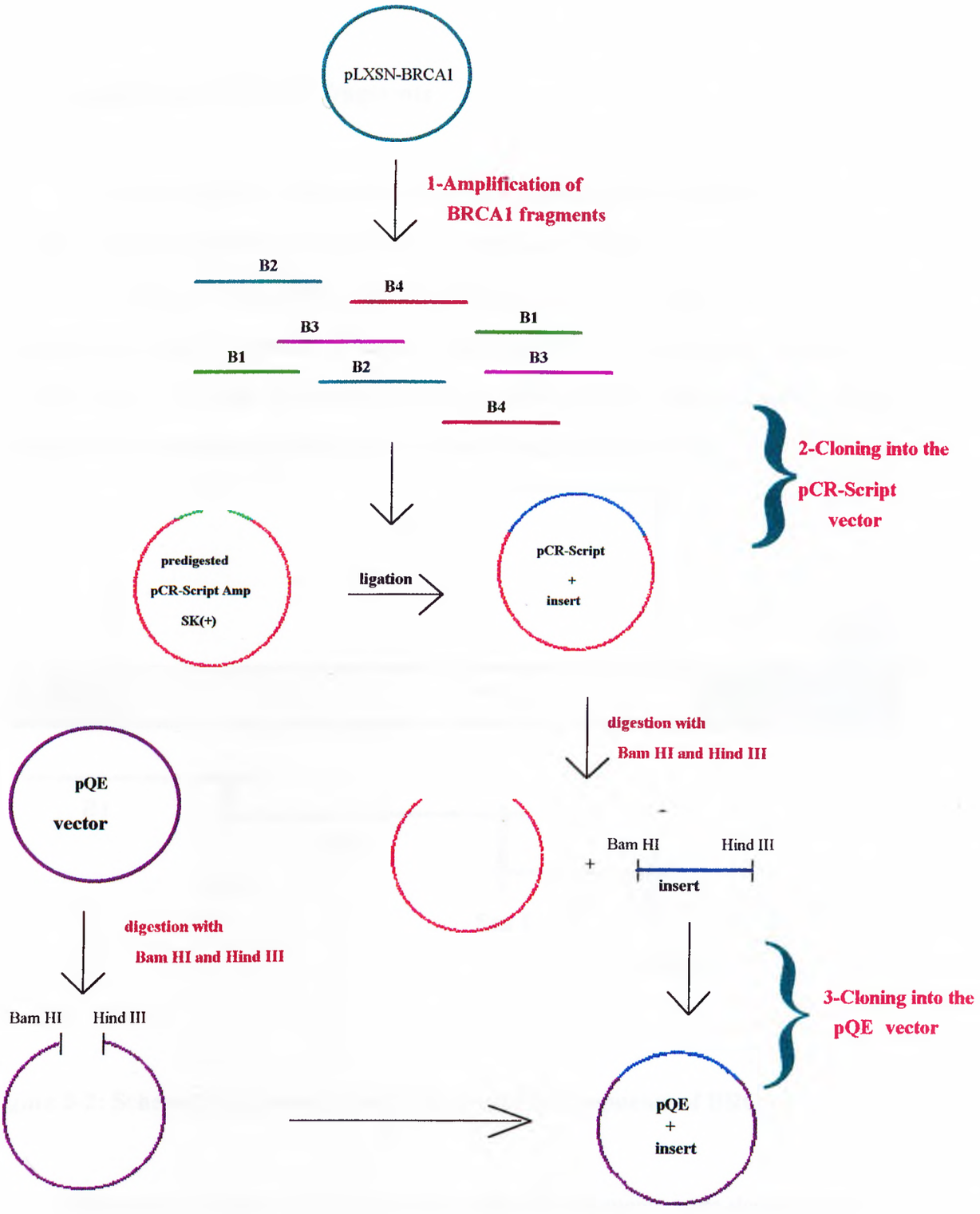


Figure 3-1: Summary of the strategy followed in this study

3-1 Amplification of BRCA1 fragments

We have designed to clone BRCA1 in four overlapping fragments (B1, B2, B3, and B4). All the fragments of BRCA1 were amplified by using *Pfu* DNA polymerase. *Pfu* DNA polymerase was preferred because of the enzyme's high fidelity and in order to generate blunt ended products. Schematic representation of the amplified regions is shown in figure 3-2. In all PCR reactions BRCA1 cDNA (pLXSN-BRCA1) was used as template and the reactions products were run on 1% agarose gel at 100V.

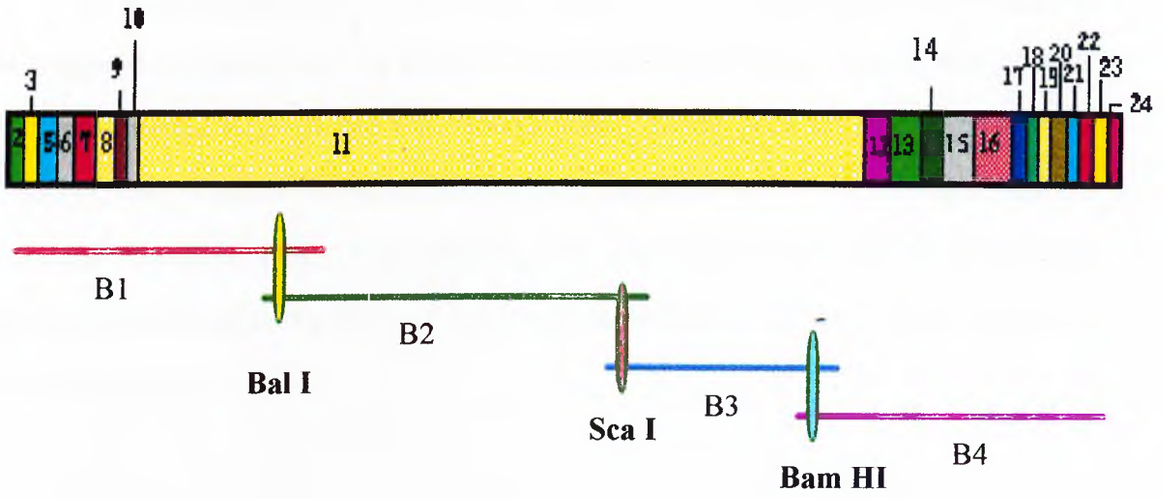


Figure 3-2: Schematic representation of the amplified fragments of BRCA1

The primers for the amplification of four BRCA1 fragments were designed such that each fragment contains a Bam HI site at its 5 prime (5') end and a Hind III site at its 3 prime (3') end. Also, the overlapping regions of the fragments contain restriction enzyme cutting sites that are known to cut BRCA1 only once (Bal I site at nucleotide

1414, Sca I site at nucleotide 3267, and Bam HI site at nucleotide 4058). These unique sites can be used to join two fragments in the future studies.

3-2 Cloning of the amplified fragments into pCR-Script Amp SK (+) vector

Each *Pfu* amplified blunt-ended PCR product was purified and cloned into the predigested pCR-Script vector. Epicurian *coli* cells were transformed with the pCR-Script-BRCA1 plasmids.

3-3 Subcloning of the B4 fragment into pQE-31

pCR-Script-BRCA1/B4 plasmid was digested with Bam HI and Hind III and the B4 fragment was taken out. As BRCA1 also has a Bam HI site at nucleotide position 4058, the internal Bam HI site was utilized instaed of the Bam HI site in the forward primer in order to put B4 in frame. B4 fragment was found to be in frame when pQE-31 was used. Therefore, pQE-31 was also digested with Bam HI and Hind III and the B4 fragment was cloned into pQE-31. A schematic representation of the cloning proedure is shown in figure 3-3.

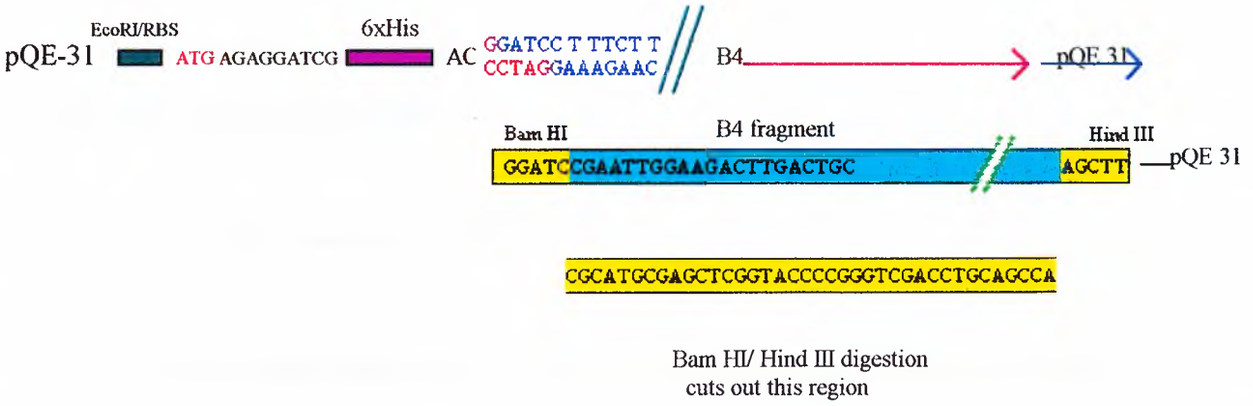


Figure 3-3 : Cloning into pQE 31

CHAPTER IV

MATERIALS AND METHODS

4-1 Polymerase chain reaction (PCR)

Polymerase chain reaction had been used during this study in order to amplify the human BRCA1 gene. The requirements for PCR are listed below (Saiki *et al.*, 1988). Pfu DNA polymerase was used to catalyze the reaction because Pfu DNA polymerase exhibits the lowest error rate of any thermostable DNA polymerase (1.3×10^{-6}). Comparison of the fidelities of different thermostable DNA polymerases are listed in table 4-1. *Pfu* DNA polymerase is a proofreading enzyme with 3' → 5' exonuclease activity, that generates blunt-ended PCR products. The buffer conditions were described by the supplier.

1-Template DNA with known flanking sequences

2-Oligonucleotides with homologous sequences to that of the template flanking region (primers)

3-DNA polymerase and appropriate buffer

4-Deoxynucleotide triphosphates

5-Temperature cycling machine

A standard 100 ul reaction set up in a 0.2 ml PCR tube

primers	0.1-1 uM of each primer (reverse and forward)
dNTP	0.2 mM diluted from the 10 mM stock
buffer	1X diluted from the 10X stock

template 10 ng of genomic or plasmid DNA

Made up to 100 ul with MilliQ water

enzyme 2.5 unit of Pfu DNA polymerase

Each reagent and tube were placed on ice. To perform a hot-start PCR the thermal cycler was preheated to approximately 90^{o}C before placing the tubes into it.

Table 4-1: Fidelity comparison of thermostable DNA polymerases

Thermostable DNA polymerases	Error rate (x 10^{-6})	Mutated PCR products (%)
Pfu DNA Polymerase	1.3	2.6
Taq DNA Polymerase	8.0	16.0
Vent DNA Polymerase	2.8	5.6
Deep *Vent* DNA Polymerase	2.7	5.4
Tfl DNA Polymerase	8.3	16.6
Tbr DNA Polymerase	9.5	19.0
UlTma DNA Polymerase	55.3	110.6

List of the synthetic oligonucleotide primer sets used in this study are summarized in table 4-2.

Table 4-2 List of the synthetic oligonucleotide primer sets used in this study

name of the primer	sequence (5'-3')	size	Tm (^{0}C)
BRC1F1	CggATCCTggATTTATCTgCTCTTCgC	27	65
BRC1R1	TAAgCTTAgCCTCATgAggATCACTgg	27	60
BRC1F2	CggATCCgAggTAgATgAATATTCTgg	27	61
BRC1R2	gAAgCTTCATTAgTACTggAACCTAC	26	51
BRC1F3	CggATCCggAACATTCAATgTCACCTg	27	67
BRC1R3	gAAgCTTAATTCCTTgTCACTCAgACC	27	57
BRC1F4	CggATCCgAATTggAAgACTTgACTgC	27	65
BRC1R4	TAAgCTTCAgTAgTggCTgTgggggg	25	61

4-2 Purification of PCR products

Purification of PCR products were done either as described below or by the MBI DNA extraction kit (#K0513).

PCR products were purified by precipitating the DNA with ammonium acetate. 1/10 volume of 10X STE buffer, equal volume of 4M ammonium acetate and 2.5 volume of 100% (v/v) ethanol were added to the PCR products sequentially. The reaction tube was centrifugated at 13000 rpm for 20 minutes at room temperature to pellet DNA. The supernatant was removed carefully and discarded. The pellet was washed with 200 ul of 70% (v/v) ice-cold ethanol. The pellet was dried and DNA was resuspended by using TE buffer.

4-3 Horizontal electrophoresis of DNA

Agarose, which is extracted from seaweed is a linear polymer. Charged molecules like DNA or RNA became oriented in an electric field and migrate towards the attracting pole of the field. Such charged polymeric molecules are also capable to migrate within the porous structure of a linear polymer, like agarose. These properties of DNA and RNA were abused to qualitatively and quantitatively analyze nucleic acids. In the presence of a good electrolitic buffer and an electric field, nucleic acids migrate towords cathode through the linear pores of polymerized agarose. The seperation capacity of such gels depend on the agarose content. Range of seperation in gels containing different amount of agarose is summarized in table 4-3.

Table 4-3 : Range of seperation in gels containing different amount of agarose

Amount of agarose in the gel (%w/v)	Efficient range of seperation of linear DNA molecules(kb)
0.3	5-20
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

Generally TAE and TBE buffers were used to define a conductive medium. These buffers have their best electrolytic ability when used as 0.5-1X solutions. Contents of these buffers are summarized in table 4-4.

Table 4-4 : Commonly used electrophoresis buffers

Buffer	Working solution	Concentrated stock solution (per liter)
Tris-acetate (TAE)	**1X: 0.04M Tris acetate** **0.001M EDTA**	**50X: 242 g Tris base** **57.1 ml Glacial acetic acid** **100 ml of 0.5 M EDTA**
Tris-borate (TBE)	**0.5X: 0.045M Tris borate** **0.001M EDTA**	**5X: 54 g Tris base** **27.5 g boric acid** **20 ml of 0.5 M EDTA**

Agarose is added at desired amount of 0.5-1 X of electrophoresis buffer (volume adjusted with dH_2O) and the slurry mixture is heated until it gets transparent. The gel apparatus must be set before and the wells that DNA will be inserted must be defined with combs that were provided with the gel apparatus. After pouring and solidification, the gel is ready to be placed in a tank filled with the same running buffer and ready for DNA to be inserted to the holes provided by the comb. A steady voltage of 50-100V is applied to the buffer and the tank is frequently checked for overheating. It is of importance to consider the place of holes in the gel and the direction of the voltage since DNA will run from (-) to (+). Visualization of DNA in the gels could be achieved

by soaking the gel in ethidium bromide solution (10ug/ml) and staining for 10 minutes. The DNA could be viewed under UV light.

4-4 DNA ligation

DNA fragments, that were cut from agarose gel and recovered by MBI DNA extraction kit (#KO513) were used for ligation. For forced cloning (where both vector and insert DNA has protruding ends) approximately 1:4 ratio of vector and insert DNA was mixed with the T4 DNA ligase buffer and 1 to 3 units of T4 DNA ligase. The reaction is carried out either 2 hours at room temperature or 16 hours at 12°C. For blunt end ligation insert:vector ratio could be increased up to 40 and preferably the reactions must be accomplished at 12°C. The reaction could be stopped directly by heating at 65°C for 10 minutes or directly used to transform competent bacterial cells.

4-5 Bacterial strains and their growth and storage

4-5.1 Bacterial strains and media

4-5.1.1 Strains of *E.coli* : Strains of *E.coli* used in this study are summarized in table 4-5.

4-5.1.2 Solid and liquid mediums :

LB medium : 0.5% Yeast extract, 1% Bacto-tryptone, 1% NaCl.

LA medium : 0.5% Yeast extract, 1% Bacto-tryptone, 1% NaCl and 1% agar. Supplemented with appropriate antibiotics.

Table 4-5 : Strains of *E.coli* used in this study

Strain	genotype	usage	reference
M15 (pREP4)	Nal s Str s *rif* s *lac*$^-$ *ara*$^-$ *gal*$^-$ *mtl*$^-$ F$^-$ *uvr*$^+$ *rec*A$^+$	Host for expression	Villajero and Zabin (1974)
Epicurian coli XL1-Blue MRF' Kan	Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac*	supercompetent cell Host for plasmid	Stratagene (#200248)
JM 109	F, *tra*D35 *lacI*^{q} Δ(*lac* Z)*m*15 *pro*AB/*rec*A1 *end*A1 *gyr*A96 (*Nal*^{r}) thi hsdR17($r^-_K m^+_K$) *sup*E44 *e*14$^-$ (*mcr*A$^-$) *rel*A1Δ(*lac-pro*AB)	propagation of the vector (pQE)	Stratagene

4-5.1.3 Antibiotics

Ampicilin and kanamycin were used in this study (Table 4-6).

Table 4-6: Concentrations of the antibiotics used in this study

	Stock solution		working concentration	
name of the antibiotic	**concentration**	**storage**	**stringent plasmids**	**relaxed plasmids**
Ampicilin	50 mg/ml in H_2O	- 20°C	20 ug/ml	60 ug/ml
Kanamycin	10 mg/ml in H_2O	- 20°C	10 ug/ml	50 ug/ml

4-5.2 Growth and storage of bacterial strains

4-5.2.1 Growth of *E. coli* strains

All strains were grown in liquid culture in LB medium and on LA medium supplemented with the appropriate antibiotics.

4-5.2.2 Storage of *E.coli* strains

Strains of *E.coli* were stored in glycerol (long term storage). Glycerol cultures of *E.coli* were prepared by adding 0.81 ml of fresh culture to 0.91 ml of sterile 80% glycerol in a sterile screw capped tube. The tubes were vortexed and were then frozen and stored at -70°C.

4-6 Cloning and subcloning of BRCA1 fragments

4-6.1 Cloning strategies

4-6.1.1 pCR Script Amp SK (+) cloning plasmid

pCR Script Amp SK (+) cloning plasmid was supplied with the pCR-Script Amp SK (+) cloning kit of Strategene (# 211190). The pCR-Script vector is a 2961 bp plasmid, derived from the pBluescript II SK (+) phagemid. This cloning vector includes an ampicilin-resistance gene, a *lac* promoter for gene expression, T3 and T7 RNA polymerase promoters for *in vitro* production of RNA, an f1 intergenic region for single-stranded DNA (ssDNA) rescue, the SK multiple cloning site, which is modified to include the *srf* I restriction-endonuclease target sequence and five conveniently located sequencing primer sites. This vector ideally works with blunt-ended PCR products. The map of pCR Script Amp SK (+) cloning plasmid is shown in figure 4-1.

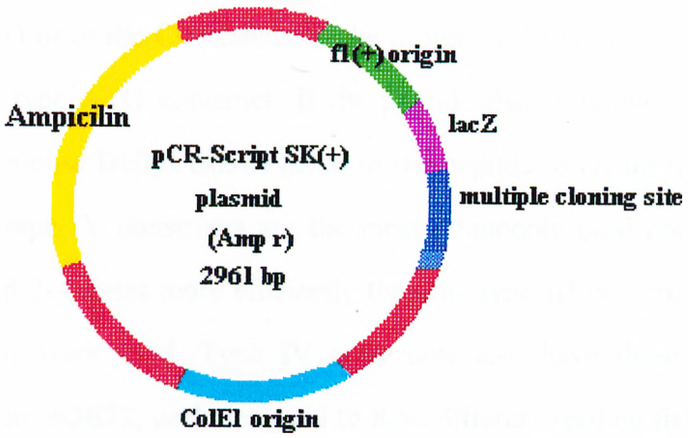


Figure 4-1: Map of the pCR Script Amp SK (+) cloning plasmid

4-6.1.2 pQE expression vectors

The pQE plasmids belong to the pDS family of plasmids and were derived from plasmids pDS56/RBSII and pDS781/RBSII-DHFRS. They contain the following elements:

A regulable promoter/operator element (consisting of the E.*coli* phage T5 promoter and two *lac* operator sequences), a synthetic ribosome binding site (RBSII), 6xHis affinity coding sequence, a multi-cloning site, translation stop codons in all three reading frames, the transcriptional terminator 't_0' from phage lambda and the replication region and the gene for β-lactamase of plasmid pBR322. A map of the pQE vector is shown in figure 4-2.

The pQE expression vectors were preferred as they provide high-level expression of proteins or peptides containing a 6xHis affinity tag in E.*coli*. The tag may be placed at

the N-terminus of the protein (Type IV constructs), C-terminus of the protein (Type III construct) or at the C-terminus of the protein utilizing its original ATG start codon, to create a type ATG construct. If the peptide that is going to be expressed is a short peptide, mouse DHFR can be fused to the peptide to create type II construct (figure 4-3). The type IV constructs are the most commonly used constructs as they are often expressed 2-4 times more efficiently than the type III constructs. In this study type IV constructs were used. Type IV constructs also have three different types, pQE30, pQE31 and pQE32, each designed to have different reading frames for the expression of the protein inframe (figure 4-2).

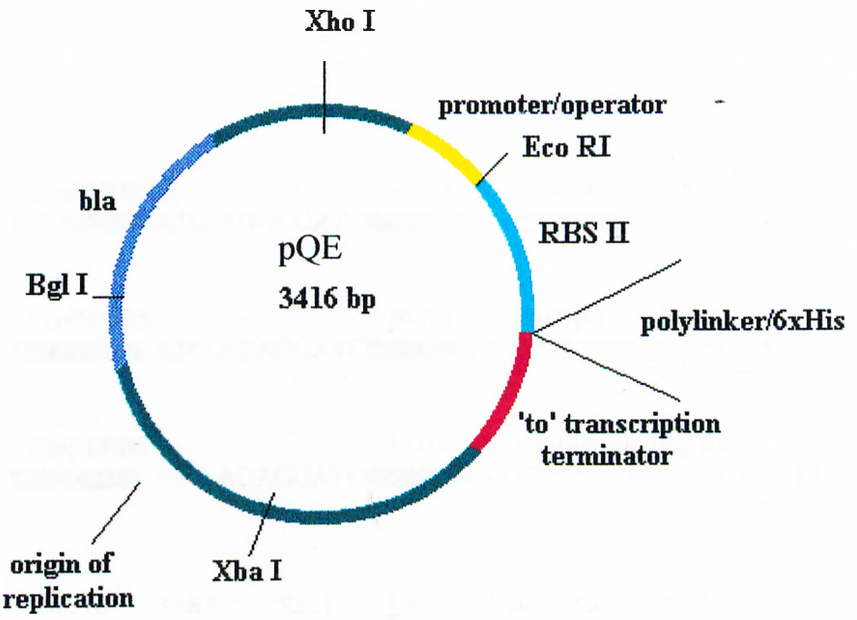


Figure 4-2: pQE vector

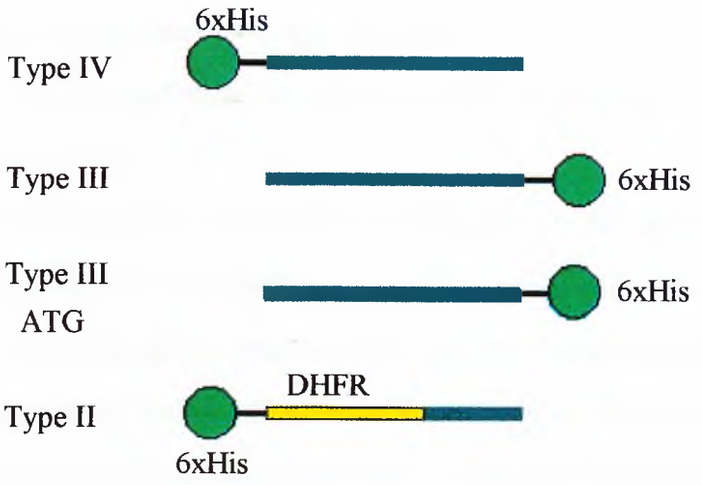


Figure 4-3: Different pQE constructs

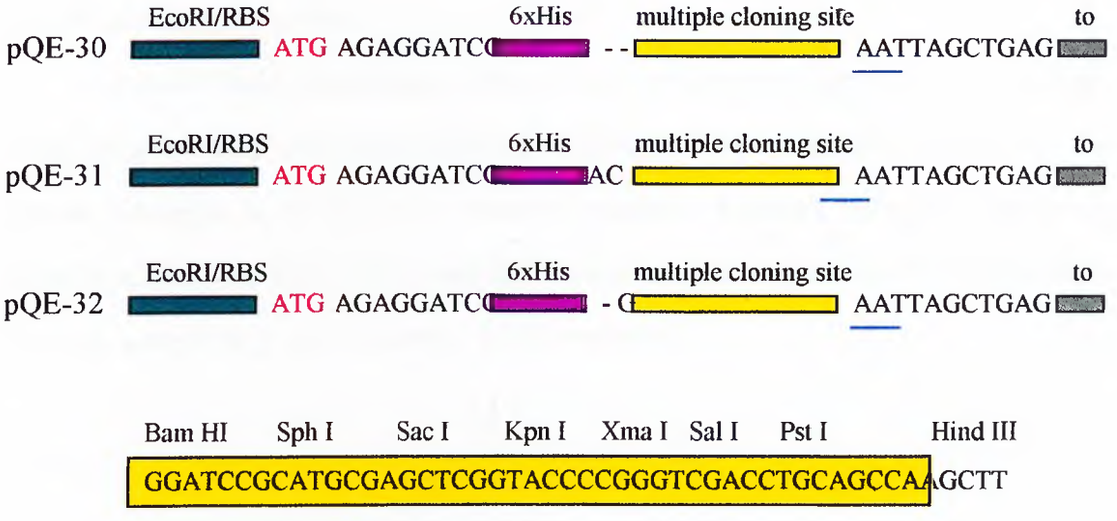


Figure 4-4 :Type IV constructs

Expression is regulated by the pREP4 repressor plasmid which carries the lacI and the kanamycin resistance gene and is present in M15 strain of E.*coli*. Expression from pQE vectors is induced by the addition of IPTG, which inactivates the repressor and clears the promoter.

The 6xHis tag allows the protein to be purified by the usage of Ni-NTA resin. The NTA ligand has four chelating sites which can interact with metal ions. NTA occupies four of the six ligand binding sites in the coordination sphere of the Ni^{2+} ion, leaving two sites free to interact with the 6xHis tag. This interaction between the 6xHis tag and Ni-NTA resin permits the purification of proteins at approximately 99% purity.

4-6.2 Growth of plasmids in transformed bacteria

Double stranded DNA was manipulated by the introduction of plasmid vectors into bacterial host cells. The existence of ampicilin and/or kanamycin resistance gene in each of these vectors was exploited to select the transformed bacterial cells during growth of bacterial cultures.

Bacterial cells transformed with plasmid vectors were streaked onto LBagar plates supplemented with the antibiotics at indicated concentrations in table 4-5 and grown overnight at 37°C in the inverted position. A single bacterial colony was transferred into 5 ml LB broth containing the appropriate concentration of the antibiotic by using a sterile loop, and rotated at 37°C overnight.

4-7 Preparation of competent cells

Cells were made competent using a modification of the $CaCl_2$ method described by Maniatis *et al.*, 1982.

5 ml LB was inoculated using a single colony from a freshly grown plate of the *E. coli* strain to be used, and was incubated at 37°C for approximately 2 hours, until the culture was slightly turbid. The culture was then cooled on ice for 5 minutes, 1 ml aliquots were added to microcentrifuge tubes and then the cells were pelleted by centrifugation (1 minute at 13,000 rpm). The cells were resuspended in 0.5 ml of 50 mM $CaCl_2$ by gentle vortexing, before being placed on ice for 30 minutes. The cells were pelleted by centrifugation (1 minute at 13,000 rpm), and the supernatant was discarded. The pellet was resuspended in 0.1 ml of $CaCl_2$ with gentle vortexing. The cells were then stored on ice until required for transformation. Alternatively competent cells were prepared and stored at -70°C until required. 500 ml of LB was seeded with a 10 ml of overnight culture and grown to an OD_{600}= 0.6. Cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4°C, before being incubated on ice for 20 minutes. The cells were harvested as before, resuspended in 25 ml of 50 mM $CaCl_2$ /20% glycerol and aliquoted into microcentrifuge tubes before being frozen. Samples were stored at -70°C and were viable for at least 2 months. Cells were thawed on ice prior to the addition of DNA.

50 mM $CaCl_2$

73.5 g $CaCl_2.2H_2O$

Add H_2O to 100 ml

Filter sterilized using a disposable filter, or autoclaved.

4-8 Transformation of bacterial cells

The DNA to be transformed (usually a 1ul of ligation mixture or approximately 100 ng of plasmid DNA) was added to the 100 ul of competent cells, mixed gently and incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 90 seconds and chilled by placing on ice for 2 minutes. 1 ml of prewarmed LB was then added and the suspension was incubated at 37°C for 1 hour. Each sample was pelleted by centrifugation at 13,000 rpm for 2 minutes, resuspended in 100-200 ul of LB and plated onto selective medium and incubated overnight at 37°C to allow the growth of the transformants.

If the vector-host system used featured α-complementation of β-galactosidase to enable for blue/white selection of recombinants, then 25 ul of a 20 mg/ml stock solution of X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) in dimethylformamide and 20 ul of a 20 mg/ml stock solution of IPTG (Isopropylthio-β-D-galactoside) in dH_2O were added to the agar plates prior to spreading the cells. If the host was DH5αMCR then IPTG was omitted, since this strain lacks the *lac* repressor and hence expression from *Plac* promoter is constitutive.

4-9 Small scale preparation of plasmid DNA (Mini-preparation)

This protocol is based on the alkaline lysis method of Birnboim and Doly (1979).

The transformed bacterial strain containing the plasmid of interest was grown at 37°C overnight in 5 ml of LB+antibiotic. 1.5 ml of the bacterial culture was pelleted by centrifugation for 1 minute (bench-top microfuge, 13,000 rpm) in a 1.5 ml microfuge tube. After the discartion of the supernatant, the cells were resuspended in 0.1 ml of ice-cold solution I and stored for 5 minutes at room temperature. 0.2 ml of solution II was mixed by inversion, the tube was then stored on ice for 5 minutes. Bacterial

chromosomal DNA and proteins were precipitated by the addition of 0.15 ml of ice-cold solution III. The mixture was left on ice for 5 minutes, then centrifugated in a bench-top centrifuge for 5 minutes to pellet the host DNA and proteins. The supernatant was mixed with an equal volume of phenol-chloroform (1:1) and centrifugated in a bench-top microfuge for 3 minutes to separate the two phases. The top phase was removed and plasmid DNA precipitated by mixing it with 2.5 volumes of 95% ethanol, and pelleted by centrifugation for 10 minutes (bench-top microfuge, 13,000 rpm) after keeping the mixture at 4°C for 15 minutes. The supernatant was discarded and the pellet was left for 15-20 minutes at room temperature to dry and then resuspended in 20-30 ul of TE buffer containing 10 ug/ml RNase A. Samples were stored at 4°C.

This procedure yields approximately 3 ug of DNA.

Solution I

50 mM glucose

25 mM Tris·Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 minutes at 10 lb/square in. on liquid cycle, and stored at 4°C.

Solution II

0.2 NaOH (freshly diluted from 10 N stock)

1% SDS

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

TE buffer

pH 7.4	10mM Tris·Cl (pH 7.4)
	1mM EDTA (pH 8.0)
pH 7.6	10mM Tris·Cl (pH 7.6)
	1mM EDTA (pH 8.0)
pH 8.0	10mM Tris·Cl (pH 78.0)
	1mM EDTA (pH 8.0)

4-10 Purification of plasmid DNA using Wizard tips (Midiprep)

All midi-preparations were carried out by using the kit supplied by Promega (Catalog number:A7510)

4-11 Purification of plasmid DNA by equilibrium centrifugation in caesium chloride-ethidium bromide gradients (Maxi-preparation)

4-11.1 Preparation of large scale bacterial culture

The bacterial strain containing the plasmid of interest was first grown in a 30 ml culture with necessary antibiotic until late log phase (OD_{600} of 0.6) then inoculated into 500 ml LB or Terrific Broth medium for 10-12 hours (2.5 ml of a solution of chloramphenicol (34 mg/ml in ethanol) could be added when OD_{600} of 0.4 is reached in order to increase the yield from low copy number plasmids.). The cells were harvested by centrifugation at 4000 rpm for 15 minutes at $4^{o}C$ in a Sorvall GS3 rotor (or equivalent). The supernatant was discarded and allow to drain away in a upside down position. Cell were resuspended in 100 ml of ice cold STE.

STE :

0.1 M NaCl

10 mM Tris.Cl(pH:8.0)

1 mM EDTA(pH:8.0)

Cells were collected again by centrifugation as described above.

4-11.2 Lysis by alkali

This protocol is based on the alkaline lysis method of Birnboim and Doly (1979) and the solutions were described above in the mini preparation section.

The bacterial pellet was resuspended and washed in 18 ml solution I (in the presence of chloramphenicol cells should be resuspended in 10 ml and the instructions in parentheses should be followed).

- 2 ml (1 ml) of freshly prepared solution of lysozyme was added (10 mg/ml in 10 mM Tris.Cl (pH:8.0))
- 40 ml (20 ml) of freshly prepared solution II was added , the top of centrifuge tube was closed and the bottle was inverted several times. The bottle was kept at room temperature for 5-10 minutes.
- 20 ml (15 ml) of ice cold solution III was added, the top of the bottle was closed and shaked several times. The bottle was stored on ice for 10 minutes.
- The cell lysate was centrifuged at 4000 rpm for 15 minutes at 4°C. The rotor must stop without braking.
- The supernatant was filtered through 4 layers of cheesecloth into a 250 ml centrifuge bottle. 0.6 volume of isopropanol was added and mixed well, in order to precipitate the nucleic acids. The bottle was kept at room temperature for 10 minutes.
- Nucleic acids were recovered by centrifugation at 5000 rpm for 15 minutes at room temperature in a Sorvall GS3 rotor (or equivalent). Salt may precipitate if the

centrifugation is performed at 4°C. The supernatant was poured off gently and the bottle was inverted so as to allow all fluid to drain away. The pellet and the walls of the bottle was washed with 70% ethanol at room temperature. After draining off ethanol, by the help of a pasteur pipette attached to vacuum, beads of liquid that were attached to the walls of the bottle were removed. The inaccessible ethanol was left to evaporate at room temperature. The pellet was dissolved in 3 ml of TE (pH 8.0) and become ready for centrifugation in CsCl-ethidium bromide gradients.

4-11.3 Purification of closed circular DNA by equilibrium centrifugation in CsCl-ethidium bromide gradients

The volume of the DNA solution must be measured exactly and for every milliliter, 1 g of solid CsCl must be added to the solution. It is feasible to warm the solution to 30°C to allow easy dissolution of the salt. The mixture was centrifuged at 8000 rpm for 5 minutes in order to separate the proteins complexed with ethidium bromide and pure nucleic acids. The clear red solution at lower phase contains nucleic acids and must be transferred to a Beckman Quick seal tube or equivalent. For this process, a disposable syringe could be used. It is very important to seal the centrifuge tube correctly and not to overload the tube. The density gradients were centrifuged at 80000 rpm for 16 hours at 20°C. Two bands appear after centrifugation in the center of the gradient and upper band contains the linear bacterial chromosome and nicked plasmid DNA, whereas the lower and thicker band corresponds to the circular plasmid DNA. The deep red pellet at the bottom of the tube consists of ethidium bromide-RNA complexes. For the collection of DNA bands, first a 21-gauge hypodermic needle was inserted into the top of the tube to allow air to enter. For minimizing the possibility of contamination, first the upper band was collected. The surface of the tube was cleaned with ethanol and a piece of Scotch Tape was attached to the outside of the tube. An 18-gauge hypodermic

needle was inserted just below the upper band and the upper band was collected. As collection was finished, the needle was plugged and a second needle was inserted just below the lower band of DNA and the viscous solution was collected. The ethidium bromide must be removed from the DNA solution by extracting at least 6 times an equal volume of isoamyl alcohol. The DNA must also be cleared away from CsCl so the final solution of DNA is diluted with 3 volumes of water and 2 volumes of ethanol (total volume is 6X of the original) and precipitated by centrifugation at 10000g for 15 minutes at 4^{o}C. This method yields 1-5 mg of plasmid DNA.

4-12 Quantification of double strand DNA

The amount of DNA in samples were determined by reading the absorbency of the samples at 260 nm. An OD260 of 1 corresponds to a concentration of 50 ug/ml for double stranded DNA, 40 ug/ml for single stranded DNA and 20 ug/ml for oligonucleotides (Maniatis *et al.*, 1982).

4-13 Restriction analysis of plasmid DNA

DNA restriction reactions were carried out in a total volume of 20-100 ul with 5-10 units of restriction enzyme. 1ul of boiled RnaseA (1mg/ml) was used where appropriate. The volume of the reaction buffers were arranged to be 1X and the enzyme volume was never greater than 1/10th of the reaction volume so as to prevent star activity due to the high glycerol concentration.

4-14 Induction of expression, lysis of bacteria and purification of proteins

Protein purification can be done under denaturing and non-denaturing conditions.

4-14.1 Small scale protein purification under denaturing conditions :

Bacteria harboring expression plasmid was cultured overnight in 5-10 ml LB with adequate selection of antibiotic. Induction of the cells were done with 0.4-2 mM IPTG for 5 hours. Cells were harvested by centrifugation at 20000 rpm for 2 minutes. The cell pellet was dissolved in 2 ml of buffer B.

The suspension was vortexed in order to lyse the cells, During this step frothing should be avoided. The suspension get clear when lysis was complete. The sample quickly loaded to the column prepared and equilibrated before.

4-14.1.1 Preparation and equilibration of the column

The column was prepared from a 10 ml syringe. The opening of the syringe was plugged with cotton glass and wetted with dH_2O. 200-400 ul 50% Ni-NTA agarose was placed into the column (It is very important not to plug very much glass cotton that could prevent flow of the fluids by gravitational force.). After allowing the Ni-NTA agarose to settle, 2 ml of buffer B was added and allowed to flow by gravity. It is very important not to allow the column dry, so as the flow ends, 200 ul of buffer B was placed into the column and the opening of the column was plugged. The column was equilibrated in this position for 30 min. at room temperature before loading.

4-14.1.2 Loading and purification of the sample

100 ul aliquot was taken from total lysate (named fraction 1) and kept at -20°C for SDS-PAGE analysis of proteins. The rest of the total lysate was loaded on the pre-equilibrated column. The fluid let flow by gravity. 6xHis tagged proteins were supposed to bind the column and the rest of the proteins were supposed to flow down and produce the second fraction named; flowthrough. Washing the column 3 times with 2 ml of buffer C was essential to remove any unspecific bound proteins from the column. These elutions gained as a result of the 3 washes were called fractions 3,4 and 5. Finally the 6xHis tagged proteins were removed from the column by 1 ml of buffer D (named fraction 6).

If desired all the proteins that bind the column could be eluted with strip buffer (named fraction 7), which removes the Ni^{++} ions from the column so the proteins bound the Ni^{++} ions. It is of importance that the stripped columns can not be used again.

The fractions can be analyzed by SDS-PAGE protein electrophoresis.

Buffer A :

8 M Urea

300 mM NaCl

50 mM NaH_2PO_4

Buffer B : same as buffer A but the overall pH of this buffer is adjusted to 8.0 with HCl.

Buffer C : Same as buffer A but pH adjusted to 6.3 with HCl.

Buffer D : Buffer C containing 400 mM imidazole.

Strip buffer : 0.1-0.5 M EDTA pH : 8.0

4-14.2 Large Scale Protein purification under non-denaturing conditions

100 ul of bacteria which had been inoculated from a colony on agar plate harboring the expression vector was inoculated into 20 ml of LB media including the appropriate antibiotic and incubated overnight at 37°C. The concentration of antibiotic could be increased in order to minimize the plasmid loss. 10 ml of this culture was then used to inoculate 500 ml of fresh LB media+antibiotic. Bacteria were grown until the absorption at 600 nm reach 0.7-0.9. IPTG is added to a final concentration of 2 mM and incubation was further continued for 5 hours.

Cells were harvested by spinning at 5000 rpm for 10 minutes at 4°C, supernatant was discarded. The pellet was resuspended in 5-volumes of solubilization buffer and homogenized in the homogenizer for 5 times of 90 second periods. It is very important to keep the samples on ice between each homogenization to avoid protein degradation.. Samples were left on a rotative shaker in a refrigerated room for 20 minutes. An aliquot of 0.5 ml was taken, and soluble and insoluble fractions were separated by centrifugation at 15,000 rpm for 5 minutes at 4°C. Both fractions were kept at -70°C. The remaining suspension was treated with liquid nitrogen and 37°C iteratively (freeze-thaw) 5 times. An aliquot of 0.5 ml was taken, and soluble and insoluble fractions were separated by centrifugation at 15,000 rpm for 5 minutes at 4°C. Both fractions were kept at -70°C. The remaining suspension was sonicated in ice cold water for 2 minutes. An aliquot of 0.5 ml was taken, and soluble and insoluble fractions were separated by centrifugation at 15,000 rpm for 5 minutes at 4°C. Both fractions were kept at -70°C. The remaining suspension was centrifugated at 4°C for 10 minutes at 13,000 rpm. The supernatant and the pellet was freezed down to -70°C.

4-14.2.1 Preparation and equilibration of the column

Preparation of the column was described above in 'small scale purification of proteins under denaturing conditions' section, but since this is a large scale purification, 1 ml of 50% Ni-NTA agarose is placed in the column and equilibrated with 2 ml of solubilization buffer without lysozyme (Buffer 2).

4-14.2.2 Loading and purification of the sample

2.5 ml of supernatant from the supernatant was applied to the column and allowed to flow with gravity. This fraction was named as flowthrough and the 6-His tagged proteins were supposed to bind the column. The column was washed 2 times with buffer 2 and 1 times with buffer 3. The wash steps produced the fractions W1, W2 and W3 and supposed to clear all the non-specific bindings, whereas the 6-His tagged proteins were supposed to retain. The elution of tagged proteins were done in 2 steps, eluting with 300 ul of buffer 4 and 300 ul of buffer 5. The fractions obtained as total lysate, flowthrough, W1, W2, W3 and buffer 4 and buffer 5 could be run in a SDS-PAGE gel.

The Ni-NTA Spin Kit was also used for the purification of proteins. (# AW 96003)

Buffer 1 (Solubilization Buffer)

50 mM Sodium phosphate (NaH_2PO_4) pH:8

300 mM Sodium Chloride (NaCl)

1mg/ml lyzozyme (freshly added)

Buffer 2

50 mM Sodium phosphate (NaH2PO4) pH:8

300 mM Sodium Chloride (NaCl)

Buffer 3

Buffer 2 with 20mM imidazole

Buffer 4

Buffer 2 with 250mM imidazole

Buffer 5

Buffer 2 with 500mM imidazole

4-15 SDS-Polyacrylamide gel electrophoresis of proteins

The glass plates were assembled according to the manufacturer's instructions (BioRad). The volume of the gel mold was determined according to the information provided by the manufacturer (BioRad). In an Erlenmeyer flask, the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel was prepared. Effective range of separation of SDS-PAGE gels due to different acrylamide concentrations are summarized in table 4-7 and concentrations of components of the resolving gel at different concentrations are summarized in table 4-8.

Table 4-7 Effective range of separation of SDS-PAGE gels:

Acrylamide concentration (%)	Linear range of separation (kD)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

Without delay, the mixture was swirled rapidly and the acrylamide solution was poured into the gap between the glass plates. Sufficient space (the length of the teeth of the comb plus 1 cm.) for the stacking gel was left. The acrylamide solution was overlayed by using a pasteur pipette with 0.1 % SDS (for gels containing <8% acrylamide) or isobutanol (for gels containing >10% acrylamide). The gel was placed in a vertical position at room temperature. After polymerization was complete, the overlay was poured off and the top of the gel was washed several times with deionised water to remove any unpolymerized acrylamide. As much fluid as possible was drained from the top of the gel and then any remaining water was removed with the edge of a paper towel.

Table 4-8 Solution of preparing resolving gels for Tris-glycine SDS-PAGE

Solution components	Component Volumes (ml)							
6%	**5 ml**	**10 ml**	**15 ml**	**20 ml**	**25 ml**	**30 ml**	**40 ml**	**50 ml**
dH_2O	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
30% mix	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.004	0.008	0.012	0.016	0.020	0.024	0.032	0.040
8%								
dH_2O	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% mix	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.030
10%								
dH_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% mix	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5

10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020
12%								
dH_2O	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% mix	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020
15%								
dH_2O	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% mix	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020

Stacking gel was prepared in a disposable plastic tube at an appropriate volume and at desired acrylamide concentration. Concentrations of components of the resolving gel at different concentrations are summarized in table 4-9. Without delay, the mixture was swirled rapidly and the stacking gel solution was poured directly onto the surface of the polymerized resolving gel. The comb was immediately inserted into the stacking gel,

being careful to avoid trapping air bubbles. The gel was placed in a vertical position at room temperature. While the stacking gel was polymerizing, the samples to be loaded were prepared by heating them to 100°C for 3 minutes in 1X SDS gel-loading buffer to denaturate the proteins. After polymerization was complete, the comb was removed carefully. By using a squirt bottle, the wells were washed with deionized water to remove any unpolymerized acrylamide.

Table 4-9 Solution of preparing 5% stacking gels for Tris-glycine SDS-PAGE

Solution components	**Component Volumes (ml)**							
5% gel	**1 ml**	**2 ml**	**3 ml**	**4 ml**	**5 ml**	**6 ml**	**8 ml**	**10 ml**
dH_2O	0.68	1.4	2.1	2,7	3.4	4.1	5.5	6.8
30% mix	0.17	0.33	0.50	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0,25	0.38	0.50	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% APS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

The gel was mounted in the electrophoresis apparatus. Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. The bubbles that were trapped at the bottom of the gel between the glass plates were removed by a bent hypodermic needle attached to a syringe. 100-200 ug of protein was loaded in a predetermined order into the wells. The electrophoresis apparatus was attached to an

electric power supply and the gel was run at a voltage that is approximately 8V/cm, After the dye front has moved to the resolving gel, the voltage was increased to 15 V/cm, until the bromophenol blue reaches the bottom of the resolving gel. Then the power supply was turned off.

The glass plates were removed from the electrophoresis apparatus and placed on a paper towel. By using a spatula, the plates were pried apart. Orientation of the gel was marked by cutting a corner from the bottom.

1X SDS gel-loading buffer

50 mM Tric·Cl (pH 6.8)

100mM dithiothreitol (DTT)

2% SDS (electrophoresis grade)

0.1% bromophenol blue

10% glycerol

1X SDS gel-loading buffer lacking DTT can be stored at room temperature. DTT should be added, just prior to use, from 1 M stock.

Tris-glycine electrophoresis buffer

25 mM Tris

250 mM glycine (electrophoresis grade)

0.1% SDS

30% mix (Acrylamide and bis-acrylamide solution)

A stock solution of 29% (w/v) acrylamide and 1% (w/v) bis-acrylamide. Solution was stored in dark bottles at $4^{o}C$.

10% SDS

A 10% (w/v) stock solution was prepared in deionized water

APS

A small amount of 10% stock solution was prepared in deionized water and stored at 4°C.

4-16 Transfer of proteins from SDS-polyacrylamide gels to solid supports

As the SDS-polyacrylamide gel was approaching the end of its run, six pieces of Whatman 3MM paper and one piece of transfer membrane (Millipore-Immobilon-P) was cut to the exact size of the SDS-SDS-polyacrylamide gel by wearing gloves because oil and secretions from the skin may prevent the transfer of proteins from the gel. Matching of the sizes of these papers and transfer membrane is very important because if the paper or membrane is larger than the gel, the overhanging edges of the paper and the membrane will touch the charged plates, causing a short circuit that will prevent the transfer of protein from the gel. One corner of the membrane was marked with a soft-lead pencil.

The membrane was left in methanol for 5 minutes and then washed with deionized water and soaked into transfer buffer for 15-20 minutes. Meanwhile the Whatman 3MM papers were soaked into a shallow tray containing a small amount of transfer buffer and kept shaking for 15-20 minutes.

Wearing gloves, the transfer apparatus was set as follows:

- 3 layers of Whatman 3MM paper that have been soaked in transfer buffer was put onto the plate which will be negatively charged (cathode). It is important to squeeze out any air bubbles.

- The transfer membrane was placed onto the Whatman 3MM papers. (The transfer membrane should be exactly aligned and the air bubbles trapped between it and the Whatman 3MM paper should be squeezed out .)

- The glass plates holding the SDS-polyacrylamide gel was removed from the electrophoresis tank, and the gel was transferred to a tray of deionized water.

- SDS-polyacrylamide gel was placed onto the transfer membrane. Any trapped air bubbles were squeezed out with a gloved hand.

- 3 layers of Whatman 3MM paper were placed to the top of the sandwich (this side will be positively charged during the transfer (anode side).)

The upper plate of the apparatus which will be the cathode during the transfer. The electrical leads of the apparatus were connected to the power supply and the transfer was carried out at a current of 0.65 mA/square. cm of the gel for a period of 1.5-2 hours.

The electric current was turned of at the end of the run time and the transfer apparatus was disassembled from top downward, peeling off each layer in turn. The gel was transferred to a tray containing Coomassie Brilliant Blue and stained in order check if the transfer is complete or not. The bottom left-hand corner of the membrane was cut as insurance against obliteration of the pencil mark.

4-17 Staining SDS-polyacrylamide gels with Coomassie Brilliant Blue

Polypeptides separated by SDS-polyacrylamide gels were fixed with methanol:glacial acetic acid and stained with Coomassie Brilliant Blue R250.

0.25 g of Coomassie Brilliant Blue R250 was dissolved in 90 ml of methanol:H_2O (1:1 v/v) and 10 ml of glacial acetic acid. In order to remove any particulate material, the solution was filtered through a Whatman No. 1 filter. The gel was immersed in at least 5 volumes of staining solution and was placed on a slowly rotating platform for a minimum of 4 hours at room temperature.

At the end of staining, the stain was removed and saved for future use. The gel was destained by soaking in the methanol-acetic acid solution without the dye (90 ml of methanol:H_2O (1:1 v/v) and 10 ml of glacial acetic acid), on a slowly rotating platform for 4-8 hours at room temperature, changing the destaining solution three or four times.

destaining for 24 hours allows as little as 0.1 ug of protein to be detected as a single band.

After destaining, gels can be stored indefinitely in water or can be dried.

4-18 Staining proteins immobilized on solid surfaces with Ponceau S

The membrane onto which proteins were transferred was washed with deionized water and then soaked into Ponceau S. When bands of proteins become visible, the membrane was washed in several changes of deionized water at room temperature. The positions of proteins used as molecular-weight standards were marked.

Ponceau S

10% glacial acidic acid

5% Ponceau S

dH_2O

4-19 Immunological detection of immobilized proteins (Western Blotting)

After staining the proteins immobilized on transfer membrane with Ponceau S, the membrane was washed gently with deionized water and neutralized with the blocking buffer for 5 minutes. In order to inhibit non-specific binding sites, the membrane was immersed in the blocking solution for at least 30 minutes. We used the mouse monoclonal antibody from Calbiochem (#OP94). Primary antibody was diluted as recommended by the supplier in blocking solution and was kept at room temperature for at least one hour on a slowly rotating platform. Afterwards the membrane was washed for three times, once for 15 minutes and twice for 5 minutes, with blocking buffer.

Following the washes the membrane was incubated in the blocking solution containing the secondary antibody (peroxidase-conjugated rabbit anti-mouse immunoglobin-DAKO) diluted as recommended by the supplier for 1 hour and then washed for three times, once for 15 minutes and twice for 5 minutes, with the blocking buffer. Finally the membrane was washed with deionized water and become ready for development.

Blocking Buffer

0.1 % Tween-20 in 1X PBS

Blocking Solution

5% milk powder in 0.1% Tween 20-PBS solution

1X PBS

8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0,24 g KH_2PO_4

Volume was adjusted to 1 liter

pH was adjusted to 7.4

4-20 Detection of proteins immobilized on membranes by using the ECL Western Blotting kit (Amersham Life Science) (Catalog no: RPN 2106)

Detection of proteins immobilized on membranes were done by using the ECL Western Blotting kit (Amersham Life Science) (Catalog no: RPN 2106)

CHAPTER V

RESULTS

5-1 Introduction

Results are explained in the following order:

- Amplification of BRCA1 fragments by PCR
- Cloning of BRCA1 fragments into pCR-Script Amp SK(+) cloning vector
- Plasmid DNA isolation be miniprep
- Confirmation of the positive clones by restriction enzyme analysis
- Maxiprep of the pQE vectors
- Subcloning of B4 into the pQE-31 vector
- Plasmid DNA isolation be miniprep
- Confirmation of the positive clones by restriction enyme analysis
- Conformation of the expression and purification of BRCA1 C-terminal (B4) encoded polypeptide by Western Blotting

Results of the restriction enyme analysis are shown following the miniprep results for each fragment.

5-2 Amplification of BRCA1 fragments

The names of the primers and the region that they amplify are shown in table 5-1 and the schematic representation of the amplified regions is shown in figure 5-2.

Table 5-1: Names of the primers and the regions that they amplify

name of the primer	name of the fragment	amplified region (nt)	length of the fragment	amplified region (aa)
BRCF1-BRCR1	B1	121-1436	1316 bp	1-439
BRCF2-BRCR2	B2	1371-3286	1915 bp	419-1052
BRCF3-BRCR3	B3	3131-4138	1008 bp	1005-1341
BRCF4-BRCR4	B4	4023-5711	1688 bp	1301-1863

The PCR conditions used during the amplification reactions are listed below:

B1: 95°C for 7 min.; (95°C for 1 min.; 56°C for 1 min.; 72°C for 3.00 min) x 35; 72°C for 10 min.; 4°C

B2: 95°C for 7 min.; (95°C for 1 min.; 56°C for 1 min.; 72°C for 4.30 min.) x 35; 72°C for 10 min.; 4°C

B3: 95°C for 7 min.; (95°C for 1 min.; 56°C for 1 min.; 72°C for 2.46 min.) x 35; 72°C for 10 min.; 4°C

B4: 95°C for 7 min.; (95°C for 1 min.; 57°C for 1 min.; 72°C for 3.30 min.) x 35; 72°C for 10 min.; 4°C

The results of the PCR reactions are shown in figure 5-1 and 5-2.

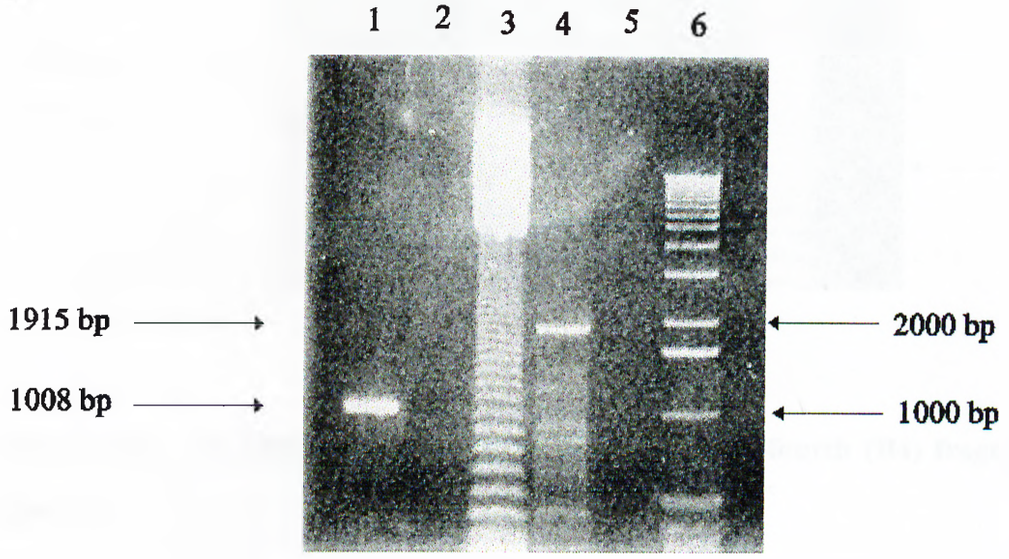


Figure 5-1: Amplification results of the second (B2) and third (B3) fragments of BRCA1

(**Lane 1**: B3; **Lane 2**: B3 negative control; **Lane 3**: 123 bp DNA ladder (Sigma); **Lane 4**: B2; **Lane 5**: B2 negative control; **Lane 6**: 1 kb DNA ladder); (7 ul of 50 ul)

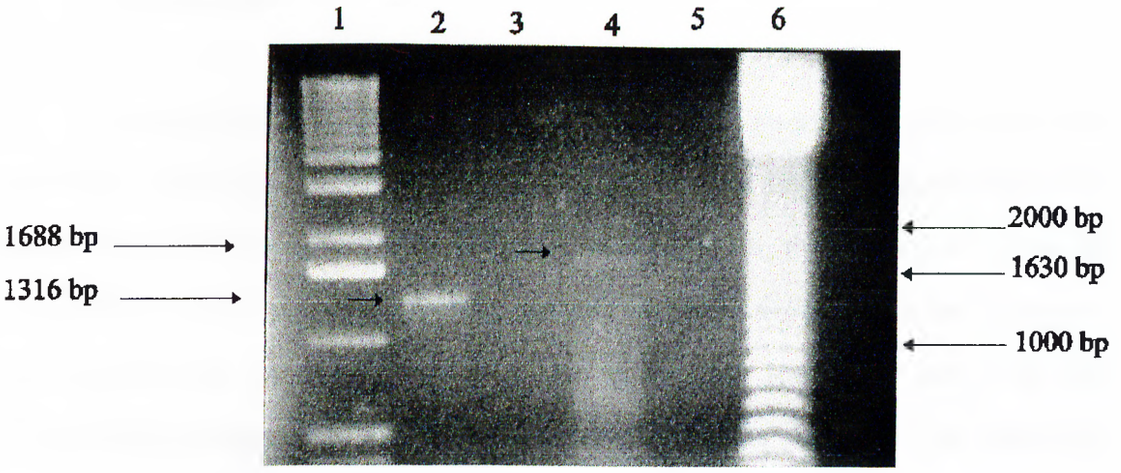


Figure 5-2: Amplification results of the first (B1) and fourth (B4) fragments of BRCA1

(**Lane1**:1 kb DNA ladder; **Lane 2**: B1 ; **Lane 3**: B1 negative control; **Lane 4**: B4; **Lane 5**: B4 negative control; **Lane 6**: 123 bp DNA ladder); (7 ul of 50 ul)

The expected lengths of the PCR products are shown in table 5-1. Amplification reactions resulted in the production of BRCA1 fragments of the expected length with the absence of non-specific bands, indicating the accuracy of the PCR conditions. As control, the same reactions were carried out in the absence of template DNA in order to show that the bands are not the results of any contamination.

5-3 Cloning of the 4 different PCR fragments of BRCA1 into the pCR-Script cloning vector

The amplified BRCA1 fragments were purified and inserted into the pCR-Script Amp SK(+) cloning vector (Stratagene, #211190). The *Pfu* amplified, blunt ended PCR products were incubated with the predigested cloning vector, the restriction enzyme *Srf* I (1 ul from 5 U/ul stock) and T4 DNA ligase (1 unit from 4U/ul stock). Both enzymes were supplied by Strategene. Srf is a rare cutter that specifically cuts from the GCCC/GGGC sequence to generate blunt ended products. Usage of the restriction enzyme in the ligation reaction maintained a high-steady state concentration of the digested vector.

The cloning procedure is summarized in figure 5-3.

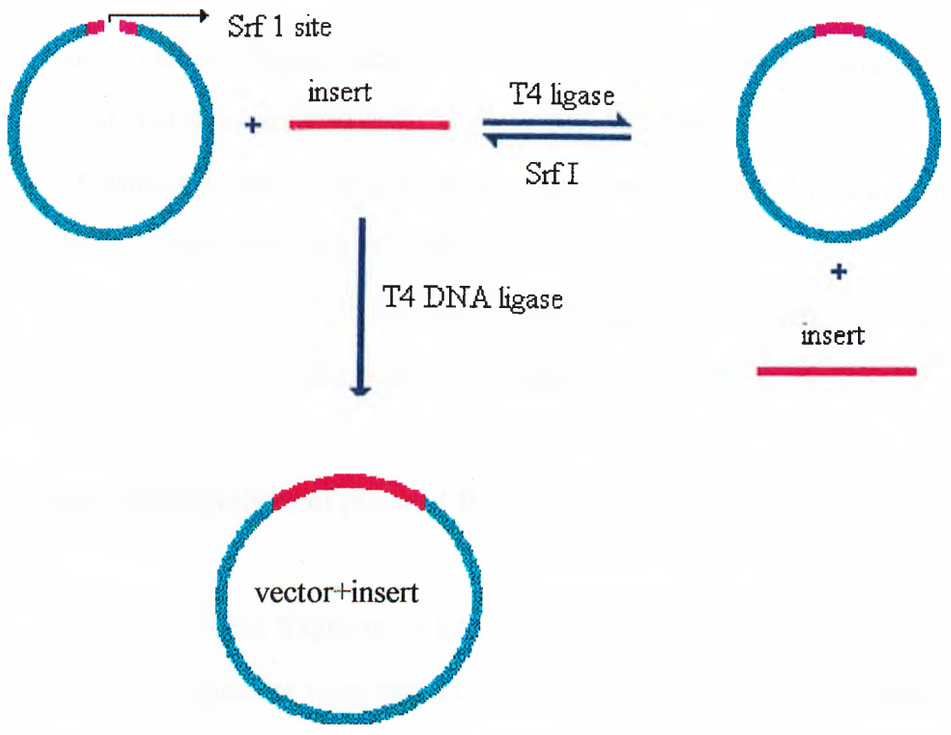


Figure 5-3: A summary of the ligation protocol

5-4 Minipreps of plasmid DNA

Following the transformation of the ligation products into the Epicurian Coli XL1-Blue MRF' Kan supercompetent cells (Stratagene), the positive clones were selected on the basis of blue/white selection on IPTG - X-Gal LB-agar plates. The IPTG-Xgal LB-Agar plates were prepared by spreading 40 ul of 2% X-Gal (dissolved in dimethylformamide) and 25 ul of 100 mM IPTG (dissolved in dH_2O) 1 hour before the inoculation of bacteria, as IPTG can be toxic to cells if it is not absorbed by the LB-agar. The white colonies were the ones harboring the insert and the blue color indicates the

absence of the insert because in such colonies *lacZ* gene is still intact and the IPTG in the medium induces this gene to produce β-galactosidase. β-galactosidase in the medium uses X-gal, and this reaction results in the formation of blue color.

Plasmids in the supercompetent cells were purified by using the miniprep technique explained in the materials and methods section.

The plasmids with the insert were expected to run slower on 1% agarose gel. The results of the minipreps are shown in figures 5-4, 5-5, 5-11, 5-13, 5-15.

5-5 Restriction analysis of plasmid DNA

Presence of the fragment of interest in the plasmid was confirmed by restriction analysis. All the reactions were carried out by using 300-500 ng of plasmid DNA and 5-10 U of each enzyme, in the appropriate buffer.

The results of the reactions are shown in figures 5-6, 5-7, 5-10, 5-12, 5-14, 5-16.

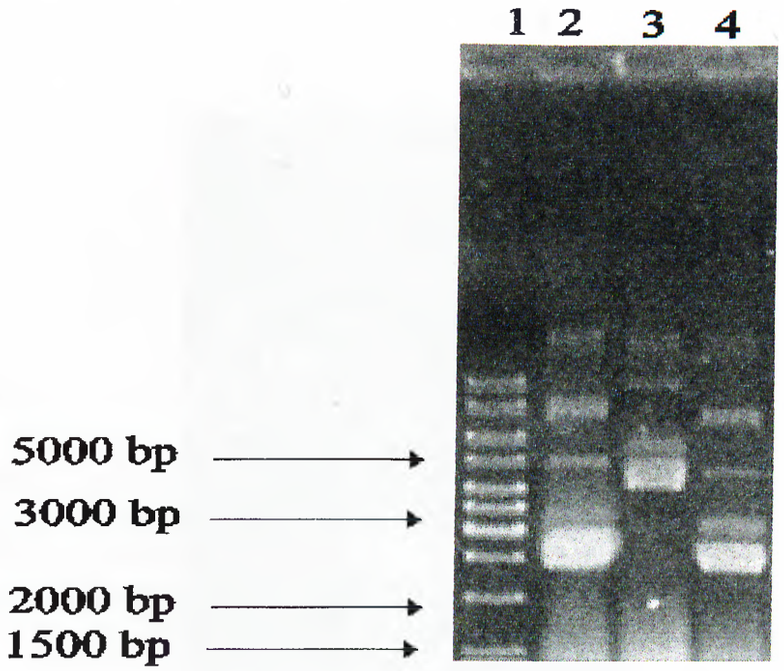


Figure 5-4: Miniprep result of the clones that are expected to carry the first fragment (B1)

(**Lane 1**: 1 kb DNA ladder (MBI), **Lane 2** : B1-clone5, **Lane 3** : B1-clone 6, **Lane 4** : B1-clone 7); (4 ul of 25 ul)

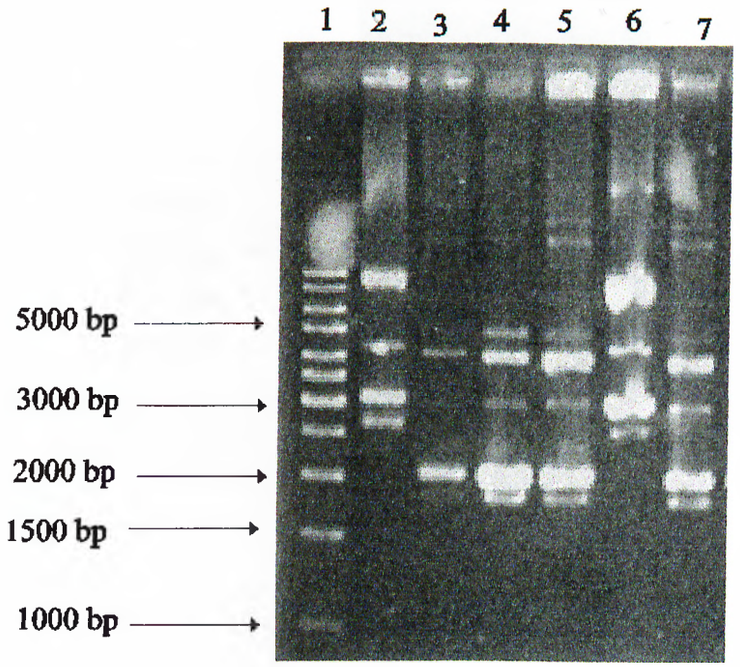


Figure 5-5: Miniprep result of the clones that are expected to carry the first fragment (B1)

(**Lane 1**: 1 kb DNA ladder (MBI), **Lane 2**: B1-clone 6, **Lane 3**: B1-clone 8, **Lane 4**: B1-clone 9, **Lane 5**: B1-clone 10, **Lane 6**: B1-clone 11, **Lane 7** : B1-clone 12); (4 ul of 25 ul)

As the shift of the bands indicate the presence of the insert, among the 13 clones tested, only two of them were said to be positive (clones 6 and 11). The bands seen at smaller lengths indicate the presence of different conformations of plasmid DNA, occurred due to the alkaline treatment of DNA in the second step of the DNA purification.

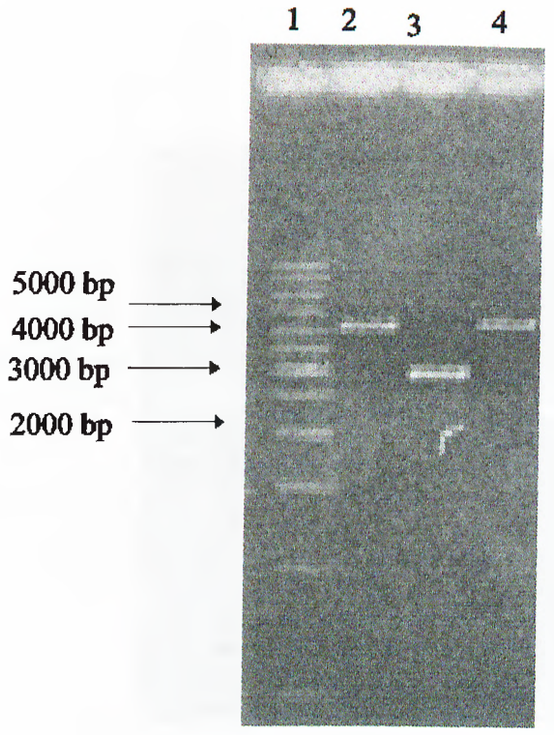


Figure 5-6 : Restriction enzyme digestion of the DNA obtained from the minipreps of the clones carrying the first fragment (B1)

(**Lane 1**: 1 kb DNA ladder (MBI), **Lane 2** : B1-clone 6- Hind III cut, **Lane 3** : B1-clone 1-Hind III cut, **Lane 4** : B1-clone 11- BamHI cut) (10 ul reaction volume loaded)

The digestions of the plasmid DNA obtained from clones 1, 6 and 11, showed that the shift in the run pattern of DNAs indicate the presence of the insert in the plasmids. Clone number 1 was used as a negative control. As expected, vector without the BRCA1 fragment run approximately at 3000 bp (2961 bp) and the vectors with the insert run approximately at 4000 bp (4277 bp).

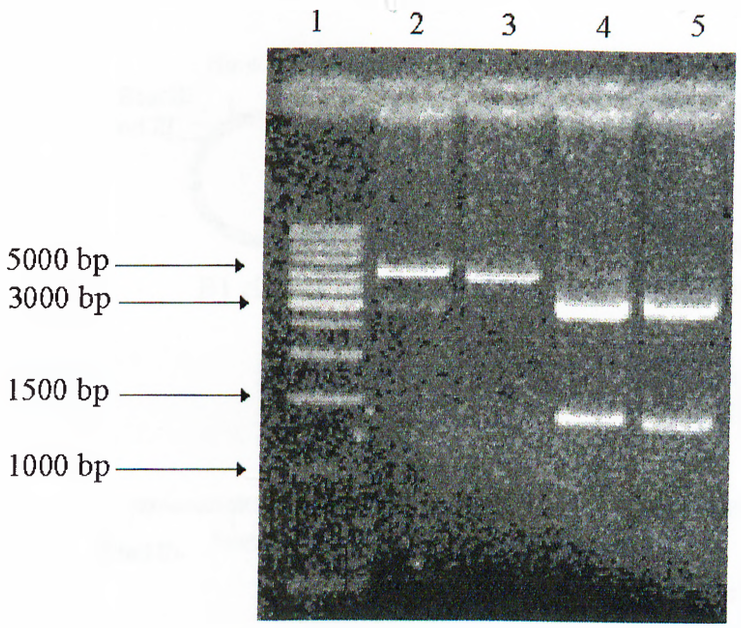


Figure 5-7 : Restriction enzyme digestion of the DNA obtained from the minipreps of the clones carrying the first fragment (B1)

(**Lane 1**: 1 kb DNA ladder, **Lane 2** : B1-clone 6- Hind III cut, **Lane 3** : B1-clone 6- Hind III and BamHI cut, **Lane 4** : B1-clone 11- Hind III cut, **Lane 5** : B1-clone 11- Hind III and BamHI cut); (10 ul reaction volume loaded)

As a result of this experiment two bands were expected. The different digestion patterns of the two clones indicate that the BamHI site of the first fragment was somehow lost and that the fragments were inserted in opposite orientations into the vector, as shown in figure 5-8. The presence of the insert was further confirmed by digesting the vector with Sal I and Sca I (Figure 5-9). These two enzymes cut only from the multiple cloning site of the vector and the first fragment of BRCA1 do not have the recognition sites for these enzymes. Results of these digestions are shown in figure 5-10.

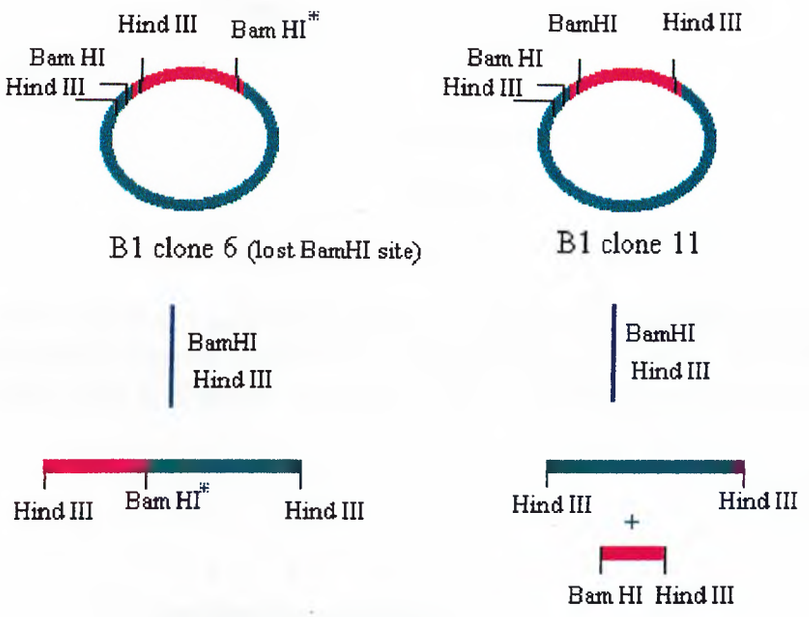


Figure 5-8 : The two orientations of the B1 insert in pCR-Script cloning vector.

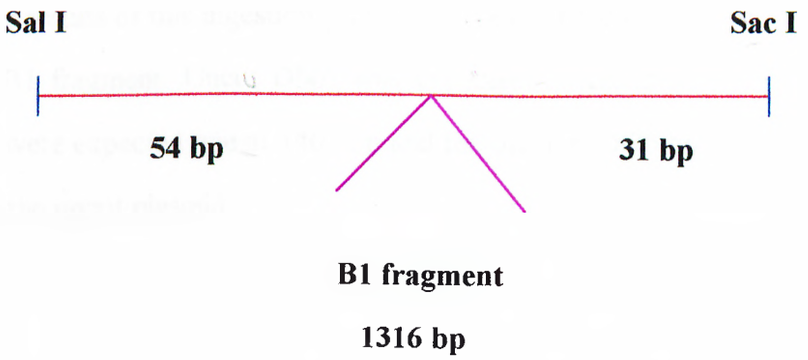


Figure 5-9: The Sac I and Sal I restriction sites on the pCR-Script cloning vector.
(If the insert is present length of the digestion product will be: 1316+85=1401 bp and when this region is digested out length of the remaining vector will be 2961-85=2876 bp)

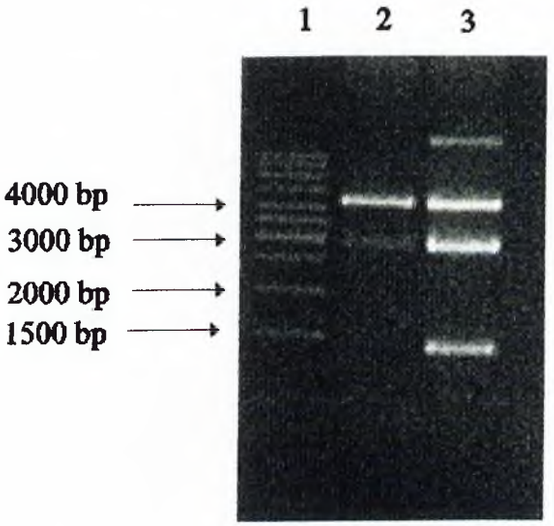
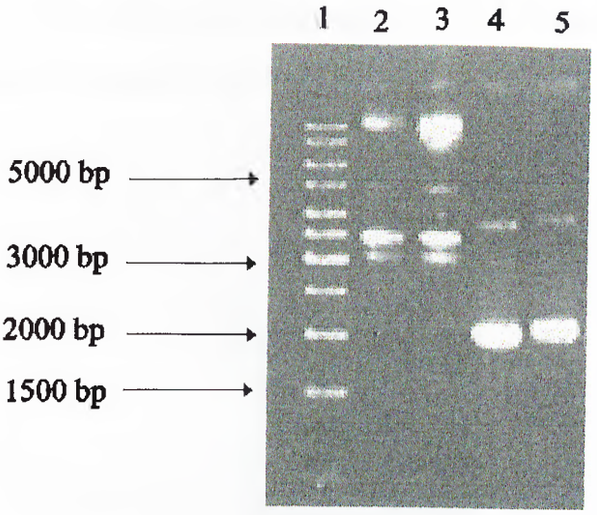


Figure 5-10 : Digestion of the DNAs obtained from clones B1-6 and B1-11 with Sac I and Sal I

(**Lane 1**: 1 kb DNA ladder (MBI), **Lane 2**: B1-clone 6, **Lane 3**: B1-clone 11); (10 ul reaction volume loaded)

The results of this digestion proved that both of the clones carry the vectors that carry the B1 fragment. Uncut DNA was expected to run at 4277 bp and two other fragments were expected one at 1401 bp and the other at 2876 bp. The band of 4277 bp represents the uncut plasmid.

A-



B-

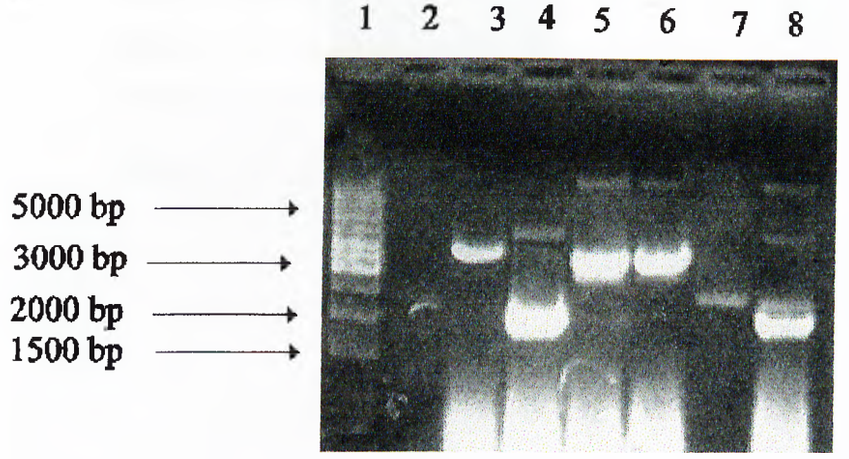
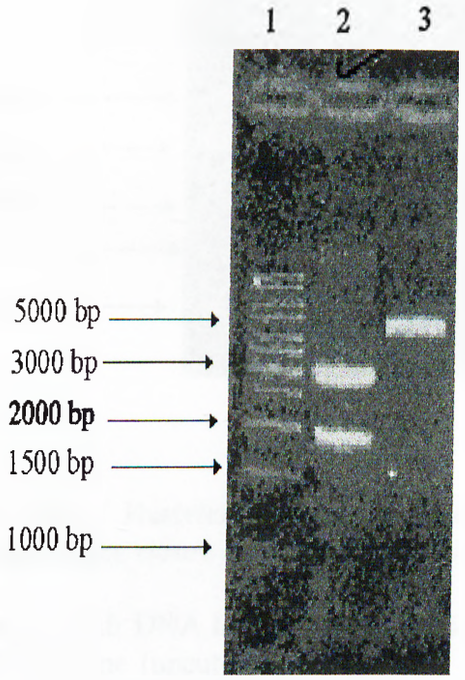


Figure 5-11 : Miniprep result of the clones that are expected to carry the second fragment (B2)

A:(**Lane 1**: 1 kb DNA ladder (MBI), **Lane 2**: B2-clone 1, **Lane 3**: B2-clone 2, **Lane 4**: B2-clone 3)
B:(**Lane 1**: 1 kb DNA ladder (MBI), **Lane 2**: empty, **Lane 3**: B2-clone 4, **Lane 4**: B2-clone 5, **Lane 5**: B2-clone 6, **Lane 6**: B2-clone 7, **Lane 7**: B2-clone 8, **Lane 8**: B2-clone 9); (4 ul of 25 ul)

Five of the nine clones (clones 1, 2, 3, 5 and 6) were thought to be positive and digested by Bam HI and Hind III.

A:



B:

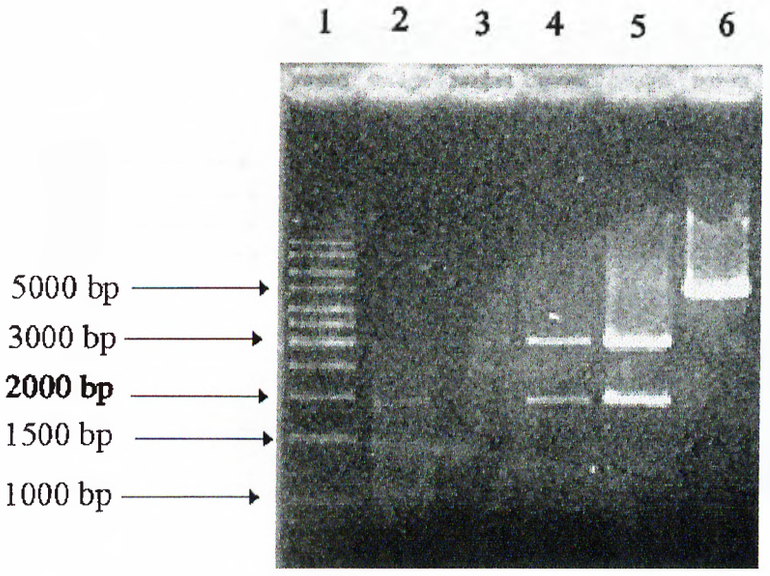


Figure 5-12 : Restriction enzyme digestion of the DNA obtained from the minipreps of the clones carrying the second fragment (B2)

A:(**Lane 1**: 1 kb DNA ladder (MBI), **Lane 2**: B2-clone-1- Hind III and BamHI cut, **Lane 3**: B2-clone 1uncut-control)
B:(**Lane1**: 1 kb DNA ladder (MBI), **Lane 2**: B2-clone-2, **Lane 3**: empty, **Lane 4**: B2-clone 4-Hind III and BamHI cut, **Lane 5**: B2-clone 6-Hind III and BamHI cut. **Lane 6**: B2-clone 6-uncut-control); (10 ul reaction volume loaded)

Results of the restriction enzyme digestion showed the presence of B2 harboring plasmid in the colonies indicated above.

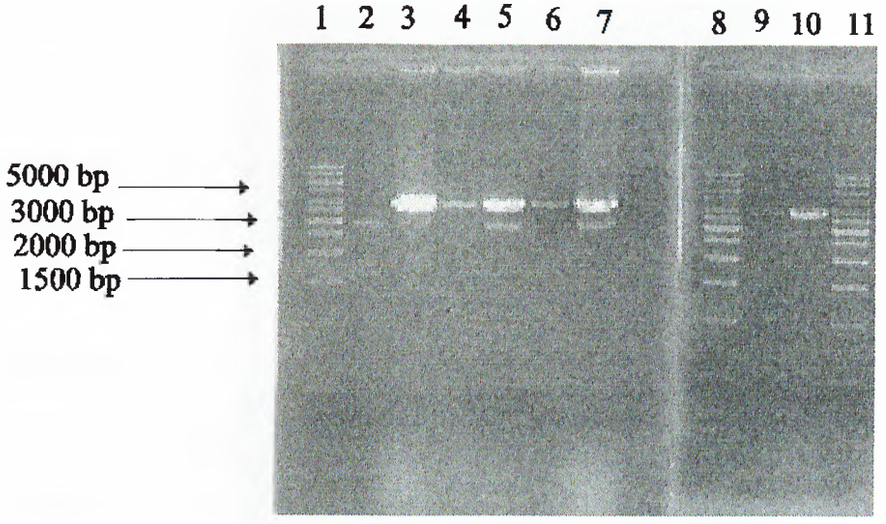


Figure 5-13 : Miniprep results of the clones that are expected to carry the third fragment (B3)

(**Lane 1:** 1 kb DNA ladder (MBI), **Lane 2:** empty vector-control, **Lane 3:** B3-clone 1, **Lane 4:** B3-clone 2, **Lane 5:** B3-clone 3, **Lane 6:** B3-clone 4, **Lane 7:** B3-clone5, **Lane 8:** empty, **Lane 9:** 1 kb DNA ladder (MBI), **Lane 10:** B3-clone 6. **Lane 11:** B3-clone 7, **Lane 12:** 1 kb DNA ladder (MBI)); (4 ul of 25 ul)

Comparison of the lengths of DNA fragments in lanes 3, 4, 5, 6, 7, 10, and 11 with the empty plasmid control in lane 2, suggested that all the clones were positive. Restriction enzyme analysis of the positive fragments with Hind III and Bam HI showed that the insert was B3.

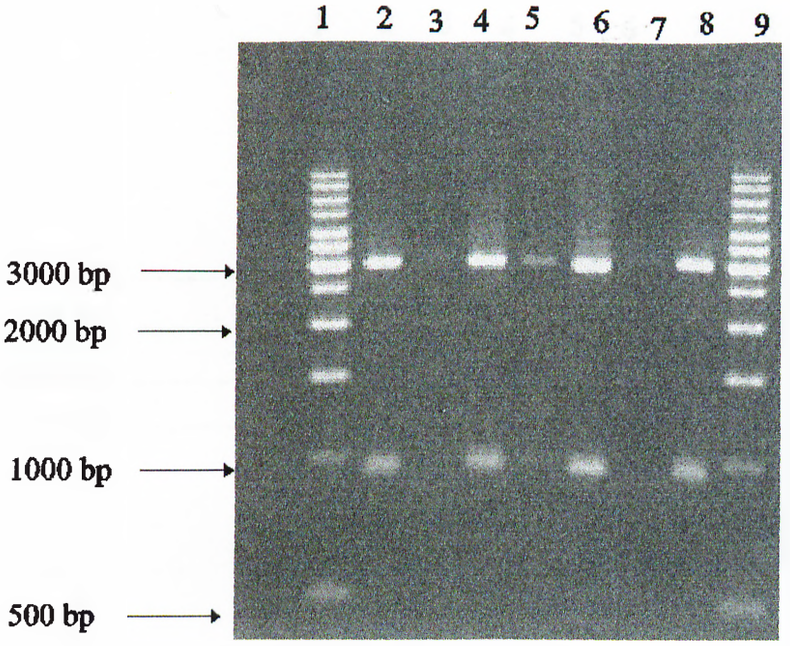


Figure 5-14: **Restriction enzyme digestion of the DNA obtained from the minipreps of the clones carrying the third fragment (B3)**

(**Lane1**: 1 kb DNA ladder (MBI), **Lane 2**: B3-clone 1, **Lane 3**: B3-clone 2, **Lane 4**: B3-clone 3, **Lane 5**: B3-clone 4, **Lane 6**: B3-clone 5, **Lane 7**: B3-clone 6, **Lane 8**: B3-clone 7, **Lane 9**: 1 kb DNA ladder (MBI); (10 ul reaction volume loaded)

Results of the restriction enzyme digestion showed the presence of B3 harboring plasmid in the colonies indicated above.

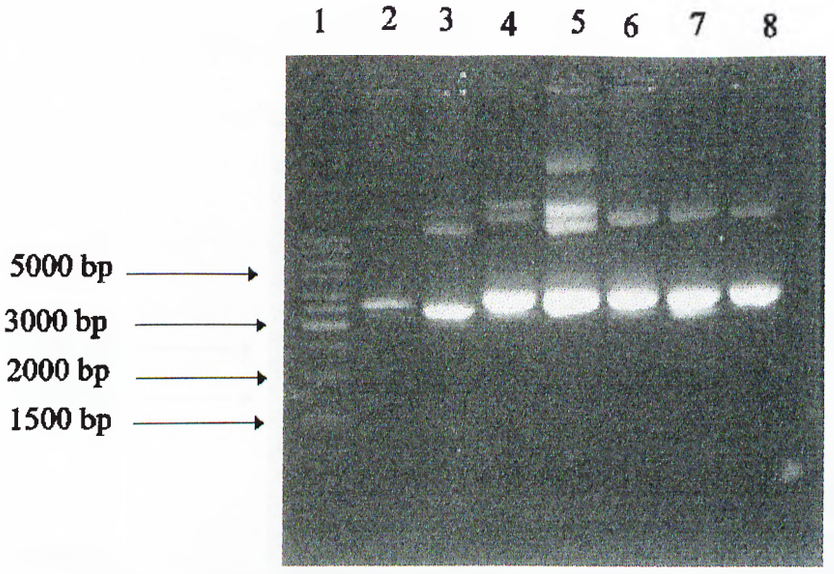


Figure 5-15: Miniprep results of the clones that are expected to carry the fourth fragment (B4)

(**Lane 1:** 1 kb DNA ladder (MBI), **Lane 2 and 3**: control minipreps , **Lane 4:** B4-clone 1, **Lane 5:** B4-clone 2, **Lane 6:** B4-clone 3, **Lane 7:** B4-clone 4, **Lane 8:** B4-clone 5) ; (4 ul of 25 ul)

Results of the miniprep indicated that all of the five clones are harboring plasmids with an insert and in order to show that the insert is B4, the plasmids were digested with Bam HI and Hind III.

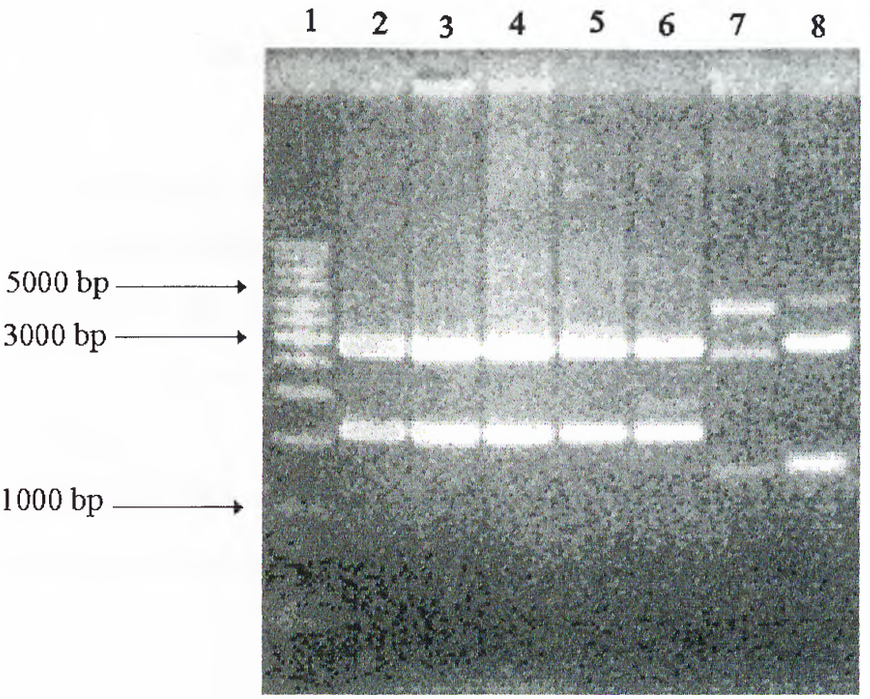


Figure 5-16: **Restriction enzyme digestion of the DNA obtained from the minipreps of the clones carrying the fourth fragment (B4)**

(**Lane1**: 1 kb DNA ladder (MBI), **Lane 2**: B4-clone 1, **Lane 3**: B4-clone 2, **Lane 4**: B4-clone 3, **Lane 5**: B4-clone 4, **Lane 6**: B4-clone 5); (10 ul reaction volume loaded)

Restriction enzyme digestion of the positive fragments with Bam HI and Hind III showed that the insert was B4.

5-6 Purification of plasmid DNA by equilibrium centrifugation in caesium chloride-ethidium bromide gradients (Maxiprep)

The maxipreps of the pQE expression vectors were done as described in the materials and methods section. All three types of the pQE vectors: pQE 30, 31 and 32 were first amplified in the host JM109 and then following the miniprep (figure 5-17) and restriction analysis (figure 5-18), the vectors were purified in large quantities by maxiprep.

The DNA yield was about 2 ug/ul (total 2 mg DNA). The results of the maxipreps are shown in figure 5-19.

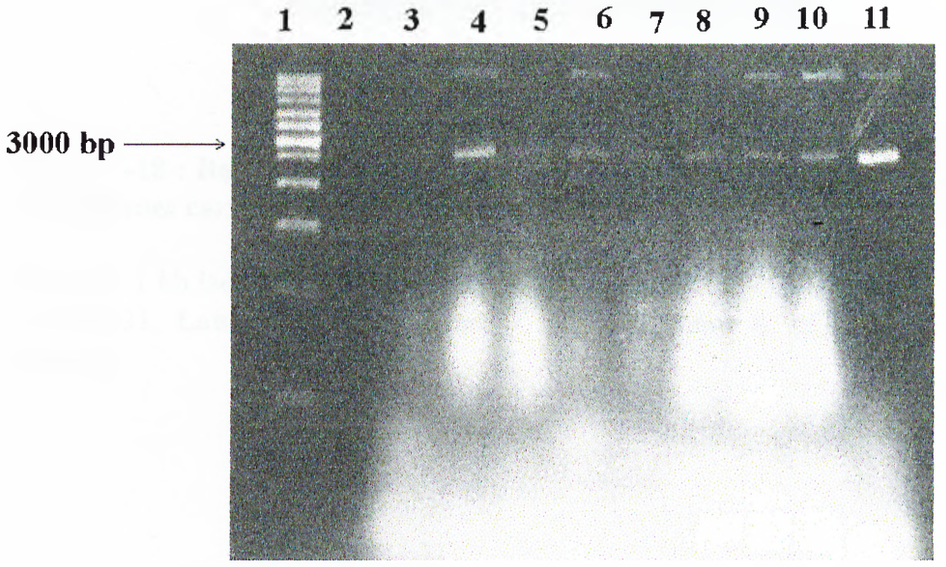


Figure 5-17 : Miniprep results of the clones that are expected to carry the PQE vectors(Lane 1: 1 kb ladder (MBI), **Lane 2**: empty, **Lane3**: pQE 30, **Lane 4**: pQE 30, **Lane 5**: pQE 30, **Lane 6** pQE 31, **Lane 7**: pQE 31, **Lane 8**: pQE 31, **Lane 9**: pQE 32, **Lane 10**: pQE 32, **Lane 11**: pQE 32); (4 ul of 25 ul)

The expected uncut size of pQE was expected to be approximately 2500 bp, (cut size is 3460 bp).

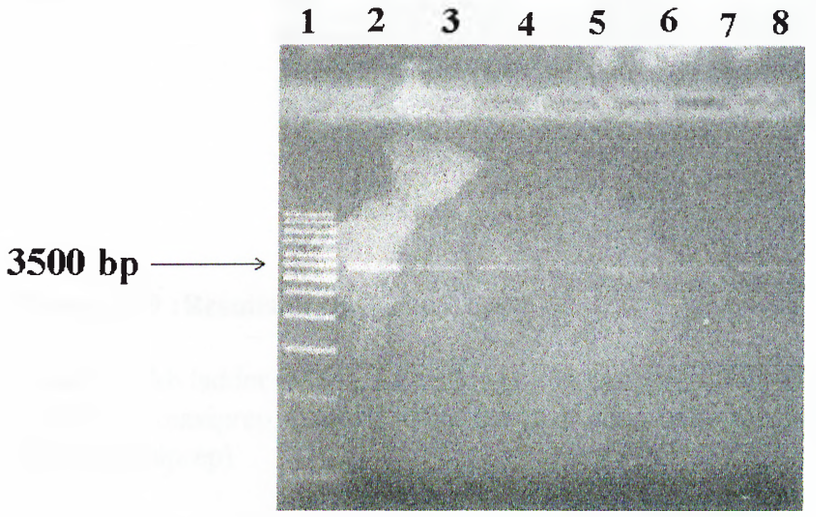


Figure 5-18 : Restriction enzyme analysis of the DNA obtained from the minipreps of the clones carrying the pQE vector.

(**Lane 1**: 1 kb ladder (MBI), **Lane 2**: pQE 30, **Lane3**: pQE 30, **Lane 4**: pQE 30, **Lane 5**: pQE 31, **Lane 6**: pQE 31, **Lane 7**: pQE 32, **Lane 8**: pQE 32); (10 ul reaction volume)

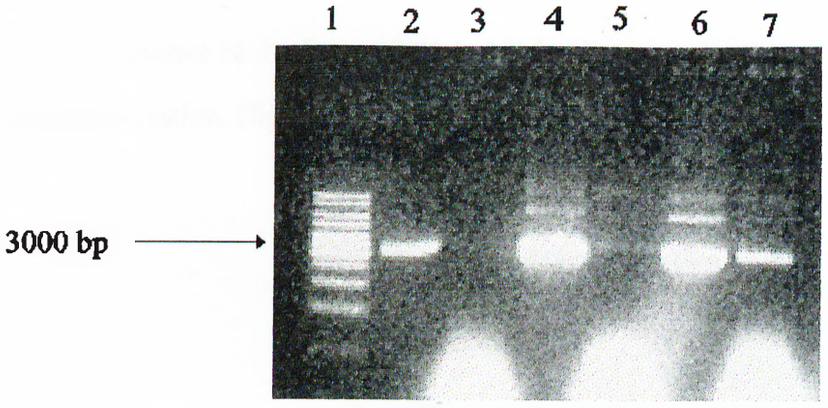


Figure 5-19 :Results of the maxipreps

(**Lane 1**: 1 kb ladder (MBI), **Lane 2**: pQE 30 maxiprep, **Lane3**: pQE 30 miniprep, **Lane 4**: pQE 31 maxiprep, **Lane 5**: pQE 31 miniprep, **Lane 6**: pQE 32 maxiprep, **Lane 7**: pQE 32 miniprep)

These results indicated the presence of a pure and high yield DNA without RNA contamination.

5-7 Subcloning of the C-terminal of BRCA1 into the expression vector pQE

The C-terminal region of BRCA1 (B4) was subcloned into pQE31. For this purpose the DNA extracted from pCR-Script-B4 harboring cells by midiprep was digested with Bam HI and Hind III enzymes in order to cut out the B4 fragment (Figure 5-20) and the B4 fragment was recovered by eluting the band of interest from 1% agarose gel. Similarly pQE31 was digested with the two enzymes and the vector was eluted from 1% agarose gel by using the MBI DNA extraction kit (# K05132). The ligation and transformation reactions were carried out as described in the materials and methods section. For transformation, M15 cells were used, as these cells contain another plasmid coding for the repressor (prep4). After transformation cells were grown in LB-medium containing both ampicilin and kanamycin.

Presence of the B4+pQE31 in M15 cells were shown by miniprep and restriction enzyme digestion. (figure 5-21 and figure 5-22)

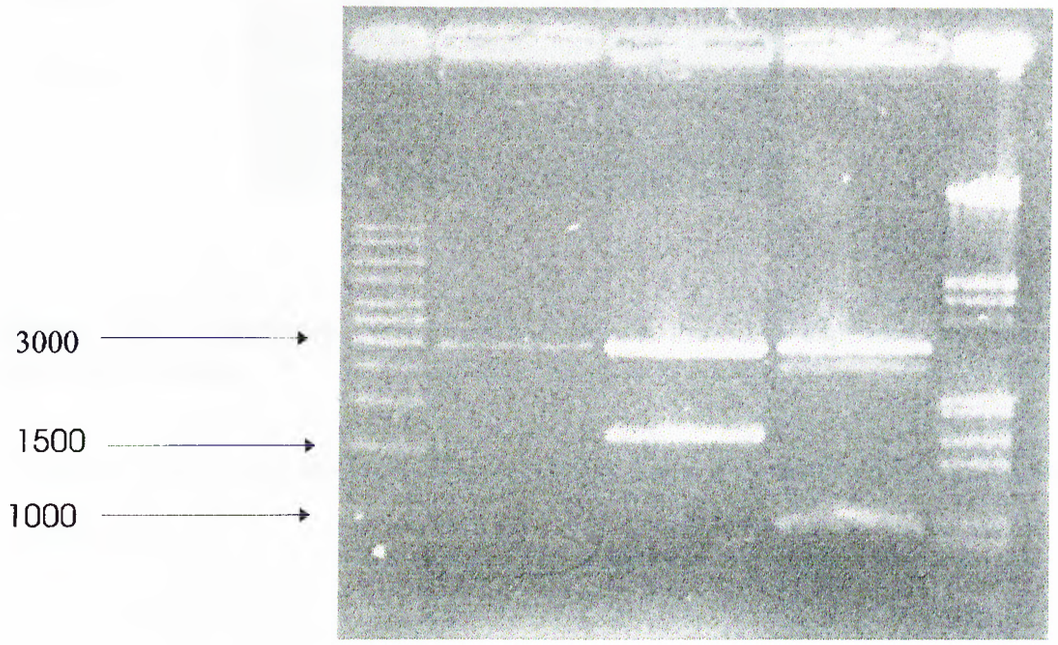


Figure 5-20 : Restriction enzyme digestion of B4+pCR-Script vector with Bam HI and Hind III.

(**Lane 1**: 1 kb marker (MBI), **Lanes 2 and 3**: control digestions, **Lanes 4 and 5**: B4 Bam HI and Hind III cut, **Lanes 6 and 7**: control digestions, **Lane 8**: (λ DNA/ EcoRI-Hind III-MBI).

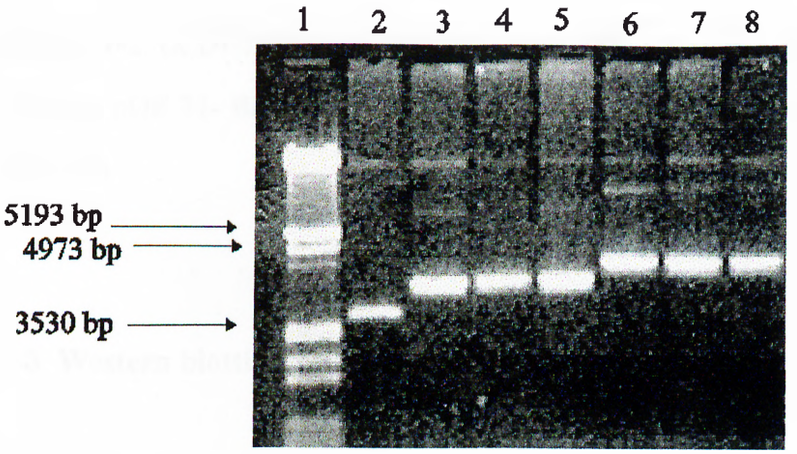


Figure 5-21 : Miniprep results of the clones that are expected to carry the B4+PQE31 vector

(**Lane 1**: (λ DNA/Eco RI- Hind III digest-MBI), **Lane 2-5**: control minipreps, **Lane 6-8**: B4 in pQE)

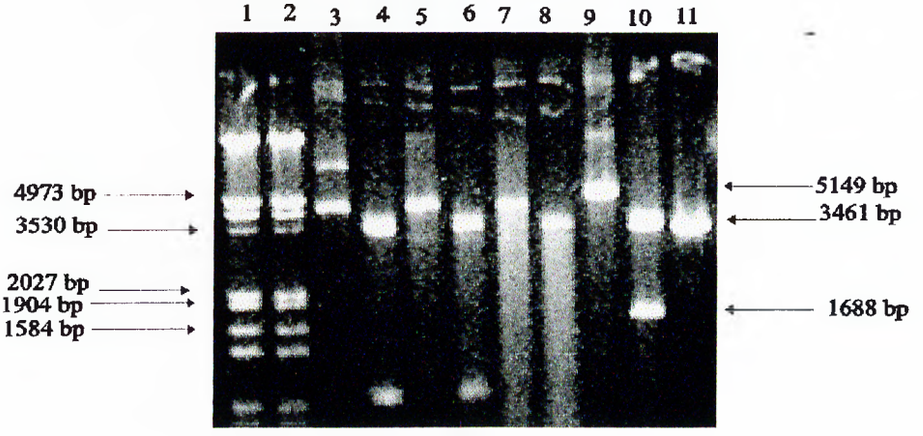


Figure 5-22 : Restriction enzyme digestion of B4+pQE31 vector with Bam HI and Hind III.

(**Lanes 1-2**: (λ DNA/Eco RI- Hind III digest-MBI), **Lanes 3-8**: control digestions, **Lane 9**: B4 in pQE 31- Bam HI cut, **Lane 10**: B4 in pQE 31- Bam HI-Hind III cut, **Lane 11**: pQE 31)

5-8 Western blotting

The pQE+B4 harboring M15 cells were grown and induced, and the protein (carboxyl terminal of BRCA1) was purified as described in the materials and methods section. Induction of the B4 fragment was shown by loading the non-induced and induced samples to the gel (figure 5-23). Although most of the protein content in the cell was found to be insoluble (5-24), a small-scale purification was performed under denaturating conditions and a large-scale purification was performed under non-denaturating conditions. Western blot analysis of the proteins with the BRCA1 antibody, BRCA1-3 (#OP94) revealed a purified band at 73 kD (figure 5-25).

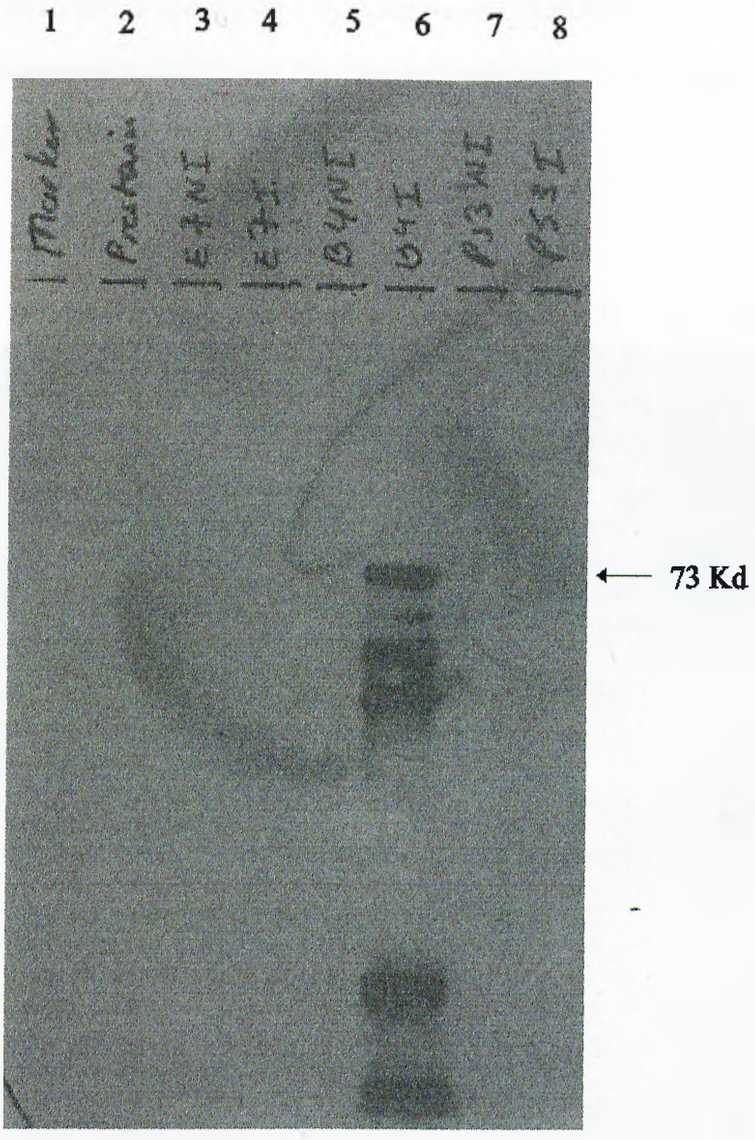


Figure 5-23 : Induced and non-induced samples

(**Lane 1**: molecular weight marker, **Lane 2**: prestained molecular weight marker, **Lane 3**: C-terminal of the BRCA2 protein (E7)-non-induced, **Lane 4**: C-terminal of the BRCA2 protein (E7)-induced, **Lane 5**: B4-non-induced, **Lane 6**: B4-induced, **Lane 7**: p53 non-induced, **Lane 8**: p53 induced)

BRCA2 fragments and p53 were used as negative controls, for the specificity of the antibody. Presence of the bands only in lane6 indicated that the expression of the B4 fragment was induced by IPTG and that the antibody BRCA1-3 do not cross-react with any of the tested proteins.

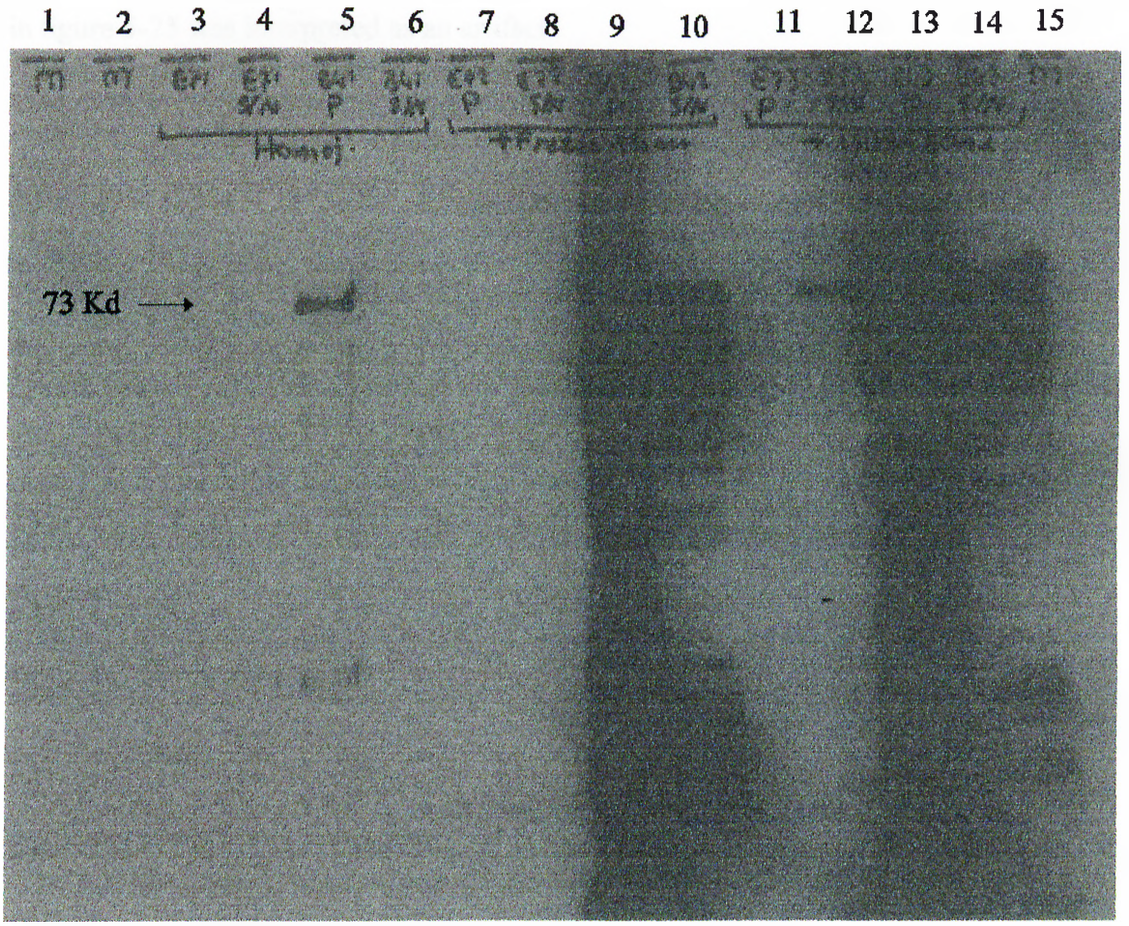


Figure 5-24 : Soluble and the insoluble fractions of the protein

(M: molecular weight marker (lanes 1 and 15), M: prestained molecular weight marker (lane 2); P=insoluble fraction; S/N+soluble fraction)

For non-denaturating purification of the proteins, cells were lysed in three steps. E71 P, E71-S/N, B41 P and B41-S/N represent the samples obtained as a result of homogenizing the samples for 5 times; E72 P, E72-S/N, B42 P and B42-S/N represent the samples obtained as a result of freeze-thaw; and finally E73 P, E73-S/N, B43 P and B43-S/N represent the samples obtained as a result of ultrasonification. The results indicated that the most efficient way of lysis was freeze-thaw. The band seen in lane 12 in figure 5-25 was interpreted as an artifact.

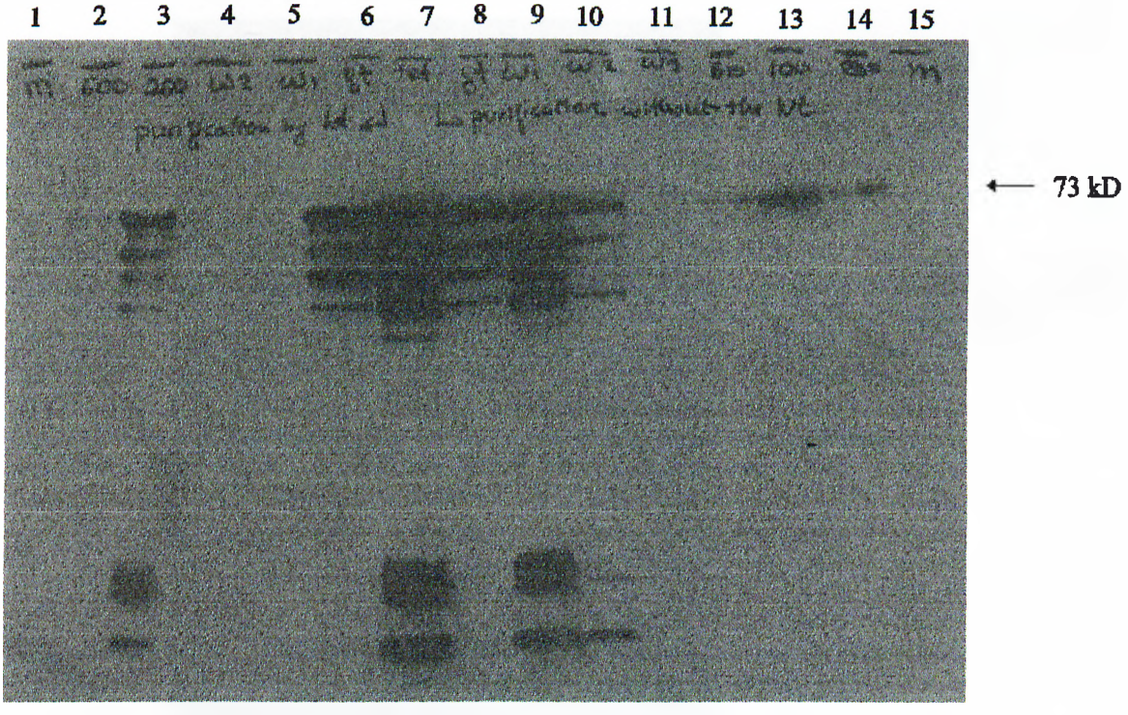


Figure 5-25 : Purified BRCA1 C-terminal protein (1)

(M: molecular weight marker (lane1), M: prestained molecular weight marker (lane15); Lanes 2-6 : the samples obtained from the purification performed by using the Quigen Ni-NTA resin kit ; lane 7: total lysate; and lanes 8-14 : the samples obtained

from the purification performed as explained in materials and methods section.)

These results indicated that the protein was most efficiently eluted by 250 mM imidazole. The wash steps in the second part of the gel (lanes 9-11) were performed with 20 mM imidazole and this amount of imidazole is believed to wash away all the non-specific bindings.

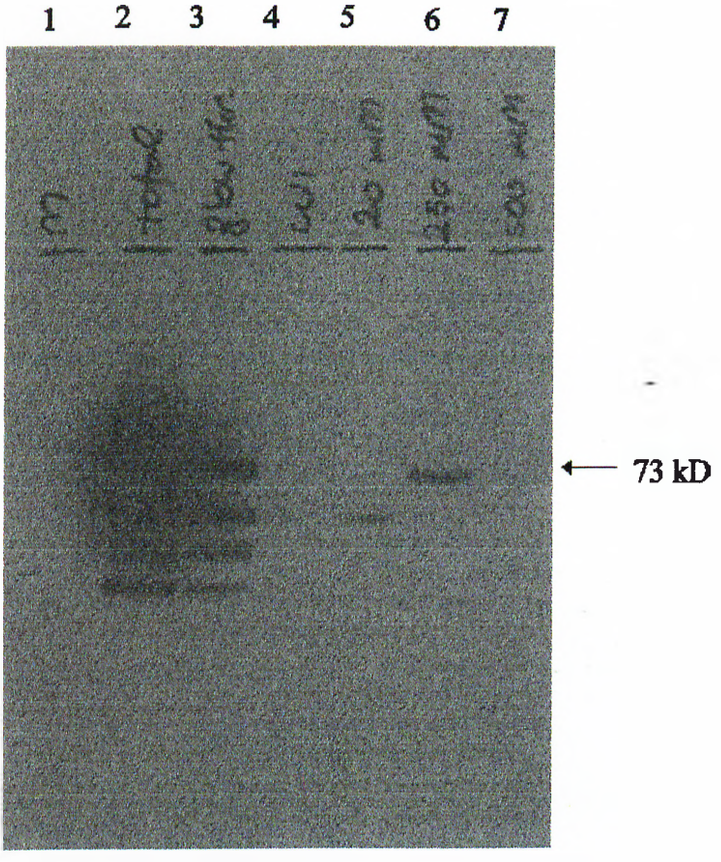


Figure 5-26 : Purified BRCA1 C-terminal protein (2)

(M: molecular weight marker (lane1))

These results indicated that the protein was most efficiently eluted by 250 mM imidazole and that usage of 20mM imidazole elutes all the non-specific bindings supporting the results of the previous experiment.

CHAPTER VI

DISCUSSION AND FUTURE PERSPECTIVES

The BRCA1 gene and its protein product have been the subjects of intensive investigation because of its important role in human breast and ovarian cancers. In spite of numerous studies conserning the regulation of BRCA1 expression and the function of its protein products, there are still lots of unknowns.

BRCA1 gene is composed of 22 coding exons which are transcribed into 7.8 kb transcript (Miki *et al.*,). Identification of shorter transcripts, indicates the presence of alternative splicing (Lu *et al.*, 1996); (Xu *et al.*, 1995). Alternative splicing may play significant roles in modulating the biological functions of a number of proteins. Some splice variants of BRCA1 were shown to lack exon 11 which harbors two important nuclear localization signals. Absence of nuclear localization signals results in the cytoplasmic localization of the protein. Thus, presence of different BRCA1 transcripts encoding proteins with different motifs, may play a significant role in modulating the subcellular localization and therefore the physiological functions of BRCA1.

BRCA1 was found to be 220 kD phosphoprotein that is phosphorylated in a cell cycle dependent manner. Many line of evidence indicated that BRCA1 is a tumor supressor gene. Recently identified BRCT domain in the BRCA1 protein suggested that BRCA1 may be involved in DNA repair. It has also been identified that BRCA1 expression was very during proliferation and differentiation.

All these data suggest different roles for BRCA1. Thus the exact function of BRCA1 remains to be elucidated. Therefore, this study was directed towards the construction of expression vectors, that produce different regions of BRCA1.

In this study we planned to optimize a strategy in order to express and purify BRCA1 encoded proteins in E.*coli* as purified proteins are needed for such studies. For this purpose we have chosen the carboxyl terminal of the protein. Carboxyl terminal of the protein emerges as the most important region of BRCA1 as 90% of mutations of the BRCA1 gene results in premature translation. Also the presence of the BRCT motif in this regions confers further importance to this region.

Future studies will include the expression and purification of the other three fragments of BRCA1 (B1, B2, and B3), which are already present in the cloning vector pCR-Script Amp SK (+). It will also be possible to join these fragments with each other by using the single cutter sites present in the overlapping regions and thus produce larger proteins. It will also be possible to produce antibodies against each of the fragment and if desired against the combinations of the fragments. Production of antibodies against small fragments of BRCA1 will provide more specificity to the antibody, such that we will be able to know the region of the BRCA1 which is recognized by that particular antibody.

Antibodies are important and useful tools in molecular biology. Many cellular localization studies of BRCA1 were carried out by using polyclonal and monoclonal antibodies. One of the essential features of antibodies is the specificity of the antibody. Usage of antibodies that cross-react with other proteins in a cell may give rise to false positive results as in the case of granin expectations of BRCA1 (Chen *et al.*, 1995).

Presence of purified protein products will also be useful in studies conserning the identification of different functional domains of the BRCA1 encoded proteins.

CHAPTER VII

REFERENCES

Abel K.J., Xu J., Yin G.Y., Lyons R.H., Meisler M.H., Weber B.L., "Mouse Brca1: localization sequence analysis and identification of evolutionarily conserved domains." Hum Mol Genet , vol: 4, pp: 2265-2273, 1995.

Anderson J. J, Tiniakos D. G., McIntosh G. G., Autzen P., Henry J. A., Thomas M. D., Reed J., Horne G. M., Lennard T. W., Angus B., Horne C. H.., "Retinoblastoma protein in human breast carcinoma: immunohistochemical study using a new monoclonal antibody effective on routinely processed tissues." Journal of pathology, vol:180, pp:65-70, 1996.

Aprelikova O., Kuthiala A., Bessho M., Ethier S., Liu E.T., "BRCA1 protein level is not affected by peptide growth factors in MCF10A cell line." Oncogene, vol:13, pp:2487-2491, 1996.

Baumann P., Benson F. E., West S. C., "Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions *in vitro*." Cell, vol:87, pp:757-766, 1996.

Berns E. M., Foekens J. A., van Staveren I. L., van Putten W. L., de Koning H. Y., Portengen H., Klijn J. G., "Oncogene amplification and prognosis in breast cancer: relationship with systemic treatment." Gene, vol:159, pp:11-18, 1995.

Bienstock R. J, Darden T., Wiseman R., Pedersen L., Barrett J. C., "Molecular modeling of the amino-terminal zinc ring domain of BRCA1." Cancer Research, vol:56, pp:2539-2545, 1996.

Birnboim H. C., Doly J., "A rapid alkaline extraction procedure for screening recombinant plasmid DNA." Nucl. Acids. Res., vol:7, pp:1513-1523, 1979.

Bishop D. K., "RecA homologues Dmc1 and Rad51 interact to form multiple nuclear complexes prior to meiotic chromosome synapsis." Cell, vol:79, pp:1081-1092, 1994.

Borresen A. L., "Role of genetic factors in breast cancer susceptibility." Acta Oncol., vol:31, pp:151-155, 1992.

Bowcock A. M., "Molecular cloning of BRCA1: a gene for early onset familial breast and ovarian cancer." Breast cancer research and treatment, vol:28, pp:121-135, 1993.

Brugarolas J., Jacks T., "Double indemnity: p53, BRCA and cancer." Nature Medicine, vol:3, pp:721-722, 1997.

Callebaut I., Mornon J. P., "From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair." FEBS Letters, vol:400, pp:25-30, 1997.

Castilla L. H., Couch F. J., Erdos M. R., Hoskins K. F., Calzone K., Garber J. E., Boyd J., Lubin M. B., Deshano M. L., Brody L. C., Collins F. S., Weber B. L., "Mutations in the BRCA1 gene in families with early-onset breast and ovarian cancer." Nature Genetics, vol:8, pp:387-391, 1994.

Chen Y., Chen C. F., Riley D. J., Allred D. C., Chen P. L., Von Hoff D., Osborne C. K., Lee W. H. "Aberrant subcellular localization of BRCA1 in breast cancer." Science, vol:270, pp:789-791, 1995.

Chen Y., Farmer A. A., Chen C. F., Jones D. C., Chen P. L., Lee W. H., "BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner." Cancer Res, vol:56, pp:3168-3172, 1996.

Clarke R., Dickson R. B., Lippman M. E., "Hormonal aspects of breast cancer: growth factors, drugs, and stromal interactions." Crit. Rev. Oncol. Hematol., vol:12, pp:1-23, 1992.

Claus E. B., Thompson W. D., Risch N., Genetic analysis of breast cancer in the cancer and steroid hormone study." American Journal of Human Genetics, vol:48, pp:232-241, 1991.

Coene E., Van Ooostveldt P., Willems K., Van Emmelo J., DePotter C. R., "BRCA1 is localized in cytoplasmic tube-like invaginations in the nucleus." Nature Genetics, vol:116, pp:122-124, 1997.

Collins N., McManus R., Wooster R., Mangion J., Seal S., Lakhani S. R., "Consistent loss of the wild type allele in breast cancers from a family linked to the BRCA2 gene on chromosome 13q12-13." Oncogene, vol:10, pp:1673-1675, 1995.

Davidoff A. M., Kerns B. J. M., Iglehart J. D., Marks J. R., "Maintenance of p53 alterations throughout breast cancer progression." Cancer Research, vol:51, pp:2605-2610, 1991.

Easton D. F., Bishop D. T., Ford D., Crockford G., P. and Breast Cancer Linkage Consortium, "Genetic linkage analysis in familial breast and ovarian cancer. Results from 214 families." American Journal of Human Genetics, vol:52, pp:678-701, 1993.

Ed. Erlich H. A. pp:7-16., Stockton Press "The design and optimization of the PCR. In PCR technology"

Ford D. and Easton D. F. "The genetics of breast and ovarian cancer" Br. J, Cancer, vol:72, pp:805-812, 1995.

Ford D., Easton D. F., Bishop D. T., Narod S. A., Goldgar D. E., "Risks of cancer in BRCA1-mutation carriers:Breast Cancer Linkage Consortium." Lancet, vol:343, pp:692-695, 1994.

Friedman L. S., Ostermeyer E. A., Szabo C. I., Dowd P., Lynch E. D., Rowell S. E., King M. C., "Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families." Nature Genetics, vol:8, pp:399-404, 1994.

Futreal P. A., Liu Q. Y., Shattuck-Eidens D., Cochran C., Harshman K., Tavtigian S., Bennett L. M.., Haugen-Strano A., Swensen J., Miki Y., Eddington K., McClure M., Frye C., Weaver-Feldhaus J., Ding W., Gholami Z., Soderkvist P., Terry L., Jhanwar S., Berchuck A., Iglehart J. D., Marks J., Ballinger D. G., Barrett J. C., Skolnick M. H., Kamb A., Wiseman R., "Brca1 mutations in primary breast and ovarian carcinomas." Science, vol:266, pp:120-122, 1994.

Gayther S. A., Warren W., Mazoyer S., Russell P. A., Harrington P. A., Chiano M., Seal S., Hamoudi R., Rensburg E. J. van., Dunning A. M., Love R., Evans G., Easton D., Clayton D., Stratton M. R., Ponder B. A. J., "Germline mutations of the BRCA1 gene in breast and ovarian cancer families provide evidence for a genotype-phenotype correlation." Nature Genetics, vol:11, pp:428-433, 1995.

Gillett C., Fantl V., Smith R., Fisher C., Bartek J., Dickson C., Barnes D, Peters G., "Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining." Cancer Research, vol:54, pp:1812-1817, 1994.

Gowen L. C., Johnson B. L., Latour A. M., Sulik K. K., Koller B. H., "Brca1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities." Nature Genetics, vol:12, pp:191-194, 1996.

Gudas J. M., Nguyen H., Li T., Cowan K. H., "Hormone-dependent regulation of BRCA1 in human breast cancer cells." Cancer Research, vol:55, pp:4561-4565, 1995.

Hakem R., de la Pompa J. L., Sirard C., Mo R., Woo M., Hakem A., Wakeham A., Potter J., Reitmair A., Billia F., Firpo E., Hui C. C., Roberts J., Rossant J., Mak T. W., "The tumor suppressor gene Brca1 is required for embryonic cellular proliferation in the mouse." Cell, vol:85, pp:1009-1023, 1996.

Hakem R., de la Pompa J. L., Elia A., Potter J., Mak T. W., "Partial rescue of Brca1 early embryonic lethality by p53 or p21 null mutation." Nature Genetics, vol:16, pp:298-302, 1997.

Hall J. M., Lee M. K., Newman B., Morrow J. E., Anderson L. A., Huey B, King M. C., "Linkage of early-onset familial breast cancer to chromosome 17q21." Science, vol:250, pp:1684-1689, 1990.

Hamann U., Herbold C., Costa S., Solomayer E. F., Kaufmann M., Bastert G., Ulmer H. U., Frenzel H., Komitowski D., " Allelic imbalance on chromosome 13q: evidence for the involvement of BRCA2 and RB1 in sporadic breast cancer." Cancer Research, vol:56, pp:1988-1990, 1996.

Holt J. T., Thompson M. E., Szabo C., Robinson-Benion C., Arteaga C. L., King M. C., Jensen R. A., "Growth retardation and tumour inhibition by BRCA1." Nature Genetics, vol:12, pp:298-302, 1996.

Holmberg L., Baron J. A., Byers T., Ohlander E. M., Zack M., Adami H.O., "Alcohol intake and breast cancer risk: effect of exposure from 15 years of age." Cancer Epidemiol. Biomarkers Prev., vol:4, pp:843-847, 1995.

Huttner W. B., Gerdes H. H., Rosa P., "The granin (chromogranin/secretogranin) family" Trends Biochem. Sci., vol:16, pp:27-30, 1991.

Jensen R. A., Thompson M. E., Jetton T. L., Szabo C. I., van der Meer R., Helou B., Tronick S. R., Page D. L., King M. C., Holt J. T. "BRCA1 is secreted and exhibits properties of a granin." Nature Genetics, vol:12, pp:303-308, 1996.

Kelsell D. P., Black D. M., Bishop D.T., Spurr N. K, "Genetic analysis of the BRCA1 region in a large breast-ovarian family- refinement of the minima; region containing BRCA1." Human Molecular Genetics, vol:2, pp:1823-1828, 1993.

Knekt P., Jarvinen R., Seppanen R., Pukkala E., Aroma A., "Intake of dairy products and the risk of breast cancer." Br. J. of Cancer., vol:73, pp:687-691, 1996.

Koonin E. V., Altschul S. F., "BRCA1 protein products: functional motifs." Nature Genetics, vol:13, pp:266-267, 1996.

Kozak M., "An analysis of vertebrate mRNA sequences: intimations of translational control." Journal of Cell Biology, vol:115, pp:887-903, 1991.

Lane T. F., Deng C., Elson A., Lyu M. S., Kozak C. A., Leder P., "Expression of Brca1 is associated with terminal differentiation of ectodermally and mesodermally derived tissues in mice." Genes and Development, vol:9, pp:2712-2722, 1995.

Lee E. Y, H., To H., Shew J. Y., Bookstein R., Scully P., Lee W. H., "Inactivation of the retinoblastoma susceptibility gene in human breast cancer." Science, vol:241, pp:218-221, 1988.

Lee S., Elenbaas B., Levine A., Griffit J., "p53 and its 14 kD C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches." Cell, vol:81, pp:1013-1020, 1995.

Levine A. J., Momand J., Finlay C. A., "The p53 tumor suppressor gene" Nature, vol:351, pp:453-456, 1991.

Li F. P., Fraumeni J. F., "Soft tissue sarcomas, breast cancer, and other neoplasms: a familial syndrome?" Annuals of International Medicine, vol:71, pp:747-751, 1969.

Lindblom A., Skoog L., rotstein S., Werelius B., Larsson C., Nordenskjold M. "Loss of heterozygosity in familial breast carcinomas." Cancer Research, vol:53, pp:4356-4361, 1993.

Lu M., Conzen S. D., Cole C. N., Arrick B. A., "Characterization of functional messenger RNA splice variants of BRCA1 expressed in nonmalignant and tumor-derived breast cells." Cancer Research, vol:56, pp:4578-4581, 1996.

Lynch H. T., Lynch J., Conway T., Watson P., Feunteun J., Lenoir G., Narod S., Fitzgibbons R. Jr., "Hereditary breast cancer and family cancer syndromes." World Journal of Surgery, vol:18, pp:21-31, 1994.

Maldonato E., Shiekhattar R., Sheldon M., Cho H., Drapkin R., Rickert P., Lees E., Anderson C. W., Linn S., Reinberg D., "A human RNA polymerase II complex associated with SRB and DNA repair proteins." Nature, vol:381, pp:86-89, 1996.

Maniatis T., Fritsch E. F., Sambrook J., "Eds.Molecular Cloning: A Laboratory Manual" Cold Spring Harbor Press, New York

Marks J. R., Huper G., Vaughn J. P., Davis P. L., Norris J., McDonnell D. P., Wiseman R. W., Futreal P. A., Iglehart J. D., "BRCA1 expression is not directly responsive to estrogen." Oncogene, vol:14, pp:115-121, 1997.

Marquis S. T., Rajan J. V., Wynshaw-Boris A., Xu J., Yin G. Y., Abel K. J.,Weber B. L., Chodosh L. A., "The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues." Nature Genetics, vol:11, pp:17-26, 1995.

Marchetti A., Buttitta F., Girlando S., Dalla Palma P., Pellegrini S., Fina P., Doglioni C., Bevilacqua G., Barbareschi M., "MDM2 gene alterations and MDM2 protein expression in breast carcinomas." Journal of Pathology, vol:175, pp:31-38, 1995

Marcus J. N., Watson P., Page D. L., Narod S. A., Lenoir G. M., Tonin P., Linder-Stephenson L., Salerno G., Conway T. A., Lynch H. T., "Hereditary breast cancer: pathobiology, prognosis, and BRCA1 and BRCA2 gene linkage." Cancer, vol:77, pp:697-709, 1996.

McCann A. H., Kirley A., Carney D. N., Corbally N., Magee H. M., Keating G., Dervan P. A., "Amplification of the MDM2 gene in human breast cancer and its association with MDM2 and p53 protein status." British Journal of Cancer, vol:71, pp:981-985, 1995.

Miki Y., Swensen J., Shattuck-Eidens D., Futreal P. A., Harshman K., Tavtigian S., Liu Q., Cochran C., Bennett M., Ding W., Bell R., Rosenthal J., Hussey C., Tran T., McClure M., Frye C., Hattier T., Phelps R., Haugen-Strano A., Katcher H., Yakumo K., Gholami Z., Shaffer D., Stone S., Bayer S., Wray C., Bogden R., Dayananth P., Ward J., Tonin P., Narod S., Bristow P. K., Norris F. H., Helvering L., Morrison P., Rosteck P., Lai M., Barrett J. C., Lewis C., Neuhausen S., Cannon-Albright L., Goldgar D., Wiseman R., Kamb A., Skolnick M. H., "A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1." Science, vol:266, pp:66-71, 1994.

Momand J., Zambetti G. P., Olson D. C., George D., Levine A. J., "The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation." Cell, vol;69, pp:1237-1245, 1992.

Monteiro A. N., August A., Hanafusa H., "Evidence for a transcriptional activation function of BRCA1 C-terminal region." Proc. Natl. Acad. Sci. U S A, vol:93, pp:13595-13599, 1996.

Moser A. R., Mattes E. M., Dove W. F., Lindstrom M. J., Haag J. D., Gould M. N., "ApcMin, a mutation in the murine Apc gene, predisposes to mammary carcinomas and focal alveolar hyperplasias." Proc. Natl. Acad. Sci. U S A, vol:90, pp:8977-8981, 1993.

Nagai M. A., Yamamato L., Salaorni S., Pacheco M. M., Brentani M. M., Barbosa E. M., Brentani R. R., Mazoyer S., Smith S. A., Ponder B. A., "Detailed deletion mapping of chromosome segment 17q12-21 in sporadic breast-tumors." Genes chromosomes and cancer, vol:11, pp:58-62, 1994.

Nandi S.,Guzman R. C., Yang J., "Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis." Proc. Natl. Acad. Sci. USA., vol:92, pp:3650-3657, 1995.

Narod S. A., Feunteun J., Lynch H. T., Watson P., Conway T., Lynch J., Lenoir G. M. "Familial breast-ovarian cancer locus on chromosome 17q12-23." Lancet, vol:338, pp:82-83, 1991.

Neuhausen S., Marshall C. J., "Loss of heterozygosity in familial tumors from 3 Brca1-linked kindreds." Cancer Research, vol:54, pp:6069-6072, 1994.

Neuhausen S., Swensen J., Miki Y., Liu Q., "A P1-based physical map of the region from D17S776 to D17S78 containing the breast-cancer susceptibility gene Brca1." Human Molecular Genetics, vol:3, pp:1919-1926, 1994.

Newman B., Austin M.A., Lee M., King M. C., "Inheritance of breast cancer: evidence for autosomal dominant transmission in high risk families." Proc. Natl. Acad. Sci. USA, vol:85 , pp:3044, 1988.

Offit K., Gilewski T., McGuire P., Schluger A., Hampel H., Brown K., Swensen J., Neuhausen S., Skolnick M., Norton L., Goldgar D., "Germline BRCA1 185delAG mutations in Jewish women with breast cancer." Lancet 1996, vol:347, pp:1643-1645, 1996.

Parker S. L., Tong T., Borden S., Wingo P. A., "Cancer statistics" CA. Cancer J. Clin., vol:65, pp:5-27, 1996.

Phelan C. M., Lancaster J. M., Tonin P., Gumbs C., Cochran C., Carter R., Ghadirian P., Perret C., Moslehi R., Dion F., Faucher M. C., Dole K., Karimi S., Foulkes W., Lounis H., Warner E., Goss P., Anderson D., Larsson C., Narod S., Futreal P. A., "Mutation analysis of the BRCA2 gene in 49 site-specific breast cancer families." Nature Genetics, vol:13, pp:120-122, 1996.

Rajan V. J., Wang M., Marquis S. T., Chodosh L. A., "BRCA2 is coordinately regulated with BRCA1 during proliferation and differentiation in mammary epithelial cells." Proc. Natl. Acad. Sci. USA., vol:93, pp:13078-13083, 1996.

Rajan V. J., Marquis S. T., Gardner H. P., Chodosh L. A., "Developmental expression of BRCA2 colocalizes with BRCA1 and is associated with proliferation and differentiation in multiple tissues." Developmental Biology, vol;184, pp:385-401, 1997.

Rao V. N., Shao N., Ahmad M.,Reddy E. S. P., "Antisense RNA to the putative tumor suppressor gene BRCA1 transforms mouse fibroblasts." Oncogene, vol:12, pp:523-528, 1996.

Rubin E., Farber J. L., "PATHOLOGY", J. B. Lippincott Press, 1988.

Ruffner H., Verma I. M., "BRCA1 is a cell cycle-regulated nuclear phosphoprotein." Proc. Natl. Acad. Sci. U S A., vol:94, pp:7138-7143, 1997.

Sager R., "Tumor suppressor genes: the puzzle and the promise." Science, vol:246, pp:1406-1412, 1989.

Saiki, R. K.,Gelfand, D. H., Stoffel, S., and Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K.B., Erlich, H. A., "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase." Science, vol:239, pp:487-491, 1988.

Schildkraut J. M., Risch N., Thompson W. D., "Evaluating genetic association among ovarian, breast and endometrial cancer: evidence for a breast/ovarian cancer relationship." American Journal of Human Genetics, vol:45, pp:521-529, 1989.

Schutte M., da Costa L. T., Hahn S. A., Moskaluk C., Hoque A. T., Rozenblum E., Weinstein C. L., Bittner M., Meltzer P. S., Trent J. M., Yeo C. J., Hruban H. R., Scott E. K., "Identification by representational difference analysis of a homozygous deletion in pancreatic carcinoma that lies within the BRCA2 region." Proc. Natl. Acad. Sci. U S A, vol:92, pp:5950-5954,1995.

Scully R., Ganesan S., Brown M., De Caprio J. A., Cannistra S. A., Feunteun J.,Schnitt S., Livingston D. M., "Location of BRCA1 in human breast and ovarian cancer cells." Science, vol:272, pp:123-126, 1996.

Scully R., Chen J., Plug A., Xiao Y., Weaver D., Feunteun J., Ashley T., Livingston D. M. (a), "Association of BRCA1 with RAD51 in mitotic and meiotic cells." Cell, vol:88, pp:265-275, 1997.

Scully R., Anderson S. F., Chao D. M., Wei W., Ye L., Young R. A., Livingston D. M., Parvin J. D. (b), "BRCA1 is a component of the RNA polymerase II holoenzyme." Proc.Natl. Acad.Sci., vol:94, pp:5605-5610, 1997.

Shao N., Chai Y. L., Shyam E., Reddy P., Rao V. N., "Induction of apoptosis by the tumor suppressor protein BRCA1." Oncogene, vol:13, pp:1-7, 1996.

Shattuck-Eidens D., McClure M., Simard J., Labrie F., Narod S., Couch F., Hoskins K., Weber B., Castilla L., Erdos M., Brody L.,Friedman L., Ostermeyer E., Szapo O., King M. C., Jhanwar S., Offit K., Norton L., Gilewski T., Lubin M., Osborne M., Black D., Boyd M., Steel M., Ingles S., Haile R., Lindblom A., Olsson H., Borg A., Bishop T., Solomon E., Radice P., Spatti G., Gayther S., Ponder B., Warren W., Stratton M., Liu Q., Fujimura F., Lewis C., Skolnick M. H., Goldgar D. E., "A collaborative survey of 80 mutations in the BRCA1 breast and ovarian cancer susceptibility gene: implications for presymptomatic testing and screening." JAMA, vol:273, pp:535-541, 1995.

Shinohara A., Ogawa H., Ogawa T., "Rad51 protein involved in repair and recombination in *Saccharomyces cerevisiae* is a RecA-like protein." Cell, vol:69, pp:457-470, 1992.

Siziopikou K. P., Prioleau J. E., Harris J. R., Schnitt S. J., "Bcl-2 expression in the spectrum of preinvasive breast lesions." Cancer, vol:77, pp:499-506, 1996.

Slamon D. J., Godolphin W., Jones L. A., Holt J. A., Wong S. G., Keith D. E., Lewin W. J., Stuart S. G., Udove J., Ullrich A., Press M. F., "Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer." Science, vol:244, pp:707-712, 1989.

Smith M. L., Chen I-T., Zhan Q., O'Connor P. M., Fornace A.J,Jr., "Involvement of the p53 tumor suppressor in repair of u.v.-type DNA damage." Oncogene, vol:10, pp:1053-1059, 1995.

Smith S. A., Easton D. G., Evans D. G. R., Ponder B. A. J., "Allele losses in the region 17q12-21 in familial breast and ovarian cancer involve the wild-type chromosome." Nature Genetics, vol:2, pp:128-131, 1992.

Sobol H., Stoppa-Lyonner D., Bressac-de-Paillerets B., Peyrat J. P., Kerangueven F., Janin N., Noguchi T., Eisinger F., Guinebretiere J. M., Jacquemier J., Birnbaum D., "Truncation at conserved terminal regions of BRCA1 protein is associated with highly proliferating hereditary breast cancers." Cancer Research, vol:56, pp:3216-3219, 1996.

Stanbridge E. J., "Human tumor suppressor genes." Annual Reviews on Genetics, vol:24, pp:615-657, 1990.

Struewing J. P., Abeliovich D., Peretz T., Avishai N., Kaback M. M., Collins F. S., Brody L. C., "The carrier frequency of the Brca1 185del AG mutation is approximately 1 percent in Ashkenazi-Jewish Individuals." Nature Genetics, vol:11, pp:198-200, 1995.

Swift M., Chase C. L., Morrell D., "Cancer predisposition of ataxia-telangiectasia heterozygotes." Cancer Genetics and Cytogenetics, vol:46, pp:21-27, 1990.

Swift M., Reitnauer P. J., Morrell D., Chase C. L., "Breast and other cancer sites in families with ataxia-telangiectasia." New England Journal of Medicine, vol:316, pp:1289-1294, 1987.

Takahashi H., Behbakht K., Mcgovern P. E., Chiu H. C., Couch F. J., Weber B. L., Friedman L. S., King M. C., "Mutation Analysis of the Brca1 gene in ovarian cancers." Cancer Research, vol:55, pp:2998-3002, 1995.

Tashiro S., Kotomura N., Shinohara A., Tanaka K., Ueda K., Kamada N., "S phase specific formation of the human Rad51 protein nuclear foci in lymphocytes." Oncogene, vol:12, pp:2165-2170, 1996.

Tavtigian S. V., Simard J., Rommens J., Couch F., Shattuck-Eidens D., Neuhausen S., Merajver S., Thorlacius S., Offit K., Stoppa-Lyonnet D., Belanger C., Bell R., Berry S, Bogden R., Chen Q., Davis T., Dumont M., Frye C., Hattier T., Jammulapati S., Janecki T., Jiang P., Kehrer R., Leblanc J. F., Mitchel J. T., McArthur-Morrison J., Nguyen K., Peng Y., Samson C., Schroeder M., Snyder S. C., Steele L., Stringfellow M., Stroup C., Swedlund B., Swensen J., Teng D., Thomas A., Tran T., Tranchat M., Feldhaus-Weaver J., Wong A. K. C., Shizuya H., Eyfjord J. E., Albright-Cannon L., Fabrie F., Skolnick M. H., Weber B., Kamb A.,Goldgar D. E., "The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds." Nature Genetics, vol:12, pp:333-337, 1996.

Terasawa M., Shinohara A., Hotta Y., Ogawa H., Ogawa T., "Localization of RecA-like recombinations proteins on chromosomes of lily at various meiotic stages." Genes and Development, vol:9, pp:925-934, 1995.

Thakur S, Zhang HB, Peng Y, Le H, Carroll B, Ward T, Yao J, Farid LM, Couch F. J., Wilson RB, Weber BL., "Localization of BRCA1 and a splice variant identifies the nuclear localization signal." Mol. Cell. Biol., vol:17, pp:444-452, 1997.

Thompson A. M., Morris R. G., Wallace M., Wylie A. H., Steel C. M., Carter D. C. "Allele loss from 5q21 (APC/MCC) and 18q21 (DCC) and DCC mRNA expression in breast cancer." Br. J. Cancer, vol:68, pp:64-68, 1993.

Thompson M. E., Jensen R. A., Obermiller P. S., Page D. L., Holt T. J., "Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression." Nature Genetics, vol:9, pp:444-450, 1995.

Thompson M. E., Zimmer W. E., Haynes A. L., Valentine D. L., Forss-Petter S., Scammel J. G., "Prolactin granulogenesis is associated with increased secretogranin expression and aggregation in the golgi apparatus of GH4C1 cells." Endocrinology, vol:131, pp:318-326, 1992.

Thorlacius S., Tryggvadottir L., Olafsdottir G. H., Jonasson J. G., Ogmundsdottir H. M., Tulinius H., Eyfjord J. E., "Linkage to BRCA2 region in hereditary male breast cancer." Lancet, vol:346, pp:544-545, 1995.

Thorlacius S., Olafsdottir G., Tryggvadottir L., Neuhausen S., Jonasson J. G., Tavtigian S. V., Tulinius H., Ogmundsdottir H. M., Eyfjord J. E., "A single BRCA2 mutation in male and female breast cancer families from Iceland with varied cancer phenotypes." Nature Genetics, vol:13, pp:117-119, 1996.

Tsuzuki T., Fujii Y., Sakumi K., Tominaga Y., Nakao K., Sekiguchi M., Matsushiro A., Yoshimura Y., Morita T., "Targeted distruption of the Rad51 gene leads to lethality in embryonic mice." Proc. Natl. Acad. USA, vol:93, pp:6236-6240, 1996.

Vijer M. J. van de., "Molecular Genetic Changes in human breast cancer." Advances in Cancer Research, vol:61, pp:25-56. 1993.

Vogelstein B. and Kinzler K. W., "p53 function and dysfunction." Cell, vol:70, pp:523-526, 1992.

Wang J. Y. Y., Knudsen E. S., Welch P. J., "The retinoblastoma tumor suppressor protein." Advances in Cancer Research., vol:64, pp:25-83, 1994.

Weber-Chappuis K., Bieri-Burger S., Hurlimann J., "Comparison of prognostic markers detected by immunohistochemistry in male and female breast carcinomas." European Journal of Cancer, vol:10, pp:1686-1692, 1996.

Weinberg R., "The action of oncogenes in the cytoplasm and nucleus." Science, vol;230, pp:770-776, 1985.

Wendy M. Mars and Grandy F. Saunders., "Chromosomal abnormalities in human breast cancer." Cancer and Metastasis Reviews, vol:9, pp:35-43, 1990.

Wilson C. A., Payton M. N., Elliott G. S., Buaas F. W , Cajulis E. E., Grosshans D., Ramos L., Reese D. M., Slamon D. J., Calzone F. J., "Differential subcellular localization, expression and biological toxicity of BRCA1 and the splice variant BRCA1-Δ11b." Oncogene, vol:14, pp:1-16, 1997.

Wilson C. A., Payton M. N., Pekar S. K., Zhang K., Pacifici R. E., Gudas J. L., Thukral S, Calzone F. J., Reese D. M., Slamon D. I., "BRCA1 protein products: antibody specificity..." Nature Genetics, vol:13, pp:264-265, 1996 .

Wooster R., Zou Z., Pirollo K., Blattner W., Chang E. H. A., "A germline mutation in the androgen receptor gene in two brothers with breast cancer and Reifenstein syndrome." Nature Genetics, vol:2, pp:132-134, 1992.

Wooster R., Neuhausen S. L., Mangion J., Quirk Y., Ford D., Collins N., Collins N., Nguyen K., Seal S., Tran T., Averill D., Fields P., Marshall G., Narod S., Lenoir G. M., Lynch H., Feunteun J., Devilee P., Cornelisse C. J., Menko F. H., Daly P. A., Ormiston W., McManus R., Pye C., Lewis C. M., Cannon-Albright L. A., Peto J., Ponder B. A. J., Skolnick M. H., Easton D. F., Goldgar D. E., Stratton M. R., "Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13." Science, vol:265, pp:2088-2090, 1994.

Wooster R., Bignell G., Lancaster., Swift S., Seal S., Manglon J., Collins N., Gregory S., Gumbs C., Micklem G., Barfoot R., Hamoudi R., Patel S., Rice C., Biggs P., Hashim Y., Smith A., Connor F., Arason A., Gudmundsson J., Ficenec D., Kelsell D., Ford D., Tonin P., Bishop D. T., Spurr N. K., Ponder B. A. J., Eeles R., Peto J., Devilee P., Cornelisse Cees., Lynch H., Narod S., Lenoir G., Egilsson V., Barkadottir R. B., Easton D. F., Bentley D. R., Futreal P. A.., Ashworth A., Stratton M. R., "Identification of the breast cancer susceptibility gene BRCA2." Nature, vol:378, pp:789-791, 1995.

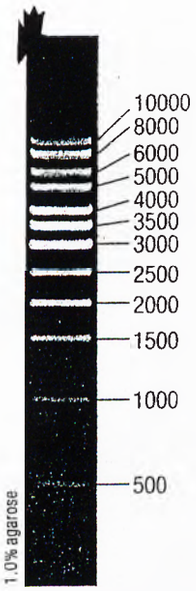
Wu L. C., Wang Z. W., Tsan J. T., Spillman M. A., Phung A., Xu X. L., Yang M. C. W., Hwang L. Y., Bowcock A. M., Baer R., "Identification of a RING protein that can interact in vivo with the BRCA1 gene product." Nature Genetics, vol:14, pp:430-440, 1996.

Wu X., Bayle J. H., Olson D., Levine A. J., "The p53-mdm-2 autoregulatory feedback loop." EMBO Journal, vol;12, pp:461-468, 1993.

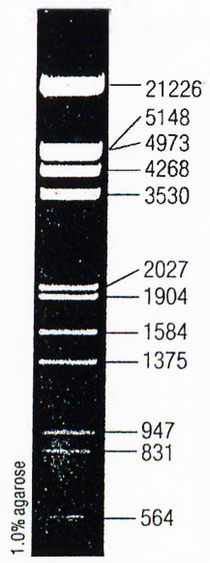
Xu C. F., Brown M. A., Chambers J. A., Griffiths B., Nicolai H., Solomon E., "Distinct transcription start sites generate two forms of BRCA1 mRNA." Human Molecular Genetics, vol:4, pp:2259-2264, 1995.

APPENDIX

The DNA ladders used in this study.



1kb DNA Ladder



λ DNA/*Eco*RI+*Hin*dIII

DNA LADDER (123 bp)