Human lymphoblastoid cell lines expressing mutant p53 exhibit decreased sensitivity to cisplatin-induced cytotoxicity.

by

Beata Kuzniar

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Institute of Medical Science
University of Toronto

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ABSTRACT

Human lymphoblastoid cell lines expressing mutant p53 exhibit decreased sensitivity to cisplatin-induced cytotoxicity.

Degree of Masters of Science, 1997

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Cisplatin is a chemotherapeutic agent commonly used, though with variable results, in treatment of recurrent non-Hodgkin's lymphoma. With the aim of obtaining definite evidence for p53 involvement in cisplatin-dependent apoptosis in human B lymphocytes we have designed an experimental system where human lymphoblastoid cells were transfected with expression vectors containing p53 cDNA mutated at either codon 135 or 246. The cells were subjected to cisplatin treatment or γ-radiation and observed for changes in cell cycle arrest and apoptosis. We found that compared to the parental cell line, cells overexpressing mutant p53 (either 246val or 135ser) exhibited decreased apoptosis in response to γ-radiation or cisplatin as measured by: propidium iodide (PI) staining of the cellular DNA (cell cycle analysis) and decrease in PARP (poly ADP-ribose polymerase) cleavage as detected by western blotting. Interestingly the cells expressing mutant p53(135ser) protein were less resistant to cisplatin induced apoptosis than the p53(246val)-bearing cell line. A significant decrease in the G1/S arrest assayed by bromodeoxyuridine and PI staining (cell cycle/proliferation assay) was also observed in response to
irradiation and cisplatin in cell lines expressing either of the mutant p53 constructs. In contrast to the increase in p21/Waf1 seen after irradiation, no significant induction of the cell cycle inhibitor p21/Waf1 was observed after cisplatin treatment in either wild-type or mutant p53 expressing cell lines. There was also no increase in basal levels of Bcl-2 protein in wild type or mutant p53 expressing cells in response to cisplatin or irradiation. Unexpectedly, following cisplatin treatment we observed an increase in mutant p53 RNA steady state levels in addition to increased levels of p53 protein. Taken together these results suggest a role for p53 in cisplatin induced cytotoxicity. In addition increases in the level of mutant p53 RNA after irradiation or cisplatin treatment suggest that these agents may not only stabilize wild type p53 protein but also induce or stabilize mutant p53 RNA. Finally these results indicate that both irradiation and cisplatin should be used with caution in the treatment of lymphoid tumours bearing mutations of p53.
ACKNOWLEDGMENTS

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PUBLICATION

Selected results from this thesis have been submitted for publication.
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<td>aa</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>arg</td>
<td>arginine</td>
</tr>
<tr>
<td>AT</td>
<td>ataxia telangiectasia</td>
</tr>
<tr>
<td>BCS</td>
<td>bovine calf serum</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt's lymphoma</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>cdc, cdk</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CK</td>
<td>casein kinase</td>
</tr>
<tr>
<td>cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DLCL</td>
<td>diffuse large cell lymphoma</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>EBNA</td>
<td>Epstein-Barr virus nuclear antigen</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>FL</td>
<td>follicular lymphoma</td>
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<tr>
<td>GADD</td>
<td>growth arrest and DNA damage-inducible gene</td>
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<tr>
<td>gly</td>
<td>glycine</td>
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<td>his</td>
<td>histidine</td>
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<td>HPV</td>
<td>human papillomavirus</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ICE</td>
<td>interleukin-1β-converting enzyme</td>
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<td>IGIF</td>
<td>interferon γ inducing factor</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun-N terminal kinase</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>LCL</td>
<td>lymphoblastoid cell line</td>
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<tr>
<td>LMP</td>
<td>latent membrane protein</td>
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<tr>
<td>LP</td>
<td>leader protein</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>multi drug resistance</td>
</tr>
<tr>
<td>met</td>
<td>methionine</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<td>PARP</td>
<td>poly-ADP-ribose polymerase</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PI</td>
<td>propidium iodide</td>
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<td>pro</td>
<td>proline</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RNase</td>
<td>ribonuclease</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>ser</td>
<td>serine</td>
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<tr>
<td>TCGF</td>
<td>T cell growth factor</td>
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<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>U</td>
<td>uracil</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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val valine
CHAPTER 1: INTRODUCTION

1.1. Introductory overview and rationale.

Cisplatin is used as a chemotherapeutic agent in combination therapy of non-Hodgkin's lymphoma, as well as ovarian, head, neck, cervical and other carcinomas with varied success. Although there is inferential evidence that cisplatin induced apoptosis is mediated through p53, there is no definitive proof of this. Our experimental model utilized isogenic B cell lymphoblastoid cell lines (LCLs) differing only in the presence of specific p53 mutations. Unlike other experimental systems the LCLs are not transformed and non-tumourigenic.

Because of the importance of cisplatin as a cytotoxic agent in treatment of non-Hodgkin's lymphoma and because of the variability of response to this agent we felt it important to conclusively determine whether p53 mutations may mediate cisplatin resistance.


Non-Hodgkin's lymphoma is a malignancy of lymphoid tissue affecting mature B cells. Approximately 50,000 new cases of Non-Hodgkin's lymphoma are diagnosed each year in U.S.A. (Aisenberg, 1995). About 40% of those constitute low grade type such as follicular lymphoma (FL) which occurs as asymptomatic and widely disseminated disease characterized by spontaneous regressions in up to 23% of cases studied (Horning & Rosenberg, 1984). Within 5 to 10 years, more than 50% of cases of the low grade malignancy transform into a high grade, aggressive
and lethal disease an example of which is diffuse large cell lymphoma (DLCL) (Connors, 1989). In fact it is possible for a single patient to harbor two different types of lymphoma at different sites.

Different genetic abnormalities have been associated with different subtypes of lymphoma (Aisenberg, 1995; Horio et al., 1993). There is some evidence that this may occur as a mistake at the time of physiologic VDJ recombination in B lymphocytes (Bakhshi et al., 1987; Tsujimoto et al., 1985). These events usually involve oncogenes translocated to antigen receptor genes: immunoglobulin genes in B cell lymphomas and T cell receptor genes in T cell lymphomas. Oncogenes implicated in lymphomagenesis include c-myc (transcriptional regulator), bcl-1 (mitotic regulator cyclin D), bcl-2 (regulator of apoptosis), bcl-3 (inhibitor of transcription factor NF-κβ), and bcl-6 (regulator of cell proliferation and differentiation). Extensive evidence suggests that lymphomagenesis is a multistep process. It has been proposed that the initial transforming event contributing to the development of FL is a translocation between chromosomes 14 and 18 (t(14;18)) which results in juxtaposition of a bcl-2 protooncogene on 18q21 to the highly transcribed immunoglobulin heavy chain locus on 14q32 (Bakhshi et al., 1987; Tsujimoto & Croce, 1986). The effect of this translocation is overproduction of the bcl-2 protein. Since bcl-2 by itself in not transforming it is thought that a secondary genetic event must occur in order for the malignant phenotype to develop (Hockenbery, 1994). Rearrangements of c-myc and bcl-6, as well as loss of p53 function have been suggested as the additional genetic events involved in the progression from FL to DLCL (Aisenberg, 1995; Farrugia et al., 1994).
1.2.b. Treatment of non-Hodgkin's lymphoma.

The complex treatment of non-Hodgkin's lymphoma must take under consideration the histologic subtype, stage, spread pattern and other characteristics of the disease and the patient (Connors, 1989). The aggressive disease is treated with multi-agent chemotherapy but 15 to 30% of patients fail to achieve remission and 20 to 50% of those who do still may relapse (Velasquez et al., 1988). Combination of cisplatin with other drugs are the treatment of choice in most patients with relapsed aggressive histology lymphoma often subsequently consolidated with high dose therapy and progenitor stem cell transplantation. Such cisplatin combination treatments include DHAP-cisplatin with cytosine arabinose (Ara-C) and dexamethasone (Velasquez et al., 1988), or ESHAP (etoposide, methylprednisolone, cytarabine, and cisplatin) (Rodriguez et al., 1995). The apparent synergistic effect of these drugs resulted in 25 to 29% overall survival at two years of follow-up in these studies. However, the remissions were generally not durable and relapses are ongoing.

1.2.c. Biological effects of cisplatin.

Cisplatin (cis-dichlorodiamminoplatinum II) causes platination of DNA (Roberts & Thomson, 1979) which leads to inter and intrastrand cross-linking (Eastman, 1985; Eastman, 1987) as well as protein-DNA crosslinks (Zamble & Lippard, 1995). It has been suggested that cytotoxicity of cisplatin is a result of the inhibition of DNA and RNA polymerases and likely other proteins by these adducts (Zamble & Lippard, 1995). As a result of treatment with cisplatin the rate of DNA synthesis is reduced and often cells become arrested in the G2/M stage of the cell cycle (Ormerod et al., 1994). Several studies have demonstrated that certain
tumour-derived cell lines exposed to high doses of cisplatin die by apoptosis which is preceded by the induction of p53 protein in a dose dependent manner (Allday et al., 1995a; Zhang et al., 1996). Increased apoptosis in response to cisplatin by gene transfer of wild type p53 in p53-null human lung cancer cell lines (Fujiwara et al., 1994) suggested that p53 is involved in the cell line's sensitivity to the drug. Association between p53 mutations and cisplatin resistance has been also suggested by studies of ovarian carcinoma, (Perego et al., 1996), lymphoma, (Fan et al., 1994) and other tumour cell lines.

1.3. Human p53 tumour suppressor gene in health and disease.

1.3.a. p53: gene and protein structure, and regulation.

The human p53 gene is located on a single locus on chromosome 17 p13 (Isobe et al., 1986; Miller et al., 1986). It encompasses 20 kb of DNA and is split into 11 exons where the noncoding exon 1 is separated from exon 2 by a 10 kb intron (Lamb & Crawford, 1986). Several potential transcription initiation sites have been identified within the p53 gene sequence but the minimum p53 promoter required for basal p53 promoter activity was pinpointed to an 85 base pair region upstream of exon 1 (Tuck & Crawford, 1989). Although p53 regulation has been shown to be mostly achieved by post translational mechanisms (Kastan et al., 1991; Maltzman & Czyzyk, 1984), translational (Fu & Benchimol, 1997; Fu et al., 1996; Mosner et al., 1995), transcriptional (Bálint & Reisman, 1996; Sun et al., 1995) and post transcriptional regulation have also been invoked (Voelkerding et al., 1995).
The p53 mRNA is 2.6 kb and contains a large 3' untranslated region (3'UTR) (Harlow et al., 1985; Matlashewski et al., 1984). The 3'UTR of other genes has been implicated in mRNA localization (Gavis & Lehmann, 1994) and determination of the length of the poly(A) tail (Sheets et al., 1994). Recent reports have implicated the p53 3'UTR in the regulation of the p53 mRNA translation (Fu & Benchimol, 1997; Fu et al., 1996). A negative regulatory element containing a U-rich sequence followed by an Alu-like element was identified on a 330 nucleotide region at the distal end of the human p53 3'UTR and found to repress translation of mRNA in vivo and in vitro. It was postulated that the interaction of this region with repressor proteins prevents initiation of translation. The human p53 3'UTR was also found to participate in translational regulation of the p53 following γ-irradiation (Fu & Benchimol, 1997).

The 393 amino acid long p53 protein consists of three structural domains including a highly charged acidic amino terminus encompassing the first 80 amino acids, a hydrophobic proline rich domain between residues 80 and 150, and a highly charged basic carboxy terminal region from amino acid 319 to 393 (reviewed by (Prokocimer & Rotter, 1994)). The functional domains of p53 protein (Figure 1.1) include the N-terminal transactivation region, sequence-specific DNA binding region (aa 100-293), nuclear localization sequence (aa 316-325) and oligomerization region (aa 319-360) (reviewed by (Harris, 1996)). The transactivation domain contains a very strong transcriptional activation sequence (Fields & Jang, 1990) which is responsible for the p53-regulated expression of many important genes. Recent studies have suggested that this transactivation activity is regulated by the C terminus of the protein (Mundt et al., 1997). The ability of p53 protein to bind a specific consensus DNA sequence (El-
Deiry et al., 1992; Kern et al., 1991) confers its function as a transcription factor. P53 also possesses nonspecific nucleic acid binding ability which maps to the residues 320-393 in the C terminus (Bakalkin et al., 1995). This site, which allows the molecule to bind short single stranded DNA fragments or DNA ends, was postulated to be involved in the regulation of the sequence specific DNA binding and is necessary for the p53 function in response to DNA damage (Bakalkin et al., 1995; Bayle et al., 1995; Reed et al., 1995).

P53 is an unstable protein with a half life of 20 to 35 minutes (Maltzman & Czyzyk, 1984) which becomes stabilized and accumulates in the nucleus via its nuclear localization sequence during the S phase of the cell cycle (Shaulsky et al., 1990) or in response to DNA damage (Kastan et al., 1991; Lu & Lane, 1993; Maltzman & Czyzyk, 1984). The protein likely functions in the cell in a tetrameric configuration (Friedman et al., 1993; Milner et al., 1991). If one of the alleles is mutated in a cell the mutant protein may exert a "dominant negative" effect on the wild type by participating in these multimeric complexes (Hachiya et al., 1994; Milner & Medcalf, 1991; Milner et al., 1991). p53 protein can also interact with some of the proteins encoded by DNA tumour viruses for instance adenovirus E1B p55 protein (Debbas & White, 1993), human papilloma virus (HPV) E6 protein (Werness et al., 1990) and simian virus 40 large tumour antigen (SV40 largeT-ag) (Horning & Rosenberg, 1984; Tsujimoto et al., 1985) which leads to the disruption of p53 function and allows for viral transformation. One of many cellular proteins that can associate with p53 protein is MDM 2 (murine double minute gene 2), a nuclear protein which is not only transactivated by p53 but is also a negative regulator of its activity (Chen et al., 1996; Piette et al., 1997; Wu et al., 1993). P53 can
also bind many transcription factors and replication protein A (reviewed by (Selivanova & Wilman, 1995)), as well as protein kinases which may be involved in modulating p53's activity via phosphorylation (Herrmann et al., 1991; Milner et al., 1990; Stürzbecher et al., 1990).

**Figure 1.1. Schematic representation of p53 molecule.** The human p53 protein consists of 393 amino acids with functional domains, evolutionarily conserved domains and regions designated as mutational hotspots. Functional domains include the transactivation region (diagonally striped block), sequence-specific DNA binding region (amino acids 100-293), nuclear localization sequence (amino acids 316-325, vertically striped block). Evolutionarily conserved domains include amino acids 17-29, 97-292, and 324-352 (black areas). There are seven mutational hotspots and evolutionarily conserved regions within the large conserved domain (amino acids 130-142, 151-164, 171-181, 193-200, 213-223, 234-258, and 270-286, checkered blocks). Vertical lines above the schematic represent missense mutations; lines below schematic represent non-missense mutations (nonsense, frameshift, splicing, and silent). Adapted from C.C. Harris, 1996.
1.3.b. Biological and biochemical functions of p53 protein.

P53 is an active component of a DNA damage response pathway activated by ionizing radiation (Kuerbitz et al., 1992; Lowe et al., 1993b) and cytotoxic agents (Fujiwara et al., 1994; Lowe et al., 1993a) as well as other growth arrest signals such as hypoxia (Graeber et al., 1996; Graeber et al., 1994). In response to genotoxic stress p53 protein levels rapidly increase (Kastan et al., 1991; Lu & Lane, 1993). The activation/induction of p53 results in either growth arrest in G1/S or G2/M phase or apoptosis (Guillouf et al., 1995a; Guillouf et al., 1995b; Kastan et al., 1992; Maltzman & Czyzyk, 1984). Cell cycle arrest is mainly mediated by p21/Waf1, transactivated by p53 (El-Deiry et al., 1993). It has been postulated that cell cycle arrest allows time for DNA repair prior to DNA replication and mitosis thus preventing passing on mutations to the next generation of cells while apoptosis rids the organism of cells with severely damaged DNA. The only data so far supporting this model is the correlation between enhanced gene amplification and loss of functional p53 in REFs which was also accompanied by lack of cell cycle arrest (Livingstone et al., 1992). This observation was confirmed by wild-type p53-mediated restoration of cell cycle control and inhibition of gene amplification in the same experimental system (Yin et al., 1992).

Transcriptional activation of the human bax gene by p53 is likely directly connected to the p53-dependent mediation of apoptosis (Miyashita & Reed, 1995).

P53 also appears to be involved in the regulation of DNA transcription and/or repair machinery (reviewed by (Götz & Montenarh, 1995; Selivanova & Wilman, 1995)). It was found to form complexes with and possibly modulate the activity of numerous helicases such as the protein
subunits of the basal transcription-repair complex TFIIH (Leveillard et al., 1996; Wang et al., 1994; Wang et al., 1996), other cellular helicases (Sakurai et al., 1994) as well as a viral helicase, SV40 large T-ag (Stürzbecher et al., 1988). By modulating the activity of these helicases p53 may exert a regulatory function on the cell cycle. P53 can also interact with and induce GADD45 (growth arrest and DNA damage-inducible gene) a protein involved in DNA repair (Kastan et al., 1992; Smith et al., 1994; Zhan et al., 1994). It has been suggested that p53 may play a direct role in the repair of damaged DNA due to its ability to bind single stranded DNA and to catalyze DNA renaturation and DNA strand transfer (Bakalkin et al., 1995; Bakalkin et al., 1994).

Developmental studies suggest that functional p53 is not essential for a normal development of an organism since p53-null mice develop fully and produce fertile gametes (Donehower et al., 1992). Recent studies however reveal that a fraction of p53-deficient embryos exhibit a range of abnormalities in their nervous system (Sah et al., 1995). High incidence of tumour development in 6 months old p53-null mice strongly suggests that p53 has an important role in prevention of neoplastic transformation (Donehower et al., 1992).

Recent studies have shown that p53 has a direct role in senescence and when mutated it can significantly prolong the life span of human diploid cells (Bond et al., 1995). p53 protein has also been implicated in the process of differentiation in hematopoietic cell lineages (reviewed by (Prokocimer & Rotter, 1994; Selivanova & Wilman, 1995)). When constitutively expressed, p53 induced either progression in differentiation or apoptosis in these cell lines (Aloni-Grinstein et al., 1993; Rotter et al., 1993).
Biochemical functions of p53 are likely regulated by phosphorylation (reviewed by (Harris, 1996; Steegenga et al., 1996)). Differentially phosphorylated isoforms of p53 have been detected in the cell (Merrick et al., 1995) and the phosphorylation status of p53 was found to vary depending on the subcellular localization of the protein (Martinez et al., 1991). At least six different serines which are the targets of phosphorylation have been identified on the p53 molecule (reviewed by (Meek, 1994)). The N-terminal serines have been implicated in the p53's transactivation function (Mayr et al., 1995) while two C-terminal phosphorylation sites play a role in the DNA binding activity of the protein (Hupp et al., 1992; Wang & Prives, 1995). Various kinases have been reported to phosphorylate p53 in vitro at specific sites. That includes cdc-2, cdk-2, casein kinases (CK) I and II, DNA-dependent protein kinase I (DNA-PK I), protein kinase C, mitogen-activated protein kinase (MAP kinase), JNK1 and others (reviewed by (Meek, 1994)). However their involvement in the p53 regulatory pathway in vivo has not been completely elucidated. One good candidate for a p53 specific kinase is the recently described ATM (ataxia telangiectasia mutated) protein which contains a domain homologous to a family of PI-3 protein kinases and which is required for accumulation of p53 in response to radiation (Oren & Prives, 1996).

1.3.c. p53 in cell cycle arrest.

DNA damage occurs routinely and has to be dealt with in the regular course of the cell cycle. The repair of damaged DNA is important in prevention of malignant transformation of the cell. One option for the cell is to pause in its replication to allow the DNA repair machinery to rectify
the injury. In response to certain types of DNA damage cells have been shown to arrest their cycle either prior to DNA synthesis (G1:S arrest) or prior to segregation of their chromosomes (G2 arrest) (reviewed by (Leonard et al., 1995)).

P53 has been shown to play a major role in G1/S checkpoint brought about by DNA damage induced by ionizing radiation or chemotherapeutic drugs (Kastan et al., 1991). The growth arrest function of p53 was shown to depend on its ability to act as a sequence-specific transcriptional activator (Crook et al., 1994; Leiter et al., 1996). One of the many genes which is induced by p53 is p21/WAF1/CIP1 which plays an important role in the growth arrest pathway (El-Deiry et al., 1993; Harper et al., 1993). p21 protein binds to and inhibits the function of cyclin-dependent kinases thereby preventing the phosphorylation of pRb (retinoblastoma) family of proteins (Slebos et al., 1994). The hypo-phosphorylated pRb binds to and inhibits the transcriptional activity of the E2F family of transcription factors necessary for the induction of such cell cycle regulators as cyclins E and A, cdc 2 and others, as well as gene products required for DNA synthesis such as DHFR, thymidine kinase and others (DeGregori et al., 1995; Li et al., 1997; Spitkovsky et al., 1997) thus blocking the progression of cells from G1 to S phase of the cell cycle (Xiong et al., 1993). p21 also binds to and inhibits activity of PCNA necessary for the activation of DNA polymerase δ thus blocking its function in DNA replication but not interfering with DNA repair (Li et al., 1996; Waga et al., 1994).

Transcriptional activation by p53 of other genes such as gadd 45 (growth-arrest and DNA damage inducible gene) may also play a role in DNA damage activated growth arrest (Kastan et al., 1992). Gadd 45 gene product is necessary for the repair of damaged DNA (Smith et al., 1996).
It can also bind PCNA (Smith et al., 1994) and interact with p21 (Kearsey et al., 1995). It is likely that the competition for PCNA between gadd 45 and p21 contributes to the regulation of the cell cycle (Chen et al., 1995).

MDM 2 (murine double minute gene 2) is another protein which is transcriptionally activated by p53 following DNA damage (Barak et al., 1993). It however is involved in the rescue of cells from G1:S arrest rather than induction of G1:S arrest. MDM 2 was shown to bind to p53 transactivation domain and abrogate the protein's cell cycle arrest and apoptotic activities, which suggested a function as a negative feedback regulator of p53 allowing re-entry into the cell cycle (Chen et al., 1996; Thut et al., 1997). MDM 2 is also able to target p53 for rapid proteolytic degradation (Kubbutat et al., 1997; Piette et al., 1997).

Cyclin G, a possible subunit of cyclin dependent kinase, was also shown to be activated by p53 and although a good candidate for the involvement in cell cycle control (Okamoto & Beach, 1994; Zauberman et al., 1995) so far it has been shown to promote cell growth rather than arrest (Smith et al., 1997).

It has also been suggested that p53 may induce G2/M arrest as shown by experiments in a murine pre-B cell line and rat embryo fibroblasts (Aloni-Grinstein et al., 1995; Stewart et al., 1995). Recent data implicated a new p53 (and bcl-2) associated protein 53BP2 which was proposed to induce a G2/M arrest by direct interaction with p53 (Naumovski & Cleary, 1996).

1.3.d. P53 in apoptosis.

The apoptotic pathway induced by the activation of p53 has not been
completely elucidated and is still under active investigation. It is accepted that G1:S arrest and apoptosis are distinct functions of p53 which can be separated; by generating mutants deficient in one but not the other (Rowan et al., 1996), by uncoupling apoptosis and arrest through the action of cytokines (Lin & Benchimol, 1995) or by coexpression with p53 of specific inhibitors of apoptosis such as bcl-2 protein (Guillouf et al., 1995a). Different cell systems respond differently to specific DNA damaging agents and while some arrest their cycle, others die by apoptosis (reviewed by (Götz & Montenarh, 1995; Leonard et al., 1995)). Which path is followed by the induction of p53 does not appear to be always directed by the extent of DNA damage but more commonly by the physiological setting and presence or absence of specific growth factors.

Transactivation function of p53 was found important in p53-induced apoptosis in some experimental systems (Sabbatini et al., 1995; Yonish-Rouach et al., 1995). The implication of the p53-inducible growth arrest gene p21 in DNA damage-induced apoptosis has been suggested by a correlation between mutations in p21 and abrogation of apoptosis in human bladder cancer cell lines (Kawasaki et al., 1996). On the other hand, because p21-null mice were not defective in p53-induced apoptosis, its role has to be further investigated (Deng et al., 1995). So far the most promising targets for p53-mediated apoptosis response genes are: bax, induced, and bcl-2, down regulated by p53 (Miyashita et al., 1994; Selvakumaran et al., 1994). As bax modulates the survival functions of bcl-2 (Oltvai et al., 1993) p53 could favour the induction of apoptosis through the regulation of bax. Other p53-inducible genes may also play a role in apoptosis. For instance enhanced gadd 45 expression was correlated with cell death in one system (Canman et al., 1995). Another likely p53 targeted
gene is Fas/apo-1, a well known member of receptor/ligand tumor necrosis factor family, which provides a model for apoptosis controlled by cell surface receptors, and which was shown to be upregulated by p53 in human lung cancer cell line (Owen-Schaub et al., 1995). Recent experiments using hepatoma cell line suggest that drug-induced apoptosis is mediated, at least in part, by p53-dependent stimulation of the fas/apo-1 receptor/ligand system (Muller et al., 1997).

A recent finding that may shed some light on the way that p53 promotes apoptosis was isolation of several genes induced before the onset of p53 dependent apoptosis (Polyak et al., 1997). The analysis of the transcripts predicted that many of these genes encoded proteins that could generate or respond to oxidative stress. These experiments suggested that by the induction of redox-related genes p53 may generate reactive oxygen species, which in turn participate in the oxidative degradation of mitochondrial components culminating in cell death.

1.3.e. Alterations of p53 in human cancer.

P53 tumor suppressor gene is one of the most commonly altered genes found in human cancer including solid tumours of brain, breast, lung, colon, stomach and skin (Bártek et al., 1991; Hollstein et al., 1991; Nigro et al., 1989), different types of myeloid leukemias (Bi et al., 1993; Slingerland et al., 1991), as well as lymphoproliferative disorders such as Burkitt's lymphoma (BL), non-Hodgkin's lymphoma and lymphoid leukemias (Farrugia et al., 1994; Gaidano et al., 1991; Li et al., 1995; Sugimoto et al., 1991). It has been proposed, however that p53 alterations alone do not result in malignant phenotype but rather additional oncogenic events are required for the development of cancer. That conclusion was
drawn when p53-null nude mice were observed to develop spontaneous lymphomas and sarcomas in the first 6 months of life (Donehower et al., 1992). It is likely that loss of p53 function predisposes the cell to accumulating further mutations which, when unchecked could lead to malignant transformation.

In most tumours examined wild type p53 function is lost due to a combination of point mutations, allelic loss and deletions involving both alleles (reviewed by Lane, 1994)). More than 90% of these alterations fall within evolutionarily conserved region which encompasses exons 5 through 8 (Figure 1), and which is a part of a functional domain involved in sequence specific DNA binding (reviewed by Harris, 1996; Prokocimer & Rotter, 1994)). Most analyses have focused on the region including exons 5 through 8 since mutations in surrounding regions are rare (Hollstein et al., 1991). The mutant protein is usually present in high quantities in tumour cells while the wild type protein is sometimes undetectable due to a very short half-life.

P53 alterations were found to be of prognostic value in many types of cancer studied (Horio et al., 1993; Thor et al., 1992). A recent study in aggressive B cell lymphoma reported that p53 mutations predict a poor response to chemotherapy and short survival (Ichikawa et al., 1997). They found that a rate of complete remission decreased from 76% in patients with wild type p53-bearing tumour to 27% in those with mutant p53. These results are in agreement with suggestions that p53 mutations may be involved in the development of drug resistance (Lowe et al., 1993a) especially since mutant p53 protein was found to transactivate a promoter of the multidrug resistance gene 1 (MDR 1) while wild type protein repressed it (Chin et al., 1992).
1.4. Apoptosis.

1.4.a. Morphology and biochemical events involved in apoptosis.

Apoptosis or programmed cell death is a highly organized form of cellular suicide which occurs at specific times during development of an organism or in response to specific stimuli such as DNA damage and growth factor or cytokine withdrawal (reviewed by Götz & Montenarh, 1995; Samali et al., 1996). Apoptosis is a feature of normal embryonic development. For example, approximately half of the neurons produced in the development of the nervous system die by apoptosis. In addition tissue homeostasis, such as that of the hematopoietic system, is maintained by apoptosis (reviewed by Vermes & Haanen, 1994). In the development of the immune system immature self-reactive T cells or T cells that bind to antigen not associated with MHC complex, or B cells producing antibodies of lower affinity for a specific antigen also die by apoptosis.

Programmed cell death is a process consisting of genetically controlled sequence of morphological and biochemical events leading to cell death but, unlike necrosis, not inducing an inflammatory response. The characteristic morphology of apoptosis represents an end point to this series of events. Early in the execution phase of apoptosis margination and condensation of chromatin occurs in the nucleus (known as pyknosis), followed by cell volume shrinkage, membrane blebbing, nuclear fragmentation and finally disintegration of the cellular structure into apoptotic bodies (reviewed by Götz & Montenarh, 1995)). These fragmented cells are targeted by professional phagocytes for removal from circulation. This recognition is mediated through numerous biochemical
changes in the plasma membrane of the dying cell such as alterations of carbohydrates to attract macrophages which then bind via vitronectin receptors (CD36). Another significant change in the membrane of cells dying by apoptosis is exposure of molecules normally confined to the interior of the cell e.g. externalization of phosphatidylserine (PS) as a result of the loss of membrane phospholipid asymmetry. This event was utilized as a method of detection of apoptosis due to the development of an anti-PS antibody called Annexin V (Koopman et al., 1994).

A recent finding potentially important in understanding the biochemistry of apoptosis, suggested an ability of anti-apoptotic protein bcl-2 to form specific channels through the lipid membranes (Schendel et al., 1997). The in vitro experiments showed that pH and membrane acidity-dependent channel formation by purified recombinant bcl-2 protein was a property of two core hydrophobic α-helices. It was proposed that bcl-2-formed channel opening and closing in vivo which may be modulated by pH, may be intended for transport of an ion or protein resulting in protection of the cell from apoptosis. The same group suggested that pro-apoptotic protein bax, which also was found to form channels in vitro, may transport the same molecules but in opposite direction.

The active processes triggered in the cell dying by apoptosis include DNA, ribosomal RNA (rRNA) and protein degradation (reviewed by (Samali et al., 1996)). DNA fragmentation is a biochemical hallmark of later stages of apoptosis in many cells. When visualized by gel electrophoresis it results in a characteristic "ladder" pattern which was placed after the cell shrinkage in the time course of the committed apoptosis (Wesselborg & Kabelitz, 1993). The DNA becomes cleaved between nucleosomes producing fragments at first large, sized 50-200 kbp
DNA ladder pattern detectable in later phases of apoptosis is mostly a result of cleavage of internucleosomal linker DNA producing fragments of the size of DNA wrapped around a histone octamer. A number of endonucleases have been identified which can produce the "ladder" pattern of DNA in isolated nuclei in different cell systems (reviewed by (Hale et al., 1996)). Their common features include inhibition by zinc, dependence on calcium and magnesium ions and induction of double strand breaks resulting in free 3'-OH. In some experimental systems during apoptosis a specific cleavage of 28S rRNA was also observed, while 18S rRNA remained intact (Samali et al., 1996).

Endoproteolytic cleavage of many proteins by a number of different proteases, including serine proteases, calpains and proteasomes is another apoptosis-specific event observed ubiquitously. It appears to disable critical repair and homeostatic processes as well as disassemble structural components of the cell. The most studied of those proteases are ICE (interleukin-1β converting enzyme)-like proteases also known as caspases (cysteinyl aspartate-specific proteases).

1.4.b. Effectors of apoptosis.

Caspases have been implicated as the effectors of apoptosis in all known instances of programmed cell death (reviewed by (Leist & Nicotera, 1997; Porter et al., 1997; Rowan & Fisher, 1997)). They were discovered by their homology to a C. elegans death gene CED-3. Overexpression of caspases in mammalian or insect cells leads to death by apoptosis. These proteases exist in the cell as inactive precursors and must be activated by cleavage by other proteases. When active they in turn cleave other proteins
(death substrates) inactivating them in most cases, though activation of some substrates was also observed (reviewed by (Nicholson & Thornberry, 1997; Porter et al., 1997; Thor et al., 1992)). The first member of caspase family described was ICE (caspase-1), a cystein protease responsible for the proteolytic cleavage of cytokine precursors pro-interleukin 1β (proIL-1β) and interferon-γ inducing factor (IGIF) to their biologically active forms. Nine more ICE-like proteases have been identified so far (summarized in Table I). A phylogenetic analysis of the caspases suggests that based on their homology to either CED 3 or ICE they can be subdivided into two subfamilies but within each subfamily functional divisions also exist (Table II). Alternatively, the caspases can be divided into three groups based on their own tetrapeptide specificity for cleavage (P₁ is always an Aspartate residue). Within each group the amino acid preferences are very similar or even identical, suggesting function redundancy for at least some of these enzymes.
<table>
<thead>
<tr>
<th>Caspase</th>
<th>Other names</th>
<th>Subfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>ICE</td>
<td>ICE</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>Nedd2, ICH-1</td>
<td>CED-3</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>CPP32, Yama, apopain</td>
<td>CED-3</td>
</tr>
<tr>
<td>Caspase-4</td>
<td>ICE&lt;sub&gt;rel&lt;/sub&gt;-II, TX, ICH-2</td>
<td>ICE</td>
</tr>
<tr>
<td>Caspase-5</td>
<td>ICE&lt;sub&gt;rel&lt;/sub&gt;-III, TY</td>
<td>ICE</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>Mch2</td>
<td>CED-3</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>Mch3, ICE-LAP3, CMH-1</td>
<td>CED-3</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>MACH, FLICE, Mch5</td>
<td>CED-3</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>ICH-LAP6, Mch6</td>
<td>CED-3</td>
</tr>
<tr>
<td>Caspase-10</td>
<td>Mch4</td>
<td>CED-3</td>
</tr>
</tbody>
</table>

Table I. Members of the caspase family. Subfamily is determined by the closest homology to either ICE or CED-3. Table adapted from (Nicholson & Thornberry, 1997).

<table>
<thead>
<tr>
<th>Specificity group / caspase</th>
<th>Consensus cleavage site (P&lt;sub&gt;4&lt;/sub&gt;-P&lt;sub&gt;1&lt;/sub&gt;)</th>
<th>Proposed role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td>Maturation of multiple pro-inflammatory cytokines.</td>
</tr>
<tr>
<td>Caspase-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WEHD</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td>Cleavage of DxxD apoptotic substrates.</td>
</tr>
<tr>
<td>Caspase-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DExD</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td></td>
<td>Activation of group II caspases, activation of other group III caspases, cleavage of non-DxxD apoptotic structures.</td>
</tr>
<tr>
<td>Caspase-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(IVL)ExD</td>
<td></td>
</tr>
</tbody>
</table>

Table II. Specificities and proposed biological functions for caspases.

Three major groups of caspases varying by tetrapeptide specificities play different roles in inflammation and apoptosis. Table adapted from (Nicholson & Thornberry, 1997).
A nuclear enzyme, poly-ADP-ribose polymerase (PARP) is one of the first death substrates to be cleaved during apoptosis yielding an 85 kDa inactive fragment (reviewed by (Nicholson & Thornberry, 1997; Porter et al., 1997; Thor et al., 1992)). CPP32 or caspase-3 was the enzyme identified to be responsible for this cleavage which results in the separation of the catalytic from the DNA binding domain, although Caspase-7 has also been implicated. PARP was suggested to play a role in DNA repair and suppression of the activity of an apoptotic endonuclease. PARP cleavage is commonly used as a marker of apoptosis in mammalian cells.

DNA-dependent protein kinase (DNA-PK) is another DNA repair enzyme whose catalytic subunit is inactivated by proteolytic cleavage mediated by caspase-3 during apoptosis. The cell cycle regulator retinoblastoma (Rb) protein was also identified to be a substrate for caspase-3 and likely several other yet unidentified caspases (Jänicke et al., 1996). It was found to be cleaved at different functionally important sites likely by different caspases depending on the apoptotic stimulus. The protein component of the U1 small nuclear ribonucleoprotein (U1-70) also becomes cleaved early in apoptosis in some systems, likely resulting in inhibition of gene expression due to negative effect on mRNA splicing and transport (Song et al., 1996). Caspase-3 is also a candidate for mediating this cleavage. Another interesting caspase 3 cleavage substrate is mdm 2 protein which normally is bound to p53 retaining it in cytoplasm, and whose cleavage may allow the entry of p53 into the nucleus and thus its functional activation (Chen et al., 1997; Erhardt et al., 1997). Many new caspase substrates have emerged with proposed roles in DNA repair, DNA fragmentation, cell cycle progression, cytoskeletal integrity and many others.
Proteolysis of important structural proteins involved in cellular integrity was also shown to occur via caspases (reviewed by (Cohen, 1997; Porter et al., 1997)). Examples of such proteolysis are the cleavage of nuclear lamins which are a part of intermediate filament meshwork contributing to nuclear envelope integrity, as well as the cleavage of Gas 2, a part of actin microfilament network constituting cytoskeleton. The cleavage of lamins was found to precede DNA fragmentation and was proposed to contribute to chromatin condensation and nuclear shrinkage (Rao et al., 1996). Caspase-6 together with other members of the family were implicated in these events. The proteolysis of Gas 2 likely contributes to cellular shape changes observed in apoptosis.

Recent findings suggest that some caspase substrates may become activated by cleavage during apoptosis (reviewed by (Porter et al., 1997)). Protein kinase Cδ, for instance, is cleaved at the onset of apoptosis most likely by caspase-3 which activates its catalytic subunit (Ghayur et al., 1996). The exact function of PKCδ in cell death is not yet clear though its involvement in cellular signaling is likely. The activation of certain transcription factors by caspase-induced cleavage during apoptosis has also been observed (Pai et al., 1996). That includes sterol regulatory element binding proteins whose activation induces biosynthesis of cholesterol which may in turn contribute to the preservation of the integrity of the plasma membrane, characteristic of apoptosis.

1.4.c. Regulators of apoptosis.

A group of proteins involved in positive and negative regulation of apoptosis includes a variety of protooncogenes, tumour suppressor gene products and cell cycle regulators (reviewed by (Hale et al., 1996; Rowan
The Bcl-2 protooncogene, first isolated inhibitor of apoptosis, is able to prolong life of cells deprived of growth factors or insulted with DNA damaging agents. It was originally found in a translocation involving an immunoglobulin heavy chain locus in human follicular lymphoma. A whole family of Bcl-2 related proteins has been isolated by virtue of two closely conserved domains termed BH1 and BH2 (Williams & Smith, 1993). These domains have been conserved throughout evolution and mutations within these regions alter the function of the proteins (Yin et al., 1994). The extensively studied Bcl-2 family of genes, although homologous to *C. elegans* apoptosis inhibitor ced-9, includes both inhibitors and activators of cell death. Bcl-x<sub>L</sub>, a larger splicing variant of Bcl-x protein as well as mcl-1 protein (expressed in myeloid cells undergoing differentiation), function in a similar way to their homolog Bcl-2. A smaller splicing variant of Bcl-x, Bcl-x<sub>S</sub>, not only is a positive regulator of apoptosis, but also can override the inhibitory effects of Bcl-2 (Boise et al., 1993). Bax and Bak, also members of human Bcl-2 family, constitute other examples of accelerators of cell death induced by growth factor withdrawal (IL-3) and can reverse Bcl-2 imposed protection. The expression of Bax and Bcl-2 proteins was found to be regulated by p53: positively for bax and negatively for bcl-2 (Miyashita et al., 1994). The members of Bcl-2 family of proteins exist as homodimers and heterodimers. Bcl-2, for example, besides forming homodimers, can heterodimerize with Bcl-x<sub>L</sub>, Bcl-x<sub>S</sub>, Bax and Mcl-1. It is likely that the regulation of the apoptotic response in the cell occurs through the ratio of the apoptosis promoters and suppressors present. How Bcl-2 prevents apoptosis has not been completely elucidated but several theories are available. It has been suggested that Bcl-2 may inhibit the release of
calcium ions from the endoplasmic reticulum to the cytosol or that it may modulate transport of proteins through the nuclear pores (reviewed by Hale et al., 1996). This possible function of Bcl-2 is supported by recent evidence for its ability to form channels through lipid bilayers (Schendel et al., 1997). It may also act by simply preventing Bax from inducing apoptosis. It is likely that Bcl-2 functions in more than one way to exert its anti-apoptotic effects.

Another group of genes involved in modulation of apoptosis are certain protooncogenes not related to the Bcl-2 family as well as tumour suppressor genes (e.g. p53 discussed earlier), which were found to induce cell death when overexpressed in some experimental systems (reviewed by Harrington et al., 1994; Rowan & Fisher, 1997). Protooncogenes c-Myc and c-Myb involved in the regulation of cell proliferation and differentiation were shown to accelerate apoptosis under specific conditions such as T cell growth factor β-1 (TGFβ1)-induced growth inhibition of myeloid leukemia cells (Hoffman & Liebermann, 1994). C-Myc can also induce proliferation of cells when its expression is deregulated but the decision to proliferate or die depends on the extracellular survival factors present. Adenovirus proteins E1A and E1B are other examples of modulators of apoptosis as the E1A oncogene can promote either proliferation or apoptosis, while E1B prevents cell death. Both c-Myc and E1A-induced apoptosis appear to be p53 dependent (reviewed by Rowan & Fisher, 1997).
1.5. Lymphoblastoid cell lines: molecular and phenotypic properties.

As a model for B cell malignancy we sought to recapitulate B cell neoplasia by introducing relevant oncogenes into B lymphocytes. However, because primary B cells do not survive well in culture it was necessary to infect these with Epstein-Barr virus to create a workable model for human B cell malignancy. Infection of human B cells with transforming Epstein-Barr virus (EBV) *in vitro* results in polyclonal activation and immortalization of the cells but not malignant transformation (reviewed by (Nilsson & Klein, 1982)). *In vivo* EBV infection results in a spontaneous outgrowth of B cell clones harbouring the virus. *In vitro* transformed lymphoblastoid cells (LCLs) resemble normally activated B lymphoblasts but express latent viral proteins responsible for the immortalization of the cells and for the maintenance of the virus in a latent episomal form. At least nine EBV proteins are usually expressed by LCLs including six EBV nuclear antigens (EBNA 1, 2, 3A, 3B, 3C and LP (leader protein)) and three latent membrane proteins LMP 1, 2A and 2B (reviewed by (Ichikawa *et al.*, 1997)). Similar patterns of EBV protein expression are seen in EBV-associated B cell lymphomas in immuno-compromised patients but more restricted expression patterns are associated with other EBV-associated cases of lymphoma (Hodgkin's disease, Burkitt's lymphoma, etc.). EBV-harbouring Burkitt's lymphoma-derived cell lines express only one viral protein; EBNA 1.

As a result of the expression of viral proteins, the induction of cellular RNA, protein and DNA synthesis occurs. LMP 1 and EBNA 2 were found to induce the expression of the bcl-2 gene (an inhibitor of apoptosis) thus possibly preventing programmed death of the host cell
EBNA 5 was reported to bind to p53 and Rb proteins \textit{in vitro} which may interfere with their functions (Szekely \textit{et al.}, 1993). However, EBV present in the immortalized LCLs does not appear to block the p53 activated DNA damage response pathway as the cells show functional p53 and remain sensitive to DNA-damaging chemotherapeutic drugs such as cisplatin, actinomycin D and adriamycin (Allday \textit{et al.}, 1995b).

The phenotypic properties of LCLs are similar between different cell lines, however secondary changes of the phenotype may occur upon prolonged passaging (over 6 months) (reviewed by (Nilsson & Klein, 1982)). The individual cells are motile and variable in shape. They adhere to each other in culture forming characteristic clusters. The doubling time of LCLs is usually around 30 hrs and they cannot survive in low cell densities (less than $10^4$ cells/ml). LCLs do not constitute transformed cell lines as they do not form tumours when injected subcutaneously into nude mice or do not form colonies in agarose \textit{in vitro} (Nilsson \textit{et al.}, 1977).
CHAPTER 2: MATERIALS AND METHODS.

2.1. Cell lines and culture conditions.

Human lymphoblastoid cell line GM607 was obtained from Dr. L. Rubin (University of Toronto, Toronto, ON). It is a non-transformed B cell line which has been immortalized with Epstein-Barr virus (Allday et al., 1995b). GM607 transfected with a pRCCMV vector (GM607/pRCCMV) behaves in the same way as GM607 and is used as a control cell line in these assays. It was generated as a vector only control for other types of experiments and mutant p53 constructs (mut p53/pCEP4) were trasfected into it. Bcl-2/pCEP4, a bcl-2 protein overexpressing cell line, was generated by the transfection of GM607 with a bcl-2/pRCCMV construct and after subcloning this cell line was retransfected with a pCEP4 vector. Human diffuse large cell lymphoma cell lines used in the study included: OCI LY1, LY2 and LY8 C3 (Tweeddale et al., 1987) and were obtained from Dr. H. Messner (Princess Margaret Hospital, Toronto, ON ). LY1 carries a t(14;18) translocation resulting in overexpressed bcl-2 protein and both its p53 alleles are mutated (arg→his 158, cys→gly 176) resulting in high levels of p53 protein. LY2 carries a single mutant p53 allele (cys→phe 176) and expresses high levels of p53 protein. Human osteosarcoma p53-null cell line SAOS 2 was kindly provided by Dr. S. Benchimol (OCI, Toronto, ON).

All non-adherent cells were cultured in RPMI 1640 (Wisent, St. Bruno, Que.) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 15% bovine calf serum (BCS), (Wisent, Que.) in the presence of 5% CO₂ at 37°C. GM607/pRCCMV was grown in the above media but with addition of 750 μg/ml of G418 (geneticin sulfate,
GIBCO BRL, Gaithersburg, MD) while the mutant p53-expressing cell lines, vector only control (pRCCMV/pCEP4) and bcl-2 overexpressing control (bcl-2/pCEP4) were cultured with both 750 µg/ml G418 and 150 µg/ml hygromycin (GIBCO BRL). For some of the apoptosis assays (wherever indicated) the 15% BCS contained in the growth media was replaced by B-27 supplement (GIBCO BRL). The adherent SAOS 2 cells were cultured in DMEM (GIBCO BRL) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% bovine calf serum (Wisent).

2.2. Plasmids and constructs.

pCEP4 (Invitrogen Corp., San Diego, CA) is a CMV promoter based and hygromycin resistance conferring shuttle vector. The EBV origin of replication and the expression of EBNA-1 allow high copy episomal replication of the vector. It was used to express mutant p53 cDNAs (Farrugia et al., 1994; Slingerland & Benchimol, 1991; Slingerland et al., 1993). The p53 cDNAs were blunt-ended and ligated into PvuII site of the pCEP4 vector. pRCCMV (Invitrogen Corp.), a CMV promoter-driven shuttle vector carrying G418 resistance gene was used for the overexpression of bcl-2 cDNA to create an apoptosis resistant control (bcl-2/pCEP4). The 0.9 kb bcl-2 cDNA was digested out of an EcoRI site in a pB4 plasmid, blunt-ended and subcloned into pRCCMV which had been digested with NotI and then blunt-ended. All ligations were performed using T4 DNA ligase (GIBCO BRL). The ligated products were electroporated into XL1-Blue Escherichia coli using the Gene Pulser system (Bio Rad, Hercules, CA) and the colonies were screened by Southern blotting. Large quantities of the plasmids were produced using

2.3. Establishment of mutant p53 variants.

GM607/pRCCCMV cells were electroporated with either pCEP4 vector or one of the following mutant p53 constructs: pCEP4/p53(246val), pCEP4/p53(135ser) or pCEP4/p53(273his) where mutant p53 cDNA (1.8 kb, 1.8 kb and 1.6 kb long respectively, Figure 4) had been originally cloned from AML cell lines (Slingerland & Benchimol, 1991; Slingerland et al., 1993), as well as pCEP4/p53(282pro), pCEP4/p53(158his) or pCEP4/p53(176gly) where mutant p53 cDNA (1.3 kb long in each case) came from DLCL cell lines (Farrugia et al., 1994). 3x10⁷ cells in 0.75 ml of PBS buffer (5.4 mM KCl, 2.8 mM Na₂HPO₄, 2.9 mM KH₂PO₄ and 0.3 M NaCl, pH 7.3) were mixed with 50-100 µg of plasmid DNA contained in 100 µl of PBS, pulsed at 700 V and 25 µF and incubated at 5% CO₂ and 37°C for 48 hrs in non-selective media. The cells were then plated out at 10⁴, 10³ and 10² cells per well in 96 well plates in media with the addition of hygromycin at 750 µg/ml and grown for 2-3 weeks. Colonies were expanded off the plates on which less than 30% of the wells contained growing cells, and were then screened by western blot. Bcl-2/pCEP4 control cell line was generated in similar manner but bcl-2 cDNA was expressed via a pRCCCMV vector.

2.4. Transient transfection assay.

Approximately 8x10⁵ SAOS 2 cells were plated in each of 25 cm² flasks and grown overnight at 37°C and 5% CO₂ till 50-80% confluent. For each of the flasks 2.5 µg of mutant p53 construct DNA diluted into 250 µl
of serum-free DMEM was combined with 7.5 µl of LIPOFECTAMINE™ reagent (GIBCO BRL) that had been diluted into 250 µl of serum-free DMEM. The mixture was incubated at RT for 30 min. 2.5 ml of serum-free DMEM was mixed with the LIPOFECTAMINE™/DNA mixture and it was poured onto SAOS 2 cells (in each flask) which had been washed with 10 ml of serum-free DMEM. The cells were incubated at 37°C and 5% CO₂ for 4 hrs. The media was removed and replaced with complete media and the cells were incubated for approximately 24 or 48 hrs before protein isolation.

2.5. Drug treatment and irradiation.

Cells growing in log phase were subcultured at 3x10⁵ cells/ml and 24 hrs later they were either irradiated with 50 Gy of γ-radiation or cisplatin (Bristol Laboratories, Montreal, Que) was added to a final concentration of 33 µM. For cell cycle and proliferation assays cells were incubated for 24 hrs after irradiation or addition of cisplatin. For western and northern blot analysis cells were lysed either before or at specific time points after irradiation or addition of cisplatin.

2.6. Protein isolation and immunoblotting.

Cells were lysed in a lysis buffer composed of 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA (pH 8), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 0.23 µg/ml aprotinin, by incubation on ice for 15-30 min. Debris was sedimented by centrifugation at 14,000 rpm at 4°C for 10 min and 10 µl of each supernatant was added to 5 µl of sample buffer containing 0.188 M Tris/HCl (pH 6.8), 6% SDS, 30% glycerol, 15% 2-mercaptoethanol and 0.015% of bromophenol blue.
Proteins were resolved at 150 V for 1.5 h on polyacrylamide, Tris-Glycine precast gels: 4-12% gradient or 10% discontinuous gels (Novex, San Diego, CA) in Tris-Glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) using XCell II™ Mini Cell gel runner (Novex). The resolved proteins were electrophoretically transferred at RT for 3 hrs to PVDF membrane (Millipore, Mississauga, ON) using XCell II™ blot module (Novex). Membranes were blocked for 2 hrs at room temperature in a buffer composed of 50 mM Tris, 140 mM NaCl, and 0.1% Tween-20 and containing 4% BSA. They were probed for 2 hrs with the primary and then for 1 hr with the secondary antibody diluted in the above buffer but containing 1% BSA. The antibody staining was visualized using the "Renaissance" fluorescein labeling system (DuPont, Boston, MA) and exposed to an X-ray film (BIOMAX™MR, Kodak, Rochester, NY). The intensity of the fluorescent signals was quantified using IPLabGel densitometric software. The p53 and bcl-2 densitometric values were normalized to actin or PCNA levels (as indicated) to control for variations in sample loading.

The primary antibodies used included: mouse anti-human p53 (pAB1801, PharMingen, San Diego, CA), hamster anti-human bcl-2 (6C8, PharMingen), mouse anti-human p21/Waf1 (Calbiochem, Cambridge, MA), mouse anti-human PARP (C2-10, obtained from Dr. G. Poirier, U.of Laval, Que), mouse anti-actin (Amersham, Arlington Heights, Ill) and mouse anti-PCNA (Oncogene Science Inc., Cambridge, MA). The secondary antibodies used: sheep anti-mouse HRP (1:5000, Amersham) and rabbit anti-hamster HRP (1:5000, Amersham).
2.7. RNA isolation and northern blotting.

Total RNA was isolated using TRIZOL® reagent (GIBCO BRL). 10⁷ cells were centrifuged for 5 min at 1,000 rpm and 4°C and lysed in TRIZOL® (1 ml per 5x10⁶ cells). The lysates were incubated for 5 min at room temperature (RT). 0.2 ml of chloroform was added per 1 ml of TRIZOL® and the tubes were shaken vigorously for 15 sec and incubated at RT for 3 min. The samples were centrifuged at 12,000g for 15 min at 4°C. The upper (aqueous) phase was removed and RNA was precipitated from it by addition of 0.5 ml of isopropyl alcohol per every 1 ml of TRIZOL® used initially. Samples were incubated at RT for 10 min and centrifuged at 12,000g and 4°C for 10 min. The supernatant was removed and the RNA pellet was washed with 75% ethanol by vortexing and recentrifugation for 5 min at 7,500g and 4°C. The supernatant was discarded and the RNA pellet was air-dried, dissolved in 0.5% SDS (made in RNAse-free water), incubated for 10 min at 55°C and the absorption at 600 nm (A₂₆₀) was read using a spectrophotometer and used to calculate the concentration of RNA in the sample. 10 μg of RNA was added to an equal volume of loading buffer (final concentration of 50% formamide, 6% paraformaldehyde, 0.02% XC-bromophenylblue and 3-(N-morpholino) propanesulfonic acid (MOPS)). The sample was then electrophoretically size fractionated in a 6% formaldehyde, MOPS on a 1% agarose gel and transferred onto a Zeta-probe nylon membrane (Bio Rad, Hercules, CA) by overnight capillary transfer in 10X SSC (30 M sodium chloride, 3 M trisodium citrate). The membrane was cross-linked in a UV stratalinker (Stratagene, LaJolla, CA), prehybridized and hybridized to an appropriate ³²P α-labeled probe, then washed and exposed to an X-ray film (Kodak) at -70°C. All technical procedures followed were provided by the
manufacturer of Zeta-probe. The intensity of the hybridization signals was quantitated using IPLabGel densitometric software. The p53 densitometric values were normalized to actin levels to control for variations in sample loading.

Probes used included: 1.0 kb cDNA fragment specific for human β-actin gene and 1.3 kb BamHI/HindIII p53 cDNA fragment originally isolated from LY8 C3 cell line and cloned into pCEP4 vector.

2.8. Cell cycle analysis.

5 x10^6 cells were spun down for 5 min at 1,000 rpm and 4°C, and washed with PBS buffer and fixed in 80% ethanol on ice for 1 hr. They were centrifuged at 3,000 rpm, washed with PBS and then with a propidium iodide staining buffer (PIB: PBS buffer with the addition of 0.12% Triton X-100 and 0.12 mM EDTA). The cells were resuspended in RNaseA/PIB (50-100 µg/ml, Boehringer Mannheim Canada, Mississauga, ON) and incubated at 37°C for 45 min. PI solution (5 mg/ml propidium iodide dissolved in PIB) was added at 50 µg/ml and the reactions were incubated at room temperature in the dark for at least 1 hr. The analysis was performed on the day of the assay using a flow cytometer FACScalibur (Becton Dickinson, Mountain View, CA).

2.9. Cell cycle/proliferation assay.

5-8 x10^6 cells were incubated for 3 hrs with 18 µg/ml of bromodeoxyuridine (BrdU) added to the media, at 37°C and 5% CO₂. The cells were spun down for 5 min at 1000 rpm and 4°C and washed twice with 10 ml of IFA buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 4% BCS and 0.1% NaN₃). The cells were resuspended in 5 ml of pre-chilled (-20°C)
70% ethanol and fixed on ice for 30 min. Fixed cells were centrifuged for 10 min at 1,400 rpm at 4°C, pellets were washed once with cold PBS buffer and resuspended in 0.5 ml of autoclaved water. The samples were then transferred into microtubes containing 16 μl of 0.1N HCl, heated at 97°C for 10 min and then chilled on ice for 10 min. Samples were washed twice with 1 ml of IFA/0.5% Tween-20 (centrifuging at 1,400 rpm, 4°C for 10 min) and resuspended in 70% ethanol for storage at -20°C (for up to 1 week). Following storage cell pellets were washed twice with 1 ml of IFA/0.5% Tween-20 (centrifuging at 1,400 rpm, 4°C for 10 min) and resuspended in a final volume of 1 ml of IFA buffer. 0.5 μg of fluorescinated anti-BrdU antibody (BR-3, Cedarlane Laboratories LTD, Hornby, ON) was added to each sample and allowed to bind by incubation on ice for 30 min. Cell pellets were washed twice with IFA/0.5% Tween-20 and resuspended in 0.5 ml of IFA buffer containing RNAseA (Boehringer Mannheim Canada). After 15 min incubation at 37°C PI solution was added at 50 μg/ml (described in section 2.7) and samples were incubated for at least 1 hr at 4°C before flow cytometric analysis (see section 2.7).
CHAPTER 3: RESULTS

3.1. Generation of mutant p53 expressing lymphoblastoid cell lines and of a bcl-2 overexpressing control cell line.

Recombinant plasmids containing two different mutant p53 cDNAs: cys→ser135 and met→val246 were constructed by ligating the mutant p53 cDNAs into pCEP4 expression vectors (Figure 3.1.a). The constructs were transfected into the lymphoblastoid cell line GM607/pRCCMV and the independent clones expressing high levels of p53 protein as assessed by western blotting were isolated (Figure 3.1.b). All of the clones were shown to express similar levels of the p53 protein, from 14 to 24 times higher than the vector control (Figure 3.1.b). The presence of the recombinant RNA transcript in two independent p53 overexpressing clones transfected with pCEP4/p53(246val) construct confirmed that mutant protein was indeed overexpressed in those clones (Figure 3.1.c). Both mutant p53 protein and RNA in the variants were present at levels comparable to the mutant p53 expressed by three independent human diffuse large cell lymphoma (DLCL) cell lines previously shown to contain mutant p53 (Farrugia et al., 1994), although there was some variability in the protein expression between these DLCL cell lines.

Bcl-2 overexpressing lymphoblastoid cell line was generated to be used as an apoptosis resistant control. Recombinant pRC/CMV plasmid containing bcl-2 cDNA (Figure 3.2.a) was transfected into the parental GM607 cell line and with the help of western blot screening the independent clones overexpressing Bcl-2 protein were isolated. One of the clones, expressing 3.6 times more Bcl-2 protein than the vector control,
was chosen for further studies (Figure 3.2.b). P53 expression remained unchanged upon overexpression of bcl-2 gene.

We have also attempted to express other mutant p53 constructs in the lymphoblastoid system. Recombinant expression plasmids containing the following mutant p53 cDNAs: arg→his273 cloned from an AML cell line (Slingerland & Benchimol, 1991; Slingerland et al., 1993), as well as arg→pro282, arg→his158 and cys→gly176 cloned from DLCL cell lines (Farrugia et al., 1994) were cloned in a similar manner to the previously described mutant p53 constructs (Figure 3.1.a). Western and northern blots of the transfectants generated using the above constructs did not however reveal any clones overexpressing transgenic p53 (data summarized in Table III). To test the ability of our constructs to be expressed in a mammalian system and to exclude a possibility of a toxic effect of the proteins, transient transfection assays were performed in a p53-null human osteosarcoma cell line (SAOS 2). The western blot assay performed 24 hrs after transfection indicated that only SAOS 2 cells that received pCEP4/p53(246val) or pCEP4/p53(135ser) constructs expressed high levels of p53 protein (Figure 3.4).
Figure 3.1. Structure of the mutant p53 expression construct (a) and its expression in transfected lymphoblastoid GM607/pRCCMV cell lines at the protein (b) and RNA (c) level. P53 protein and RNA levels in the lymphoblastoid cell transfectants were compared to those of the control parental cell line transfected with pCEP4 only (lane 1). Protein lysate equivalent to 10^5 cells was loaded in each lane of the protein gel. 10 μg of total RNA was run in each lane of the RNA gel. Actin expression and transcription levels were shown as those of an unrelated control housekeeping gene. LY1, LY2 and LY8 C3 are diffuse large cell lymphoma (DLCL) cell lines carrying only mutant p53 genes. The levels of p53 expression in each cell line, normalized to actin levels, are shown below each lane of the western blot.
a) \textbf{mut p53pCEP4}

\begin{center}
\includegraphics[width=0.7\textwidth]{a.png}
\end{center}

\textbf{Hygromycin P TK} $\rightarrow$ \textbf{TK pA} $\rightarrow$ \textbf{mut p53 cDNA} $\rightarrow$ \textbf{P CMV} $\rightarrow$ \textbf{SV 40 pA} $\rightarrow$ \textbf{EBNA-1}

b) \textbf{p53/actin protein levels:}

\begin{center}
\begin{tabular}{cccccccc}
66 kDa - & 0.04 & 0.68 & 0.96 & 0.56 & 0.64 & 0.58 & 0.36 & 1.35 \\
46 kDa - & p53 & & & & & & & \\
& actin & & & & & & & \\
\end{tabular}
\end{center}

c) \textbf{pCEP4 val246c10 val246c14}

\begin{center}
\includegraphics[width=0.7\textwidth]{c.png}
\end{center}

\textbf{endogenous p53 (2.6 kb)} $\rightarrow$ \textbf{transgenic p53 (1.8 kb)} $\rightarrow$ \textbf{actin (2 kb)}
Figure 3.2. Structure of the Bcl-2 expressing construct (a) and its expression in the transfected GM607 lymphoblastoid cell line (b). Bcl-2 protein levels in the transfectant (bcl-2 c5-5, lane 3) were compared to those of the parental (GM607, lane 1) and the control cell line transfected with the pRC/CMV vector only (pRC/CMV, lane 2). Protein lysate equivalent to $10^5$ cells was loaded in each lane of the protein gel. The membranes were hybridized with anti-PCNA antibody to show the expression of an unrelated housekeeping gene, and anti-p53 antibody to examine expression of p53 in the Bcl-2 overexpressing cell line. The levels of Bcl-2 protein were normalized to the PCNA protein levels and are shown below each lane.
Figure 3.3. Structure and sizes of the p53 cDNAs isolated from AML and DLCL cell lines. Primer sequences p53 P₁ and P₂ and cloning procedures for the mutants isolated from DLCL cell lines were described by Farrugia et al, 1994. Mutant p53 constructs originated from AML cell lines were described by Slingerland et al, 1991. Sequence distances are shown in base pairs below the p53 cDNA (not drawn to scale). ORF and 3' UTR designate an open reading frame and a 3' untranslated region respectively.
<table>
<thead>
<tr>
<th>construct used for transfection</th>
<th># of transfections performed</th>
<th>% of hygromycin resistant cells</th>
<th># of clones overexpressing p53 / # of clones assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCEP4/p53(246val)</td>
<td>2</td>
<td>0.01</td>
<td>22 / 24</td>
</tr>
<tr>
<td>pCEP4/p53(135ser)</td>
<td>1</td>
<td>0.01</td>
<td>6 / 7</td>
</tr>
<tr>
<td>pCEP4/p53(273his)</td>
<td>1</td>
<td>0.4</td>
<td>0 / 37</td>
</tr>
<tr>
<td>pCEP4/p53(158his)</td>
<td>1</td>
<td>0.01</td>
<td>0 / 10</td>
</tr>
<tr>
<td>pCEP4/p53(176gly)</td>
<td>1</td>
<td>0.1</td>
<td>0 / 18</td>
</tr>
<tr>
<td>pCEP4/p53(282pro)</td>
<td>1</td>
<td>0.1</td>
<td>0 / 10</td>
</tr>
</tbody>
</table>

Table III. The summary of transfection data accumulated in the process of generation of mutant p53 expressing lymphoblastoid cell lines. Mutant p53 expression constructs (column 1) were used for the transfection of GM607/pRCCMV cell line either once or twice (column 2). Cells were plated at a density range of $10^2$-$10^4$ cells per well. The percent of hygromycin resistant clones (column 3) was calculated by the ratio of the number of wells containing growing colonies in the 96 well plate seeded at the lowest cell density to the total number of cells seeded on that plate. The hygromycin resistant clones were screened by western blotting for the expression of p53 protein (summarized in column 4).
**Figure 3.4.** The expression of p53 protein resulting from transient transfection of mutant p53 constructs into SAOS 2 cell line. Western blot shows the expression of p53 and PCNA (an unrelated, housekeeping protein) assayed 24 hrs after transient transfection. The p53 levels in cells transfected with each of the indicated mutant p53 constructs (lanes 3-8) were compared to those in the cells transfected without the addition of DNA (lane 1) or with pCEP4 vector alone (lane 2). LY8 C3 is a control diffuse large cell lymphoma cell line known to express mutant p53 protein. Protein lysate equivalent to $10^5$ cells was loaded in each lane of the protein gel.
3.2. Effects of cisplatin and ionizing radiation on G1:S arrest in mutant p53 overexpressing lymphoblastoid variants.

Incorporation of the deoxynucleotide analog bromodeoxyuridine (BrdU) into cells allows visualization of cells actively synthesizing DNA and thus permits an accurate measurement of the cell population going through S phase. Combined with the PI staining of the cellular DNA this proliferation/cell cycle assay is an accepted method for quantitation of G1:S arrest (Peacock et al., 1995). Both irradiation and cisplatin induced marked G1:S arrest in the parental lymphoblastoid cell line (Figure 3.5). The reduction in G1:S arrest in the mutant p53 variants with respect to the wild-type p53 control was calculated using the following formula:

\[ 100\% - \left( \frac{G1:S\,\,\,\text{arrest\,\,of\,\,the\,\,mutant\,p53\,clone}}{G1:S\,\,\,\text{arrest\,\,of\,\,the\,\,wild\,type\,p53\,control}} \right) \times 100\% \]

where G1:S arrest was calculated using the formula shown in the legend of Table IVa. Compared to the control cell line pRCCMV/pCEP4, irradiation of the two independent mutant p53(246val) clones with 50 Gy assayed 24 hrs later in four independent experiments (Table IVa) resulted in between 12% to 69% reproducible reductions in G1:S arrest (from the lowest to the highest reduction value). Similarly, mutant p53(135ser)c3 showed a 27% to 81% reduction in radiation-induced G1:S arrest in three independent experiments. Treatment with 33 μM cisplatin resulted in 14% to 68% reduction in G1:S arrest of the p53(246val)-expressing cell lines and 6% to 82% in the p53(135ser) variant as compared to the control in four and three independent experiments respectively. Surprisingly our measurements demonstrate the occurrence of substantial G1:S arrest even in the cell lines expressing mutant p53 although, as described earlier, to a lesser extent that in the wild type p53 control. In a single independent experiment the mutant only p53-
expressing DLCL cell lines (LY1 and LY8 C3) were found not to undergo G1:S arrest after irradiation with up to 50 Gy unlike the wild type p53-expressing tumour (AML3) and lymphoblastoid (GM 607) cell lines (Table IVb).

Various doses of radiation and cisplatin have been tested in experiments similar to those described above (data not shown). The doses used here have been chosen as the ones which produced the clearest and the most reproducible results.
Figure 3.5. G1/S arrest of pRCCMV/pCEP4 lymphoblastoid cell line in response to cisplatin and ionizing radiation. The proliferation / cell cycle assay was performed 24 hrs after the exposure of the cells to 33 μM cisplatin or 50 Gy of γ-radiation. The cells were pulsed with bromodeoxyuridine (BrdU) for 3 hrs, stained with anti-BrdU antibody (FL1-H) and with propidium iodide (FL2-A). The measurement was obtained by flow cytometric assessment of 10,000 cells.
Table IV. G1:S arrest induced by cisplatin and γ-radiation is reduced in the lymphoblastoid transfectants expressing mutant p53. a). The summary of G1:S arrest data for mutant p53-expressing lymphoblastoid transfectants following 33 µM cisplatin treatment or irradiation with 50 Gy. b). The summary of G1:S arrest data for tumour cell lines expressing either mutant only p53 (LY 1, LY8 C3) or wild type p53 (AML 3) as well as wild type p53 bearing LCL (GM 607) followin irradiation. G1:S arrest was calculated using the following formula: G1:S ratio of treated cells divided by G1:S ratio of untreated cells. G1 refers to the % of cells present in the G1:G0 and S refers to the % of cells present in the S phase of the cell cycle which were obtained from the cell cycle/proliferation assay as described in the legend of Fig 3.5.
3.3. Effects of cisplatin and ionizing radiation on apoptosis in mutant p53 overexpressing lymphoblastoid variants.

When stained with propidium iodide, cells undergoing apoptosis often appear as a subdiploid population, due to the loss of oligonucleotide size DNA fragments by DNase digestion in the early stages of programmed cell death. Others have shown flow cytometry of propidium iodide stained cells to be a quantitative method for assessment of apoptosis (Nicoletti et al., 1991; Zinzani et al., 1994).

Lymphoblastoid mutant p53(246val) transfectants and the control cell lines cultured in the presence of 15% BCS and 33 μM cisplatin for 24 hrs were assayed for the percent of apoptotic cells present in the cell population. In the representative experiment shown in Figure 3.6, approximately 2.5 times fewer apoptotic cells were observed in the mutant p53 bearing cell line population than in the wild type p53 control cell line pRCCMV/pCEP4. The clonally related Bcl-2 overexpressing cell line (bcl-2/pCEP4) exhibited 5 times fewer apoptotic cells and the human DLCL cell line (LY8 C3) - 6.5 times fewer apoptotic cells than the wild type p53 control. Induction of apoptosis by irradiation under similar conditions gave much less reproducible results (data not shown).

To test if the BCS present in the media contributes to the lack of reproducibility the experiment was repeated in the absence of serum. The cell lines were passaged for 24 hrs in media containing 1X B-27 supplement which replaced the serum, and then 33 μM cisplatin was added. 24 hrs later apoptosis was assayed by PI staining (Figure 3.7). Approximately 4-13 times fewer apoptotic cells were observed in the mutant p53(246val) cell line population and 2 times less in the mutant p53(135ser) compared to the wild type p53 control cell line...
pRCCMV/pCEP4. As expected the clonally related bcl-2 overexpressing lymphoblastoid cell line (bcl-2/pCEP4) and the human DLCL cell line exhibit even lower levels of apoptosis upon cisplatin treatment. Irradiation shown by others to induce apoptosis through p53 dependent pathway (Lowe et al., 1993b), similarly resulted in lower levels of apoptosis in the mutant p53 or Bcl-2 expressing cell lines as well as the DLCL cell line. Compared to the wild type control the population of apoptotic cells after irradiation was approximately 4 times less for p53(246val) and 2-3 times less for p53(135ser) expressing cell lines as well as over 20 times less for either Bcl-2-overexpressing variant or DLCL cell line. Other DLCL cell lines showed similar behaviour to that of LY8 C3 although there was noticeable variability between cell cycle profiles of the DLCL cell lines in response to cisplatin or radiation (data not shown). The lymphoma cell lines in general displayed lower basal and induced levels of apoptosis than the mutant p53 variants which we generated likely due to the fact that the former express only mutant p53 protein (i.e. no wild type) in conjunction with other genetic abnormalities.
Figure 3.6. Decrease in cisplatin-induced apoptosis in mutant p53 overexpressing cell lines cultured in the presence of 15% bovine calf serum as measured by cell cycle assay. Control (pRCCMV/pCEP4) cell line, two independent clones of mutant p53-transfected GM607/pRCCMV cells, a Bcl-2 overexpressing control (bcl-2/pCEP4) and a DLCL cell line (LY8 C3) were incubated with 33 μM cisplatin for 24 hrs in 15% BCS-containing media. Cell cycle was assayed by propidium iodide staining of the DNA and by flow cytometric analysis of 10,000 cells. The percent of cells present in the subdiploid population is displayed above each histogram.
Figure 3.7. Decrease in cisplatin or γ-radiation induced apoptosis in mutant p53 overexpressing cell lines cultured in the presence of B-27 supplement as measured by cell cycle assay. Control (pRCCMV/pCEP4) cell line, four independent clones of mutant p53-transfected GM607/pRCCMV cells, a Bcl-2 overexpressing control (bcl-2/pCEP4) and DLCL cell line (LY8C3) after being passaged once in B-27 supplement-containing media, were incubated with 33 μM cisplatin for 24 hrs or treated with 50 Gy of radiation. Cell cycle was assayed 24 hrs later by propidium iodide staining of the DNA and by the flow cytometric analysis of 10,000 cells. The percent of cells present in the subdiploid population is displayed above each histogram.
3.4. Induction of PARP cleavage by cisplatin and γ-radiation in mutant p53 overexpressing lymphoblastoid transfectants and in the lymphoma cell lines.

PARP inactivation by proteolytic cleavage is an early event in apoptosis that is easily detected by western blotting (Casciola-Rosen et al., 1996; Gu et al., 1995). In wild-type p53 expressing cell lines the 85 kDa product of that cleavage was apparent even prior to exposure to cisplatin probably indicating basal levels of apoptosis (Figure 3.8.a and 3.8.b). The intensity of the 85 kDa band started to increase 8 hrs after cisplatin treatment and was accompanied by a decrease in the intensity of the band representing uncleaved 115 kDa protein. As expected this cleavage was markedly reduced in the clonally related variant overexpressing bcl-2 protein (bcl-2/pCEP4; Figure 3.8.c). In all of the mutant p53 expressing transfectants the cleavage product band was considerably less intense than that seen in the parental pRCCMV/pCEP4 clone and only appeared around 24 hrs post-treatment. The background levels of PARP cleavage product were also less intense in the DLCL cell lines than in the lymphoblastoid cell line (Figure 3.8.d). In addition there was considerably less cleavage product at 24 hrs in the DLCL cell lines than in the control lymphoblastoid cell line. Similar results were observed in two independent experiments.

Irradiation with 50 Gy in two independent experiments produced very similar data to those resulting from cisplatin treatment. The cleavage of PARP was clearly apparent by 24 hrs from irradiation only in the wild type p53 cell line (pRCCMV/pCEP4) but completely abrogated in the mutant p53(246val) transfected cell line during the time period tested (Figure 3.9). Similarly the Bcl-2 overexpressing control (bcl-2/pCEP4) as
well as DLCL cell lines (LY8 C3, LY1) show no apparent PARP cleavage at 24 hrs following irradiation.
Figure 3.8. Decreased cleavage of PARP in response to cisplatin in lymphoblastoid cell lines expressing mutant p53. Western blot analysis of PARP cleavage followed over a 24 to 48 hr period after treatment with 33 μM cisplatin. Parental cells (pRCCMV/pCEP4) were compared to two clones carrying the mutant p53(246val) construct (a) and one clone expressing the mutant p53(135ser) construct (b). A lymphoblastoid cell line overexpressing Bcl-2 (bcl-2/pCEP4) (c) and two diffuse large cell lymphoma cell lines known to carry p53 mutations (LY8C3 and LY1) were compared to the lymphoblastoid cell lines (d). Expression of actin was used as a loading control. Cellular equivalent of $10^5$ cells was loaded in each lane.
Figure 3.9. Decreased cleavage of PARP in response to γ-radiation in lymphoblastoid cell lines expressing mutant p53. Western blot analysis of PARP cleavage followed over a 24 hr period after treatment with 50 Gy of γ-radiation. Parental cells (pRCCMV/pCEP4) were compared to two clones carrying the mutant p53(246val) construct. A lymphoblastoid cell line overexpressing Bcl-2 (bcl-2/pCEP4) and two diffuse large cell lymphoma cell lines known to carry p53 mutations (LY8C3 and LY1) were compared to the lymphoblastoid cell lines. Expression of actin was used as a loading control. Cellular equivalent of $10^5$ cells was loaded in each lane.
3.5. Alterations in the levels of p53, Bcl-2 and p21/Waf1 proteins in the mutant p53 transfectants following treatment with cisplatin and γ-radiation.

Cisplatin treatment resulted in substantial increases in p53 protein levels over 48 hrs in both the wild type control (pRCCMV/pCEP4) and three independent mutant p53-transfected lymphoblastoid variants (Figure 3.10.a and b) as well as (surprisingly) over 24 hrs in DLCL cell lines (Figure 3.10.c). In contrast to that found after irradiation (Figure 3.11) only minimal if any induction of p21/Waf1 was seen in wild type clones and no induction was seen in mutant clones and DLCL cell lines. No significant change in Bcl-2 protein levels was observed in any of the cell lines studied. All experiments were repeated twice with similar results.

In spite of somewhat confusing presence of a smaller protein band cross-binding to the p21/Waf1 signal, we concluded that γ-radiation treatment of the wild-type lymphoblastoid control (pRCCMV/pCEP4) resulted in an expected increase of the p53 and p21/Waf1 protein levels assayed over 24 hrs, although no significant change in the levels of bcl-2 protein was seen (Figure 3.11). P21/Waf1 protein levels also increased in the mutant p53(246val)-bearing transfectant but did not reach the same levels up to 24 hrs post-irradiation as in the wild-type control. Similarly to the results with cisplatin, the levels of p53 protein surprisingly increased in both of the mutant p53(246val) transfectants as well as the DLCL cell lines but did not result in induction of p21/Waf1 protein in the DLCL cell lines.
Figure 3.10. Alterations in the expression of p53, Bcl-2 and p21 proteins in the mutant p53-bearing lymphoblastoid cell lines induced by treatment with cisplatin. Western blot analysis of the p53, Bcl-2 and p21/Waf1 protein expression was performed over a 24 or 48 hr period following addition of 33 μM cisplatin. The lymphoblastoid cells bearing pRCCMV and pCEP4 plasmids (pRCCMV/pCEP4) were used as a wild type p53 control and were compared to three independent clones expressing mutant p53: two p53(246val) clones (a) and one p53(135ser) clone (b) as well as two DLCL cell lines (LY8 C3 and LY1) (c). The expression of actin was used as loading control. Cellular equivalent of 10^5 cells was loaded in each lane.
### a) pRCCMV/pCEP4 vs. pRCCMV/p53(246val)c10 vs. pRCCMV/p53(246val)c14

<table>
<thead>
<tr>
<th>Time (hr)</th>
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<th>pRCCMV/p53(246val)c14</th>
</tr>
</thead>
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<tr>
<td>48</td>
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</tbody>
</table>

- **p53**
- **actin**
- **bcl2**
- **p21/Waf1**

### b) pRCCMV/pCEP4 vs. pRCCMV/p53(135ser)c3

<table>
<thead>
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</tr>
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</tbody>
</table>

- **p53**
- **actin**
- **bcl2**
- **p21/Waf1**

### c) pRCCMV/pCEP4 vs. LY8 C3 vs. LY1

<table>
<thead>
<tr>
<th>Time (hr)</th>
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<th>LY8 C3</th>
<th>LY1</th>
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</tbody>
</table>

- **p53**
- **actin**
- **bcl2**
- **p21/Waf1**
Figure 3.11. Alterations in the expression of p53, Bcl2 and p21 proteins in the mutant p53-bearing lymphoblastoid cell lines induced by treatment with γ-irradiation. Western blot analysis of the p53, Bcl-2 and p21/Waf1 protein expression was performed over a 24 hour period following irradiation with 50 Gy. The lymphoblastoid cells bearing pRCCMV and pCEP4 plasmids (pRCCMV/pCEP4) were used as a wild type p53 control and were compared to two independent clones expressing mutant p53(246val) construct as well as two DLCL cell lines (LY8 C3 and LY1). The expression of actin was used as loading control. Cellular equivalent of 10⁵ cells was loaded in each lane.

Because irradiation and cisplatin resulted in an increase in the p53 protein levels in not only the control cell line but also in the mutant p53 transfectants (Figures 3.10 and 3.11) we wished to determine whether in fact mutant p53 protein was induced in the lymphoblastoid variants. Surprisingly, we found that cisplatin treatment induced a 10 fold increase in the levels of transfected mutant p53 RNA within the 24 hr time course as visualized by the increase in the intensity of the lower, 1.8 kb band of transgenic RNA in the mutant p53 bearing cell clones (Figure 3.12 and Table V). A small increase in wild type p53 levels was seen although its quantification was difficult as it was partially masked by the very strong signal of the mutant p53 RNA band.

We recognize limitations to this quantitation assessment. We did not have a titration curve to demonstrate that our values were in the linear range of the readout, nevertheless these results suggest a relative increase in the basal levels of total transgenic p53 RNA within 24 hrs of the exposure to cisplatin.
Figure 3.12. Increase in the level of mutant p53 RNA in the lymphoblastoid cell lines transfected with p53(246val) construct following treatment with cisplatin. Cells were incubated after addition of 33 μM cisplatin for 8 or 24 hours at which time total RNA was isolated. 10 μg of RNA was fractionated by electrophoresis. Arrows are pointing to the wild type (endogenous) and mutant (transgenic) p53 transcripts as well as actin RNA which was used as a loading control.
Table V. The level of transfected mutant p53 RNA in the lymphoblastoid cell lines increases following treatment with cisplatin. Summary of the changes in mutant p53 RNA levels following 33 µM cisplatin treatment of two independent clones of the lymphoblastoid cell lines transfected with p53(246val). The numbers represent intensity of the bands obtained by densitometric analysis where the p53 RNA levels were normalized to the respective actin levels.

<table>
<thead>
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<th>cell line</th>
<th>0</th>
<th>8</th>
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</tr>
</thead>
<tbody>
<tr>
<td>pRCCMV/p53(246val) c10</td>
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<td>0.5</td>
<td>2.2</td>
</tr>
<tr>
<td>pRCCMV/p53(246val) c14</td>
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CHAPTER 4: DISCUSSION

Cisplatin is an important cytotoxic drug used in the treatment of many malignancies including non-Hodgkin's B cell lymphoma. Cisplatin treatment induces apoptosis and there is evidence that this apoptosis may be dependent on a functional p53 tumour suppressor gene (Allday et al., 1995a; Fujiwara et al., 1994). Recently Perego et al. isolated cisplatin resistant mutants of an ovarian carcinoma cell line that were found to have acquired a mutation of p53 (Perego et al., 1996). These mutants however may have gained other genetic aberrations which could have contributed to cisplatin resistance in addition to the p53 mutation. The issue of whether cisplatin can induce apoptosis in cancers with p53 mutations thus remains an important unresolved question with clinical relevance.

4.1. Establishment of the experimental system to study the involvement of p53 protein in cisplatin-induced apoptosis.

To determine the importance of p53 mutations in cisplatin resistance we generated clonally derived variants of a lymphoblastoid cell line that differed only by introduced mutant p53 cDNAs. We expressed separately two different p53 mutations in our system, both of which were previously shown to transform rat embryo fibroblasts in a dominant negative manner (Slingerland & Benchimol, 1991; Slingerland et al., 1991). Our data shows that these mutated p53 proteins were expressed in our clones and that these mutations were indeed functional and dominant. That is, we demonstrated that, as expected, there was a reduction in the G1:S arrest after irradiation in all mutant cell lines tested. Others have shown that G1:S arrest after irradiation is dependent upon intact wild type p53 (Kuerbitz et al., 1992).
In addition we demonstrated that clones expressing these mutant p53 genes underwent less apoptosis than the parental clone in response to irradiation and that there was a diminished induction of p21/Waf1 after irradiation in clones expressing mutant p53.

4.2. Cisplatin-induced apoptosis is dependent on functionally intact p53 gene and its extent varies with the kind of mutation.

Having established the validity of our experimental system we then assessed the response of the various clones to cisplatin exposure. We found that whereas the parental cell line underwent apoptosis in response to cisplatin, there was significantly less apoptosis in the variant lines engineered to express either p53 mutation. To be convinced of the correctness of this observation we measured apoptosis using two different methodologies: propidium iodide (PI) staining for detection of a subdiploid population and cleavage of PARP. Concordant results were obtained using both techniques although the clones expressing p53(246val) mutant were more resistant to cisplatin than the clones expressing the p53(135ser) as measured by PI staining assay. These results clearly demonstrate that apoptosis induced by cisplatin is induced through a p53 pathway and that this apoptosis may be abrogated to some degree in cells harboring mutations of p53. We can thus add genotoxic stress induced by cisplatin to the increasing list of stimuli that induce apoptosis through a p53 pathway (Fujiwara et al., 1994; Graeber et al., 1996; Graeber et al., 1994; Kuerbitz et al., 1992; Lowe et al., 1993a; Lowe et al., 1993b).

These results also suggest that different mutations may interfere with cisplatin activation of the p53 pathway to different degrees possibly because specific mutations may affect dominant negative property of the mutant
protein. Alternatively, our results could be explained by the difference in the level of p53 expression between the two different mutants, where the clone expressing lower levels of p53 also exhibits less resistance to genotoxic treatments. Less resistance to apoptosis was reproducibly seen in the two clones containing the p53(135ser) mutation. This mutation was also less transforming when coexpressed in rat embryo fibroblasts with an activated ras cDNA (Slingerland & Benchimol, 1991). Others have shown that different p53 mutations may have different effects on the p53 pathway. For example, different p53 mutations had varying effects on a colon carcinoma cell line's ability to undergo G1:S arrest (Pocard et al., 1996).

We believe in the validity of our results because we measured apoptosis by two different assays. Several methods are available to identify apoptosis in cell systems and whole tissues. The most common are qualitative techniques such as detection of structural changes by light or electron microscopy which allows an easy discrimination between apoptotic and necrotic cells (Wyllie, 1985). The observation that double stranded DNA is often, but not always, cleaved into oligonucleosomal-length fragments in apoptotic cells has prompted visualization of "DNA ladders" by electrophoretic separation of DNA cleaved at internucleosomal sites (Wyllie et al., 1984) and the development of TUNEL staining (TdT mediated dUTP nick end labelling) of apoptotic cells (Gorczyca et al., 1993). Both microscopic observations and DNA-ladder pattern are difficult to quantitate apoptosis (reviewed by (Goldsworthy et al., 1996)). Analysis of "DNA ladders" because it does not evaluate apoptosis in intact cells, does not allow one to determine proportion of normal versus apoptotic cells. Flow cytometry, one of the assays chosen by us, has been used to assay cell death in numerous experimental systems especially in the cells of the
immune system and has been found an excellent method for discrimination and quantification of apoptosis which is easy to perform and requires few cells for analysis (Darzynkiewicz et al., 1992; Telford et al., 1994). The PI stained subdiploid DNA peak as a result of DNA degradation is one of the characteristics of apoptosis visualized by flow cytometry. Another assay we chose, the detection of PARP cleavage is an ubiquitous event characteristic of late apoptosis, easy to detect and, as we found, of high reproducibility (Gu et al., 1995; Nicoletti et al., 1991). New methods of assessment are however emerging. One of them and with great potential is antibody (Annexin V) mediated recognition of phosphatidylserine uniquely and ubiquitously exposed at the surface of an apoptotic cell (Koopman et al., 1994).

As a result of cell cycle experiments in the presence of bovine calf serum or replacing the serum with B-27 supplement we concluded that serum may interfere with the outcome of induction of apoptosis under certain conditions. This result was not completely unexpected as serum contains known and unknown cytokines and other growth factors which could influence the signaling in the apoptotic pathway. This is further substantiated by a recent discovery of a factor present in fetal calf serum, capable of suppressing Fas-mediated apoptosis in a T cell line (Kikuchi et al., 1995). Another group reported that the presence of even low concentration of fetal or bovine calf serum in the growth media of neuronal cell line prevented apoptosis (Budhram-Mahadeo et al., 1994). Our experiments suggest that the use of B-27 may contribute to the reproducibility and clarity of the results of apoptosis assays but it should be tested in other cell cycle and apoptosis assays.
4.3. Increase in mutant p53 RNA levels in response to cisplatin.

An unexpected observation resulting from our investigations was that p53 protein levels increased after irradiation and cisplatin treatment in the mutant lymphoblastoid clones as well as in the wild type clone. Similar results were observed following DNA-damaging insults using three DLCL cell lines expressing only mutated p53. To determine whether wild type or mutant p53 protein was being induced in the lymphoblastoid transfectants, we assessed the mRNA levels of one of the mutant clones, namely p53(246val) after treatment with cisplatin. We found that cisplatin induced significant increases in the transfected mutant p53 mRNA. Because the mutant p53 cDNAs were being expressed by a strong viral promoter (CMV) which is not known to be responsive to DNA damage inducing ionizing radiation (Cheng & Iliakis, 1995), it is likely that RNA stabilization was accounting for the increased steady state levels of the mutant p53 RNA. The validity of that assumption, however should be tested by confirming that this vector is not cisplatin-inducible. The increase in the transgenic p53 mutant RNA level may be responsible for the increase in the p53 protein levels although this correlation should be further investigated.

Increased p53 protein levels in response to genotoxic stress have been shown to be mostly related to translational mechanisms (Kastan et al., 1991; Maltzman & Czyzyk, 1984) although enhanced transcription has also been suggested (Kastan et al., 1991; Kondo et al., 1996; Voelkerding et al., 1995). Further experiments to better understand the role of RNA stabilization in the regulation of wild type and mutant p53 RNA are underway. However, these preliminary experiments suggest that the detrimental effects of functionally altered p53 could be further exaggerated
by increasing mutant p53 levels in response to genotoxic stress such as cisplatin.

4.4. P53-mediated control of cell cycle and apoptosis-regulating genes.

Whereas p21/Waf1 protein levels increased in the wild type lymphoblastoid clone after irradiation we did not detect an increase in p21/Waf1 after cisplatin treatment. This is contrary to previous reports where cisplatin induced p21/Waf1 protein within six hours in human foreskin fibroblasts (Hawkins et al., 1996). This occurred despite a significant increase in induction of p53 protein after cisplatin treatment. The biochemical explanation for the lack of p21/Waf1 induction in response to cisplatin is unclear but possibly cisplatin treatment favours p53 mediated apoptosis and the increase in p21/Waf1 protein levels is too low to be detected in our system or G1:S arrest is induced by a p21 independent mechanism.

Contrary to a report of a p53-driven control of expression of the bcl-2 gene in murine leukemia cell line (Miyashita et al., 1994) but in agreement with another study performed in murine bone marrow-derived cytokine-dependent cells (Canman et al., 1995), we did not observe down-regulation of bcl-2 protein associated with increased p53 levels following DNA damaging treatments. Neither do we see increased expression levels of bcl-2 in the cells bearing mutant p53. Since another study reported no change in the levels of bcl-2 in lymphoblastoid cell lines exposed to cisplatin (Allday et al., 1995a) these discrepancies are clearly modelspecific. It is possible that the use of temperature-inducible p53 construct in
the murine system triggers different response pathways than the DNA damaging treatments.

4.5. Possible existence of RNA sequences positively regulating RNA stability.

Our attempts to express four other p53 mutant constructs in the lymphoblastoid system proved unsuccessful. The transient transfection assay suggested that our inability to isolate variants expressing those constructs was not simply due to their detrimental effects on the cells that may bear them, as this assay was performed within 24 hrs from the transfection. Perhaps the answer lies in the size differences of the mutant p53 cDNAs (see Figure 4). It may not be a coincidence that the p53 mutant cDNAs which we were able to express were both of the same size (approximately 1.8 kb) while the others were all reduced in length (1.3 kb). It is possible that a part of the cDNA is missing which is vital to either the stability of the transcript or the event of transcription itself. The existence of RNA stabilizing sequences has been suggested previously in other cell systems (reviewed by (Wickens, 1993)). One such example is the stem-loop forming motif of the iron response element (IRE) found in the 3' UTR of transferrin receptor mRNA, which controls RNA stability by providing a binding site for an IRE-binding protein (reviewed by (Klausner et al., 1993). We performed a preliminary sequence analysis on the 3' UTR segment missing from the mutant p53 constructs which we were unable to express, using a GCG/Wisconsin package analysis software. This analysis revealed presence of numerous motifs which potentially could form stem loop structures (data not shown) and numerous binding sites for
various proteins. Further and more specific analysis of that segment should be performed to clarify this issue.

4.6. Conclusions.

In conclusion we have shown that cisplatin induces G1:S arrest and apoptosis in human lymphoblastoid cell lines and that these effects are dependent on a functional p53 tumour suppressor gene. Lymphoblastoid cell lines expressing mutant p53 protein undergo less G1:S arrest and apoptosis that clonally related wild type p53 cell lines. Moreover our preliminary data encourage further investigation of the effect of cisplatin on the mutant and wild type p53 protein and RNA.

4.7. Future directions.

The experimental system we devised could be used in studies of development of lymphoid malignancies and a possible function of cooperation between p53 and various protooncogenes. The lymphoid and non-transformed nature of the cell line will likely yield more biologically and possibly clinically relevant observations than studies using the common target cell lines frequently employed in transfection assays (e.g. REF, NIH 3T3 etc.). Our system would also allow further studies on mechanisms and biochemistry of apoptosis.

The issue of p53 RNA stabilization is an interesting one and should be investigated starting with direct sequence comparisons between the six mutant p53 cDNA constructs. The DNA fragments missing from the constructs we were unable to express should be assayed for the presence of important protein binding sites or secondary structure formation which may be implicated in RNA transcription or stabilization. A deletional
analysis of this part of 5'UTR would likely provide answers to the question of the involvement of this region in the expression of p53. Results of such investigation may also clarify the role of transcriptional control mechanisms in the induction of p53 and could uncover novel mechanisms of RNA stabilization and the importance of these mechanisms in regulation of mutant p53 molecules. The latter is an issue of utmost clinical importance and may suggest strategies to overcome mutant p53 expression and concomitant resistance to cytotoxic agents in lymphoid and other malignancies.

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