

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

CENTRE FOR NEWFOUNDLAND STUDIES

**TOTAL OF 10 PAGES ONLY
MAY BE XEROXED**

(Without Author's Permission)

K.A. NAGENDRA PRASAD



**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**

By

© K.A. Nagendra Prasad, B.V.Sc.

**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 [³H] 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, Willfried Marzluff, Heinz-Kurt Hoeckkoppel, 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. Ganesh 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, Sianti, 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, Aji 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.

TABLE OF CONTENTS

ABSTRACT	iii
ACKNOWLEDGEMENTS	vi
TABLE OF CONTENTS	ix
LIST OF FIGURES & TABLES	xiv
LIST OF ABBREVIATIONS	xvii

CHAPTER 1 INTRODUCTION

Growth factor signal transduction and cell cycle regulation

1.1. Growth factors and cancer	1
1.2 Growth factor signal transduction	
1.2.1 Receptors and immediate early events	3
1.2.2 Ras - a downstream mediator	5
1.2.3 Ras and Grb2-Sos1	7
1.2.4 Phosphoinositol turnover-PLC	8
1.2.5 Raf1 kinase	9
1.2.6 Phosphoinositol 3'kinase	10
1.3 G proteins in signal transduction	11
1.4 Cell cycle	13
1.4.1 Cell cycle check points	13
1.4.1.1 G2-M	14
1.4.1.2 G1-S	15
1.4.2 Growth inhibitory mechanisms - yeast model	16
1.5 RB -The paradigm for tumour suppressor function	18
1.5.1 RB in normal cells - The paradox	20
1.5.2 TGF- β and RB	21
1.6 Epidermal growth factor	23
1.6.1 EGF and growth inhibition in MDA-468: An intriguing system	24
1.6.2 Working hypothesis	28

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals	29
2.1.2 Radioisotopes	29
2.1.3 Cell culture reagents	30
2.1.4 Antibodies	30
2.1.5 Probes for Northern blot analysis	31
2.1.6 Oligonucleotides	32
2.1.7 CAT assay reagents	33
2.1.8 Molecular cloning and hybridization reagents	33

2.2 Methods

2.2.1 Cell culture	34
2.2.2 Flow cytometric analysis of DNA content	35
2.2.3 DNA/protein synthesis assays	36
2.2.4 Radiolabelling of probes for Northern blot analysis	37
2.2.5 Northern blot analysis	37
2.2.6 Immunofluorescence experiments	38
2.2.7 Metabolic labelling	39
2.2.8 Western blot analysis	40
2.2.9 Immunoprecipitation	41
2.2.10 Nuclear extract preparation	42
2.2.11 Nuclear extract preparation from radiolabelled cells	43
2.2.11.1 Immunoprecipitation from nuclear extracts	43
2.2.12 Electrophoretic mobility shift assays	44
2.2.13 Construction of p53-responsive plasmids	46
2.2.14 DNA-transfection: Calcium phosphate precipitation method	49
2.2.15 Chloramphenicol acetyl transferase assays	49

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

3.1	Introduction	52
3.1.1	Flow cytometry	52
3.1.2	Cell cycle-dependent gene expression	53
3.2	Results	54
3.2.1	Cell cycle analysis	54
3.2.2	DNA synthesis assays	61
3.2.3	Protein synthesis assays	64
3.2.4	Alterations in gene expression	64
3.3	Discussion	69

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

4.1	Introduction	76
4.1.1	Background	76
4.1.2	p53 history - a tortuous story	77
4.1.3	p53 in cancer	78
4.1.4	Wild-type p53: biological function	80
4.1.4.1	Cell cycle regulation	80
4.1.4.2	Apoptosis and "guardian of the genome"	83
4.1.5	Wild-type p53: Biochemical function	85
4.1.5.1	Ancillary replication factor	85
4.1.5.2	p53 as a transcription factor	86
4.1.6	Mutations in p53: Functional consequences	87
4.1.6.1	Arg273His - an atypical mutant: "Pseudo wild-type"	89
4.1.7	Working hypothesis	90
4.2	Results	92
4.2.1	Northern blot analysis	92
4.2.2	Western blot analysis	92
4.2.3	Pulse-chase experiments	97

4.2.4	p53 protein synthesis	97
4.2.5	Immunofluorescence studies	104
4.2.6	EGF effects on phosphorylation status of p53	111
4.2.7	Nuclear translocation of p53	116
4.3	Discussion	116

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

5.1	Introduction	127
5.1.1	Background	127
5.1.2	p53 - A transcription factor	127
5.1.2.1	Transactivation by p53	127
5.1.2.2	Specific DNA binding sites for p53	128
5.1.2.3	Regulation of transcription by p53	130
5.1.3	DNA binding ability of p53 ^{273.His}	132
5.1.4	Transcriptional activation by p53 ^{273.His}	133
5.1.5	Working hypothesis	134
5.2	Results	135
5.2.1	p53 ^{273.His} forms specific complexes with CON and FRA	135
5.2.2	Reactivity of p53-DNA complexes with anti-p53 antibodies	141
5.2.2.1	Reactivity with PAb 421	141
5.2.2.2	Reactivity with conformation specific antibodies (PAb 240 & PAb 1620)	144
5.2.2.3	Reactivity with PAb 1801	147
5.2.3	Effect of PAb 1620 on formation and stability of p53-DNA complexes	147

5.2.4	Transcriptional activity of p53 ^{273.His}	153
5.2.5	Effect of EGF on transactivation by p53 ^{273.His}	156
5.3	Discussion	159

CHAPTER 6 SUMMARY AND FUTURE DIRECTIONS

6.1	Background	170
6.2	General experimental strategy	170
6.3	Results and conclusion	171
6.4	Implications of altered p53 ^{273.His} conformation and function in EGF-dependent G1 arrest of MDA-468 cells	173
6.4.1	p53 and cell cycle	173
6.4.2	Novel function for p53 ^{273.His} in MDA-468 cells	174
6.5	Future directions	176
6.5.1	Confirmation of the putative critical role of p53 ^{273.His}	176
6.5.1.1	Induction of p53-deficiency	176
6.5.1.2	Inhibiting p53 activity	177
6.5.2	Identification of putative novel p53-binding sequences in MDA-468 cells	178
6.5.3	Cloning and identification of genes altered by p53	179

REFERENCES	181
------------	-----

LIST OF FIGURES AND TABLES

FIG. 2.1	p53-responsive CAT-constructs.	48
FIG. 3.1	Effect of EGF (10^{-8} M) on proliferation of MDA-468 cells.	56
FIG. 3.2	Cell cycle analysis.	58
FIG. 3.3	Effect of EGF on DNA synthesis of MDA-468 cells.	63
FIG. 3.4	Effect of EGF on total protein synthesis.	66
FIG. 3.5	Effect of EGF on cell cycle-dependent gene expression.	68
FIG. 4.1	Effect of EGF on p53 steady state mRNA levels.	94
FIG. 4.2	Effect of EGF on p53 steady state protein levels.	96
FIG. 4.3	Effect of EGF on p53 stability.	99
FIG. 4.4	Examination of EGF effect on p53 protein synthesis.	101
FIG. 4.5	Examination of short-term EGF effect on p53 protein synthesis.	103
FIG. 4.6	Examination of subcellular localization of p53 in response to EGF.	106
FIG. 4.7	Specificity of EGF-effects on PAb 240 reactivity.	109

FIG. 4.8 Examination of phosphorylation status of p53 in response to EGF.	113
FIG. 4.9 Phosphorylation status of <u>nuclear</u> -p53 in response to EGF.	116
FIG. 4.10 Effect of EGF on nuclear levels of <u>de-novo</u> synthesized p53.	118
FIG. 5.1 Analysis of DNA-binding ability of p53 ^{273.His} in response to EGF - I.	137
FIG. 5.2 Analysis of DNA-binding ability of p53 ^{273.His} in response to EGF - II.	140
FIG. 5.3 Analysis of immunoreactivity of p53-DNA complex from control and EGF-treated cells - I.	143
FIG. 5.4 Analysis of immunoreactivity of p53-DNA complex from control and EGF-treated cells - II.	146
FIG. 5.5 Effect of PAb 1620 on formation of p53-DNA complex in the presence of PAb 421.	149
FIG. 5.6 Effect of PAb 1620 on formation and stability of p53-DNA complex in the presence of PAb 421.	152

FIG. 5.7 Examination of p53-response element-mediated transcriptional activity.	155
FIG. 5.8 Effect of EGF on p53-response element-mediated transcriptional activity.	158
Table 3.1 The cell cycle distribution of MDA-468 cells in response to EGF.	60

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	<u>Mitogen activated/extracellular signal regulated kinase kinase</u>
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	Phosphatidylinositol 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
RJC	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 & Schlessenger, 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 (PLC γ) (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; Lerner *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 phosphatidylinositol 4,5-bisphosphate (PIP₂), phosphatidylcholine etc. The hydrolysis is mediated by a class of enzymes collectively called phospholipases (PL). Phospholipase C- γ (PLC γ) 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 (Ulrich & 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 functional 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

(Battagay *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 $1-2 \times 10^6$ /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 $1-2 \times 10^6$ /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 hyperproliferation 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 suggest 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, 1991; 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 MgCl₂. Vinblastine was dissolved in methanol to a concentration of 0.5 µg/µl. Stock solution of aphidicolin. (1 µg/µl) 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-³H],(sp.act. 82.4 Ci/mmol); adenosine 5' [α-³²P] triphosphate, (sp.act. 6000 Ci/mmol) were purchased from NEN/DuPont Research Products (Mississauga, Ontario). Cytidine 5'-[α-³²P]-triphosphate (sp.act. 300 Ci/mmol) was obtained from Amersham. L-[³⁵S] 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_2O (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 $\mu g/ml$, 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; IgG₁) a human-specific antibody, reactive with both wild-type and mutant p53 (Banks *et al.*, 1986), PAb 240 (Ab 3; IgG₁) 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 supernatant fluid) , human wild-type p53-specific antibody (Milner *et al.*, 1987) was provided as a gift by Dr.Hoehkoppel, 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'-GGACATGCCCGGGCATGTCC-3' is self-annealed to obtain blunt-ended double-strand-CON. A 33-base sequence 5'-TTCTCCTTGCTGGACTTGCCTGGCCTTGCCIT-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'-CCTTGCTGGACTTGCCTGGCCTTGCCITTTCT-3' was annealed with 5'-AGAAAAGGCAAGGCCAGGCAAGTCCAGGCAAGG-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 Sall, 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 $\mu\text{g}/\text{ml}$ 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 (4×10^3 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 3×10^5 cells/100 mm plates. After 24 h of equilibration, EGF was added to a final concentration of 10^{-8} M. The medium \pm 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 ($1 \mu\text{g/ml}$) 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 $100 \mu\text{g/ml}$ 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 3×10^4 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, [^3H] thymidine (2.5 $\mu\text{Ci/ml}$) 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 \pm 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 $\mu\text{Ci/ml}$) 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 $[\alpha\text{-}^{32}\text{P}]\text{-dATP}$ 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 \pm 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 \pm 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 $5\mu\text{g/ml}$ 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 an identical manner except that fixing was carried out with 3% BSA/PBS at 37°C . For the experiments with aphidicolin, cells were treated ($5\mu\text{g/ml}$ 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 ($100 \mu\text{Ci/ml}$) was carried out for approximately 10 h (overnight) at the end of EGF-treatment (Fig. 4.4).
- ii) Labelling with ^{35}S -methionine ($100 \mu\text{Ci/ml}$) 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 ($100 \mu\text{Ci/ml}$) in methionine-free media, followed by extensive washing with complete media and incubation was continued \pm 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 $\text{H}_3\text{-}^{32}\text{P}_0_4$ (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 μ g/ml Leupeptin, 10 μ g/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 μ g 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 μ g/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 MgCl₂).

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. $\text{H}_3\text{-}^{32}\text{P}\text{O}_4$ 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 $\mu\text{g}/\text{ml}$ Leupeptin, 10 $\mu\text{g}/\text{ml}$ Aprotinin, 1 mM Phenylmethylsulfonyl fluoride [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 Eppendorf 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 μ l 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

Senai-confluent plates (approx. 3×10^6 cells/100 mm dish) after 24 h of plating were further incubated \pm 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 $\mu\text{g}/\text{ml}$ leupeptin, 100 $\mu\text{g}/\text{ml}$ 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 \pm EGF (10^{-8} M) were labelled with ^{35}S -methionine or $\text{H}_3\text{-}^{32}\text{PO}_4$ 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 $\mu\text{g}/\text{ml}$ leupeptin, 100 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$ leupeptin, 100 $\mu\text{g}/\text{ml}$ 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 MgCl₂, 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 ³²P-dATP (³²P-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 (1/10th 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.H₂O. 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 MgCl₂, 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 (0.045 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 $\text{Sma} \text{I}$ site of the vector pBLCAT2 (Fig. 2.1). To facilitate this procedure, pBLCAT2 was first digested with Sall 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 Sall 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-caesium 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

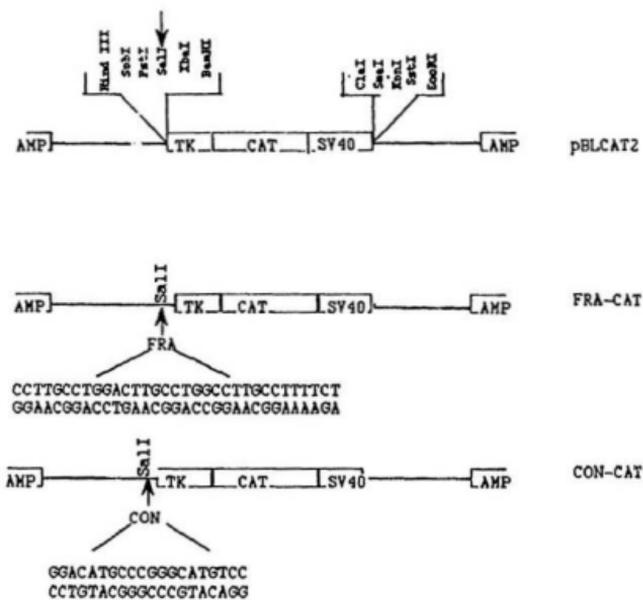


FIG. 2.1 A schematic diagram of p53-responsive CAT-constructs

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 μ l of 2 M CaCl_2 , 5-10 μ g DNA (CAT-constructs 5 μ g, and internal control RSV- β gal 2 μ g) and $\text{d.H}_2\text{O}$ up to 500 μ l. Also prepared 500 μ l of 2X BES-buffered saline (50 mM N,N'-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid {BES}, 280 mM NaCl, 1.5 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; 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 \pm 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 \pm 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 μ l 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 μ l of the cell extract was mixed with 20 μ l CAT-assay pre-mix (4 mM Acetyl CoA, 1 μ Ci 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 μ l 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; Lalonde, 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 ($1\mu\text{g/ml}$), 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 3×10^5 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.

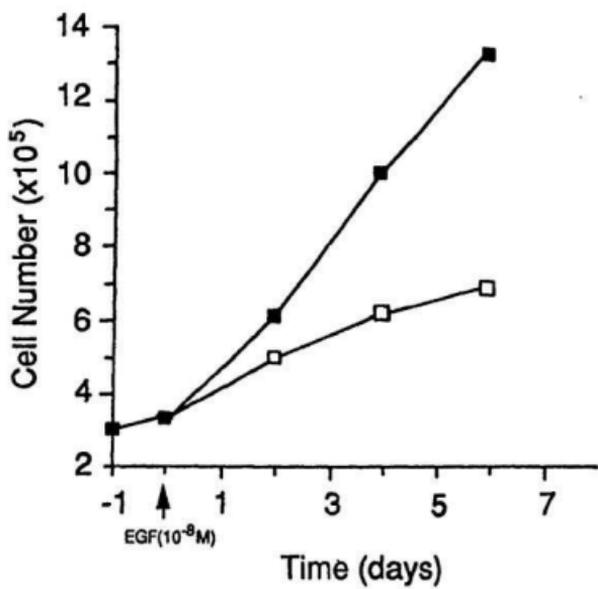


FIG. 3.2 Cell cycle analysis. Representative FACS-generated, DNA histograms of MDA-468 cells, cultured in the absence (A,C) and in the presence of EGF (10^{-8} M) (B,D), after 4 days of treatment. The control, as well as EGF-treated cells, were stained with mithramycin, and 20,000 cells were analyzed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

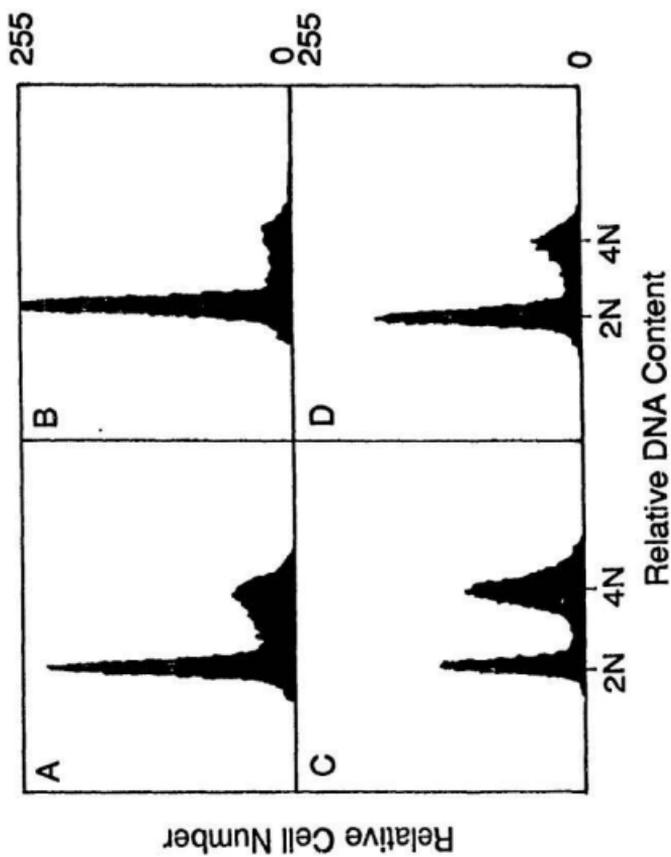


Table. 3.1 The cell cycle distribution of MDA-468 cells in response to EGF. The DNA histograms generated from the FACS analysis of EGF-treated, as well as control untreated cells, from the experiments described under Fig.3.2. legend and in Materials and Methods, were analyzed and the fraction of cells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

^aThe 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 G₁, S and G₂/M were estimated by computer analysis as outlined in Materials and Methods.

^bData are % of total cells analyzed; mean ± S.E.M. from a minimum of 5 experiments in each case.

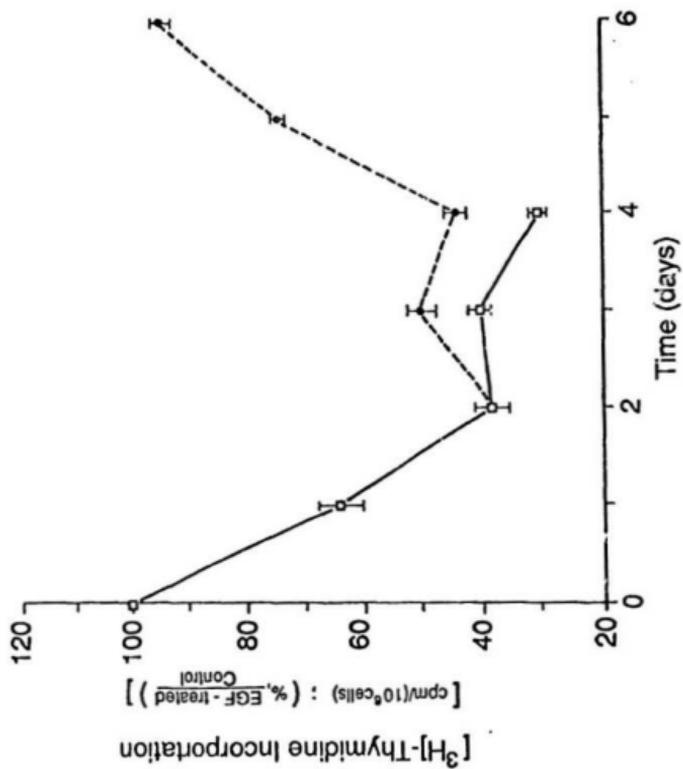
^cVinblastine (1μg/ml) 24 hr. prior to assay.

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 [³H] 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 3×10^4 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 [^3H] thymidine ($2.5 \mu\text{Ci/ml}$) 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, \pm std.error.of mean from three individual experiments. \square --- \square EGF (10^{-8} M) treated cells; \bullet --- \bullet Cells from which EGF was removed after 48 h of EGF treatment.



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 3×10^4 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 $\mu\text{Ci/ml}$) 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.

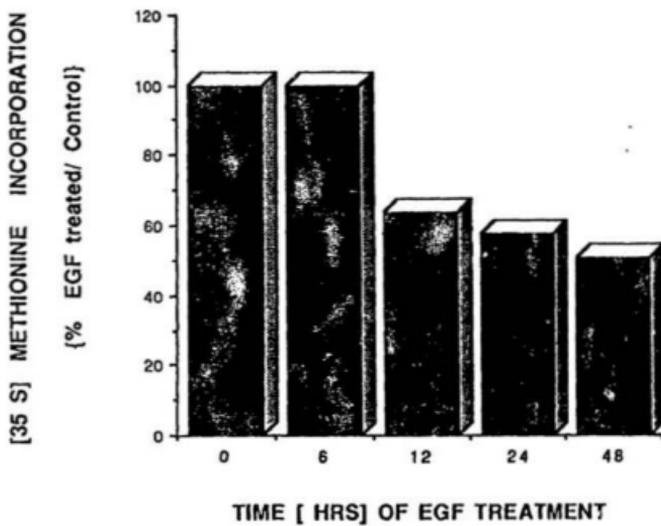


FIG. 3.5 Effect of EGF on cell cycle dependent gene expression. Exponentially growing cells were serum starved for four days (lane 1), following which the cells were further incubated with the media containing serum, in the absence (lanes 2,3) and in the presence of 10^{-8} M EGF (lanes 4,5), for indicated 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 μ g 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.



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 [³H] 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 [³H] 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. [³H] 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 & Beach, 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 (Lavigne *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; Zuberma *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 & Bracley, 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 $\mu\text{g}/\text{lane}$) 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.

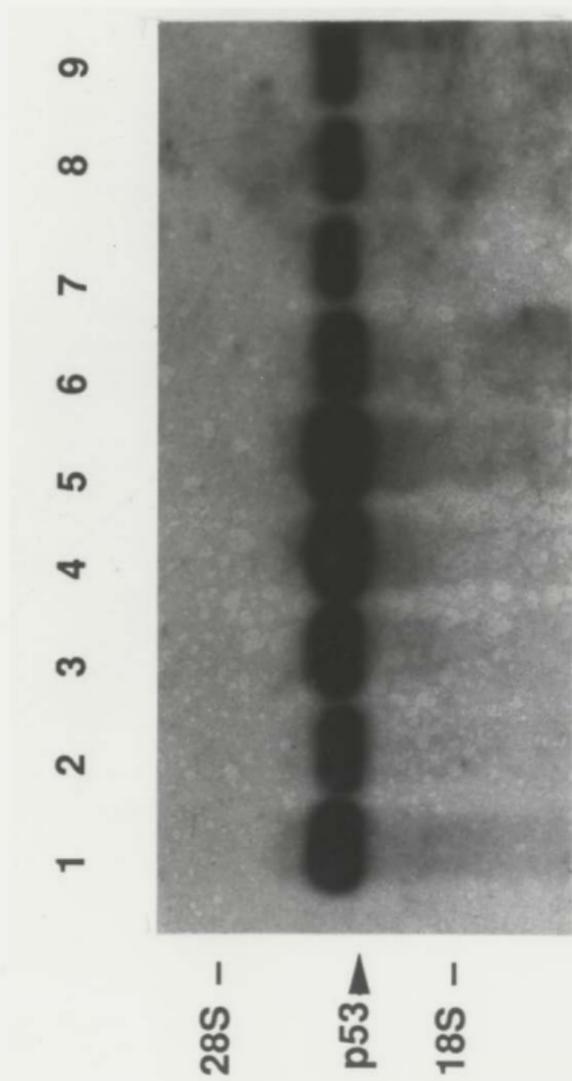
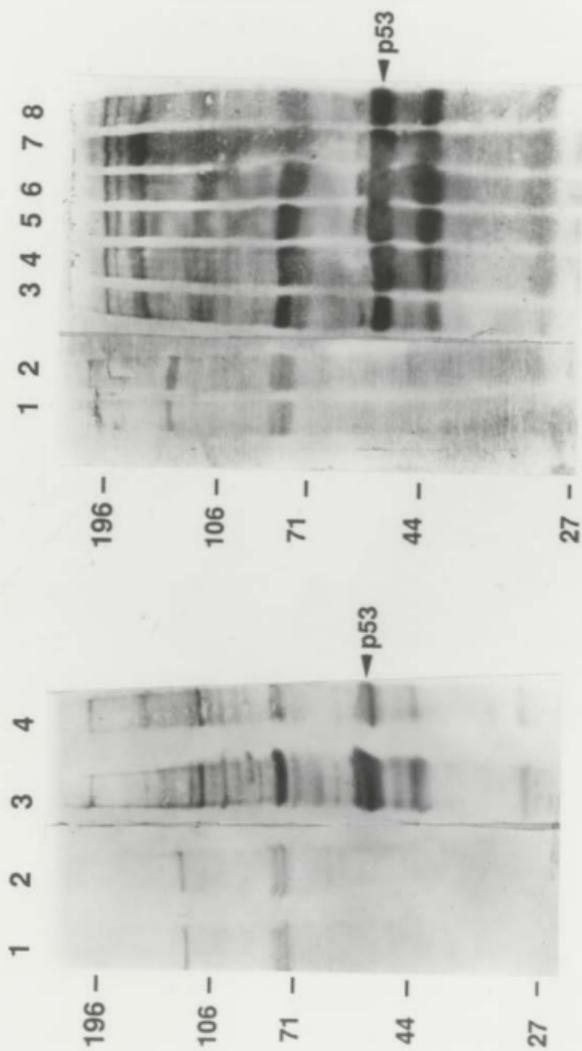


FIG. 4.2 Effect of EGF on p53 steady state protein levels. The total protein samples (100 $\mu\text{g}/\text{lane}$) isolated from MDA-468 cells cultured \pm EGF (10^{-8} M), were separated on an 8% SDS-PAGE. The p53 protein levels were examined by Western blot analysis utilizing alkaline-phosphatase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



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 ³⁵S-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 ³⁵S-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 (1×10^6 cells/100mm plate) were labelled overnight (10 h) with ^{35}S -methionine (100 $\mu\text{Ci/ml}$). 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.

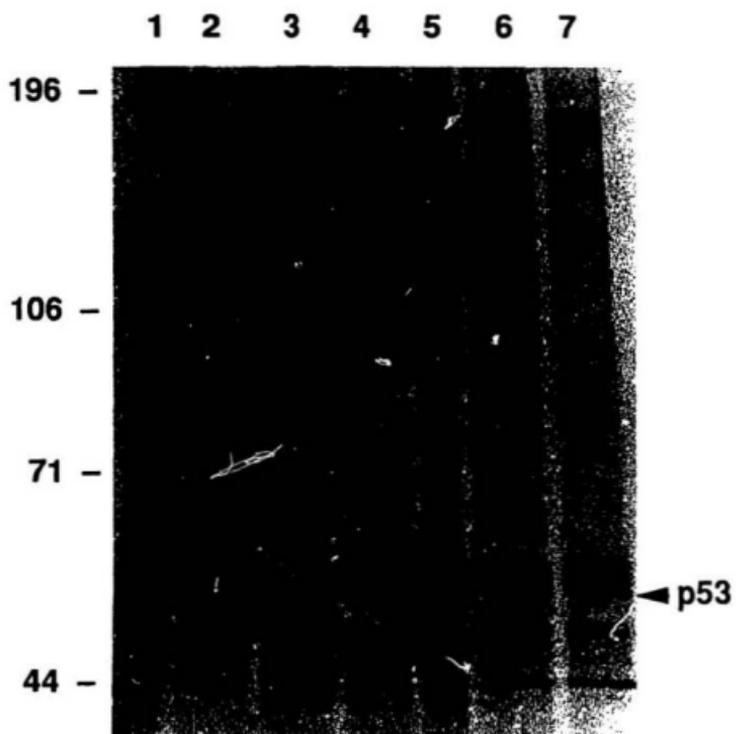


FIG. 4.4 Examination of EGF effect on p53 protein synthesis. MDA-468 cells, after 24 h of plating, were treated with \pm EGF (10^{-8} M) for the indicated intervals. Labelling was carried out after the indicated duration of EGF-treatment with ^{35}S -methionine (100 $\mu\text{Ci/ml}$) for approx. 10 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.

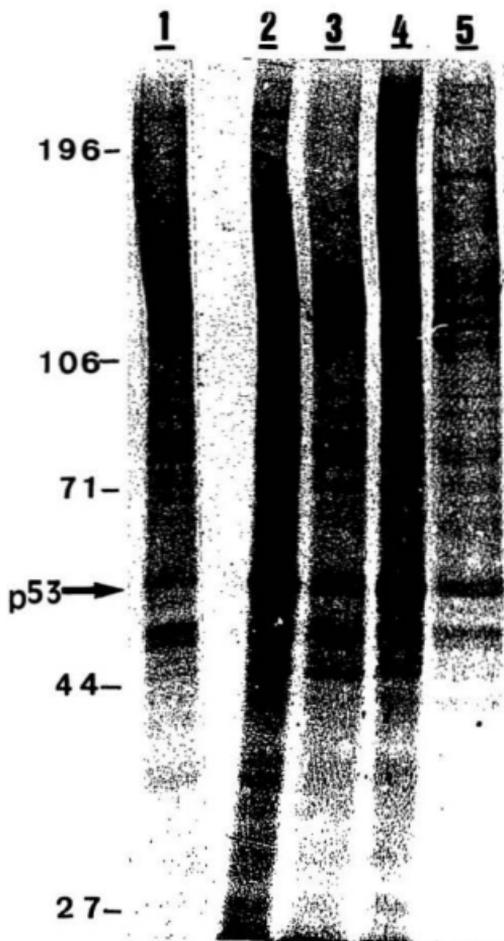
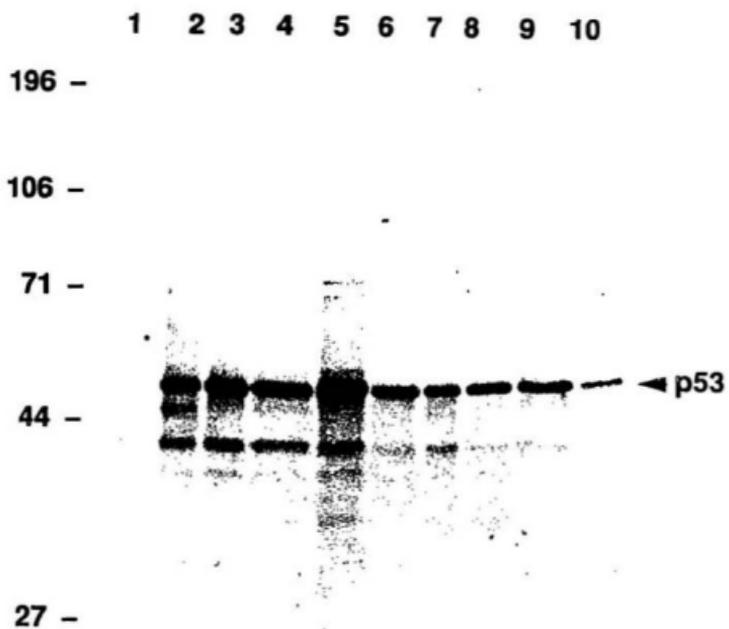


FIG. 4.5 Examination of short-term EGF effect on p53 protein synthesis. MDA-468 cells were treated with EGF (10^{-8} M) for the indicated intervals. Labelling with ^{35}S -methionine (100 $\mu\text{Ci/ml}$) was carried out during the last 3 h of incubation. Cells were then lysed and immunoprecipitated 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.



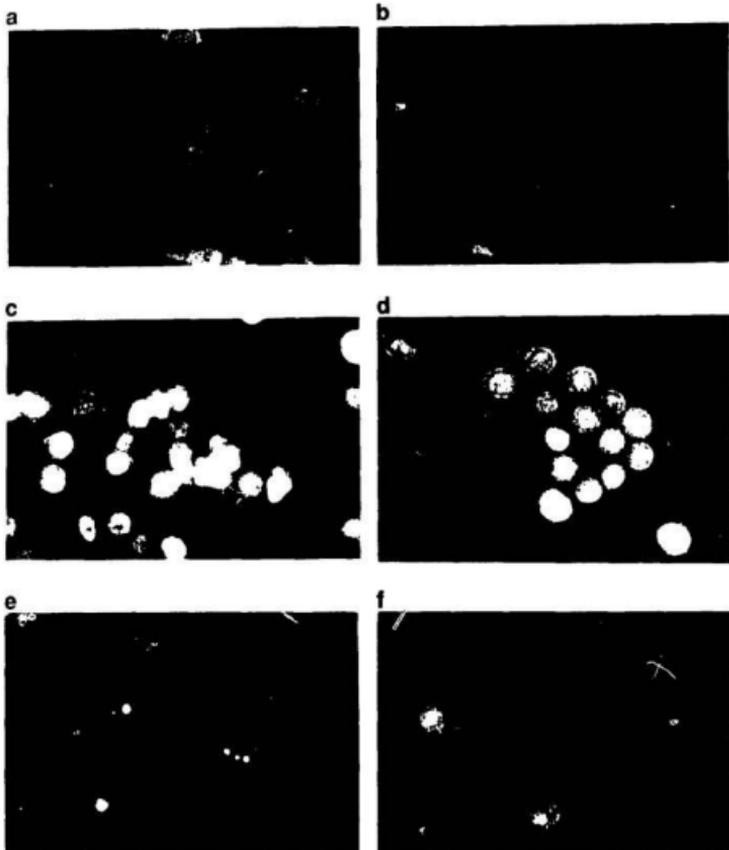
synthesized p53 protein levels were significantly lower after 24 h of EGF treatment (Fig.4.4; lane 3) no immediate effect was obvious until 12 h (Fig.4.5; lane 10). This reduction in p53 protein synthesis following EGF addition likely reflects the reduction in total protein synthesis which occurred between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



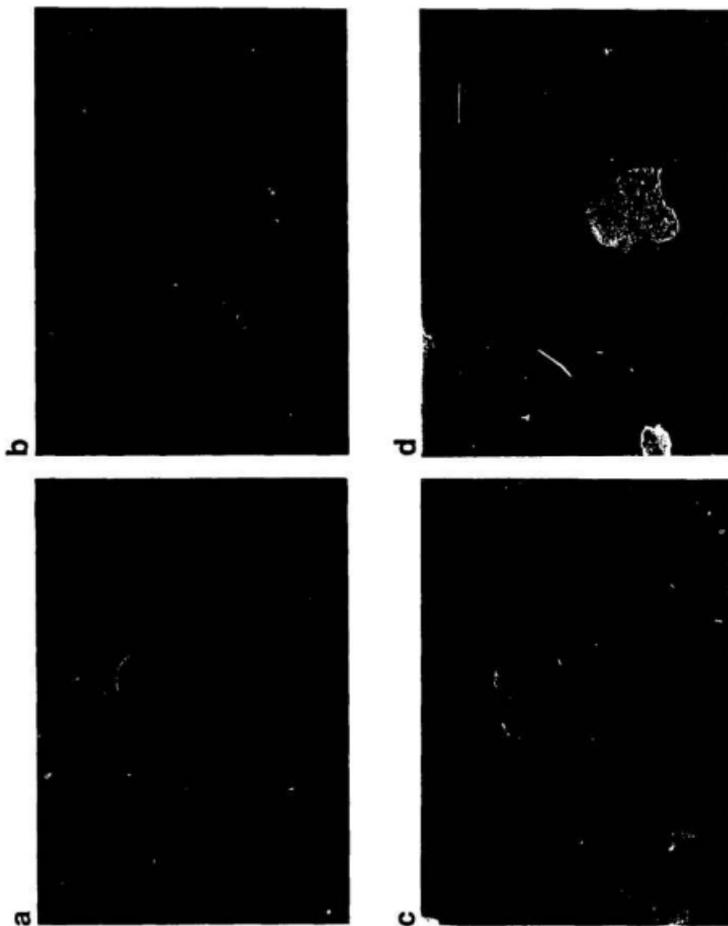
In serum-starved cells, antibodies PAb 1801 and PAb 240 displayed a characteristic nuclear signal along with bright cytoplasmic staining, which remained unaltered upon stimulation with serum. The purpose of serum starvation was to minimise the background staining with p53 antibodies so that any EGF effects 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 \pm EGF (10^{-8} M) for additional 24 h. Cells were then examined by immunofluorescence with PAb 240 (mutant-specific anti-p53; 5 μ g/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 \pm aphidicolin (5 μ g/ml) were examined by immunofluorescence by PAb 240. Panel c - untreated cells; panel d - cells treated with aphidicolin for 24 h.



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 $\mu\text{g/ml}$) 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 (1×10^6 cells/100mm plate) were treated with EGF (10^{-8} M) for the indicated periods. Cells were labelled with $H_3\text{-}^{32}P0_4$ (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 μ g 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.

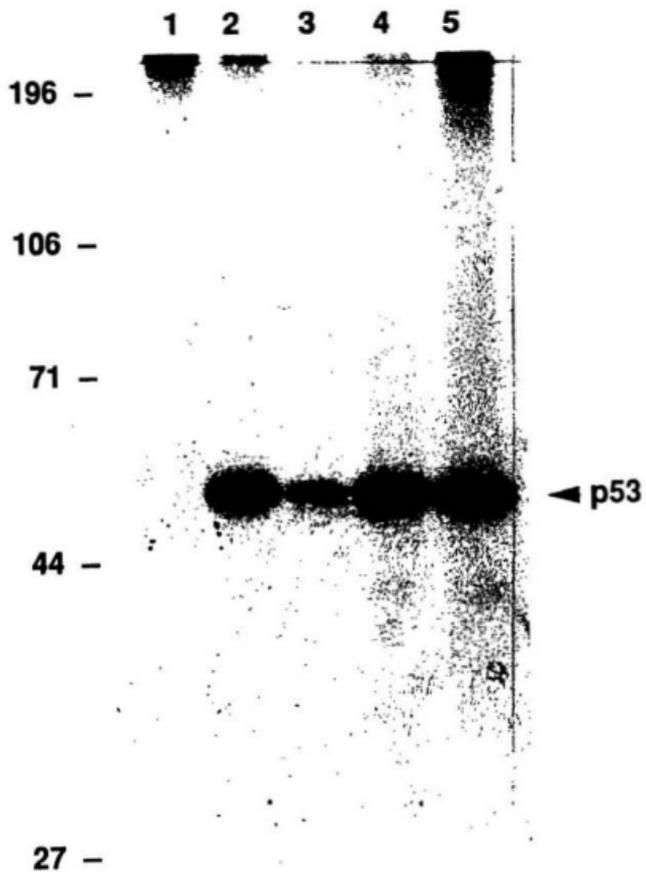
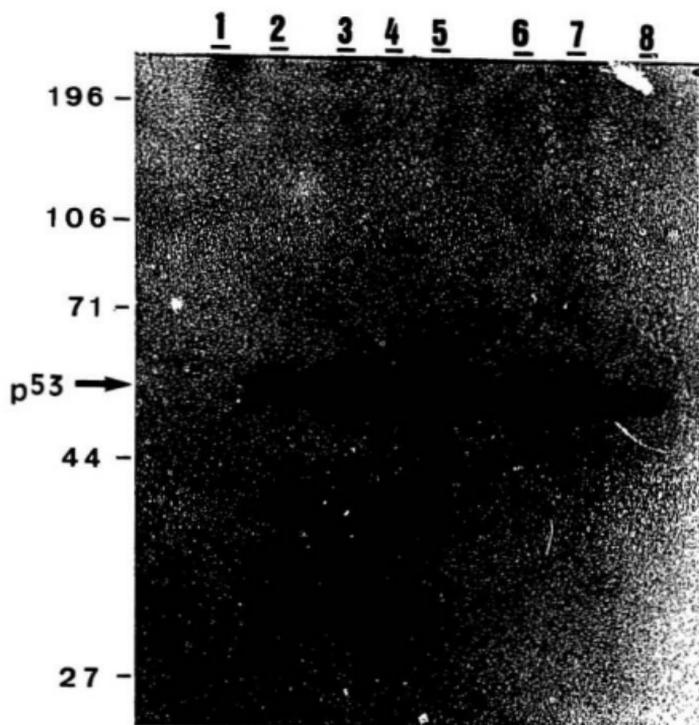


FIG. 4.9 Phosphorylation status of nuclear-p53 in response to EGF. MDA-468 cells (1×10^6 cells/100mm plate) were treated with EGF (10^{-8} M) for the indicated periods. Cells were labelled with $H_3\text{-}^{32}P\text{O}_4$ (0.25 mCi/ml) for the final 2 h during EGF-treatment. Nuclear extracts of labelled-cells 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.



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).

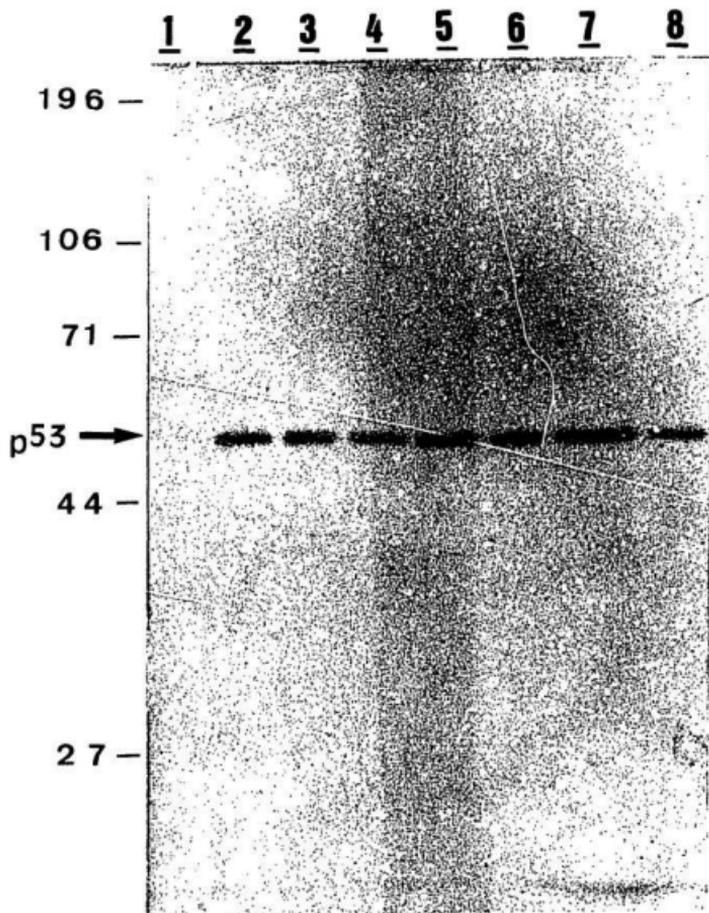
4.2.7 Nuclear translocation of p53

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 (1×10^6 cells/100mm plate) were treated with EGF (10^{-8} M) for the indicated periods. Cells were labelled with ^{35}S -methionine ($100 \mu\text{Ci/ml}$) 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 $1 \mu\text{g}$ 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.



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 ³²P-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 ³⁵S-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 1st 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

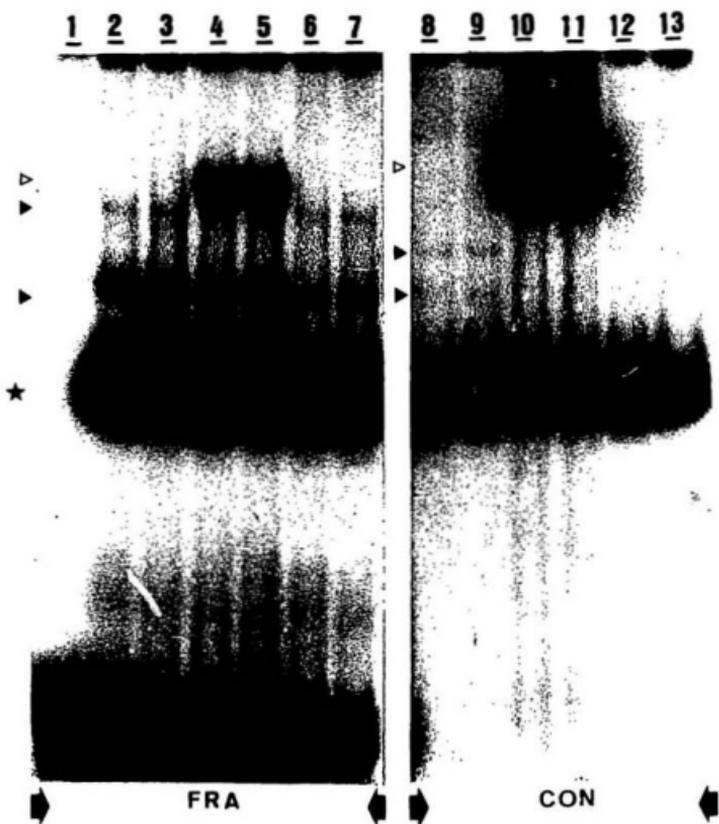
binding ability was determined by electrophoretic mobility shift assays (EMSAs). Further, we asked whether or not EGF modifies the transcription from a minimal promoter bearing a TATA box motif, and from promoters containing these p53 response elements, in MDA-468 breast cancer cells. The experimental 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

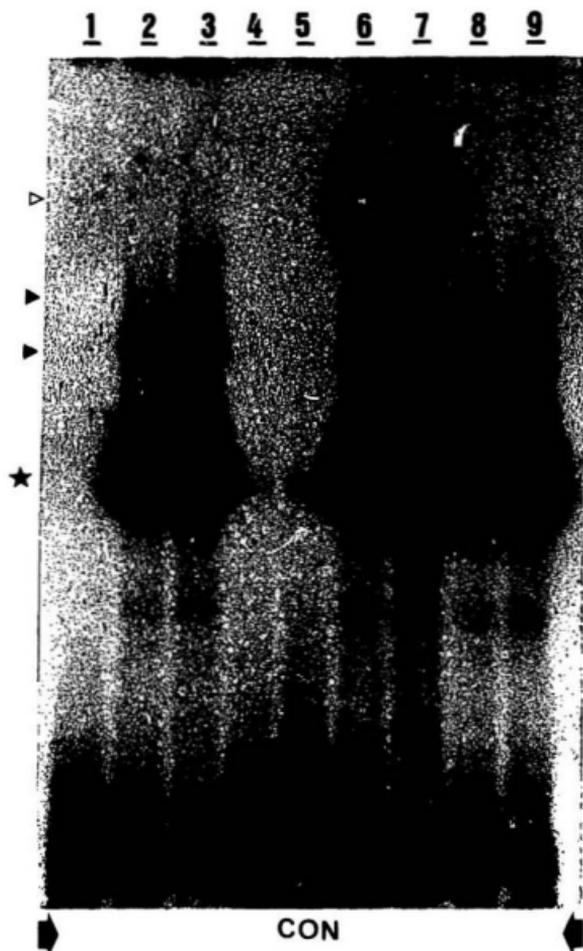
FIG. 5.1 Analysis of DNA-binding ability of p53^{273.His} in response to EGF - I. Nuclear extracts (3 μ g) from untreated/control (even numbered lanes), and EGF-treated (odd numbered lanes) MDA-468 cells were analyzed for mobility shifts of end-labelled oligos representing p53-specific sites. Lane 1 - 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 μ g) - lanes 4, 5, 10 & 11, and PAb 1620 (1.0 μ g protein equivalent of hybridoma supernatant 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.



(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 supernatant 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 μ g) 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 μ g) - lanes 6 & 7 and PAb 1620 (1.0 μ g protein equivalent of hybridoma supernatant 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.



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 μ g), 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.

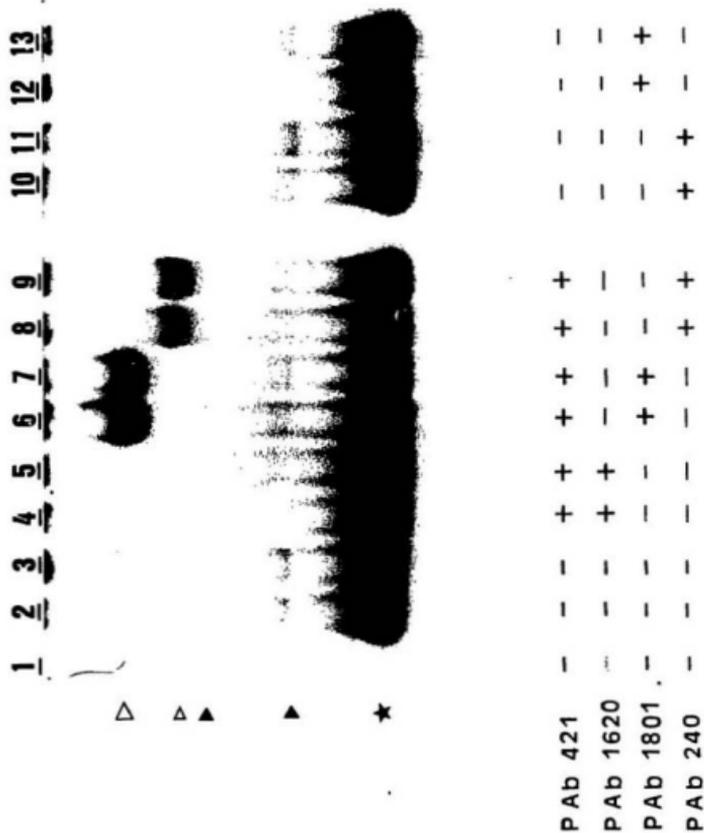
5.2.2 Reactivity of p53-DNA complexes with anti-p53 antibodies

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 supernatant fluid), PAb 1801 (0.1 μg), and PAb 240 (0.1 μg). The figure is a 24 h exposure of the autoradiograph. \star 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.



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 & Claderwood, 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 supernatant 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.

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



△

△

▲

▲

★

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 supershifted 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 not 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 supernatant 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



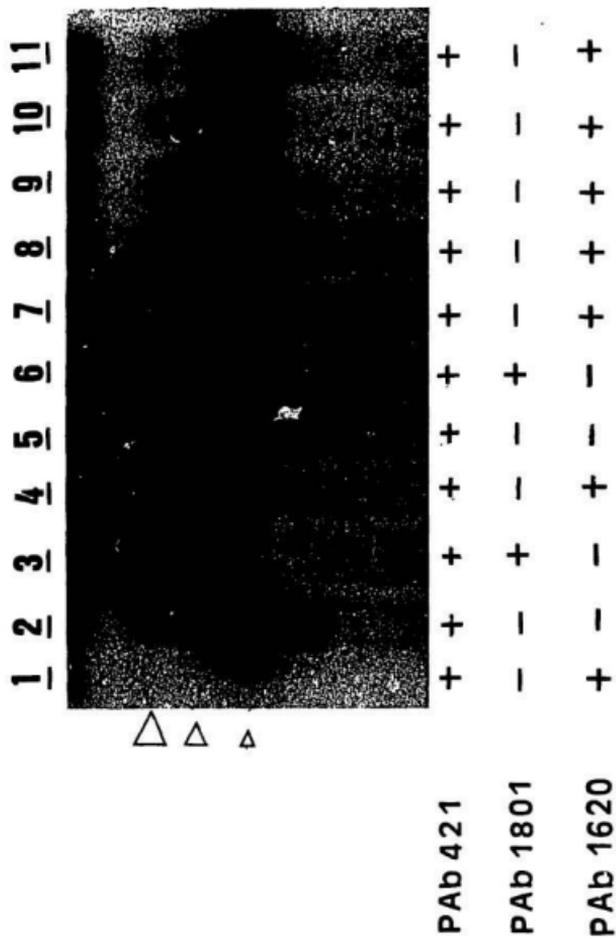
△ △ △

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 cligo (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 supernatant 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.



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.

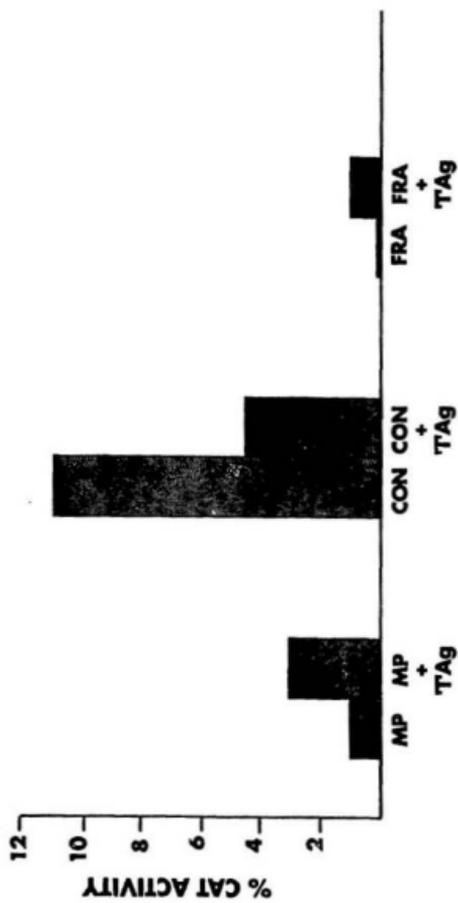
Taken together, the DNA binding studies demonstrate that endogenous p53^{273.His} of MDA-468 cells specifically interacts with both FRA and CON sequences. Furthermore, EGF treatment enhances this interaction. The reactivity of p53-DNA complexes with various antibodies indicate a sequence-dependent conformation 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 (2×10^6 cells/100mm plate) were transfected with 5 μ g 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 μ g/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 μ g/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.

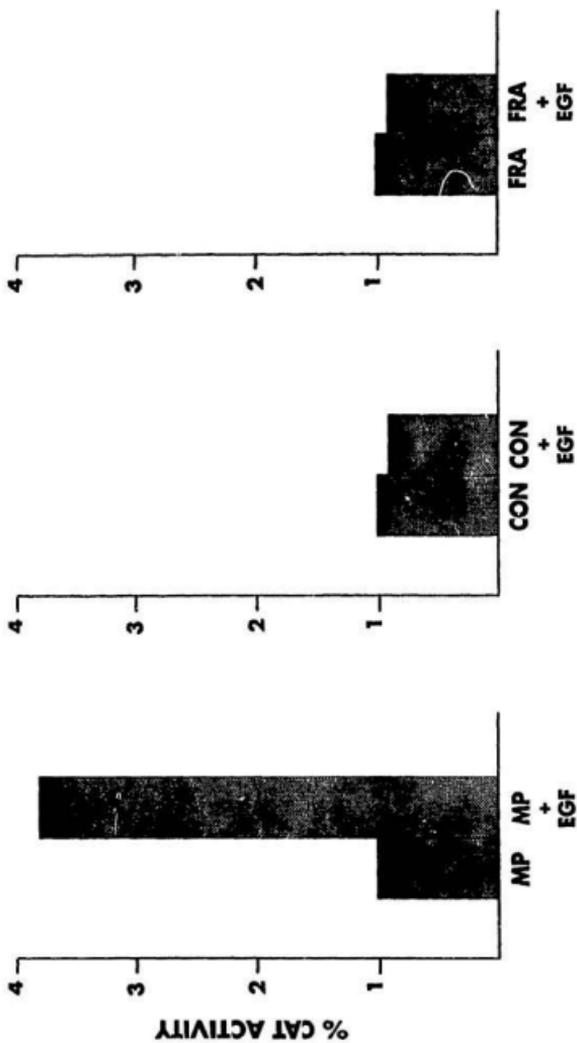


and FRA in the absence of PAb 421, it was essential to determine the functional consequences of the observed DNA binding activity. To this end, the p53 response DNA elements were cloned upstream of a minimal promoter regulating a chloramphenicol acetyl transferase (CAT) reporter gene (pBLCAT2). This 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 (2×10^6 cells/100mm plate) were transfected with 5 μ g 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 \pm 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 μ g/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.



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 PyF,Py-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 & Claderwood, 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 1 BP 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 Saos2 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 particularly 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 *trans*-forming (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-Deiry *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.

REFERENCES

- Aaronson, S.A. (1991) Growth factors and cancer. Science, 254:1146-1153.
- Ackerman, P., Glover, C.V.C., and Osheroff, N. (1990) Stimulation of casein kinase II by epidermal growth factor: Relationship between the physiological activity of the kinase and the phosphorylation state of its β subunit. Proc.Natl.Acad.Sci.USA, 87:821-825.
- Addison, C., Jenkins, J.R. and Sturzbecher, H-W. (1990) The p53 nuclear localization signal linked to a p34^{cdc2} kinase motif. Oncogene, 5:423-426.
- Agoff, S.N., Hou, J., Linzer, D.I.H., and Wu. B. (1993) Regulation of the human hsp70 promoter by p53. Science, 259:84-87.
- Angel, P., and Karin, M. (1991) The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. Biochem.Biophys.Acta, 1072: 129-157.
- Asaoka, Y., Nakamura, S., Yoshida, K., and Nishizuka, Y. (1992) Protein kinase C, calcium and phospholipid degradation. TIBS, 17:414-417.
- Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P., and Cantley, L.C. (1989) PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell, 57:167-175.
- Baker, S.J., Markowitz, S., Fearon, E.R., Wilson, J.K.V. and Vogelstein, B. (1990) Suppression of human colorectal carcinoma cell growth by wild-type p53. Science, 249:912-915.
- Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., vanTuinen, P., Ledbetter, D.H., Barker, D.F., Nakamura, Y., White, R., and Vogelstein, B. (1989) Chromosome 17 deletions and p53 mutations in colorectal carcinomas. Science, 244:217-221.
- Banks, L., Matlashewski, G., and Crawford, L. (1986) Isolation of human-p53-specific monoclonal antibodies and their use in the studies of human p53 expression. Eur.J.Biochem., 159:529-534.
- Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, Y., and Schlessinger, J. (1993) SH3 domains direct cellular localization of signalling molecules. Cell, 74:83-91.

- Barak, Y., Juven, T., Haffner, R., and Oren, M.** (1993) *mdm2* expression is induced by wild-type p53 activity. *EMBO J.*, 12:461-468.
- Barford, D.** (1991) Molecular mechanisms for the control of enzymatic activity by protein phosphorylation. *Biochem. Biophys. Acta.*, 1133:55-62
- Bargonetti, J., Friedman, P.N., Kern, S.E., Vogelstein, B., and Prives, C.** (1991) Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. *Cell.* 65:1083-1091.
- Barnard, J.A., Lyons, R.M., and Moses, H.L.** (1990) The cell biology of transforming growth factor β . *Biochem. Biophys. Acta.*, 1032:79-87.
- Barnes, D.W.** (1982) Epidermal growth factor inhibits growth of A431 epidermoid carcinoma in serum-free cell culture. *J. Cell Biol.*, 93:1-4.
- Bartek, J., Iggo, R., Gannon, J., and Lane, D.P.** (1990) Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene.* 5:893-899.
- Battagay, E.J., Raines, E.W., Selfert, R.A., Bowen-Pope, D.F., and Ross, R.** (1990) TGF- β induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. *Cell.* 63:515-524.
- Bischoff, J.R., Friedman, P.N., Marshak, D.R., Prives, C., and Beach, D.** (1990) Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. *Proc. Natl. Acad. Sci. USA.*, 87:4766-4770.
- Bishop, J.M.** (1987) The molecular genetics of cancer. *Science.* 235:305-311.
- Bjorge, J.D., Chan, T.O., Antezak, M., Kung, H.J., and Fugita, D.J.** (1990) Activated type I phosphatidylinositol kinase is associated with the epidermal growth factor (EGF) receptor following EGF stimulation. *Proc. Natl. Acad. Sci. USA.*, 90:3816-3820.
- Blenis, J.** (1993) Signal transduction via the MAP kinases: Proceed at your own RSK. *Proc. Natl. Acad. Sci. USA.*, 90:5889-5892.
- Bradford, M.M.** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein dye binding. *Anal. Biochem.* 72:248-254.

Buss, J.E., Kudlow, J.E., Lazar, C.S., and Gill, G.N. (1982) Altered EGF-stimulated protein kinase activity in variant A431 cells with altered growth responses to EGF. Proc.Natl.Acad.Sci.USA, 79:2574-2578.

Carpenter, G., and Cohen, S. (1976) Human epidermal growth factor and the proliferation of human fibroblasts. J.Cell.Physiol., 88:227-238.

Carpenter, G., and Cohen, S. (1990) Epidermal growth factor. J.Biol.Chem., 265:7709-7712.

Carpenter, G., Nishibe, S., Todderud, G., Ball, R., and Wahl, M. (1991) Activation of second messenger pathways by epidermal growth factor. In: Origins of Human Cancer: A comprehensive Review. Brugge, J., Curran, T., Harlow, E., and McCormick, F., eds. Cold spring Harbor laboratory Press, New York, 255-263.

Casey, G., Lo-Hsueh, M., Lopez, M.E., Vogelstein, B., and Stanbridge, E.J. (1991) Growth-suppression of human breast cancer cells by the introduction of a wild-type p53 gene. Oncogene, 6:1791-1797.

Chang, L.M.S., and Bollum, F.J. (1971) Deoxynucleotide polymerising enzymes of calf thymus gland. J.Biol.Chem., 246:909-916.

Chang, F., and Herskowitz, I. (1990) Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. Cell, 63:999-1011.

Chang, L-S., Pater, M.M., Hutchinson, N.I., and Mayorca, G.D. (1984) Transformation by purified early genes of simian virus 40. Virology, 133:341-353.

Chardin, P., Camonis, J.H., Gale, N.W., Aelst, L.V., Schlessinger, J., Wigler, M.H., and Bar-Sagi, D. (1993) Human Sos1: A guanine nucleotide exchange factor for Ras that binds to GRB2. Science, 260:1338-1343.

Chen, J.-Y., Funk, W.D., Wright, W.E., Shay, J.W., and Minna, J.D. (1993b) Heterogeneity of transcriptional activity of mutant p53 proteins and p53 DNA target sequences. Oncogene, 8:2159-2166.

Chen, P., Scully, P., Shew, J., Wang, J.Y.J., and Lee, W. (1989) Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell, 58:1193-1198.

- Chen, P.-L., Chen, Y., Bookstein, R., and Lee, W.-H.** (1990) Genetic mechanisms of tumor suppression by the human p53 gene. Science, **250**:1576-1580.
- Chen, X., Farmer, G., Zhu, H., Prywes, R., and Prives, C.** (1993a) Cooperative DNA binding of p53 with TFIID (TBP): a possible mechanism for transcriptional activation. Genes Dev., **7**:1837-1849.
- Chen, Y., Chen, P.-L., Arnaiz, N., Goodrich, D., and Lee, W.-H.** (1991) Expression of wild-type p53 in human A673 cells suppress tumorigenicity but not growth rate. Oncogene, **6**:1799-1805.
- Chin, K.-V., Ueda, K., Pastan, I., and Gottesman, M.M.** (1992) Modulation of activity of the promoter of the MDR1 gene by Ras and p53. Science, **255**:459-464.
- Chodish, L.A., Olesen, J., Hahn, S., Baldwin, A.S., Guarente, L., and Sharp, P.A.** (1988) A yeast and a human CCAAT-binding proteins have heterologous subunits that are functionally interchangeable. Cell, **53**:25-35.
- Church, J.G., Mills, G.B., and Buick, R.N.** (1989) Activation of Na⁺/H⁺ antiport is not required for epidermal growth factor-dependent gene expression, growth inhibition or proliferation in human breast cancer cells. Biochem.J., **257**:151-157.
- Church, J.G., Richardson, V.J., and Lockwood, A.G.** (1992) Atypical receptor-mediated signal transduction events in the EGF-dependent growth-inhibited cell line, MDA-468. J.Cell.Physiol., **153**:373-380.
- Church, J.G. and Buick, R.N.** (1988) G-protein-mediated epidermal growth factor signal transduction in a human breast cancer cell line. Evidence for two intracellular pathways distinguishable by pertussis toxin. J.Biol.Chem., **263**:4242-4246.
- Clepham, D.E., and Neer, E.J.** (1993) New roles for G-protein $\beta\gamma$ -dimers in transmembrane signalling. Nature (London), **365**:403-406.
- Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L., and Wyllie, A.H.** (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature (London), **362**:849-852.
- Cohen, P.** (1992) Signal-integration at the level of protein kinases, protein phosphatases and their substrates. TIBS, **17**:408-413.

- Cohen, S., Carpenter, G., and King, L.Jr. (1980) Epidermal growth factor-receptor-protein kinase interactions: Copurification of receptor and epidermal growth factor enhanced phosphorylation activity. J.Biol.Chem., **255**: 4835-4842.
- Collins, S., Caron, M.G., and Lefkowitz, R.J. (1992) From ligand binding to gene expression: new insights into the regulation of G-protein-coupled receptors. TIBS, **17**:37-39.
- Cook, S.J., and Wakelam, M.J.O. (1992) Phospholipases C and D in mitogenic signal transduction. Rev.Physiol.Biochem. Pharmacol., **119**:13-45.
- Cooper, J.A., and Whyte, P. (1989) RB and the cell cycle: entrance or exit? Cell, **58**:1009-1011.
- Coppock, D.L. and Pardee, A.B. (1987) Control of thymidine kinase mRNA during the cell cycle. Mol.Cell.Biol., **7**:2925-2932.
- Coughlin, S.R., Escobedo, J.A., and Williams, L.T. (1989) Role of phosphoinositol kinase in PDGF receptor signal transduction. Science, **243**:1191-1194.
- Crews, C.M., and Erikson, R.L. (1993) Extracellular signals and reversible protein phosphorylation: What to mek of it all. Cell, **74**:215-217.
- Crissman, H.A., and Tobey, R.A. (1974) Cell cycle analysis in 15 minutes. Nature (London), **184**: 1297-1298.
- Cross, F., Roberts, J., and Weintraub, H. (1989) Simple and complex cell cycles. Annu.Rev.Cell Biol., **5**:341-395.
- Crouch, M.F., Belford, D.A., Milburn, P.J., and Hendry, I.A. (1990) Pertussis toxin inhibits EGF-, phorbol ester-, and insulin-stimulated DNA synthesis in BALB/c3T3 cells. Evidence for postreceptor activation of Gi α . Biochem.Biophys.Res. Commun., **167**:1369-1376.
- Dalton, S. (1992) Cell cycle regulation of human cdc2 gene. EMBO J., **11**:1797-1804.
- Darnell, J., Lodish, H., and Baltimore, D. (1986) Viruses as agents of transformation: Oncogenes. In: Molecular Cell Biology, Scientific American Books, 1059-1060.
- Deb, S., Jackson, C.T., Subler, M.A., and Martin, D.W. (1992) Modulation of cellular and viral promoters by mutant human p53 proteins found in tumor cells. J.Virol.,

66:6164-6170.

DeCaprio, J.A., Ludlow, J.W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, Huang, C., and Livingston, D.M. (1989) The product of retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell, 58:1085-1095.

DeClue, J.E., Zhang, K., Redford, P., Vass, W.C., and Lowy, D.R. (1991) Suppression of src transformation by overexpression of full-length GTPase activating protein (GAP) or the GAP C-terminus. Mol.Cell.Biol., 11:2819-2825.

Deppert, W., Buschhausen-Denker, G., Patschinsky, T., and Steinmeyer, K (1990) Cell cycle control of p53 in normal(3T3) and chemically transformed (Meth A) mouse cells. II.Requirement for cell cycle progression. Oncogene, 5:1701-1706.

Di Fiore, P.P., Pierce, J.H., Fleming, T.P., Hazan, R., Ullrich, A., King, C.R., Schlessinger, J., and Aaronson, S.A. (1987) Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. Cell, 51:1063-1070.

Diller, L., Kassel, J., Nelson, C.E., Gryka, M.A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S.J., Vogelstein, B., and Friend, S.H. (1990) p53 functions as a cell cycle control protein in osteosarcomas. Mol.Cell.Biol., 10:5772-5781.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery Jr, C.A., Butel, J.S., and Bradley, A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature (London), 356:215-221.

Donehower, L.A., and Bradley, A. (1993) The tumor suppressor p53. Biochem.Biophys.Acta., 1155:181-205.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Duvare, S.G., Robbins, K.C., Aaronson, S.A., and Antoniadis, H.N. (1983) Simian sarcoma virus 'onc' gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science, 221:275-276.

Downing, J.R., Shurtleff, S.A., and Sherr, C.J. (1991) Peptide antisera to human colony stimulating factor 1 receptor detect ligand induced conformational changes and a binding sites for phosphatidylinositol 3'-kinase. Mol.Cell.Biol., 11:2489-2495.

- Downing, J.R., Margolis, B.L., Zilberstein, A., Ashmun, R.A., Ullrich, A., Sherr, C.J., and Schlessinger, J. (1989) Phospholipase C- γ , a substrate for PDGF receptor kinase is not phosphorylated on tyrosine during the mitogenic response to CSF 1. EMBO J., 8:3345-3350.
- Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and Waterford, M. (1984) Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature (London), 307:521-527.
- Downward, J., Graves, J.D., Warne, P.H., Rayter, S., and Cantrell, D.A. (1990) Stimulation of p21^{ras} upon T-cell activation. Nature (London), 346:719-723.
- Draetta, G. (1990) Cell cycle control in eukaryotes: molecular mechanisms of cdc2 activation. TIBS, 15:378-383.
- Draetta, G., and Beach, D. (1988) Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. Cell, 54:17-26.
- Dutta, A., Ruppert, J.M., Aster, J.C., and Winchester, E. (1993) Inhibition of DNA replication factor RPA by p53. Nature (London), 365:79-82.
- Dyson, N., Howley, P.M., Munger, K., and Harlow, E. (1989) The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science, 243:934-936.
- Egan, S.E., Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M., and Weinberg, R.A. (1993) Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature (London), 363:45-51.
- El-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W., and Vogelstein, B. (1992) Definition of a consensus binding site for p53. Nature Genetics, 1:41-49.
- Elion, E.A., Grisafi, P.L., and Fink, G.R. (1990) FUS3 encodes a cdc2+/CDC28-related kinase required for the transition from mitosis to conjugation. Cell, 60:649-664.
- Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O., and Oren, M. (1989) Wild-type p53 can inhibit oncogene-mediated focus formation. Proc.Natl.Acad.Sci. USA, 86:8763-8767.

Eliyahu, D., Goldfinger, N., Pinhasi-Kimhi, O., Shaulsky, G., Shurnik, Y., Aral, N., Rotter, V., and Oren, M. (1988) Meth A fibrosarcoma cells express two transforming mutant p53 species. Oncogene, 3:313-321.

Eliyahu, D., Raz, A., Gruss, P., Givol, D., and Oren, M. (1984) Participation of p53 cellular tumor antigen in transformation of normal embryonic cells. Nature (London), 312:646-649.

Ellis, C., Moran, M., McCormick, F., and Pawson, T. (1990) Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature (London), 343:377-381.

Escobedo, J.A., Navankasattusas, S., Kavanaugh, M., Milfay, D., Fried, V.A., and Williams, L.T. (1991) cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF- β receptor. Cell, 65:75-82.

Fairman, M.P., and Stillman, B. (1988) Cellular factors required for multiple stages of SV40 DNA replication *in vitro*. EMBO J., 7:1211-1218.

Fang, F., and Newport, J.W. (1991) Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. Cell, 66:731-742.

Farmer, G., Bargonetti, J., Zhu, H., Friedman, P., Prywes, R., and Prives, C. (1992) Wild-type p53 activates transcription *in vitro*. Nature (London), 358:83-88.

Fields, S. (1990) Pheromone response in yeast. TIBS, 15:270-273.

Fields, S., and Jang, S.K. (1990) Presence of a potent transcription activating sequence in the p53 protein. Science, 249:1046-1048.

Filmus, J., Pollack, M.N., Cailleau, R., and Buick, R.N. (1985a) MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. Biochem.Biophys.Res.Comm., 128:898-905.

Filmus, J., Pollack, M.N., Cairncross, J.G., and Buick, R.N. (1985b) Amplified, overexpressed and rearranged epidermal growth factor gene in a human astrocytoma cell line. Biochem.Biophys.Res.Comm., 131:207-215.

Filmus, J., Benchimol, S., and Buick, R.N. (1987a) Comparative analysis of the involvement of p53, c-myc and c-fos in epidermal growth factor-mediated signal transduction. Exp.Cell Res., 169:554-559.

Filmus, J., Trent, J.M., Pollack, M.N., and Buick, R.N. (1987b) Epidermal growth factor gene-amplified MDA-468 breast cancer cells and its nonamplified variants. Mol.Cell.Biol., 7:251-257.

Finlay, C.A., Hinds, P.W., and Levine, A.J. (1989) The p53 proto-oncogene can act as a suppressor of transformation. Cell, 57:1083-193.

Fiscella, M., Ullrich, S.J., Zambrano, N., Shields, M.T., Lin, D., Lees-Miller, S.P., Anderson, C.W., Mercer, W.E. and Appella, E. (1993) Mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit the cell cycle progression. Oncogene, 8:1519-1528.

Foord, O.S., Bhattacharya, P., Reich, Z., and Rotter, V. (1991) A DNA binding domain is contained in the C-terminus of wild-type p53 protein. Nucl.Acids Res., 19: 5191-5198.

Foxwell, B.M.J., Barrett, K., and Feldmann, M. (1992) Cytokine receptors: structure and signal transduction. Clin. exp. immunol., 90:161-169.

Freshney, R.I. (1987) Physical methods of cell separation. In: Culture of Animal Cells: A Manual of Basic Technique, Second edition, Alan R. Liss, Inc., New York. 155-168.

Fritsche, M., Haessler, C., and Brandner, G. (1993) Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene, 8:307-318.

Furk, W.D., Pak, D.T., Karas, R.H., Wright, R.H., and Shay, J.W. (1992) A transcriptionally active DNA-binding site for human p53 protein complexes. Mol.Cell.Biol., 12:2866-2871.

Gale, N.W., Kaplan, S., Lowenstein, E.J., Schlessinger, J., and Bar-Sagi, D. (1993) Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. Nature (London), 363:88-92.

Gallie, B.L., Squire, J.A., Goddard, A., Gunn, J.M., Canton, M., Hinton, D., Zhu, X.P., Phillips, R.A. (1990) Mechanisms of oncogenesis in retinoblastoma. Lab.Invest.,

62:394-408.

Gannon, J.V., Greaves, R., Iggo, R., and Lane, D.P. (1990) Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J. 9:1595-1602.

Gardner, A.M., Vaillancourt, R.R., and Johnson, G.L. (1993) Activation of mitogen activated protein kinase/extracellular signal-regulated kinase kinase by G protein and tyrosine kinase oncoprotein. J.Biol.Chem. 268:17896-17901.

Gibbs, J.B. (1991) GAP & Farnesyl protein transferase: Potential anti-Ras targets. In: Origins of Human Cancer: A comprehensive Review. Brugge, J., Curran, T., Harlow, E., and McCormick, F., eds. Cold spring Harbor laboratory Press, New York, 319-326.

Gibbs, J.B., Marshall, M.S., Scolnick, E.M., Dixon, R.A.F., and Vogel, U.S. (1990) Modulation of guanine nucleotide bound to Ras in NIH 3T3 cells by oncogenes, growth factors, and the GTPase activating μ -protein (GAP). J.Biol.Chem. 265: 20437-20442.

Gill, G., and Lazar, C. (1981) Increased phosphotyrosine contents and inhibition of proliferation in EGF-treated A431 cells. Nature (London), 293:305-307.

Gill, G.N., Bertics, P.J., Thompson, D.M., Weber, W., and Cochet, C. (1985) Structure and regulation of the epidermal growth factor receptor. In Cancer cells, Feramisco, J., Ozanne, B., and Stiles, C.(ed) Cold spring Harbor laboratory, Cold spring harbor, N.Y., 3:11-18.

Gill, G.N., Kawamoto, T., Cochet, C., Le, A., Sato, D., Masui, H., MacLeod, C., and Mendelsohn, J.(1984) Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor stimulated tyrosine protein kinase activity. J.Biol.Chem. 259: 7755-7760.

Gill, G.N., Buss, J.E., Lazar, C.S., Lifshitz, A., and Cooper, J.A. (1982) Role of epidermal growth factor-stimulated protein kinase in control of proliferation of A431 cells. J.Cell.Biochem. 19:249-257.

Gilman, A.G. (1987) G proteins: Transducers of receptor-generated signals. Annu.Rev.Biochem. 56:615-649.

Ginsberg, D., Oren, M., Yaniv, M., and Piette, J. (1990) Protein binding elements in

the promoter region of the mouse p53 gene. Oncogene, 5:1285-1290.

Ginsberg, D., Michael-Michalovitz, D., Ginsberg, D., and Oren, M. (1991a) Induction of growth arrest by a temperature-sensitive p53 mutant is correlated with increased nuclear localization and decreased stability of the protein. Mol.Cell.Biol., 11:582-585.

Ginsberg, D., Mechta, F., Yaniv, M., and Oren, M. (1991b) wild-type p53 can down-modulate the activity of various promoters. Proc.Natl.Acad.Sci.USA., 88:9979-9983.

Giordano, A., Lee, J.H., Scheppler, J.A., Herrmann, C., Harlow, E., Deuschle, U., Beach, D., and Franza, Jr.,B.R. (1991) Cell cycle regulation of histone H1 kinase activity associated with the adenoviral protein E1A. Science, 253:1271-1275.

Goodrich, D.W., Wang, N.P., Qian, Y., Lee, E.Y.-H.P. and Lee, W. (1991) The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell, 67:293-302.

Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982) Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol.Cell.Biol., 2:1044-1051.

Goulian, M., Goulian, S.H., Codd, E.E., and Blumenfeld, A.Z. (1973) Properties of oligonucleotides that determine priming activity with *Escherichia Coli* deoxyribonucleic acid polymerase I. Biochemistry, 12:2893-2901.

Graff, J.M., Gordon, J.I., and Blackshear, P.J. (1989) Myristoylated and nonmyristoylated forms of a protein are phosphorylated by protein kinase C. Science, 246:503-506.

Gusterson, B.A. (1992) Identification and interpretation of epidermal growth factor and c-erbB-2 overexpression. Eur.J.Cancer, 28:263-267.

Halazonetis, T.D., Davis, L.J., and Kandil, A.N. (1993) Wild-type p53 adopts a 'mutant'-like conformation when bound to DNA. EMBO J., 12:1021-1028.

Hall, A. (1990) ras and GAP - Who's controlling whom? Cell, 61:921-923.

Hall, A. (1992) Signal transduction through small GTPases-A tale of two GAPs. Cell, 69:389-391.

- Hall, F.L., Braun, R.K., Mihara, K., Iino, Y.T., Berndt, N., Carbonaro-Hall, D.A., and Vulliamy, P.R. (1991) Characterization of the cytoplasmic proline-directed protein kinase in proliferative cells and tissues as a heterodimer comprised of p34^{cdc2} and p58^{cyclin A}. J.Biol.Chem., 266:17430-17440.
- Hall, P.A., McKee, P.H., Menage, H du P., Dover, R., and Lane, D.P. (1993a) High levels of p53 protein in UV-irradiated normal human skin. Oncogene, 8:203-207.
- Hall, F.L., Williams, R.T., Wu, L., Wu, F., Carbonaro-Hall, D.A., Harper, J.W., and Warburton, D. (1993b) Two potentially oncogenic cyclins, cyclin A and cyclin D1, share common properties of subunit configuration, tyrosine phosphorylation and physical association with the Rb protein. Oncogene, 8:1377-1384.
- Hameel, P.A., Gallie, B.L., and Phillips, R.A. (1992) The retinoblastoma protein and cell cycle regulation. TIG, 8: 180-185.
- Han, M., Golden, A., Han, Y., and Sternberg, P.W. (1993) *C.elegans* lin-45 raf gene participates in let-60 ras-stimulated vulval differentiation. Nature (London), 363:133-139.
- Harlow, E., Crawford, L., Pim, D.C., and Williamson, N.M. (1981) Monoclonal antibodies specific for simian virus 40 tumor antigens. J.Virol., 39:861-869.
- Harlow, E. (1992) For our eyes only. Nature (London), 359:270-271.
- Hartwell, L.H., and Weinert, T.A. (1989) Checkpoints: Controls that ensure the order of cell cycle events. Science, 246:629-634.
- Harvey, M., Sands, A.T., Weiss, R.S., Hegi, M.E., Wiseman, R.W., Pantazis, P., Giovannella, B.C., Tainsky, M.A., Bradley, A., and Donehower, L.A. (1993) In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. Oncogene, 8:2437-2467.
- Heldin, C.H., and Westermark, B. (1989) Growth factors as transforming proteins. Eur.J.Biochem., 184:487-496.
- Hepler, J.R., and Gilman, A.G. (1992) G proteins. TIBS, 17:383-387.

Herskowitz, I., and Chang, F. (1991) Arrest of yeast cell cycle by a negative growth factor: Antagonism of a G1 cyclin (CLN2) by FAR1. In: Origins of Human Cancer: A comprehensive Review. Brugge, J., Curran, T., Harlow, E., and McCormick, F., eds. Cold spring Harbor laboratory Press, New York, 35-43.

Hinds, P., Finlay, C., and Levine, A.J. (1989) Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J.Virol., **63**:739-746.

Hinds, P.W., Finlay, C.A., Quartin, R.S., Baker, S.J., Fearon, E.R., Vogelstein, B., and Levine, A. (1990) Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the 'hot spot' mutant phenotypes. Cell Growth Different., **1**:571-580.

Hirschhorn, R.R., Marashi, F., Baserga, R., Stein, J., and Stein, G. (1984) Expression of histone genes in a G1-specific temperature-sensitive mutant of the cell cycle. Biochemistry, **23**:3731-3735.

Ho, Z., Brinton, B.T., Greenblatt, J., Hassel, J.A., and Ingles, C.J. (1993) The transactivator proteins VP16 and GAL4 bind replication factor A. Cell., **73**:1223-1232.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991) p53 mutations in human cancers. Science, **253**:49-53.

Hoppe-Seyler, F., and Butz, K. (1993) Repression of endogenous p53 transactivation function in HeLa cervical carcinoma cells by human papillomavirus type 16 E6, human mdm2 and mutant p53. J.Virol., **67**:3111-3117.

Howard, A., and Pelc, S.R. (1951) Nuclear incorporation of P³² as demonstrated by autoradiographs. Exp.Cell Res., **2**:178-187.

Huang, H.-J., Yee, J.-K., Shew, J.-Y., Chen, P.-L., Bookstein, R., Friedmann, T., Lee, E.Y.-H.P., Lee, W.-H. (1988) Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Nature (London), **242**:1563-1566.

Huberman, J.A. (1981) New views of the biochemistry of eucaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase α . Cell, **23**:647-648.

Hudziak, R.M., Schlessinger, J., and Ullrich, A. (1987) Increased expression of the putative growth factor receptor p185^{HER2} causes transformation and tumorigenesis of NIH3T3 cells. Proc.Natl.Acad.Sci.USA., **84**:7159-7163.

- Hunter, T., and Pines, J. (1991) Cyclins and cancer. Cell, 66:1071-1074.
- Hunter, T. and Cooper, J.A. (1985) Protein-tyrosine kinases. Annu.Rev.Biochem. 53:897-930.
- Hupp, T.R., Meek, D.W., Midgley, C.A., and Lane, D.P. (1992) Regulation of the specific DNA binding function of p53. Cell, 71:875-886.
- Hurt, M.M., Pandey, N.B., and Marzuff, W.F. (1989) A region in the coding sequence is required for high level expression of murine histone H3 gene. Proc.Natl.Acad.Sci.USA, 86:4450-4454.
- Imamura, K., Dianoux, A., Nakamura, T., and Kufe, D. (1990) Colony-stimulating factor activates protein kinase C in human monocytes. EMBO J., 9:2423-2429.
- Izant, J.G., and Weintraub, H. (1984) Inhibition of thymidine kinase gene expression by anti-sense RNA: A molecular approach to genetic analysis. Cell, 36:1007-1015.
- Jackson, P., Bos, E., and Braithwaite, A.W. (1993) Wild-type mouse p53 down-regulates transcription from different virus enhancer/promoters. Oncogene, 8:589-597.
- Jaskulski, D., Gatti, C., Travali, S., Calabretta, B., and Baserga, R. (1988) Regulation of proliferating nuclear antigen cyclin and thymidine kinase mRNA levels by growth factors. J.Biol.Chem., 263:10175-10179.
- Johnson, P., Gray, D., Mowat, M., and Benchimol, S. (1991) Expression of wild-type p53 is not compatible with continued growth of p53-negative tumor cells. Mol.Cell.Biol., 11:1-11.
- Johnson, M.T., Read, B.A., Monku, A.M., Papas, G., and Johnson, B.A. (1986) A convenient, new method for desalting, deproteinising, and concentrating DNA and RNA. Biotechniques, 4:64-70.
- Kaelin, W.G. Jr., Pallas, D.C., DeCaprio, J.A., Kaye, F.J., and Livingston, D.M. (1991) Cellular proteins that can interact specifically with the retinoblastoma susceptibility gene product. In: Origins of Human Cancer: A comprehensive Review. Brugge, J., Curran, T., Harlow, E., and McCormick, F., eds. Cold spring Harbor laboratory Press, New York, 423-429.
- Karin, M., and Smolnik, T. (1992) Control of transcription factors by signal transduction pathways: the beginning or the end. TIBS, 17:418-422.

Kastan, M.B., Zhan, Q., El-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W., Plunkett, B.S., Vogelstein, B., and Fornace, Jr., A.J. (1992) A mammalian cell cycle check point pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell, **71**:587-597.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. (1991) Participation of p53 protein in the cellular response to DNA damage. Cancer Research, **51**:6304-6311.

Kato, J-Y., Matsushime, H., Heibert, S.W., Ewen, M.E., and Sherr, C.J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev., **7**:331-342.

Kawamoto, T., Sato, J.D., Le, A., Polikoff, J., Sato, G.H., and Mendelsohn, J. (1983) Growth stimulation of A431 cells by epidermal growth factor: Identification of high affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. Proc.Natl.Acad.Sci.USA, **80**:1337-1341.

Kawamoto, T., Mendelsohn, J., Le, A., Sato, G.H., Lazor, C.S., and Gill, G.N. (1984) Relation of epidermal growth factor receptor concentration to growth of human epidermoid carcinoma A431 cells. J.Biol.Chem., **259**:7761-7766.

Kelly, K., Cochran, B.H., Stiles, C.D., and Leder, P. (1983) Cell specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell, **35**:603-610.

Kern, S.E., Kinzler, K.W., Baker, S.J., Nigro, J.M., Rotter, V., Levine, A.J., Friedman, P., Prives, C., and Vogelstein, B. (1991a) Mutant p53 proteins bind DNA abnormally in vitro. Oncogene, **6**:131-136.

Kern, S.E., Kinzler, K.W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelstein, B. (1991b) Identification of p53 as a sequence specific DNA-binding protein. Science, **252**:1708-1711.

Kern, S.E., Pietenpol, J.A., Thiagalingam, S., Seymour, A., Kinzler, K.W., and Vogelstein, B. (1992) Oncogenic forms of p53 inhibit p53-regulated gene expression. Science, **256**:827-830.

Kim, S-J., Wagner, S., Liu, F., O'Reilly, M.A., Robbins, P.D. and Green, M.R. (1992) Retinoblastoma gene product activates expression of the human TGF- β 2 gene through transcription factor ATF-2. Nature (London), **358**:331-334.

- Kley, N., Chung, R.Y., Fay, S., Loeffler, J.P., and Seizinger, B.R. (1992) Repression of the basal c-fos promoter by wild-type p53. Nucl.Acid.Res., 20:4083-4087.
- Koch, C.A., Anderson, D., Moran, M.F., Ellis, C., and Pawson, T. (1991) SH2 and SH3 domains: Elements that control interactions of cytoplasmic signalling proteins. Science, 252:668-674.
- Kolch, W., Heidecker, G., Lloyd, P., and Rapp, U.R. (1991) Raf-1 protein kinase is required for the growth of induced NIH 3T3 cells. Nature (London), 349:426-428.
- Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahldl, H., Mischak, H., Finkenzellers, G., Marme, D., and Rapp, U.R. (1993) Protein-kinase C γ activates RAF-1 by direct phosphorylation. Nature (London), 364:249-252.
- Krupinski, J., Rajaram, R., Lakonishok, M., Benovic, J.L., and Cerlone, R.A. (1988) Insulin-dependent phosphorylation of GTP-binding proteins in phospholipid vesicles. J.Biol.Chem., 263:12333-12341.
- Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V., and Kastan, M.B. (1992) Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc.Natl.Acad.Sci.USA., 89:7491-7495.
- Kypta, R.M., Goldberg, Y., Ulug, E.T., and Courtneidge, S.A. (1990) Association between the PDGF receptor and members of the src family tyrosine kinases. Cell, 62:481-492.
- Laiho, M., DeCaprio, J.A., Ludlow, J.W., Livingston, D.M., and Massague, J. (1990) Growth inhibition by TGF- β linked to suppression of retinoblastoma protein phosphorylation. Cell, 62: 175-185.
- Lalande, M. (1990) A reversible arrest point in the late G1 phase of the mammalian cell cycle. Exp.Cell Res., 186: 332-339.
- Lane, D.P. and Beach, D. (1990) p53: Oncogene or anti-oncogene? Genes Dev., 4:1-8.
- Lane, D.P. (1992) p53, guardian of the genome. Nature (London), 358:15-16. (News and Views).
- Lane, D.P., and Crawford, L.V. (1979) T antigen is bound to a host protein in SV40-transformed cells. Nature (London), 278:245-249.

Larner, A.C., David, M., Feldman, G.M., Igarashi, K-I., Hackett, R.H., Webb, D.S.A., Sweitzer, S.M., Petricoin III, E.F., Finbloom, D. (1993) Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. Science, **261**:1730-1733.

Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. (1991) Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell, **66**:305-315.

Lau, L.F., and Nathans, D. (1987) Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: Coordinated regulation with *c-fos* or *c-myc*. Proc.Natl.Acad.Sci.USA, **84**:1182-1186.

Lavigne, A., Maltby, V., Mock, D., Rossant, J., Pawson, T., and Bernstein, A. (1989) High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. Mol.Cell.Biol., **9**:3982-3991.

Lechner, M.S., Mack, D.H., Finicle, A.B., Crook, T., Vousden, K.H., and Laimins, L.A. (1992) Human papillomavirus E6 proteins bind p53 *in vivo* and abrogate p53-mediated repression of transcription. EMBO J., **11**:3045-3052.

Lee, E.Y.-H.P., Chang, C.-Y., Hu, N., Wang, Y.J., Lai, C.-C., Herrup, K., Lee, W.-H., and Bradley, A. (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature (London), **359**:288-294.

Lee, W.-H., and Lee, E.Y.-H.P. (1991) The retinoblastoma gene: A prototypic model for tumor suppression. In Origins of Human Cancer: A comprehensive Review. Brugge, J., Curran, T., Harlow, E., and McCormick, F., eds. Cold spring Harbor laboratory Press, New York, 413-421.

Lees-Miller, S.P., Chen, Y.R., and Anderson, C.W. (1990) Human cells contain a DNA-activated protein kinase that phosphorylates simian virus 40 T antigen, mouse p53 and the human Ku autoantigen. Mol.Cell.Biol., **10**:6472-6481.

Lefkowitz, R.J. (1993) G protein-coupled receptor kinases. Cell, **74**:409-412.

Levine, A.J., Momand, J., and Finlay, C.A. (1991) The p53 tumour suppressor gene. Nature (London), **351**:453-456.

Lewin, B. (1990) Driving the cell cycle: M phase kinase, its partners, and substrates. Cell, **61**:743-752.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993) Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature (London), **363**:85-88.

Liang, P., and Pardee, A.B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science, **257**:967-971.

Lifshitz, A., Lazar, C.S., Duss, J.E., and Gill, G.N. (1983) Analysis of morphology and receptor metabolism in clonal variant A431 cells with differing growth responses to epidermal growth factor. J.Cell.Physiol., **115**:235-242.

Lin, D., Shields, M., Uilrich, S.J., and Appella, E. (1992) Growth arrest induced by wild-type p53 protein blocks cells prior to or near the restriction point in late G1 phase. Proc.Natl.Acad.Sci.USA, **89**:9210-9214.

Linzler, D.I.H., and Levine, A.J. (1979) Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40 transfected cells and uninfected embryonal carcinoma cells. Cell, **17**:43-52.

Liscovitch, M. (1992) Crosstalk among multiple signal-activated phospholipases. TIBS, **17**:393-399.

Liu, Y.C., Marraccino, R.L., Keng, P.C., Bambara, R.A., Lord, E.M., Chow, W.G., and Zain, S.B. (1989) Requirement for proliferating cell nuclear antigen expression during stages of the chinese hamster ovary cell cycle. Biochemistry, **18**:2967-2974

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A., and Jacks, T. (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature (London), **362**:847-849.

Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Uilrich, A., Skolnik, Y., Bar-Sagi, D., and Schlessinger, J. (1992) The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signalling. Cell, **70**:431-442.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J.Biol.Chem., **193**:265-275.

Lowy, D.R., Zhang, K., De Clue, J.E., and Williams, B.M. (1991) Regulation of p21^{ras} activity. TIG, **7**:346-351.

Luckow, B., and Schutz, G. (1987) CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucl. Acid. Res., 15:5490.

Ludlow, J.W., DeCaprio, J.A., Huang, C.-M., Lee, W.-H., Paucha, E., and Livingston, D.M. (1989) SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell, 56:57-65.

MacLeod, C.L., Luk, A., Castagnola, J., Cronin, M., and Mendelsohn, J. (1986) EGF induces cell cycle arrest of A431 human epidermoid carcinoma cells. J.Cell.Physiol., 127:175-182.

Margolis, B., Rhee, S.G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., and Schlessinger, J. (1989) EGF induces phosphorylation of phospholipase C-II: A potential mechanism for EGF receptor signalling. Cell, 57:1101-1107.

Margolis, B., Zilberstein, A., Franks, C., Felder, S., Kreamer, S., Ullrich, A., Rhee, S.G., Skorecki, K., Schlessinger, J. (1990) Effect of phospholipase C- γ overexpression on PDGF-induced second messengers and mitogenesis. Science, 248:607-610.

Marshall, C.J. (1991) How does p21^{ras} transform cells? TIG, 7:91-95.

Martinez, J., Georgoff, I., Martinez, J., and Levine, A.J. (1991) Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Genes Dev., 5:151-159.

Matuoka, K., Fukami, K., Naakanishi, O., Kawai, S., and Takenawa, T. (1988) Mitogenesis in response to PDGF and bombesin abolished by microinjection of antibody to PIP2. Science, 239:640-643.

Maxwell, S.A., and Roth, J.A. (1993) Binding of cellular proteins to a conformational domain of tumor suppressor protein p53. Oncogene, 8:3421-3426.

McCormick, F. (1990) GAP as ras effector or negative regulator? Molecular Carcinogenesis, 3:185-187.

Meek, D.W., Simon, S., Kikkawa, U., and Eckhart, W. (1990) The p53 tumour suppressor protein is phosphorylated at serine 389 by casein kinase II. EMBO J., 9:3253-3260.

- Mercer, W.E., Avignolo, C., and Baserga, R. (1984) Role of the p53 protein in cell proliferation as studied by microinjection of monoclonal antibodies. Mol.Cell.Biol., 4:276-281.
- Mercer, W.E., Shields, M.T., Amin, M., Salve, G.J., Appella, E., Romano, J.W., and Ulirsch, S.J. (1990) Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc.Natl.Acad.Sci.USA, 87:6166-6170.
- Mercer, W.E., Shields, M.T., Lin, D., Appella, E., and Ulirsch, S.J. (1991) Growth suppression induced by wild-type p53 protein is accompanied by selective down-regulation of proliferating-cell nuclear antigen expression. Proc. Natl. Acad.Sci.USA, 88:1958-1962.
- Merlino, G.T., Xu, Y.H., Ishii, S., Clark, A.J.L., Semba, K., Toyoshima, K., Yamamoto, T., and Pastan, I. (1984) Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. Science, 224: 417-419.
- Michalovitz, D., Halevy, O., and Oren, M. (1991) p53 mutations: gains or losses? J.Cell.Biochem., 45:22-29.
- Miller, C.W., Imai, Y., Aslo, A., Li, L., and Koeffler, P. (1992) Sublocalization of transcriptional activation domain of p53. Proc.American.Associ.Cancer.Res., 386 (Abstract)
- Miller, C.W., Chumakov, A., Said, J., Chen, D.L., Aslo, A., and Koeffler, P.H. (1993) Mutant p53 proteins have diverse abilities to oligomerize and activate transcription. Oncogene, 8:1815-1824.
- Milne, D.M., Palmer, R.H., and Meek, D.W. (1992) Mutation of the casein kinase II phosphorylation site abolishes the anti-proliferative activity of p53. Nucl.Acid.Res., 20:5565-5570.
- Milner, J., Cook, A., and Sheldon, M. (1987) A new anti-p53 monoclonal antibody, previously reported to be directed against the large T antigen of simian virus 40. Oncogene, 1:453-455.
- Milner, J., Cook, A., and Mason, J. (1990) p53 is associated with p34^{cdc2} in transformed cells. EMBO J., 9:2885-2889.

- Milner, J. and Watson, J.V.** (1990) Addition of fresh medium induces cell cycle and conformation changes in p53, a tumour suppressor protein. Oncogene, 5:1683-1690.
- Milner, J.** (1991) The role of p53 in the normal control of cell proliferation. Curr.Op.Cell Biol., 3, 282-286.
- Milner, J., Medcalf, E.A., and Cook, A.C.** (1991) Tumor suppressor p53: Analysis of wild-type and mutant p53 complexes. Mol.Cell.Biol., 11:12-19.
- Milner, J. and Medcalf, E.A.** (1991) Co-translation of activated mutant p53 with wild type drives the wild-type p53 protein into the mutant conformation. Cell, 65:765-774.
- Milner, J., Chan, Y.S., Medcalf, E.A., Wang, Y., and Eckhart, W.** (1993) Partially transformed T3T3 cells express high levels of mutant p53 in the 'wild-type' immunoreactive form with defective oligomerization. Oncogene, 8:2001-2008.
- Molloy, C.J., Bottaro, D.P., Fleming, T.P., Marshall, M.S., Gibbs, J.B., and Aaronson, S.A.** (1989) PDGF induction of tyrosine phosphorylation of GTPase activating protein. Nature (London), 342:711-714.
- Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J.** (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53 mediated transactivation. Cell, 69:1237-1245.
- Montminy, M.** (1993) Trying on a new pair of SH2s. Science, 261:1694-1695.
- Moran, M., Polakis, P.G., McCormick, F., Pawson, T., and Ellis, C.** (1991) Protein-tyrosine kinase regulates the phosphorylation, protein interactions, subcellular distribution and activity of p21^{ras} GTPase-activating protein. Mol.Cell.Biol., 11:1804-1812.
- Morrison, D.K., Kaplan, D.R., Escobedo, J.A., Rapp, U.R., Roberts, T.M., and Williams, L.T.** (1989) Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation of the PDGF β -receptor. Cell, 58:649-657.
- Moses, H.L., Yang, E.Y., and Pietenpol, J.A.** (1990) TGF- β stimulation and inhibition of cell proliferation: New mechanistic insights. Cell, 63:245-247.
- Mosner, J., and Deppert, W.** (1992) Conformational analysis of p53 in resting and concanavalin A-stimulated mouse lymphocytes. Oncogene, 7:661-666.

- Motukura, T., and Arnold, A.** (1993) Cyclins and oncogenesis. Biochem.Biophys.Acta, **1155**:63-78.
- M'owat, M., Cheng, A., Kimura, N., Bernstein, A., and Benchimol, S.** (1985) The arrangements of the cellular p53 gene in erythroleukaemic cells transformed by Friend virus. Nature (London), **314**:633-636.
- Muller, R., Bravo, R., Burckhardt, J., and Curran, T.** (1984) Induction of c-fos gene and protein by growth factor precedes activation of c-myc. Nature (London), **312**: 716-720.
- Muller, R., Mumberg, D., and Lucibello, F.C.** (1993) Signals and genes in the control of cell-cycle progression. Biochem.Biophys.Acta, **1155**:151-179.
- Muller, W.J., Sinn, E., Pattengale, P.K., Wallace, R., and Leder, P.** (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell, **54**:105-109.
- Neer, E.J., and Clapham, D.E.** (1988) Roles of G protein subunits in transmembrane signalling. Nature (London), **333**:129-134.
- Nigro, J.M., Baker, S.J., Preisinger, A.C., Jessup, J.M., Hostetter, R., Cleary, K., Bigner, S.H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F.S., Weston, A., Modali, R., Harris, C.C., and Vogelstein, B.** (1989) Mutations in the p53 gene occur in diverse human tumour types. Nature (London), **342**:705-708.
- Nishibe, S., Wahl, M.L., Hernandez-Sotomayor, S.M.T., Tonks, N.K., Rhee, S.G., and Carpenter, G.** (1990) Increase of the catalytic activity of phospholipase C- γ 1 by tyrosine phosphorylation. Science, **250**:1253-1256.
- Nishida, E., and Gotch, Y.** (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. TIBS, **18**:128-131.
- O'Rourke, R.W., Miller, C.W., Kato, G.J., Simon, K.J., Chen, D., Dang, C.V., and Koeffler, P.H.** (1990) A potential transcriptional activation element in the p53 protein. Oncogene, **5**:1829-1832.
- Obersler, P., Floch, P., Ramsperger, U., and Stahl, H.** (1993) p53-catalyzed annealing of complementary single-stranded nucleic acids. EMBO J., **12**:2389-2396.
- Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D., and Vogelstein, B.** (1992)

Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature (London), **358**:80-83.

Oren, M. (1992) p53: the ultimate tumor suppressor gene? FASEB J., **6**:3169-3176.

Oren, M., Maltzman, W., and Levine, A.J. (1981) Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. Mol.Cell.Biol., **1**:101-110.

Pardee, A.B. (1989) G1 events and regulation of cell proliferation. Science, **246**:603-608.

Pathak, S., Siciliano, M.J., Cailleau, R., Wiseman, C.L., and Hsu, T.L. (1979) A human breast adenocarcinoma with chromosome and isoenzyme markers similar to those of the HeLa cell line. J.Natl.Cancer Inst., **62**:263-271.

Pawson, T., and Gish, G.D. (1992) SH2 and SH3 domains: From structure to function. Cell, **71**:359-362.

Pawson, T. (1988) Non catalytic domain of cytoplasmic protein tyrosine kinase : Regulatory elements in signal transduction. Oncogene, **3**:491-495.

Pazin, M.J., and Williams, L.T. (1992) Triggering signalling cascades by receptor tyrosine kinases. TIBS, **17**:374-378.

Picksley, S.M., Meek, D.W., and Lane, D.P. (1992) The conformational change of a murine temperature-sensitive p53 protein is independent of change in phosphorylation status. Oncogene, **7**:1649-1657.

Pietenpol, J.A., Holt, J.T., Stein, R.W., and Moses, H.L. (1990a) TGF- β suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. Proc.Natl.Acad.Sci.USA., **87**:3758-3762.

Pietenpol, J.A., Stein, R.W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R.M., Pittelkow, M.R., Munger, K., Howley, P., and Moses, H.L. (1990b) TGF- β inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell, **61**:777-785.

Pines, J. (1993) Cyclins and cyclin-dependent kinases: take your partners. TIBS, **18**:195-197.

Prasad, K.A.N. and Church, J.G. (1991) EGF-dependent growth inhibition in MDA-

468 human breast cancer cells is characterised by late G1 arrest and altered gene expression. Exp.Cell Res., 195:20-26.

Price, B.D., and Claderwood, S.K. (1993) Increased sequence-specific p53-DNA binding activity after DNA damage is attenuated by phorbol esters. Oncogene, 8:3055-3062.

Quartin, R.S., Finlay, C.A., Hinds, P.W., Baker, S.J., Fearon, E.R., Vogelstein, B. and Levine, A.J. (1991) Mutant p53 DNA clones from human colon carcinomas cooperate with ras to transform primary rat cells. In: Origins of Human Cancer: A comprehensive Review. Brugge, J., Curran, T., Harlow, E., and McCormick, F., eds. Cold spring Harbor laboratory Press, New York, 609- 615.

Ragimov, N., Krauskopf, A., Navot, N., Rotter, V., Oren, M., and Aloni, Y. (1993) Wild-type but not mutant p53 can repress transcription initiation in vitro by interfering with the binding of basal transcription factors to the TATA motif. Oncogene, 8:1183-1193.

Raycroft, L., Schimdt, J.R., Yoas, K., Hao, M., and Lozano, G. (1991) Analysis of p53 mutants for transcriptional activity. Mol.Cell.Biol., 11:6067-6074.

Raycroft, L., Wu,H., and Lozano, G. (1990) Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science, 249:1049-1051.

Reed, S.I. (1991) G1- specific cyclins: in search of an S-phase promoting factor. TIG, 7:95-99.

Reich, N.C., Oren, M., and Levine, A.J. (1983) two distinct mechanisms regulate the levels of cellular tumor antigen. Mol.Cell.Biol., 3:2143-2150.

Reich, N.C., and Levine, A.J. (1984) Growth regulation of a cellular tumour antigen, p53, in non-transformed cells. Nature (London), 308:199-201.

Reifenberger, G., L.a, L., Ichimura, K., Schmidt, E.E., and Collins, V.P. (1993) Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations. Cancer Research, 53:2736-2739.

Robinson, L.C., Gibbs, J.E., Marshall, M.S., Segal, I.S., and Tatchell, K. (1987) CDC25: A component of the RAS-adenylate cyclase pathway in Saccharomyces cerevisiae. Science, 235: 1218-1221.

- Roehl, H.H., and Conrad, S.E.** (1990) Identification of G1-S-phase regulated region in the human thymidine kinase gene promoter. Mol.Cell.Biol., **10**:3834-3837.
- Rovinski, B., and Benchimol, S.** (1988) immortalization of rat embryo fibroblasts by the cellular p53 oncogene. Oncogene, **2**:44: 452.
- Ruff-Jamison, S., Chen, K., and Cohen, S.** (1993) Induction by EGF and interferon- γ of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. Science, **261**:1733-1736.
- Sambrook, J., Fritsch, E.F., and Maniatis, T.** (1989) Molecular Cloning: A laboratory Manual. 2nd edition. Cold Spring Harbor Press.
- Santhanam, U., Ray, A., and Sehgal, P.B.** (1991) Repression of the interleukin 6 gene promoter by p53 and the retinoblastoma susceptibility gene product. Proc.Natl.Acad.Sci.USA., **88**:7605-7609.
- Sarnow, P., Ho, Y.S., Williams, J., and Levine, A.J.** (1982) Adenovirus E1b-58kD tumor antigen and SV40 large T antigen are physically associated with the same 54kD cellular protein in transformed cells. Cell, **28**:387-394.
- Sato, E., Usheva, A., Zambetti, G.P., Momand, J., Horikoshi, N., Weinmann, R., Levine, A.J., and Shenk, T.** (1992) Wild-type p53 binds to the TAT λ -binding protein and represses transcription. Proc.Natl.Acad.Sci.USA., **89**:12028-12032.
- Schausky, G., Ben-Ze'ev, A., and Rotter, V.** (1990) Subcellular distribution of the p53 protein during the cell cycle of Balb/c 3T3 cells. Oncogene, **5**:1707-1711.
- Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J., and Howley, P.M.** (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes degradation of p53. Cell, **63**:1129-1136.
- Schlessinger, J.** (1993) How Receptor tyrosine kinases activate Ras. TIBS, **18**:273-275.
- Segawa, K., Minowa, A., Sugawara, K., Takano, T., and Hanaoka, F.** (1993) Abrogation of p53-mediated transactivation by SV40 large T antigen. Oncogene, **8**:543-548.
- Settleman, J., Narasimhan, V., Foster, L.C., and Weinberg, R.A.** (1992) Molecular cloning of cDNAs encoding the GAP-associated protein p190: Implications for a

signalling pathway from Ras to the nucleus. Cell, 69:539-549.

Seuwen, K., and Pouyssegur, J. (1992) G protein-controlled signal transduction pathways and the regulation of cell proliferation. Adv.Cancer Res., 58:75-94.

Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B., and Costa, J. (1992) Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA, 89:4495-4499.

Sheikh, M.S., Shao, Z.-M., Hussain, A., and Fontana, J.A. (1993) The p53-binding protein MDM2 gene is differentially expressed in human breast carcinoma. Cancer Research, 53:3226-3228.

Shio, Y., Yamamoto, T., and Yamaguchi, N. (1992) Negative regulation of Rb expression by the p53 gene product. Proc.Natl.Acad.Sci.USA, 89:5206-5210.

Shohat, O., Greenberg, M., Reisman, D., Oren, M., and Rotter, V. (1987) Inhibition of cell growth mediated by plasmids encoding p53 anti-sense. Oncogene, 1:277-283.

Silvennoinen, O., Schindler, C., Schlessinger, J., and Levy, D.F. (1993) Ras-independent growth factor signalling by transcription factor tyrosine phosphorylation. Science, 261:1736-

Skolnik, E.Y., Lee, C.-H., Batzer, A., Vicentini, L.M., Zhou, M., Daly, R., Myers, Jr., M.J., Backer, J.M., Ullrich, A., White, M.F., and Schlessinger, J. (1993) The SH2/SH3 domain containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of ras signalling. EMBO J., 12:1929-1936.

Slingerland, J.M., Jenkins, J.R., and Benchimol, S. (1993) The transforming and suppressor functions of p53 alleles: effects of mutations that disrupt phosphorylation, oligomerization and nuclear translocation. EMBO J., 12:1029-1037.

Smith, M.R., DeGudicibus, S.J., and Stacey, D.W. (1986) Requirement for c-ras protein during viral oncogene transformation. Nature (London), 320:540-543.

Sprague, Jr. G.F. (1991) Signal transduction in yeast mating. TIG, 7:393-398.

Steinmeyer, K., Maacke, H., and Deppert, W. (1990) Cell cycle control by p53 in normal (3T3) and chemically transformed (Meth A) mouse cells. I. Regulation of p53 expression. Oncogene, 5:1691-1699.

- Stryker, L.** (1986) Cyclic GMP cascade of vision. Annu.Rev. Neurosci., 9:87-119.
- Stürzbecher, H.-W., Brain, R., Maimets, T., Addison, C., Rudge, K., and Jenkins, J.R.** (1988) Mouse p53 blocks SV40 DNA replication in vitro and downregulates T antigen DNA helicase activity. Oncogene, 3:405-413.
- Stürzbecher, H.-W., Maimets, T., Chumakov, P., Brain, R., Addison, C., Simanis, V., Rudge, K., Philp, R., Grimaldi, M., Court, W., and Jenkins, J.R.** (1990) p53 interacts with p34^{cdc2} in mammalian cells: implications for cell cycle control and oncogenesis. Oncogene, 5:795-801.
- Subler, M.A., Martin, D.W., and Deb, S.** (1992) Inhibition of viral and cellular promoters by human wild-type p53. J.Virol., 66:4757-4762.
- Suzuki-Sekimori, R., Matuoka, K., Nagai, Y., and Takenawa, T.** (1989) Diacylglycerol, but not inositol 1,4,5-triphosphate accounts for platelet-derived growth factor-stimulated proliferation of BALB 3T3 cells. J.Cell.Physiol., 140:432-438.
- Tabor, S., Huber, H.E., and Richardson, C.C.** (1987) Escherichia coli thioredoxin confers processivity on the DNA polymerase activity of the gene 5 protein of bacteriophage T7. J.Biol.Chem., 262:16212-16223.
- Tarunina, M., and Jenkins, J.R.** (1993) Human p53 binds DNA as a protein homodimer but monomeric variants retain full transcription transactivation activity. Oncogene, 8:3165-3174.
- Taylor, C.W., and Marshall, I.C.B.** (1992) Calcium and inositol 1,4,5-triphosphate receptors: a complex relationship. TIBS, 17:403-407.
- Thelen, M., Rosen, A., Nairn, A.C., and Aderem, A.** (1991) Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. Nature (London), 351:320-322.
- Trahey, M., and McCormick, F.** (1987) A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science, 238:542-545.
- Travali, S., Ku, D.H., Rizzo, M.G., Ottavio, L., Baserga, R., and Calabretta, B.** (1989) Structure of the human gene for the proliferating cell nuclear antigen. J.Biol.Chem., 264:7466-7472.

- Travali, S., Koniecki, J., Petralia, S., and Baserga, R. (1990) Oncogenes in growth and development. FASEB J., 4:3209-3214.
- Tsukada, T., Tomooka, Y., Takai, S., Ueda, Y., Nishikawa, S., Yagi, T., Tokunaga, T., Takeda, N., Suda, Y., Abe, S., Matsuo, I., Ikawa, Y., and Aizawa, S. (1993) Enhanced proliferation potential in culture of cells from p53-deficient mice. Oncogene, 8:3313-3322.
- Ullrich, S.J., Mercer, W.E., and Appella, E. (1992) Human wild-type p53 adopts a unique conformational and phosphorylational state in vivo during growth arrest of glioblastoma cells. Oncogene, 7:1635-1643.
- Ullrich, S.J., Sakaguchi, K., Lees-Miller, S.P., Fiscella, M., Mercer, W.E., Anderson, C., and Appella, E. (1993) Phosphorylation at Ser-15 and Ser-392 in mutant p53 molecules from human tumors is altered compared to wild-type p53. Proc.Natl.Acad.Sci.USA., 90:5954-5958.
- Ullrich, A., and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. Cell, 61:203-212.
- Unger, T., Nau, M.M., Segal, S., and Minna, J.D. (1992) p53: a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. EMBO J., 4:1383-1390.
- Vogelstein, B. (1990) A deadly inheritance. Nature (London), 348: 681-682 (News and Views).
- Vogelstein, B. and Kinzler, K.W. (1992) p53 function and dysfunction. Cell, 70:523-526.
- Vogelstein, B. and Kinzler, K.W. (1993) The multi-step nature of cancer. TIG, 9:138-141.
- Vojtek, A.B., Hollenberg, S.M., and Cooper, J.A. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell, 74:205-214.
- Wang, N.P., To, H., Lee, W.-H., and Lee, W.-H.P. (1993) Tumor suppressor activity of RB and p53 genes in human breast cancer cells. Oncogene, 8:279-288.
- Weinberg, R.A. (1991a) Tumor suppressor genes. Science, 254:1138-1145.

Weinberg, R.A. (1991b) Oncogenes, tumor suppressor genes, and cell transformation: Trying to put it all together. In: Origins of Human Cancer: A comprehensive Review. Brugge, J., Curran, T., Harlow, E., and McCormick, F., eds. Cold spring Harbor laboratory Press, New York, 1-16.

Weinberg, R.A. (1990) The retinoblastoma gene and cell growth control. TIBS, 15:199-202.

Weintraub, H., Hauschka, S., and Tapscott, S.J. (1991) The MCK enhancer contains a p53 responsive element. Proc. Natl. Acad. Sci. USA, 88:4570-4571.

Werness, B.A., Levine, A.J., and Howley, P. (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science, 248:76-79.

White, L., Ortega, J.A., Ying, K.L. (1985) Acute non-lumphyocytic leukemia following multimodality therapy for retinoblastoma. Cancer, 55:496-498.

Whyte, P., Williamson, N.M., and Harlow, E. (1989) Cellular targets for transformation by the adenovirus E1A proteins. Cell, 56:67-75.

Wilcock, D. and Lane, D.P. (1991) Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature (London), 349:429-431.

Willumsen, B.M., Papageorge, A.G., Kung, H-F., Bekesi, E., Robbins, T., and Johnson, M., Vass, W.C., and Lowy, D.R. (1986) Mutational analysis of a ras catalytic domain. Mol. Cell. Biol., 6:2646-2654.

Wittenberg, C., Sugimoto, K., and Reed, S.I. (1990) G1-specific cyclins of *S.cerevisiae*: Cell cycle periodicity, regulation by mating pheromone, and association with the p34^{CDC28} protein kinase. Cell, 62:225-237.

Wong, G., Muller, O., Clark, R., Conroy, L., Moran, M.F., Polakis, P., and McCormick, F. (1992) Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. Cell, 69:551-558.

Wu, X., Bayle, J.H., Olson, D., and Levine, A.J. (1993) The p53-mdm-2 autoregulatory feedback loop. Genes Dev., 7:1126-1132.

Yatani, A., Okabe, K., Halenbeck, R., McCormick, F., and Brown, A.M. (1990) *ras* p21 and GAP inhibit coupling of muscarinic receptors to atrial K⁺ channels. Cell,

61:769-776.

Yew, P.R., and Berk, A.J. (1992) Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature (London), 357:82-85.

Yuan, J.-N., Liu, B.-H., Lee, H., Shaw, Y.-T., Chiou, S.-T., Chang, W.-C., and Lai, M.-D. (1993) Release of the p53-induced repression on thymidine kinase promoter by single p53-binding sequence. Biochem.Biophys.Res.Commun., 191:662-668.

Zambetti, G.P., Bargonetti, J., Walker, K., Prives, C., and Levine, A.J. (1992) Wild-type p53 mediates positive regulation of gene expression, through a specific DNA sequence element. Genes Dev., 6:1143-1152.

Zastawny, R.L., Salvino, R., Chen, J., Benchimol, S., and Ling, V. (1993) The core promoter region of the P-glycoprotein gene is sufficient to confer differential responsiveness to wild-type and mutant p53. Oncogene, 8:1529-1535.

Zauberman, A., Barak, Y., Ragimov, N., Levy, N., and Oren M. (1993) Sequence-specific DNA binding by p53: identification of target and lack of binding to p53-MDM2 complexes. EMBO J., 12:2799-2808.

Zerrahn, J., Deppert, W., Weidemann, D., Patschinsky, T., Richards, F., and Milner, J. (1992) Correlation between the conformational phenotype of p53 and its subcellular location. Oncogene, 7:1371-1381.

Zhang, X.-F., Settleman, J., Kyriakis, J.M., Takeuchi-Suzuki, E., Elledge, S.J., Marshall, M.S., Bruder, J.T., Rapp, U.R., and Avruch, J. (1993) Normal and oncogenic p21^{ras} proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature (London), 364:308-313.

Zentella, A., Weis, F.M., Ralph, D.A., Laiho, M., and Massague, J. (1991) Early gene responses to transforming growth factor- β in cells lacking growth suppressive RB function. Mol.Cell.Biol., 11:4952-4958.



