STUDY OF EPIDERMAL GROWTH FACTOR-MEDIATED GROWTH INHIBITION IN MDA-468 HUMAN BREAST CANCER CELLS: A ROLE FOR THE TUMOUR SUPPRESSOR p53 IN CELL CYCLE ARREST

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By

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A thesis submitted to the School of Graduate Studies
in partial fulfilment of the requirements for
the degree of Doctor of Philosophy

Division of Basic Medical Sciences
Faculty of Medicine
Memorial University of Newfoundland

January, 1994

St.John's Newfoundland

Youth and beauty vanish, life and wealth vanish, name and fame vanish, even the mountains crumble into dust. Friendship and love vanish. Truth alone abides. God of truth, be Thou alone my guide !

-- *Swāmi Vivēkānanda.*

This thesis is dedicated to my family and friends

ABSTRACT

Epidermal growth factor (EGF), in pharmacological concentrations, inhibits the cellular proliferation of MDA-468 human breast cancer cells. In this study, we characterized this unusual phenomenon by means of cell cycle and Northern blot analysis. Following EGF treatment, cell number in G1 phase increased, with a concomitant depletion of cells in S and G2/M phases of the cell cycle, as revealed by flow cytometric analysis of DNA content. DNA synthesis, as measured by incorporation of [^{3}H] thymidine, was reduced to about 35% of that measured in control cells after 48 hours of EGF treatment, confirming the earlier observation of G1 arrest. Moreover, DNA synthesis returned to normal following the removal of EGF from the growth arrested cells. Northern blot analysis revealed that EGF treatment did not alter the induction of early G1 marker, c-myc, nor expression of the late G1 markers, proliferating cell nuclear antigen and thymidine kinase. However, EGF treatment resulted in the downregulation of p53 and histone 3.2 steady-state mRNA levels. Increased levels of these gene transcripts are observed at the G1/S boundary and in S phase, respectively. These results indicated that EGF reversibly blocks the cell cycle of MDA-468 cells at the G1/S boundary.

The observation of lowered mRNA levels of p53 (a point mutant, $p53^{273.His}$) led us to hypothesize its possible involvement in EGF-mediated G1 arrest. The wild-type p53 is generally regarded as a tumour suppressor and mutations in p53 are commonly seen in a wide variety of cancers. Since it has been suggested that this

particular mutant $p53^{273.His}$, might have gained an alternative function and act positively to enhance cell proliferation, we hypothesized that EGF-induced G1 arrest might be mediated by changes induced in $p53^{273.His}$. In order to test this hypothesis, an in-depth analysis of EGF effect on $p53^{273.His}$ was undertaken.

In our studies, no immediate effects of EGF-treatment were observed with regard to mRNA and protein levels, protein stability, and protein synthesis of $p53^{273.His}$ in MDA-468 cells. Interestingly, an EGF-dependent altered conformation of p53 was indicated by immunofluorescence studies. These experiments demonstrated a decreased PAb 240 (mutant-specific anti-p53) reactivity of nuclear $p53^{273.His}$ in EGF-treated cells, while PAb 1801 and PAb 1620 (pan-specific and wild-type-specific anti-p53 antibodies respectively) continued to detect the nuclear presence of $p53^{273.His}$. Further studies indicated a decreased phosphorylation of $p53^{273.His}$ in EGF-treated cells. The EGF-dependent conformation shift and lowered phosphorylation levels of nuclear $p53^{273.His}$ were detected early enough to be attributed as causative of EGF-mediated cell cycle arrest.

In order to obtain further confirmation for the observed EGF-dependent altered conformation, and to test its functional significance in terms of transcriptional regulation by $p53^{273.His}$, DNA-binding and transactivation assays were performed. We detected specific complexes of $p53^{273.His}$ with both CON and FRA oligonucleotides, two of the known p53-DNA binding sites. Furthermore, in transient transfection assays, these sequences mediated p53-specific transcriptional modulation,

namely transactivation through CON, and repression through FRA. These experiments, indicated a distinct function for $p53^{273.His}$ in MDA-468 cells. Interestingly enough, EGF-treatment of MDA-468 cells, resulted in increased DNA-binding ability of $p53^{273.His}$ to both CON and FRA. In addition, EGF potentiated p53-mediated transcription from a minimal promoter. Taken together, this study, has provided significant insights into EGF-mediated growth inhibition in MDA-468 breast cancer cells and furnished enough evidence to implicate the involvement of an endogenous mutant p53 in EGF signal transduction. Furthermore, the data presented, suggest a novel and unique function for $p53^{273.His}$ which may be cell-type specific.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Jon Church, for his patient guidance during the entire period of my Ph.D. training and in preparation of this manuscript. Thanks to Dr. George Carayanniotis, for his input in this research project as a member of the research supervisory committee and for the critical reading of the thesis draft. I would also like to extend my acknowledgements to Drs. Peter Howard, and Ban Younghusband, for their useful guidance as supervisory committee members for various durations. Thanks to Drs. Verna Skanes, Roger Green, Thomas Michalak, and Keith Egger for serving as members of the comprehensive examination committee. I am grateful to the School of Graduate Studies, the Faculty of Medicine, Memorial University of Newfoundland and the National Cancer Institute of Canada for the financial support during this training period.

In addition, I would like to acknowledge the receipt of a number of reagents as gifts from the following researchers. Thanks to Drs. Ron Buick, George Carayanniotis, Thomas Michalak, Alan Pater, Mary Pater, Renato Baserga, Susan Conard, William Marzluff, Heinz-Kurt Hoechkoppel, and Samuel Benchimol. I am also thankful to Drs. Gary Paterno and Laura Gillespie for their input in this project from time to time.

My sincere gratitude goes to Dr. Chet Michalaski, former Assistant Dean of Research and Graduate Studies, Faculty of Medicine, for his understanding and

support especially during tough times. I was fortunate to receive support, both technical and moral, from a number of friends for the last four and a half years. I am thankful to all those people including Drs. Harikrishna Nakshatri, Srinivasa Dhanakoti, Shivaramakrishna Prasad, Ramadas Gowda, Narasimha Swamy, Rakesh Mittal, Umesh Kumar, and Kouichiro Tsutsusmi. My special thanks to Drs. Rakesh Mittal and Umesh Kumar for their interest in this research project and for the helpful discussions/input which was of immense importance to me and to my project. And thanks to all those who made this place feel like home.

In addition, I would like to express my gratitude to all my teachers for their guidance. In particular I am thankful to Mr. Ganesha Shastri Sringeri, who taught me English for the first time at Kunchebyle Elementary School, Mr. Ananthaiah Hande Udupi, Head Master of The UKB High School and Maths teacher, Dr. C.V. Bhat, Professor of Maths at MGM college, Udupi. I am highly grateful to Dr. A.V. Rai, Former Director of Instruction, and Professor of Genetics at Veterinary College, Bangalore, Dr. R. Raghavan, Professor of Veterinary Microbiology, Dr. R. Narayana, Professor of Pharmacology, Dr. Sheshadri, Professor of Pathology, Dr. Chandramouli, Professor of Anatomy, and Dr. Subba Rao, Professor of Meat Science, for their inspiration and guidance during my career as a student of veterinary science. In addition, I was fortunate enough to be guided by a number of individuals with high moral standards and principles. My sincere thanks to all those people especially, Dr. Thippeswamy, Dr. Sathyanarayana, Dr. Narayana Bhat, Mr. Arun Joshi, & Mr.

Sudheer (Bangalore), and Mr. Raghavendra Kamath, Mr. Chandru & Mr. Nirmal Kumar (Udupi).

Lastly, I once again wish to acknowledge the love and support from my family, Amma, Appa, Shanti, Sheela, Shruti, Dattatreya and Suresh, and from my extended family of best friends in Bangalore Drs. Basavaraja Naregal (Basya), Ravindra Jahagirdhar (Jaggu; now in Regina, Saskatchewan), Nagabhushan (Kyatha), Manoj Kumar (now in College Park, Maryland), Harshakumar Shetty, Jayant Deshpande (Pande), Suresh Belliyappa (Kutti), Nadeem, Shashikanth, Ajit kumar, Rajeev Shetty, Raveendra Hegde (Yabbaddhu), Prasanna (Belura), Divakara (LTTE), Ranganathaiah, Thammaiah (Thimma), Keshava, Raghavendra Bhatta, Eshwaran (now deceased), Darur, Srinivasa (Haga), Murulidhara, Murulikrishna (Appu), Sampath Kumar, Basavanagowda Patil & Jayaprakash and Mr. Ravi, Mr. Shankar, Mr. Gajendra, & Mr. Vijay of Gangenahalli. Constant encouragement and support from these people was invaluable in my training . I am indebted to them all.

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LIST OF ABBREVIATIONS

β-gal	β-Galactosidase
AP1	Activator protein 1
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl transferase
CBF	CCAAT binding factor
cdc	Cell division control
CDK	Cyclin-dependent kinase
CK II	Casein kinase II
CMV	Cytomegalovirus
CON	Consensus sequence
CREBP	cAMP response element binding protein
DNAPK	DNA (double strand)-dependent protein kinase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal regulated kinase
FACS	Fluorescence-activated cell sorting/sorter
FITC	Fluorescein isothiocyanate
FRA	Fragment A
G protein	Guanine nucleotide binding protein
G1/G2	Gap 1/2 (cell cycle phases)
GADD	Growth arrest/DNA damage
GAP	GTPase activating protein
GEF/GRF	Guanine nucleotide exchange/release factor
Grb2	Growth factor receptor binding (protein) 2
HPV	Human papilloma virus
hSos1	Human (homolog) Son of sevenless
IL	Interleukins
IP3	Inositol triphosphate
IRS1	Insulin receptor substrate 1
M-phase	Mitotic phase (a cell cycle stage)
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MARCKS	Myristylated alanine rich C kinase substrate
MCK	Muscle creatinine kinase
MDM2	Mouse double minute 2
MDR1	Multiple drug resistance 1

MEK	Mitogen activated/extracellular signal regulated kinase kinase
MPF	Mitosis/Maturation promoting factor
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositol 3' kinase
PIP2	Phosphotidylinositol 4,5-biphosphate
PKA	Protein kinase A (cAMP dependent protein kinase)
PKC	Protein kinase C
PL	Phospholipase
RB	Retinoblastoma (susceptibility)
RCE	Retinoblastoma control element
REF	Rat embryo fibroblasts
RGC	Ribosomal gene cluster
RSK	Ribosomal S6 subunit kinase
RSV	Rous sarcomavirus
S-phase	Synthesis (of DNA) phase (a cell cycle stage)
SH2, and SH3	Src homology (domains) 2 and 3
SPF	S (DNA synthesis) phase promoting factor
STP	Signal transfer particle
SV40	Simian Virus 40
T antigen	Tumour antigen
TAF	TBP associated factors
TBP	TATA binding protein
TCA	Trichloroacetic acid
TFIID	Transcription factor II D
TGF	Transforming growth factor
TK	Thymidine kinase
TLC	Thin layer chromatography

1. INTRODUCTION

GROWTH FACTOR SIGNAL TRANSDUCTION AND CELL CYCLE REGULATION

1.1 GROWTH FACTORS AND CANCER

The normal regulation of growth and the proliferation of embryonic and adult tissue involves a cascade of molecular events in response to the external environment. Growth factors are short polypeptide hormones known to mediate the interaction of a cell with its microenvironment or immediate surroundings. These factors behave as regulators of cell proliferation, and influence cellular differentiation. In general, growth factors function through interaction with a specific cell membrane receptor protein. This interaction initiates a series of molecular events through which the signal passes to the nucleus (Reviewed in Ullrich & Schlessinger, 1990; Pazin & Williams, 1992). In the nucleus, such a signal is generally considered to be responsible for alterations in cellular transcription, leading to the synthesis of proteins essential for cell proliferation or differentiation.

Cancer, the uncontrolled proliferation of body cells, is widely considered to involve the accumulation of genetic aberrations in a progressive manner (Vogelstein & Kinzler, 1993). These genetic changes, as revealed from studies of a wide variety of cancers, often affect one or more components of growth factor signal transduction pathways (Aaronson, 1991). For example, overexpression of the epidermal growth factor receptor (EGFR) is commonly observed in breast cancer as a result of gene

amplification (Gusterson, 1992). Ras, the product of a cellular proto-oncogene c-ras, believed to mediate many receptor-generated signals, is frequently found in activated form in a number of tumours (Bishop, 1987). Studies with transforming viruses have provided further insight into the relevance of growth factors in tumorigenesis. Several transforming retroviruses encode products which are highly homologous to various growth factors or their receptors in activated forms. For example, the v-sis oncogene of simian sarcoma virus encodes a protein similar or almost identical to the active form of platelet derived growth factor (PDGF)(Doolittle et al., 1983); v-erbB2 oncogene of avian erythroblastosis virus encodes a truncated form of the EGFR (Downward et al., 1984). This line of evidence suggested that constitutively active components of growth factor signal transduction pathways may be the molecular mechanism of viral transformation (Darnell et al., 1986).

Similarly, several components of signal transduction pathways possess transforming potential when overexpressed in active forms under in vitro conditions. Cellular counterparts of many transforming oncogenes such as raf, src, and ras, have turned out to be important elements in growth factor signalling (Heldin & Westermark, 1989). Overexpressed EGFR-like $p185^{HER2/neu}$ transforms rodent fibroblasts (Hudziak et al., 1987), whereas, a mutant/activated form induces mammary tumours in mice (Muller et al., 1988). Furthermore, recently Egan et al. (1993), reported that a Drosophila gene, Sos (Son of sevenless) believed to be a modulator of Ras, transforms rodent fibroblasts (See also Section 1.2.3). Accordingly, growth

factors and the components of the signal transduction pathways are an important part of cancer research. A thorough understanding of molecular mechanisms of growth factor signal transduction will translate into a better knowledge of tumorigenesis and may ultimately lead to the development of strategies for efficient cancer therapy and prevention.

1.2 GROWTH FACTOR SIGNAL TRANSDUCTION

1.2.1. Receptors and immediate early events

As introduced in Section 1.1, growth factors initiate a cellular response by means of binding to cell-surface receptors. Several growth factors, including the well-studied platelet derived growth factor (PDGF) and epidermal growth factor (EGF), interact with receptors bearing tyrosine kinase activity (Ullrich & Schlessinger, 1990). Some other members of the growth factor family, bind to proteins lacking such enzymatic function. Examples of such receptors are transforming growth factor (TGF)-β receptors I and II, receptors for cytokines like interleukin (IL) 2, IL6 etc (Foxwell et al., 1992). Nevertheless, receptors with tyrosine kinase activity are a primary focus of research. Signal transduction mediated by receptor tyrosine kinases is discussed in detail below.

The receptor tyrosine kinases are membrane-localized proteins with intrinsic tyrosine kinase activity. These proteins have an amino-terminal extracellular domain linked by a short trans-membrane domain to a cytoplasmic carboxyl-terminal domain. The catalytic function of the receptor resides in the cytoplasmic domain, whereas the

extracellular portion is responsible for the ligand binding. Receptors for various growth factors differ in structure and the occurrence and distribution of receptors depends on tissue type, and perhaps, determines the differential responsiveness of cells to various growth factors.

Research so far has revealed several cellular events occurring immediately following receptor binding (Reviewed in Ullrich & Schlissenger, 1990; Pazin & Williams, 1992). Briefly, binding induces receptor oligomerization and activates receptor tyrosine kinase activity leading to an intermolecular receptor autophosphorylation. This is thought to facilitate the binding and phosphorylation of other cellular proteins to the activated receptor. Such a complex of proteins in association with the activated receptor is termed a "signalling complex" (signal transfer particle; STP). Some of the proteins associated with such a signalling complex have been identified. They include GTPase activating protein (GAP) of Ras (Molloy et al., 1989; Ellis et al., 1990), phospholipase C-γ (PLCγ)(Margolis et al., 1989), phosphoinositol 3' kinase (PI3K) (Coughlin et al., 1989; Bjorge et al., 1990), Raf1, the product of raf1 oncogene (Morrison et al., 1989), and c-src and related cytoplasmic tyrosine kinases (Kypta et al., 1990). Recent studies have demonstrated that many of these proteins contain stretches of amino acids having homology to the non-catalytic regions of c-src tyrosine kinase. Src homology (SH) domains, are lengths of 100 (SH2) or 60 (SH3) amino acids, which mediate specific protein-protein interactions (Pawson,1988; Carpenter et al., 1991; Koch et al., 1991; Pawson & Gish,

1992). SH2 domains can interact with short peptide regions containing phosphorylated tyrosine, while SH3 domains are known to interact with proline-rich regions of proteins. A number of studies (Koch et al., 1991; Lowenstein et al., 1992; Montminy, 1993) have indicated that these domains are essential for the interaction of cellular proteins and activated growth factor receptors. Further, SH3 domains are believed to regulate the cellular localization of proteins through their interaction with components of cytoskeleton (Bar-Sagi et al., 1993). More recently, several studies identified a cellular protein, p91 as a substrate for activated growth factor and cytokine receptors (Silvennoinen et al., 1993; Ruff-Jamison et al., 1993; Larner et al., 1993). p91 is a SH2 domain containing protein and apparently acts as a transcription factor (Montminy, 1993). These studies, combined with suggested nuclear functions of GAP-associated proteins (p190 and p62; section 1.2.2), have raised the hope that perhaps a search for a direct link between growth factor receptor-associated proteins and nuclear events has finally succeeded (Hall, 1992).

1.2.2 Ras- a downstream mediator

Ras, a proto-oncogene product, is a member of the small guanine nucleotide binding protein (G protein) family. In cells, Ras occurs in either GTP-bound or GDP-bound form. Ras.GTP is active whereas GDP-bound Ras is considered to be inactive (Marshall, 1991). The switch from GTP- to GDP-bound form is catalyzed by the intrinsic GTPase activity of Ras itself. The importance of Ras in growth factor signal transduction was clearly demonstrated in a study from Smith et al. (1986). In their

report, the authors introduced specific antibodies against Ras into cells and studied their effect on mitogenesis and transformation by various growth factors and oncogenes. The antibodies effectively blocked growth factor-induced mitogenic responses indicating that Ras was an important downstream mediator.

A cellular protein, GAP, enhances the intrinsic GTPase activity of Ras several fold (Trahey & McCormick, 1987). GAP, by activating Ras-GTPase activity, promotes the conversion of Ras.GTP to Ras.GDP, thereby negatively regulating Ras function (Gibbs et al., 1990; DeClue et al., 1991). GAP associates with activated growth factor receptors and undergoes tyrosine phosphorylation (Molloy et al., 1989). Since increased levels of Ras.GTP were reported in growth factor-stimulated cells, it has been suggested that interaction with activated receptor may temporarily inactivate GAP (Downward et al., 1990). One hypothesis is that receptor-mediated inactivation of GAP allows accumulation of Ras in an active state leading to the stimulation of mitogenic events (Moran et al., 1991; Reviewed in Hall, 1990; McCormick, 1990; Lowy et al., 1991).

On the other hand, GAP is also implicated as a downstream effector of Ras. Loss-of-function mutations in the 'effector region' of Ras, believed to be responsible for its biological function, also disrupt its interaction with GAP (Willumsen et al., 1986). In some instances, both Ras and GAP are required to elicit a response, as in case of regulation of atrial K^+ channels by muscarinic receptors (Yatani et al., 1990). Furthermore, characterization of two GAP-associated proteins,

p62 and p190 indicates their possible role in mRNA processing (Wong et al., 1992), and in transcriptional regulation respectively (Settleman et al., 1992). Taken together, these reports support the notion of GAP as a downstream effector of Ras.

1.2.3 Ras and Grb2-Sos1

Elevated levels of Ras.GTP in response to growth factors also suggest the possible existence of an activation factor for Ras, since conversion of Ras.GDP to Ras.GTP is brought about by the exchange of guanine nucleotides. Indeed, in the lower eukaryotes, S.cerevisiae and Drosophila, proteins with such a function have been identified. These factors, CDC25 of yeast (Robinson et al., 1987) and Son of sevenless (Sos) of flies, are generally termed guanine nucleotide exchange/release factors (GEF/GRF). Mammalian homologs of Sos/CDC25 have been identified in mouse and humans (Li et al., 1993; Chardin et al., 1993). Further studies have identified their association with activated growth factor receptors mediated by a cellular protein termed growth factor receptor binding protein 2 (Grb2; Lowenstein et al., 1992). Grb2 has a SH2 domain flanked on either side by SH3 domains. The SH2 domain of Grb2 mediates its interaction with the activated growth factor receptors (Li et al., 1993; Skolnik et al., 1993). The SH3 domains of Grb2 are essential for its interaction with Sos1. Overexpression of Grb2 enhances Ras.GTP levels (Gale et al., 1993). Thus, Grb2 acts like an adaptor molecule linking an activated growth factor receptor to Ras. This molecular interaction leads to increased cellular Ras.GTP levels. Furthermore, Drosophila Sos transforms rodent fibroblasts

(Egan et al., 1993) indicating a role for a Ras-activator (Sos1) in mitogenesis.

1.2.4 Phosphoinositol turnover - PLC

Growth factor binding to a relevant receptor in many instances increases the cellular levels of inositol triphosphates (IP3) and diacylglycerol (Reviewed in Cook & Wakelam, 1992). IP3 enhances the release of Ca^{++} from the intracellular stores (Reviewed in Taylor & Marshall, 1992), which in turn stimulates the activity of several Ca^{++}-dependent protein kinases and biochemical reactions. The elevated levels of IP3 and diacylglycerol occur as a result of growth factor-dependent increases in the hydrolysis of membrane-associated phospholipids such as phosphotidylinositol 4,5-biphosphate (PIP_2), phosphotidylcholine etc. The hydrolysis is mediated by a class of enzymes collectively called phospholipases (PL). Phospholipase C-γ (PLCγ) is known to form a complex with activated growth factor receptors (Margolis et al., 1989). Elevated levels of inositol phosphates have been shown to be a pre-requisite for mitogenesis in some instances (Matuoka et al., 1988). A molecular interaction of PLC-γ with an activated receptor is thought to increase the enzymatic activity of PLC-γ. This view was corroborated by in vitro studies (Nishibe et al., 1990). The phospholipid metabolites generated are thought to function as second messengers in various systems. However, there is evidence contradicting this conclusion (Downing et al., 1989; Margolis et al., 1990). For instance, Margolis et al. (1990), observed that overexpression of PLC-γ did not alter the rate of DNA synthesis despite the increased intracellular IP3 and Ca^{++} levels.

On the other hand, diacylglycerol in conjunction with increased cytoplasmic Ca^{++} levels appears to stimulate protein kinase C (PKC) activity (Reviewed in Asaoka et al., 1992). PKC acts on a variety of cellular proteins modulating their function. The importance of diacylglycerol and PKC to mitogenesis has been demonstrated by the stimulation of cell cycle entry upon microinjection of diacylglycerol into BALB/c 3T3 cells (Suzuki-Sekimori et al., 1989). Transcription factor activator protein 1 (AP1) activity is one example where protein kinase C-dependent dephosphorylation of AP1 subunits increases its activity (Angel & Karin, 1991). Myristylated alanine rich C kinase substrate (MARCKS) is an actin binding protein whose cellular localization is regulated by PKC (Graff et al., 1989; Thelen et al., 1991). PKC also phosphorylates growth factor receptors. This perhaps acts as part of a negative feed-back mechanism for downregulating receptor activity (Ullrich & Schlessinger, 1990).

1.2.5 Raf1 kinase

Raf1 is a serine/threonine kinase often found in association with activated growth factor receptors (Morrison et al., 1989). Activation of Raf1 is observed in response to growth factors. It has also been reported that Raf1 and Ras interact through physical complex formation. Ras acts upstream of Raf1 since dominant negative mutants of Ras block receptor-mediated activation of Raf1. Raf1 preferentially binds to Ras.GTP over Ras.GDP (Zhang et al., 1993; Vojtek et al., 1993). A similar cascade has been identified in the nematode C.elegans (Han et al.,

1993). Taken together, activation of Ras by growth factors leads to its association with and activation of Raf1. The Raf1 activation mechanism is still unclear. Interestingly enough, an α isomer of protein kinase C also stimulates Raf1 by phosphorylation (Kolch et al., 1993).

Mitogen activated protein kinases (MAPK) were identified as the name indicates, by virtue of their activation by several mitogens (Reviewed in Nishida & Gotch, 1993). MAP kinases are known to act downstream of Ras and Raf1. MAP kinases are regulated by phosphorylation on both tyrosine and threonine residues. Raf1 activates a dual specific kinase, MAPK kinase (also known as MEK) which in turn activates MAP kinases. Cloning of several MAPKKs and MAPKs suggests the possible existence of multiple protein kinase cascades. The recruitment and extent of activation of several kinases may determine the ultimate response to growth factors and other mitogens. Several of the MAPK substrates include those regulating early gene responses including Jun, Elk1, and those involved in protein synthesis such as ribosomal S6 subunit kinase (Rsk) etc. Identification of MAPK substrates and the study of the effect of such an interaction is an active area of current research (Blenis, 1993).

1.2.6 Phosphoinositol 3' kinase

Phosphoinositol 3'kinase (PI3K) is yet another component of the signalling complex. Association of PI3K with specific phosphotyrosine residues of receptor and non-receptor tyrosine kinases has been reported (Escobedo et al., 1991). PI3K is

comprised of two subunits of 85 kDa (p85) and 110kDa (p110). p85 contains two SH2 domains and is responsible for specific interactions with activated receptor and non-receptor kinases. This enzyme phosphorylates the D-3 position of the inositol ring, producing phosphatidylinositol (PI) 3-phosphate, PI 3,4-diphosphate, PI 3,4,5-triphosphate and PI- 1,3,4,5, tetraphosphate. The latter three products have been associated with growth factor stimulation of cells (Auger et al., 1989) and may act as important components in yet-to-be identified signalling pathways.

Taken together, these observations (detailed in this section, 1.2) indicate that growth factors recruit and modulate the activity of several cellular protein kinases through different mechanisms. The substrates of these enzyme are likely to be critical components of cell proliferation and differentiation.

1.3 G PROTEINS IN SIGNAL TRANSDUCTION

A classic G protein is hetero-trimeric, consisting of α, β and γ subunits. G proteins couple membrane bound receptors for mitogens, hormones and neurotransmitters to different enzymes located intracellularly (Hepler & Gilman, 1992). A prototypical example is G protein coupling of β-adrenergic receptors to adenylate cyclase which in turn modulates synthesis of cAMP. cAMP specifically activates a cAMP-dependent protein kinase (Protein kinase A). Protein kinase A can affect the activity of transcription factors and perhaps other regulatory cellular proteins (Collins et al., 1992). Several mitogens such as thrombin and bombesin are known to act through receptors coupled with G proteins. G proteins also activate

different isoforms of phospholipases leading to increased phosphoinositol turnover (Seuwen & Pouyssegur, 1992; Liskovitch, 1992).

Ligand binding to a receptor leads to a change in receptor conformation. This induces coupling to G proteins. The interaction leads to the activation of G proteins. As in case of Ras, the α subunit of heterotrimeric G-proteins is bound to GTP in activated form. Upon receptor coupling and GTP binding, the α subunit disassociates from the $\beta\gamma$ dimer and the receptor. Free α subunit then finds and activates the appropriate enzyme in the membrane (Reviewed in Gilman, 1987; Stryer,1986). Evidence exists to show that even the disassociated $\beta\gamma$ dimer is active as a modulator of enzyme activities (Clapham & Neer, 1993). The downregulation of G protein activity is mediated by the conversion of α subunit-bound GTP to GDP by intrinsic GTPase function.

Recent reports indicate that G protein-coupled receptors activate MAPK in a Raf1-independent manner (Gardner et al., 1993). This also seems to demonstrate the possibility of the existence of multiple signal transduction pathways leading to the same final target molecule. In many instances of growth factor signalling, involvement of G proteins has been demonstrated (Krupinski et al., 1988; Church & Buick, 1988; Crouch et al., 1990). Recently, G proteins have also been shown to interact with receptors with tyrosine kinase activity (Lefkowitz, 1993). This, in combination with reports that question the sufficiency of known cellular events such as phosphoinositol turnover (Imamura et al., 1990; Downing et al., 1989 & 1991) and GAP regulation

of Ras (Church et al., 1992) in mitogenesis, suggest that mitogenesis can be mediated through multiple pathways.

1.4 CELL CYCLE

Howard and Pelc first described the cell cycle in 1951, and this cellular event has been intensely studied since (Reviewed in Cross et al., 1989). The eukaryotic cell cycle is subdivided into four distinct phases. Two of the phases are characterized by readily observable events, DNA synthesis and mitosis. The period of DNA replication is termed synthetic phase (S phase) and the mitotic stage is known as M phase. Separating these two periods are two phases that were initially characterized simply as time "gaps"; gap 1 (G1 phase) preceding the S phase; gap 2 (G2 phase) separating S phase and M phase. Detailed analyses of the cell cycle have now revealed that these two gaps are functional periods during which important decision-making processes regarding cell proliferation take place. Cells can enter a third time period of quiescence , termed G0, following a final round of mitosis, when exposed to extreme and inappropriate growth conditions or upon differentiation. However, transformed cells have reduced growth requirements due to the deregulation of cell cycle control mechanisms, and they seldom enter G0 (Reviewed in Pardee, 1989).

1.4.1 Cell cycle check points

The cell cycle can be envisioned as a complex set of inter-connected molecular events leading to DNA replication, and ultimately, cell division. Several distinct check points are believed to be essential for coordinated cell cycle progression (Reviewed

in Hartwell & Weinert, 1989). Among these, controls at G1-S and at G2-M are crucial.

1.4.1.1 G2-M

Cell cycle progression from G2 to M phase is known to be controlled by an active serine/threonine kinase complex, initially termed mitosis/maturation promoting factor (MPF), whose catalytic component is a 34 kD protein, generally known as $p34^{cdc2}$ kinase (cdc2), initially described as the product of the cdc2 gene of the yeast, Schizosaccharomyces pombe (Reviewed in Draetta, 1990; Lewin, 1990). Homologs of the cdc2 gene product have been identified in several species including humans (Draetta & Beach, 1988). The kinase activity is dependent on the association of $p34^{cdc2}$ with another class of proteins known as cyclins. Levels of these proteins vary in a cell cycle-dependent manner, hence the term cyclins. The cdc2/cyclin complex phosphorylates a number of cellular proteins, modulating their function. For instance, phosphorylation of histone H1 by cdc2 appears to induce chromosome condensation; phosphorylation of nuclear lamins by cdc2 precedes the dissolution of nuclear envelope. A list of other cdc2 substrates include SV40 T antigen, the retinoblastoma gene product (RB), and p53. The functiona' consequences of many of these interactions remain unclear.

Kinase activity of cdc2 peaks just prior to mitosis, initiating mitotic events, and its rapid inactivation allows the cell to exit from mitosis. Site-specific phosphorylation of cdc2 by other cellular kinases also determines the activity of the kinase complex.

In addition, an association with different cyclins in a stage-specific manner may determine the substrate specificity of the kinase. Studies have indicated the existence of stage-specific cyclins and these data correlate with a differential function of $p34^{cdc2}$ kinase (Reviewed in Hunter & Pines, 1991; Motokura & Arnold, 1993; Muller et al., 1993).

1.4.1.2 G1-S

Research also indicates the presence of more than one cdc2 kinase, and at least in frogs, evidence has confirmed that the G1-S transition is controlled by a different cdc2 kinase than that of G2-M (Fang & Newport,1991). Steven Reed, a prominent yeast biologist, has proposed the possible existence of an S-phase promoting factor (SPF), acting at the G1-S boundary, or more appropriately, at various check points in G1, regulating the initiation of DNA replication (Reviewed in Reed, 1991). This hypothesis seems correct since a number of cdc2-related kinases and their cyclin partners have been isolated. Hence, they are now termed cyclin-dependent kinases (CDK). CDK1 is the original $p34^{cdc2}$ kinase, while other cdc2-relatives are named CDK2 through 5. Many of the CDKs bind differentially to the different cyclins and interact in a unique manner with a variety of cellular proteins. In frogs, CDK2 and not CDK1, is essential for DNA replication (Fang & Newport, 1991).

In S.cerevisiae (budding yeast), a critical check point in G1 (START) is regulated by a homolog of $p34^{cdc2}$, CDC28. Activity of CDC28 at START is

determined by its association with three novel G1 cyclins (Wittenberg et al., 1990). Other recently identified cyclins include C-, D1-, D2-, D3-, E- and F-type cyclins. Currently, the importance of each of these cyclins in cell cycle regulation is under investigation (known CDKs and cyclins are listed in Pines, 1993; Motokura & Arnold, 1993).

1.4.2 Growth inhibitory mechanisms - yeast model

To achieve regulated cell proliferation, all the events described above, must be well coordinated. Any perturbation in these molecular events might result in growth aberrations. Cells do not commit to DNA replication or to mitosis until certain requirements such as cell size, are met. In other words, the cell cycle is a delicate balance of positive and negative controls. At specific points in cell cycle, negative regulation will be relieved while concomitant positive regulatory mechanisms drive cell proliferation (Hartwell & Weinert, 1989).

Yeast is one of the preferred eukaryotic systems to study the cell cycle. In particular, Saccharomyces cerevisiae (budding yeast) serves as an excellent model , since major cell cycle decisions are made in G1, comparable to mammalian cells, unlike fission yeast and amphibian embryonic cells in which mitotic control is primary (Sprague, Jr., 1991). The budding yeast forms specialized gametes for conjugation, proliferate by means of budding and may enter a quiescent G0 stage under limiting growth conditions. The major check point in late G1 is termed START. Once a cell commits to continued cell division by proceeding beyond START, it will do so even

under adverse conditions such as nutrient limitations. Thus START serves as a control point analogous to restriction point (R point - a stage after which cells commit to DNA synthesis and have minimum requirements for mitogens and protein synthesis; Muller et al., 1993) in higher eukaryotes.

A classic example of a growth inhibitory mechanism can be provided by the phenomenon of growth arrest induced by mating pheromone in the budding yeast, Saccharomyces cerevisiae (Fields, 1990; Herskowitz & Chang, 1991). These short polypeptide pheromones induce G1 arrest at START in yeast of the opposite mating type by inducing the expression of certain genes that interfere in cell cycle progression. The pheromone signal transduction pathway involves G protein-mediated activation of a cascade of protein kinases culminating in G1 arrest. A number of gene products taking part in this pathway have been identified. They function in the order STE5, STE11/STE7/FUS3/KSS1 (these kinases are redundant in function), and STE12 (a transcription factor). Such a cascade is believed to culminate in pheromone-dependent alteration of cellular transcription (Reviewed in Sprague, Jr., 1991). So far, at least two such pheromone-inducible genes, FAR1 and FUS3 have been identified and were demonstrated to act by inhibiting G1 cyclins (Chang & Herskowitz, 1990; Elion et al., 1990). In higher eukaryotes, though such a direct mechanism has yet to be discovered, there is wide speculation that the tumour suppressor genes may act in a similar manner. Since the objective of this study is to elucidate the molecular mechanisms involved in inhibition of proliferation of human

tumour cells, a detailed account of the role of the best studied and prototypic tumour suppressor, retinoblastoma gene, is given below.

1.5 RB - THE PARADIGM FOR TUMOUR SUPPRESSOR FUNCTION

Each non-lethal genetic abnormality acquired by a potentially tumorigenic cell is believed to confer a selective advantage for cell proliferation. This results in the clonal outgrowth of tumour cells. Such changes may either induce the production of gene products that promote mitosis, as in the case of activating mutations in proto-oncogenes, or nullify one or more negative regulatory pathways, exemplified by allelic loss followed by mutational inactivation of tumour suppressor genes (Bishop, 1987).

Interestingly enough, intense research aimed at delineating the molecular mechanisms underlying the initiation and progression of cancer, has revealed that many proto-oncogenes and tumour suppressor genes are key components of regulatory pathways in the normal cell cycle (Travali et al., 1990). The product of the retinoblastoma gene, $p110^{Rb}$ (RB), a nuclear phosphoprotein and a classic example of a tumour suppressor, has been implicated in regulating the progression of cells from G1 phase to S phase of the cell cycle (Hamel et al., 1992). Complete loss of RB function is considered to be the molecular basis of retinoblastoma, a rare form of childhood cancer of the retina. Elegant experiments employing cloned RB genes have demonstrated its function as a negative regulator of cell proliferation (Huang et al., 1988; Goodrich et al., 1991). RB is differentially phosphorylated in a cell cycle-dependent manner. More heavily phosphorylated forms occur in late G1 and S phase,

while early G1 cells contain relatively under-phosphorylated forms of RB (Chen et al., 1989). Viral transforming proteins such as SV40 T antigen preferentially associate with the under-phosphorylated form of RB (Ludlow et al., 1989). Taken together, it appears that the apparent negative cell cycle regulation by RB may be blocked by phosphorylation or through complex formation with viral transforming proteins, permitting unrestricted G1-S progression (Weinberg, 1991b; Hamel et al., 1992).

Efforts to understand the RB function in normal cells have provided evidence for its involvement in transcriptional regulation. RB represses transcription from E2F and DRTF1 transcription factors by means of a physical association (Reviewed in Weinberg, 1991a & 1991b). Several genes including c-myc, c-fos, Rb1, and TGF-β have been shown to be negatively regulated by RB, apparently in a sequence-dependent manner through a putative Retinoblastoma Control Element (RCE) in their promoters. Since the loss of Rb function is a common feature of majority of cancers studied (Lee & Lee, 1991), perhaps in normal cells Rb negatively regulates the cell cycle progression by inhibiting certain cell cycle regulatory components. This view has been getting some attention recently as Dalton (1992), reported repression of the cdc2 promoter by Rb. In contrast, RB may also act positively to enhance transcription as demonstrated in case of the Sp1 transcription factor (Kim et al., 1992). However, direct DNA binding of RB has not been demonstrated in these instances, implying that effects may be the result of protein-protein interactions. In support of this, RB has been reported to interact with a number of cellular proteins

including cyclins A, D2 and D3, Myc and several uncharacterized polypeptides (Giordano et al., 1991; Kaelin, Jr. et al., 1991; Kato et al., 1993; Hall et al., 1993b). Though $p33^{cdk2}$ also appears to be a component of an RB-cyclin complex, the interaction is likely of an indirect nature, mediated by cyclins. Thus, a cell cycle-dependent complex formation of RB with cyclins and CDKs and subsequent phosphorylation of RB may act as a mechanism to inactivate RB function at particular stages of the cell cycle (Weinberg, 1991b; Hamel et al., 1992).

1.5.1 RB in normal cell - The paradox

A story of RB research would not be complete without mentioning the paradox it presents. Despite the fact that RB is ubiquitously expressed in a variety of normal cells and functions as a cell cycle regulator in experimental situations, germ-line mutations in RB predispose only to certain tumours, including those of retina, bone and soft-tissues. In principle, a germ-line mutation in one of the alleles for Rb results in the presence of a single wild-type allele in all body cells. In theory, all the actively proliferating somatic cells must then be exposed to the same rate of mutation in the existing normal Rb allele. However, in individuals with RB germ-line mutations, tumours arising from highly proliferative tissues like gut, skin, or haematopoietic system, are extremely rare (White et al., 1985; Gallie et al., 1990). This puzzling tissue specificity is further compounded by the observation that RB-deficient mice fail to complete term. This mid-term lethality was characterized by defects in the central nervous system and haematopoietic system (Lee et al., 1992).

Nevertheless, it is clear that a number of regulated cell divisions have taken place allowing the embryo to reach mid-term before the manifestation of RB deficiency.

These observations have raised a fundamental question about the role of RB in normal cells in vivo. It is essential to clarify the function of RB in normal cell cycle progression. In light of the reports regarding RB-deficient mice, Ed Harlow comments " The suggestion that RB has an essential role in all normal cell divisions must now be abandoned - it just isn't that important ". This view was further extended to propose a critical role for RB in differentiation (Harlow, 1992). However, this contradicts the convincing evidence implicating RB in cell cycle regulation. To explain such a paradox, several arguments have been put forward. Most convincing of them all is that of redundancy. Given the putative critical nature of the G1 check point it is clearly plausible that more than one protein may be acting in the same manner. This may also explain the relatively narrow tissue specificity of RB tumours as only those cells in which RB function is rate-limiting may become tumorigenic but not those with functional RB-like redundancy (Hamel et al., 1992).

1.5.2 TGF-β and RB

Transforming growth factor-β (TGF-β) is a growth factor with contrasting effects on cell proliferation. Broadly speaking, TGF-β is regarded as a growth-stimulator for mesenchymal cells and as a growth-inhibitor for epithelial cells (Reviewed in Moses et al., 1990). TGF-β apparently acts as an indirect mitogen for mesenchymal cells (smooth muscle cells) by inducing PDGF-AA autocrine secretion

(Battegay et al., 1990). Moreover, it was demonstrated that the stimulation occurs only at low concentrations, and at higher concentrations TGF-β actually inhibits the muscle cell proliferation. The growth inhibition was correlated with decreased expression of the PDGFR subunit which mediates the responses to PDGF-AA.

TGF-β mediated growth inhibition in epithelial cells under both in vitro and in vivo conditions has been studied extensively as well (Barnard et al., 1990; Moses et al., 1990). TGF-β modulates the expression of several proto-oncogenes such as c-myc, junB and some of the genes involved in the production of extracellular matrix such as plasminogen activator inhibitor 1 (PAI-1) and fibronectin. There is convincing evidence that c-myc expression is necessary for the proliferation of keratinocytes which are sensitive to growth inhibition by TGF-β. TGF-β rapidly downregulates c-myc both at the level of RNA and protein (Pietenpol et al., 1990a). This event was suggested to be responsible for TGF-β-induced growth inhibition. Interestingly enough, TGF-β fails to inhibit cells that are transformed by DNA tumour viruses. This observation led to the suggestion that a common protein may be targeted by both viruses and TGF-β. RB is one of the best candidates since the DNA tumour viruses are known to bind RB and presumably block its anti-proliferative activity. Moreover, the negative cell cycle regulation exerted by RB is operative in early G1 phase (Cooper & Whyte, 1989). Further experiments have clearly indicated the involvement of RB in TGF-β signalling mechanism. In transient transfection assays, tumour antigens of DNA viruses blocked the TGF-β effects on c-myc expression,

whereas mutants of tumour antigens that are defective in RB binding were unable to do so (Pietenpol et al., 1990b). It has also been shown that TGF-β inhibits phosphorylation of RB, and this was initially proposed to be the molecular mechanism of TGF-β-mediated growth inhibition (Laiho et al., 1990). However, the kinetics of TGF-β-induced suppression of c-myc precedes that of RB phosphorylation. Similarly, TGF-β-induced alteration in c-myc, junB and PAI-1 mRNA levels in S-phase cells in which RB is presumably inactive (Zentella et al., 1991). In addition, the authors note that cells with mutant non-functional RB were equally responsive to TGF-β. These reports have raised the concern that the preliminary hypothesis implicating RB in TGF-β-mediated growth arrest may not be universal and may well be cell/tissue-specific. Nevertheless, in some cell-types, convincing evidence establishes RB as an downstream effector of TGF-β, mediating the growth inhibition.

1.6 EPIDERMAL GROWTH FACTOR

Epidermal growth factor (EGF), is an important member of the growth factor family (Cohen et al., 1980; reviewed in Hunter & Cooper, 1985; Carpenter & Cohen, 1990). EGF is a short polypeptide, 53 amino acids long, which binds to a cell surface protein of molecular weight 170 kD termed the EGF receptor (EGFR). Elevated levels of EGFRs were reported to be associated with several types of cancers, including breast cancer, and were believed to be responsible for decreased growth factor and hormone dependence of such tumours. EGFR also mediates the signals from another ligand, transforming growth factor (TGF)-α, secreted by many

transformed cells and modulating their growth in an autocrine fashion.

Due to its prominence in cancer biology (Heldin & Westermark, 1989; Gusterson, 1992), research has been able to provide valuable information regarding the molecular mechanisms of EGF-mediated signal transduction. As outlined earlier, upon binding to EGFR, EGF induces receptor oligomerization and activates receptor tyrosine kinase activity. This leads to receptor auto-phosphorylation, and at least in some cells, the formation of a signalling complex which may include GAP, PI3K, PLC-γ (Carpenter et al., 1991) and Grb2-Sos1 (Schlessinger, 1993). The precise molecular linkage of the activated EGFR with nuclear events mediating cell proliferation or differentiation, remain obscure.

1.6.1 EGF and growth inhibition in MDA-468: An intriguing system

EGF is generally a potent mitogen for a variety of cells, both in culture and in vivo (Cohen et al., 1980). Elevated levels of EGFRs are commonly associated with breast cancers (Gusterson, 1992), indicating a critical role for EGF in transformation. It has been suggested that increased EGFR levels may confer a growth advantage in situations of decreased EGF concentrations (Gill et al., 1985). Studies with a cell line MDA-468 (or MDA-MB-468), established from pleural perfusion of a breast cancer patient (Pathak et al., 1979), further supports the argument that EGF acts as an important determinant of tumour cell proliferation. MDA-468 cells, express large numbers of EGFRs, about 1-2 X 10^6/cell, as a result of gene amplification for the EGFR (Filmus et al., 1985a, and 1987b). Further studies demonstrated that EGF, in

fact, induced inhibition of cellular proliferation at concentrations above 1 nM (Filmus et al., 1985a). Interestingly, a similar observation was made with another cell-system, A431, a human epidermoid carcinoma cell line (Gill & Lazar, 1981). A431 cells were also shown to overexpress EGFRs upto 1-2 X 10^6/cell (Merlino et al., 1984). Studies with A431 cells, indicated that EGF-induced growth inhibition at nanomolar concentrations, while stimulating cell proliferation at picomolar concentrations (Kawamoto et al., 1983)

Initial studies on this unusual phenomenon of growth inhibition induced by EGF suggested a possible relationship between the overexpression of the EGFR in these cells with the arrest in cell proliferation. This view was bolstered by the observation that a reduction in the number of available receptors either due to specific antibody binding (Kawamoto et al., 1984), or due to the loss of gene amplification (Filmus et al., 1985b; Gill et al., 1982), resulted in growth stimulation in response to EGF. The selection for cells resistant to EGF-mediated growth inhibition, led to the isolation of several variant EGF-resistant clones of both A431 and MDA-468 cells (Buss et al., 1982; Filmus et al., 1987b). Studies with these variant clones indicated a correlation between number of EGFRs expressed and the degree of EGF-mediated growth inhibition Taken together, it was thought that expression of EGFRs over a threshold level result in growth-inhibitory response to nanomolar concentrations of EGF. This is consistent with the notion that growth inhibition might be a result of cellular energy depletion, since overexpression of EGFRs might be

expected to amplify the entire signal transduction pathway. This may utilize all the available cellular energy within a short period of time leading to the cessation of further proliferative responses (Kawamoto et al., 1984).

On the other hand, a thorough review of the literature reveals several inconsistencies. For example, clone #29, a variant of the A431 cell line, expresses about 10^6 EGFR/cell, yet is growth-stimulated by EGF even at nanomolar concentrations (Gill et al., 1984; Kawamoto et al., 1984). Moreover, it was shown that experimental reconstitution of large numbers of EGFRs to levels similar to that in MDA-468 cells in cells originally expressing few or no EGFRs, resulted in hyper-proliferation and a transformed phenotype in response to nanomolar concentrations of EGF (Di Fiore et al., 1987). Detailed analysis of EGF binding and receptor metabolism in parental and variant clones of A431 cells revealed no significant shifts in the rates of EGF binding to EGFR, internalization of EGFR, and the receptor down-regulation regardless of final outcome of EGF treatment (Lifshitz et al., 1983). Church et al. (1989), have reported that biochemical events, like cytoplasmic alkalinization through the Na^+/H^+ antiport, believed to be an obligatory requirement for mitogenesis, are neither necessary nor sufficient for EGF-mediated growth response in MDA-468 cells. Furthermore, Church and Buick (1988), demonstrated that EGF-induced growth inhibition in MDA-468 cells can be blocked by pertussis toxin without altering the EGFR number. Pertussis toxin is known to inhibit the function of a subclass of G proteins by ADP-ribosylation. G-proteins act as amplifiers

in the signal transduction pathways of several hormones and growth promoting agents (Section 1.3; reviewed in Neer & Clapham, 1988). Church and Buick (1988), suggested the existence of more than one, and perhaps parallel, signal transduction pathways and the involvement of a G-protein in at least one pathway obligatory for the growth inhibitory response of MDA-468 cells to EGF. These reports suggests that over-expression of EGFRs alone may not be necessary or sufficient to induce growth inhibition in response to pharmacological concentrations of EGF.

Although, EGF-mediated growth inhibition in EGFR-overexpressing cells such as MDA-468 and A431 is an unusual phenomenon, interesting parallels can be drawn with other systems utilized to study growth-inhibitory mechanisms. In yeast, S. cerevisiae, pheromones (analogous to growth factors) induce a G1 arrest to facilitate mating (Section 1.4.2). The pheromone signal transduction pathway is mediated by a G protein (Sprague, Jr., 1991). Interestingly, involvement of a G protein in EGF signal transduction in MDA-468 cells is documented as well (Church & Buick, 1988). Since studies with S.cerevisiae indicated that pheromones modulate the expression of cell cycle regulatory genes (Herskowitz and Chang, 1991), a similar pathway involving EGF in MDA-468 cells may affect one or more components of cell cycle regulation. Furthermore, similar to TGF-β-dependent proliferative responses (Section 1.5.2), EGF elicits a bimodal response from MDA-468 and A431 cells, namely growth stimulation at low (picomolar) concentrations, and growth inhibition at high (nanomolar) concentrations (Gill & Lazar, 1981; Kawamoto et al., 1984; Filmus et

al., 1985a). TGF-β is known to modulate the function of a tumour suppressor gene RB, and this effect is believed to be important in growth inhibition of keratinocytes (Section 1.5.2). It is conceivable then, EGF may target a tumour suppressor gene in MDA-468 cells to mediate the growth inhibitory effects. These suggestions while speculative at best, were considered in formulating the following hypothesis.

1.6.2 Working hypothesis

This study was intended to investigate the molecular mechanisms involved in EGF-induced growth inhibition in MDA-468 cells. The working hypothesis for this study is that,

> **" growth inhibition is mediated , not by EGFR over-expression per se, but by an altered signalling mechanism in cells that are growth inhibited by EGF. This may involve EGF-mediated activation of an existing protein (perhaps a kinase, phosphatase or protease), transcription and translation of a proliferation suppressing gene and/or intervention in functioning of a growth promoting gene/gene product ".**

Our initial approach to this problem was simple and straight forward. Preliminary studies focused on the effect of EGF on cell cycle progression with an aim to localize the EGF actions to a particular stage in the cell cycle. This would then be followed by examining the role of genes/gene products thought to be involved at that stage in cell cycle progression.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Mithramycin, vinblastine and aphidicolin were purchased from Sigma Chemical Company. (St.Louis, Missouri). A 10 X stock solution of mithramycin was made in aqueous 25% ethanol containing 1 mg/ml mithramycin and 150 mM $MgCl_2$. Vinblastine was dissolved in methanol to a concentration of 0.5 $\mu g/\mu l$. Stock solution of aphidicolin (1 $\mu g/\mu l$) was prepared in 70% ethanol. Epidermal growth factor (EGF) was obtained from Collaborative Research Inc. (Bedford, Massachusetts), and prepared as a stock solution of concentration 3.3 X 10^{-6} M. Scintillation cocktail, Aquasol 2 was purchased from NEN (Mississauga, Ontario). Protein A-Sepharose from Amersham (Oakville, Ontario), was prepared as a 50% solution in PBS. A 10% solution of fixed Staphylococcal (S.aureus) cell suspension (Omnisorb) was purchased from Calbiochem (San Diego, California). A protein assay dye reagent was purchased from Bio-Rad (Mississauga, Ontario).

2.1.2 Radioisotopes

Thymidine [methyl-^{3}H],(sp.act. 82.4 Ci/mmol); adenosine 5' [α-^{32}P] triphosphate, (sp.act. 6000 Ci/mmol) were purchased from NEN/DuPont Research Products (Mississauga, Ontario). Cytidine 5'-[α-^{32}P]-triphosphate (sp.act. 300 Ci/mmol) was obtained from Amersham. L-[^{35}S] methionine of in vivo cell labelling

grade in aqueous solution (sp.act. 1028 Ci/mmol) was purchased from Amersham. Inorganic phosphate as H_3-$^{32}P0_4$ in H_20 (carrier free) was obtained from ICN Radiochemicals (St.Laurent, Quebec). Chloramphenicol, D-threo-[1,2- ^{14}C] (sp.act 60 Ci/mmol) was purchased from ICN as well.

2.1.3 Cell culture reagents

Leibovitz-15 (L-15) media, modified with L-glutamine was from ICN/Flow Laboratories (Mississauga, Ontario), fetal bovine serum was purchased from ICN/Flow and Gibco Labs (Burlington, Ontario). Penicillin & streptomycin 10,000 IU/ml and 10,000 μg/ml, were obtained from ICN/Flow. Materials for tissue culture were purchased regularly as follows; 75 cm^2 flasks from Falcon (distributed by Becton Dickinson, Mississauga, Ontario), and Nunc, 100 mm dishes from Falcon, and Corning, 6-well plates from Falcon, and chambered glass slides from Nunc.

2.1.4 Antibodies

PAb 1801 (Ab 2; IgG_1) a human-specific antibody, reactive with both wild-type and mutant p53 (Banks et al., 1986), PAb 240 (Ab 3; IgG_1) a mutant-specific and conformation-dependent antibody (Gannon et al., 1990), and PAb 421 (Ab 1; IgG_{2a}) a mammalian p53-specific antibody reactive with both wild-type and mutant forms of p53 (Harlow et al., 1981), were obtained from Oncogene Science (Manhasset, New York). PAb 1620 (obtained as hybridoma supernatent fluid) , human wild-type p53-specific antibody (Milner et al., 1987) was provided as a gift by Dr.Hoechkoppel, Ciba Geigy, Switzerland. A mouse monoclonal antibody, IgG_{2a},

raised against an MHC-class II antigen (anti I-A^k) was provided by Dr. George Carayanniotis, Memorial University of Newfoundland, and was used as an antibody control in Western blot, immunoprecipitation and immunofluorescence experiments. This antibody does not react with human MHC antigens. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse, $F(ab)'_2$-specific IgG, for use in immunofluorescence experiments was purchased from Jackson Immunochemicals (distributed by Bio/Can Scientific, Mississauga, Ontario).

2.1.5 Probes for Northern blot analysis

Cloned cDNAs for histone 3.2, proliferating cell nuclear antigen (PCNA), and thymidine kinase (TK) were used as probes. For histone 3.2, H3.2-614, a subclone of plasmid MM614, with XbaI-BglII fragment of MM614 cloned into PUC18 (Hurt et al., 1989), a generous gift from Dr. W. Marzluff (Dept. of Chemistry, The Florida State University, Tallahassee, FL), was used. A construct, p3-Bam-Full PCNA n.2, containing a cDNA for the full length human PCNA gene, along with its promoter, kindly provided by Dr.R. Baserga (Dept. of Pathology, Temple University, Philadelphia, Pennsylvania), was employed as a probe for PCNA (Travali et al., 1989). To probe mRNA for the TK gene expression, the plasmid, pSp65-Bma.Sma.TK, a gift from Dr. S. Conrad (Dept. of Microbiology and Public Health, Michigan State University, East Lansing, MI), containing a 1.2 kb BamHI-SmaI fragment from within the TK cDNA cloned into pSp65 was used (Roehl & Conrad,1990). Oligonucleotide probes were employed for p53 (a 40-mer; Oncogene

Science), c-myc (a 30-mer; NEN/DuPont), and α-tubulin blots (a 30-mer; Clontech).

2.1.6 Oligonucleotides

The following oligos were employed for electrophoretic mobility shift assays (EMSA) and construction of p53-responsive elements. The oligos 5'-GTCCGGACATGCCCGGGCAT-3' and 5'-GGACATGCCCGGGCATGTCC-3' were annealed to obtain a double stranded fragment with 5'-overhang, corresponding to the CON fragment (Funk et al., 1992) and the latter oligo 5'-GGACATGCCCGGGC ATGTCC-3' is self-annealed to obtain blunt-ended double-strand-CON. A 33-base sequence 5'-TTCTCCTTGCCTGGACTTGCCTGGCCTTGCCTT-3' and 5'-AGAAAAGGCAAGGCCAGGCAAGTCCAGGCAAGG-3' were annealed to obtain a double stranded fragment with 5' overhang, corresponding to the bases 106-138 of fragment A (FRA)(Kern et al., 1991b).5'-CCTTGCCTGGACTTGCCTGGC CTTGCCTTTTCT-3' was annealed with 5'-AGAAAAGGCAAGGCCAGGCAAG TCCAGGCAAGG-3' to obtain blunt-ended double-stranded FRA. The individual oligos were synthesized and obtained from OLIGOS ETC.Inc. (Wilsonville, Oregon). A double stranded oligo with sequence corresponding to an NF1 binding sequence 5'-AACCTAATTGCATATTTGGCATAAGGTTT -3', for use as a non-specific competitor in electrophoretic mobility shift assays, was a kind gift from Drs. A and M. Pater, Memorial University of Newfoundland.

2.1.7 CAT assay reagents

The vector, pBLCAT2, contains a chloramphenicol acetyl transferase gene under the control of the herpes simplex virus (HSV) thymidine kinase (TK) promoter (Luckow & Schutz, 1987) was a gift from Drs. A and M. Pater. A polylinker region upstream of the promoter region allowed us to clone p53-responsive elements in front of the TK promoter generating CON-CAT and FRA-CAT. An expression construct containing SV40 T antigen cDNA regulated by SV 40 promoter/enhancer regions (Chang et al., 1984) was also generously provided by Drs. A and M. Pater, Memorial University of Newfoundland.

N,N,-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) for DNA transfection studies, was from Calbiochem. Acetyl coenzyme A (Acetyl CoA) was purchased from Sigma Chemical Co. Plates for thin layer chromatography (TLC) were purchased from Fisher Sci. (Ottawa, Ontario).

2.1.8 Molecular cloning and hybridization reagents

Restriction enzymes including SalI, PstI, and SmaI were purchased from Gibco/BRL. The source for modifying enzymes such as T4 polynucleotide kinase and T4 DNA ligase was Gibco/BRL, and for AMV reverse transcriptase was Pharmacia (Baie d' Orfe, Quebec). Appropriate reaction buffers were provided along with the above enzymes. Calf liver 28 S and 18 S ribosomal RNA standards were obtained from Amersham. Nylon membranes (NYTRAN) for Northern blot analysis were from Schleicher & Schuell (distributed by Mandel Sci.Co.), nitrocellulose membranes

(Optibind) for Western blot analysis were from Mandel Scientific Co. (Keene, NH) The sources for the following reagent-kits were, Stratagene (La Jolla, California) for a total RNA-isolation kit, and a random primer kit (PRIME-IT); NEN/DuPont for an end-labelling kit; Gibco/BRL for a nick-translation kit, and Amersham for a Western blot detection kit for use with mouse monoclonal antibodies.

2.2 METHODS

2.2.1 Cell culture

MDA-468, a human breast cancer cell line (A kind gift from Dr. Ron Buick, Ontario Cancer Institute, Toronto), was maintained in Leibovitz-15 modified medium supplemented with 10% FBS, 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were routinely grown in 100 mm plates and or 75 cm^2 flasks. As cell cycle distribution and EGF effects were greatly influenced by cell density as well as culture conditions (Gill & Lazar, 1981), the seeding density was adjusted ($4x10^3$ cells/cm^2; Gill & Lazar, 1981; Lifshitz et al., 1983) so that the cells were always kept under 50% confluency throughout the experiments and the culture medium was changed every 48 h. A concentration of 10^{-8} M of EGF was employed in our studies. It has been demonstrated by several groups that, this concentration (10^{-8} M) of EGF produces maximum growth inhibition (Gill & Lazar, 1981; Barnes, 1982; Lifshitz et al., 1983; Filmus et al., 1985 and 1987b)

2.2.2 Flow cytometric analysis of DNA content

Cells were plated at an initial density of 3x 10^5 cells/100 mm plates. After 24 h of equilibration, EGF was added to a final concentration of 10^{-8} M. The medium ± EGF was changed every 48 h. At 48 h intervals, cells were harvested by trypsinization, washed once with phosphate buffered saline (PBS), and fixed with 25% ethanol. Duplicate cultures were further treated with vinblastine (1μg/ml) for 24 h prior to harvesting. Fixed cells were stored at 4°C prior to flow cytometric analysis.

One hour prior to assay, the cells were centrifuged at 1000 rpm in a table top centrifuge (model IEC-HN-SII/ DAMON-IEC), and resuspended in a solution containing 100 μg/ml mithramycin and 15 mM $MgCl_2$ in aqueous 25% ethanol (Crissman & Tobey, 1974). After 20 min of incubation on ice, samples were filtered through glass wool and passed through a 26-gauge needle to remove clumps, transferred to rinsed glass test tubes and held on ice until analysis was carried out in a Coulter EPICS-C fluorescence-activated cell sorter (FACS) fitted with a 5-watt argon laser, set at an excitation wavelength of 457 nm (Crissman & Tobey, 1974). Approximately 20,000 cells were analyzed per sample. The fraction of cells in different phases of the cell cycle was determined by "DNAFIT" analysis, using Coulter Cytologic Software which follows a multirectangular model for S phase and calculates the percentage of cells in G1, S and G2/M fractions.

2.2.3 DNA/Protein synthesis assays

MDA-468 cells were grown in 6-well plates at a seeding density of 3 X 10^4 cells/well. Twenty-four hours after plating, EGF was added to a final concentration of 10^{-8} M. At the indicated intervals, following EGF addition, [^{3}H] thymidine (2.5 μCi/ml) was added and incubation was continued for 24 h prior to harvesting. At the end of this time, triplicate samples of labelled cells were washed twice with PBS, harvested by trypsinization, and precipitated by ice-cold 10% trichloroacetic acid (TCA). The precipitate was filtered using glass fibre filters and washed 3X with 10% TCA. The TCA precipitable radioactivity was counted by liquid scintillation in a Beckman counter (Model LS 3801) using 5 ml of scintillation cocktail (Aquasol 2; NEN) per sample. At each interval, cells from unlabelled parallel cultures were counted in duplicates, using a haemocytometer. The incorporated radioactivity was standardized to 10^6 cells. Throughout the experiment, media ± EGF was changed every 48 h. To observe the effect of EGF withdrawal, the media was removed after 48 h of EGF treatment and the monolayer was washed with PBS and further incubated with fresh media without EGF. DNA synthesis was then measured in a manner identical to that described above.

To examine EGF effect on total protein synthesis, cells that are plated and EGF-treated as described for DNA synthesis experiments, were labelled for 1 h with ^{35}S-methionine (25 μCi/ml) prior to harvesting. Harvesting of cells, TCA precipitation and standardization of counts were carried out as described above.

2.2.4 Radiolabelling of probes for Northern blot analysis

The cDNA probes were ^{32}P labelled using [α-^{32}P]-dATP and a random priming technique. For this purpose, PRIME-IT, a random primer kit (Stratagene) was used. The technique is essentially the same as described by Goulian et al. (1973), and Tabor et al. (1987). Oligonucleotide probes were radiolabelled by means of a 3' end labelling technique employing a kit (NEN/Dupont) which is essentially similar to the one described by Chang & Bollum (1971), and Johnson et al. (1986).

2.2.5 Northern blot analysis

Total RNA was isolated from MDA-468 cells ± EGF, using a guanidinium thiocyanate-phenol-chloroform single step extraction method (RNA isolation kit; Stratagene). 20 μg of total RNA was denatured with glyoxal/DMSO and electrophoresed in a horizontal 1% agarose slab gel. The RNA was then transferred to a nylon membrane (NYTRAN) in neutral buffer, 0.01 M NaH_2PO_4 ,and hybridized to a radiolabelled probe under the standard conditions as described by Sambrook et al.(1989). The 28 S and 18 S ribosomal RNA from calf liver were used as markers. The prehybridization, hybridization and washing for cDNA probes were carried out under standard high stringency conditions essentially as described earlier (Sambrook et al., 1989). For oligonucleotide probes, the conditions were as per the specifications of membrane manufacturer. Autoradiography was performed at -70°C using Kodak XAR film.

2.2.6 Immunofluorescence experiments

Cells grown on chambered glass slides were serum starved for 48 h before treating with complete medium with serum ± EGF. At the indicated intervals, monolayers on the slides were washed 3X with PBS, and fixed with methanol:acetone (1:1) at -20 °C for 10 min. After fixing, slides were either air dried and stored at -70° C or stained immediately. Cells were washed 3X with PBS and blocked with 3% bovine serum albumin in PBS (PBS/BSA) for 30 min at room temperature (RT). After rinsing 3X with PBS, cells were incubated at RT for 60 min with either a control antibody (anti-MHC) or an anti-p53 antibody (PAb 1801 or PAb 240) at 5μg/ml concentration in PBS/BSA. After 3 washes with PBS, slides were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG ($F(ab)'_2$ specific; Jackson Immunochemicals) at 1:50 dilution in PBS/BSA for 30 min at RT. Following staining, cells were washed extensively with PBS followed by a final rinse with double distilled water, mounted with 50% glycerol and observed under a fluorescent microscope. Photographs were taken using Kodak ektachrome 400 film.

For PAb 1620 staining, cells were prepared essentially in a identical manner except that fixing was carried out with 3% BSA/PBS at 37°C. For the experiments with aphidicolin, cells were treated (5 μg/ml final concentration) for 24 h prior to fixing.

2.2.7 Metabolic labelling

In vivo labelling with ^{35}S-methionine for protein synthesis experiments was done as follows. Cells were treated with 10^{-8} M EGF after 24 h of plating.

i) Labelling with ^{35}S-methionine (100 μCi/ml) was carried out for approximately 10 h (overnight) at the end of EGF-treatment (Fig .4.4).

ii) Labelling with ^{35}S-methionine (100 μCi/ml) was carried out during the last 3 h of EGF treatment, in methionine-free media (Fig.4.5).

At the end of this period, cells were lysed and immunoprecipitated using PAb 1801 as described below (2.2.9).

For pulse-chase experiments, cells were labelled overnight (12-15 h) with ^{35}S-methionine (100 μCi/ml) in methionine-free media , followed by extensive washing with complete media and incubation was continued ± EGF in complete media with serum. Cells were lysed at the indicated intervals and immunoprecipitated with PAb 1801 as described below (2.2.9).

For phosphorylation experiments, cells were incubated with H_3-$^{32}P0_4$, (0.25 mCi/ml) in phosphate-free DMEM media with 20 mM HEPES [pH 8.0] during the final 3 h of EGF treatment. At the end of labelling, cells were lysed and immunoprecipitated using the appropriate antibody as described below in 2.2.9.

2.2.8 Western blot analysis

Appropriately treated cells were harvested by trypsinization. After washing once with PBS, cells were resuspended with cell-homogenization buffer (20 mM Tris.HCl [pH7.4], 5 mM Ethylene glycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA), 1 μg/ml Leupeptin, 10 μg/ml Aprotinin, 1 mM Phenylmethylsulfonylfluoride [PMSF]). Cell suspensions were then subjected to five cycles of ultrasonification (30 sec ON and 30 sec OFF). Complete cell lysis was confirmed by microscopic examination. Protein concentrations were then determined by Lowry's colorimetric assay (Lowry et al., 1951).

100 μg of total protein was run on 8% SDS-PAGE, transferred to nitrocellulose (Optibind, Mandel Sci.Co.) using a semi-dry transfer method (Hoefer). The nitrocellulose filter was incubated overnight in 10% dried milk membrane blocking agent (Blotting detection kit, Amersham) at 4°C with constant rotation. Western blot analysis was then performed with PAb 1801, followed by an alkaline-phosphatase detection system (Amersham). The primary anti-p53 antibody, PAb 1801 was used at 10 μg/ml for 1 h at room temperature, and the second antibody, biotinylated goat anti-mouse IgG, was used at 1:250 dilution for 20 mins at room temperature. Followed by incubation with streptavidin-alkaline phosphatase conjugate (1:3000) for 20 mins. The enzyme substrate was prepared by adding 1 drop of Nitro-blue tetrazolium, and 5-Bromo-4-chloro-3-indolyl phosphate in dimethyl formamide to 10 ml of diethanolamine buffer (100 mM diethanolamine [pH 9.5], 5 mM $MgCl_2$).

The filter was incubated with the above enzyme substrate for 10-20 mins followed by thorough washing. TBS buffer (20 mM Tris.HCl [pH 7.6], 137 mM NaCl) was used for washing filter in between incubations and for diluting the reagents. 0.1 % of Tween 20 in TBS was used to prepare blocking solution.

2.2.9 Immunoprecipitation

Cells were washed 2X with PBS before harvesting. ^{35}S-methionine labelled cells were harvested by trypsinization. H_3-$^{32}PO_4$ labelled cells were harvested by scraping with the lysis buffer. Trypsinized cells were lysed with 0.5 ml lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM Ethylene glycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid [EGTA], 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 1 mM Phenylmethylsulfonylfluoride [PMSF], 200 μM Sodium orthovanadate, 10 mM Tetrasodium pyrophosphate, 100 mM Sodium fluoride; {the last three components were included in experiments studying protein phosphorylation} Margolis et al., 1989) for 30 min on ice, and centrifuged 30 min at 14,000 rpm in an Eppendrof Microfuge (Model 5415C) at 4°C. Cells harvested by scraping with lysis buffer were also incubated on ice for 30 min prior to centrifugation. The supernatant was recovered and treated with 1-2 μg of p53 antibody for at least 1 h at 0°C followed by 30 μl of 50% protein A-Sepharose (or a 10% Staphylococcal cell suspension, Omnisorb by Calbiochem, where indicated) with rotation at 4°C. The immunoprecipitates were then recovered by centrifugation for 30 sec at 14,000 rpm, washed 3X with 0.5 ml of wash buffer (20 mM HEPES

[Ph 7.5], 10% Glycerol, 0.1% Triton X-100, 150 mM NaCl, 1 mM Sodium orthovanadate; Margolis et al., 1989) followed by a final wash in PBS and resuspension in 30 μl of 10 X SDS-digestion buffer. The samples were then boiled for 5 min, pelleted, and the supernatant was recovered and electrophoresed on SDS-PAGE. The samples were always adjusted for equal radioactivity. At the end of electrophoresis, gels were dried in a Bio-Rad gel drier, and autoradiographed using Kodak XAR film at -70°C.

2.2.10 Nuclear extract preparation

Semi-confluent plates (approx. 3 X 10^6 cells/100 mm dish) after 24 h of plating were further incubated ± EGF (10^{-8} M) for the indicated durations. At the end of the treatment, plates were washed 3X with ice-cold PBS. The cells were scraped with 1.5 ml of Buffer A per 100 mm plate (20 mM Hepes [pH 7.6], 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 10 μg/ml leupeptin, 100 μg/ml aprotinin, 1 mM PMSF; Lassar et al., 1991). The cell suspension was then centrifuged at 2000 rpm at 4°C in a microfuge. The pellet (nuclei) was resuspended in 0.5 ml of Buffer B (essentially identical to Buffer A except NaCl at 0.5 M concentration). The suspension was rocked gently for 1 h at 4°C followed by centrifugation at 10,000 rpm for 5 min. The supernatant was recovered and the protein concentrations of the extracts were estimated using a Bio-Rad protein assay dye reagent (Bradford, 1976). The nuclear extract was stored in small aliquots at -70°C.

2.2.11 Nuclear extract preparation from radiolabelled cells

The cells cultured ±EGF (10^{-8} M) were labelled with ^{35}S-methionine or H_3-$^{32}PO_4$ during the last 2 h of incubation prior to harvesting. At the end of the labelling period, cells were washed 3X with ice-cold PBS followed by scraping with 10 mM EDTA [pH 8.0] in PBS. Cells were pelleted by centrifugation at 2000 rpm at 4°C. The pellet were resuspended in Buffer B (components are listed in section 2.2.10) at 50 µl/ pellet from one 100 mm plate. The suspension was gently rocked at 4°C for 1 h followed by centrifugation at 10,000 rpm for 5 min at 4°C. The supernatant was subjected to scintillation counting. Equivalent counts were used for immunoprecipitation experiments.

2.2.11.1 Immunoprecipitation from nuclear extracts

Equivalent counts of each sample of extracts as prepared in 2.2.11, in 40 µl volume (Volume adjusted with Buffer B of 2.2.10) was incubated with 350 µl of nuclear extract immunoprecipitation buffer (10 mM Hepes [pH 7.6], 250 mM NaCl, 0.25% NP-40, 5 mM EDTA, 10 µg/ml leupeptin, 100 µg/ml aprotinin, 1 mM PMSF; Lassar et al., 1991), 1.0 µg of anti-p53 antibody and 25 µl of 50% protein A-Sepharose. The incubation was carried out at 4°C for 90 min with gentle rocking. The immunoprecipitates were then recovered by centrifugation at 10,000 rpm for 2 min at 4°C. The Sepharose pellet was washed 4X with RIPA buffer (10 mM Tris.HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 µg/ml leupeptin, 100 µg/ml aprotinin, 1 mM PMSF; Lassar et al., 1991). The samples were

then boiled with 10 X SDS-digestion buffer for 5 min and centrifuged. The supernatant was separated on a 10% SDS-PAGE. The gels were dried at 80°C for 1 h and exposed to Kodak XAR film at -70°C.

2.2.12 Electrophoretic mobility shift assays

The EMSA is a standard technique to test and identify the proteins that specifically bind to known DNA sequences. The principle of the assay basically involves a binding reaction, comprising a radiolabelled, double-stranded DNA fragment of known sequence and a protein source such as nuclear extracts or purified proteins. The reaction mixture is then separated on a non-denaturing gel. Since unbound labelled DNA migrates faster than the protein-DNA complexes in a native gel, if any proteins bind to DNA, shifts in the mobility of labelled-DNA fragments can be detected because of the bound proteins. The specificity of the reaction can be assessed through specific competition by antibodies or by unlabelled DNA fragments.

Equimolar concentrations of oligos corresponding to CON or FRA (section 2.1.6) were mixed in annealing buffer (40 mM Tris.HCl [pH7.5], 20 mM $MgCl_2$, 50 mM NaCl; Sequenase buffer, United States Biochemical Corporations, Cleveland, Ohio). The mixture was then heated to 70°C for 5 mins and allowed to cool slowly to 4°C, to yield corresponding double strand DNA elements. 0.5 μg of double stranded oligos (CON or FRA) with 5'-overhang, were incubated with 20 units of AMV reverse transcriptase, 5 μl dNTP mix (excluding dATP or dCTP, depending on the isotope used/BRL nick-translation kit), 5.0 μl ^{32}P-dATP (^{32}P-dCTP was used for CON only,

in some experiments) in reverse transcriptase buffer at 37°C for 1 h. The oligos were then precipitated with ethanol ($1/10^{th}$ volume of 3 M Sodium acetate [pH 5.4], and 2 volumes of absolute ethanol) at -70°C for 1 h. Precipitated/labelled oligos were recovered by centrifugation at 14,000 rpm for 30 mins at 4°C. Labelled oligos were washed twice with 70% ethanol, air dried for 15-30 mins, resuspended with sterile d.H_20. Aliquots of the samples were counted in a scintillation counter.

The binding reactions were performed with nuclear extracts equivalent of 3-5 μg of protein in binding buffer A (1 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 7% glycerol, 10 mM HEPES [pH 7.4]; protocol was a kind-gift from Dr. S. Benchimol, Ontario Cancer Institute, Toronto), sonicated salmon sperm DNA 1.0 μg, and 25,000 cpm of end-labelled probe. The reaction was carried out at room temperature for 30 mins. The binding reactions for experiments studying effects of PAb 1620 on p53-DNA complexes (Fig. 5.5 & 5.6) were performed in binding buffer B (12% glycerol, 12 mM HEPES [pH7.9], 4 mM Tris.HCl [pH 7.9], 60 mM KCl, 1 mM EDTA, 0.6 mM DTT, bovine serum albumin 300 μg/ml; Chodish et al., 1988), Appropriate antibodies were incubated with nuclear extract prior to the binding reaction for 15 min on ice, added at the beginning of the reaction, or added at the end of the binding reaction for 30 more minutes of incubation on ice as indicated. Appropriate competitors were included in the binding reaction wherever indicated. The complexes were resolved on a 4% native polyacrylamide gel. A pre-run at 100 V was performed for 2 h at 4°C with buffer recirculation followed by the electrophoresis of the

samples for 3-4 h with 0.5 X TBE (O.045 M Tris borate, 0.001 M EDTA [pH 8.0]) at 4°C with buffer recirculation. At the end of the run, the gels were dried at 80° for 1 h and exposed to Kodak XAR film at -70°C.

2.2.13 Construction of p53-responsive plasmids

Equimolar concentrations of appropriate oligos (Section 2.1.6) were annealed as described above (Section 2.2.12) to obtain blunt-ended double strand oligos. These, oligos corresponding to CON and FRA were 5' phosphorylated using T4 kinase and ATP (Gibco/BRL). 5'-phosphorylated oligos were inserted into the SalI site of the vector pBLCAT2 (Fig. 2.1). To facilitate this procedure, pBLCAT2 was first digested with SalI restriction enzyme producing protruded termini, which were filled in by reverse transcription (AMV reverse transcriptase from Pharmacia). Thus blunt-ended pBLCAT2 was incubated with calf intestinal phosphatase (37°C for 1 h) in order to dephosphorylate its 5' termini. 5' phosphorylated, blunt-ended oligos were incubated overnight at 16°C, with SalI digested, blunt-ended and dephosphorylated pBLCAT2 and T4 DNA ligase. The ligated plasmids were then used to transform competent-E.coli (strain XL-Blue) cells. The drug (ampicillin)-resistant colonies were screened for inserts by restriction analysis. Clones with inserts were then grown on a larger scale to prepare sufficient amounts of plasmids. The plasmids were isolated using ethidium bromide-cesium chloride centrifugation method (Sambrook et al., 1989). The constructs were tested once again by restriction analysis and amount of DNA present was estimated by spectro-photometry.

FIG. 2.1 **A schematic diagram of p53-responsive CAT-constructs**

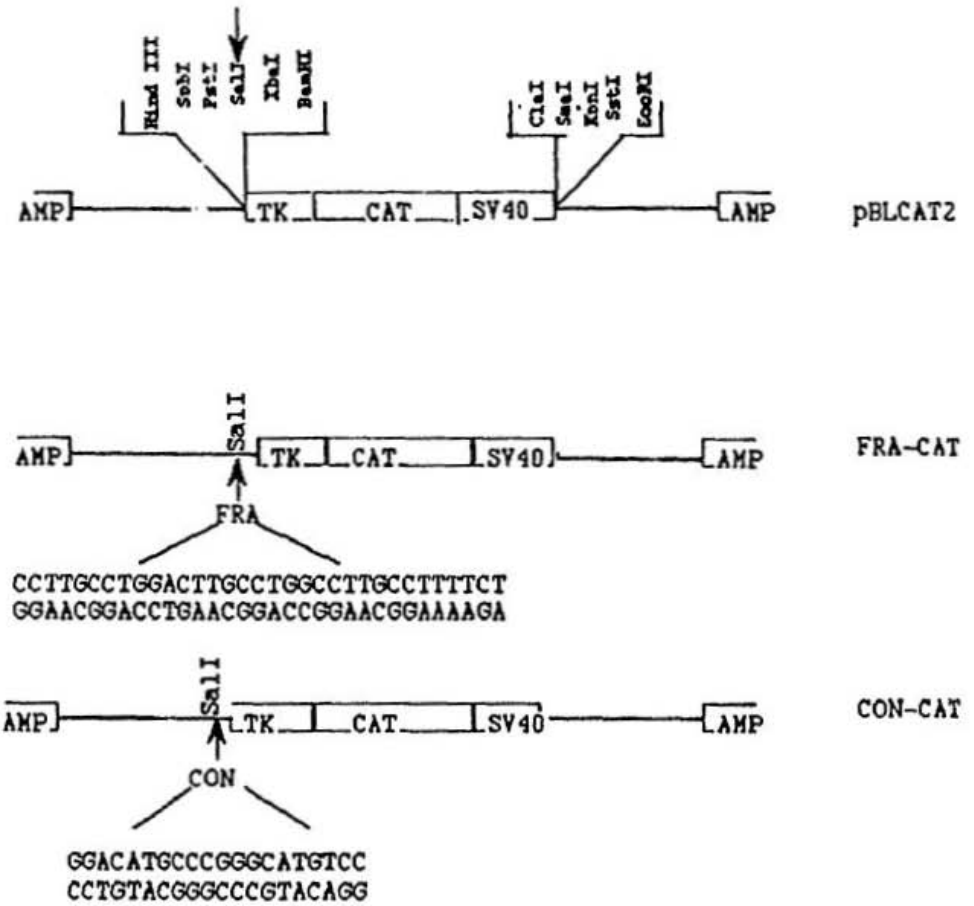


FIG. 2.1 A schematic diagram of p53-responsive CAT-constructs

2.2.14 DNA transfection - Calcium phosphate precipitation method

Semi-confluent plates, 24 h after plating, were employed for transfection. Cells received fresh medium with serum 3-4 h before transfection to ensure the optimum growth. A solution containing appropriate plasmids used for transfection was prepared as follows. For a 100 mm plate, 62 μl of 2 M $CaCl_2$, 5-10 μg DNA (CAT-constructs 5 μg, and internal control RSV-β gal 2 μg) and d.H_2O up to 500 μl. Also prepared 500 μl of 2X BES-buffered saline (50 mM N,N,-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid{BES}, 280 mM NaCl, 1.5 mM Na_2HPO_4 .$2H_20$; Sambrook et al., 1989) per 100 mm plate. The calcium phosphate precipitate was then allowed to form by mixing the DNA solution with BBS. The solution was then vortexed and kept at RT for 15 mins. The precipitate (1.0 ml/100 mm plate) was pipetted gently onto the cells. Incubation was continued at 37°C. 18-24 h later, cells were washed twice with serum free medium or PBS. The plates received normal medium ± EGF (10^{-8} M) with serum and were incubated for another 48 h. Cells were harvested and the extracts were used for assaying CAT activity as described below.

2.2.15 Chloramphenicol acetyl transferase assay

Transfected cells were harvested at the end of incubation with media ± EGF (48 h) with Tris.EDTA.NaCl (0.04 mM Tris.HCl [pH 7.4], 1 mM EDTA, 150 mM NaCl). Cells were collected by scraping and were transferred to chilled microfuge tubes. Cells were then pelleted by brief centrifugation at 4°C. The pellets were resuspended in 100 μl of 0.25 M Tris.HCl [pH7.8]. The cell suspension was subjected

to three rounds of freezing and thawing (liquid nitrogen for 3 min and 37°C for 3 min). The supernatant was recovered after a brief centrifugation at 4°C for 5 min. 20 μl of the cell extract was mixed with 20 μl CAT-assay pre-mix (4 mM Acetyl CoA, 1 μCi ^{14}C-Chloramphenicol, 0.7 M Tris.HCl [pH7.8]; Gorman et al., 1982). The reaction was carried out at 37°C for 1 h. At the end of the incubation, 0.5 ml of ethyl acetate was added and the samples were centrifuged for 30 sec. The upper organic phase was recovered carefully and dried in a SpeedVac under vacuum for 30-60 min. 15 μl of ethyl acetate was then added to each tube, mixed thoroughly by vortexing. The samples were charged onto a Kodak TLC plate. TLC was conducted in chloroform:methanol (95:5) until the liquid reached 1" below the top. TLC plates were then exposed to Kodak XAR film. CAT activity was measured as % conversion of chloramphenicol to acetyl-chloramphenicol. This was done by counting radioactivity from portions of TLC plate corresponding to both unconverted and converted forms in a Beckman scintillation counter.

Aliquots of cell extracts were estimated for protein concentrations by Bio-Rad protein-estimation kit. CAT activities were then standardized to protein concentrations. An internal control, RSV-β gal was used in all the experiments. The β-galactosidase activity (as described by Sambrook et al., 1989), however, seemed to be influenced by EGF-treatment. In our experiments, we observed an approximate 50% increase in β-galactosidase activity in EGF-treated samples. Standardization of CAT values in case of EGF-effects on a minimal promoter, to both protein

concentrations and β-galactosidase activities, gave the results qualitatively similar to the presented data. However, this procedure, when followed for CON-CAT and FRA-CAT, gave values that are 50% lower upon EGF-treatment, as compared to untreated cells. These differences more or less corresponded to the EGF-dependent increase in β-galactosidase activity, hence they were not included in the results.

3. CHARACTERIZATION OF EGF-INDUCED GROWTH ARREST IN MDA-468 HUMAN BREAST CANCER CELLS

3.1 INTRODUCTION

3.1.1 Flow cytometry

This phase of the study of EGF-mediated growth inhibition was carried out in order to have a clear picture of events at the cellular level. The effect of EGF on cell cycle progression was studied by flow cytometry. Flow cytometry for cell cycle analysis requires the cells to be stained with a dye that binds to DNA. The stained cells are then passed in a liquid stream through a sensing area and a laser beam of specific wavelength. The DNA-bound dye fluoresces at a particular wavelength, producing light scatter. This scattered light is collected by detectors and produces an electronic signal which is proportional to the amount of light being scattered. The strength of the scattered light depends on the amount of dye present in the cell. The amount of dye incorporated into DNA will be proportional to the amount of DNA present in the cell. Thus the signals produced are directly proportional to the cellular DNA content. As cells move from G1 to S the 2N DNA content starts increasing and becomes 4N by the end of S phase. So, with flow cytometry, it is possible to differentiate populations of cells in G1, with 2N DNA content, and G2/M with 4N DNA content. The S phase cells contain DNA in between 2N and 4N (Freshney, 1987).

3.1.2 Cell cycle-dependent gene expression

Cell cycle studies of EGF-mediated growth inhibition in A431 human epidermoid carcinoma cells by MacLeod et al. (1986), indicated that the cell cycle was reversibly blocked at G1 and G2 phases. Cell cycle studies will help in locating the specific EGF-induced event responsible for the arrest by providing information concerning the temporal location of the blockade in the cell cycle. Furthermore, since progression of cells through the cell cycle is strictly governed at a number of control points by the modulated expression of a variety of cell cycle-dependent genes in G1 and G2 (Pardee, 1989), examination of the expression of these genes can be used to further characterize the growth arrest. The expression of the proto-oncogene c-myc is known to be induced both in early G1 and in response to EGF (Kelly et al., 1983; Muller et al., 1984). Similarly, other genes are expressed at higher levels late in G1. For example, in serum-stimulated cells the transcription of proliferating cell nuclear antigen (PCNA), an ancillary factor for DNA polymerase δ, increases rapidly in late G1 (Liu et al., 1989), followed closely by increased mRNA levels for thymidine kinase (TK), an enzyme involved in salvage pathways for nucleotide biosynthesis (Coppock & Pardee,1987). Elevated levels of p53 expression occur at the G1/S boundary (Reich & Levine, 1984; Lalande, 1990). The histone genes, whose products are necessary for organization of chromatin, show higher mRNA levels in S phase of the cell cycle and their expression is believed to be coupled with DNA synthesis (Hirschhorn et al., 1984). In this study, we have characterised the EGF-induced growth inhibition in

MDA-468 human breast cancer cells in terms of cell cycle distribution and cell cycle-dependent gene expression.

3.2 RESULTS

3.2.1 Cell cycle analysis

Treatment of MDA-468 cells with 10^{-8} M EGF resulted in marked growth inhibition when compared to control, untreated cells (Fig.3.1), confirming earlier studies (Filmus et al., 1985a). Flow cytometric analysis of MDA-468 cells revealed perturbations in the cell cycle distribution in response to the growth inhibitory concentrations of EGF. Figure 3.2 shows representative DNA histograms after 4 days of EGF treatment. EGF treatment resulted in accumulation of cells in G1 phase of the cell cycle with a concomitant depletion of cells in S and G2/M phases as compared to the untreated cells (Fig.3.2A and 3.2B). Table 3.1 shows the average percentage of cells in different phases of the cell cycle following EGF treatment of 2-6 days in duration, from a minimum of five experiments. The data indicate that EGF treatment has resulted in arrest of the cell cycle in G1 phase. To confirm this apparent G1 arrest, cells were treated with vinblastine (1μg/ml), a mitotic inhibitor. Vinblastine inhibits polymerization of mitotic spindles, thereby arresting the cells in mitosis and blocking the re-entry of cells into G1. In cells progressing from G1 to mitosis, vinblastine would block the M to G1 transition, resulting in depleted G1 and an increase in the number of cells in M phase. As expected, the control cells displayed a greater G2/M population, with depleted G1 and S phases, upon

FIG. 3.1 **Effect of EGF (10^{-8} M) on proliferation of MDA-468 cells.** Cells were seeded at $3x10^5$ cells per 100mm plate and treated with EGF after 24 h of plating. Cells were harvested at 48 h intervals after EGF treatment, by trypsinization,and counted using a haemocytometer as described in Materials and Methods. ■--■ control cells; □--□ EGF-treated cells.

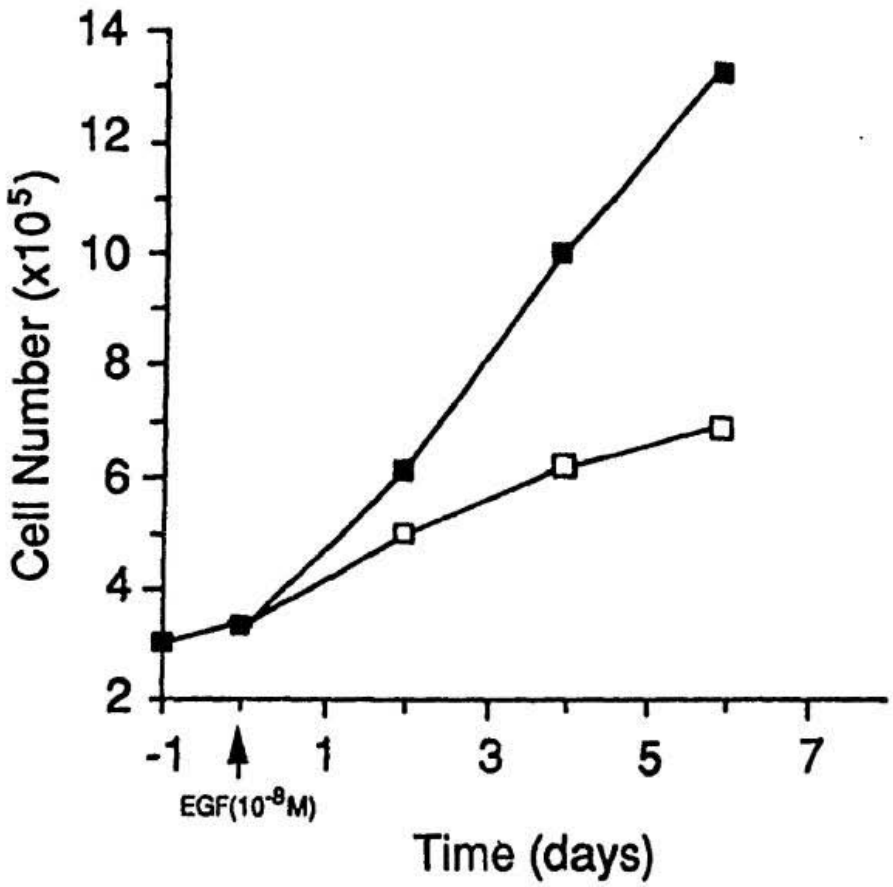

14
12
10
8
6
4
2
Cell Number (x10^5)
-1
1
3
5
7
EGF(10^{-8}M)
Time (days)

FIG. 3.2 **Cell cycle analysis.** Representative FACS-generated, DNA histograms of MDA-468 cells, cultured in the absence (A,C) and in the presence of EGF (10^{-8} M) (B,D), after 4 days of treatment. The control, as well as EGF-treated cells, were stained with mithramycin, and 20,000 cells were analyzed by flow cytometry as described in Materials and Methods. Duplicate cultures (C,D) were incubated with vinblastine for 24 h prior to analysis. Media ± EGF were changed every 48 h and the seeding density was adjusted for optimum EGF effects (As detailed in 2.2.1).

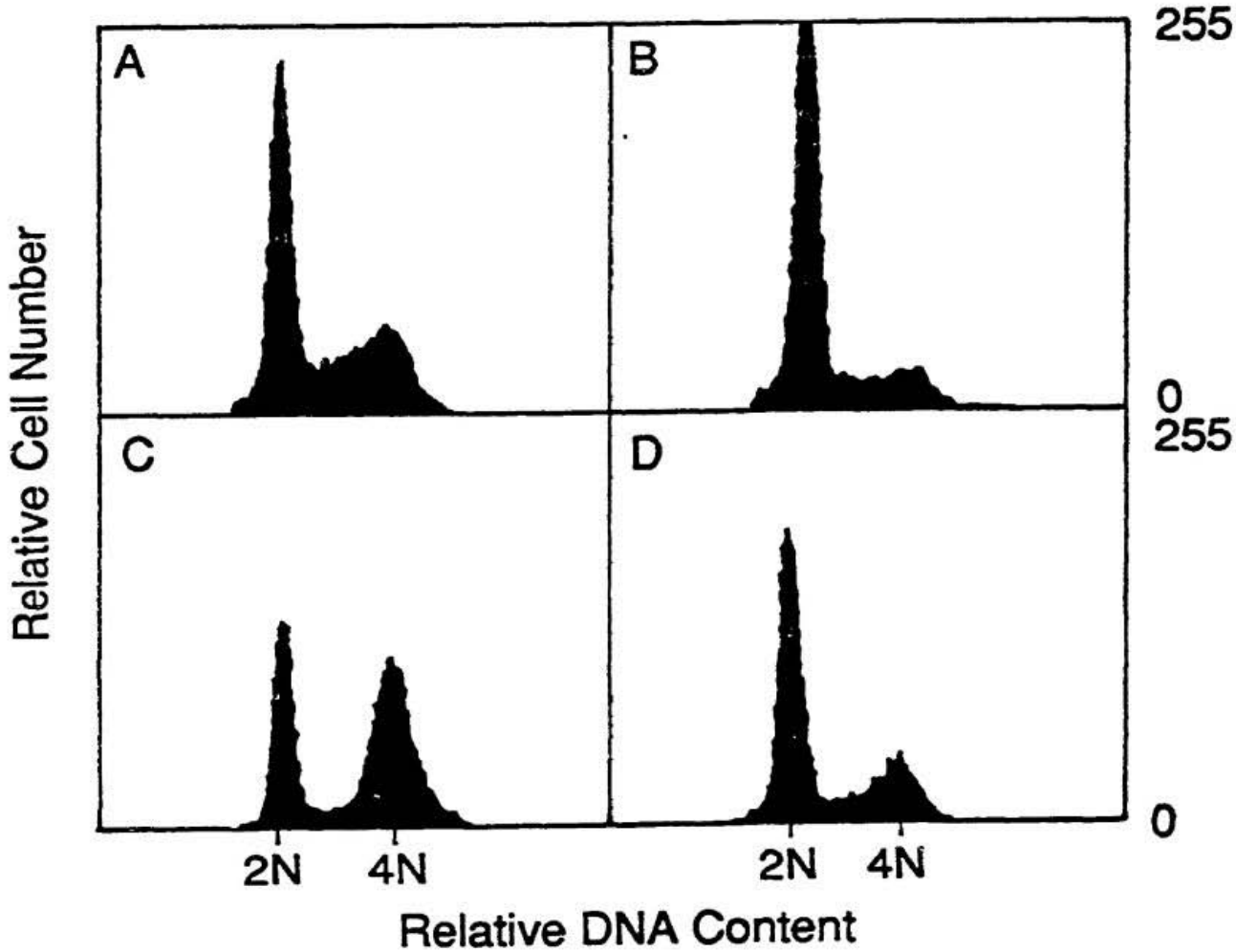

A
B
C
D
255
0
255
0
Relative Cell Number
2N
4N
2N
4N
Relative DNA Content

Table. 3.1 **The cell cycle distribution of MDA-468 cells in response to EGF.** The DNA histograms generated from the FACS analysis of EGF-treated, as well as control untreated cells, from the experiments described under Fig.3.2. legend and in Materials and Methods, were analyzed and the fraction of cells in G1, S and G2/M were estimated as outlined in Materials and Methods.

Length of EGF Treatment (days)	G_1[a]	S	G_2/M
0	44.1 ± 4.6[b]	29.8 ± 5.6	26.0 ± 6.0
0 + V[c]	15.2 ± 5.4	8.2 ± 1.5	76.7 ± 4.8
2	50.7 ± 2.7	26.9 ± 4.2	20.4 ± 5.7
2 + V[c]	34.0 ± 4.6	26.73 ± 8.2	39.1 ± 9.6
4	60.6 ± 3.1	18.9 ± 2.5	20.5 ± 3.7
4 + V[c]	56.5 ± 3.4	11.5 ± 3.5	32.1 ± 4.8
6	58.4 ± 3.2	20.8 ± 2.2	20.9 ± 3.0
6 + V[c]	51.6 ± 3.4	18.4 ± 3.6	28.7 ± 3.9

[a]The DNA histograms generated from FACS analysis of control and EGF-treated (10^{-8}M) cells from experiments as described in Materials and Methods, were collected and the fraction of cells in G_1, S and G_2/M were estimated by computer analysis as outlined in Materials and Methods.

[b]Data are % of total cells analyzed; mean ± S.E.M. from a minimum of 5 experiments in each case.

[c]Vinblastine (1μg/ml) 24 hr. prior to assay.

vinblastine treatment for 24 h (Figs. 3.2A and 3.2C; Table 3.1). However, EGF-treated cells retained a significantly higher G1 population even after vinblastine treatment (Figs.3.2B and 3.2D; Table 3.1) confirming the earlier observation that EGF blocked the cell cycle in G1 phase.

3.2.2 DNA synthesis assays

The cell cycle arrest, observed as an accumulation of cells in G1, could be detected by flow cytometric analysis after 4 days of EGF treatment (Table 3.1). However, it is likely that EGF-induced changes at the molecular level are initiated at a point significantly earlier than this. To address this question, DNA synthesis by MDA-468 cells in response to EGF was studied. Figure 3.3 displays the effect of EGF on DNA synthesis, as measured by the incorporation of [^{3}H] thymidine. There was a significant reduction in DNA synthesis which could be detected as early as 24 h after EGF addition. This drop assumed a plateau after 48 h, which corresponded to approximately 35% of the DNA synthesis as measured in control cells. After 48 h of exposure, the removal of EGF from the medium resulted in the return of DNA synthesis levels to normal, i.e., similar to untreated cells, in about 72 h (Fig.3.3). These findings suggests that EGF reversibly blocks DNA synthesis and cell cycle progression.

FIG. 3.3 **Effect of EGF on DNA synthesis of MDA-468 cells.** Cells were plated at $3x10^4$ cells per well in 6-well plates, and were treated with EGF for the indicated intervals after 24 h of equilibration. Cells were labelled with [^{3}H] thymidine (2.5 μCi/ml) and incubated for 24 h prior to harvesting. At the end of labelling, cells were harvested by trypsinization and TCA-precipitable counts were determined. To correct for varying cell numbers during the course of the experiment, parallel unlabelled cultures were counted using a haemocytometer, and the incorporated radioactivity was standardized to 10^6 cells. The data are shown as percentage of incorporation of control cultures in the absence of EGF, ± std.error.of mean from three individual experiments. □---□ EGF (10^{-8} M) treated cells; ●---● Cells from which EGF was removed after 48 h of EGF treatment.

[^{3}H]-Thymidine Incorporation
[cpm/(10^6cells) : (%, $\frac{\text{EGF - treated}}{\text{Control}}$)]
120
100
80
60
40
20
0
2
4
6
Time (days)

3.2.3 Protein synthesis assays

A time course study of EGF effects on total protein synthesis was carried out. To this end, cells were labelled with ^{35}S-methionine in the presence or absence of EGF for various intervals. At the end of this treatment, cells were harvested and TCA-precipitable radioactivity was measured. Fig. 3.4 depicts the results from such an experiment. It is clear that EGF downmodulates total protein synthesis by about 12 h of treatment. Up to 6 h EGF had no significant effect on protein synthesis. Maximum effect of about 50% inhibition, was approached by 12 h and reached a plateau thereafter. Approximately 50% of protein synthesis was observed in growth-inhibited cells even after 48 h of EGF treatment. The drop in the total protein synthesis was the earliest detectable cellular event associated with growth inhibition.

3.2.4 Alterations in gene expression

Since FACS analysis indicated that EGF induces growth arrest during G1, we examined the levels of transcription of certain genes which are known to be regulated in G1 phase of the cell cycle. We examined the mRNA levels for c-myc, an early G1 marker, PCNA, TK and p53 as late G1 markers and histone 3.2 as a S phase specific marker.

Northern blot analysis of total RNA under various EGF-treatment conditions is shown in Fig.3.5. Cells were serum starved for 4 days prior to the EGF-treatment in an attempt to minimise the background expression levels due to serum. While serum starvation dramatically reduced the c-myc expression, no significant

FIG. 3.4 **Effect of EGF on total protein synthesis.** Cells were plated at $3x10^4$ cells per well in 6-well plates, and were treated with EGF (10^{-8} M) for the indicated intervals after 24 h of equilibration. Cells were labelled with ^{35}S-methionine (25 μCi/ml) and incubated for 1 h prior to harvesting. At the end of labelling cells were harvested by trypsinization and TCA-precipitable counts were determined. To correct for varying cell numbers during the course of the experiment, parallel unlabelled cultures were counted using a haemocytometer, and the incorporated radioactivity was standardized to 10^6 cells. The data are shown as percentage of incorporation of control cultures in the absence of EGF. The figures are from a representative experiment carried out in duplicate.

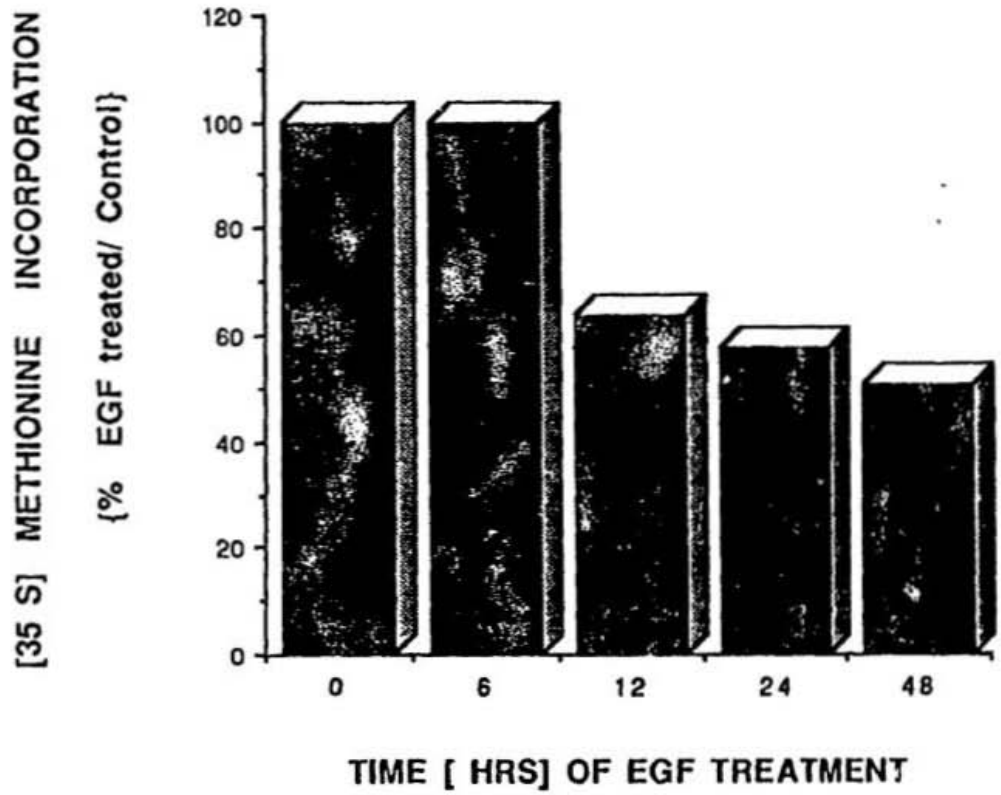
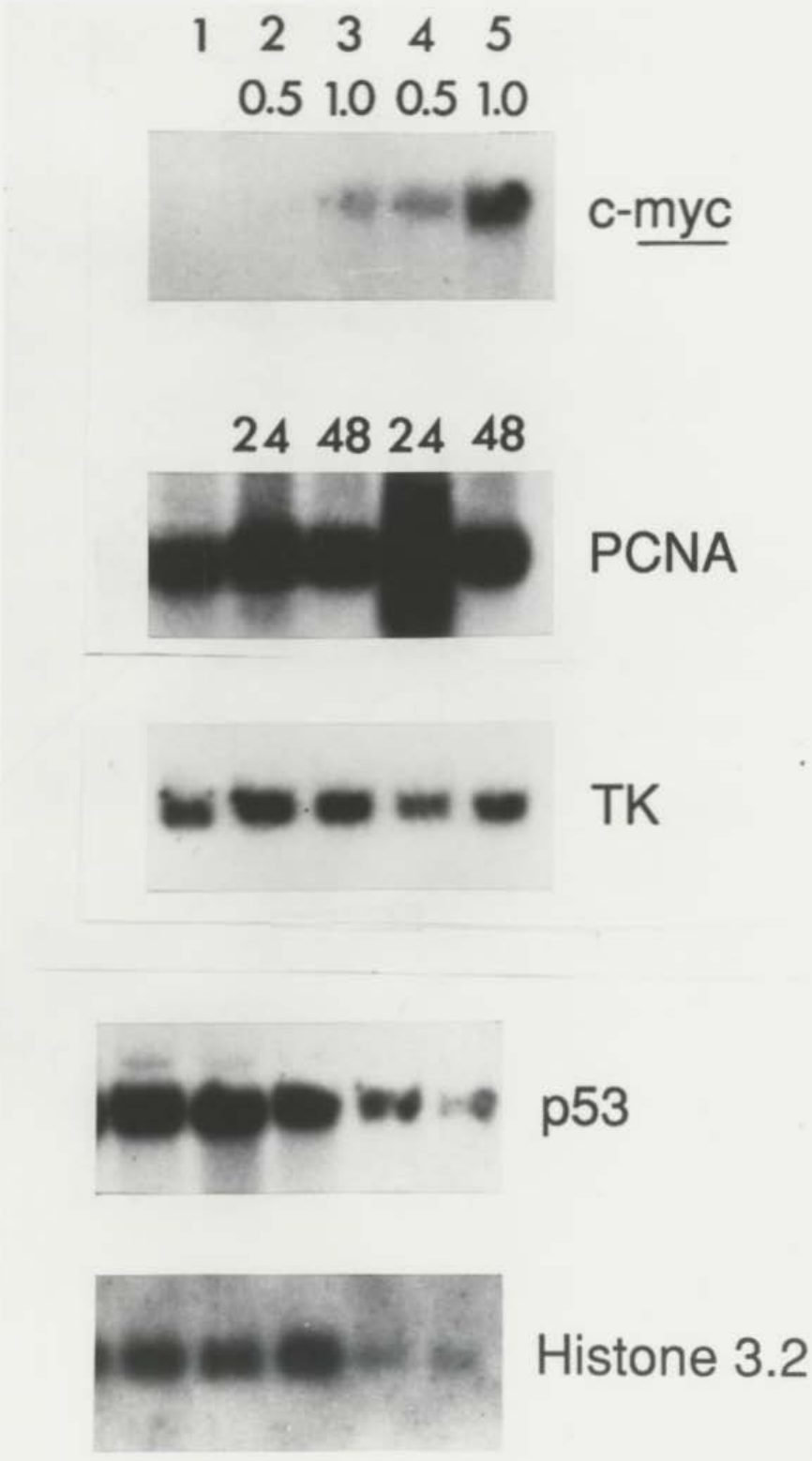

[35 S] METHIONINE INCORPORATION
{% EGF treated/ Control}
120
100
80
60
40
20
0
0
6
12
24
48
TIME [HRS] OF EGF TREATMENT

FIG. 3.5 **Effect of EGF on cell cycle dependent gene expression.** Exponentially growing cells were serum starved for four days (lane 1), following which the cells were further incubated with the media containing serum, in the absence (lanes 2,3) and in the presence of 10^{-8} M EGF (lanes 4,5), for indicated durations (The time points tested for c-myc expression were 0.5 h-lanes 2 & 4-, and 1 h-lanes 3 & 5; rest were checked at intervals of 24 h- lanes 2 & 4, and 48 h- lanes 3 & 5). 20 μg of total RNA was applied to each lane, and gene expression was examined by Northern blot analysis with [^{32}P] labelled probes as outlined (Section 2.2.4). Calf liver 28 S and 18 S RNA were used as standards.


1 2 3 4 5
0.5 1.0 0.5 1.0
c-myc
24 48 24 48
PCNA
TK
p53
Histone 3.2

downmodulation was observed for other markers. The EGF treatment of MDA-468 cells resulted in marked alterations in the transcription of these cell cycle-dependent genes. As reported earlier (Filmus et al., 1987a), c-myc was induced by serum alone and its expression was further enhanced in EGF-treated cells. Higher levels of mRNA for PCNA were also observed after 24 h of EGF treatment. However, this effect was transient since cells treated for longer periods, i.e., for 48 h showed similar levels as compared to control cells. The steady state mRNA level of the TK gene was unaltered in response to EGF treatment. Another late G1 marker, p53, a tumour suppressor gene, believed to play a role in the G1 to S transition, was downregulated in response to EGF. The low levels of mRNA for histone 3.2, indicated a considerable reduction in the number of cells entering S phase. The variations in expression seen in these blots were confirmed by measuring levels of the proliferation-independent gene, α-tubulin, as control for RNA loaded (Not shown).

3.3 DISCUSSION

Flow cytometric analysis of DNA content in MDA-468 cells growth-inhibited by pharmacological concentrations (10^{-8} M) of EGF indicated that the cell cycle was blocked in G1 phase. This evidence was corroborated and extended by the demonstration of its reversibility, and by examining [^{3}H] thymidine uptake in EGF-treated cells. MDA-468 may contain a subpopulation of cells which are resistant to EGF-induced growth inhibition. It has also been observed that MDA-468 cells are heterogenous with respect to number of EGFRs expressed per cell. In fact, this has

allowed the isolation and characterization of variant cell lines that behave in more normal, proliferative manner in the presence of exogenous EGF (Filmus et al., 1987b). This phenomenon may account for the fact that the DNA histograms do not show complete depletion of S and G2/M phase cell populations (Fig.3.2; Table 3.1), and for the observation that the rate of DNA synthesis does not go below 30% of control in [^{3}H] thymidine incorporation assays (Fig.3.3), even when both experiments were carried out over 6 days of EGF treatment (Table.3.1). The reversible nature of the event demonstrates that it is not a function of EGF-mediated toxicity affecting cell viability. This conclusion was corroborated by EGF effects on total protein synthesis. Although, EGF-treated cells displayed immediate reduction in overall protein synthesis in 6-12 h, these cells continue to display significant levels of protein synthesis as compared to untreated cells. This clearly confirms that these cells are completely viable. In fact, more direct evidence for the viability of cells growth-inhibited by EGF has already been reported by MacLeod et al. (1986), by a dye-exclusion test. They observed that more than 90% of A431 cells were viable under the culture conditions used, and there was no significant difference between the viability of untreated and EGF-treated cells.

The manifestations of EGF-mediated growth inhibition are slightly different, depending upon whether it is examined by means of FACS analysis or by [^{3}H] thymidine uptake. Specifically, the decrease in DNA synthesis rates can be observed after only 24 h of EGF treatment, whereas 4 days were required before pronounced

differences in cell cycle distribution were observed. The exact reason for this difference is obscure, but a number of characteristics of both the cell line used and the assays performed may account for it. The approximate doubling time of MDA-468 cells observed under our experimental conditions was about 48 h or more (Fig.3.1). It is known that in a normal cell cycle distribution of an asynchronous cell population, approximately 40-50% of cells will be in different stages of G1 and at least 8 h of continuous EGF presence is essential before its effect on DNA synthesis is observed (Carpenter & Cohen, 1976). The ability to detect an accumulation in G1 over and above control levels will then be expected to take a reasonably long time. Partial synchronization of MDA-468 cell population by serum starvation had little effect on the observed EGF-effects on cell cycle progression (Not shown). This was not unexpected considering the ability of MDA-468 cells to grow under minimal serum requirement. Moreover, EGF-treatment apparently allows the first cell cycle to complete before causing growth inhibition (Gill & Lazar, 1981) perhaps due to the requirement for the continuous presence of EGF for more than 8 h. These observations may explain why release from serum starvation resulted in a more or less asynchronous population. Other more efficient methods of synchronization typically involve intervention in the cell cycle by means of chemicals were not thoroughly tested for fear of any unknown effects on the EGF-mediated growth inhibition.

It is conceivable that cloning of MDA-468 cells may improve the EGF-effects quantitatively, we expected to see measurable changes even without cloning, since EGF produces a detectable effect in a heterogenous population. However, cloning homogenous population of MDA-468 cells was being carried out simultaneously by others in the laboratory. On the other hand, the levels of EGFR expression may change in a cell depending on its exposure to EGF/culture conditions and hence may result in heterogeneity even in a homogenous population over few passages (Gill & Lazar, 1981; Lifshitz et al., 1983). However, we were able to detect early effects of EGF at the molecular level. [^{3}H] thymidine incorporation measures changes in the rate of cellular proliferation by assaying a more precise molecular biological event, i.e., DNA synthesis. As such, it is not surprising that changes here occur prior to those that are manifested by alterations in the distribution of cell populations. However, by examining G1- and S-phase specific gene expression, we were able to assay the effect of EGF treatment on cellular proliferation in an even more precise manner.

As cells enter G1 phase, increases at the level of transcription of a number of genes are observed. These initial G1-phase-specific changes in gene expression include rapid alterations in the expressions of a number of "immediate early genes" (Lau & Nathans, 1987), including c-fos and c-myc (Kelly et al., 1983; Muller et al., 1984). While both can be induced within 90 min of serum or growth factor addition to either G0-arrested or cycling cells (Church & Buick, 1988), the function of these

gene products in the cellular responses to EGF in MDA-468 cells is not understood. Nonetheless, the EGF-mediated augmentation of c-myc mRNA levels shown here (Fig.3.5) clearly demonstrates that the growth factor initiates early signal transduction events in the MDA-468 cell line, and the block in cell growth occurs after passage into G1. As cells continue through G1 and approach S phase, elevations in transcription levels of genes which encode enzymes for DNA synthesis including TK and PCNA (a cofactor for DNA polymerase δ) are observed. In addition, the expression of the tumour suppressor gene, p53, has been shown to increase in late G1 phase (Reich & Levine, 1984; Lalande,1990). In MDA-468 cells, treated with growth inhibitory concentrations of EGF, there was a transient induction of PCNA expression (Fig.3.5). These data suggest that the cells are progressing into late G1, although the significance of higher mRNA levels for PCNA of such short duration is not clear. TK mRNA levels remained unaltered under the same treatment. It has been previously shown that TK gene expression can not be augmented by EGF (Jaskulski et al., 1988) and that TK mRNA levels can be regulated by both transcriptional and post-transcriptional mechanisms (Coppock & Pardee, 1987). However, the fact that TK expression in EGF-treated cells is not reduced relative to untreated cells, suggests cell cycle transit up to late G1. Histone 3.2, being a core histone, is involved in chromatin organization and is expressed at higher levels in S phase (Hirschhorn et al., 1984). In fact, the reduced histone 3.2 mRNA levels further corroborate our FACS data showing EGF-dependent depletion of S and G2/M cell

populations. Lastly p53 expression was down regulated in MDA-468 growth-inhibited cells, indicating that the EGF-induced block in cell cycle transit occurs prior to p53 expression, i.e., at the G1/S boundary.

While the actual mechanism of EGF-mediated growth inhibition remains elusive, we believe that data reported here, combined with that available in the literature, do provide us with some important clues. Firstly, our data suggest that the EGF-induced block in MDA-468 cell cycle occurs only in G1. This differs substantially from similar work carried out in A431 cells (MacLeod et al., 1986), where EGF-dependent growth inhibition was due to cell cycle transit interruption in both G1 and G2 phases. While, the precise reason for this difference between A431 and MDA-468 cells are unclear, it strengthens the notion that overexpression of EGFRs alone, a common feature of these two cell lines, is unlikely to be the sole mechanism responsible for the EGF-induced growth inhibition. Secondly, it is likely the down-regulation of the structural protein, histone 3.2, is a result of cell cycle transit blockade rather than the cause. As causative, inhibition of histone 3.2 transcription may lead to accumulation of cells in S phase due to its requirement for genome organization. However, given that there is no evidence suggesting a cell cycle regulatory role for histone, the observed decreased mRNA levels of histone 3.2, were interpreted as decreased transit of cells through S phase.

On the other hand, our data indicating an EGF-dependent down-regulation of p53 gene transcription may be more important. Several reports clearly demonstrated that a tumour suppressor, RB can act as a mediator for TGF-β induced growth inhibition (Section 1.5.2). MDA-468 cells are homozygously deleted for RB, and possess a single allele for p53 with a point mutation at codon 273 (Bartek et al., 1990). Interestingly, p53, another well-studied tumour suppressor gene, has also been implicated in cell cycle regulation at G1-S transition (Reviewed in Levine et al., 1991). Furthermore, reports suggest a possible role for p53 in TGF-β mediated growth inhibition similar to RB, in a variety of cell types (Ginsberg et al., 1990). Given the total lack of RB function in MDA-468 cells, the presence of a dominant mutant allele of p53 in MDA-468 cells (Nigro et al., 1989; Bartek et al., 1990; Chen et al., 1990), combined with the proposed G1 regulatory role by p53, we suggest that EGF effects may be mediated by the mutant p53. The molecular pathways may be similar to that of TGF-β and RB in keratinocytes (Section 1.5.2). We pursued this line of evidence further to delineate a role of p53 in EGF-mediated G1 arrest in MDA-468 cells.

4. AN INVESTIGATION OF EGF EFFECTS ON p53 IN MDA-468 HUMAN BREAST CANCER CELLS: IMPLICATIONS FOR G1 ARREST

4.1 INTRODUCTION

4.1.1 Background

The previous studies of EGF-mediated growth inhibition in MDA-468 cells provided an essential basis for the experiments detailed in this chapter. Specifically, the observation that EGF-treated MDA-468 cells display lower levels of mRNA for a mutant p53 (Fig.3.5, page 68) suggested that this may play a role in growth inhibition. Wild-type p53 is regarded as a tumour suppressor and is believed to function as a negative regulator of cell proliferation in late G1 phase, whereas certain mutations in p53 are oncogenic (Reviewed in Lane & Benchimol, 1990; Levine et al.,1991; Michalovitz et al., 1991; Donehower & Bradley, 1993). It is well documented that RB, a prototypic tumour suppressor may be involved in growth factor(TGF-β)-induced growth inhibition (Section 1.5.2). However, RB is not likely to be critical in MDA-468 cells, since they are homozygously deleted for RB (Bartek et al., 1990). In a model similar to TGF-β and RB, we hypothesized a putative role for p53 in EGF-induced growth inhibition.

4.1.2 p53 history - a tortuous story

Lane and Crawford, in 1979, identified p53 as a protein found in association with the transforming protein T antigen of DNA tumour virus SV40 (Lane & Crawford, 1979; Linzer & Levine, 1979). p53 is a nuclear phosphoprotein, and draws its name from its apparent molecular weight. There are several regions of the p53 protein which are highly conserved among different species, including humans, rodents and amphibians. These conserved domains are therefore believed to be critical in determining p53 function (Vogelstein & Kinzler, 1992; Donehower & Bradley, 1993).

Studies to characterize p53 indicated that p53 alone could immortalize rat embryo fibroblasts and could cooperate with an activated ras gene in transformation experiments (Jenkins et al., 1984; Eliyahu et al., 1984; Rovinski & Benchimol, 1988). This led to the suggestion that cellular p53 may function as an oncogene. However, this initial notion of p53 as an oncogene was in contrast to the observation of Benchimol and others that several of the tumour-derived cell lines were devoid of any detectable p53 protein (Mowat et al., 1985). In addition, attempts to reproduce the results from the previous transformation studies were not successful using a cDNA clone isolated from normal cells (Finlay et al., 1989). These contrasting views were finally reconciled when the cDNA clones used for the initial studies were found to carry mutations (Eliyahu et al., 1988; Hinds et al., 1989). More thorough investigations from a number of groups established that normal (wild-type) p53

lacked any transforming ability. Interestingly, p53 cDNA clones from normal cells suppressed the transformation by other oncogenes (Finlay et al., 1989; Eliyahu et al., 1989). Furthermore, several groups reported that over-expression of exogenous wild-type p53 in tumour cell lines was actually incompatible with cell proliferation (Mercer et al., 1990; Baker et al., 1990; Chen et al., 1990; Chen et al., 1991; Johnson et al., 1991). This was cited as the explanation for the inability to obtain stable clones of cells expressing wild-type p53 (Diller et al., 1990). Taken together, these data conclude p53 as a 'tumour suppressor' analogous to RB (Section 1.5).

4.1.3 p53 in cancer

In 1989, Vogelstein and colleagues discovered that the p53 gene was frequently affected in colorectal cancers (Baker et al., 1989). This observation has been extended to a variety of other malignancies (Nigro et al., 1989). Deletions, and more commonly, missense mutations in the p53 gene, have been reported in a majority of tumours examined (Vogelstein, 1990; Hollstein et al., 1991). Numerous reports published prior to this had indicated that the p53 gene was mutated in many tumour-derived cells as well (Listed in Quartin et al., 1991). These observations together with the earlier reports that wild-type p53 was incompatible with tumour cell proliferation (Mercer et al., 1990; Baker et al, 1990; Diller et al, 1990), provided strong support to the notion that wild-type p53 was actually a tumour suppressor, and its loss by deletion or inactivation through mutation was associated with transformation and malignancy. This was further corroborated by the observation that transgenic mice

bearing exogenous mutant p53 were highly susceptible to tumorigenesis and developed multiple tumours early in life (Lavigueur et al., 1989).

Another line of evidence comes from the study of DNA tumour viruses. Since p53 was first identified by its association with T antigen of SV40, explanations had been sought for such an interaction. Interestingly enough, different proteins of other tumour viruses interact with p53 as well. For instance, E6 protein of high risk human papilloma viruses HPV 16/HPV 18 (Werness et al., 1990), and E1b protein of Adenovirus 5 (Sarnow et al., 1982), were known to interact with p53. Moreover, proteins of these viruses were known to bind to another prototypic tumour suppressor, RB (DeCaprio et al., 1988; Whyte et al., 1989; Dyson et al., 1989). This led to the suggestion that viral proteins may be eliminating a restraint on cell proliferation through binding and inactivation of tumour suppressors such as p53 and RB. This view was further confirmed when mutants of these viral proteins which failed to bind RB or p53, were found to be defective in transformation assays. The molecular mechanisms underlying such viral protein and p53 interaction are different for each virus. It is known that SV40 T antigen increases the steady state levels of p53, whereas E6 of the HPVs enhances its degradation (Oren et al., 1981; Reich et al., 1983; Scheffner et al., 1990). SV40 T antigen, E1b of Ad5 and E6 of the HPVs were reported to inhibit p53-mediated transactivation function (Segawa et al., 1993; Yew & Berk, 1992; Lechner et al., 1992). These reports define a molecular mechanism by which DNA viruses bring about transformation through inactivation

of negative regulators (p53, RB) of cell proliferation.

4.1.4 Wild-type p53: Biological function

4.1.4.1 Cell cycle regulation

A large body of evidence supports the hypothesis that p53 regulates cellular entry into S phase and progression of DNA replication (Mercer et al., 1984). Schaulsky et al. (1990), demonstrated a cell cycle-dependent alteration in subcellular localization of p53 in normal fibroblasts by means of an immunofluorescence technique. The authors observed that p53 enters the nucleus just prior to the G1/S transition and remains in the nucleus during S phase. In contrast, p53 was absent in the nuclei of mitotic cells. Reports have indicated the involvement of p53 in both normal and transformed cell G1/S transitions (Steinmeyer et al, 1990; Deppert et al., 1990). Studies with a temperature-sensitive mutant p53 protein, further strengthen this finding. The $p53^{135.Val}$ mutant is transforming in association with ras at 37°C, but suppresses transformation at 32.5°C. In addition, cells transfected with this mutant were shown to be growth inhibited in late G1 phase at 32.5°C. The cell cycle block was reversible upon temperature shift to 37°C. Furthermore, employing conformation-specific antibodies, it has been demonstrated that a majority of this temperature-sensitive form of p53 was in mutant conformation (PAb 240 reactive) at 37°C, whereas the wild-type form (PAb 246 reactive) predominated at 32.5°C. Such temperature-induced shift in the conformation of p53, followed by the relocation of such an altered p53 into the nucleus, was attributed as the cause of G1 arrest in

the cell cycle (Ginsberg et al, 1991a; Martinez et al., 1991; Michalovitz et al, 1991).

Milner & Watson (1990), reported loss of certain wild-type-specific epitopes (recognized by PAb 246) of p53 in quiescent cells, stimulated by serum to enter the cell cycle. They suggested that the alteration in p53 protein conformation lead to loss of a negative regulatory function. Milner, in 1991, proposed a conformational hypothesis to explain the biology of p53. According to this theory (Milner, 1991), the regulation of p53 biological function occurs at the level of protein conformation. By means of certain post-translational modifications, endogenous wild-type p53 can assume a 'promoter' form to stimulate cell cycle progression but it also can become a 'suppressor' to negatively regulate the cell cycle. In tumour cells, mutations alter the ability of p53 to undergo such conformational shifts and these mutant forms of p53 are permanently locked in 'promoter' form. Milner's group provided evidence that mutant p53 can force the wild-type form to assume a mutant conformation upon co-translation, suggesting a mechanism for the complete loss of wild-type p53 function with the mutation in only a single allele (Milner & Medcalf, 1991). While this theory has been rigorously tested, the available data are still debatable. Evidence both in support, and in contrast, is equivocal (Zerrahn et al., 1992; Mosner & Deppert, 1992).

An additional link to cell cycle regulation is provided by the fact that p53 is known to be a substrate for $p34^{cdc2}$kinase (CDK1) in vitro (Bischoff et al., 1990). CDK1 is believed to be an important regulator of the eukaryotic cell cycle (Section

1.4.1.1). Studies by Milner et al.(1990), indicated a physical association between p53 and $p34^{cdc2}$ in the interphase stage of transformed cells. Phosphorylation by CDK1 also seems to direct nuclear translocation of p53 (Addison et al.,1990). Other kinases, such as the DNA-dependent protein kinase (DNAPK) (Lees-Miller et al., 1990) and casein kinase II (Meek et al., 1990) are known to phosphorylate p53 as well. Mutations in one of the casein kinase II sites (serine 386) was shown to abolish the anti-proliferative activity of p53 (Milne et al., 1992). In contrast, a series of experiments performed by Slingerland et al. (1993), tested the effect of mutations in specific p53 domains, including the CDK1 sites, the nuclear localization signals, the oligomerization domain and so on, in transformation and transformation-suppression assays, and raised concerns about the significance of the above kinases in p53 function. None of these mutations affected the function of p53 to a significant extent. One clear finding, however, was that monomeric forms of p53 are capable of transformation suppression, whereas oligomerization was essential only for transformation activity. This raises the possibility that different molecular mechanisms are responsible for separate p53 functions. It still remains unclear how this would take place in a normal cell.

Cell cycle regulation by p53 resides in late G1 phase, prior to a restriction point (R point; Lin et al., 1992). Several reports have now established that both in normal, and tumour cells, loss of p53 function abrogates a check point operating in late G1 (Harvey et al., 1993; Tsukada et al., 1993; Kuerbitz et al., 1992). The kinetics

of p53 expression precede that of G1-S progression in synchronized cell populations (Deppert et al., 1990; Steinmeyer et al., 1990). In summary, p53 seems to be a key component of cell cycle regulation specifically in G1, prior to the R point (Donehower & Bradley, 1993).

4.1.4.2 Apoptosis and "guardian of the genome"

The seemingly well-accepted concept of p53 as a negative regulator of cell cycle, was recently challenged by the observations from transgenic mice studies carried out by Allan Bradley's group. Bradley and colleagues developed a p53-deficient strain of mice by a homologous recombination-based gene knock-out technique (Donehower et al., 1992). These mice, even without a functional p53 protein, developed normally to full term without any gross abnormalities. However, the p53- deficient offspring were all dead by 10 months of age. Increased tumour susceptibility was observed, as 75% of these animals developed tumours in various tissues as early as 6 months of age. Based on these data, p53 can be interpreted as non-essential for regulation of normal cell cycle progression. Instead, p53 deficiency may manifest as a long-term effect.

This prompted Lane to propose an alternative function for p53. As he suggests in his "Guardian of the Genome" theory, in a normal cell, p53 operates as a negative regulator, activated by DNA damage (Lane, 1992). Signals from damaged DNA are thought to act on p53 which in turn responds by activating a distinct set of genes. These gene products may be responsible for DNA repair as well as transient

inhibition of replication and cell cycle progression. Evidence to this end is beginning to accumulate. A variety of DNA damage-inducing agents including γ-radiation, X-rays, and cisplatin induce the accumulation of p53 protein by a post-transcriptional mechanism (Kastan et al., 1991; Kastan et al., 1992; Fritsche et al., 1993; Hall et al., 1993a). Certain genes associated with DNA damage such as GADD45 (growth arrest DNA damage) are specifically induced by wild-type p53 upon irradiation (Kastan et al., 1992). Therefore, p53 seems to be an important effector of a DNA-damage response pathway responsible for maintaining genomic integrity. Following a genomic insult, p53 activity is upregulated, which in turn blocks cell cycle progression in G1. In turn this allows the cell to repair the genomic damage. Lack of such a function in cells of p53 deficient mice, or in tumour cells with mutant p53, may allow the cells to proliferate despite the damaged DNA causing accumulation of genetic aberrations. This is reasoned to be a factor in tumorigenesis in young p53-deficient mice (Lane, 1992; Oren, 1992).

In certain cell types, however, such a DNA-damage response pathway seems to result in a different end point. These cells undergo programmed cell death, or 'apoptosis' in response to DNA damage-inducing agents. While, this response has been shown to be mediated by more than one pathway, cellular p53 is known to be an important mediator in at least one of the mechanisms (Shaw et al., 1992; Lowe et al., 1993; Clarke et al., 1993). Thus, evidence is accumulating that p53 is a protein required for determination of cell fate upon a genomic insult. It suggests p53 acts as

a "gate-keeper" of sorts, mediating apoptosis or a transient halt in G1-S progression, depending on the signals received from the damaged DNA.

4.1.5 Wild-type p53: Biochemical function

4.1.5.1 Ancillary replication factor

It is interesting to note that p53 was reported to associate with replication origins along with other known accessory proteins involved in DNA replication (Willcock & Lane, 1991). This prompted the authors to speculate a possible role for p53 in DNA replication. p53 has RNA-binding activity and promotes annealing of single stranded RNAs and DNAs (Oberosler et al., 1993). This activity was originally observed as an anti-helicase activity which blocked SV40 T antigen-mediated DNA replication (Stürzbecher et al., 1988). The notion that p53 may have a role in DNA replication was bolstered by a recent report that replication protein A (RPA) was associated with and inhibited by wild-type and mutant forms of p53 (Dutta et al., 1993). RPA is a single-stranded DNA binding protein complex, believed to be essential for unwinding of DNA origins and initiation of DNA replication (Fairman & Stillman, 1988). The significance of such an interaction is yet to be determined.

4.1.5.2 p53 as a transcription factor

Some authors have suggested p53 may act as a transcription factor. Research indicates the presence of a potent transcriptional activation domain in the amino-terminal region of p53 and that some mutant alleles may have lost this ability (Raycroft et al., 1990; O'Rourke et al., 1990; Fields & Jang, 1990). A DNA binding

domain has also been identified at the carboxyl terminal domain of wild-type p53 (Foord et al., 1991). Furthermore, DNA binding of wild-type p53 has been observed in a sequence-specific manner as well (Kern et al., 1991b, Bargonetti et al., 1991; Funk et al., 1992; Zuberman et al., 1993). Taken together, one of the biochemical roles for p53 appears to be that of a transcription factor. Recently, certain genes regulated by p53 have been discovered. Kastan et al. (1992), reported the p53-mediated induction of GADD45 in response to γ-irradiation. Several reports list various promoters affected by p53 including those associated with proliferating cell nuclear antigen (PCNA)(Mercer et al., 1991; Subler et al., 1992), long terminal regions of Rous sarcoma (RSV) and cytomegalo virus (CMV), SV40 (Jackson et al., 1993), retinoblastoma (Shiio et al., 1992), mdm2 (Barak et al., 1993), interleukin-6 (Santhanam et al., 1991), β-actin, c-fos, c-jun, p53 (Deb et al., 1992; Ginsberg et al., 1991b; Kley et al., 1992) muscle creatine kinase (MCK) (Weintraub et al., 1991) and MDR1 (Chin et al., 1992). Furthermore, p53 has been reported to interact with components of basal transcription apparatus, such as TATA binding protein (TBP) and CCAAT binding factor (CBF) (Seto et al., 1992; Ragimov et al., 1993; Agoff et al., 1993). Therefore, it is conceivable that p53 can alter the expression of specific genes. Those specifically altered may depend on cell-type. In a given cell-type, some promoters may be more sensitive to p53 than others. p53-specific gene expression in cells and their role in G1-S progression or in tumour suppression remains to be elucidated.

4.1.6 Mutations in p53: Functional consequences

Studies performed by several investigators indicated that different mutant alleles of the p53 gene have different biological and biochemical properties (Reviewed in Levine et al.,1991; Michalovitz et al., 1991; Oren, 1992; Donehower & Bradley, 1993). However, in general, most of them are transforming along with the ras oncogene, fail to bind SV40 T antigen, have longer half-lives, and possess different conformations than the wild-type protein. A majority of the mutants fail to react with PAb 1620 , whereas they are recognized by another antibody PAb 240. These antibodies have been well characterized and this pattern of differential antibody-reactivity of p53 has been extensively utilized (Milner et al., 1987; Finlay et al., 1988; Gannon et al., 1990; Michalovitz et al., 1990; Milner & Medcalf, 1990; Milner et al., 1991; Levine et al., 1991; Martinez et al., 1991). Several of the above reports have tested and validated the interpretation of p53 phenotype based on antibody-reactivity by sequencing the gene. Therefore it has been a common practice in p53 research to base conclusions concerning the phenotype based on antibody reactivity, even though rare exceptions to this pattern are known (Gannon et al., 1990; Bartek et al., 1990).

The observation that a majority of tumour cells carry mutations in p53 led to several hypotheses to explain the possible mechanisms of inactivation of p53. The most well-accepted one is that of dominant-negative action of mutants over wild-type p53 (Reviewed in Vogelstein & Kinzler, 1992; Oren, 1992). Briefly, mutant p53

molecules are capable of forming oligomers with wild-type ones, thus interfering with their function (Milner et al., 1991; Milner & Medcalf, 1991). This could either retain the wild-type p53 in the cytoplasm, or simply the oligomers with mutant molecules lack the normal function. This theory was used extensively as an explanation for the toleration of a single wild-type p53 allele along with a mutant p53 allele in some types of transformed cells (Oren, 1992). The oligomerization/sequestration theory implying near total inactivation of wild-type p53 by a mutant allele, however, failed to explain the need for the loss of remaining wild-type p53 allele, commonly observed during the later stages of tumour progression. More detailed studies raised concern as oligomerization-defective p53 molecules seemed to be equally effective as transformation suppressors, as compared to the oligomerization-competent ones (Slingerland et al., 1993). Therefore, a convincing theory regarding the functional consequences of mutations in p53 gene remains to be delineated. In support of this notion are some of the recent systematic studies which revealed that different mutations have different functional consequences as well (Chen et al., 1993b; Miller et al., 1993). Therefore, it may be essential to functionally test for the effect of changes in the p53 gene to fully understand its role in transformation and tumour progression.

On the other hand, interaction with other cellular proteins may determine some functions of p53. Recently, one such cellular protein has been identified (Momand et al., 1992; Oliner et al., 1992). The cloning and further characterization

of this protein, MDM2 (mouse double minute 2), delineated a pathway regulating p53 function. It was shown that mdm2 can be induced by p53 at the transcriptional level (Wu et al., 1993; Barak et al, 1993). MDM2 can interfere with p53 transactivation function through physical binding (Momand et al., 1992), which completes an auto-regulatory loop. Several types of tumour cells tolerating wild-type p53, have been reported to overexpress MDM2 (Reifenberger et al., 1993). There may be other cellular factors affecting p53 function in several ways. More recently, several of these cellular proteins associated with p53 were identified (Maxwell & Roth, 1993). Further characterization of these interactions is essential for total understanding of p53 and its role in cancer.

4.1.6.1 Arg 273 his - an atypical mutant: "Pseudo wild-type"

In MDA-468 cells, studies have demonstrated the presence of a single allele for p53 with a point mutation at codon 273 (Nigro et al.1989; Bartek et al., 1990). This mutation has resulted in substitution of the amino acid arginine for histidine. Histidine 273 mutant p53 ($p53^{273.His}$) appears to have peculiar properties, unlike the majority of mutant p53 proteins. It is more analogous to wild-type in its ability to bind SV40 T antigen (Levine et al., 1991), to transactivate heterologous promoters when expressed as a GAL4 fusion protein (Fields & Jang, 1990), to react with wild-type specific antibody 1620 (Milner et al., 1987) and most interestingly, its nuclear localization irrespective of cell cycle stage (Bartek et al., 1990; Ginsberg et al., 1991a). However it has a longer half-life (>7-8 h), and possesses transforming

potential in co-operation with activated ras (Hinds et al., 1990), features that are shared by other mutant alleles (Reviewed in Levine et al, 1991, Michalovitz et al., 1991). Moreover, $p53^{273.His}$ has been reported to have reduced non-specific DNA binding and to be unable to bind sequences specifically recognized by wild-type p53 (Kern et al., 1991a; Kern et al., 1991b, Bargonetti et al., 1991). However, these reports are based on comparison studies with wild-type p53 under in vitro conditions and have not addressed the possibility of an altered sequence-specificity for mutant p53. Sequences recently identified are recognized by certain mutant p53 molecules to the same extent as the wild-type p53 for transcriptional activity. For instance, $p53^{273.His}$ retains the ability to transactivate through a consensus sequence identified by Funk et al, (1992).

Upon an extensive review of the literature, it is clear that the effect of mutations in p53 is still unclear. There exists a definite likelihood that some of the transforming mutant alleles of p53 remain active as transcription factors inducing the expression of genes essential for cell cycle progression, and in turn, drive cell proliferation.

4.1.7 Working hypothesis

In the context of a pseudo wild-type p53 ($p53^{273.His}$) in MDA-468 cells, observations of its lowered mRNA levels in EGF-treated MDA-468 human breast cancer cells suggested that specific EGF-mediated alteration in putative proliferation-promoting p53 function might be responsible for the G1 arrest. Although it is

plausible that EGF signal transduction may not involve p53, given the known G1 regulatory role of p53, the presence of a functional mutant p53 in MDA-468 cells, and evidence for the existence of similar molecular pathways mediating growth inhibition (TGF-β and RB; Section 1.5.2), we considered it would be essential to test this hypothesis.

> **" We envision a distinct function for $p53^{273.His}$ in MDA-468 cells, one that is essential for their cell cycle progression. Any modulation of such an activity by EGF would arrest the cell cycle of MDA-468 cells".**

To examine this possibility, we initiated an in-depth analysis of EGF effects on p53 in MDA-468 human breast cancer cells. It was reported earlier that no significant changes in p53 transcription were observed within 2 h of EGF-treatment (Filmus et al., 1987a). It is conceivable that EGF-dependent alterations in p53 function may instead occur at translational/post-translational levels. Therefore, we studied various aspects of p53 expression that may have functional consequences including steady state protein levels, protein synthesis, protein stability, subcellular localization and phosphorylation state.

4.2 RESULTS

4.2.1 Northern blot analysis

A time course experiment utilizing Northern blot analysis (Fig.4.1) indicated the down-regulation of p53 mRNA levels are not apparent until at least 18 h after treatment with EGF begins. Reprobing of the filter with α-tubulin, revealed that the decreased signal in lanes 2, 6 and 7 are actually due to reduced amounts of RNA loaded in those lanes (Not shown). Our earlier studies indicated that one of the most readily measurable indicators of growth inhibition was a decrease in overall protein synthesis. This drop occurs significantly within 6-12 h of EGF treatment (Fig.3.4). Therefore, to be causative any change in p53 must precede, or be simultaneous with, this period. Therefore, it is apparent that EGF effects on p53 steady state mRNA levels may not be causative for G1 arrest.

4.2.2 Western blot analysis

Examination of cellular protein levels for p53 was carried out by Western blotting using PAb 1801, a human-specific monoclonal antibody known to react with both wild-type and mutant p53 (Banks et al., 1986). PAb 1801 reacted specifically with at least three proteins other than p53. While a 40 kd band may be a by-product of p53, no clear explanations for other higher molecular weight bands are known. Cells treated with EGF for at least 48 h contained significantly lower levels of p53 (Fig.4.2 Left panel). However, there was no apparent alteration in the levels of p53 in response to shorter EGF treatment intervals (Fig .4.2 Right panel). The protein

FIG. 4.1 **Effect of EGF on p53 steady state mRNA levels.** Total RNA (20 μg/lane) isolated from untreated cells (lanes 1-5), and EGF (10^{-8} M)-treated cells (lanes 6-9) were examined by Northern blot analysis. An end-labelled p53 oligo probe was utilized for hybridization. The durations of EGF-treatment tested were 6 h (lane 6), 12 h (lane 7), 18 h (lane 8) and 24 h (lane 9). The corresponding control samples for 0 h, 6 h, 12 h, 18 h and 24 h (lane 1-5 respectively) were also examined. Calf liver 28 S and 18 S RNA were used as standards. The arrow indicates the p53 transcript of approx. 3.0 kb.

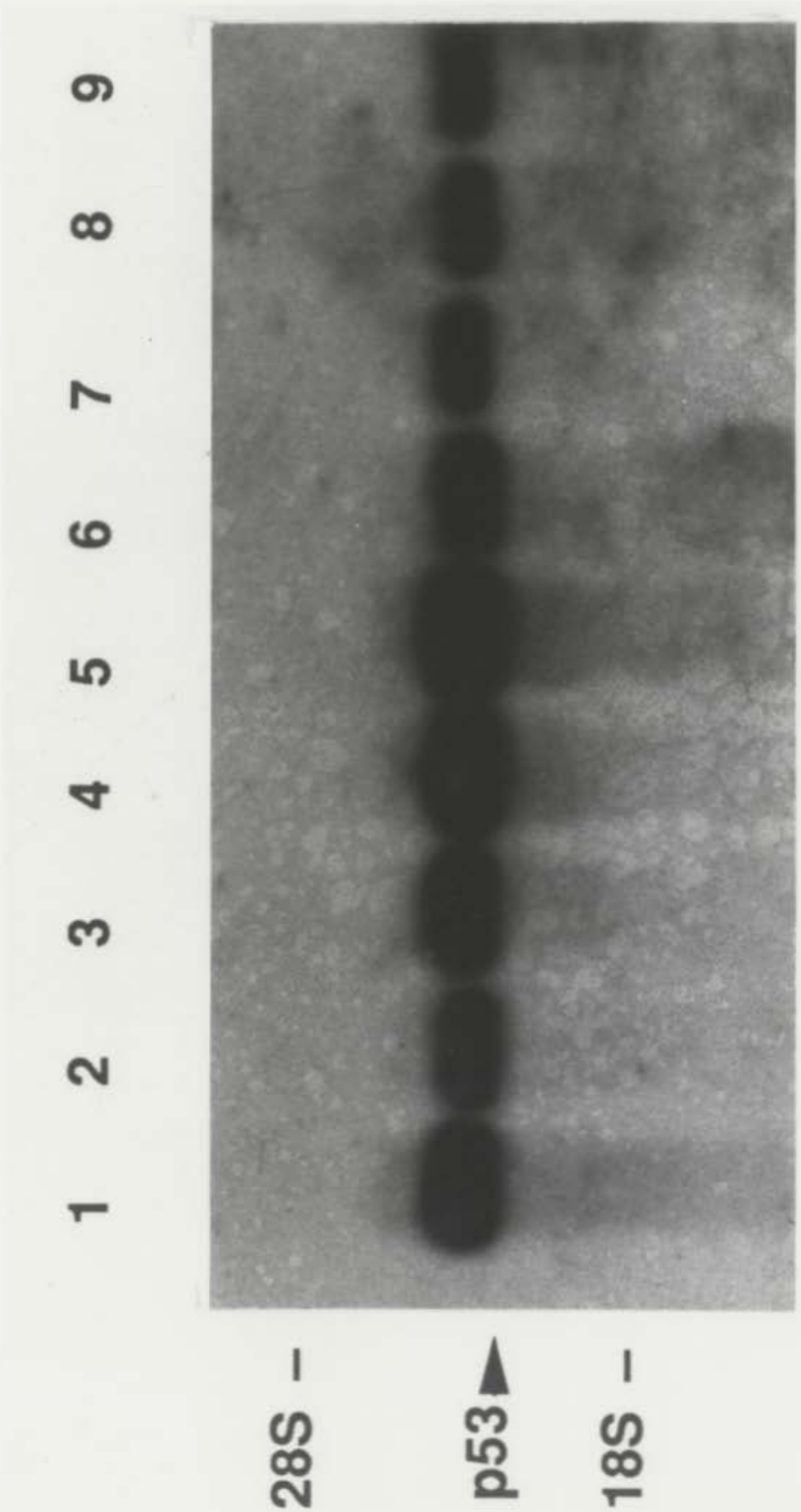
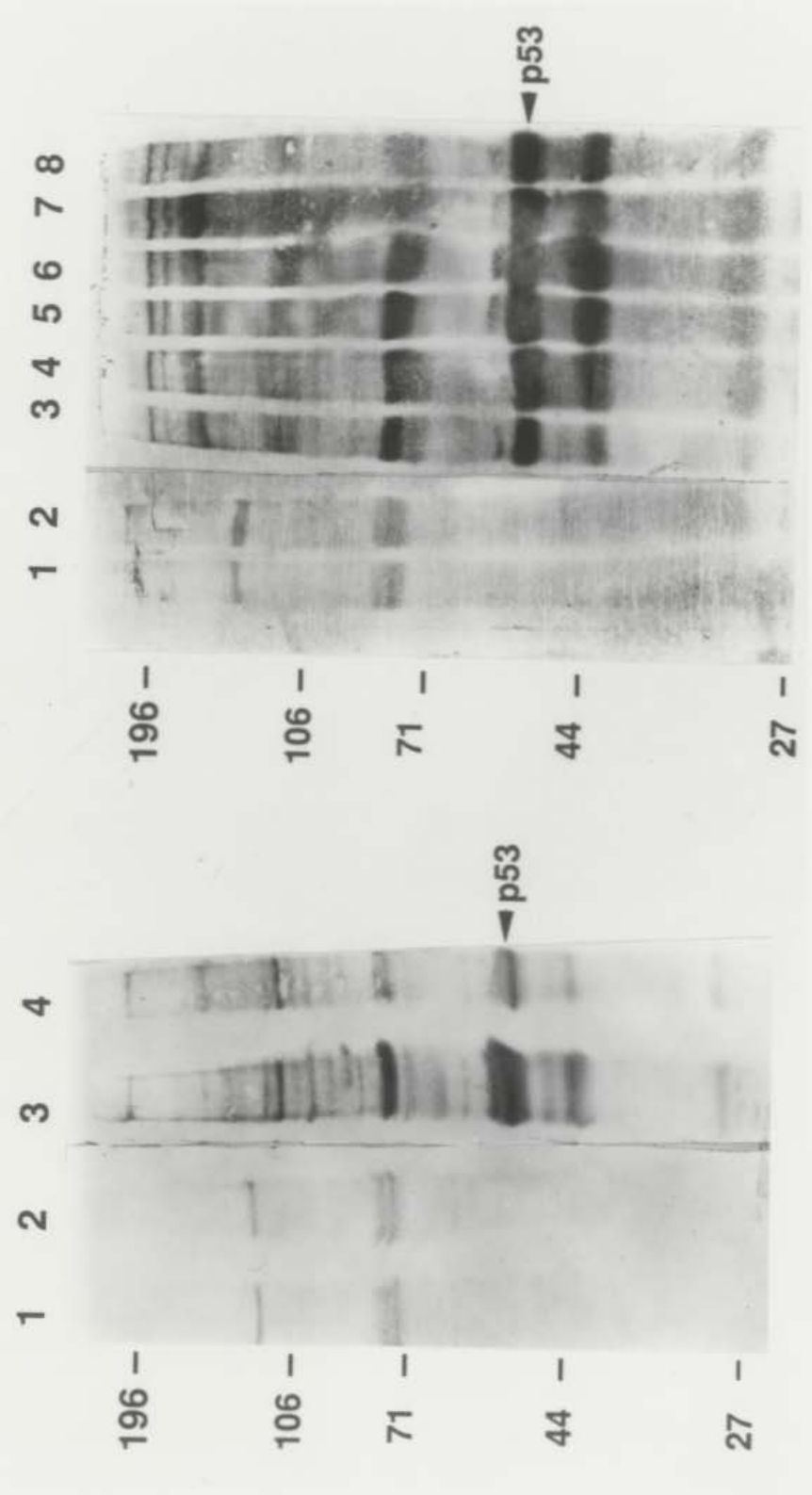

1 2 3 4 5 6 7 8 9
28S –
p53
18S –

FIG. 4.2 **Effect of EGF on p53 steady state protein levels.** The total protein samples (100 μg/lane) isolated from MDA-468 cells cultured ± EGF (10^{-8} M), were separated on an 8% SDS-PAGE. The p53 protein levels were examined by Western blot analysis utilizing alkaline-phosphatase detection system.

Left panel: Samples, in lanes 1 & 3 are from control/untreated cells; in lanes 2 & 4 are from cells, EGF-treated for 48 h.

Right Panel: Samples are from cells, EGF-treated for, 0 h-lanes 1 & 3; 3 h-lane 4; 6 h-lane 5; 9 h-lane 6; 12 h-lane 7; and 24 h-lanes 2 & 8.

Lanes 1 & 2 of both panels were blotted with a control antibody (anti-MHC) while, lanes 3 & 4 of left panel and lanes 3-8 were blotted with PAb 1801 (anti-p53). The arrow indicates the specific band corresponding to p53.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106 –
71 –
44 –
27 –
p53
1 2 3 4 5 6 7 8
196 –
106 –
71 –
44 –
27 –
p53

levels seem to reflect decreased mRNA levels observed after about 18-24 h of EGF treatment. A drop in p53 protein levels might be expected to take considerable time to be apparent given the long half-life (>7-8 h; Hinds et al., 1990) of this protein.

4.2.3 Pulse-chase experiments

Though data on protein levels are indirect evidence for the absence of any dramatic effect on protein stability, a confirmation can be made by a pulse-chase experiment. Standard pulse-chase experiments clearly indicated no shift in the protein stability in response to EGF treatment (Fig. 4.3). Any significant shift in the stability of p53 would have resulted in alterations in the amounts of immunoprecipitated-labelled- p53. A chase of up to 24 h, following over-night labelling of cells with ^{35}S-methionine, revealed that equal amounts of labelled p53 protein were present as indicated by immunoprecipitation with PAb 1801, irrespective of EGF treatment (Fig.4.3; compare lanes 3,5,7 with 2,4 and 6 respectively). Therefore, EGF treatment has not affected the stability of the p53 protein. The multiple bands observed other than p53 were apparently due to the non-specific adsorption to Staphylococcal cells used to recover immunoprecipitates.

4.2.4 p53 protein synthesis

Since we were interested in detecting any and all effects of EGF on cellular p53 status, possible changes in p53 protein synthesis were investigated as well. Cells, briefly labelled with ^{35}S-methionine in the presence or absence of EGF, were sonicated and homogenates were immunoprecipitated with PAb 1801. Though newly

FIG. 4.3 **Effect of EGF on p53 stability.** MDA-468 cells ($1x10^6$ cells/100mm plate) were labelled overnight (10 h) with ^{35}S-methionine (100 μci/ml). The labelling was followed by a incubation in ^{35}S-methionine-free media in the absence (lanes 2, 4, & 6) or in the presence (lanes 3, 5, & 7) of EGF (10^{-8}M), for the indicated intervals. At the end of this incubation (chase), cells were lysed and immunoprecipitated with PAb 1801 (anti-p53). Equivalent counts of the immunoprecipitated samples were then separated on an 8% SDS-PAGE. The durations of the chase tested were, 0 h-lane 1; 6 h-lanes 2 & 3; 8 h-lanes 4 & 5; 24 h-lanes 6 & 7. The arrow indicates the specific band corresponding to p53.

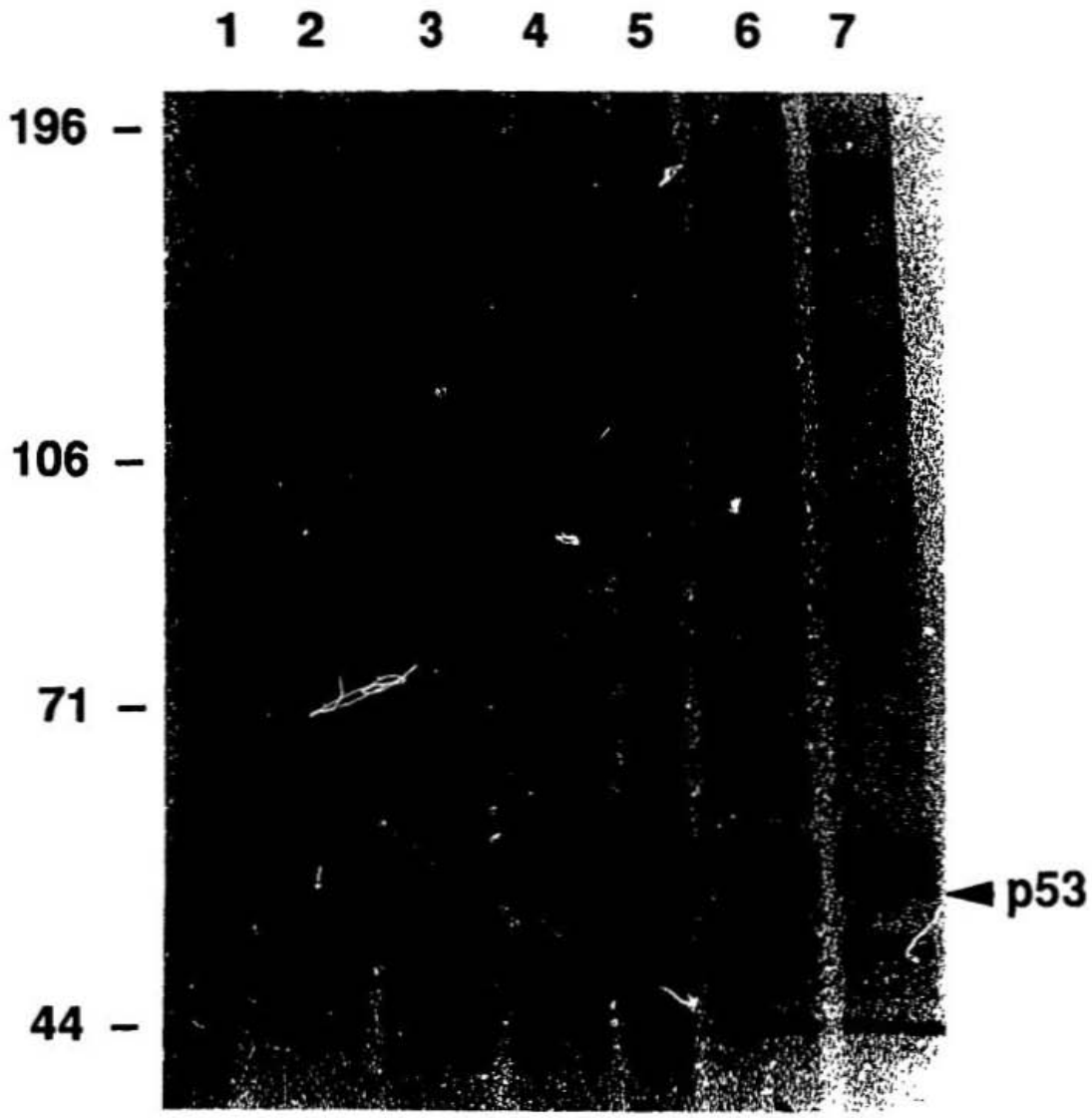
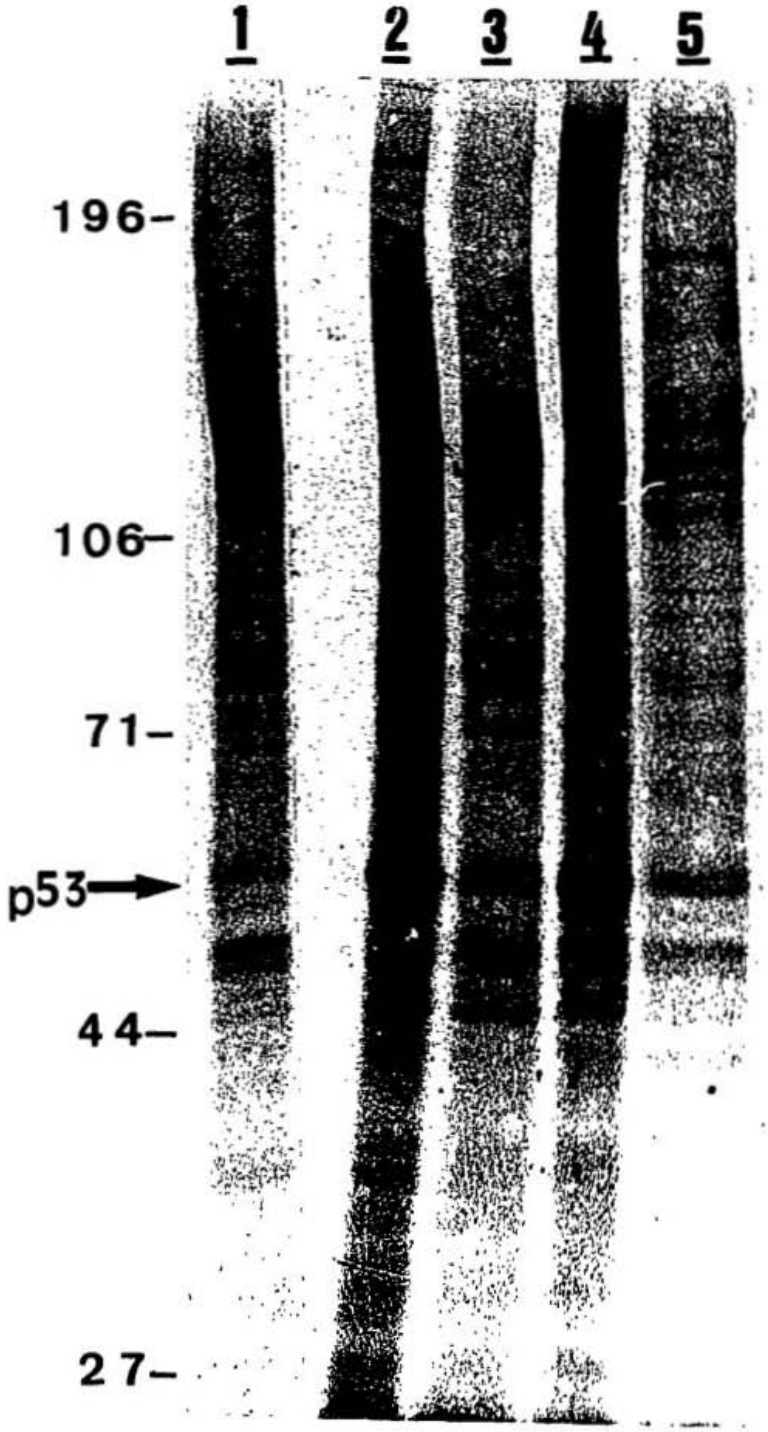

1 2 3 4 5 6 7
196 –
106 –
71 –
44 –
p53

FIG. 4.4 **Examination of EGF effect on p53 protein synthesis.** MDA-468 cells, after 24 h of plating, were treated with ± EGF (10^{-8} M) for the indicated intervals. Labelling was carried out after the indicated duration of EGF-treatment with ^{35}S-methionine (100 μCi/ml) for approx. 10 h (overnight). Labelled cells were then lysed, immunoprecipitated with 2 μg of either a control antibody (anti-MHC; lane 1) or PAb 1801 (anti-p53; lanes 2-5). Immunoprecipitates were collected by Staphylococcal cell-suspension (10% Omnisorb). Equivalent counts of samples were separated on a 8% SDS-PAGE. Lanes 2 & 4 represent samples from 24 h and 48 h untreated cells respectively, lanes 3 & 5 represent samples from 24 h and 48 h EGF-treated cells respectively. Sample in lane 1 was from 24 h untreated cells. The arrow indicates the specific band corresponding to p53.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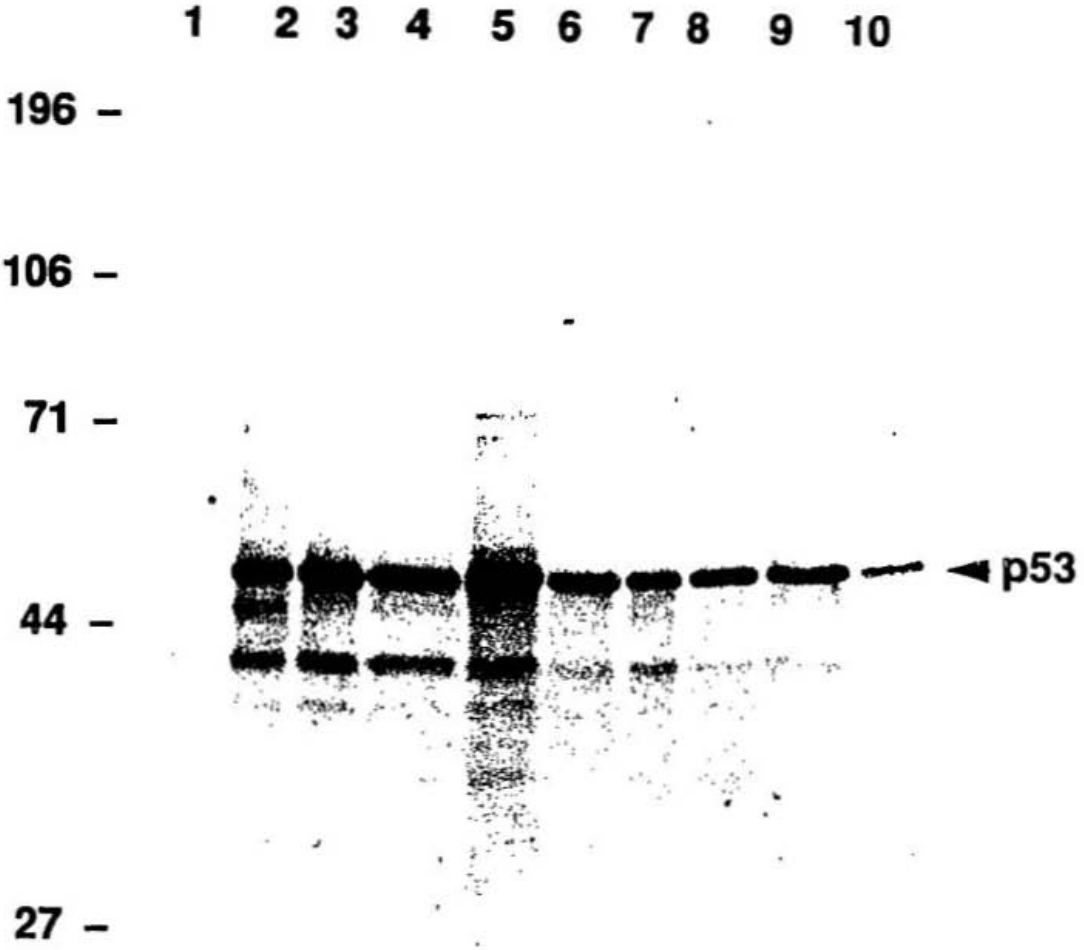
106–
71–
p53
44–
27–

FIG. 4.5 **Examination of short-term EGF effect on p53 protein synthesis.** MDA-468 cells were treated with EGF (10^{-8} M) for the indicated intervals. Labelling with ^{35}S-methionine (100 μCi/ml) was carried out during the last 3 h of incubation. Cells were then lysed and immunoprecipitated with 1 μg of either a control antibody (anti-MHC; lane 1), or PAb 1801 (anti-p53; lanes 2-10). The samples collected on protein A-Sepharose, were adjusted to equivalent counts and separated on a 10% SDS-PAGE. Lanes 1 & 2 - 0 h sample labelled prior to the beginning of EGF-treatment. Lanes 3, 5, 7 & 9 correspond to 3 h, 6 h, 9 h & 12 h samples from untreated cells. Lanes 4, 6, 8, & 10 correspond to 3 h, 6 h, 9 h & 12 h samples from EGF-treated cells. The arrow indicates the specific band corresponding to p53.


1 2 3 4 5 6 7 8 9 10
196 –
106 –
71 –
44 –
27 –
p53

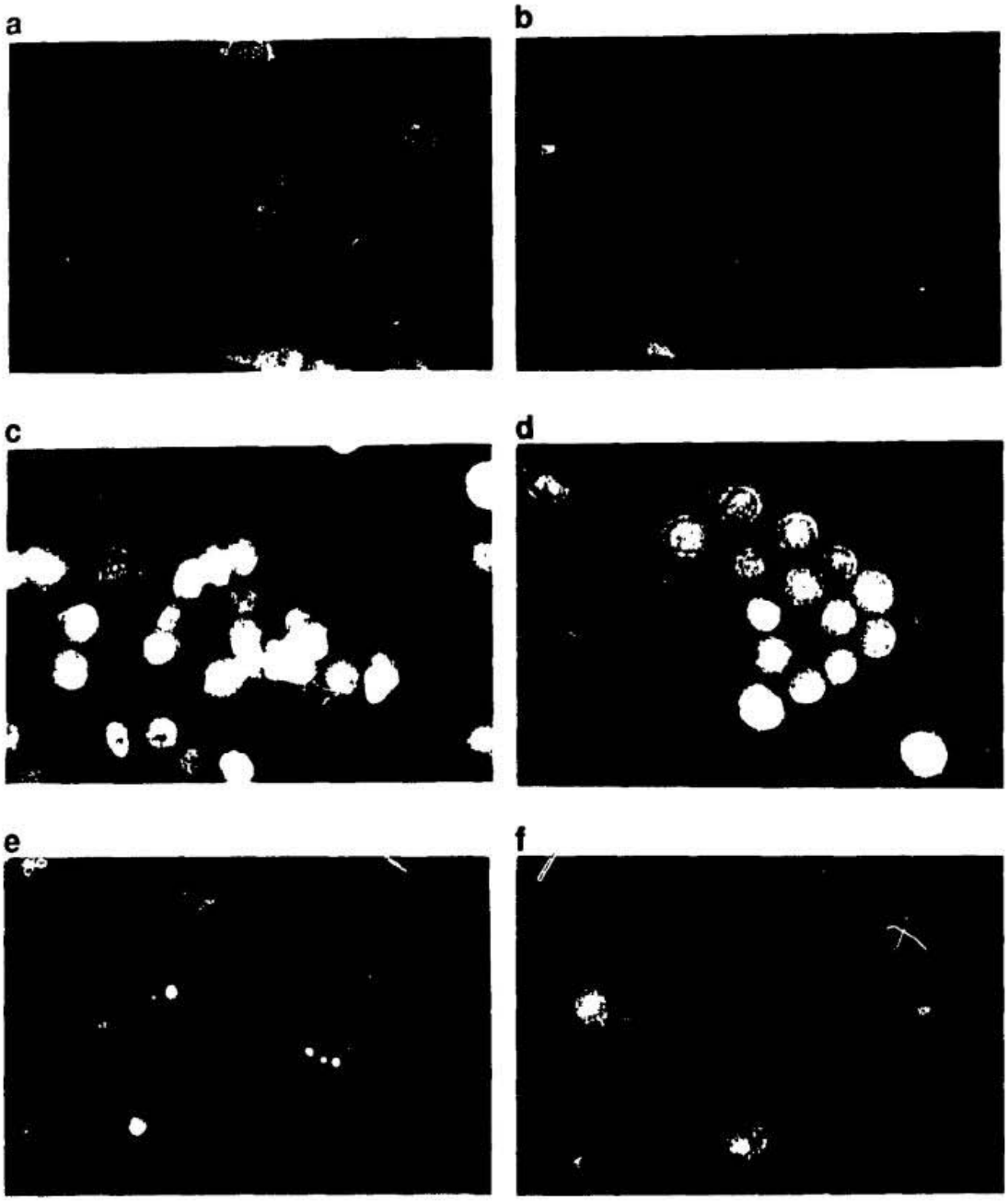
synthesized p53 protein levels were significantly lower after 24 h of EGF treatment (Fig.4.4; lane 3) no immediate effect was obvious until 12 h (Fig.4.5; lane 10). This reduction in p53 protein synthesis following EGF addition likely reflects the reduction in total protein synthesis which occurred between 6-12 h following EGF-treatment (Fig.3.4).

Thus, the data from Northern blot, Western blot, pulse-chase, and protein synthesis experiments, indicate that EGF has no immediate effect on p53 status at the level of transcription, translation or stability that might be attributed as a cause of EGF-mediated G1 arrest.

4.2.5 Immunofluorescence studies

The proposed cellular site of action for p53 is the nucleus and it has been well documented that the function of p53 can be affected by its subcellular localization (Section 4.1.4.1). Hence, we examined the subcellular localization of p53 in MDA-468 cells in cycling and EGF-treated conditions. To this end, we made use of three monoclonal antibodies in immunofluorescence studies: a human-specific PAb 1801 which reacts with both wild-type and mutant p53 (Banks et al., 1986); PAb 240 which specifically reacts with mutant p53 (Gannon et al., 1990); and, the wild-type specific PAb 1620 (Milner et al., 1987). All these antibodies are known to react with the p53 protein in MDA-468 cells, both in immunoprecipitation and immunofluorescence reactions (Bartek et al., 1990; Milner et al., 1987). These antibodies produce a strong nuclear staining in immunofluorescence reactions.

FIG. 4.6 **Examination of subcellular localization of p53 in response to EGF.** Serum starved MDA-468 cells grown on glass slides were incubated with serum containing medium ± EGF (10^{-8} M) for 24 h. At the end of this duration, cells were fixed with methanol:acetone (1:1), blocked with 3% BSA/PBS, and incubated with the anti-p53 antibodies (5 μg/ml). Cells were then stained with FITC-conjugated anti-mouse immunoglobulin (1:50). The p53 localization was detected by fluorescent microscope at 125X (40X x 1.25X x 2.5X) magnification. Panels a, c, & e were untreated cells; panels b, d, & f were EGF-treated cells. Primary anti-p53 antibodies used were panels a & b - PAb 240 (mutant-specific); panels c & d - PAb 1801 (pan-specific); panels e & f - PAb 1620 (wild-type-specific). Parallel chambers were stained with no primary antibody and with a irrelevant antibody (anti-MHC) as controls in every individual experiment (Not shown). Staining with PAb 240 was repeated over 12 times (independent of each other), and consistent staining pattern was observed.


a
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f

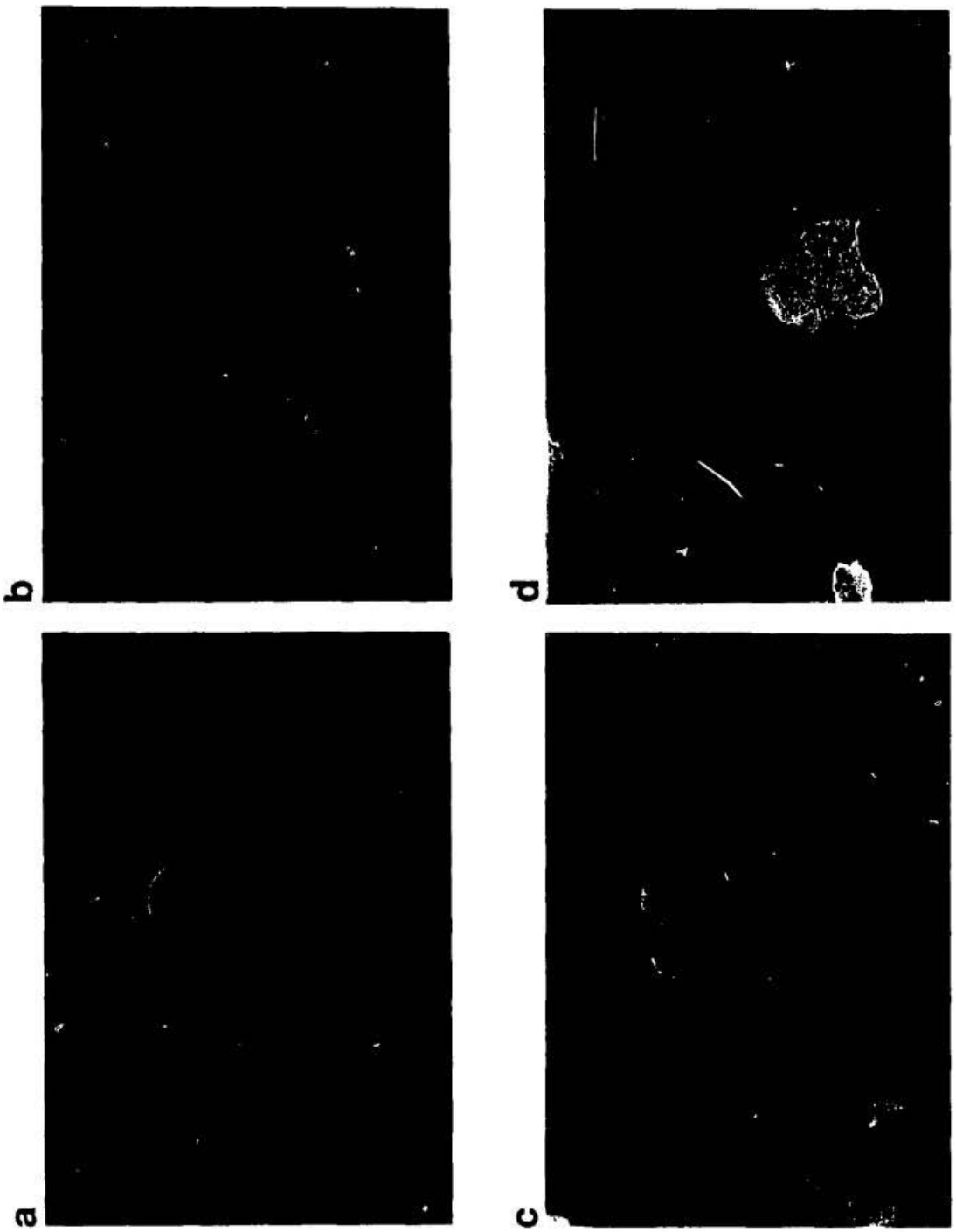
In serum-starved cells, antibodies PAb 1801 and PAb 240 displayed a characteristic nuclear signal along with bright cytoplasmic staining, which remained unaltered upon stimulation with serum. The purpose of serum starvation was to minimise the background staining with p53 antibodies so that any EGF effects would be easily detected. The experiments were carried out with or without serum starvation and no changes in the staining pattern was observed. However, the addition of EGF to the media elicited a differential staining pattern with the two antibodies. With PAb 1801, EGF treatment did not significantly alter the nuclear signal though the pattern appeared to be slightly different and less intense than that of untreated, proliferating cells (Fig.4.6; c and d). The cells were examined up to 72 h in the presence or absence of EGF. The results were consistent and no significant shifts in the intracellular distribution were observed during this period. On the other hand, PAb 240, a mutant-specific antibody, failed to produce nuclear signals in EGF-treated cells, while staining cytoplasm to an extent similar to that of control cells (Fig.4.6; compare b with a). This intriguing lack of nuclear staining became apparent as early as 6 h post-EGF treatment and was observed in 70-75% of the population within 24 h of EGF treatment. Control untreated cells continued to display the characteristic nuclear signal along with cytoplasmic staining. The observation was consistent over a number of repeated experiments (not less than 12 repetitions).

Withdrawal of EGF from the media after 48 h of EGF treatment resulted in reappearance of the nuclear signal in 18-24 h (Fig.4.7; a and b). This observation is

FIG. 4.7 **Specificity of EGF-effects on PAb 240 reactivity.**

Panels a & b : MDA-468 cells grown on glass slides were treated with EGF (10^{-8} M) for 48 h. After 48 h, medium with EGF was removed, cells were washed with PBS and further incubated with fresh medium ± EGF (10^{-8} M) for additional 24 h. Cells were then examined by immunofluorescence with PAb 240 (mutant-specific anti-p53; 5 μg/ml). Panel a - cells continued to receive EGF (total duration of EGF-treatment 72 h); panel b - cells from which EGF was withdrawn for 24 h.

Panels c & d : MDA-468 cells treated with ± aphidicolin (5 μg/ml) were examined by immunofluorescence by PAb 240. Panel c - untreated cells; panel d - cells treated with aphidicolin for 24 h.


b
d
a
c

consistent with the observed resumption of DNA synthesis following EGF withdrawal after 24-48 h (Fig.3.3), and confirms the reversible nature of the cell cycle arrest. To test whether this alteration in staining pattern with PAb 240 is specific to EGF or is an indirect result of growth inhibition, another G1 arrest- inducing agent, aphidicolin was employed. Aphidicolin is a specific inhibitor of DNA polymerase-α and induces a late G1 block in the cell cycle (Huberman, 1981; Lalande, 1990). In MDA-468 cells, treatment with aphidicolin (5 μg/ml) for 24-48 h resulted in an 80% drop in DNA synthesis, indicating that a majority of cells are cell cycle arrested. Immunofluorescence of such growth arrested cells with PAb 240 did not reveal any significant shift in the p53 nuclear staining pattern (Fig.4.7; c and d). This observation confirmed that the loss of PAb 240 reactivity in response to EGF was not a non-specific reaction to G1 arrest but specific to the EGF treatment.

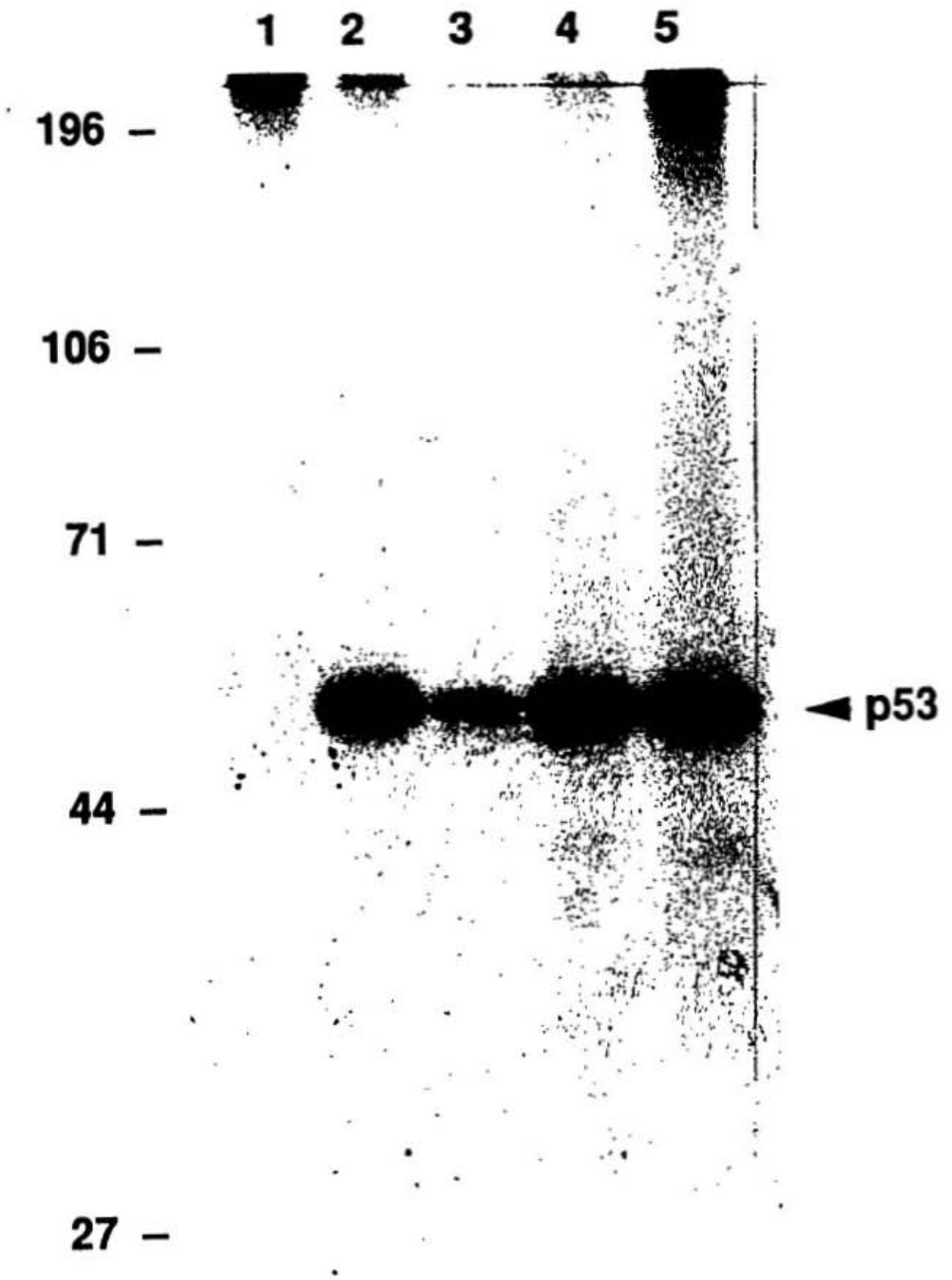
The contrasting results obtained with PAb 1801 and PAb 240 immunofluorescence of EGF-treated MDA-468 cells were intriguing. While PAb 1801 clearly indicated the presence of p53 in the nucleus of EGF-treated cells, this was not detected by PAb 240. We wished to determine, whether another conformation-specific antibody PAb 1620 would react with the nuclear p53 in EGF-treated cells. PAb 1620, a wild-type specific antibody, stained only nuclei and in particular, the nucleoli, with high intensity in the absence of EGF (Fig.4.6; e). This observation is similar to those by Benchimol and colleagues in cells transfected with p53-expression constructs (Slingerland et al., 1993). EGF treatment did not further alter the staining

pattern (panel "f" of Fig.4.6 displays increased nuclear staining, however was not a consistent observation upon repetition). Taken together then, the immunofluorescence data demonstrated that EGF-treated cells continued to carry nuclear p53 that is reactive with PAb 1801 and PAb 1620 but not with PAb 240. The data indicates a EGF-induced shift in the conformation of nuclear p53. Alternatively, it can be interpreted as decreased nuclear translocation of a population of p53 that is reactive with PAb 240, assuming the existence of two conformationally distinct p53 populations in MDA-468 cells as suggested by Bartek et al., (1990).

4.2.6 EGF effects on phosphorylation status of p53.

Immunofluorescence studies indicated a shift in the nuclear localization or a possible change in conformation of p53. Since protein phosphorylation is believed to alter both, in directing nuclear translocation of p53 and altering protein conformation (Addison et al., 1990; Barford, 1991; Karin & Smeal, 1992), the effect of EGF on the phosphorylation status of p53 was studied. Metabolic labelling of cells with ^{32}P-H_3PO_4 and subsequent immunoprecipitation of p53 from whole cell extracts with PAb 1801 revealed significant differences in the phosphorylation levels of p53 in response to EGF (Fig.4.8). EGF caused a dramatic reduction in amounts of p53 phosphorylation within 3 h (Fig.4.8; lane 3). However, at 6 h post-treatment, EGF-treated cells displayed similar p53 ^{32}P content as compared to untreated, control cells (Fig.4.8; lane 4 and 5). Immunoprecipitation from whole cell extracts may have diluted any changes associated with nuclear p53. Therefore, we further tested

FIG. 4.8 **Examination of phosphorylation status of p53 in response to EGF.** MDA-468 cells ($1x10^6$ cells/100mm plate) were treated with EGF (10^{-8} M) for the indicated periods. Cells were labelled with H_3-$^{32}P0_4$ (0.25 mCi/ml) for final 3 h during EGF-treatment. EGF-treated cells and the corresponding untreated/control cells were then lysed, and immunoprecipitated with 2 μg of either a control antibody (anti-MHC) or PAb 1801 (anti-p53) antibodies. Immunoprecipitates were collected on protein A-Sepharose beads, adjusted to equivalent counts and separated on a 10% SDS-PAGE. (Immunoprecipitations conducted with extracts from equal number of control and EGF-treated cells displayed qualitatively similar picture). The arrow indicates the specific band corresponding to p53. Lanes 1 & 2 - untreated cells 3 h; lanes 4 - untreated cells 6 h; lanes 3 & 5 - EGF-treated cells
3 h & 6 h respectively.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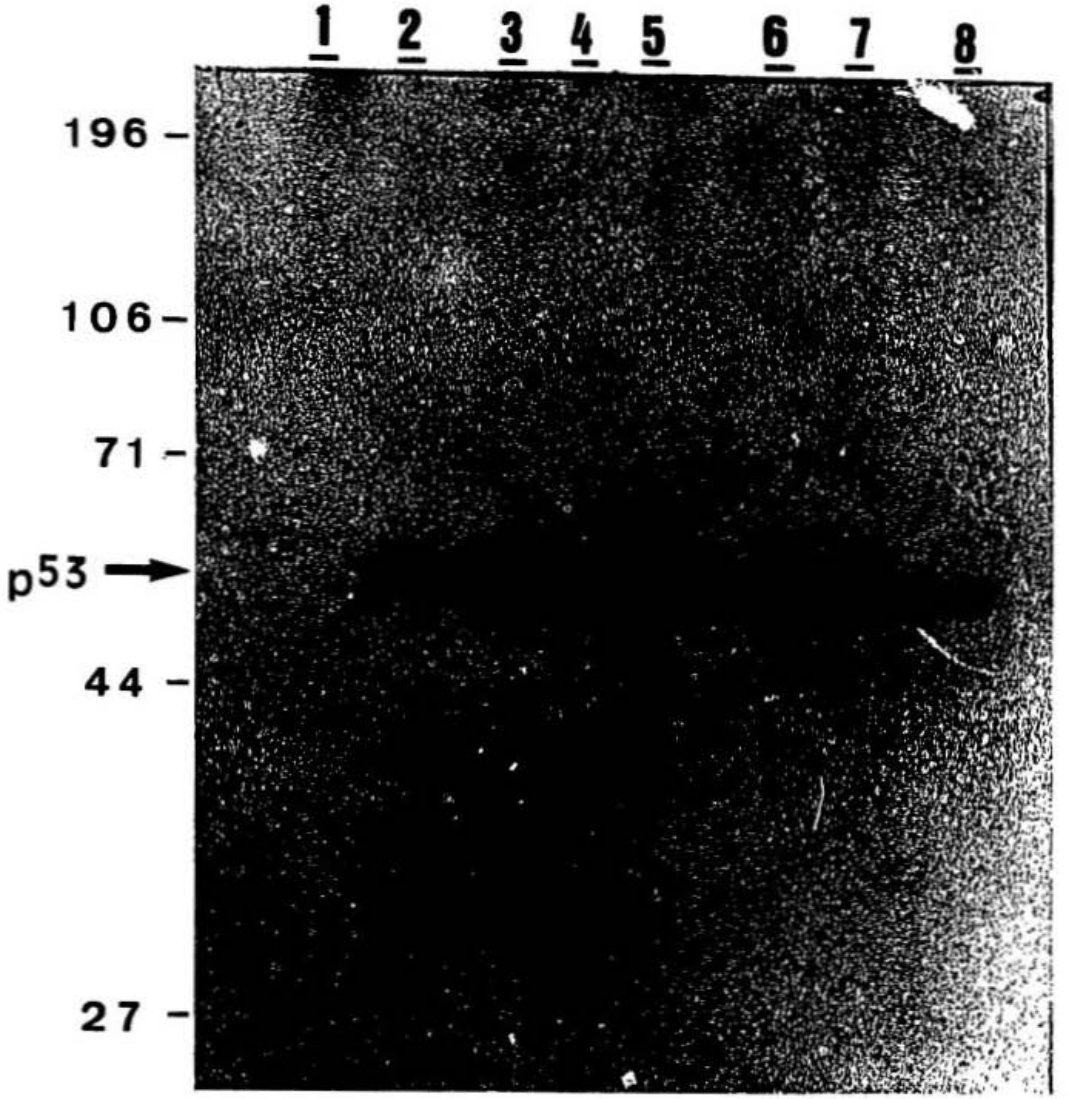
p53

FIG. 4.9 **Phosphorylation status of nuclear-p53 in response to EGF.** MDA-468 cells ($1x10^6$ cells/100mm plate) were treated with EGF (10^{-8} M) for the indicated periods. Cells were labelled with H_3-$^{32}P0_4$ (0.25 mCi/ml) for the final 2 h during EGF-treatment. Nuclear extracts of labelled-cells were prepared as described in Chapter 2 (2.2.11). Equivalent counts of the extracts were used for immunoprecipitation with 1 μg of either a control antibody (anti-MHC; lane 1), or PAb 1801 (anti-p53; lanes 2-8). The protein A-Sepharose collected samples were then separated on a 10% SDS-PAGE. The arrow indicates the specific band corresponding to p53. Lanes 1 & 2 - 0 h (labelled for 2 h prior to the commencement of EGF-treatment); lanes 3, 5, & 7 - untreated cells corresponding to 2 h, 4 h, and 6 h, respectively; lanes 4, 6, & 8 - EGF-treated cells corresponding to 2 h, 4 h, and 6 h, respectively.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phosphorylation of nuclear p53 alone. H_3-$^{32}PO_4$-labelled nuclear extracts were immunoprecipitated with PAb 1801 (Fig.4.9). This revealed that nuclear p53 was phosphorylated to a lesser extent upon EGF-treatment compared to untreated cells. This was obvious within 4 h of EGF addition (Fig.4.9; lanes 5 and 6) and consistent even after 6 h (Fig.4.9; lanes 7 and 8).

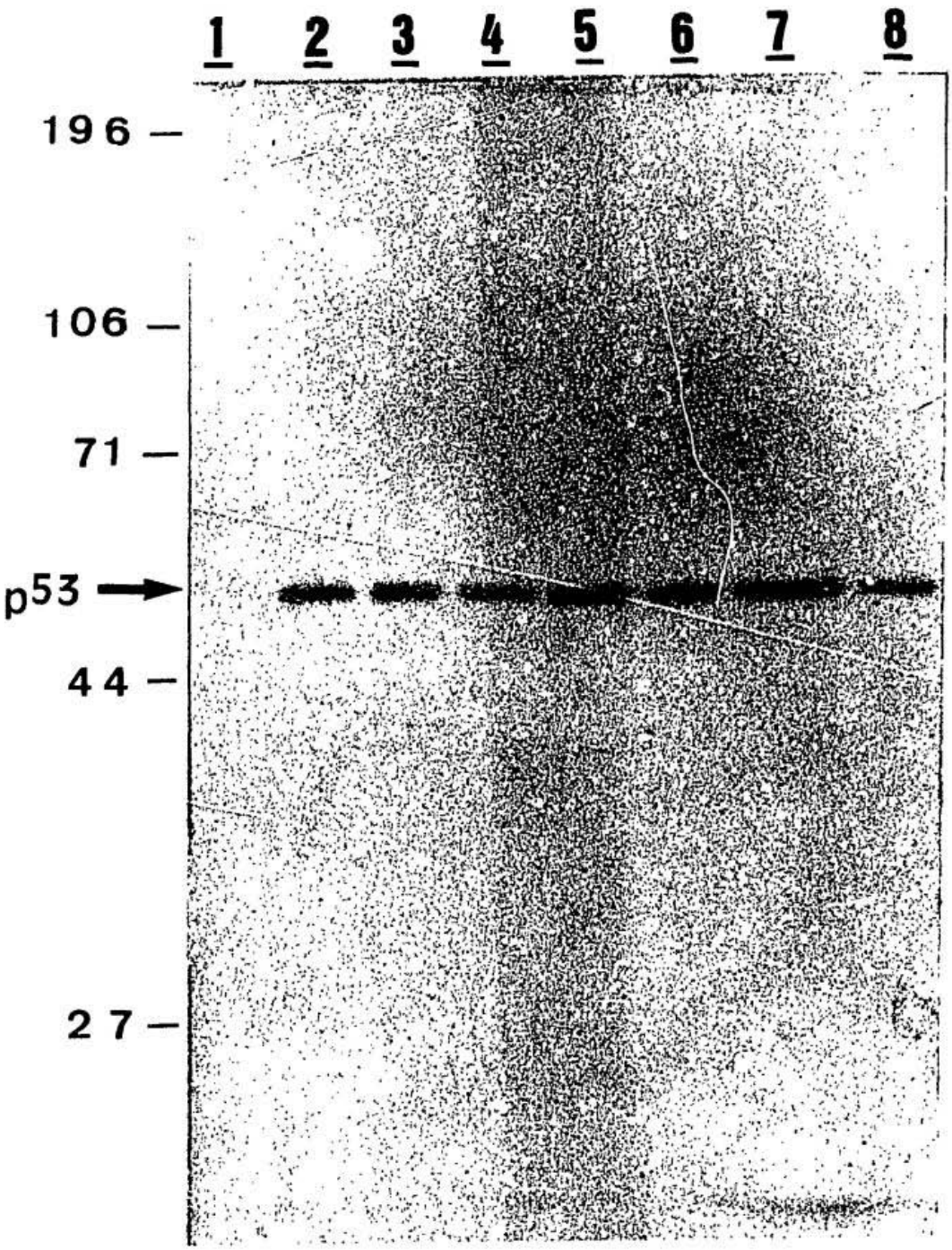
4.2.7 Nuclear translocation of p53

The changes in p53 phosphorylation observed early during the course of EGF treatment may be responsible for EGF-induced conformational change. On the other hand, this may simply reflect an altered nuclear translocation of a population of p53 (PAb 240 reactive) resulting in low levels of nuclear p53 phosphoprotein in EGF-treated cells. To test whether EGF induces a block in nuclear translocation, p53 immunoprecipitations with PAb 1801 from nuclear extracts prepared from cells briefly labelled with ^{35}S-methionine, during the last 2 h of EGF-treatment, were performed. Results (Fig.4.10) failed to demonstrate any significant differences in the nuclear content of the newly synthesized (therefore, labelled) p53 protein up to 6 h after EGF-treatment. We interpret this as indicative of no significant changes in the nuclear translocation of the newly synthesized p53 in the presence of EGF.

4.3 DISCUSSION

We have previously characterized EGF-mediated growth inhibition in MDA-468 cells as a reversible G1 arrest at or near the G1/S boundary in the cell cycle (Chapter 3; Prasad & Church, 1991). Observations of lowered mRNA levels for p53

FIG. 4.10 **Effects of EGF on nuclear levels of de-novo synthesized p53.** MDA-468 cells ($1x10^6$ cells/100mm plate) were treated with EGF (10^{-8}M) for the indicated periods. Cells were labelled with ^{35}S-methionine (100 μCi/ml)) for the final 2 h during EGF-treatment. Nuclear extracts of labelled-cells were prepared as described in Chapter 2 (2.2.11). Equivalent counts of the extracts were used for immunoprecipitation with 1 μg of either a control antibody (anti-MHC; lane 1), or PAb 1801 (anti-p53; lanes 2-8). The protein A-Sepharose collected samples were then separated on a 10% SDS-PAGE. The arrow indicates the specific band corresponding to p53. Lanes 1 & 2 - 0 h (labelled for 2 h prior to the commencement of EGF-treatment); lanes 3, 5, & 7 - untreated cells corresponding to 2 h, 4 h, and 6 h, respectively; lanes 4, 6, & 8 - EGF-treated cells corresponding to 2 h, 4 h, and 6 h, respectively.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in EGF-treated cells and the documented G1 regulatory role of p53 (Section 4.1.4.1) led us to hypothesize its involvement in EGF-mediated G1 arrest. MDA-468 cells are homozygously deleted for the retinoblastoma gene and harbour a single allele for p53 with a point mutation at codon 273 (Nigro et al., 1989; Bartek et al., 1990). This left us with a distinct possibility that loss of negative regulation by RB and wild-type p53, together with an added dominant oncogenic function elicited by the persistent mutant p53 allele, may be driving the cell cycle of MDA-468 cells continuously. Perhaps EGF, through modulation of this mutant p53, might induce the G1 arrest. This portion of the study was initiated to investigate the possibility of p53 being the target for EGF in MDA-468 cells.

We observed the down-regulation of mRNA levels for p53 after about 18 h of EGF treatment (Fig.4.1). However, an immediate EGF effect observed in MDA-468 cells was reduction in overall protein synthesis, which was maximum within 12 h of treatment (Fig.3.4). Therefore, the effect on p53 mRNA levels does not likely constitute a cause of growth inhibition. Moreover, our experiments indicate little alteration in p53 protein levels or stability, in response to EGF (Fig. 4.2 & 4.3). This was a reasonable observation considering the apparent over-expression and longer half life (>7-8 h) of mutant p53 (Hinds et al., 1990). p53 protein synthesis was found to be reduced within 12 h post-treatment with EGF. However, as mentioned earlier, the reduction in overall protein synthesis was maximum prior to this period. Thus, the reduction in p53 protein synthesis may simply reflect the decrease in overall protein

synthesis as a result of growth inhibition.

Our results make it clear that any change in p53 judged a putative cause of growth inhibition must then be post-translational and would be expected to reverse the function of the native p53 protein. Here we report such a change could be reflected in an altered antibody-reactivity of nuclear p53, as detected by a mutant-specific antibody, PAb 240, in response to EGF (Fig.4.6; a and b). This occurs early enough to be attributed as causative, and is reversible upon withdrawal of EGF (Fig.4.7; a and b). Moreover, this observation is specific to EGF-treatment and not a non-specific result of cell cycle arrest as indicated by immunofluorescence of aphidicolin-treated cells (Fig.4.7; c and d). Considered alone, this might simply be interpreted as a block in nuclear translocation of mutant p53 by EGF, thereby removing an essential driving force for cell cycle progression. However, results with a pan-specific antibody, PAb 1801, adds another dimension to the observation. Detection of nuclear p53 with PAb 1801 in EGF-treated cells raised an important question as to whether or not p53 so detected was in the wild-type conformation, being non-reactive with mutant-specific PAb 240. Indeed, nuclear p53 was recognized and detected by a wild-type specific antibody PAb 1620, both in untreated and EGF-treated cells. Intriguingly, PAb 1620 stained nucleoli preferentially in untreated cells with only a faint staining of the rest of the nucleus. There was absolutely no cytoplasmic staining with PAb 1620. In EGF-treated cells however, increased staining of the entire nucleus was apparent. Since immunofluorescence is only semi-

quantitative at best, we can not readily interpret this as a major shift in the abundance of p53 with wild-type conformation. However, it was clear that in EGF treated cells, nuclear p53 retains only a wild-type specific (detected by PAb 1620) epitope and not a PAb 240 reactive one.

These intriguing observations led us to hypothesize a molecular mechanism that may be responsible for EGF-induced growth inhibition in MDA-468 cells. EGF clearly induces a change in the conformation of nuclear p53, leading to a loss of a species expressing the mutant specific epitope. MDA-468 cells may therefore have two distinct populations of p53 molecules, both mutant and wild-type conformations, despite the presence of single, genotypically mutant allele. A dynamic equilibrium between the two in the nucleus would determine the G1-S progression. EGF treatment might be envisioned to shift the equilibrium inhibiting cell cycle progression. This type of phenomenon is not unprecedented (Bartek et al., 1990; Milner, 1991). Furthermore, Milner et al (1993), have reported that certain types of transformed cells carry high levels of phenotypically wild-type forms of p53 despite the presence of mutations in p53 gene.

It is conceivable that a post-translational modification of the protein such as phosphorylation, may mediate the above changes (Addison et al., 1990; Barford, 1991; Ullrich et al., 1992). Indeed, immunoprecipitation of p53 from ^{32}P-orthophosphate labelled MDA-468 cells, indicated a considerable reduction in the levels of p53 phosphoprotein upon EGF treatment. Under the conditions used for

gel-electrophoresis no mobility shift was observed due to altered phosphorylation. To our knowledge, there are no reports indicating such a mobility shift for p53 upon differential phosphorylation in 1-D gels, unlike RB. Serine 15, a potential site for DNA PK, has shown to be differentially phosphorylated in mutants, yet no major change in apparent molecular weights were observed between mutants and wild-type p53 (Ullrich et al., 1993). Similarly, other reports indicate differential site-specific phosphorylation of mutants and wild-type p53 in 2-D gels, yet no mobility shifts were detected in 1-D gels (Ullrich et al., 1992; Milner et al., 1993). This, along with no changes in p53 levels as indicated by ^{35}S-methionine labelled cell-immunoprecipitations, led us to suspect that the decreased phosphorylation of p53 was significant. It is conceivable that these changes may not be reflected in a major shift in the molecular weight. A Lower level of phosphorylation of nuclear p53 in EGF-treated cells is an important indicator of the possible molecular mechanism of altered p53 conformation and function. The identification of the sites involved, along with the possible kinases affected in this EGF-induced phenomenon, will facilitate the delineation of the molecular pathways involved.

We do not, however, have any direct molecular confirmation of the existence of two populations of p53 in MDA-468 cells. PAb 240 weakly reacts with $p53^{273.His}$ in immunoprecipitation experiments. While $p53^{273.His}$ is reactive with the wild-type specific antibody, PAb 1620, we precipitated similar amounts of protein with PAb 1620 in each of three sequential immunoprecipitations. Similar sequential

immunoprecipitation from the nuclear extract failed to bring down detectable amounts of protein with conformation-specific antibodies (both PAb 1620 and PAb 240), perhaps due to the higher salt concentrations employed for preparation of nuclear extracts.

Alternatively, $p53^{273.His}$ in MDA-468 cells may be in a distinct/unique conformation. This may explain its peculiar properties. $p53^{273.His}$ might retain its wild-type conformation, as recognized by PAb 1620, but may also have an altered conformation relevant to other epitopes. This might result in a poorly-recognizable PAb 240 epitope. The observed differences in PAb 240 immunofluorescence could be an indication of further change in the conformation of $p53^{273.His}$. Moreover, loss of PAb 240 reactivity might occur as a result of epitope masking due to post-translational modifications such as phosphorylation. We do not think this is the case, however, since p53 can be immunoprecipitated from whole cell extracts with PAb 240, albeit poorly, from both untreated and EGF-treated cells (Not shown). Therefore, we favour the conformational change explanation, given that there is no evidence for the existence of two populations of p53 in MDA-468 cells or epitope masking by phosphorylation.

The literature concerning the ultimate functional consequences of mutations in p53, remains obscure. The initial notion of a mutant p53 inactivating its wild-type counterpart upon co-existence in the cell has recently been refuted by functional assays. It was reported that p53 monomers are effective as transformation

suppressors (Slingerland et al., 1993). In a separate report it was shown that oligomerization-defective mutants are transcriptionally active, although their DNA binding ability was very low (Tarunina and Jenkins, 1993). Moreover, certain mutants remain functional, indicating that not all the mutations culminate in loss of function of p53. For instance, $p53^{273.His}$ binds at least one of the identified p53 response elements (CON from Funk et al., 1992), and retains the ability to transactivate transcription through it (Chen et al., 1993b).

Many studies aimed at elucidating biochemical functions of wild-type p53, have included $p53^{273.His}$ in the experiments, since it is one of the commonly observed ("hot-spot") mutations in tumour cells (Levine et al.,1991; Vogelstein & Kinzler, 1992). This approach provided a large body of information regarding the effect of Arg273His mutation in p53. Interestingly enough, $p53^{273.His}$ potentiates the transcription from a GAL4-p53 (wild-type) fusion protein in co-transfection experiments (Miller et al., 1993). This was shown to be mediated by oligomerization of the mutant p53 and the fusion protein. Thus, it is possible that in case of co-existence in a cell, $p53^{273.His}$ may increase certain activities of a wild-type p53. However, $p53^{273.His}$ apparently inhibits the DNA binding of wild-type p53 alone to some other DNA sequences (Kern et al., 1991b; Bargonetti et al.,1991). It is clear that sequence specificity for p53 DNA binding is very relaxed. Many of the sequences identified by CASTing, or antibody hybridization, seem to be affected by the presence of certain antibodies employed in the technique. The sensitivity of certain

DNA sequences may be determined in a cell type-dependent manner. Such sequences are difficult to identify. Thus, as Oren suggests, there remains a possibility that p53, acts through unidentified response sequences (Oren, 1992). On the other hand, p53 can also mediate its effect through interaction with components of basal transcription machinery such as TBP and CBF (Sato et al., 1992; Agoff et al., 1993). Effects of $p53^{273.His}$ on these aspects of p53 functions are unclear at present.

Given the G1 regulation by p53 (Section 4.1.4.1), we hypothesize that $p53^{273.His}$ function is critical for cell cycle progression. Our results demonstrate that EGF-mediated changes in p53 conformation may be through altered phosphorylation. We further speculate that this change might have profound effects on $p53^{273.His}$ function. This in turn might mediate a G1 block in the cell cycle of MDA-468 cells. A similar, novel role for $p53^{273.His}$, was proposed by Benchimol and colleagues recently (Slingerland et al., 1993). They observed that this mutation is weakly transforming in rat embryo fibroblasts (REF) along with EJ-ras, depending on the promoter used for its expression. Furthermore, they observed that, $p53^{273.His}$ retained certain wild-type like characteristics such as nuclear localization and suppression of transformation induced by ras and E7 transforming protein of human papilloma virus. This and the previous observation of $p53^{273.His}$-induced transformation of p53-null Saos2 cells (Chen et al., 1990), together, prompted the authors to speculate a novel function for $p53^{273.His}$. We believe that a novel role may be determined by the cell-type rather than by the intrinsic properties of the protein itself. Experiments

conducted to test this hypothesis is described in the following chapter.

5. ANALYSIS OF p53 FUNCTION AS A TRANSCRIPTION FACTOR IN RESPONSE TO EGF IN MDA-468 CELLS.

5.1 INTRODUCTION

5.1.1 Background

A detailed analysis of the effects of EGF on p53 in MDA-468 cells has indicated a shift in the conformation of nuclear p53 molecules. Specifically, we suggest that G1-S progression is essentially dependent upon p53 function, and in turn on its conformation. It is essential, however, to obtain confirmation for the EGF-dependent change in the p53 conformation by more sensitive techniques. In addition, functional information concerning the observed shift in conformation of p53 molecules upon EGF treatment of MDA-468 cells will be invaluable in understanding how it is involved in EGF-mediated G1 arrest. In this chapter, we attempt to address this by means of a detailed study of specific DNA binding and transcriptional activation by $p53^{273.His}$ in MDA-468 cells.

5.1.2 p53 - a transcription factor

5.1.2.1 Transactivation by p53

Studies aimed at elucidating the biochemical activities of p53 provided conclusive proof for its role as a transcription factor. The ability of a cellular protein to regulate transcription specifically resides in its intrinsic DNA-binding activity in a

sequence-dependent manner and potential to modulate transcription upon binding to such sequences. Initial work from Kern et al. (1991a), indicated non-specific DNA binding activity intrinsic to p53. The authors also noted that a series of mutants of p53 have reduced or no DNA binding activity. In addition, two groups independently reported that the amino-terminal acid-rich region of p53 possessed a potent transcriptional activation domain (Raycroft et al., 1990; Fields and Jang, 1990). These investigators made use of chimeric proteins generated by fusing the amino-terminal domain of p53 to the DNA-binding domain of GAL4, a yeast transcription factor. This fusion protein transactivated a template with GAL4 sites. In these functional assays also, some of the mutants tested negative for transcriptional activity. Further characterization of the domains required for transactivation narrowed the region to 22 amino acids located between codons 20 and 42 (Unger et al., 1992; Miller et al., 1992). Thus, these studies provided sufficient information to suggest a biochemical role for p53 as a transcription factor.

5.1.2.2 Specific DNA-binding sites for p53

The above reports elicited further attempts to obtain confirmatory evidence for a regulatory role of p53 in transcription. This led to the identification of DNA-sequences specifically bound by p53. Vogelstein and colleagues (Kern et al., 1991b), reported identification of two separate sequences as specific-sites for p53 DNA-binding. These sequences were found in association with ribosomal gene clusters (RGC) which contained two or more TGCCT repeats. The TGCCT repeats appeared

to be important for specific interaction with p53. More or less simultaneously, Weintraub and co-workers, in their study of transcriptional regulation of the muscle creatinine kinase (MCK) gene, observed that wild-type p53 activated transcription through MCK enhancer/promoter sequences (Weintraub et al., 1991). The p53-mediated transactivation was later demonstrated to be dependent on a 50-base sequence within the MCK enhancer/promoter and was bound specifically by wild-type p53 (Zambetti et al., 1992). Interestingly enough, the 50-base sequence contained two TGCCT repeats as identified by Kern et al. (1991b). These studies were soon followed by a number of others identifying additional specific sites for p53-mediated transcriptional regulation (Funk et al., 1992; El-Diery et al., 1992; Zauberman et al., 1993). Attempts to identify genomic p53-responsive elements, indicated a loose consensus sequence required for DNA binding (El-diery et al., 1992). It appears p53 binds to two repeats of the sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0-13 bases. The role of p53 in transcriptional regulation was further confirmed by in vitro studies employing immuno-purified p53 protein and a sequence, associated with ribosomal gene clusters (RGC), conforming to the above consensus (Farmer et al., 1992).

Detailed analysis of p53 revealed that the carboxyl-terminal domain is required for DNA-binding activity (Foord et al., 1991), and this was found to be influenced by a variety of cellular mechanisms (Hupp et al., 1992), including phosphorylation, tryptic digestion, and antibody (PAb 421)-binding. A 30-amino acid deletion of the

carboxyl-terminal, partial cleavage by trypsin, binding of PAb 421 to a carboxyl-terminal epitope, phosphorylation by casein kinase II, all resulted in activation of the DNA-binding ability of p53. The authors suggested that these and perhaps other as yet unknown modifications, may convert the latent DNA-binding activity of cellular p53 into an active one. Intriguingly, it was also observed that cellular p53, in nuclear extract preparations, bound to DNA whereas in vitro translated-p53 failed to do so (Funk et al., 1992). This observation strengthens the notion of Hupp et al. (1992), that transcriptional activity of p53 may be influenced by interaction with other cellular factors. Such a regulation of p53 activity by biochemical events in a cell-type dependent manner, is an attractive hypothesis, but, remains to be confirmed.

5.1.2.3 Regulation of transcription by p53

Since the first indications that p53 could act as a transcription factor, a plethora of studies have reported the effects of p53 on various promoters (Ginsberg et al., 1991b; Santhanam et al., 1991). Wild-type p53 actively potentiates transcription from promoters with p53-responsive elements. Examples of cellular genes activated by p53 include MCK, GADD45 and mdm2 (Zambetti et al., 1992; Kastan et al., 1993, Wu et al., 1993). As mentioned earlier, a 50-base sequence in the 5' region of MCK gene is a p53-responsive element (Zambetti et al., 1992). In the case of GADD45, a sequence distantly related to a p53-response element, was identified in the I^{st} intron (Kastan et al., 1993). The 5' regulatory regions of the mdm2 gene also contain a sequence related to a p53-response element (Wu et al., 1993).

In the absence of p53-responsive elements, wild-type p53 represses transcription. p53 was reported to downregulate transcription from various promoters such as those associated with the genes coding for PCNA (Mercer et al., 1991; Subler et al., 1992), c-fos, c-jun, β-actin, heat shock protein 70 (hsp 70), p53 (Ginsberg et al., 1991b; Kley et al., 1992) multiple drug resistance (MDR)(Chin et al., 1992), RB (Shiio et al.,1992), interleukin-6 (Santhanam et al., 1991), viral regulatory regions of Rous sarcoma (RSV) and cytomegalo viruses (CMV), SV 40 (Jackson et al., 1993), human immunodeficiency (HIV), herpes simplex (HSV 1) viruses (Deb et al., 1992; Subler et al., 1992). Recently, it was demonstrated that wild-type p53 binds to the TATAA binding protein (TBP), a component of transcription factor IID (TFIID) (Sato et al., 1992). It is this interaction that is thought to be responsible for the almost universal repression of transcription by wild-type p53. More recent studies, however, have indicated an enhanced binding of p53 to a p53-responsive element in the presence of TBP (TFIID) (Chen et al., 1993a). Wild-type p53 inhibited DNA binding of TBP but not that of TFIID. Thus, specific interactions with basal transcriptional machinery may be important for the observed effects of p53. This notion is supported by a report demonstrating interaction of p53 with CCAAT binding factor (CBF) involved in the repression of hsp70 gene expression (Agoff et al., 1993).

In summary, wild-type p53 is capable of transactivation through p53-responsive elements and inhibiting transcription from promoters lacking such sequences. However, while mutations in p53, in general, fail to demonstrate transcriptional regulatory activity (Raycroft et al., 1991; Kern et al., 1992), exceptions, once again, have been described. $p53^{248.Trp}$ and $p53^{273.His}$ have been shown to be equally active as wild-type from certain p53-response elements (Raycroft et al., 1991; Chen et al., 1993b). Since, MDA-468 cells harbour $p53^{273.His}$, reports concerning its DNA-binding and transactivation are discussed below in detail.

5.1.3 DNA binding ability of $p53.^{273.His}$

The mutant $p53^{273.His}$ was initially thought to be unable to specifically bind DNA. The sequences first identified by Kern et al. (1991b) and Bargonetti et al. (1991), were not bound by $p53^{273.His}$. In fact, the authors observed that $p53^{273.His}$ interfered with the DNA-binding of wild-type p53 under in vitro conditions. The authors hypothesized that alterations in DNA-binding may be the ultimate effect of mutations leading to the loss-of-function phenotype. Interestingly, a consensus sequence described by Funk et al. (1992), was bound by $p53^{273.His}$ equally well as wild-type p53. This observation underscores the necessity for a cautious interpretation of results and care in generalization. Thus, it is conceivable, that certain p53 mutants may remain active through sequences that remain to be identified.

5.1.4 Transcriptional activation by $p53^{273.His}$

One of the preliminary studies that demonstrated the existence of a potent transactivation domain in p53 actually utilized $p53^{273.His}$ (Fields & Jang, 1990). The study clearly indicated that this particular point mutation has not affected the transactivation function of p53. However, later studies with the specific p53-response elements identified by Kern et al. (1991b), and Bargonetti et al. (1991), argued that the Arg273His mutation had detrimental effects on p53 sequence-specific DNA binding and transcription (Farmer et al., 1992). In contrast, $p53^{273.His}$ was demonstrated to transactivate transcription through specific-binding to the sequence (CON) identified by Funk et al. (Chen et al., 1993b), to an extent similar to wild-type p53. Another report of particular interest described the effect of $p53.^{273.His}$ on the transcriptional activity of GAL4-p53 fusion proteins (Miller et al., 1993). In this study, the authors showed that co-expression of $p53^{273.His}$, enhanced the transactivation function of GAL4-p53 (wild type) by five-fold. The authors went on to demonstrate that this was mediated by oligomerization of the fusion protein and the mutant p53. Thus, it appears that $p53^{273.His}$, can indeed bind specifically to DNA, can modulate transcription independently, and can alter the transactivation function of wild type p53 as well.

5.1.5 Working hypothesis

Since immunofluorescence experiments have demonstrated that EGF-treated cells display loss of PAb 240 epitope in nuclear p53, while retaining PAb 1620 reactivity (Fig.4.6), we hypothesized that EGF may be shifting the p53 conformation to wild type form. This putative EGF-induced change in p53 conformation, perhaps due to altered phosphorylation (Fig. 4.8 & 4.9; and Section 4.3), might result in an altered p53 function. In order to confirm an EGF-mediated alteration in p53 conformation, as well as to determine any concomitant EGF-dependent change in function of p53 as a transcription factor, two different p53- responsive sequences have been utilized. They are the CONsensus sequence described by Funk et al. (1992), and fragment A (FRA) as reported initially by Kern et al. (1991b). As mentioned above, $p53^{273.His}$ has been shown to bind to and to transactivate from the CON sequence alone.

" We hypothesized that an EGF-induced change in p53 conformation could be detected by different binding characteristics of $p53^{273.His}$ to the two specific DNA binding sites. This EGF-induced p53 activity, may be responsible for the induction of genes associated with the G1 arrest".

To test the transcriptional activity of p53 through CON and FRA, its DNA

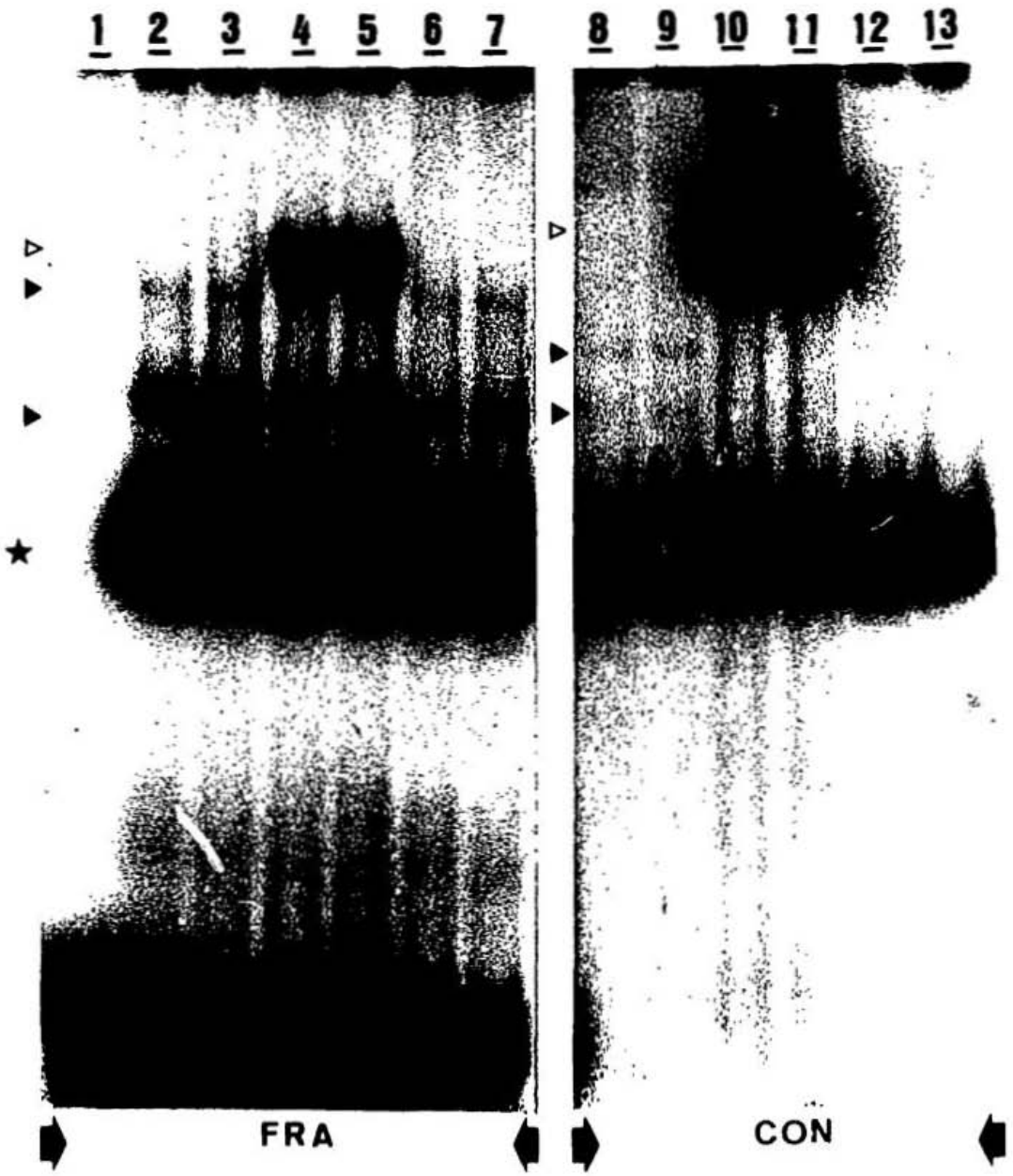
binding ability was determined by electrophoretic mobility shift assays (EMSAs). Further, we asked whether or not EGF modifies the transcription from a minimal promoter bearing a TATA box motif, and from promoters containing these p53 response elements, in MDA-468 breast cancer cells. The experimental approach chosen basically detects any EGF-dependent functional alterations in $p53^{273.His}$ mediated from two of the known p53-responsive elements. The alternative explanation for the observed changes in PAb 240 reactivity (Fig.4.6) and decreased phosphorylation (Fig.4.8 & 4.9) upon EGF treatment, phosphorylation-mediated epitope masking, may also result in altered transcriptional activity of p53, since it has been well established that phosphorylation can modulate the activity of a number of transcription factors (Angel & Karin, 1991; Barford, 1992; Karin & Smeal, 1992). The experiments used here, would detect any functional consequences of EGF-dependent altered phosphorylation of $p53^{273.His}$ in MDA-468 cells.

5.2 RESULTS

5.2.1 $p53^{273.His}$ forms specific complexes with CON and FRA

Nuclear extracts from MDA-468 cells were tested for the ability to form specific complexes with the p53 response elements. Equal amounts of protein from nuclei of untreated or EGF-treated MDA-468 cells were incubated with end-labelled double-stranded oligos corresponding to CON and FRA. A 6 h EGF treatment was employed in all the EMSAs since we wished to detect any functional alteration in p53 that might occur prior to the earliest manifestations of growth inhibition

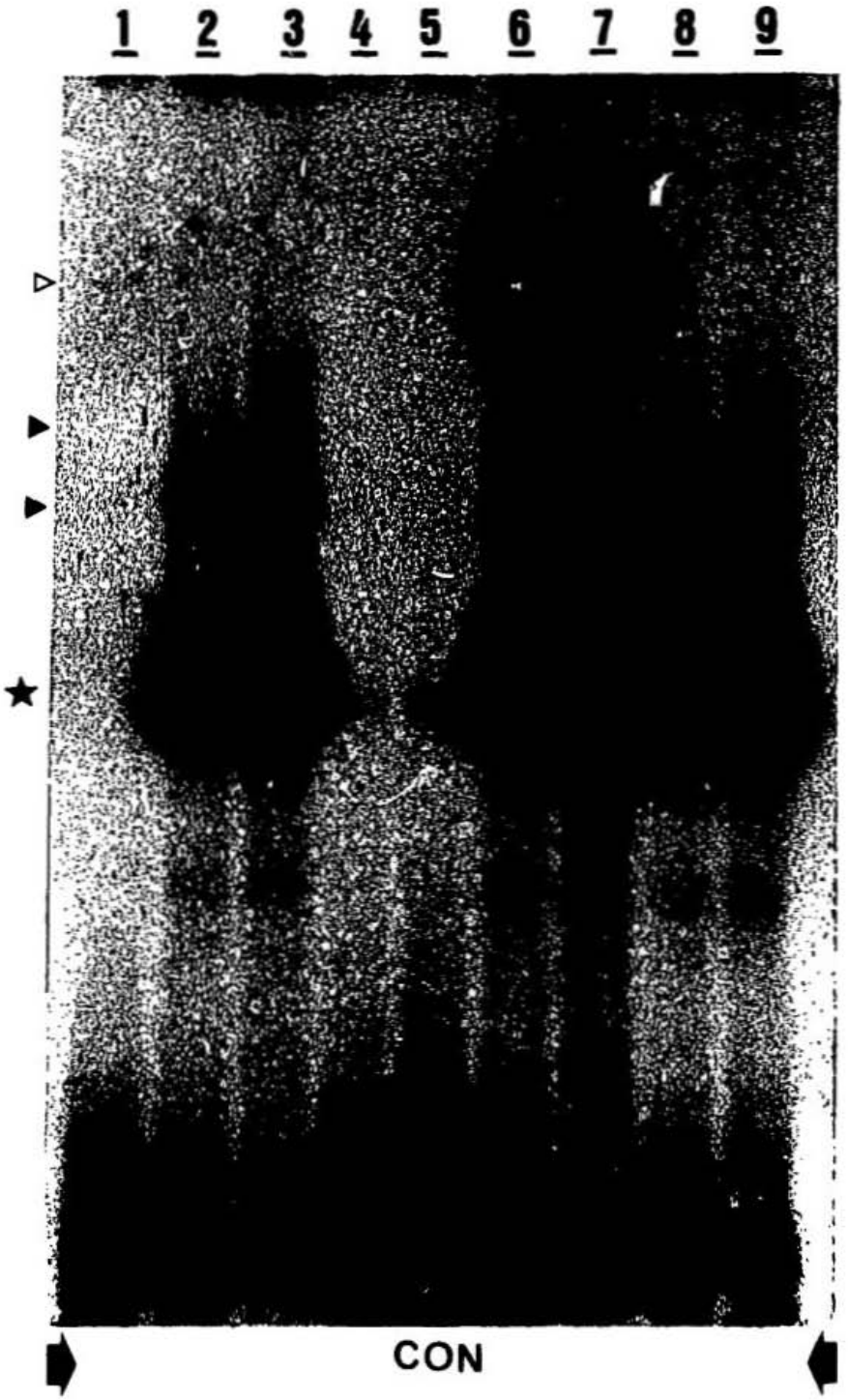
FIG. 5.1 **Analysis of DNA-binding ability of $p53^{273.His}$ in response to EGF - I.** Nuclear extracts (3 μg) from untreated/control (even numbered lanes), and EGF-treated (odd numbered lanes) MDA-468 cells were analyzed for mobility shifts of end-labelled oligos representing p53-specific sites. Lane 1 - blank with no nuclear extract; lanes 2, 3 & 8, 9 - no further additions; the anti-p53 antibodies included in the binding reaction are PAb 421 (0.1 μg) - lanes 4, 5, 10 & 11, and PAb 1620 (1.0 μg protein equivalent of hybridoma supernatent fluid) - lanes 6, 7, 12, & 13. The figure is an 18 h exposure of the autoradiograph. ★ is a non-specific complex which was competed out by unlabelled oligo corresponding to NF1 site(Not included). Further this complex did not react with any of the four anti-p53 antibodies tested. Solid arrow heads indicates specific p53-DNA complexes, and antibody- supershifted complexes are denoted by open arrow heads. Free probe has run off the gel.


1 2 3 4 5 6 7
8 9 10 11 12 13
FRA
CON

(downregulation of total protein synthesis; Fig.3.4, page 66). In contrast to published reports, we measured comparable levels of mobility shifts for both CON and FRA in MDA-468 nuclear extracts (Fig 5.1). The fastest migrating complex (denoted by an ★) was non-specific since none of the anti-p53 antibodies altered its mobility and was completely competed out with excess of cold/unlabelled non-specific NF1 double-stranded oligo (Not shown). However, the two slower migrating complexes (denoted by solid arrow heads) contained p53 molecules. This was confirmed by addition of anti-p53 antibodies to the reaction. PAb 421 not only supershifted the complexes (denoted by open arrow heads) but also significantly enhanced the DNA binding of p53 (Fig 5.1, lanes 4 & 5; lanes 10 & 11). This increase in DNA binding was more pronounced with the CON probe than with FRA. PAb 1620 (wild-type specific/ conformation-dependent) was used at 1.0 μg concentration as the reagent was a hybridoma supernatent fluid and not a purified form unlike other antibodies used in the study. Inclusion of PAb 1620, however, slightly reduced the specific complexes (Fig.5.1, lanes 6 & 7, lanes 12 & 13). Increased concentrations of up to 5 μg of PAb 1620 abolished the specific complexes (Not shown)

Interestingly, EGF treatment enhanced the formation of specific complexes with both CON and FRA. In the case of FRA, the faster running complex was more pronounced (The lower solid arrow head; compare lane 3 with 2), whereas with CON, the slower migrating band was increased in EGF-treated cells (The upper solid arrow head; compare lane 8 & 9). We went on to test further whether increased

FIG. 5.2 **Analysis of DNA-binding ability of $p53^{273.His}$ in response to EGF - II.** Nuclear extracts (5 μg) from untreated/control (even numbered lanes), and EGF-treated (odd numbered lanes) MDA-468 cells were analyzed for mobility shifts of an end-labelled oligo representing p53-specific site, CON. Lane 1 - blank with no nuclear extract; lanes 2 & 3 - no further additions; the anti-p53 antibodies included in the binding reaction are PAb 421 (0.1 μg) - lanes 6 & 7 and PAb 1620 (1.0 μg protein equivalent of hybridoma supernatent fluid) - lanes 8 & 9; and 50 fold excess unlabelled CON - lanes 4 & 5. The figure is a 3 day exposure of the autoradiograph. ★ is a non-specific complex. Solid arrow heads indicates specific p53-DNA complexes, and antibody-supershifted complexes are denoted by open arrow heads. Free probe has run off the gel.


1 2 3 4 5 6 7 8 9
CON

concentrations of nuclear extract proteins from untreated cells would correspondingly enhance the slower migrating complex. Fig 5.2 is an over-exposed autoradiograph of an EMSA with CON carried out with approximately twice the protein-concentration of that used in Fig.5.1. This, however, did not significantly enhance the slower migrating complex in untreated cells (Fig.5.2; upper solid arrow head; lane 2). Another interesting observation was that both p53-CON and p53-FRA complexes from EGF-treated cells were relatively more resistant to PAb 1620-induced abolition than that from untreated cells. (Fig.5.1; compare lanes 6 & 7 and lanes 12 & 13). PAb 1620 was included from the beginning of the reaction, and at the concentrations used (1 μg), we anticipated that it would interfere with the binding to a similar extent in spite of the increased DNA binding observed upon EGF-treatment. This led us to conduct further characterization of the complexes.

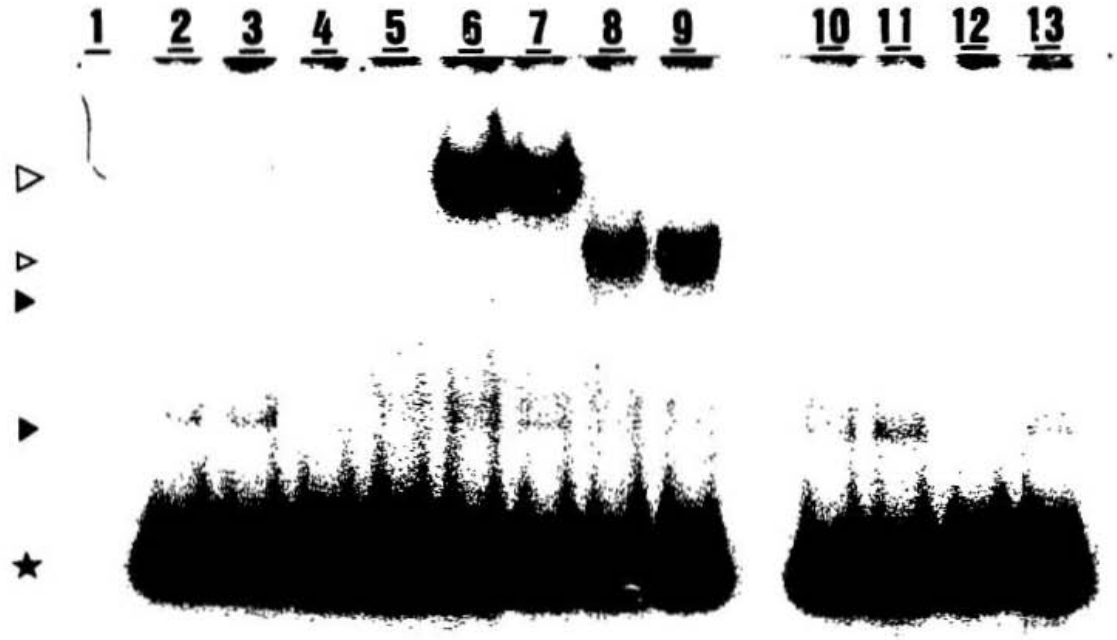
5.2.2 Reactivity of p53-DNA complexes with anti-p53 antibodies

After clearly identifying the specificity of p53 complexes in EMSAs, it was of interest to determine the nature of these complexes. To this end, a series of EMSAs in the presence of different p53 antibodies alone, or in combination, were performed.

5.2.2.1 Reactivity with PAb 421

PAb 421 supershifted the complexes in both control and EGF-treated cells to the same extent (open arrow head in Fig 5.1, lanes 4 & 5; lanes 10 & 11; Fig.5.2, lanes 6 & 7). The antibody supershift was associated with an increase in DNA-binding by several fold. This had been reported earlier (Hupp et al., 1992). In fact, many

FIG. 5.3 **Analysis of immunoreactivity of p53-DNA complex from control and EGF-treated cells - I.** Nuclear extracts (3 μg) from untreated/control (even numbered lanes), and EGF-treated (odd numbered lanes) MDA-468 cells were analyzed for mobility shifts of an end-labelled oligo representing p53-specific site, FRA. Lane 1 - blank with no nuclear extract; lanes 2 & 3 - no further additions; the anti-p53 antibodies included in the binding reaction are PAb 421 (0.1 μg), PAb 1620 (1.0 μg protein equivalent of hybridoma supernatent fluid), PAb 1801 (0.1 μg),and PAb 240 (0.1 μg). The figure is a 24 h exposure of the autoradiograph. ★ is a non-specific complex. Solid arrow heads indicates specific p53-DNA complexes, and antibody-supershifted complexes are denoted by open arrow heads. Free probe has run off the gel. The gap between lane 9 and 10 is an unloaded lane.



PAb 421	−	−	−	+	+	+	+	+	+	−	−	−	−
PAb 1620	−	−	−	+	+	−	−	−	−	−	−	−	−
PAb 1801	−	−	−	−	−	+	+	−	−	−	−	+	+
PAb 240	−	−	−	−	−	−	−	+	+	+	+	−	−

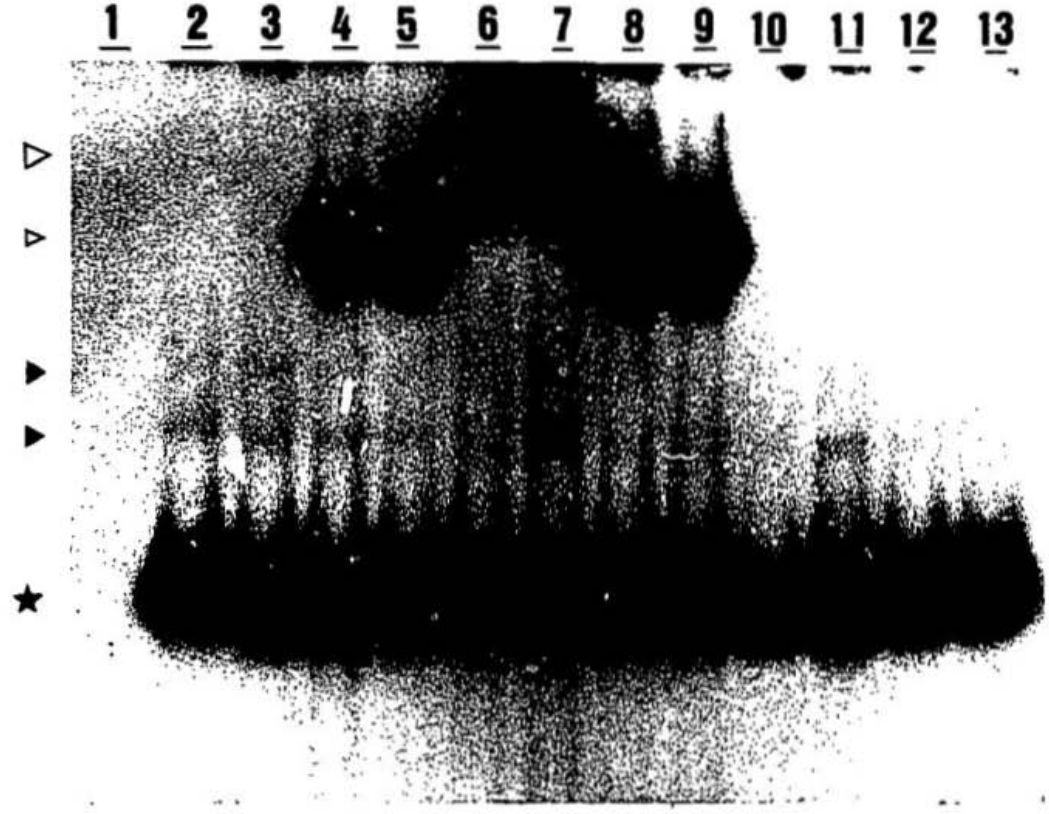
laboratories have used PAb 421 in their reactions in order to detect any mobility shifts at all (Hupp et al., 1992; Funk et al., 1992; Price & Cladenwood, 1993; Hopp-Sleyer & Butz, 1993). The presence of PAb 421 in the reactions did not, however, highlight any major difference between untreated and EGF-treated cells.

5.2.2.2 Reactivity with conformation-specific antibodies (PAb 240 and PAb 1620)

PAb 240 interfered with the specific p53-DNA interaction resulting in decreased complex formation (Fig. 5.3 & 5.4, lanes 10 & 11). The interference was observed to a greater extent in untreated cells (lane 10) than in EGF-treated cells (lane 11). Incubation with PAb 240 in concentrations five times higher than that used in Fig.5.3 and 5.4, almost completely abolished the specific complexes from untreated, but not from EGF-treated cells (Not shown). No supershift was observed with PAb 240. This was confirmed with longer exposures (Not shown).

PAb 1620 had similar effects on p53-DNA complexes (Fig.5.1, lanes 6 & 7 and 12 & 13; Fig.5.2, lanes 8 & 9). The EGF effect of making complexes more resistant to abolition was observed with both CON and FRA. However, when PAb 240 or PAb 1620, was included with PAb 421 in the binding reaction, a similar EGF-dependent reduction in antibody-reactivity with p53-DNA complexes was only observed with FRA (Fig. 5.3, lanes 8 & 9 and lanes 4 & 5 respectively). However, no such differential antibody reactivity was observed with CON, as PAb 421 greatly enhanced the p53 binding alone (Fig.5.4, lanes 8 & 9 and lanes 4 & 5).

FIG. 5.4 **Analysis of immunoreactivity of p53-DNA complex from control and EGF-treated cells - II .** Nuclear extracts (3 μg) from untreated/control (even numbered lanes), and EGF-treated (odd numbered lanes) MDA-468 cells were analyzed for mobility shifts of an end labelled oligo representing p53-specific site, CON. Lane 1 - blank with no nuclear extract; lanes 2 & 3 - no further additions; the anti-p53 antibodies included in the binding reaction are PAb 421 (0.1 μg), PAb 1620 (1.0 μg protein equivalent of hybridoma supernatent fluid), PAb 1801 (0.1 μg),and PAb 240 (0.1 μg). The figure is a 24 h exposure of the autoradiograph. ★ is a non-specific complex. Solid arrow heads indicates specific p53-DNA complexes, and antibody-supershifted complexes are denoted by open arrow heads. The free probe has run off the gel.



PAb 421	–	–	–	+	+	+	+	+	+	–	–	–	–
PAb 1620	–	–	–	+	+	–	–	–	–	–	–	–	–
PAb 1801	–	–	–	–	–	+	+	–	–	–	–	+	+
PAb 240	–	–	–	–	–	–	–	+	+	+	+	–	–

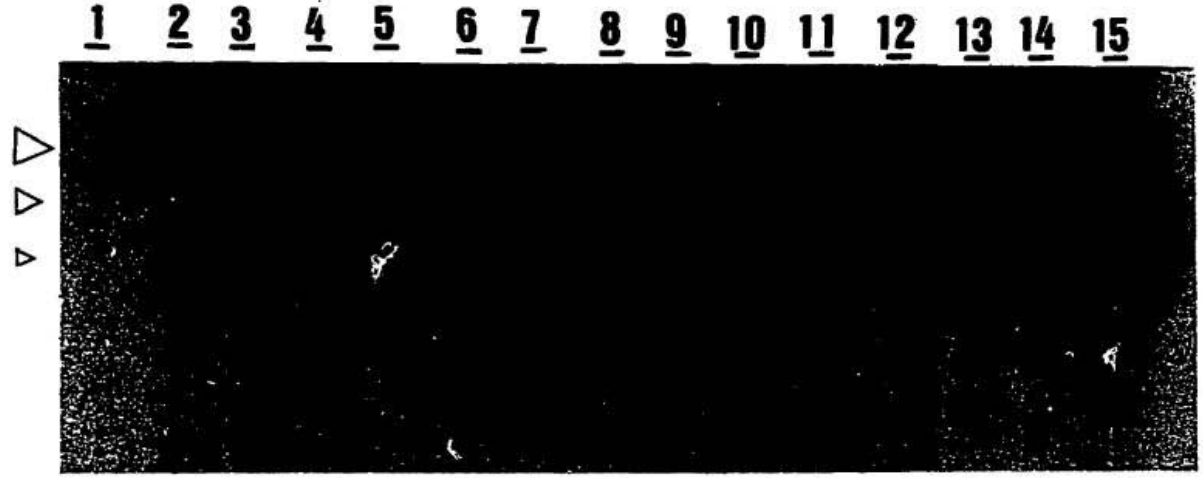
5.2.2.3 Reactivity with PAb 1801

PAb 1801 supershifted CON-p53 complexes without increasing the binding (lower/smaller open arrow head; Fig 5.4, lanes 12 & 13). No such shift was observed with FRA-p53 complexes (Fig.5.3, lanes 12 & 13). In fact, PAb 1801 abolished the specific FRA-p53 complexes. This was confirmed with longer exposures (Not shown). Inclusion of PAb 421 along with PAb 1801, double shifted the complexes (Upper/larger open arrow head ; Fig.5.3 & 5.4, lanes 6 & 7). No difference in the magnitude of this double shift was observed upon EGF-treatment (Fig.5.3 & 5.4, lanes 6 & 7).

5.2.3 Effect of PAb 1620 on formation and stability of p53-DNA complexes

Since PAb 421 enhanced the DNA binding, we tested the effects of various anti-p53 antibodies on p53-CON complexes in the presence of PAb 421 (Fig.5.5). We used CON for this purpose, since the p53-CON complexes, unlike p53-FRA, showed little EGF-dependent change in the presence of PAb 421 and a conformation-specific antibody (Fig 5.4; lanes 4 & 5 and 8 & 9). In Fig.5.5 only super-shifted complexes are shown for the sake of simplicity. The lower/smaller arrow head (lane 3 & 10) indicates the p53-CON-PAb 421 complex. Inclusion of PAb 1801 in the binding reaction along with PAb 421, double shifted the complex (Upper/larger arrow head, lanes 4 & 11). EGF treatment did nor produce any major change in this pattern (Compare lanes 4 & 11).

FIG. 5.5 **Effects of PAb 1620 on formation of p53-DNA complex in the presence of PAb 421.** Nuclear extracts (5 μg) from untreated/control (lanes 2-8), and EGF-treated (lanes 9-15) MDA-468 cells were analyzed for mobility shifts of end-labelled oligo representing p53-specific site, CON. Lane 1 - blank with no nuclear extract; lanes 2 & 9 - no further additions; the anti-p53 antibodies included in the binding reaction are PAb 421 (0.1 μg), PAb 1620 (1.0 μg protein equivalent of hybridoma supernatent fluid), PAb 1801 (0.1 μg),and PAb 240 (0.1 μg); unlabelled CON in 50 fold excess (lanes 6 &13), and in 100 fold excess (lanes 7 & 14); unlabelled non-specific competitor (sequence described in Chapter 2.1.6; page 32) in 50 fold excess (lanes 8 & 15). The competitors (PAb 1801, PAb 1620, PAb 240 and unlabelled oligos) were pre-incubated for 15' on ice, with the nuclear extract. Followed by addition of PAb 421 and incubation with the binding buffer & labelled CON (50,000 cpm) for 30' at RT. All reactions were incubated for 30' on ice after the binding reaction. The figure is a 24 h exposure of the autoradiograph. Only antibody supershifted complexes (open arrow heads) are shown.

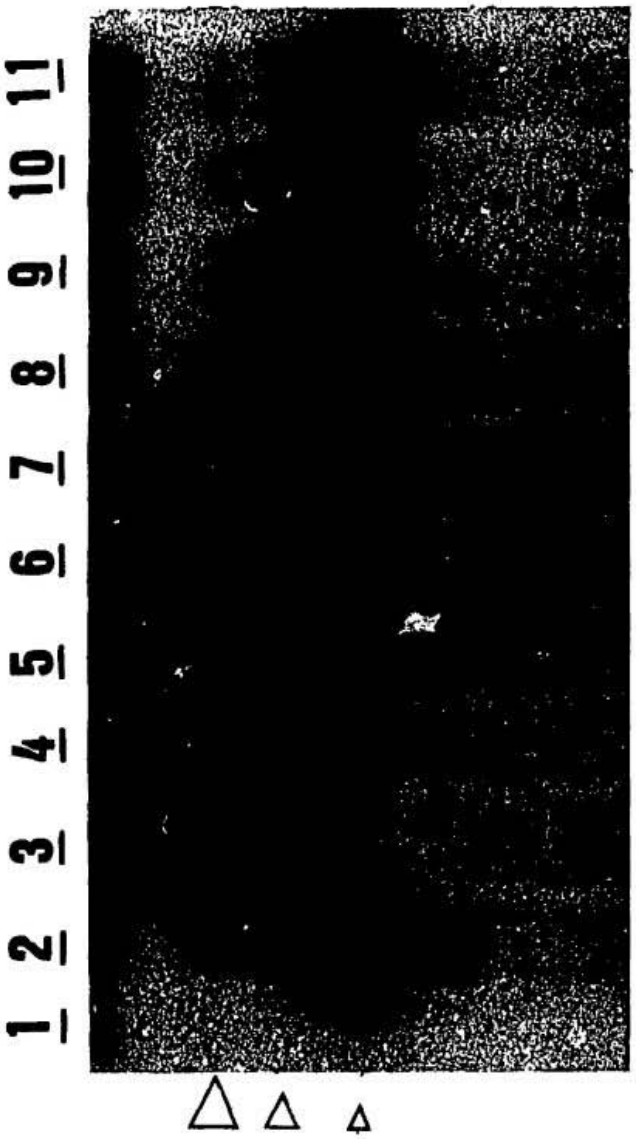


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
PAb 421	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+
PAb 1620	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–
PAb 1801	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–
NON-SP. COMP.	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+
SP.COMP.	–	–	–	–	–	+	+	–	–	–	–	–	+	+	–

In contrast, when PAb 1620 was incubated along with PAb 421 and CON, an intermediate shift of part of the complex was observed (middle arrow head, lane 5). The total amount of p53-DNA complexes inclusive of different species in the presence of PAb 1620, was considerably lower than the total amount supershifted with PAb 421 (compare lane 3 and lane 5, and with the double shifted band by PAb 1801; lane 4). This indicated that some of the p53-CON-PAb 421 complex was abolished by PAb 1620. Incubation with PAb 240 resulted in a similar intermediate complex. In this case, however, the remainder of the primary complex was not significantly altered (PAb 240 experiment was not shown). The intermediate shift may be due to the interaction of antibodies with a part of the complex. In EGF-treated cells, incubation with PAb 1620 produced a similar pattern as untreated cells (middle arrow head, lane 12). However, the lower complex (p53-CON-PAb 421) remained largely unaffected (lower/smaller arrow head, lane 12) i.e., the total amount of p53-antibody complex was not reduced in the presence of EGF, indicating reduced reactivity of the p53 present to PAb 1620 upon binding to CON. This observation is consistent with a conformational shift in p53 upon EGF-treatment. The specificity of the complexes was confirmed with incubation with an excess of unlabelled CON (lanes 6, 7, 13, and 14) and a non-specific oligo (lanes 8 & 15).

In our experiments, we observed that both sequence of addition and the incubation conditions seemed to influence the EGF effect on PAb 1620 reactivity with the p53-CON-PAb 421 complexes. Further studies (Fig 5.6) indicated that if PAb

FIG. 5.6 **Effect of PAb 1620 on formation and stability of p53-DNA complex in the presence of PAb 421.** Nuclear extracts (5 μg) from untreated/control (lanes 2-4, 8, & 10), and EGF-treated (lanes 5-7, 9, & 11) MDA-468 cells were analyzed for mobility shifts of an end-labelled oligo representing p53-specific site, CON. Lane 1 - blank reaction with no nuclear extract; the anti-p53 antibodies included in the binding reaction are PAb 421 (0.1 μg), PAb 1620 (1.0 μg protein equivalent of hybridoma supernatent fluid), & PAb 1801 (0.1 μg). PAb 421 was included in the binding reaction simultaneously with the probe (CON; 50,000 cpm). PAb 1801 (lanes 3 & 6), and PAb 1620 (lanes 4 & 7) were pre-incubated with nuclear extracts (15' on ice). In other reactions, PAb 1620 was added simultaneously with the probe and PAb 421 (lanes 8 & 9), or included after the binding reaction (30' at RT). All the reactions were further incubated for 30' on ice. The figure is a 24 h exposure of the autoradiograph. Only antibody supershifted complexes (open arrow heads) are shown.


1 2 3 4 5 6 7 8 9 10 11
PAb 421 + + + + + + + + + + +
PAb 1801 − − + − − + − − − − −
PAb 1620 + − − + − − + + + + +

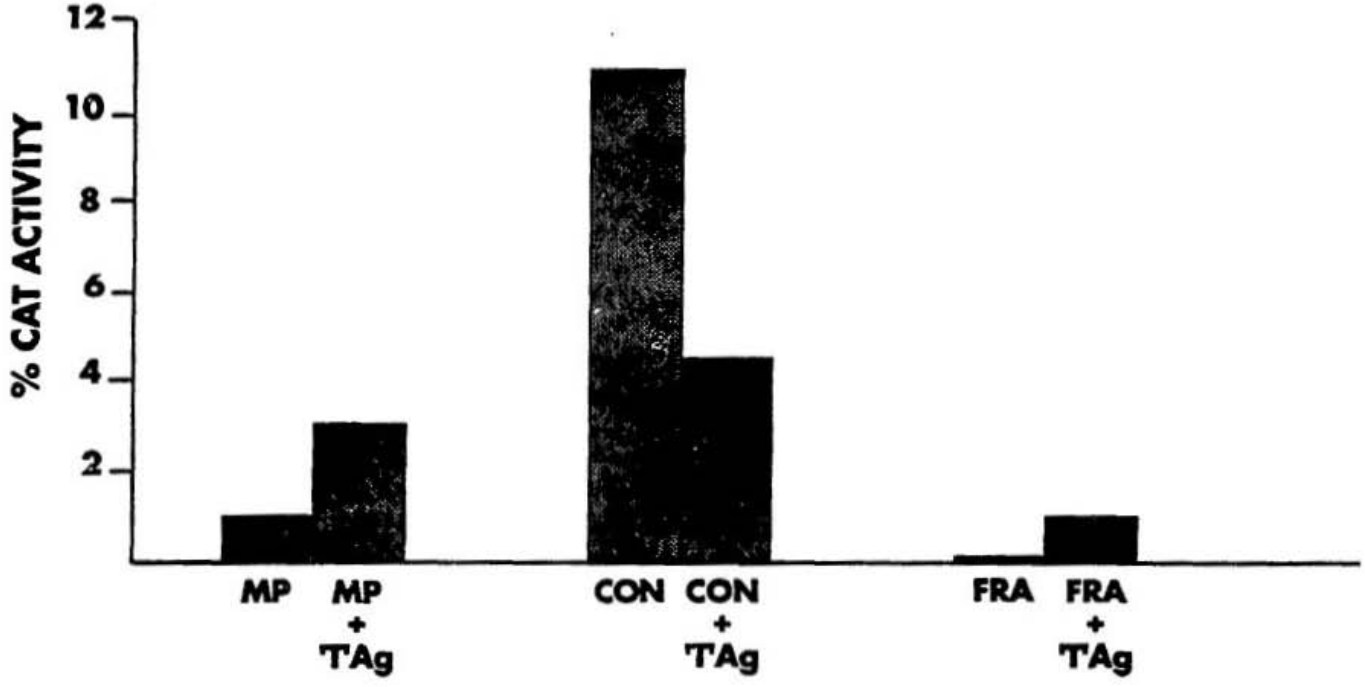
1620 was added simultaneously with PAb 421 there seemed to be little difference between untreated and EGF-treated samples, in terms of PAb 1620 reactivity with the complexes (lanes 8/untreated, and 9/EGF-treated). A pre-incubation of PAb 1620 produced a moderate difference (lanes 4/untreated, and 7/EGF-treated), whereas inclusion of PAb 1620 after the formation of specific DNA complexes with PAb 421 had the greatest effect on the complexes (lanes 10/untreated, and 11/EGF-treated). This again, seems to indicate that EGF decreases the portion of p53 in wild-type-like conformation, in a complex with CON and PAb 421.

Taken together, the DNA binding studies demonstrate that endogenous $p53^{273.His}$ of MDA-468 cells specifically interacts with both FRA and CON sequences. Furthermore, EGF treatment enhances this interaction. The reactivity of p53-DNA complexes with various antibodies indicate a sequence-dependent conformation of p53 induced upon DNA binding; FRA-p53 is abolished by PAb 1801 (Fig 5.3; lane 12 & 13) while CON-p53 is supershifted (Fig 5.4; lanes 12 & 13). In addition, EGF treatment resulted in lower reactivity of FRA-p53 (Fig 5.3; lanes 4 & 5) and PAb 421-CON-p53 to PAb 1620 (Fig 5.5 and Fig 5.6).

5.2.4 Transcriptional activity of $p53^{273.His}$

Most of the information concerning the function of $p53^{273.His}$ is from transfection studies employing exogenous p53 constructs. Therefore, we wished to establish the transcriptional activation function of endogenous $p53^{273.His}$ in MDA-468 cells. Since we were able to observe comparable levels of DNA binding to both CON

FIG. 5.7 **Examination of p53-response element-mediated transcriptional activity.** MDA-468 cells ($2x10^6$ cells/100mm plate) were transfected with 5 μg of minimal promoter-CAT (MP-CAT/pBLCAT2), the CON-CAT, and FRA-CAT as described in Chapter 2 (2.2.13; Fig.2.1, page 48). SV40 T antigen (5 μg/100mm plate) was co-transfected as a control. CAT activity was measured 48 h post-transfection and was equalized to protein concentrations. RSV-β gal construct (2 μg/100mm plate) was used as internal control. Standardization of CAT values to β gal activity was not done as it appeared that both EGF and T antigen affected RSV promoter significantly. The % conversion (CAT activity) by MP-CAT was standardized to 1.0 and the remaining values were correspondingly adjusted.

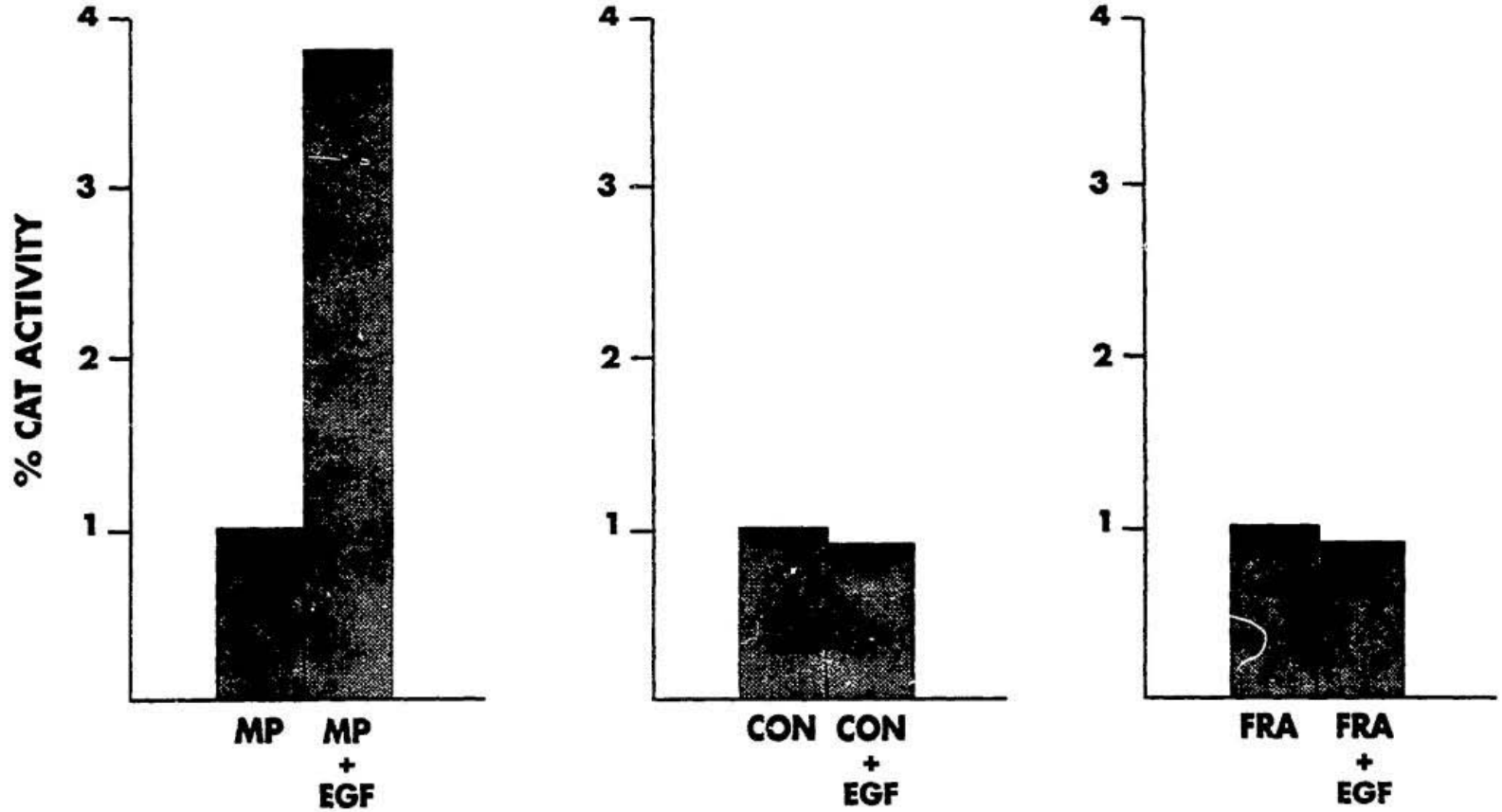

% CAT ACTIVITY
12
10
8
6
4
2
MP
MP + TAg
CON
CON + TAg
FRA
FRA + TAg

and FRA in the absence of PAb 421, it was essential to determine the functional consequences of the observed DNA binding activity. To this end, the p53 response DNA elements were cloned upstream of a minimal promoter regulating a chloramphenicol acetyl transferase (CAT) reporter gene (pBLCAT2). This vector contains a minimal promoter with a TATA box from the herpes simplex virus thymidine kinase gene (Luckow & Schutz, 1987). In transient transfection assays carried out in MDA-468 cells, CON sequences conferred about an 11-fold activation of transcription whereas FRA sequences displayed about a 10-fold repression of transcription compared to that from vector alone (Fig. 5.7). This was an interesting observation given that no FRA-mediated modulation of transcription has been reported, although previous reports have indicated transactivation by $p53^{273.His}$ through CON (Chen et al., 1993b). Co-transfection of SV40 T antigen cDNA driven by the SV40 enhancer/promoter blocked both activation and repression, confirming the involvement of p53 in this process.

5.2.5 Effect of EGF on transactivation of $p53^{273.His}$

After establishing a function of $p53^{273.His}$ in MDA-468 cells , it was essential to determine whether or not EGF- induced changes in DNA binding were reflected in altered transcriptional activity of $p53^{273.His}$. EGF treatment (48 h) of MDA-468 cells, transiently transfected with pBLCAT2 alone, consistently resulted in increased CAT activity. The average figures are shown in Fig.5.8. There was an approximate 4-fold activation of transcription through the minimal promoter in the presence of

FIG.5.8 **Effects of EGF on p53-response element-mediated transcriptional activity.** MDA-468 cells ($2x10^6$ cells/100mm plate) were transfected with 5 μg of minimal promoter-CAT (MP-CAT/pBLCAT2), the CON-CAT, and FRA-CAT as described in Chapter 2 (2.2.13; Fig 2.1, page 48). The cells were incubated ± EGF (10^{-8} M) for 48 h after transfection. CAT activity was measured 72 h post-transfection and was equalized to protein concentrations. RSV-β gal construct (2 μg/100mm plate) was used as internal control. Standardization of CAT values to β gal activity was not done as it appeared that EGF affected RSV promoter significantly. The % conversion (CAT activity) by individual construct in the absence of EGF, was standardized to 1.0 and the remaining values were correspondingly adjusted.


% CAT ACTIVITY
4
3
2
1
MP
MP + EGF
CON
CON + EGF
FRA
FRA + EGF

EGF. We observed no such EGF-induced change in the presence of SV40 T antigen in co-transfection experiments indicating that the activation was in fact mediated by p53 (Not shown). The vectors with p53-response elements, however, did not show any significant shifts in the CAT activity upon EGF treatment.

5.3 DISCUSSION

We have studied the effects of EGF on p53 status and function in MDA-468 human breast cancer cells. EGF in high concentrations(10^{-8} M) induces a reversible late G1 arrest, together with a reduction in steady state mRNA levels for p53. Since these cells contain an apparent gain-of-function mutant ($p53^{273.His}$) with unusual functions, an in-depth study was undertaken. In the previous chapter (Chapter 4), EGF-induced shifts in the antibody-reactivity of the p53 protein were described. The apparent conformational shift, as indicated by altered reactivity with conformation-specific antibodies, may be mediated through altered phosphorylation. In this chapter, studies to determine the biochemical function of $p53^{273.His}$ in MDA-468 cells, and the effects of EGF treatment on those functions, were outlined.

We provide evidence that endogenous $p53^{273.His}$ forms specific complexes with two of the p53 response DNA elements (CON and FRA) with relatively the same efficiency. Moreover, this DNA binding has a transcriptional modulation effect. In MDA-468 cells, $p53^{273.His}$ activates transcription from CON and represses through FRA. EMSAs demonstrated a considerable increase in the p53 DNA binding activity in response to EGF (Fig.5.1 and 5.2). Furthermore, we observed EGF increased p53

transcriptional activity through a minimal promoter, but not through CON and FRA (Fig.5.8).

The observed EGF-dependent stabilization of p53-CON-PAb 421 complexes varied with the reaction conditions (Fig.5.6). The simultaneous incubation of PAb 421 and PAb 1620 resulted in moderate or no EGF-effect at all. Pre-incubation or co-incubation with PAb 1620 may sequester a part of the population of p53 molecules, inhibiting formation of the complexes despite PAb 421-dependent increased DNA binding. We have consistently observed reduced reactivity of pre-formed p53-CON-PAb 421 complexes to PAb 1620 in EGF-treated cells, indicating that EGF induces a shift in p53 conformation that is distinguishable upon DNA binding. Recently, it was shown that p53, upon DNA binding, can lose reactivity with PAb 1620. The authors suggested that it may assume a mutant conformation (Halazonetis et al., 1993). Our observation of decreased PAb 1620 reactivity of p53-DNA complexes from EGF-treated cells (Fig.5.5 and 5.6), then, can be interpreted as a behaviour characteristic of wild-type p53. This is consistent with our immunofluorescence data (Fig.4.6) which implied an EGF-dependent loss of mutant-specific epitope (PAb 240) and persistent wild-type-specific epitope (PAb 1620).

Alternatively, the EGF-dependent reduction in PAb 1620 reactivity of p53-DNA complexes might also be due to an increased DNA-binding efficiency of p53 in response to EGF. This may have been reflected as a increased resistance to PAb 1620-induced abolition of the p53-DNA complexes. In either case, it is a

demonstration of an EGF-induced altered interaction of $p53^{273.His}$ with specific p53-response elements.

It has also been suggested that sequence-specific DNA binding itself may induce a conformational change in the p53 protein (Halazonetis et al., 1993) This flexible nature was attributed to wild-type p53, with a majority of mutants thought to be locked in a particular conformation. We suspect, however, that $p53^{273.His}$ might be more similar to wild-type. In EMSAs, the different p53-DNA complexes differentially reacted with PAb 1801. PAb 1801 abolished p53-FRA but supershifted p53-CON complexes. This is consistent with the notion that p53 may assume different conformations upon DNA binding in a sequence-dependent manner (Halazonetis et al., 1993). Thus, in the p53-CON complex, the PAb 1801 epitope remains available for the reaction, whereas in case of p53-FRA, the epitope may be required for or masked upon DNA-binding. Antibody binding to p53 in this latter case, may interrupt DNA-binding. This is an indication that p53 might assume different conformations in a sequence-dependent manner upon DNA binding. In the presence of PAb 421, a double shift of p53-FRA-PAb 421 was produced by PAb 1801 (Fig.5.3). Greater affinity of p53 to FRA in the presence of PAb 421, may overcome the interference by PAb 1801, thus retaining both PAb 421 and PAb 1801 in the p53-FRA complex. On the other hand, PAb 421 might induce a change in p53 conformation, one that makes it more efficient for DNA binding. This putative PAb 421-induced altered conformation may result in free PAb 1801 epitope. It is conceivable then, that the

PAb 1801 interaction with p53 will be tolerated, resulting in a double shift.

Another interesting observation strengthening the notion of sequence-dependent changes in p53 conformation upon DNA-binding, was the degree of PAb 1620-induced abolition of p53-DNA complexes. The PAb 1620-mediated abolition was near complete for p53-FRA-PAb 421 (Fig.5.3, lanes 4 & 5), while the same for p53-CON-PAb 421 was only partial (Fig.5.4, lanes 4 & 5). This indicates that the $p53^{273.His}$ domain with PAb 1620 epitope, may be relatively more critical for the interaction with FRA than that with CON. This is consistent with the idea that only wild-type p53 interacts with FRA, while both wild-type and some of the mutants interact with CON.

In the EMSAs, more than one specific p53-DNA complex was consistently observed. These slower migrating complexes have been reported by others before and they were believed to be higher order oligomers of p53 (Zauberman et al., 1993). In EGF-treated MDA-468 cells, p53 may be capable of forming more higher order oligomers that bind to CON, unlike untreated cells. Such higher order oligomers, however, form complexes with FRA (Fig.5.1, upper solid arrow) in the absence of EGF. While the significance of these differences, remain to be resolved, they may be important in determining the function of p53.

DNA binding studies from Miller et al., indicated that the presence of a single p53 molecule with a strong DNA binding domain was sufficient to retain dimers in a complex with DNA (Miller et al., 1993). Our EMSAs suggest that PAb 1620 and

PAb 240 binding may interfere with p53 DNA binding. Based on the suggestion from Miller et al. (1993), it is plausible that such an interaction with one, but not both, of the p53 molecules may be tolerated. In other words, let us assume that the simple p53-DNA complex is a dimer, as has been reported earlier (Hupp et al., 1992; Tarunina & Jenkins, 1993). Thus, an interaction with a dimer would explain a partial shift of the p53-CON-PAb 421 to an intermediate level by conformation-specific antibodies. Increased DNA-binding efficiency perhaps induced by PAb 421 may create a strong enough interaction of p53 with CON, that binding of an interfering antibody (PAb 1620 or PAb 240) to one of the p53 molecules, may be tolerated. This may account for an intermediate shift of p53-CON-PAb 421 complexes by PAb 1620 and PAb 240 (Fig.5.5 & 5.6). Interaction of interfering antibodies (PAb 1620 or PAb 240) with both p53 molecules of the dimer may abolish the p53-DNA interaction.

EMSA studies have indicated an increase in the DNA binding ability of p53 in EGF-treated cells. As previous studies indicated no change in the p53 protein levels immediately following EGF treatment (Chapter 4.2.2), this increased activity must be due to change in affinity, perhaps secondary to altered phosphorylation and a change in conformation. An observation linking a growth factor with p53 has not been previously reported to our knowledge. Kastan et al. (1992), demonstrated an increase in the formation of a specific complex of p53 with a DNA sequence distantly related to a p53 consensus sequence (5'- PuPuPuC(A/T)(T/A)G PyP_yPy-3') in the GADD45 gene in response to irradiation. However, this increase correlated with an

accumulation of p53 protein observed upon irradiation. Interestingly, while these complexes did not react with PAb 1801, an enhanced binding was observed with PAb 421. A more recent study demonstrated the formation of specific complexes of endogenous p53 with CON sequence (Price & Cladeiwood, 1993). The authors further reported, with the aid of PAb 421, an increased DNA-binding activity in response to irradiation. Once again however, the increased activity corresponded to increased cellular p53 protein levels. In light of this, the observation that EGF enhanced $p53^{273.His}$ DNA binding activity in the absence of increased p53 content is intriguing. This, we suggest, may be due to altered affinity of p53 for DNA upon conformational change.

Published reports in the literature concerning $p53^{273.His}$ DNA-binding and transactivation are confusing at best. It has been reported previously, that $p53^{273.His}$ was unable to bind to and transactivate from the FRA sequence (Kern et al., 1991b; Bargonetti et al., 1991; Farmer et al., 1992). In fact, $p53^{273.His}$ was found to inhibit the wild-type complex formation with this sequence (Kern et al., 1991b). In contrast, it has been shown that $p53^{273.His}$ reacts differently with CON sequence. The mutant not only binds to CON but was also able to transactivate transcription from this sequence (Funk et al., 1992; Chen et al., 1993b). Furthermore, wild-type p53 was reported to form complexes with TBP and repress transcription from a TATA box containing a minimal promoter. Recently, Carol Prives and colleagues, have reported that wild-type p53 and TFIID or purified TBP co-operate for DNA binding (Chen

et al., 1993a). The article showed that wild-type p53 inhibits TBP binding to TATA motif but not that of the TFIID holoprotein. The authors suggest that wild-type p53 mediated modulation of transcription may well be due to mechanisms other than inhibition of TBP DNA-binding. In other words, the transcriptional effect of p53 may be mediated by a modification of TFIID function through interactions other than DNA binding. The presence of p53 in the pre-initiation complex along with TBP, may alter the interaction of other TAFs (TBP associated factors) in the complex with the cellular proteins affecting transcription. These cellular proteins may be other promoter/enhancer binding proteins such as Sp1, CREBP, and CBF. The specificity and outcome of such interactions may be determined by cell-type. This may be reflected as either repression, activation or no change, depending on the type of the promoter and the cell-type.

Levine and colleagues (Zambetti et al., 1992), in their attempts to characterise the p53-response element in the MCK enhancer, observed repression of transcription from pBLCAT2 by wild-type but not by a mutant p53, introduced into p53-null Saos2 cells. They reported however, that inclusion of the MCK enhancer sequences into pBLCAT2 activated transcription upon transfection into p53-null Soas2 cells even in the absence of exogenous p53. They interpreted this observation as the actions of other cellular factors on the MCK enhancer (Zambetti et al., 1992). In BHK cells, with intact endogenous p53, exogenously introduced wild-type p53 repressed transcription from pBLCAT2 (Yuan et al., 1993). Interestingly enough, inclusion of

a p53-response element (RGC/FRA) into pBLCAT2 relieved this p53-induced repression. A mutant p53 ($p53^{135.Val}$) activated transcription from pBLCAT2 but had no effect through RGC sequences (Yuan et al., 1993).

We have observed a unique transcriptional activity of $p53^{273.His}$ in MDA-468 cells. Our experiments clearly demonstrated increased transcription from CON, and, interestingly, an active repression from FRA sequences inserted in pBLCAT2 (Fig.5.8). These observations are intriguing given the apparent sequence-dependent differences in p53 conformation indicated by the EMSAs (Fig. 5.3 & 5.4). Taken together, these data indicate that $p53^{273.His}$ can mediate specific and unique effects in a sequence-dependent manner. Inclusion of SV40 T antigen in the transfection studies was aimed at sequestering p53 from transactivation and DNA binding. $p53^{273.His}$ is known to possess T antigen binding ability similar to wild-type p53 (Levine et al., 1991). It has been demonstrated that SV40 T antigen abrogates DNA-binding and transcriptional activity of p53 (Segawa et al., 1993). Although, the effects of SV40 T antigen on $p53^{273.His}$ have not been reported, given the characteristics of $p53^{273.His}$, we assumed it to be similar to that of wild-type p53. In MDA-468 cells, SV40 T antigen co-expression abrogated transcription from CON and FRA and from the minimal promoter as well (Fig.5.7). This would seem to confirm that $p53^{273.His}$ is involved in transcriptional modulation from both minimal promoter, and p53-responsive elements.

Interestingly, EGF altered the activity of a minimal promoter. SV 40 T antigen also activated transcription from the minimal promoter in the absence of EGF (Fig.5.7). This may be due to the release of $p53^{273.His}$-mediated repression. EGF may have a similar effect on p53-mediated repression of a minimal promoter. On the other hand, this was not the case with FRA-CAT. SV 40 T antigen relieved the repression by about 10 fold, whereas, EGF had no effect on FRA-mediated repression. This implies that the molecular mechanisms of transcriptional repression by FRA may be different than that through a minimal promoter. It is conceivable that EGF-induced changes in conformation may affect only certain specific protein-protein interactions. These may include interaction with one or more components of the basal transcription machinery such as TBP, CBF or Sp1 , since the TK promoter in pBLCAT2 contains a TATAA motif, CCAAT motif and a GC rich Sp1 motif (Luckow & Schutz, 1987).

In this context, we suggest a novel function for $p53^{273.His}$ in MDA-468 cells, a combination of transactivation and repression effects. While $p53^{273.His}$ alone retains sequence-dependent DNA binding and transactivation functions, the cellular environment may determine which one of these activities is essential for that particular cell cycle stage. $p53^{273.His}$ is capable of transcriptional repression of a minimal promoter, transactivation through CON and active repression through FRA. This combination of activities may be responsible for the observed gain-of-function phenotype of this mutant. Alternatively, $p53^{273.His}$ may be exerting a critical function

through an-as-yet-unidentified sequence. This suggestion is particulary seductive because EGF apparently had little effect on the transcriptional activity through CON and FRA. Another alternative mechanism for EGF-dependent modulation of p53 function could involve its interaction with components of basal transcription machinery. It is conceivable EGF-mediated conformational change of $p53^{273.His}$ in MDA-468 cells might result in significant changes in its interaction with cellular proteins as indicated by altered transcription from a minimal promoter.

Our experiments have provided significant insights into the role of a peculiar mutant $p53^{273.His}$ in MDA-468 cell proliferation. We suggest, this novel function is essential for G1-S progression. EGF-treatment has profound effects on conformation of p53 and in turn on its transcriptional activity. This may lead to deregulation of the cell cycle events. These suggestions, though speculative, are not entirely unfounded. Several studies have clearly demonstrated the role of p53 in proliferation and tumorigenicity of MDA-468 cells. One such study indicated that exogenously introduced multiple copies of wild-type p53 abolished the focus formation ability of MDA-468 cells (Casey et al., 1991). A more careful approach utilizing retroviral-mediated single copy transfer of wild-type p53 into MDA-468 cells resulted in slightly different results (Wang et al., 1993). Wild-type p53 as a single copy had little effect on cell proliferation in culture, but inhibited anchorage-independent growth and tumorigenicity to a significant extent. Taken together, the effects of wild-type p53 appear to be dependent on its level of expression in the cell. Wild-type p53 was not

able to override a proliferative function/force in low levels, however, was able to exert negative regulation at higher levels. EGF in high concentrations, mimics the effects of high levels of wild-type p53 on proliferation and anchorage-independent growth of MDA-468 cells (Filmus et al., 1985a; Filmus et al., 1987b; Church & Buick, 1988, Church et al., 1989). Moreover, a clear indication that $p53^{273.His}$ might be of gain-of-function phenotype, supports our suggestion that $p53^{273.His}$ does have a novel role in cell proliferation.

Previous studies have indicated significant alteration of various cellular protein kinases in A431 cells upon nanomolar concentrations of EGF treatment. These kinases include CDK1 and casein kinase II (Hall et al., 1991; Ackerman et al., 1990). Interestingly enough, these enzymes are believed to be modulators of p53 activity through site-specific phosphorylation (Section 4.1.4.1). Thus, MDA-468 cells and their unusual response to high concentrations of EGF provide a useful model to study and understand the molecular mechanisms involved in EGF-mediated alterations in an endogenous mutant p53 and in turn its role in transformation and tumorigenesis.

6. SUMMARY AND FUTURE DIRECTIONS

6.1 BACKGROUND

This study was initiated with the goal of understanding the molecular pathways involved in EGF-mediated growth inhibition in MDA-468 human breast cancer cells. These cells overexpress EGFRs and are negative for estrogen receptors. While initial studies clearly demonstrated a correlation between the number of cell-surface EGFRs and a growth-inhibitory response (Kawamoto et al., 1984), more current work has demonstrated that this was clearly insufficient and altered signal transduction pathways may be more likely the cause of growth-inhibition (Di Fiore et al., 1987; Church et al., 1988; Church & Buick, 1988). The objective of this project was to delineate the signal transduction pathway that lead to an altered gene expression in MDA-468 cells upon treatment with growth inhibitory concentrations of EGF.

6.2 GENERAL EXPERIMENTAL STRATEGY

In general, the approach was to characterize the EGF-dependent growth-inhibition in MDA-468 cells. To this end, the following experiments were employed.

a) Cell cycle analysis, to determine the stage at which cells are affected by EGF. Flow-cytometric analysis of DNA was used for this purpose (Section 3.2.1).

b) Assays for DNA/protein synthesis in order to determine the effect of EGF at the molecular level. Metabolic labelling of cells with suitable radioactive precursors followed by an estimation of total DNA/protein synthesis aided in detection of

immediate effects of EGF on cellular processes (Section 3.2.2 & 3.2.3).

c) Gene-expression studies to test for the effects of EGF on cell cycle-dependent genes aimed at obtaining information essential to confirm and corroborate the data from flow cytometry. Standard Northern blot analysis of total RNA isolated from untreated and EGF-treated cells provided enough data to narrow the growth-inhibition to a small period during the cell cycle (Section 3.2.4).

d) Examination of effects of EGF on p53 status was initiated after careful deliberation of the literature and the data from the above experiments (Section 4.1). To this end a panel of monoclonal antibodies was used in immunoprecipitation, immunofluorescence, and Western blot techniques (Section 4.2).

e) Analysis of p53 function as transcription factor was carried out by testing for its DNA binding activity and transactivation abilities. For this purpose, electrophoretic mobility shift assays and DNA-transfection followed by chloramphenicol acetyl transferase assays were employed (Section 5.2).

6.3 RESULTS AND CONCLUSION

In summary, the experiments were successful in characterizing the EGF-dependent growth inhibition as a reversible late G1 arrest occurring near the G1/S boundary of the cell cycle (Section 3.2). The earliest detectable effect of EGF on cellular processes was that observed with total protein synthesis. Between 6-12 h following EGF treatment total protein synthesis dropped dramatically (about 60% of that of untreated cells). The results from the cell cycle-dependent gene expression

studies indicated the possible involvement of a tumour suppressor gene, p53, in EGF-induced G1 arrest. Since, p53 was an excellent candidate gene involved in regulation of cell proliferation near G1/S boundary, further studies were undertaken to critically examine the EGF effects on p53. The experiments to study the effects of EGF on cellular $p53^{273.His}$ indicated no significant shifts in the protein levels, protein synthesis, or stability. However, we demonstrated an altered conformation and phosphorylation status of $p53^{273.His}$ in response to EGF. Further studies demonstrated the specific DNA-binding ability of endogenous $p53^{273.His}$ to CON (Funk et al., 1992) and FRA (Kern et al., 1991b) sequences. Interestingly, $p53^{273.His}$ retains a transcription modulation effect through both CON and FRA in contrast to the earlier reports (Kern et al., 1992). EGF treatment of MDA-468 cells resulted increased DNA-binding to CON and FRA and in potentiation of transcriptional activation from a minimal promoter containing TATAA box motif.

From the above data and with the current understanding of growth factor signal transduction, cell cycle regulation and biological/biochemical roles of p53 in cancer, the following conclusions were drawn. Endogenous $p53^{273.His}$ exists in a unique conformation in MDA-468 cells. It bears a strong PAb 1620-reactive epitope (wild-type) and a weak PAb 240-reactive (mutant) epitope. p53 molecules with this unique conformation, are capable of DNA binding and transcriptional modulation. EGF in high concentrations induces a change in conformation of endogenous $p53^{273.His}$. Altered phosphorylation in response to EGF, may mediate the

conformation change. The EGF-dependent changes increase DNA binding and certain transactivation activities of $p53^{273.His}$. Taken together, we have provided evidence for an immediate effect of EGF on endogenous $p53^{273.His}$ and its function in MDA-468 cells. The results also demonstrate a novel function for $p53^{273.His}$ in MDA-468 cells. The observed unique conformation of $p53^{273.His}$ may be responsible for its distinct transcription modulation activities observed in a sequence-dependent manner.

6.4 IMPLICATIONS OF ALTERED $p53^{273.HIS}$ CONFORMATION AND FUNCTION IN EGF-DEPENDENT G1 ARREST OF MDA-468 CELLS

6.4.1 p53 and cell cycle

Wild-type p53 is known to be an important regulator of a check-point in late G1 phase of the cell cycle (Reviewed in Levine et al., 1990; Oren, 1992). Exogenously introduced p53 expression constructs induce a G1 arrest (Chen et al., 1991; Chen et al., 1990; Diller et al., 1990). This check-point apparently operates in tumour cells carrying mutant p53 as well (Steinmeyer et al., 1990; Deppert et al., 1990). Exogenously introduced $p53^{273.His}$ into p53-null Soas2 cells, were transforming (Chen et al., 1990). This, and other known peculiar properties of $p53^{273.His}$, indicate a gain-of-function effect for this mutation. Some of the additional characteristics of this mutant include retention of nuclear localization (Ginsberg et al., 1991a; Bartek et al., 1990), retention of sequence-specific DNA binding ability (Funk et al., 1992), ability to transactivate from certain sequences (Chen et al., 1993b), ability to

potentiate wild-type p53 transactivation function (Miller et al., 1993) and so on. These properties along with its increased half-life (>7-8 h; Hinds et al.,1990), may result in an altered function for $p53^{273.His}$. This could be important in cell cycle progression and transformation.

6.4.2 Novel function for $p53^{273.His}$ in MDA-468 cells

Our results clearly add to what is already known concerning the unusual functions of $p53^{273.His}$. In MDA-468 cells, $p53^{273.His}$ is capable of specific-DNA binding and sequence-dependent transactivation. The observations of this study confirmed the previously observed $p53^{273.His}$ interaction, both DNA-binding and transactivation, with the CON sequence. On the other hand, $p53^{273.His}$ from MDA-468 cells, was also capable of specific binding with FRA sequences. In addition, $p53^{273.His}$ in MDA-468 cells, actively repressed transcription from FRA sequences. Reports published so far, have demonstrated that $p53^{273.His}$ lacks these properties. This suggests a novel function for $p53^{273.His}$, perhaps influenced by cell-type specific biochemical/molecular interactions in MDA-468 cells. The interaction with other nuclear proteins involved in transcription, or with yet-to-be identified DNA sequences, may determine the ultimate effect of $p53^{273.His}$ on gene expression.

With the help of the existing information about wild-type p53 and $p53^{273.His}$, we suggest that $p53^{273.His}$ possesses a novel function in MDA-468 cells. This, as we observed, may be a combination of transactivation, and repression activities determined by specific DNA sequences. Alternatively, this novel function may well

be mediated through an-as-yet-unidentified DNA sequence. The results presented in Chapter 5, demonstrate that $p53^{273.His}$ in MDA-468 cells retained certain wild-type functions such as transactivation from CON, and repression of a minimal promoter,. In addition it has gained other functions, such as repression from FRA.

The distinct effects of $p53^{273.His}$ in a sequence-dependent manner, suggests a critical role for this mutant p53 in gene expression. We suggest such a role is essential for cell proliferation and transformation. The observed EGF-dependent increase in specific DNA-binding functions of $p53^{273.His}$ with CON and FRA, and EGF-induced modulation of $p53^{273.His}$ activity through the minimal promoter, may be indicators of an alteration in such a putative, essential role. An EGF-induced change in the conformation might be responsible for this altered $p53^{273.His}$ function. Abrogation of critical $p53^{273.His}$ functions might have profound effects on cell proliferation, resulting in G1 arrest.

The results of this study, though obtained from an established cell line, has significant relevance for the development of therapeutic approaches to cure breast cancer. By identifying the cellular genes or gene products involved in the EGF-induced cell cycle arrest, it is conceivable that it can be targeted through pharmacological means in a breast cancer patient. Indication of a role for p53 in EGF-dependent growth inhibition is intriguing, as a number of studies have indicated high incidence of p53 mutations in breast cancer cells isolated from patients. Approaches to alter/restore the function of p53 in these cells may lead to the

cessation of continuous cell proliferation.

6.5 FUTURE DIRECTIONS

6.5.1 Confirmation of the putative critical role of $p53^{273.His}$

The experiments described in this study have undoubtedly provided significant information regarding the function of endogenous $p53^{273.His}$ in MDA-468 cells. The results also indicated EGF-dependent modification of certain functions of $p53^{273.His}$. This led us to suggest a critical role for $p53^{273.His}$ in MDA-468 cell proliferation. This suggestion is based on our own observation of novel functioning of $p53^{273.His}$ in MDA-468 cells together with the existing knowledge in the literature of the possible gain-of-function phenotype of this mutation. This suggestion can be tested experimentally by the following approaches.

6.5.1.1 Induction of p53-deficiency: Anti-sense technology can be employed to create a p53-deficient status in MDA-468 cells. Several laboratories have used this approach to induce deficiency of a particular gene-of-interest (Izant & Weintraub, 1984; Shohat et al., 1987). These experiments can be carried out by introducing p53 cDNA constructs into MDA-468 cells by standard transfection procedures. The p53 cDNA cloned downstream of a strong promoter in an anti-sense orientation would allow synthesis of transcripts in an anti-sense orientation. This will remove all sense-transcripts available for translation, thus creating a p53-deficient status. Isolation of clones of stably transfected cells will further aid in testing the effects of p53 in cell

proliferation and transformation.

Since we suggest an essential role for $p53^{273.His}$ in cell cycle progression, abrogation of such a role may interfere with the selection of stable clones carrying p53 anti-sense constructs. This problem might be circumvented by employing an inducible promoter to direct anti-sense transcript synthesis. The anti-sense p53 synthesis can be temporarily induced with appropriate inducer, after the selection of clones containing transfected DNA.

6.5.1.2 Inhibiting p53 activity: In our experiments, we have observed inhibition of p53-mediated sequence-dependent transactivation by SV40 T antigen. This may interfere with the putative critical function of $p53^{273.His}$, abrogating cell cycle progression. It will be of considerable interest to study the effects of SV40 T antigen-mediated loss of p53 functions on cell cycle progression. Use of SV40 T antigen may serve as an alternative and/or corroborative study. This can be achieved by introducing cDNA constructs of SV 40 T antigen into MDA-468 cells, preferably regulated by an inducible promoter. Stable clones carrying SV 40 T antigen constructs can then be used to study the effects of T antigen-mediated abrogation of p53 transactivation functions. Standard cell proliferation assays, cell cycle analysis and measurement of DNA synthesis will indicate whether or not T antigen-mediated loss of p53 activity is critical for cell proliferation.

6.5.2 Identification of putative novel p53-binding sequences in MDA-468 cells

As we have suggested, authentic $p53^{273.His}$ function may be mediated by an unidentified sequence. The consensus binding-site for p53 indicates a very loose sequence requirement, and there may be several p53-responsive elements yet to be identified. MDA-468 cells provide an attractive source of a p53 with novel properties. They can be employed to identify other physiologically important p53-response elements. Identification of such novel p53-response DNA elements undoubtedly will provide significant insights into the function of $p53^{273.His}$. In addition this will further our understanding of the biology of wild-type p53.

The techniques employed by other groups are a reasonable point at which to start. CASTing, a technique involving immunopurification of p53 complexes with a mixture of PCR-amplifiable degenerate oligonucleotides, as described by Funk et al. (1992), or a PCR-based technique following isolation of genomic clones from MDA-468 cells bound by $p53^{273.His}$ to identify essential sequence, used by Vogelstein's group (El-Diery et al., 1992) to yield degenerate sequences can be used to detect novel sequences. These oligos can be incubated with nuclear extracts of MDA-468 cells, followed by isolation of those bound by p53 using anti-p53 antibodies. PAb 421 can be used alone or in combination with other antibodies. Amplification of the sequences bound by p53 using PCR and sequencing will identify the p53-response elements. These techniques, however, do not guarantee the isolation of those

sequences at which antibodies interfere with DNA-binding. Usage of antibodies in different combinations may overcome this problem to a certain extent.

6.5.3 Cloning/identification of genes altered by p53

EGF-dependent G1 arrest in MDA-468 cells may be mediated by altered gene expression. Such an alteration may either be mediated by a change in $p53^{273.His}$ function or alternatively, induce the observed changes in p53 itself. This can be tested by identifying genes whose expression is specifically altered in EGF-treated cells. Such an approach employing the 'differential display' technique (Liang & Pardee, 1992), is already underway in our laboratory. The identification of such genes provides more opportunities to test the p53 role in EGF-induced G1 arrest. Since, it is plausible that EGF-mediated changes causing G1 arrest could be occurring in genes other than p53, the approach suggested here will address this concern as well.

Identification of these genes will furnish important sequence information that might provide clues regarding promoter/enhancer regions. Such sequences can be tested for p53-responsive elements in mobility shift assays and transactivation assays. Alternatively, the effect of $p53^{273.His}$ on the expression of such genes can be tested in p53-null cell-systems such as Soas2 cells with exogenously introduced p53 expression constructs. The examination of expression of these genes in p53-deficient MDA-468 cells, generated by anti-sense technology, will further confirm the role of p53 in G1 arrest.

These studies will provide evidence, not only to confirm the suggested role for $p53^{273.His}$ in tumour cell proliferation, but also will be invaluable in elucidating the molecular mechanisms of EGF-mediated growth inhibition. Results from these studies will be of immense importance in identifying a pathway involving EGF, EGFR, $p53^{273.His}$, the down-stream effectors and G1 arrest.

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