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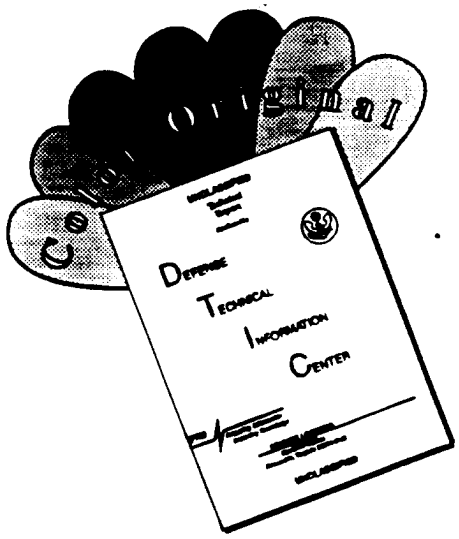

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13. ABSTRACT *(Maximum 200 words)*

We are investigating what forms of p53 bind to potential p53 DNA binding sequences *in vivo* by carrying out a systematic study of the interactions of wild-type and mutant p53 with the p53 responsive regions of genes in their native chromatin structure. Although we have not begun to investigate the *in vivo* DNA binding ability of p53 in the breast cancer cell lines, we have set up a system to identify conditions under which p53 binding sites *in vivo* demonstrate DNaseI sensitivity in the absence of wild-type p53, and resistance in the presence of p53, at the p53 responsive region of the mdm2 gene in a mouse embryo fibroblast cell line. We have identified the cytoplasmic, nuclear and whole cell levels of p53 in the breast cancer cell lines ZR75-1 (wtp53 +/-), MDA-MB-468 (273 Arg to His) and MDA-MB-157 (null for p53 by deletion)). MDA-MB-468 has a high nuclear level of p53 protein while ZR75-1 cells contain a very low level of wild-type p53 and there is no detectable p53 in MDA-MB-157. Interestingly, we have stabilized both the nuclear and cytoplasmic forms of p53 in ZR75-1 with an inhibitor of the ubiquitin proteolytic pathway.

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V Barsoum 7/27/95

PI - Signature Date

Table of Contents:

Introduction:

p53 binds to DNA and regulates transcription

The p53 tumor suppressor protein can function as a checkpoint factor (23) causing cells exposed to DNA damaging agents to arrest in G1 (21)(28). The normal function of p53 is regulated, at least in part, by the ability of the protein to bind site-specifically to DNA (55). The tumor-derived mutant p53 proteins that have been tested thus far have altered, or deficient DNA binding activity (4)(9)(30)(31), however some oncogenic p53 mutants can bind to DNA at temperatures lower than 37°C (5)(64) or to idealized p53 binding sites (26)(63). Wild-type p53 binds nonspecifically to DNA (30) (49) 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' (16)(19). Wild-type but not mutant p53 can activate transcription from specific cellular regions containing p53 binding sites both in vitro (18) and in vivo (61) (18) (19). Therefore, there is a strong connection between p53 DNA binding activity and normal p53 function. Recently several genes that are induced by high levels of wild-type p53 have been identified.

Growth arrest is presumably brought about in part by specific activation of one or more of these genes. Some of the responsive genes in which p53 binding sites have been identified include the oncogene mouse double minute 2 (mdm2) (3)(27)(43), growth arrest and DNA damage 45 (gadd45) (25)(29) (62) , and waf1/cip1 (17). The product of the 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 (17)(22). Additionally, activation of gadd45 (which is part of the DNA damage response pathway) and mdm2 (whose protein product binds to p53 and blocks p53 mediated transactivation in cell cycle checkpoint pathways (8)) may help to signal cells to growth arrest until DNA repair has taken place. The fact that the tumor derived mutant p53 proteins do not activate transcription of the genes described above (18)(32) nor induce G1 arrest (28), may account for the gene amplification which is associated with the oncogenic state (34)(59).

In addition to promoters that are activated by wild-type p53 there are also many promoters that are repressed by wild-type p53 (12)(14) (20) (45)(50)(51). Promoter regions that fall into this category are of both viral and cellular origin. Viruses containing this class of promoter include SV40 (Alwine, personal communication; Prives et. al., unpublished; (50)), herpes simplex virus thymidine kinase and UL9 promoters (12), human cytomegalovirus-immediate-early promoter (12) and the long terminal repeat regions (LTR) of HIV, Rous sarcoma virus, and human T-cell lymphotropic virus (12) (50). Some of the cellular prototypes include proliferating cell nuclear antigen (PCNA) (12), DNA polymerase alpha (38), multi drug resistance 1 (mdr1) (11), interleukin 6 (46) as well as c-fos, p53, MHC, c-jun, ß actin and Hsc70 (20). p53 can bind site specifically to SV40 DNA adjacent the SV40 origin of replication (4). This p53 binding site overlaps with binding sites for the transcription factor Sp1 and a DNA structural motif which is similar for both Sp1 and p53 binding sites has been identified (35). Although no p53 binding sites have yet been identified in the cellular genes discussed above, Sp1 binding sites are present in some and may play a part in the p53 dependent repression observed (7). Equally, if not more, intriguing is the fact that most of the viral and cellular promoters that are repressed by wild-type p53 are transcriptionally activated by mutant p53 proteins (11)(12)(14)(51). The possibility exists that in vivo both wild-type and mutant p53 can interact with promoters that contain Sp1 binding sites. In fact p53 has been shown to associate with Sp1 and this protein complex interacts with Sp1 binding sites on DNA (7).

Differential occupancy of consensus binding sites in chromatin

While some transcription factors, like GAL4 (53) and Sp1 (33), 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 (2)(44). 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 (57)). Therefore, it has been suggested that many genes are programmed during DNA replication while the nuclear chromatin assembles (58). 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 (10).

Wild-type p53 is present in low levels in normal cells (41). 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 (37). 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(13) (15)), 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 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. If this is the case, mutations in p53 might change the spectrum of growth-control genes that the protein activates.

Breast Lines available from ATCC:

All previous experiments examining the DNA binding properties of p53 proteins have been carried *in vitro*, on naked DNA, with either purified p53 protein from insect cells or bacteria or with p53 protein from crude cell extracts. We propose to study the *in vivo* ability of mutant p53 proteins to interact with sequences that are known to be bound by wild-type p53 in vitro and hope to extend the study to include regions that are known to be activated by oncogenic mutant p53 proteins.

The status of mutant p53 in many breast cancer cell lines has been identified, and utilizing these lines will, both facilitate our study as well as, yield potentially useful information for the treatment of breast cancer. A table of the p53 status in the breast cell lines available form ATCC is shown below.

p53 In Breast Cancer Cell Lines

Cell line	Mutation	Rb gene status	Ref.
BT 20	132 (region II) Lys to Glu	No gross defect	(6)
BT 549	249 (region IV) Arg to Ser	truncated mRNA	(6)
BT 474	285 (region V) Glu to Lys	No gross defect	(6)
MDA-MB-231	280 (region V) Arg to Lys	No gross defect	(6)
*MDA-MB-468	273 (region V) Arg to His	No mRNA	(40)
T 47 D	194 (III & IV) Leu-Phe	No gross defect	(40)
MCF 7	wt p53		(52)
*ZR 75.1	wtp53		(48)
*MDA-MB-157	null		(6)

* The cell lines that we have begun working with to date.

For the current project we are working to carry out a systematic study of the interactions of p53 with two genomic DNA binding sites during the course of cell cycle in various human breast cancer cell lines. These p53 responsive regions are that of the mdm-2 and gadd45 genes. The *in vivo* DNAse I sensitivity of these two regions in various breast cell lines will be compared. Additionally, we intend to work to elucidate if mutant p53 proteins derived from the breast cancer cell lines can interact with specific DNA sequences either *in vitro* or *in vivo.*

Year One with a DOD Career Development Award:

Upon completion of my first year as a new investigator I recognize, in retrospect, that my statement of work (SOW) for the current project omitted many of the tasks that I had to carry out in order to get my lab up and running. At the time that I wrote the proposal I was a postdoctoral fellow in a large laboratory that had all the necessary equipment, support personnel and lots of other researchers. When I began here at Hunter College I had empty lab space and an office. I had to begin from the bottom up, ordering everything from equipment to tips. Each task outlined in the SOW required many reagents to be prepared and additional tasks were required to set up conditions and establish controls. I also did not include in my SOW the significant amount of time necessary to train new graduate students. Some examples of tasks accomplished that were not included in my SOW are the following: I have begun training four graduate students, we have begun growing insect cells for purification of p53 protein preps, we have made and titered baculovirus stocks, we have grown monoclonal antibody cells and coupled the monoclonal antibody to protein A sepharose beads in addition to many other tasks not included in this list. The award given to me allowed for release time from teaching which has greatly assisted in the progress of my laboratory. However, it has taken us some time to get up and running. Presently I have two graduate students that are now able to work independently and consequently I have more time to do experiments.

Below is a list of the tasks from the proposal that cover months 1-12. In the results and discussion section I will explain what tasks have been carried out in preparation for the goal oriented tasks outlined in the grant proposal. This progress report documents how far my newly set up laboratory has proceeded towards completion of the tasks outlined in the SOW. In the process of beginning the work new ideas for strategies to accomplish the designated goals have been formed. These ideas are also described in this progress report.

Task 1, Establish stable breast cancer cell lines expressing temperature sensitive p53 (ts-p53val135). Months 1-24:

a. Attempts will be made to establish ts-p53val135 expressing breast cancer cell lines of BT 20, BT 549, BT 474, MDA-MB 231, MDA-MB 468, T47D, MCF-7, ZR 75-1 and MDA-MB 361.

b. Levels of p53 in the established cell lines will be examined both at the p53 permissive and restrictive temperatures.

Task 2, *in vivo* footprinting of the mdm2 and gadd45 p53 DNA binding sites on naked DNA and in unelutriated cells. Months 1-12:

a. The p53 responsive regions of the mdm2 and gadd45 genes will be amplified by PCR and cloned into plasmid vectors.

b. The cloned mdm2 and gadd45 binding sequences will be footprinted with immunopurified wild-type p53.

c. *in vivo* footprinting of the mdm2 and gadd45 p53 binding regions will be done in the breast cell lines with and without ts-p53val135, and also with the addition of chemotherapeutic agents.

Materials and Methods:

Growth Curve:

Plate 12, 60mM dishes with 1x 10^5 cells per plate. Let cells grow ON. On day one count 2 plates from 37°C and shift 6 plates to 31.5°C. On days 2 and 3 count 2 plates taken from 37°C. On days 3, 5 and 7 count 2 plates taken from 32°C. Count cells trypsinized in 1ml of trypsin, in this way the cells counted from the. Make a semi-log plot of the data.

Isolation of Protein from mammalian cell culture lines:

Whole cell lysates: Wash 100mM plate 2X with ice cold PBS
Extraction of cells on a 150mm dish will be lysed with 2ml of whole cell lysis buffer: (O.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 50mM NaCl, 25mM Tris.HCl (pH 7.5), 1mM PMSF, 50ug/ml aprotinin, 50uM 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.
Making Nuclear Extract: adapted from Cell 66 305-315 1991.
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:** 20mM Hepes, pH 7.5, 20% Glycerol, 10mM NaCl, 1.5mM Mg Cl_2, 0.2mM EDTA, 0.1% Triton X-100, 1mM DTT, 1mM PMSF, 50ug/ml aprotinin, 50uM leupeptin. **Lysis Buffer:** 8.8ml Stock and 6.2ml water. **Extraction Buffer:** 1.47 ml 5M NaCl, 8.8 ml of Stock, and 4.7 ml of water.

Immunoprecipitation from cell extracts and Western Blot Analysis:

Normalize for 50-60 mg total protein of whole cell extract before begin. Add 30-40ul 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 1ml 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).

Extraction of Nuclei and DNaseI Treatment:

This protocol was adapted from (37)Cells were grown on 150mM 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 2ml 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 2ml RSB with out PMSF 1X. Resuspend in any where from 1ml to 4ml of RSB w/o PMSF (make the lowest # of nuclei in 1ml for 4 tubes at 250ul 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: 10mM Tris pH7.4, 10mM NaCl, 3mM MgCl2 pH 7.4, 0.5% NP40, 1mM PMSF or absent. Treat with DNaseI for 10 min. at 37°C, using 250ul of nuclei in 0.1mM $CaCl_2$. Add 250ul DNaseI stop (with out DNA) and then add proteinase K to a final concentration of 400ug/ml and digest overnight at 37°C. Extract once with phenol and 3X with chloroform. **DNaseI stop:** 2M NH4 OAc, 100mM EDTA, 0.2% SDS. Southern Blot Analysis was carried out using the standard technique described in Protocols of Molecular Biology.

Results:

Task #1: Establish stable breast cancer lines expressing temperature sensitive p53 (ts-p53val135) Months 1-24.

a. Attempts will be made to establish ts-p53val135 expressing breast cancer cell lines.

Growth Curves on Breast Cancer Cell Lines MDA-MB-468, MDA-MB-157 and ZR75-1 grown at 37°C compared to the same lines grown at 32°C.

Temperature sensitive mouse p53, ts-p53val135, behaves as wild-type p53 at 32°C while at 37°C it is predominantly in a mutant conformation and is unable to carry out wild-type functions. These characteristics of ts-p53 val135 have been shown to behave as such in human cells (60). High levels of wild-type p53 can cause cells to either growth arrest at the G1/S border or to undergo apoptosis. To our knowledge temperature dependent growth curves on the breast cancer cell lines have not been reported. Therefore, in order to address the effect of temperature shift down on breast cancer cell lines we began variable temperature growth curves on some of the lines that we intend to use for creating stable ts-p53val135 lines. We have begun with one mutant p53 expressing cell line, MDA-MB-468, one wild-type p53 expressing cell line, ZR 75-1 and one p53 null cell line MDA-MB157. Growth conditions for the cells were optimized thereby finding optimal growth of MDA-MB 468 and MDA-MB 157 in DMEM + 10% FCS while ZR75.1 grows well in RPMI + 10% FCS. The variable growth temperature analysis, in these media, revealed that all three breast cancer cell lines tested are severely growth retarded at 32°C and the cells can grow if shifted back to 37°C (Fig. 1A, 1B and 1C). This result will affect our ability to score for p53 dependent growth arrest when we create ts-p53val135 stable clones.

Human cell lines are notoriously difficult to transfect and thus creating stable lines in the breast cancer cells is not expected to be a trivial exercise. Electroporation is well documented to be one of the most efficient modes of introducing foreign DNA into human cells. We are presently working out the optimal electroporation conditions for the cells described above. In light of the fact that the breast cell lines tested thus far are severely growth retarded at 32°C we have considered other means of increasing the cellular wild-type p53 content. It may be possible to increase p53 levels in breast cancer cell lines that already contain wild-type p53 by exposing the cells to specific drugs. We have analyzed endogenous p53 levels in the cell lines described above and have begun testing approaches for increasing the wild-type p53 level in ZR75-1 cells by exposure to a ubiquitin protease inhibitors (described in detail below).

Task #1 b: Levels of p53 in the established cell lines will be examined both at the p53 permissive and restrictive temperatures.

Levels of p53 Protein in Breast Cancer Cell Lines MDA-MB-468, MDA-MB-157 and ZR75-1.

We chose to begin growing three breast cancer lines with different p53 status; one producing wild-type p53 protein (ZR75-1), one producing mutant p53 protein (MDA-MB-468) and one producing no p53 protein due to a gene deletion (MDA-MB-157). We are interested in examining the p53/DNA interactions at the mdm2 responsive region, therefore it is helpful

that the level of mdm2 mRNA in two of the above cell lines has been reported (48). Although the mdm2 mRNA level for MDA-MB-157 has not been documented, the expression of mdm-2 in ZR75-1 is known to be very high while no detectable mdm-2 mRNA has been observed in MDA-MB-468. Therefore it is presumed that the wild-type p53 in ZR75-1 is activating the mdm2 gene while the mutant protein in MDA-MB-468 is not.

Although we have not yet established the breast cell lines expressing ts-p53val135 we have begun to examine the levels of p53 in the parent lines as we believe it is necessary to know the baseline P53 levels. Whole cell, nuclear and cytoplasmic extracts were prepared from the three cultured cell lines discussed above. Immunoprecipitation of p53 from the different extracts was carried out using the p53 monoclonal antibody PAb421 (derived from the growth medium of monoclonal antibody cells) coupled to protein A sepharose. Whole cell extracts from the three cell lines were normalized at a level of 1mg of total protein per immunoprecipitation and the nuclear and cytoplasmic extracts were then normalized via the number of starting cells. The immunoprecipitated 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. As shown in Fig. 2A (lanes 9 &10), p53 protein was clearly evident in the nuclear as well as whole cell extracts derived from the cell line MDA-MB-468. This is not surprising as this mutant p53 protein has previously been documented at high levels in this line (56). Interestingly, when using a lower dilution of secondary antibody at 1:1000 we were able to visualize wild-type p53 in the nuclear extract from ZR75-1 (Fig. 1B, lane 6 as indicated by the arrow on the left). We are unaware of wild-type p53 protein levels being reported previously for ZR75-1. From this detection it appears that the gene in ZR75-1 encodes an arginine at codon 72, a neutral sequence polymorphic site, which differs from the proline encoded by the mutant p53 in MDA-MB-468 (56) and results in a faster migrating form of the protein on SDS-PAGE. This is fortuitous as it allowed the low level of wild-type p53 to be visible right below the background band of heavy chain IgG at 55K (Fig. 1B, lane 6). Although the antibody was cross-linked to the solid support a substantial portion was released upon boiling.

Stabilization of Wild-type p53 in the Breast Cell Line ZR75-1.

In addition to attempting to create the ts-p53val135 breast cancer cell lines, we have decided to try various methods to stabilize the wild-type p53 present in the ZR75-1 line. The motivation for this is that if we increase the level of p53 we may be able to invoke further p53 specific mediated responses. Additionally the way in which p53 stabilization is induced (i.e.. inhibition of the ubiquitin proteolysis pathway versus the induction of DNA damage evoked by many chemotherapeutic agents) my differentially influence p53 protein activity. This will give us more insight into the regulation of the *in vivo* DNA binding properties of the protein. The wild-type p53 levels are thought to be low in cells due the fact that the protein is actively degraded by the ubiquitin-dependent pathway (47). Therefore we attempted to stabilize the p53 in ZR75-1 with an inhibitor of the ubiquitin-dependent proteolytic pathway, benzyloxycarbonyl-leucyl-leucyl-leucine aldehyde (2-LLL-CHO) (42). The reagent was synthesized by, and was a generous gift of, A. Vinitsky who works on inhibition of proteolytic activity (54). Interestingly, when 5.0 μM 2-LLL-CHO was added to the growth medium for increasing intervals of time, we observed an increase in the level of p53 present in comparison to the levels without drug after 4 and 7 hours of incubation in both nuclear extract and cytoplasmic extract (Figs 3A and 3B respectively, compare lane 1 to lanes 9 and

10). To our knowledge, this is the first time stabilization of p53 has been documented to occur in response to a ubiquitin proteolysis pathway inhibitor. The extracts from cells incubated in medium with 2.5 μM 2-LLL-CHO and 5.0 μM 2-LLL-CHO for 24 hours showed reduced levels of p53 protein (Figs 3A and 3B, lanes 2-8 and 11). Surprisingly, when the cytoplasmic and nuclear p53 proteins from ZR75-1 extracts are compared it can be seen that the migration of the protein on SDS-PAGE differs in the two extracts, with the cytoplasmic extract displaying faster migrating species than the nuclear. Possibly suggesting that the cytoplasmic species are degraded. The cytoplasmic forms decrease in their mobility with increasing time of exposure to the 2-LLL-CHO (Fig. 3B) while no change in the size of nuclear form is observed. We are presently beginning experiments to monitor the effects of DNA damage inducing chemotherapeutics: camptothecin, etoposide, actinomycin D and bleomycin, on the p53 levels in ZR75-1 cytoplasmic and nuclear cell extracts.

Task #2: *In vivo* footprinting of the mdm2 and gadd45 p53 DNA binding sites on naked DNA and in unelutriated cells. Months 1-12.

a. The p53 responsive regions of mdm-2 and gadd45 genes will be amplified by PCR and cloned into plasmid vectors.

b. The cloned mdm2 and gadd45 binding sequences will be footprinted with immunopurified wild-type p53.

When the grant proposal for this project was written I did not consider the possibility that other laboratories would have the clones I needed and that upon request I would be able to obtain them. However, I have been able to obtain both mdm2 and gadd45 clones that will be useful as probes for the southern blots that are necessarv for setting up, and determining the best conditions of, nuclear DNaseI treatment. Fig 4 is a copy of the letter I obtained from Dr. Donna George upon receipt of many different mdm2 containing clones. Fig. 5 is a copy of the letter obtained by my past mentor Carol Prives describing the gadd45 clone obtained from Dr. Albert J. Fornace (this clone I brought with me when I left Dr. Prives Lab). The described clones will facilitate this study.

We will begin using the mdm2 genomic subclone for *in vitro* footprints but will then move on to do *in vitro* footprints on naked genomic DNA utilizing the ligation mediated PCR conditions necessary for *in vivo* footprinting. This is a better control for the *in vivo* footprinting results that we intend to generate. In order to carry out the *in vitro* footprints it is necessary to have purified wild-type p53 from insect cells. Therefore we are in the process of growing insect cells, making virus stocks and characterizing our extracts from infected insect cells in order to purify the protein.

Multiplicity of infection of SF21 insect cells with wtp53 baculovirus.

Small aliquots of high titer p53 baculovirus stocks were provided by Dr. Carol Prives. We have used these to make large stocks of p53 containing baculovirus and have tested these stocks for their ability infect SF21 cells at different multiplicity's of infection. In addition we have examined the production of p53 in these infected cells 24, 48 and 72 hours after infection (Fig. 6). We found that at a MOI of 5:1 with a 48 hour infection period gave a significant level of wild-type p53 protein in the insect cell. extract. We used these infection conditions to generate enough insect cell extract containing wild-type p53 for immunopurification of the protein via PAb421 coupled to protein A sepharose beads.

Unfortunately upon our first attempt, our elution profile using 0.5M NaCl in 50% ethylene glycol proved unsuccessful, much of the protein remained attached the protein A sepharose. Previous purifications done in Dr. Prives's laboratory have shown that peptide specific for the PAb421 epitope is more successful at eluting the p53 protein off the beads. At the time when we tried our first purification we did not have PAb421 epitope peptide. We have since ordered and obtained it and have tested it for its ability to release the p53 that remained attached to the beads from the first protein purification trial. A solution of .0001g of peptide per 10 ml of 0.25M NaCl in buffer B was able to release the protein from the beads (data not shown). Therefore we are now growing up SF21 insect cells to prepare for our second purification trial.

Task #2 c: *In vivo* footprinting of the mdm2 and gadd45 binding regions will be done in the breast cell lines with and without ts-p53val135, and also with the addition of chemotherapeutic agents.

Construction of a mouse embryo fibroblast cell line containing temperature sensitive p53 Val 135.

Although we have not established the ts-p53val135 breast cancer cell lines, we have established a ts-p53 val135 mouse embryo fibroblast cell line from the parent line 10(1) that contains no endogenous p53 protein. This line is analogous to the cell line 10.1Val5 (24)), which expresses a temperature-sensitive mutant p53 protein (codon 135, Ala to Val change) in the absence of endogenous p53 because of a deletion of the p53 gene in this cell line. We call our 10(1)-ts-p53 cell line 3-4, and generated it via $CaPO_4$ transfection of 10-1 followed by selection of stable colonies in medium containing 400ug/ml of G418. The colonies were initially characterized for their ability to growth arrest at 32°C and there ability to contain high levels of p53 protein. The 3-4 line growth arrests when placed at 32°C while the parent line does not. The ability of mdm2 to be activated in the 3-4 cell line was assayed by Northern blotting polyA selected RNA from cells shifted to the permissive temperature (32°C) for four hours (Fig 7A, lane 1 as indicated by the arrow), and not from cells maintained at the non-permissive temperature (37°C) (Fig 7A, lane 2), with a probe to mdm2. The same blot was also probed for glyceraldehydephosphate dehydrogenase (GAPDH) message (1) as a control to show no p53 dependent activation of transcription of this housekeeping gene (Fig 7B). This line is being used to answer questions directly about the ability of p53 to bind to DNA *in vivo* as a number of controls are available to compare it to: 1) the parent line contains not p53, 2) the ts-p53val135 causes the 3-4 cell line to growth arrest at 32°C, 3) mdm2 mRNA is produced in the 3-4 cell line only when the cells are shifted to 32°C 4) we have been able to see high levels of p53 in the 3-4 established cell line and 5) transient transfection of a reporter plasmid containing p53 specific binding sites is activated when the cells are shifted to 32°C (data not shown).

The p53 Responsive Region of the mdm2 Gene is Highly Resistant to DNaseI Cleavage in the Presence of p53 While Being Sensitive to DNaseI in its Absence.

DNaseI treatment of the nuclear chromatin from the parental 10(1) cell line and the 3-4 cell line was compared for cells maintained at 37°C (Fig. 8) and those shifted to 32°C for 4 hrs was(Fig. 9). Nuclei were treated with increasing concentrations of DNaseI and in most

cases the chromatin showed sensitivity accordingly (see ethidium bromide staining of a 0.8% agarose gel containing the total nuclear DNA digested with NsiI (Figs 8B & 9B). Analysis of the sensitivity of the mdm2 region in chromatin was carried out by Southern blotting with an mdm2 specific probe after digestion with NsiI which is known to cut adjacent to the proposed p53 binding region (27). The Southern blot demonstrated differential sensitivity of the mdm2 region at the various levels of DNaseI tested between the chromatin from nuclei of 10(1) -vs- nuclei of 3-4 (Fig. 8A, compare lanes 3-7 with lanes 10-14). With no DNaseI, but in the presence of the restriction enzyme NsiI, a band between 4 and 5 kb is evident in both the 10(1) and 3-4 cell lines (Fig 8A lanes 2 & 10). For the cells maintained at 37°C (Fig. 8A) this band disappears in the 10(1) samples at the level of 8 μg of DNaseI (Fig 8A, lane 5) while it is still present in 3-4 samples upon the addition of 16 μg of DNaseI (Fig 8A, lane 13) and does not disappear until the level of 24 μg (Fig 8A, lane 13). Additionally a 1 kb species appears upon the addition of DNaseI and this increases in the 3-4 samples with the higher levels of DNaseI tested up to 16μg, but does not increase for the 10(1) samples. This increase in the stability of mdm2 fragments in the 3-4 cells as compared to the 10(1) cells is more pronounced when the cells are shifted to 32°C for 4 hrs (Fig 9A). Only a slight change in the sensitivity of the mdm2 region in the 3-4 cell line was observed in the cells maintained at 37°C as compared to those shifted for four hours to 32°C (compare Figs. 8 & 9 for 3-4 DNA). This is most likely due to the fact that at 37°C there is a mixture of mutant and wild-type p53 protein (codon 135, Ala to Val change) and that the protein is only totally mutant at 39.5°C (36). We must prepare samples from cells shifted to 39.5°C and also need to test the blots shown here for sensitivity of the GAPDH chromatin region by subsequently probed with a GAPDH. Preliminary data of unclear Southern blots suggest that there will be no difference for 10(1) and 3-4 samples observed on this gene. However, it is interesting to note that no mdm2 mRNA is synthesized at 37°C. Therefore while the p53 may be capable of binding to DNA at 37°C it is not able to activate transcription. We are in the process of these experiments and are beginning *in vivo* footprinting experiments on the samples of the DNA shown here.

In vivo Footprinting of Breast Cancer Cell Line DNA

We will soon begin comparative DNAse I sensitivity Southern blot analysis for p53 responsive regions within the different breast cell lines. These experiments will first be carried out in the ZR75-1 line under conditions where we are best able to stabilize the wild-type p53 and this can be compared to the results obtained in the presence of basal endogenous wild-type p53. As stated previously the conditions under which the above described breast cancer cell lines can be efficiently electroporated are presently being determined.

Conclusions:

During moths 1-12 I have succeeded in setting up my laboratory and paving the way to answer the questions addressed in the grant proposal. We have characterized the effect of temperature shift down to 32°C on the cell growth of the three breast cancer cell lines described. We have also characterize the level of wild-type p53 in ZR75-1 and mutant p53 in MDA-MB-468, two breast cancer cell lines that are characterized for mdm2 mRNA expression. Additionally we stabilized wild-type p53 in the ZR75-1 cell line by the addition of 2-LLL-COH. We have also shown that at comparable levels of total protein there is no detectable p53 in the cell line MDA-MB-157. This will enable us to begin monitoring

p53/DNA interactions directly in these lines. We are setting up conditions to construct and select the above lines as stables expressing the temperature sensitive p53 val135 mutant and have selected such a line in the mouse embryo fibroblasts cells. This cell line will give us additional comparisons in order to elucidate the way p53 activity is controlled.

We have demonstrated that the p53 responsive region of the mouse mdm-2 gene is clearly more sensitive to DNaseI in the absence of p53 than in the presence of the tumor suppressor protein. Interestingly, the effect of the protein is evident in cells maintained at 37°C (Fig. 8A), even though these cells do not express mdm2 mRNA (Fig. 7A). This suggests that there are conditions under which p53 can bind to the DNA but cannot activate transcription. This is one of the possibilities we will address in our future experiments as we are interested to see if we can purify mutant p53 as well as wild-type p53 via DNA affinity chromatography. Thus far we have generated samples that we expect to give informative *in vivo* footprints when probed by the ligation mediated PCR method (39). We have designed oligonucleotide probes to *in vivo* footprint the mdm2 responsive region (see Fig. 10) from the samples shown in Figs. 8 and 9 and presently are testing these to see if the melting temperatures predicted are in fact real in solution.

We will immunopurify baculovirus p53 as well as the mutant p53 from MDA-MB-468 in the near future. These preparations of purified proteins will allow us to carry out *in vitro* footprinting experiments as well as to screen for sequences that mutant p53 may bind to site-specifically.

In conclusion, although we have not yet completed all the tasks outlined in the SOW for month 1-12 we have made progress towards answering the major questions addressed.

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FIGURE 1A
MDA-MB-468

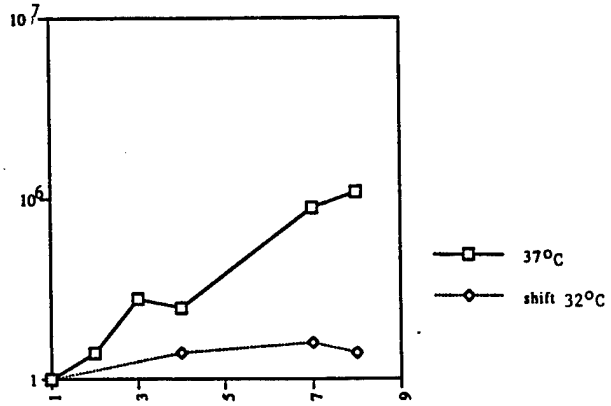


FIGURE 1B
MBA-MB-157

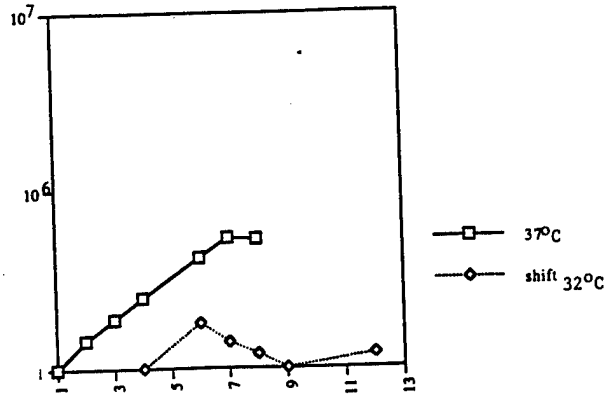


FIGURE 1C
ZR75-1

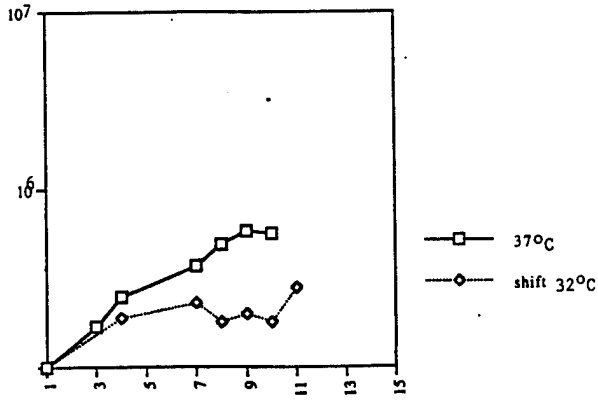
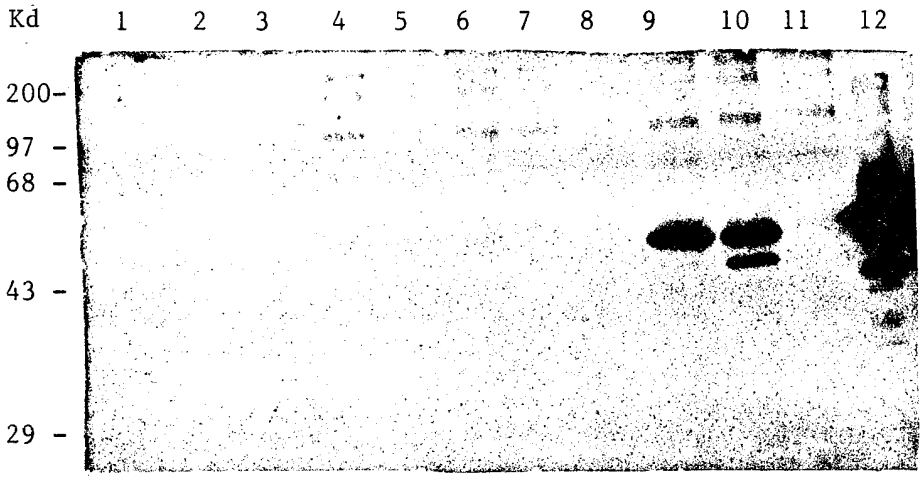


Fig.1: Growth curves of MDA-MB-468, MBA-MB-157 and ZR75-1 at 37°C and 32°C.
Growth curves were carried out as described in the methods section. **A)** Diagrams the growth curves for MDA-MB-468, **B)** diagrams the growth curves for MDA-MB-157, **C)** diagrams the growth curves for ZR75-1. For both the 32°C curves presented in B) and C) the cells were shifted back to 37°C on day 9.

FIGURE 2

A)



B)

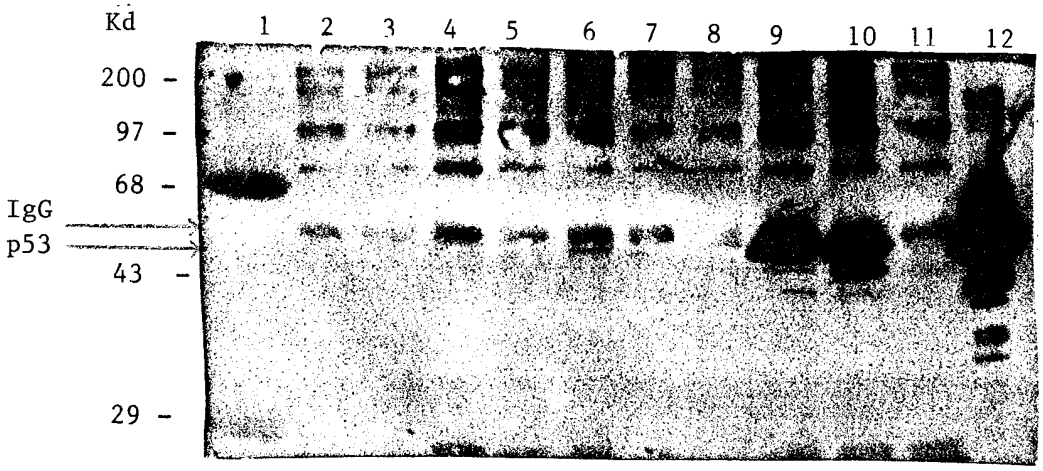


Fig. 2: Levels of p53 Protein in Breast Cancer Cell Lines MDA-MB-468, MDA-MB-157 and ZR75-1.

Immunoprecipitation was carried out as described in materials and methods. A) contained goat anti- mouse (Cappel) diluted 1:1500 while B) was with a dilution of 1:1000. Lane 1 contains prestained marker (BRL), lanes 2-4 contain MDA-MB-157 cytoplasmic, nuclear and whole cell extract respectively; lanes 5-7 contain ZR75-1 cytoplasmic, nuclear and whole cell extract respectively; lanes 8-10 contain MDA-MB-468 cytoplasmic, nuclear and whole cell extract respectively. Lane 11 contains boiled beads rocked only with PBS and lane 12 purified p53 standard. The p53 species for ZR75-1 is indicated to the left in B.

FIGURE 3

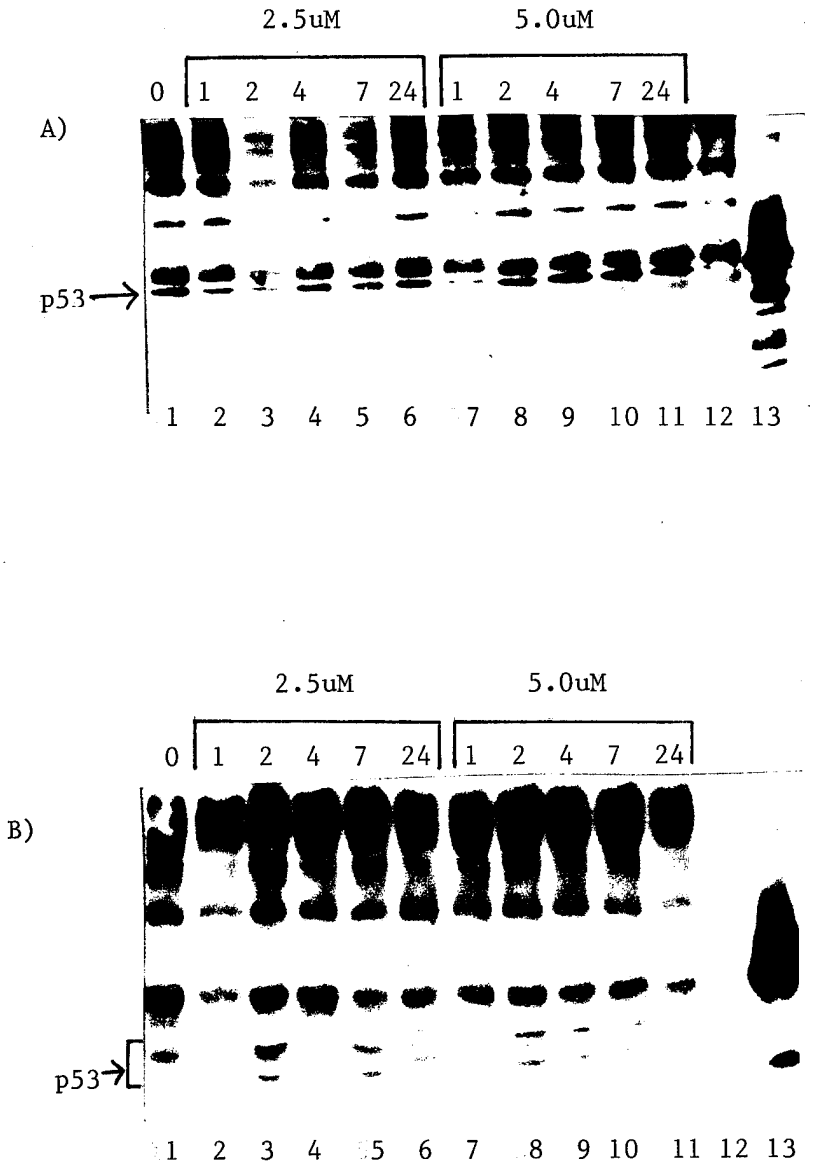


Fig. 3: Stabilization of Wild-type p53 in the Breast Cell Line ZR75-1. ZR75-1 cells were cultured in 150mm dishes at 37°C: the cells were treated with 2-LLL-CHO (2.5uM or 5.0uM) with incubation times of: 1, 2, 4, 7 and 24 hours. After the respective incubation times nuclear **A)** or cytoplasmic **B)** cell extracts were prepared and immunoprecipitated as described in methods. The products were subjected to western blot analysis, probing with a mixture of anti-p53 antibodies PAb1801, PAb 240 and PAb421. Lane 1 contains untreated extract, lanes 2-6 contain extracts from cells treated with 2.5uM 2-LLL-CHO for 1, 2, 4, 7 or 24 hours, lanes 7-11 contain extracts from cells treated with 5uM 2-LLL-CHO, Lane 12 contains extract from beads with PBS in A) and no sample in B), Lane 13 contains purified p53 from insect cells.

FIGURE 4

Letter from Dr. Donna George describing the mdm2 clones.

UNIVERSITY OF PENNSYLVANIA MEDICAL CENTER

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December 7, 1994

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Dear Jill,

I must apologize for the delay in sending the material that you requested. Some individuals have indicated to me that the mouse mdm2 cosmid clone that you requested (2A43/CV001) tends to rearrange under some growth conditions, and we have been carrying out a series of tests to make sure that our glycerol stocks and cosmid clones have not undergone such rearrangement. We have not detected any problems with this cosmid; however, you may wish to grow 2A43/pCV001 cultures in kanamycin, rather than ampicillin, and keep the culture volumes smaller (not more than 500 ml cultures).

The cosmid insert is in the bacterial strain DH5a, that has been streaked onto an LB amp agar slant. An EcoRI digest of this cosmid should yield fragments of the following size, in kb (approximately): 10 (vector), 11, 6.8, 3.0, 2.8, 2.5. The 6.8 kb fragment does not occur in the genome, but actually results from the fusion of a 4.8 kb 3' genomic fragment with a 1.5 kb fragment that was created during the construction of this cosmid. It has not affected the mdm2 expression pattern seen with transfections carried out using this clone. The insert material contains the mdm2 coding exons, as well as introns and 5' and 3' flanking material. The genomic DNA was inserted into the EcoRI site of the vector pCV001 (see EMBO J., 10:1565, 1991), which is also enclosed.

A genomic subclone is enclosed, containing a roughly 3kb EcoRI fragment, cloned into pGEM; this DNA contains mouse mdm2 promoter sequences as described in the enclosed manuscript (~~Barak~~ Juven et al.). A bacterial culture harboring this clone also has been streaked on LB amp agar.

Also enclosed is the mouse mdm2 cDNA clone 1-489 (CMV-B101). The insert is about 3 kb, contains all of the mouse coding material as well as some 5' and 3' untranslated material; it is cloned into the EcoRI site of CMV5 (see enclosed). The sequence information that we have on the B101 clone is included.

Finally, a sample of a human mdm2 cDNA, hmdm2-3 is enclosed. It has an approximately 1.3 kb insert fragment cloned into the EcoRI site of pBluescript via PCR protocols. On Southern blots, this probe hybridizes to bands about

9.5, 4.4, 4 and 2 kb in size. This cDNA contains most of the coding region, but we have not done enough of an analysis to know if it may have spliced out an exon, as do many mdm2 transcripts.

Thank you for agreeing not to distribute these clones to other investigators, and I would appreciate hearing of any results that you may obtain with them.

Sincerely,

Donna George

Donna George, Ph.D.

FIGURE 5

Letter form Dr. Albert J. Fornace describing the gadd45 clone.

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

Lab. of Molecular Pharmacology
National Cancer Institute
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email: fornace@fcrfv1.ncifcrf.gov

May 20, 1993

Dr. Carol Prives
Dept of Biological Sciences
Sherman Fairchild Center for the Life Sciences
Columbia University in the City of NY
NY, NY 10027

Dear Dr. Prives:

Per Mike Kastan's request, I am sending you the requested gadd45 clone. I request that you not distribute it without my permission and that it not be used for commercial purposes (NIH rule). Find enclosed pXR45m which is a nearly full-length Chinese hamster *gadd45* cDNA clone. It can be excised with KpnI and SacI with a resultant 1.2 kb insert fragment. The vector is Bluescript SK and the host is XL1 Blue (grow in ampicillin). After receipt streak out on an amp plate and pick a representative colony. The sequence of *gadd45* can be found in "Papathanasiou, M.A., Kerr, N., Robbins, J.H., Mc Bride, O.W., Alamo, I., Jr., Barrett, S.F., Hickson, I., and Fornace, A.J. Jr.: Induction by ionizing radiation of the gadd45 gene in cultured human cells: lack of mediation by protein kinase C. Molec. Cell. Biol. 11: 1009-1016, 1991." I have also included a map that you may find useful. Please keep me informed of any interesting results.

Best regards.

Sincerely,

Albert J. Fornace Jr., M.D.

FIGURE 6

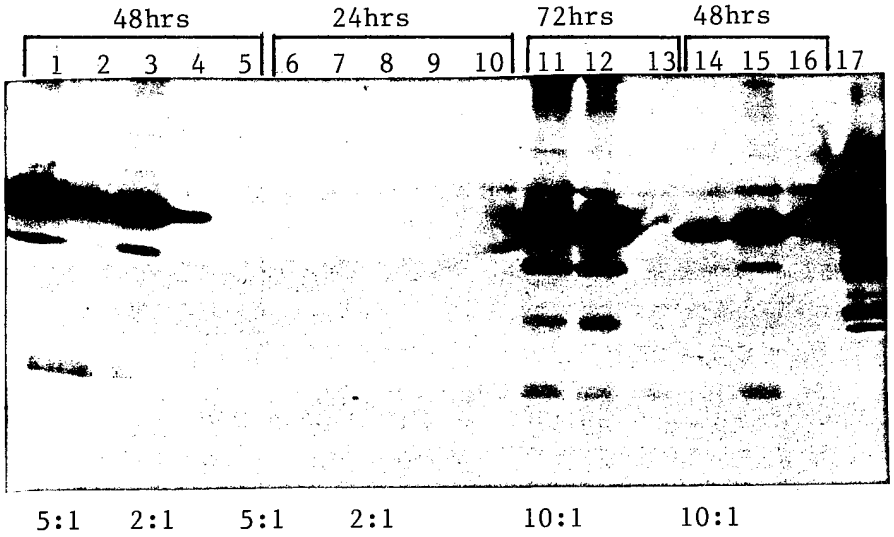


Fig. 6: Immunoprecipitation of p53 protein from SF21 insect cell extracts differentially infected with baculoviruses.
Immunoprecipitation of p53 was carried out as described in methods. Lanes 5, 10, 13 and 16 contain extract from uninfected insect cells. Insect cells were infected with baculovirus at a multiplicity of infection (MOI) of 2:1 for wild-type p53 (lanes 3 and 8) or 2:1 for His 273 mutant p53 (lanes 4 and 9) and incubated for either 48 or 24 hours as indicated. Insect cells were also infected with baculovirus at a MOI of 5:1 for wild-type p53 (lanes 1 and 6) and His 273 mutant p53 (lanes 2 and 4). and incubated for either 48 or 24 hours as indicated. Finally, insect cells were infected with baculovirus at a MOI of 10:1 for wild-type p53 (lanes 11 and 14) and His 273 (lanes 12 and 15) for either 72 or 48 hours as indicated. Lane 17 contains purified p53 from insect cells.

FIGURE 7

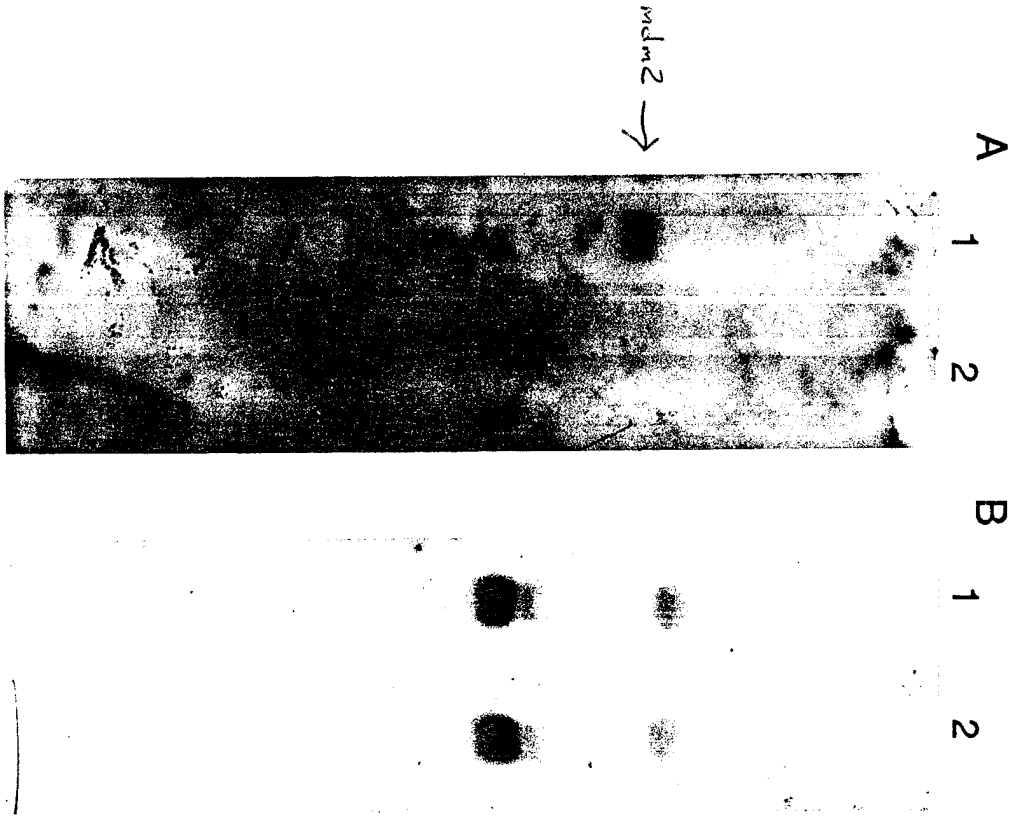


Fig. 7: Northern Analysis of mdm2 from 3-4 at 32°C -vs- 37°C.
Poly A RNA from 3-4 was isolated from cell shifted to 32C for 4 hrs (lane 1) or maintained at 37C (lane 2) and run on a 1% agarose denaturing gel. The gel was transferred and probed with A) either an mdm2 specific probe or B) a GAPDH specific probe.

FIGURE 8

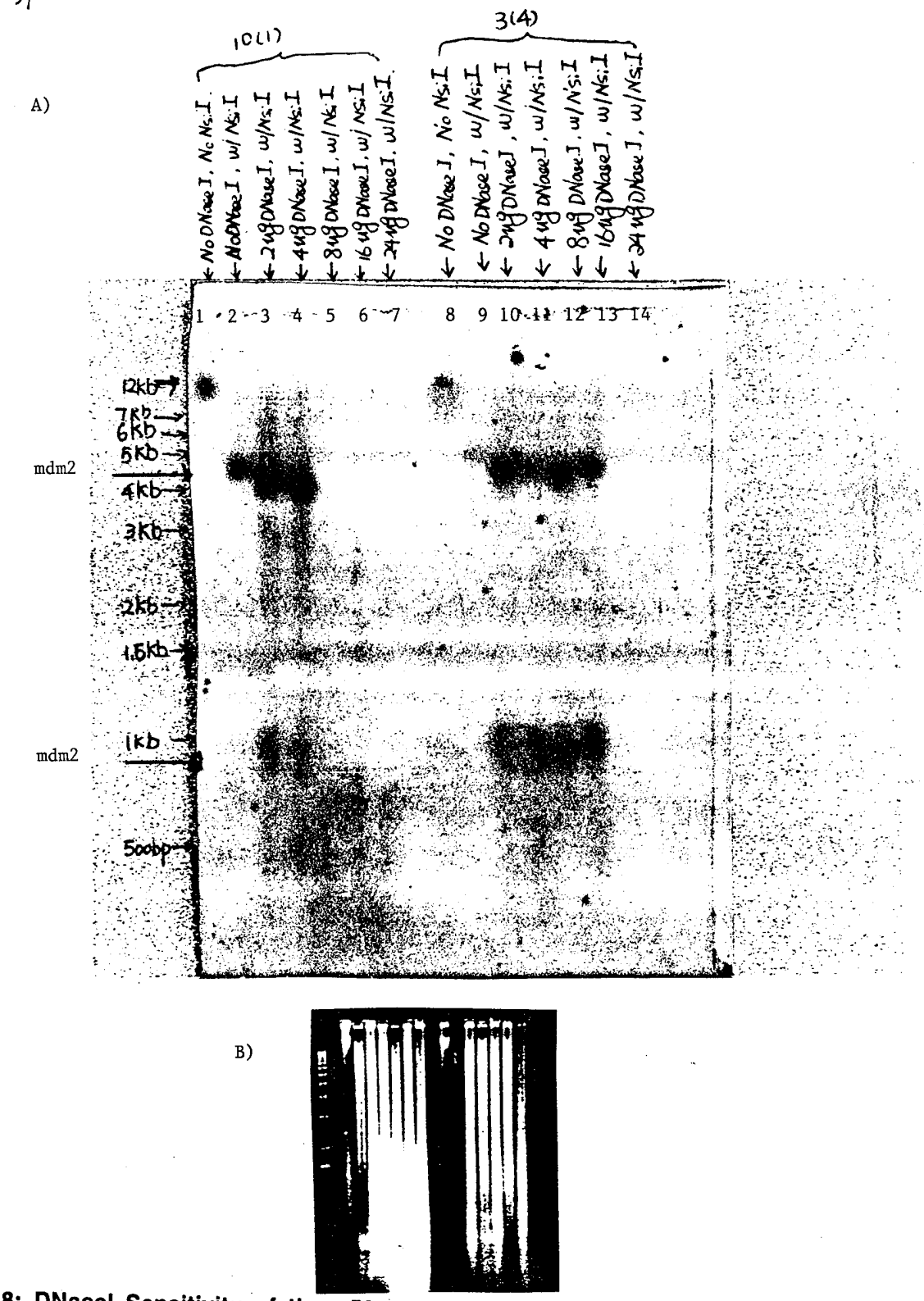


Fig. 8: DNaseI Sensitivity of the p53 responsive region of the mdm-2 gene in 10(1) and 3-4 at 37°C.

Samples were prepared as described in materials and methods. Lanes 1-7 are DNA samples from 10(1) cells. Lane 1 contains no DNaseI and no NsiI, lanes 2-7 are NsiI digested; lane 2 contains no DNaseI, lane 3 contains 2ug of DNaseI, lane 4 contains 4ug of DNaseI, lane 5 contains 8ug of DNaseI, lane 6 contains 16ug of DNaseI, lane 7 contains 24ug of DNaseI. Lanes 8-14 are DNA samples from 3-4 cells. Lane 8 contains no DNaseI and no NsiI, lanes 9-14 are NsiI digested. lane 9 contains no DNaseI, lane 10 contains 2ug of DNaseI, lane 11 contains 4ug of DNaseI, lane 12 contains 8ug of DNaseI, lane 13 contains 16ug of DNaseI, lane 14 contains 24ug of DNaseI.

C:\DATA\wmdm2.ds , Range = 1.000-49.97 Counts, 100%

FIGURE 9

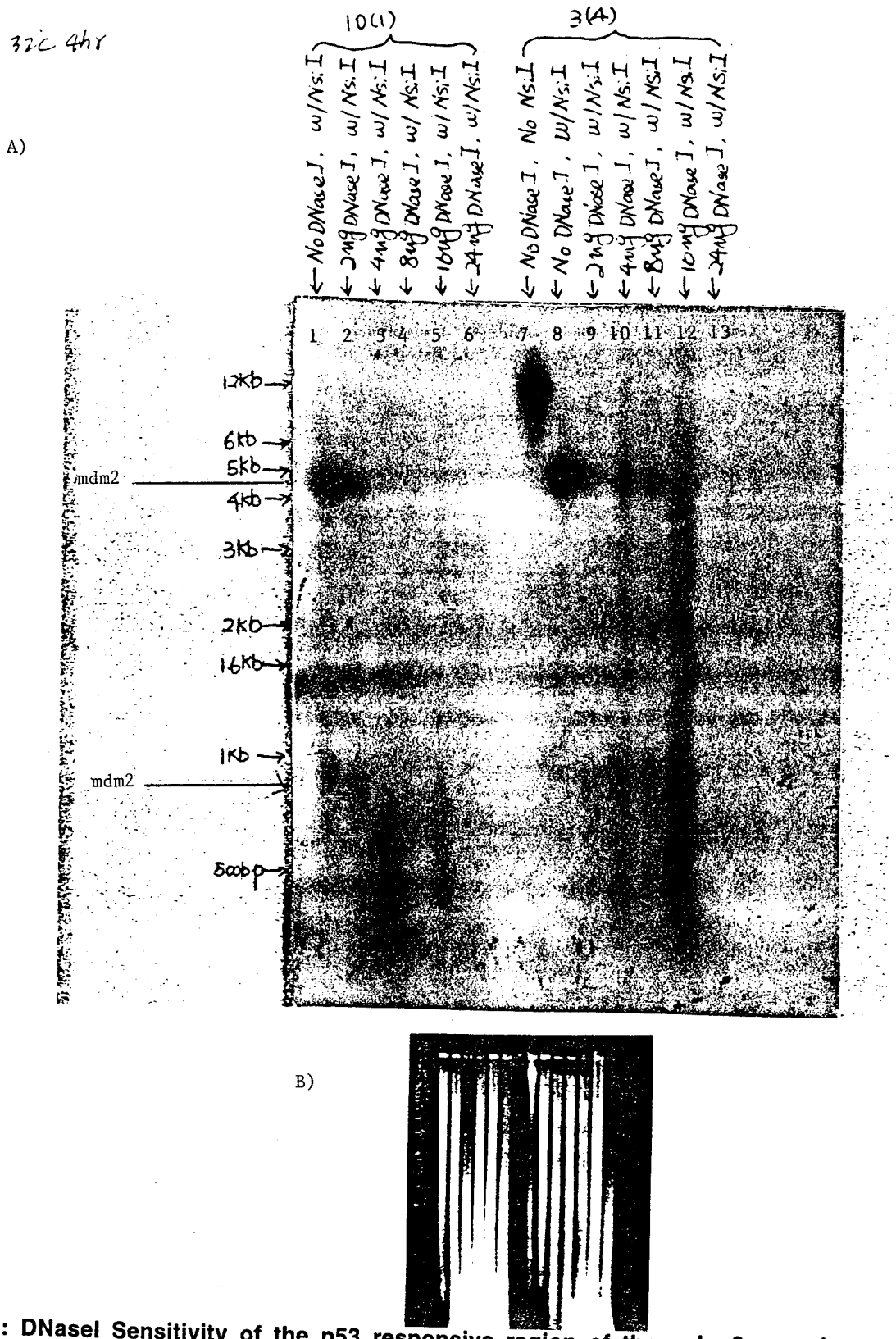


Fig. 9: DNaseI Sensitivity of the p53 responsive region of the mdm-2 gene in 10(1) and 3-4 at 32°C.

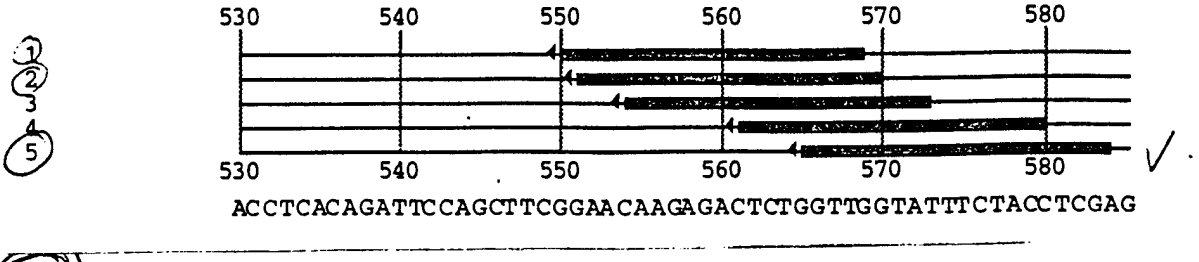
Samples were prepared as described in materials and methods. Lanes 1-6 are DNA samples from 10(1) cells. Lanes 1-6 are NsiI digested; lane 1 contains no DNaseI, lane 2 contains 2ug of DNaseI, lane 3 contains 4ug of DNaseI, lane 4 contains 8ug of DNaseI, lane 5 contains 16ug of DNaseI, lane 6 contains 24ug of DNaseI. Lanes 7-13 are DNA samples from 3-4 cells. Lane 7 contains no DNaseI and no NsiI, lanes 8-13 are NsiI digested. lane 8 contains no DNaseI, lane 9 contains 2ug of DNaseI, lane 10 contains 4ug of DNaseI, lane 11 contains 8ug of DNaseI, lane 12 contains 16ug of DNaseI, lane 13 contains 24ug of DNaseI.

Primer #1

Analysis settings:
primer size: 20 - 30
Tm (°C): 48 - 53
percent G+C content: 45 - 55
3' dinucleotide: NN

Maximum consecutive bonds allowed:
primer vs. primer (any): 4
primer vs. primer (G-C only): 2
3'-end vs. 3'-end: 2
3'-end vs. sequence: 5

FIGURE 10

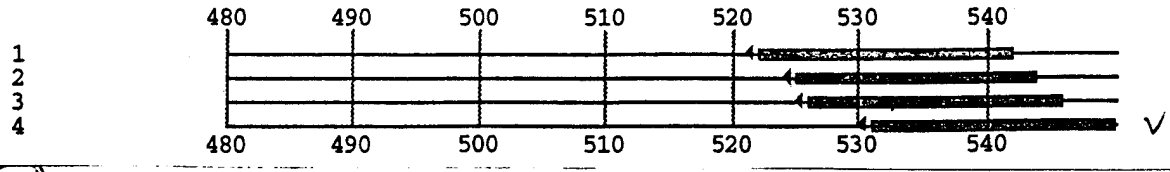


[5] 584-565 5'- TCGAGGTAGAAATACCAACC -3'
20 nt primer on minus strand
pct G+C: 45.0 Tm: 52.3

Primer #2

Analysis settings:
primer size: 18 - 25
Tm (°C): 54 - 58
percent G+C content: 45 - 55
3' dinucleotide: NN

Maximum consecutive bonds allowed:
primer vs. primer (any): 4
primer vs. primer (G-C only): 2
3'-end vs. 3'-end: 2
3'-end vs. sequence: 5

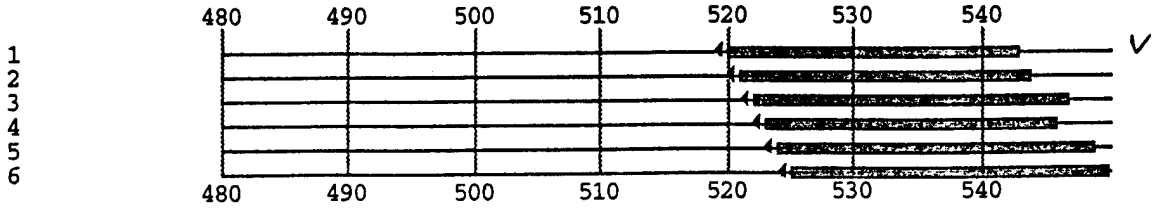


[4] 550-531 5'- CGAAGCTGGAATCTGTGAGG -3'
20 nt primer on minus strand
pct G+C: 55.0 Tm: 55.3

Primer #3

Analysis settings:
primer size: 20 - 28
Tm (°C): 60 - 68
percent G+C content: 45 - 55
3' dinucleotide: NN

Maximum consecutive bonds allowed:
primer vs. primer (any): 4
primer vs. primer (G-C only): 3
3'-end vs. 3'-end: 3
3'-end vs. sequence: 6



[1] 543-520 5'- GGAATCTGTGAGGTGCTTGCAGCA -3'
24 nt primer on minus strand
pct G+C: 54.2 Tm: 60.8

Fig. 10: Primer Choice for Ligation-Mediated PCR.
The general principles involved in ligation-mediated PCR (LM-PCR) are described by (39). Oligonucleotides should be designed as a set of three such that the region of interest in the footprinting experiment is within 200-300 base pairs form the 3' end of oligo #3 and that they have increasing melting tempertures (Tms). The program MacVector was used to select possible probes and we have chosen from that selection. The probe selected for each of the three is designated and the Tm reported.