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Abstract:

Using ligation mediated PCR genomic footprinting we observed striking p53 dependent protection of the mdm2 gene's putative p53 responce elements (REs) as well as the adjacent TATA box. Interestingly this protection pattern differed considerably from that observed with purified p53 on naked DNA. Indirect Southern blot analysis revealed constitutive nuclease-hypersensitivity at both the p53 independent (P1) and P2 promoters, indicating that both promoters are located in altered chromatin conformations that are most likely nucleosome free.

We are also actively pursuing control of p53 function throughout the cell cycle. We carry out these studies using centrifugal elutriation of ML-1 cells in order to separate the cell cycle populations. We have determined that p53 resembles cyclins in that in exponentially growing ML-1 cells p53 is present only during discrete cell cycle time points. In healthy cells the p53 level peaks at the G1/S border, however after treatment of the cells with camptothecin a surprising increase of p53 occurs only during G1 and G2/M while no increase is obseved during the S phase of the cell cycle. Interestingly, only the activated p53 in the G2/M cell cycle fractions is able to bind to the gadd45 p53 DNA binding site.

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J Bargonetti 7/11/97

PI - Signature Date

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Introduction:

The purpose of this work is to determine if different potential p53 DNA binding sites in nuclear chromatin are bound by different forms of wild-type p53 that may exist in normal and breast cancer cells; and to see if this DNA binding by p53 occurs at different times during the cell cycle. We are also working to determine if a direct DNA-mutant p53 protein interaction can occur in nuclear chromatin and if such an interaction is involved in the ability of p53 His273 to activate HIV-LTR driven transcription and activate latent HIV replication (Deb et al. 1994)(Duan et al. 1994) as well as activate transcription from a number of cellular genes (Chin et al. 1992)(Deb et al. 1992)(Dittmer et al. 1993)(Subler et al. 1994).

All previous experiments examining the DNA binding properties of p53 proteins have been carried out *in vitro*, on naked DNA, with purified p53 protein produced either in bacteria or insect cells. Experiments with mammalian derived p53 proteins on the other hand have made use of crude cell extracts. We are studying the ability of wild-type and mutant p53 proteins to interact with DNA sequences (known to be bound by wild-type p53 in cell free systems) in nuclear chromatin. The status of p53 in many breast cancer cell lines has been identified, and utilizing these lines will facilitate our study as well as generate potentially useful information for the treatment of breast cancer.

A revised statement of work for the grant proposal was accepted in August of 1996. This is the statement of work addressed in this progress report.

Background:

Biological functions of wild-type and mutant p53

The p53 gene encodes a protein which can function to suppress progression through the cell cycle in response to DNA damage (Ko and Prives 1996)(Hartwell 1992)(Lane 1992). It is present in minute concentrations in normal cells and tissues while being maintained at high levels in tumors and tumor cell lines due to post-translational stabilization (Oren et al. 1981). The p53 gene product is a phosphoprotein that is usually found in the nucleus although at times it is also found in the cytoplasm (Rotter et al. 1983) (Davis and Wynford-Thomas 1986)(Gannon and Lane 1987). p53 is not essential for viability of tumor cells as some tumor cell lines contain no functional p53 protein (Gannon and Lane 1987). Mice deficient for p53 develop normally but are susceptible to tumor formation within a short time period (Donehower et al. 1992). Studies on human tumors derived from colon, breast, brain, bone, and lung tissue, along with some studies on rodent cell transformation, suggest that the wild-type p53 gene product functions as a suppressor of neoplastic

growth, and that have demonstrated that mutation, deletion or both of the wild-type gene can inactivate the gene products ability to carry out this suppression ((Baker et al. 1990) and references within). Furthermore, several oncogenically transforming viruses have specific mechanisms to inactivate p53 function, strongly suggesting that inactivation of p53 is critical for efficient viral replication and efficient cell growth (Lane and Crawford 1979)(Linzer and Levine 1979)(Scheffner et al. 1990)(Yew and Berk 1992). Mutations in p53 can cause cells to become oncogenically transformed (reviewed in (Yew and Berk 1992)(Levine et al. 1991)&(Zambetti and Levine 1993)) and mutant p53, in cooperation with ras, can transform cells. Consistent with its role as a tumor suppressor, wild-type p53 suppresses the oncogenicity of mutant p53 plus ras in focus forming assays (Eliyahu et al. 1989) (Finlay et al. 1989). Quantitative improvements of transformation frequencies (Hinds et al. 1989) (Dittmer et al. 1993) are associated with the higher expression levels of mutant p53 protein, implying that such proteins altered amino acids impart a gain of function. It is interesting to note that the p53 gene has been conserved during evolution as demonstrated by the comparison of human, rat, mouse, frog, chicken, and bony fish cDNA clones which reveal five blocks of highly conserved sequence within the coding region (Soussi et al. 1990). Importantly, the point mutations found in several forms of human cancer (e.g. breast, lung, colon) occur predominantly in these conserved regions (Baker et al. 1990).

Wild-type p53 binds to DNA and regulates transcription

The p53 tumor suppressor protein can function as a checkpoint factor (Hartwell 1992) causing cells exposed to DNA damaging agents to arrest in G1 (Hall et al. 1993)(Kastan et al. 1991). The normal function of p53 is regulated, at least in part, by the ability of the protein to bind site-specifically to DNA (Vogelstein and Kinzler 1992). The tumor-derived mutant p53 proteins that have been tested thus far have altered, or deficient DNA binding activity (Bargonetti et al. 1991)(Chen et al. 1993)(Kern et al. 1991)(Kern et al. 1991), however some oncogenic p53 mutants can bind to DNA at temperatures lower than 37°C (Bargonetti et al. 1993)(Zhang et al. 1994) or to idealized p53 binding sites (Hupp et al. 1993)(Zhang et al. 1993). Wild-type p53 binds nonspecifically to DNA (Kern et al. 1991) (Steinmeyer and Deppert 1988) as well as specifically to diverse DNA sequences that contain two adjacent copies of the consensus sequence 5'-Pu Pu Pu C (A/T) (T/A) G Py Py Py-3' (el-Deiry et al. 1992)(Funk et al. 1992).

In response to DNA damage nuclear p53 levels rise (Kastan et al. 1991). p53 induced growth arrest or apoptosis can be brought about in part by specific activation of one or more genes. Some of the responsive genes in which p53 binding sites have been identified include the oncogene mouse double minute 2 (mdm2) (Barak et al. 1993)(Juven et al. 1993)(Perry et al. 1993), growth arrest and DNA damage 45 (gadd45) (Hollander et al. 1993)(Kastan et al. 1992) (Zhan et al. 1993),

p21/waf1/cip1 (el-Deiry et al. 1993) and bax (Miyashita and Reed 1995). The product of the p21/waf1/cip gene is a potent cyclin dependent kinase inhibitor. This presumably accounts for the ability of wild-type p53 to arrest cell cycle progression at the G1/S border before activation of the cyclin regulated p34 cdk2 kinase (el-Deiry et al. 1993)(Harper et al. 1993). Additionally, activation of gadd45 (which is part of the DNA damage response pathway) may help to signal cells to growth arrest until DNA repair has taken place. On the other hand activation of the bax gene is involved in the apoptotic signaling pathway (Miyashita et al. 1994)(Miyashita and Reed 1995). The activation of mdm2 by p53 adds another level of complexity to the issue due to the fact that the mdm2 protein product binds to p53 and blocks p53 mediated transactivation in cell cycle checkpoint pathways (Chen et al. 1994) and thus when overexpressed functions as an oncogene (Oliner et al. 1993). Additionally overexpression of mdm2 in cancer cells is achieved by different mechanisms which include increased transcription (Sheikh et al. 1993), enhanced translation (Landers et al. 1994)(He et al. 1994), gene amplification (Oliner et al. 1992)(Refenberger et al. 1993) and gene rearrangements (Leach et al. 1993).

Differential occupancy of consensus binding sites in chromatin

While some transcription factors, like GAL4 (Taylor et al. 1991) and Sp1 (Li et al. 1994), can bind specific DNA sites within a nucleosome core, others like NF1 and heat shock factor, are unable to interact with some specific DNA binding elements when the nucleosome core is assembled (Archer et al. 1991)(Pina et al. 1990). In fact it is a presupposition that in general, nucleosomes positioned over promoters are inhibitory to other proteins binding, however it has been demonstrated that this is not always the case (Reviewed in (Workman and Buchman 1993)). Therefore, it has been suggested that many genes are programmed during DNA replication while the nuclear chromatin assembles (Workman et al. 1990). If there are limiting transcription factors available in a cell then a gene that is replicated early in S-phase has more opportunity to assemble an active transcription complex than a gene that replicates late. This is because a gene that replicates early may be available for transcription factors to bind before all the early replicating portion of the genome has sequestered these factors. Additionally, transcriptional activators can stimulate eukaryotic DNA replication by modifying the outcome of the competition between initiator factors and histones for occupancy of the replication origin (Cheng et al. 1992). Interestingly the RGC site is positioned near an origin of replication (Kern et al. 1991). Finally, mutant p53 can localize to the nucleus of cells and has been observed to interact directly with nuclear matrix attachment region (MAR) sequences (Muller et al. 1996). MARs are able to organize cellular chromatin into topologically independent loops (reviewed in (Boulikas 1995)). Therefore it has been proposed by

Muller, Paulsen and Deppert that MAR-mutant p53 interactions may be involved in the mutant p53 gain of function (Muller et al. 1996).

Wild-type p53 is present in low levels in normal cells (Oren et al. 1981). Therefore it may be important for p53 to belong to the class of transcription factors that organize nucleosome structures in order to help define the p53 DNA binding sites that become active enhancer elements (McPherson et al. 1993). With this possibility in mind one could postulate that the p53 protein might bind to specific sites during S-phase, or that because the wild-type p53 protein has a short half life it may have to bind immediately in the presence of nucleosomes and therefore may only bind to sites that have nucleosome cores positioned in a particular way. However it is also possible that p53 binding sites exist in different chromatin states over the course of the cell cycle (as is the case for yeast replication origins which bind various transcription factors(Diffley et al. 1994) (Dowell et al. 1994)), and that dependent on the sequence context, p53 may choose a specific function to carry out. Additionally, if p53 is a member of the class of activators which is blocked by the presence of nucleosomes, perhaps oncogenic mutation of the protein (and/or complex formation with other proteins) may confer, on p53, the ability to bind sites that the protein normally finds inaccessible. Mutations in p53 might change the spectrum of growth-control genes that the protein activates. For example the mutant p53 proteins binding to MARs and mutant p53 complexed with Sp1 may enable mutant p53 to bind to chromatin within a nucleosome core particle.

Year Three with a DOD Career Development Award:

Upon completion of my first year as a new investigator I recognized, in retrospect, that my statement of work (SOW) for the current project omitted many of the tasks that had to be carried out in order to get the lab up and running. The new statement of work (which was accepted) follows.

Revised Statement of Work Accepted 1996

The in vivo DNA Binding Properties of Wild-type and Mutant p53 Proteins During the Course of Cell Cycle

New Task 1:

Set up a new laboratory. Months 1-12.

a. Order equipment and reagents to supply an empty laboratory.

b. Recruit and train four students.

c. Begin tissue culture facility operation and characterizing breast cancer cell lines growth conditions.

d. Set up DNaseI sensitivity and *in vivo* footprinting conditions.

e. Purification of p53 from baculovirus infected insect cells.

New Task 2 = revision of old task 1:

Establish stable breast cancer cell lines expressing temperature sensitive p53 (ts-p53val135) while working with a murine established ts-p53val135 line. Months 7-36:

a. Examine p53 level and sub-cellular localization in the established murine cell line at both the p53 permissive and restrictive temperatures. Test ts p53 effects by Northern Blotting.

b. Attempts will be made to establish ts-p53val135 expressing breast cancer cell lines of MDA-MB 468, ZR 75, MDA-MB 157, MDA-MB 361 and MCF10A.

c. Levels of p53 in the established breast cell lines will be examined both at the p53 permissive and restrictive temperatures. Test ts p53 effects by transient transfection.

New Task 3 = revised task 2:

Footprinting of the MDM-2 and GADD45 p53 DNA binding sites in nuclear chromatin of unelutriated cells and on naked DNA. Months 12-36:

a. The p53 responsive regions of the MDM-2 and GADD45 genes will be obtained from laboratories that have published the clones.

b. Gel shift analysis of p53 binding elements with nuclear and cytoplasmic extracts.

c. Southern blot using the using the murine ts p53val135 cell line to set up the system and test chromatin structure of the gene.

d. Footprinting of mdm-2 and gadd45 binding sequences in chromatin and with immunopurified p53.

e. In vivo footprinting of the MDM-2 and GADD45 p53 binding regions will be carried out in breast cell lines with and without ts-p53val135, and also on the cell lines treated with chemotherapeutic agents.

New Task 4 = revised old task 3:

***In vivo* DNA footprinting of synchronous populations of hematopoietic and breast derived cell lines. Months 12-24.**

a. Synchronous populations of the various hematopoietic and breast cell lines will be prepared by both centrifugal elutriation and drug treatment.

b. Intranuclear footprinting on the synchronous populations of cells will be carried out.

New Task 5: This task addresses a similar to question to that of the old task 4, however we will focus on one potential binding site for mutant p53 rather than searching for many. Months 20-48.

Test to see if mutant p53 His273 is able to bind to the HIV-LTR region when it is transiently transfected into the cell line MDA-MB-468.

a. Examine HIV-LTR driven transcription in breast cell lines containing different status p53 protein.

b. Footprinting of the HIV-LTR region in the nuclei of breast cell lines containing different status p53 protein in both the absence and presence of chemotherapeutic drug treatment.

c. Comparison of the proteins from different status p53 breast cell extract bound to the HIV-LTR region.

Task 6 = old task 5:

Studies on the affect of p53 on the DNA replication of the double minute chromosomes in the breast cancer cell line MDA-MB 361. Months 12-48.

a. Examine the level of MDM-2 gene amplification level in MDA-MB 361 cell lines that express ts-p53val135, both at the permissive and restrictive temperatures.

b. Make and analyze MDA-MB 361 cell fusions with normal breast cells.

c. Carry out in vivo footprinting on synchronous populations of the above cell types.

Materials and Methods:

Isolation of Protein from mammalian cell culture lines:

Whole cell lysates: Wash 100 mM plate 2X with ice cold PBS
Extraction of cells on a 150 mm dish will be lysed with 2 ml of whole cell lysis buffer: (O.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 50 mM NaCl, 25 mM Tris.HCl (pH 7.5), 1 mM PMSF, 50 ug/ml aprotinin, 50 uM leupeptin) leave for 15 min. on ice. Scrape cells and put into tube. Lysates should be spun at 15,000 rpm for 15 min., and the supernatant saved.

Nuclear Extract Preparation: Adherent cells nuclear extract was prepared as follows. Wash cells 2X with cold PBS. Add 2 ml of Lysis Buffer per Plate. Spin 2300 rpm for 5 min. - (Save the supernatant for cytoplasmic extract). Resuspend the pellet in 1.5 ml of nuclear extraction buffer. Transfer to an eppendorf tube and rock at 4°C for 60 min. Spin 10 min. in microfuge in cold room.
Lysis buffer stock: 20 mM Hepes, pH 7.5, 20% Glycerol, 10 mM NaCl, 1.5 mM Mg Cl_2, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 50 ug/ml aprotinin, 50 uM leupeptin.
Lysis Buffer: 8.8 ml Stock and 6.2 ml water. **Extraction Buffer:** 1.47 ml 5M NaCl, 8.8 ml of Stock, and 4.7 ml of water.
The procedure for the suspension ML-1 cells was adapted from (Kastan et al. 1992). The nuclei were lysed in 3 ml of nuclear lysis buffer for 1.0 x 10^9 and passed for 3 strokes through a 25 gauge needle.

Immunoprecipitation from cell extracts and Western Blot Analysis:

Normalize for total protein in the cell extract as indicated. Protein concentrations were determined using the Biorad assay reagent. Add 30-40 ul of 50% beads coupled to PAb421 to the extract. Rock 2-20 hours at 4°C in the cold room. Spin 5 minutes in the cold room. Wash beads 4X with 1 ml of RIPA buffer (150 mM $NaCl_2$, 50 mM Tris pH 7.2, 1% NP 40, 2% Na deoxycholate and 0.1% SDS). The beads were resuspended in 1X protein sample buffer and boiled. Samples were electrophoresed on a 15% SDS-PAGE and electrotransferred to nitrocellulose. The blot was probed a mixture of p53 monoclonal antibodies PAb240, PAb1801 and PAb421 and the signal was visualized after incubation with goat anti-mouse second antibody by developing with ECL solutions (Amersham).

Electroporation of Breast Cell Lines:

MDA-MB-157 and MDA-MB-468 cells are grown in DMEM with 10% fetal calf serum. ZR75-1 cells are grown in RPMI 1640 with 10% fetal calf serum. Cells were washed in medium without serum and harvested with trypsin. Electroporation at .23kV was carried out with 0.5 x $10^{(6)}$ cells/pulse in medium without serum. The amount of DNA was varied from 1 to 20 ug and selection for the different cell lines was carried out at the lowest G418 concentration determined to kill all the cells of a control un-electroporated plate.

Electrophoretic Mobility Shift Assay (EMSA):

Nuclear extracts were prepared as described above. EMSA was carried out using 0.1 pmoles of radio-labeled oligonucleotide (either a p53 super consensus sequence (SCS) binding site oligonucleotide, the DNA binding site from the gadd45 gene (gadd45), the Sp1 binding sites form the HIV-LTR (HIV) or a mutated p53 binding site oligonucleotide (Mut) and 2 ug of nuclear extract from either MDA-MB-468 or ZR75-1 cell lines maintained at 37°C. The DNA binding reactions were carried out at room temperature for 20 minutes in a 30µl volume containing 20 mM Hepes(pH 7.8), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 1 µg of sheered salmon sperm DNA and 10% glycerol in the presence of PAb 421 antibody.

Extraction of Nuclei and DNaseI Treatment and *in vivo* Footprinting:

This protocol was adapted from (McPherson et al. 1993)Cells were grown on 150 mM plates to no more than 80% confluence before shifting the temperature using 10 plates per analysis sample. Wash plates with ice cold PBS 2X. Spin down at low speed at 4°C (2500 rpm in SS34 rotor) for 10 min. Resuspend in 2 ml of RSB with PMSF. Homogenize 20 strokes and check for trypan blue exclusion. Spin down at 4000 rpm 4°C, 10 min.
Wash nuclei in 2 ml RSB with out PMSF 1X. Resuspend in any where from 1 ml to 4 ml of RSB w/o PMSF (make the lowest # of nuclei in 1 ml for 4 tubes at 250 ul per tube the rest to accordingly making sure to normalize for number of cells i.e.. estimate number of cells from confluence and count in hemocytometer). Set up reactions for DNaseI treatment.
RSB: 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl2 pH 7.4, 0.5% NP40, 1 mM PMSF or absent. Treat with DNaseI for 10 min. at 37°C, using 250 ul of nuclei in 0.1 mM $CaCl_2$. Add 250 ul DNaseI stop (with out DNA) and then add proteinase K to a final concentration of 400 ug/ml and digest overnight at 37°C. Extract once with phenol and 3X with chloroform. **DNaseI stop:** 2M NH4 OAc, 100 mM EDTA, 0.2% SDS. **Southern Blot Analysis** was carried out using the

standard technique described in Protocols of Molecular Biology. **Ligation Mediated PCR** was carried on the DNA samples as described by Mueller and Wold (Mueller and Wold 1989)

Centrifugal Elutriation of ML-1 and MANCA cells:

Centrifugal elutriation was carried as previously described for the MANCA cell line (Koff et al. 1992)(Soos et al. 1996). Elutriation of ML-1 cells has not been published, but the conditions were similar to those used for MANCA cells with the exception that the cells took a little longer to pump out because they appear to be larger.

Results:

Task #2: Establish stable breast cancer lines expressing temperature sensitive p53 (ts-p53val135) while working with a murine established ts-p53val135 line.

Task #2 a. Examine p53 level and sub-cellular localization in the established murine cell line at both the p53 permissive and restrictive temperatures. Test p53 effects by Northern Blotting.

Completed as described in the 1996 progress report.

Task #2 b. Attempts will be made to establish ts-p53val135 expressing breast cancer cell lines of MDA-MB 468, ZR 75, MDA-MB 157, MDA-MB 361 and MCF10A.

The temperature sensitive characteristics for the ts-p53 Val135 plasmid construct have been shown to behave in the same manner in human cells (Yonish-Rouach et al. 1991), therefore I assumed that screening the stable breast cancer cell lines with the ts-p53val135 selected for G418 resistance could be done by comparing the cell growth curves at the two temperatures. Unfortunately, the effect of a temperature shift down on the breast cancer cell lines MDA-MB-468 (containing p53 His273), ZR 75-1 (containing wild-type p53) and MDA-MB-157 (containing no p53 due to deletion of the endogenous gene) revealed severe growth retardation at 32°C (data was shown in 1995 progress report). All three cell lines can grow if shifted back to 37°C demonstrating that the cells are still alive, thus we concluded that we could continue trying to make the stable cell lines however screening for growth retardation can not be a primary screen for the G418 selected colonies. Therefore, we have had to test all G418 selected colonies for the level of p53 in order to score for positive colonies.

G418 resistant transfected colonies of MDA-MB 468 and ZR75-1 with p53 Val135 have been selected. 1 x 10^7 cells were transfected by electroporation at 350V with various amounts of DNA and selected with 200 mg/ml of G418. A total of 40 different clones were picked from two independent transfections with 10 mg of supercoiled plasmid encoding resistant to Neomycin and 10 mg of supercoiled plasmid containing the temperature sensitive p53 Val-135 gene. 10 clones were picked from cells transfected with 10 mg of supercoiled plasmid encoding resistant to Neomycin only. The results of screening this process are described below. We have been unable to select any MDA-MB-157 G418 resistant colonies. The MDA-MB-157 cell line appears to be more a more sensitive cell line that experiences more cell death in response to the electroporation procedure.

We will continue to trouble shoot the possible reasons why we have been unable to select this cell line.

Task #2 c: Levels of p53 in the established cell lines will be examined both at the p53 permissive and restrictive temperatures.

In order to determine if the G418 resistant colonies expressed ts p53 Val135, Western blotting with p53 specific antibodies was carried out. Nuclear extract from the 3-4 cell line containing p53 Val-135 cell line was used as a size control. Whole cell extracts from the breast cancer cells described above were normalized at a level of 20 ug of total protein per lane. The protein was analyzed by SDS-PAGE and Western blotting with a mixture of p53 monoclonal antibodies PAb421, PAb240 and PAb1801 (all of which were from the growth medium of monoclonal antibody cells). The protein bands were then visualized, after second antibody treatment, with ECL reagent from Amersham. The number of colonies screened in this way were described above. One strong ts p53 containing positive clone of MDA-MB-468 has been isolated and called 8-9 (Figure 1, lane 3); two strong ts p53 containing positive clones of ZR75-1 have been isolated and are called 1-21 and 1-18 (Figure 1, lane 8 and 9). These stable ts p53 expressing breast cancer cell line clones will enable us to analyze how wild-type and mutant p53 influence each others ability to interact with DNA sequences and co-associated proteins.

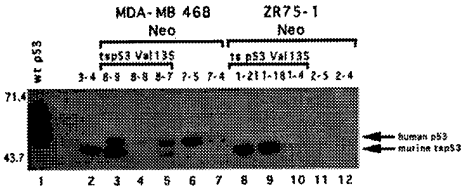

Figure 1: Stable ts p53-Val 135 clones derived from breast cancer cell line MDA-MB-468 and ZR75-1. *Whole cell lysates were prepared from the indicated cell lines and 20 ug of total protein was resolved on a 10% SDS-PAGE. The presence of p53 was detected by Western blotting with a mixture of p53 antibodies PAb 421, PAb 1801 and PAb 240. Lane 1 contains purified human wt p53. Extract from various cells lines was loaded as follows: lane 2, 3-4 (a ts p53 Val-135 stable clone derived from a mouse fibroblast cell line expressing no p53) used as a*

positive control for the size of ts p53-Val 135; lanes 3-5, lysate from MDA-MB-468 clones selected after co-transfection with a plasmid expressing ts p53 Val 135 and a plasmid expressing neomycin resistance; lanes 6-7, lysate from MDA-MB-468 clones selected after transfection with only the plasmid conferring neomycin resistance; lanes 8-10, lysate from ZR75-1 clones selected after co-transfection as above; lanes 11-12, lysate from ZR75-1 clones selected after transfection with only the plasmid conferring neomycin resistance.

Task #3 Footprinting of the MDM-2 and GADD45 p53 DNA binding sites in nuclear chromatin of unelutriated cells and on naked DNA.

Task #3 a: The p53 responsive regions of the mdm-2 and gadd45 genes will be obtained from laboratories that have published the clones.

Genomic clones of murine mdm2, human mdm2 and human gadd45 have generously been provided by Dr. Donna George (Juven et al. 1993), Dr. Moshe Oren (Zauberman et al. 1995) and Dr. Albert Fornace (Hollander et al. 1993).

Task #3 b: Gel shift analysis of p53 binding elements with nuclear and cytoplasmic extracts.

It has been demonstrated that oncogenic p53 mutants are not equivalent to functional knockouts of wtp53. Many mutant p53 proteins have de novo functions which have been categorized as oncogenic functional gains. It has not been elucidated how this gain of function occurs. One of our long term goals is to determine how mutant p53 can contribute to oncogenic transformation of cells. There is considerable data suggesting that the function of mutant p53 is promoter context dependent. This implies that mutant p53 may interact directly with the DNA as well as interacting with other factors. Mutant p53 can localize to the nucleus of cells and has been observed to interact directly with nuclear matrix attachment region (MAR) sequences. We have preliminary data which clearly demonstrates that mutant p53 His 273 present in the breast cancer cell line MDA-MB-468 binds site specifically to the p53 super consensus sequence (SCS) (Figure 2). Per ug of total nuclear extract protein more p53 binding was obtained in the mutant p53 containing cell line MDA-MB-468 then in the wild-type p53 containing cell line ZR75-1; in fact the p53 binding activity from the ZR75-1 extract was barely detectable.

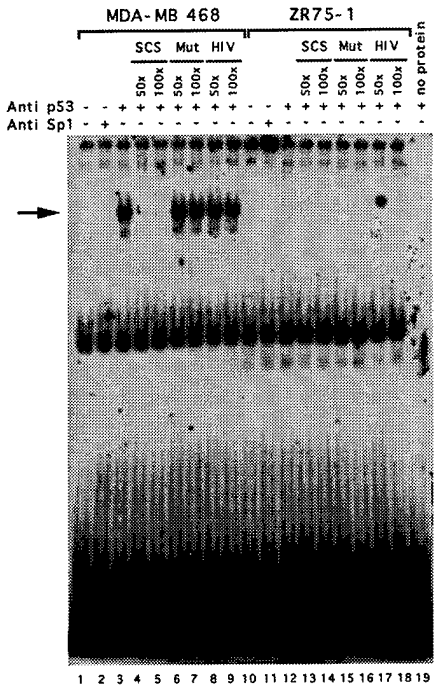

Figure 2: Sequence specific DNA binding activity of mutant p53 His 273 from the breast cancer cell lines MDA-MB-468 and ZR75-1. *EMSA was carried out using 0.15 pmoles of a p53 super consensus sequence (SCS) binding site oligonucleotide and 2 ug of nuclear extract from either MDA-MB-468 or ZR75-1 cell lines maintained at 37°C. The DNA binding reactions were carried out at room temperature for 20 minutes in a 30µl volume containing 20 mM Hepes(pH 7.8), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 1 µg of sheered salmon sperm DNA and 10% glycerol in the presence of PAb 421 antibody. Non-radioactive competitor oligonucleotide was added in 50 or 100 fold excess where indicated. SCS refers to the same p53 super consensus binding site that is ^{32}P labeled; Mut refers to an oligonucleotide that is unable to bind p53; HIV refers to an oligonucleotide containing the three Sp1 binding sites from the HIV-LTR. 20µl of each reaction mixture was resolved on a 4% acrylamide gel at 200V at 4°C. * indicates the super shift and increased binding induced by the anti-p53 antibody PAb 421.*

Activation of p53 in ML-1 and MANCA cells by DNA damaging drugs:

p53 is activated in response to DNA damage with p53 levels rising, resulting in activation of p53 target genes. DNA damaging agents increase the level of p53 in ML-1 cells (Nelson and Kastan 1994). We tested to see if two DNA damaging agents, camptothecin and zeocin, were able to increase the level of p53 protein present in the MANCA and ML-1 cells and if the p53 target genes p21/Waf1/CIP1 and GADD45 were turned on as well. Zeocin is a drug available from *invitrogen* and it acts as a direct DNA damaging agent (similar to the action of bleomycin). Interestingly we saw that both drugs were able to increase the level of p53 protein present in both the MANCA and ML-1 cells as visualized by SDS-PAGE of immunoprecipitated p53 (Figure 3B). The drug treatment with camptothecin increased the p53 levels to a greater extent than did drug treatment with

zeocin. We determined the level of p21/Waf1/CIP1 and GADD45 mRNA in the two cell lines under the identical drug treatment conditions and found that camptothecin treatment of ML-1 cells induced both p21 and GADD45 expression while zeocin only induce p21 expression (Figure 3A). However in the MANCA cells treated with drug the only activation of gene expression that resulted was increased expression of GADD45 upon camptothecin treatment. Additionally preliminary data obtained from testing of MANCA cell p53 indicates that although it does not activate p21 transcription it is in a wild-type conformation as assayed by immunoprecipitation with the mutant p53 conformation specific antibody (PAb240) (Figure 3C). We are interested in how p53 is involved in gene regulation in response to DNA damage and have determined that we should begin our studies with the ML-1 cell line and may later be able to compare it to MANCA cells for information about possible upstream signals that control p53 function.

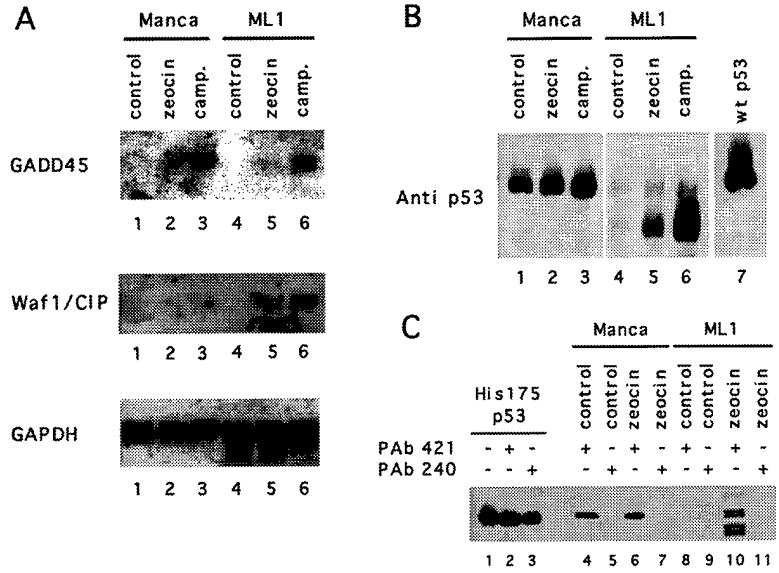

Figure 3: ML-1 and MANCA cells after treatment with DNA damaging agents. *Cells were incubated for 6 hours either with 20 uM Camptothecin or 50 ug/ml Zeocin.* ***A)*** *After treatment samples were placed on ice for 10 minutes and total cytoplasmic RNA was extracted using phenol/chloroform method and ethanol precipitated. Poly (A+) mRNA was isolated using oligo-dT spin column kit from 5'-->3' company. Two mg of Poly (A+) mRNA were electrophorised on 1% agarose denaturating formaldehyde gel and transferred onto nylon membrane in 1X TAE buffer using electrotransfer method. Blot was subsequently probed with GADD45, p21/Waf1 or GAPDH DNA probes labeled using the random primers labeling kit from BMB (probes were cleaned using sephadex G-50 columns and specific activity of the probes were higher than 10^8 cpm/ug). The blot was hybridized overnight at 42 ^oC in solution containing 50% formamide, than washed (final wash was for 1 hour at 68 ^oC) and phosphor imaging and auto radiography were performed.* ***B)*** *Western blot analysis of the p53 protein in total nuclear extract. 100ug of total nuclear extract was loaded for each sample.* ***C)*** *Western blot analysis of MANCA p53 protein immunoprecipitated with antibody 421 and 240. MANCA and ML-1 cells were treated with Camptothecin(20 um) or Zeocin(50 ug/ml) for 12 hr.. 400 ug of both cell extracts were immunoprecipitated with either anti p53 antibody 421 or 240 cross-linked to protein A sepharose beads for 2 hr. and applied to the 10% SDS-PAGE gel. Western blot was carried as described above.*

We have tested nuclear extract prepared from these drug treated cells for their ability to bind the SCS oligonucleotide as assayed by EMSA. No p53 dependent gel shift was seen for the MANCA nuclear extracts (Figure 4A). However an increase in the gel shifting ability for the ML-1 extract from both camptothecin and zeocin treated cells was observed (Figure 4A). These results are encouraging for carrying out *intra*-nuclear footprinting on elutriated cell cycle fractions of drug treated ML-1 cells. Sequencing the p53 gene in the MANCA cell line will be needed to determine if the p53 is wild-type and thus if we can compare to the ML-1 cell line to MANCA as a means of investigating upstream signals that regulate p53 level and function.

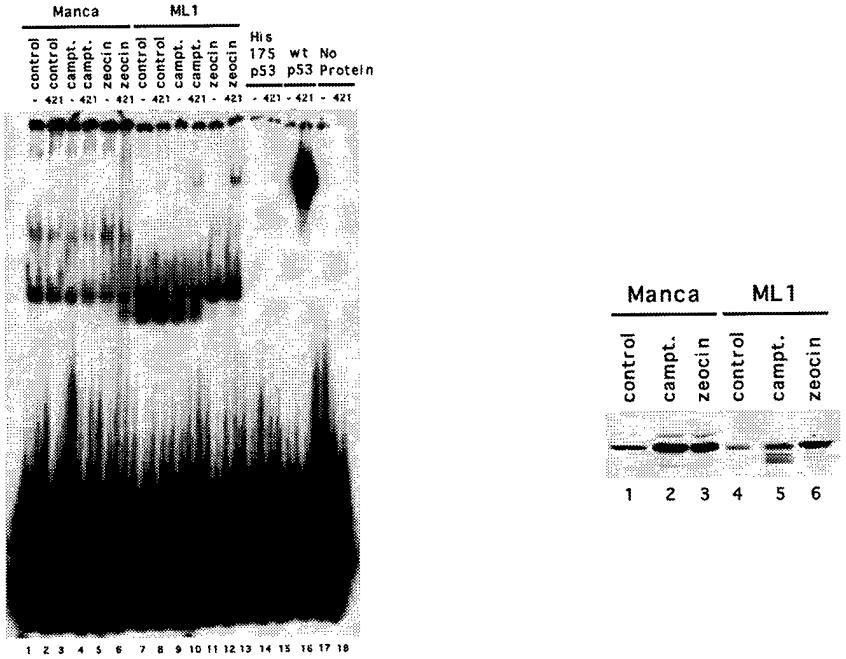

Figure 4: Western Blot and Gel shift assay of p53 from MANCA and ML-1 cells treated with Camptothecin and Zeocin. ***A)*** *Oligonucleotide contains super consensus sequence of p53 was used. 10 ug of nuclear cell extract of each of the MANCA cell without drug treatment (lane 1,2), Camptothecin treatment (lane 3,4),Zeocin treatment (lane 5,6) and ML-1 cells without drug treatment (lane 7,8), Camptothecin treatment (lane 9,10) and Zeocin treatment (11,12) were used in this assay. Mutant p53 (lane 13, 14), wild-type p53 (lane 15,16) and nuclear extract buffer (lane 17,18) were used as control. All these proteins were incubated with SCS (lane 1, 3, 5, 7, 9, 11, 13, 15, 17) or incubated with SCS and monoclonal p53 antibody 421 (lane 2, 4, 6, 8, 10, 12, 14,16,18).* ***B)*** *Western Blot Levels: Camptothecin (20 um) and Zeocin (50 ug/ml) were added to the exponential growing MANCA and Ml-1 cells. After 6 hr., nuclear extract was prepared and resolved in 10% SDS-PAGE gel. The Western blot was carried out using a mixture of PAb421, 240 and 1801 as the primary antibody and the horse radish peroxidase conjugated goat anti mouse IgG as the second antibody.*

From the intra-nuclear footprints we have determined that there is a critical window in which the p53 binding to chromatin can be observed (Figure 7 and described for task 3 d). Therefore we believe it is important to determine how long to activate the cells with the DNA damaging agents prior to footprinting. A time course for the activation of p53 DNA binding in the ML-1 cells by 20 uM camptothecin was done to determine how long we should treat the cells with drug in order to see the maximum p53 DNA binding (Figure 5). p53 specific binding monitored by EMSA of both the super consensus sequence (SCS) (Halazonetis et al. 1993) and GADD45 (Kastan et al. 1992) containing oligonucleotides showed a peaked at 6 hours that then fell after 8 hours of the drug treatment. Therefore when using the DNA damaging drug camptothecin we have chosen 6 hours of drug treatment as the optimal time point.

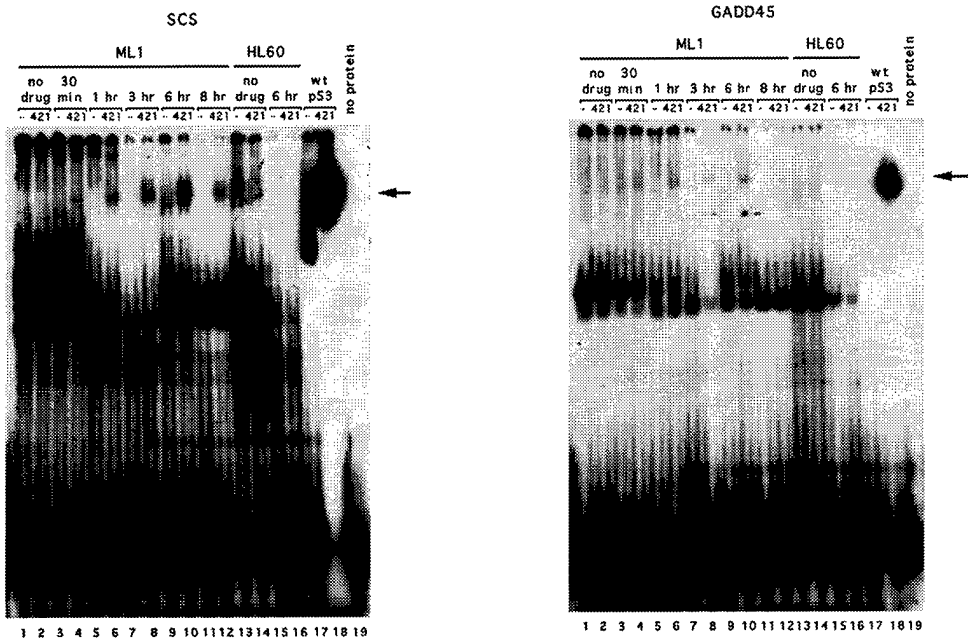

Figure 5: Electrophoretic mobility shift assay of SCS and GADD45 oligonucleotides with nuclear extract from Camptothecin treated ML-1 cells. *ML-1 cells were incubated with 20 uM camptothecin for 30 min., 1h, 3h, 6h and 8h, then placed on ice for 10 minutes. Nuclear proteins extracts were prepared according to conditions described* (Kastan et al. 1992). *7 ug of nuclear protein extract was incubated for 20 minutes at room temperature in buffer (20 mM Hepes, pH 7.8; 100 mM KCl;1 mM EDTA, pH 8.0; 1 mM DTT; 1 ug sheered salmon sperm DNA; 10% glycerol) with 150 fmol of P^{32} labeled specific primer either with or without PAb421. Samples were separated on a 4% polyacrylamide gel (gel was prerun at 100V for 15 minutes at 4 ^{o} C) at 200V for 3-3.5 hours. Gels were dried for 1 hour at 55 ^{o}C and auto radiography was performed.*

Task #3 c. Southern blot using the murine ts p53-Val135 cell line to set up the DNase I sensitivity assay and test chromatin structure of the gene.

We have investigated the interaction of p53 with the genomic p53 dependent promoter region of the mdm2 gene by comparing the *in vivo* DNase I protection of this region in two different cell lines, a p53-null cell line (10-1) and a temperature-sensitive p53-Val 135 overexpressing line (3-4). At 32°C the p53 in the 3-4 cell line is in a wild-type conformation while at 37°C the protein is conformationally mutant. The mdm2 oncogene is a p53 responsive gene which contains both a p53 independent and a p53 dependent promoter (P1 and P2 respectively) (Juven et al. 1993)(Perry et al. 1993)(Wu et al. 1993). The endogenous mdm2 gene contains two p53-response elements (REs) in its first intron (Juven et al. 1993)(Wu et al. 1993)(Zauberman et al. 1995). The MDM2 protein works as part of a feed back loop forming a protein-protein association with p53 and thereby inhibiting p53-mediated transactivation ((Momand et al. 1992)(Oliner et al. 1993)(Wu et al. 1993)). Overexpression of MDM2 can inhibit the ability of p53 to suppress transformation (Finlay 1993) as well as the ability of p53 to evoke cell cycle arrest or induce apoptosis (Chen et al. 1996). Evidence suggests that such abrogation of p53 function by MDM2 is one of the ways MDM2 exerts its oncogenic effect (Oliner et al. 1992)(Chen et al. 1996). Interestingly indirect Southern blot analysis of the promoter regions of the mdm2 gene in both cell lines revealed nuclease-hypersensitivity at both the P1 and P2 at 37°C and 32°C (Figure 6).

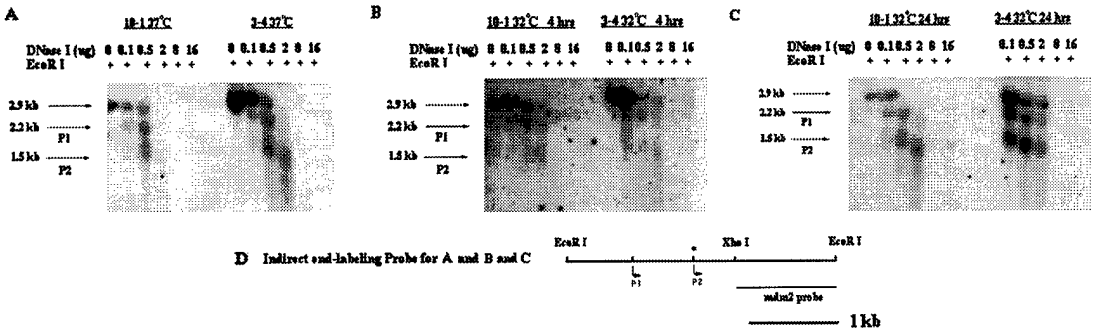

Figure 6: p53 mediated mdm2 gene activation occurs from a nucleosome free region. *DNA in 2 x 10^6 isolated nuclei was digested with increasing amounts of DNase I (0 µg, 0.1 µg, 0.5 µg, 2 µg, 8 µg, or 16 µg as indicated above each lane). Nuclei were isolated from 10-1 and 3-4 cells maintained at 37° C (A), 32° C for 4 hours (B) and 32°C for 24 hours (C). 20 ug of purified DNA was restricted with EcoRI, electrophoresed on a 0.8% agarose gel and probed with a [^{32}P] labeled EcoRI-XhoI genomic mdm2 probe fragment (shown in D). (D) The physical map of the upstream region of the murine mdm2 gene is shown. P1 and P2 are indicated.*

Using ligation mediated PCR *in vivo* footprinting we observed striking protection of both p53-REs as well as protection of the adjacent TATA box in the nuclei of 3-4 p53-Val 135 expressing cells maintained at 32°C for 4 hours but not in 3-4 cells maintained at 39°C or 37°C (this data was presented in the 1996 progress report and is shown again in Figure 7). Surprisingly this protection pattern differed considerably from that observed with purified p53 on naked DNA (data not shown). On naked DNA greater protection was evident in p53 RE1 then in p53 RE2 while in the genomic footprint the overall pattern of protection differed and greater protection was evident in p53 RE2. Gel-shift experiments and protein analysis demonstrated that the peak in p53 dependent genomic footprinting of the REs correlated with an increase in the nuclear p53 level and the p53 DNA binding activity. Although p53 binding to chromatin was demonstrated by genomic footprinting techniques, no overall nuclease sensitivity change occurred at this time point as noted by the indirect Southern blot analysis (see Figure 6 above). Phosphor imaging analysis and quantification of the DNase I induced P1 and P2 bands indicated that no significant p53 dependent change was induced at either of these promoter regions. The hypersensitive site present at the mdm2 P2 promoter indicates that this region has an altered chromatin structure. This suggests that the altered chromatin structure at the p53 dependent promoter may be important for recognition and regulation of the mdm2 chromatin. In contrast to the products of many other p53 regulated genes, Mdm2 is a positive effector of the cell cycle as opposed to being a negative effector. The fact that the mdm2 p53 REs are housed in a nucleosome free region does not indicate that all p53 binding sites are in similar structural genome locations. It is possible the altered chromatin structure evident for the p53 binding sites in the mdm2 gene may not be present at the p53 REs in genes which negatively regulate the cell cycle.

Indirect Southern blotting will be used to assess the DNaseI sensitivity of both the mdm2 and gadd45 genes in the breast cancer cell lines being used in this study, the normal breast cell line MCF12A and the hematopoietic cell lines described above. The nuclei from the breast cancer cell lines have been extracted and treated with DNaseI and an agarose gel has been used to screen the samples for increasing DNaseI digestion (data not shown). The total DNA was digested with EcoRI which is a viable enzyme for indirect Southern Blot screening of both the mdm2 gene and the gadd45 gene. The DNaseI curves were successful and we are in the process of testing to see if the mouse mdm2 indirect probe will work with the human genomic DNA. The strategy for digesting out the human gadd45 and mdm2 indirect Southern blotting probes have been designed and after we probe for the DNAse I sensitivity of the human mdm2 gene the blot will be stripped and reprobed for gadd45.

Task # 3 d. Footprinting of mdm-2 and gadd45 binding sequences in chromatin and footprinting with immunopurified p53.

Studies on the ability of p53 to bind to DNA have been carried out *in vitro*, but little is known about the ability of the protein to bind to specific target sites *in vivo*. In order to elucidate how p53 activates initiation of transcription we focused on the DNA binding of the protein to chromatin and thus carried out intra-nuclear ligation mediated PCR (LM-PCR) DNase I protection analysis. We began this analysis with one p53 responsive gene, mdm2. The region of the mdm2 gene with which p53 interacts was originally identified by co-immunoprecipitation of a specific DNA element as well as by demonstrating a minimal sequence able to confer p53 specific transactivation of a reporter construct ((Wu et al. 1993)(Juven et al. 1993)). The mdm2 oncogene is a p53 responsive gene which contains both a p53 independent and a p53 dependent promoter (P1 and P2 respectively) (Juven et al. 1993)(Perry et al. 1993)(Wu et al. 1993). The endogenous mdm2 gene contains two p53 REs in its first intron (Juven et al. 1993)(Wu et al. 1993)(Zauberman et al. 1995). The nucleotides within this region that are specifically bound by p53 and thereby protected from DNase I digestion have not been identified. We have investigated the intra-nuclear interaction of p53 with the p53 dependent promoter region of the mdm2 gene by comparing the intra-nuclear DNase I protection of this region in two different cell lines, a p53-null cell line (10-1) (Harvey and Levine 1991) and a temperature-sensitive p53-Val135 overexpressing line (3-4) (Chen et al. 1995). At 32^{o}C the p53 in the 3-4 cell line is in a wild-type conformation while at 39^{o}C the protein is conformationally mutant (Martinez et al. 1991).

We used LM-PCR footprinting analysis to define the nucleotide sequences that were protected in a p53 dependent manner in nuclei from 10-1 and 3-4 cells. Changes independent of p53 were observed in the 10-1 cells maintained at the two different temperatures of 39^{o}C and 37^{o}C. Therefore for each temperature shift and incubation duration tested we compared the protection pattern present in 10-1 cells to that present in 3-4 cells. Striking protection of the p53-REs was observed in nuclei of 3-4 cells maintained at 32^{o}C for 4 hours (Figure 7, compare lanes 7 and 8). At 39^{o}C and 37^{o}C less striking differences were observed between the 10-1 and 3-4 samples (Figure 7, lanes 1-4). Footprints in cells maintained at 32^{o}C for 2 hours showed some differences between the 10-1 and 3-4 samples with possible changes occurring at the p53-REs and the TATA box (Figure 7, lanes 5 & 6), however the most dramatic differences were evident between the 10-1 and 3-4 samples derived from cells maintained at 32^{o}C for 4 hours (Figure 7, lanes 7 & 8). The two putative p53 binding sites were protected over their downstream 3' halves in the 3-4 cell line while no protection was present in the 10-1 cell line (Figure 7, lanes 7 & 8 indicated by brackets on the right). This DNase I protected sequence in chromatin did not contain the total 20 bp consensus

homology region, however DNase I hyper cutting sites emerged adjacent to each p53 protected region suggest binding at these sequences as well. Additionally, a change in protection at the TATA box region was evident, with a hypersensitive site emerging in the middle and prolonged protection occurring over the 3' region (Figure 7B, lane 8 indicated by bracket with *). It has been shown that p53 and TFIID are able to bind cooperatively to DNA (Chen et al. 1993). The appearance of the band marked * suggests that the binding of p53 to chromatin may disrupt a nucleosome contact and thus allow TFIID and the basal transcription machinery access to the promoter. The 3-4 samples shifted to 32°C for 24 hours had reduced protection of the two p53-REs with concomitant extended protection visible over the 3' adjacent TATA box region (Figure 7, lane 10). The dynamic intra-nuclear p53 dependent changes within the mdm2 P2 promoter region over a 24 hour period suggest that the DNA binding ability of p53 and the associated proteins change over time and suggests that it is worth while monitoring the sequential interactions of proteins at p53 binding regions.

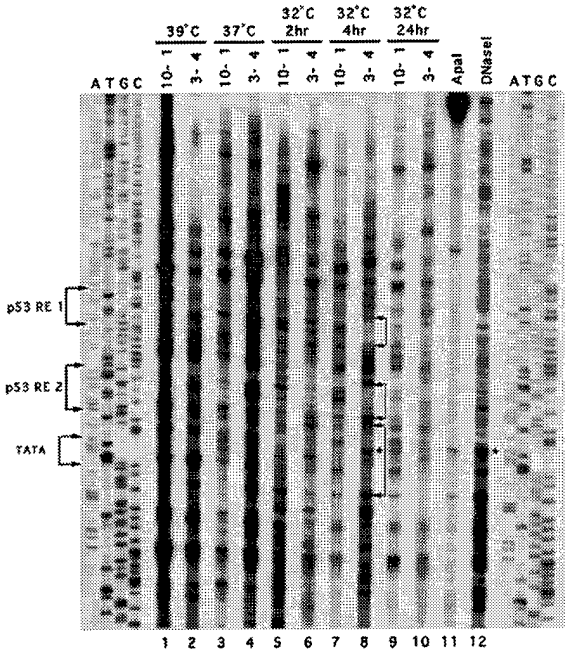

Figure 7: Ligation-mediated PCR intra-nuclear footprinting demonstrates p53 mediated protection of the p53 REs. *The published p53-REs and adjacent TATA box were identified by sequencing a genomic mdm-2 plasmid clone (lanes indicated as ATGC) and are indicated on the left. DNA in 2 x10^6 isolated nuclei from 10-1 and 3-4 cells was digested with 0.1 ug of DNase I and purified. Samples are from cells maintained at 39°C (lanes 1 and 2), 37°C (lanes 3 and 4), 32°C for 2 hours (lanes 5 and 6), 32°C for 4 hours (lanes 7 and 8) and 32°C for 24 hours (lanes 9 and 10). Purified DNA from undigested nuclei is shown in lane 11 and naked mouse genomic DNA digested with DNase I is shown in lane 12. Ligation mediated PCR was carried out followed by primer extension with [^{32}P] labeled oligonucleotide #3 which hybridized*

approximately 350 bp downstream from the P2 promoter. Samples were electrophoresed on a 6% urea sequencing gel.

In order to begin ligation mediated footprinting of the gadd45 gene the specific primers had to be ordered and tested for their predicted melting temperatures. This primer design was described in the 1996 progress report. The primers were ordered and have proven to work successfully. They have enabled us to sequence the plasmid containing the gadd45 gene as well as determine the length of time needed for the sequencing gel run. Unfortunately the first few attempts at ligation mediated PCR footprinting of the gadd45 p53 binding site region have proven unsuccessful due to technical difficulties that are presently being addressed.

p53 mediated DNase I protection of the mdm2 p53-REs is different on naked DNA

We examined the *in vitro* DNase I footprinting ability of purified p53 on the mdm2 P2 region in order to determine if the pattern of protection at the p53-REs was the same as that demonstrated *in vivo* (Figure 8). Surprisingly, the *in vitro* protection pattern demonstrated with purified p53 on naked DNA differed considerably from the genomic protection patterns that were observed. Protection of the 5' half of p53-RE1 was observed on naked DNA with p53 however no clear protection of p53-RE2 was identified (Figure 8, lanes 1& 2). The difference between the genomic footprinting pattern and the *in vitro* result suggests that the chromatin structure of the mdm2 P2 region organizes the DNA sequences into an optimal p53 DNA binding site.

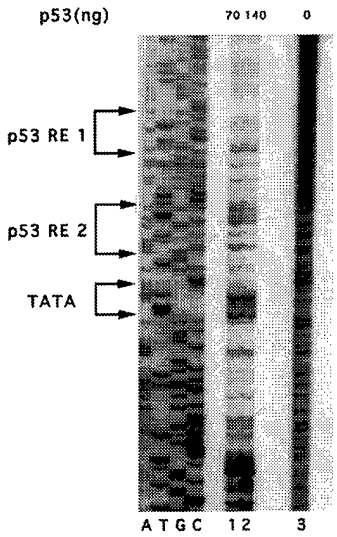

Figure 8: Protection of the p53-REs of mdm2 in vitro differs from that observed in vivo. *In vitro footprinting of a naked mdm2 DNA fragment with purified p53. The published p53-REs and adjacent TATA box were identified by sequencing genomic mdm-2 in a plasmid clone (lanes indicated as ATGC) and are indicated on the left. Increasing amounts of purified p53 were added to a complete Apa I-XhoI digest of the naked mdm2 genomic plasmid clone (lanes 1 & 2, as indicated). Footprints were visualized by primer extension using [^{32}P] labeled oligonucleotide #3 which hybridized 350 bp downstream from the TATA box. Lane 3 contains no p53 protein.*

Task #3 e. Footprinting of the mdm2 and gadd45 sites in nuclear chromatin will be carried out in breast cell lines with and without ts p53-Val135, and also in cell lines treated with chemotherapeutic agents.

Breast cancer cell lines that express ts p53-Val135 have been selected, as shown in Figure 1. DNase I sensitivity curves have been carried out in the breast cancer cell lines MDA-MB-468, MDA-MB-157 and ZR75-1. These can be used for both indirect Southern blot analysis as well as for the *in vivo* footprinting experiments. Therefore were are ready to begin generating data in this area of investigation.

Task #4 : *In vivo* DNA footprinting of synchronous populations of hematopoietic and breast derived cell lines.

Task #4 a. Synchronous populations of the various hematopoietic and breast cell lines will be prepared by both centrifugal elutriation and drug treatment.

Centrifugal elutriation of tissue culture cells allows cell cycle factions to be separated on the basis of their migration in a gradient. This method has an advantage over using drugs to block the cell cycle at specific stages because drug treatment induces a number of responses and needs time to produce the various populations of cells. In the case of p53 DNA interaction research, by the time the cells are arrested it is possible to have missed the critical moment of the interaction. We have begun our elutriation experiments using the suspension cell line ML-1 (a myeloid leukemia cell line). The ML-1 cell line contains wild-type p53 that can be activated in response to DNA damage. Elevation in the p53 level in ML-1 cells is detectable in less than 1 hour after exposure to gamma irradiation (Kastan et al. 1991) and within 2 hours after treatment with the drugs bleomycin, actinomycin D, etoposide or camptothecin (Nelson and Kastan 1994). Although the wild-type p53 status of the ML-1 cell line has been well documented no published data for a successful elutriation of this cell line exists. We have succeeded in setting up elutriation conditions for this cell line in order to carry out biochemical experiments investigating the DNA binding activity of p53 present during different stages of the cell cycle before and after the induction of DNA damage (Figures 9 & 10).

The elutriation of ML-1 cells to yield cell cycle fractions has also been successful (Figure 9). Interestingly we saw that the p53 levels in the ML-1 cell cycle fractions peaked at the G1/S border (which is one of the cell cycle check points for p53). The cell cycle distribution of p53 in the ML-1 cell line corroborates with evidence published for normal breast cells released from a G^0 block (Gudas et al. 1994). We then tested to see if the p53 in the ML-1 cell cycle fraction was capable of binding to a p53 super consensus sequence (SCS) when assayed using the electrophoretic mobility shift assay (EMSA). Over exposure of the gel was necessary in order to visualize any PAb 421 p53 specific super-shift in any of the fractions. However with over exposure we found that fraction 5 demonstrated the highest level of p53 dependent SCS gel shift ability (figure 9D). The nuclear extract prepared from the exponential growing cell population appeared to contain the same relative amount of p53 as fraction 5 when compared by Western Blot analysis (Figure 9B). This suggests that the difference seen in the EMSA normalized for total nuclear protein was due to preferential binding of p53 at the G1/S border which is the fraction 5 population.

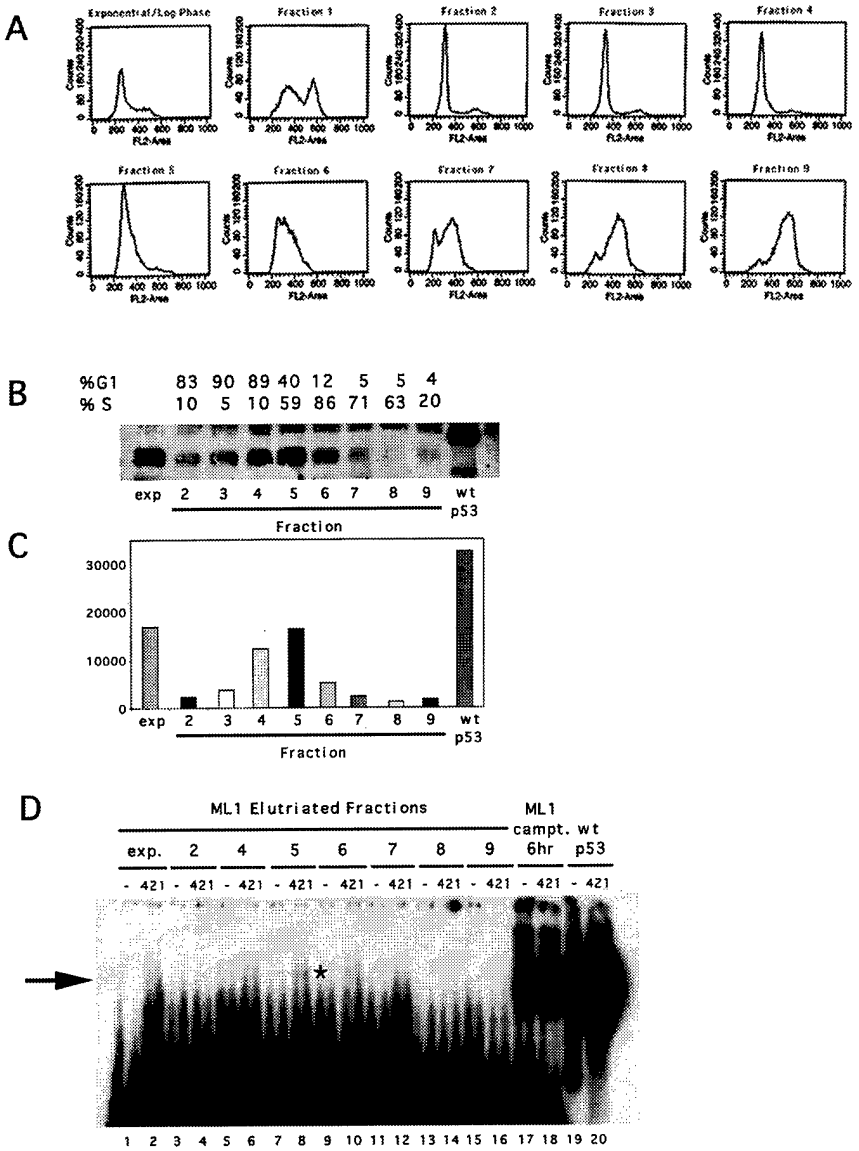

Figure 9: p53 protein levels in centrifugal elutriated ML-1 cell cycle fractions. *Four liters of ML-1 cells at 4 x $10^{(5)}$ cells/ml were elutriated using a Beckman Elutriation Rotor System.* ***A)*** *The cell cycle profile of the ML-1 elutriated fractions was determined by florescence activated cell sorter (FACS)analysis.* ***B)****Nuclear extracts were prepared and immunoprecipitated. Fraction 1 was not used because it was not cell cycle fraction enriched. The immunoprecipitated p53 was resolved by 10% SDS-PAGE and visualized by Western blot with a mixture of p53 specific antibodies PAb421, PAb240, and PAb1801 followed by detection with ECL reagent.* ***C)*** *The relative levels of p53 are presented as a histogram of pixel values determined by laser densitometer analysis and quantitation by Image QuaNT software (Version 4.1).* ***D)*** *EMSA analysis with SCS oligonucleotide the lanes are as indicated.*

Task # 4 b. Intranuclear footprinting on the synchronous populations of cells will be carried out.

Following the determination of the correct conditions for the separation of ML-1 cell cycle fractions by elutriation, we proceeded to elutriate the cells and camptothecin drug treat the individual cell cycle fractions (Figure 10) in order to determine the peak of p53 binding to the gadd45 DNA sequence. From this elutriation it was also clear that in the non drug treated ML-1 cells the peak p53 level was at the G1/S border (Figure 10A) and once again these are the fractions which show the peak binding to the SCS oligonucleotide as assessed by EMSA (data not shown). After drug treatment the p53 levels rise in fractions 2 through 4 and 7 through 8 (Figure 10B). This increase of p53 in the G1 cell cycle fractions clearly indicates that camptothecin can activate p53 during G1 although it has been shown to only induce DNA double strand breaks during S. Three fractions from non drug treated and drug treated samples were normalized for their p53 levels and tested for the ability of the p53 to bind to the gadd45 oligonucleotide (Figure 10C). The p53 specific antibody PAb421 activates the DNA binding ability of p53 and using this antibody it was clear that the p53 which was the most active for binding to the gadd45 sequence was the p53 after camptothecin drug treatment in fraction # 10 (Figure 10C, compare all other lanes to lane 12). The binding activity in fraction 10 compared to that exhibited by immunopurified p53 (compare lanes 12 and 14). It light of this data it appears that the *in vivo* footprinting of the gadd45 site should be compared in non drug treated and drug treated fraction 10 which is an S and G2/M cell cycle enriched fraction, having 31%S and 58% G2/M cells. It is difficult to see the percentage in each fraction. They are as follows: #2 (97% G1, 1% S, 2% G2/M); #3 (60% G1, 38% S, 2% G2/M), #4 (40% G1, 59% S, 1% G2/M); #5 (22% G1, 77% S, 1% G2/M); #6 (11% G1, 88% S, 1% G2/M); #7(11% G1, 80% S, 1% G2/M); #8 (10% G1, 66% S, 24% G2/M); #9 (14% G1, 45% S, 41% G2/M); #10 (11% G1, 31% S, 58% G2/M) and #11 (10% G1, 17% S, 73% G2/M).

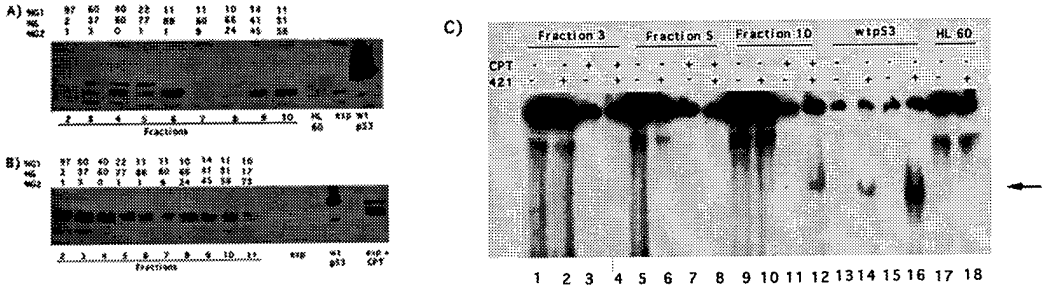

Figure 10: Camptothecin drug treatment of cell cycle fractions. *$1.2x10^9$ cells were elutriated into 11 fractions. Half of the cells from each fraction was lysed after elutriation (A). Another half was treated by Camptothecin for 6 hours and lysed (B). 95 ug of the nuclear extract were resolved by 10% SDS-PAGE gel and visualized by Western blot analysis with a mixture of p53 antibodies 421, 1801 and 240 followed by detection with ECL reagent. (C). EMSA normalized for identical p53 amounts from fractions 3, 5 and 10 with and without drug treatment was carried out with ^{32}P-labeled gadd45 oligonucleotide (lanes 1 to 12). The amount of nuclear extract added to each reaction mixture was 5.46 ug (lanes 1 and 2), 6.98 ug (lanes 3 and 4), 14 ug (lanes 5 and 6), 15.7 ug (lanes 7 and 8), 11.2 ug (lanes 9 and 10) and 16.0 ug (lanes 11 and 12). Lanes 13 to 16 contain reaction mixtures with immunopurified wt p53 (60 ng) to the ^{32}P-labeled GADD45 (13 and 14) and SCS oligonucleotides (15 and 16). 10 ug of HL60 cell nuclear extract was added to lane 17 and 18 as a negative control. The amount of p53 protein in lane 3,4,7,8,11,12 was normalized to 1.5 ng.*

Task #5 Test to see if mutant p53 His273 is able to bind to the HIV-LTR region when it is transiently transfected into the cell line MDA-MB-468.

Task # 5 a. Examine HIV-LTR driven transcription in breast cell lines containing different status p53 protein.

The ability of mutant p53 His 273 to transactivate promoters containing Sp1 binding sites has been confirmed by introducing an HIV-LTR luciferase reporter construct into the MDA 468 cells. We have results indicating that HIV-LTR directed transcription is mutant p53 dependent since co-expressing a temperature sensitive (ts) p53 in a wild type conformation negates this effect (Figure 11). This result indicates that the wt protein has a dominant effect over the mutant endogenous protein.

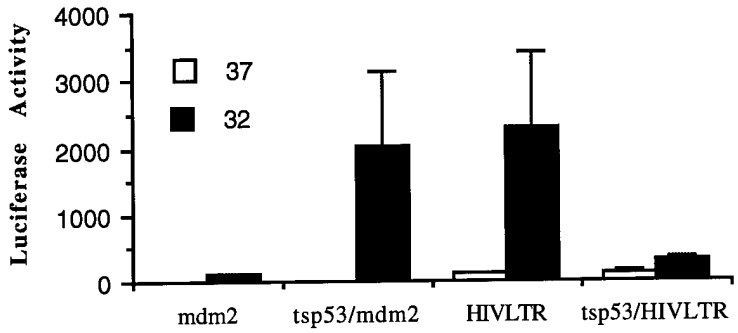

Figure 11: MDA-MB-468 transactivation of the HIV-LTR is inhibited by expressing a temperature sensitive p53 in its wild type conformation. *1 x 10^7 cells were transfected by electroporation at 350V with either 10μg of reporter plasmid (HIV-LTR or mdm2) and 10μg of carrier DNA or 10μg of reporter plasmid and 10μg of a plasmid encoding the ts p53 Val135 where indicated. At 48 hours post-transfection, cells extracts were made from cells maintained at 37°C and cells switched to 32°C for 24 hours. Data presented is the average value of duplicates. The mdm2 reporter is under regulation of the p53 binding site located in the mdm2 gene. HIV-LTR reporter is under regulation of the long terminal repeat of the HIV-1 which has three Sp1 binding sites .*

Task # 5 b. Footprinting of the HIV-LTR region in the nuclei of breast cell lines containing different status p53 protein in both the absence and presence of chemotherapeutic drug treatment.

We have not yet begun these experiments, however we will begin them as soon as possible.

Task # 5 c. Comparison of the proteins from different status p53 breast cell extract bound to the HIV-LTR region.

We have begun this study by designing an oligonucleotide containing three Sp1 binding sites found in the HIV-LTR that we called HIV. Figure 4 shows a gel retardation assay using this oligo and nuclear extracts from three breast cancer cell lines. We have been unable to detect p53 binding to this oligo using p53 antibodies 421 (Figure 12A) and 1801 (not shown). We have observed, however, that extracts from the MDA 468 cell line, which transactivate the reporter gene, bind more prominently to this oligo than extracts from the other cell lines that do not transactivate the reporter gene. This binding is not due to higher expression of Sp1 in MDA-MB-468 as detected by Western Blotting (data not shown). This oligonucleotide can also compete the binding of mutant p53 His 273 to a p53 super consensus binding site (Figure 12B) suggesting that mutant p53 can bind to this oligo and that the antibodies used do not recognize the conformation of p53 that is

bound. We are currently using other p53 antibodies to detect the binding of mutant p53 to this oligo.

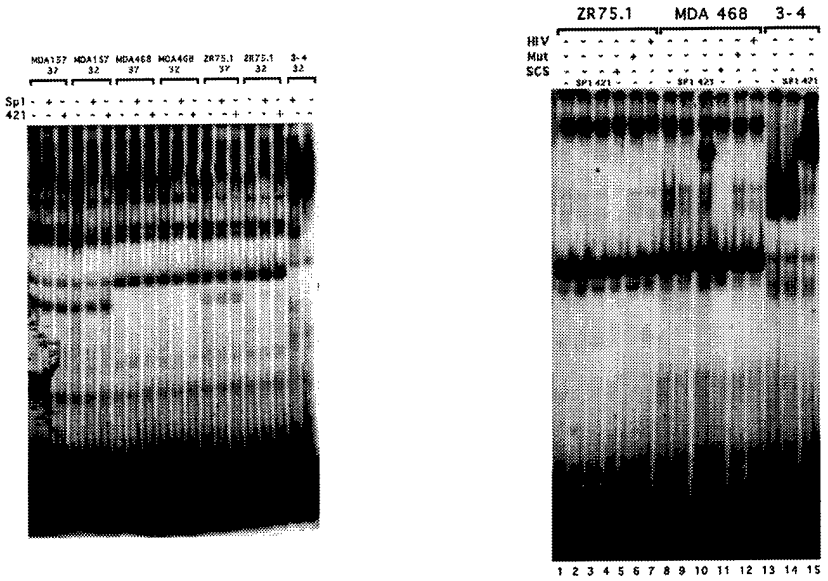

Figure 12. DNA binding assays using breast cancer cell extracts and a labeled oligo containing the three Sp1 binding sites in the HIV-LTR (A) or a labeled oligo containing a p53 super consensus sequence SCS (B). *The DNA binding reaction reactions were carry out at room temperature for 20 minutes in 30 ul total volume in a buffer containing 0.15 pmoles of oligo, 2 ug nuclear extracts, 20 mM Hepes(pH 7.8), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 1 ug of sheered salmon sperm DNA and 10% glycerol in the presence of a p53 antibody (421) or an antibody directed at the Sp1 protein (Sp1). Competitor oligonucleotide was added in 50 fold excess where indicated. 15 ul of reaction mixtures were resolved on a 4% acrylamide gel at 200V at 4°C. Mut is a mutant p53 binding site that is unable to bind p53. 3-4 is a cell line stably transfected with the temperature sensitive p53 Val 135 and is used as a control for wild type p53 binding.*

Task 6 Studies on the affect of p53 on the DNA replication of the double minute chromosomes in the breast cancer cell line MDA-MB 361.

We have begun growing this cell line however we have not initiated the tasks outlined below.

a. Examine the level of MDM-2 gene amplification level in MDA-MB 361 cell lines that express ts-p53val135, both at the permissive and restrictive temperatures.

b. Make and analyze MDA-MB 361 cell fusions with normal breast cells.

c. Carry out in vivo footprinting on synchronous populations of the above cell types.

Conclusions:

During months 24-36 we have succeeded in addressing many of the questions set forth in the grant proposal. We have selected two derivative stable breast cancer lines expressing the temperature sensitive p53-Val135 mutant. One is from the cell line MDA-MB-468 which contains endogenous mutant p53 His273 and the other is from the cell line ZR75-1 which contains endogenous wild-type p53. These cell lines will be useful for the completion of our studies while also being useful to the community involved in breast cancer research as a whole. EMSA experiments have demonstrated that the p53 mutant His273 has DNA binding activity and that this binding activity may be partially responsible for the oncogenic gain of function elicited by this protein.

We have identified that the p53 binding site region of the mdm2 gene is localized to a constitutively nucleosome free region. The has reinforced the importance of identifying the nucleosome configuration of different p53 binding sites in normal and cancer cells. We have begun these studies in breast cancer cells and are embarking on the data collection phase.

We have succeeded in isolating pure cell cycle fractions from exponentially growing hematopoeitic cell lines and have strong indications from the data that p53 activity is controlled in a cell cycle specific manner. We will investigate the intranuclear DNA binding activity of p53 to the various putative DNA binding sites over the course of the cell cycle. We are ready to carry out these experiments in breast cancer cells that contain wild-type p53, cells with oncogenic mutant p53 and cells with temperature sensitive p53 and either wild-type or mutant p53. We have also begun working with an inducible p53 cell line (TR9-7) which was described in the 1996 progress report. This work is in a very primary stage and therefore was not included in this report.

The studies carried out to date have been very informative as to how normal p53 tumor suppressor activity is controlled and how oncogenic mutant p53 is able to elicit a gain of function response. We look forward to contributing our findings to the cancer research community in the form of publications resulting from much of the data that we have presented here.

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