REGULATION OF EPIDERMAL GROWTH FACTOR GENE EXPRESSION IN T-47D HUMAN BREAST CANCER CELLS

A Thesis Presented to the

Faculty of Graduate Studies

University of Manitoba

In Partial Fulfilment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

MAN SU JOHNSON WONG

Department of Biochemistry and

Molecular Biology

Faculty of Medicine

October, 1991

ISBN 0-315-77828-8

REGULATION OF EPIDERMAL GROWTH FACTOR GENE EXPRESSION IN T-47D HUMAN BREAST CANCER CELLS

BY

MAN SU JOHNSON WONG

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

To my wife Yvonne, parents, sister and brother

To dream the impossible dream,
To fight the unbeatable foe,
To bear with unbearable sorrow,

To run where the brave dare not go.
To right the unrightable wrong,
To love pure and chaste from afar,
To try when your arms are too weary,
To reach the unreachable star.

This is my quest, to follow the star,
No matter how hopeless, no matter how far,
And to fight for a right without question or pause
To be willing to march into hell for a heavenly cause,
And I know if I only be true to this glorious quest.
That my heart will lie peaceful and calm,
When I lay to my rest,

And the world will be better for this,
That one man strong and covered with scars,
Still strove with his last ounce of courage,
To reach the unreachable star.

--the Impossible Dream

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. L. C. Murphy for her guidance and advice throughout the period of this work. Thanks also to Helmut Dotzlaw for technical assistance and help with the Mac. The critical but helpful comments and input of my committee members, Dr. J. Davie and Dr. L. M. Murphy are also gratefully appreciated.

Gratitude is also extended to the student body of the Biochemistry department which surely had made the years more fun and complete. Their support and intellectual input have always been abundant, unfailing, and inspiring. I could never forget the uncountable occasions when Ken Ng and I practically 'ruled' the department at 'peopleless' hours. Special thanks to Teresa Miller who has shown extraordinary support and encouragement. Also, the friendship of Fred Pereira and his generosity will not be forgotten. Names of other fellow students including Chris, Paul, Ketan, Denise and Mike also deserve a place here; their cheerful characters have undoubtedly brightened up many days especially during the long, dark winter! Through interactions with them and others, I have learned a great deal about science and life, the benefit from which will surely be felt in the many years to come.

The financial support from the Medical Research Council of Canada and the Manitoba Research Council are also appreciated.

ABSTRACT

Development of human breast cancer likely involves a complex interplay of hormones and growth factors. In T-47D human breast cancer cells, it has been shown unequivocally by Northern analysis that the epidermal growth factor (EGF) gene was expressed and regulated by progestins. To further examine the phenomenon, a sensitive RNAase protection assay was employed to measure EGF mRNA levels following various treatment. Treatment of T-47D cells with the synthetic progestin medroxyprogesterone acetate (MPA) was found to cause a rapid accumulation of EGF mRNA as early as 3 hr after stimulation, peaking at 12 hr with a 11-fold induction above that of control cells. In the presence of the protein synthesis inhibitor cycloheximide (CHX), MPA treatment resulted in superinduction of EGF mRNA levels (37-fold increases at 12 hr). The inability of CHX to abolish EGF induction by MPA suggested that the effect of the hormone is direct and does not require ongoing protein synthesis. Interestingly, CHX alone also stimulates EGF mRNA accumulation albeit to a much lesser extent.

To elucidate the molecular mechanisms by which MPA, MPA + CHX, and CHX regulate EGF gene expression, the effect of these treatments on EGF mRNA stability was investigated. Cells were stimulated with or without the above reagent (s) for 24 hr following which the half-life of EGF mRNA was measured after blocking transcription with actinomycin D or dichlororibofuranoyslbenzimidazole. All three treatments except MPA + CHX did not significantly alter the half-life of EGF message as compared to control untreated cells. MPA + CHX treatment resulted in a small stabilizing effect on EGF mRNA, increasing its half-life to about 1.8 fold. These data suggested that enhanced mRNA stability does not play a major role in contributing to the accumulation of EGF transcripts, and that a

transcriptional component is probably involved. With MPA treatment, transcriptional activation, as demonstrated by nuclear run-off experiments, appeared to contribute at least in part to the induced EGF mRNA levels.

In an attempt to identify regulatory DNA elements important for EGF gene expression, computer-assisted sequence analysis was initially performed to locate putative regulatory sites. The 204-22 EGF 5'-flanking region contains a TATA- and CAAT-like sequences in its promoter plus binding sites for transcription factors such as SP1, NFκB, AP-I and progesterone receptors. Functional activity of the 204-22 fragment was tested by constructing several chimeric plasmids in which the full length or part of the fragment was placed in front of the chloramphenicol acetyltransferase (CAT) gene. Transient transfection experiments using T-47D or HeLa cells (co-transfected with progesterone receptor expression vector) showed that 204-22 contains a weak and constitutive promoter activity, supporting a role for the TATA and CAAT sequences as active promoter elements. Deleting 1.5 kbp from the 5' end did not impart any changes in the fragment's strength to drive CAT expression. Moreover, no differential regulation by progestins was observed for all the constructs. These data argued against the existence of functional progesterone responsive elements (PRE) in the 3.5 kbp sequences of 204-22. Furthermore, the two putative PREs (originally identified in the uteroglobin gene promoter) identified by sequence analysis are likely to be nonfunctional in T-47D cells, being active only in a gene- and tissue-specific manner.

TABLBE OF CONTENTS

ACKNOWLEDGEMENTS i

ABSTRACT ii

TABLE OF CONTENTS iv

LIST OF FIGURES vii

LIST OF TABLES ix

ABBREVIATIONS x

INTRODUCTION 1

1. HORMONES, GROWTH FACTORS AND BREAST CANCER 1
   - 1.1 Epidemiology of Breast Cancer 1
   - 1.2 Steroid and Peptide Hormones 3
   - 1.3 Growth Factors 7

2. EPIDERMAL GROWTH FACTOR AND ITS RECEPTOR 13
   - 2.1 Biological Effects 13
   - 2.2 Physiological Functions 14
   - 2.3 Oncogenic Potential of EGF/EGF Receptor 15

3. ENDOCRINE THERAPY OF BREAST CANCER 18
   - 3.1 Antiestrogen 19
   - 3.2 Progestin 21
   - 3.3 Aminoglutethimide and others 23

4. REGULATION OF EUKARYOTIC GENE EXPRESSION: LEVELS OF CONTROL 25
   - 4.1 General Overview 25
   - 4.2 Transcription 27
     - 4.2.1 Basal Transcription by RNA polymerase II 27
     - 4.2.2 Regulated Transcription 30
       - 4.2.2.1 General introduction 30
       - 4.2.2.2 Steroid Hormone Receptors: prototypes for ligand-regulated transcription factors 35
   - 4.3 Processing of pre-mRNA 40
   - 4.4 Nucleocytoplasmic mRNA Transport 42
   - 4.5 mRNA stability 44
     - 4.5.1 General introduction 44
     - 4.5.2 Steroid-regulated mRNA stability 47
   - 4.6 Translational Control 49
   - 4.7 Post-translational Control 51

RESEARCH OBJECTIVES 54

MATERIALS AND METHODS 56

1. CELL LINES AND CULTURE CONDITIONS 56

1.1 Cell lines 56
1.2 Culture conditions 56
1.3 Routine culture procedures 57
1.3.1 Cell removal by trypsin 57
1.3.2 Cell counting 57

2. ROUTINE EXPERIMENTAL MANIPULATIONS AND ANALYSES 58

2.1 Treatments with hormones and various reagents 58
2.2 Measurement of mRNA half-life 58

3. SOUTHERN AND NORTHERN ANALYSIS 59

3.1 RNA isolation and Northern blot analysis 59
3.2 Southern blot analysis of restriction-cut DNA fragments 62
3.3 Labelling of cDNA probes with 32P-dCTP 62

4. ISOLATION AND PURIFICATION OF DNA FRAGMENTS AND PLASMIDS 63

4.1 Sources of cDNA probes and plasmids 63
4.2 Large scale plasmid preparation 63
4.3 Isolation and electrophoresis of DNA fragments 65

5. RIBONUCLEASE PROTECTION ASSAY 67

5.1 In vitro synthesis of riboprobe 67
5.2 Liquid hybridization of total RNA with riboprobe 69
5.3 Polyacrylamide gel electrophoresis 70

6. NUCLEAR RUN-ON ASSAY 71

6.1 Isolation of active nuclei 71
6.2 In-vitro elongation and isolation of hnRNA 73
6.3 Filter strip hybridization 75

7. CONSTRUCTION AND MANIPULATION OF PLASMIDS 75

7.1 Modifications of restriction enzyme-digested DNA fragments 75
7.2 Construction of reporter plasmids pGEM-CAT(S), pGEM-CAT/204, and pGEM-revCAT/204 76
7.3 Construction of reporter plasmids Basic and Enhancer-CAT/204 or 3'-204 78
7.4 Transformation of DH5a or RR1 strains of E. coli 78
7.5 Mini plasmid preparation 80

8. TRANSIENT TRANSFECTION ANALYSIS OF REPORTER GENE ACTIVITY 81

8.1 Transfection by the CaPO4/DNA precipitate method 81
8.2 Protein determination 82
8.3 Chloramphenicol acetyltransferase assay 83
8.4 β-galactosidase assay 84

RESULTS.....85

1. ACCUMULATION OF EGF MRNA AFTER TREATMENT WITH MPA, CHX AND MPA+CHX.....85

2. EFFECT OF MPA ON EGF GENE TRANSCRIPTION.....87

3. MEASUREMENT OF MRNA HALF-LIFE IN T-47D CELLS.....90

4. ANALYSIS OF 5'-FLANKING REGION OF THE EGF GENE.....101

4.1 Structural analysis of 204-22.....101
4.2 Functional analysis of 204-22.....107
4.2.1 Optimization of CAT assay.....107
4.2.2 Construction of CAT reporter plasmids carrying EGF 5'-flanking sequences.....114
4.2.3 Promoter activity in transient transfected T-47D cells.....124
4.2.4 Promoter activity in transient transfected HeLa cells.....128

DISCUSSION.....135

1. EXPRESSION OF EGF MRNA IN T-47D CELLS: SIGNIFICANCE AND PROGESTIN REGULATION.....135

2. MOLECULAR MECHANISMS OF EGF MRNA INDUCTION BY PROGESTINS, CYCLOHEXIMIDE AND PROGESTIN PLUS CYCLOHEXIMIDE.....137

3. CHARACTERIZATION OF 5'-FLANKING REGION OF THE EGF GENE.....139

REFERENCES.....144

APPENDIX.....166

LIST OF FIGURES

1. Control of gene expression in eukaryotic cells....26
2. Consensus responsive elements for nuclear receptors....37
3. Schematic representation of RNAase protection assay....68
4. Schematic representation of nuclear run-off assay....72
5. Construction scheme of plasmids pGEM-CAT (Sal or Hind), pGEM-CAT/204 and pGEM-revCAT/204....77
6. Construction scheme of plasmids Basic-CAT/204 (or 3'-204) and Enhancer-CAT/204 (or 3'-204)....79
7. Time course of the effects of MPA, MPA + CHX and CHX alone on EGF mRNA levels....86
8. Effect of MPA on transcription of the EGF gene....89
9. Time course of EGF and FAS mRNA decay in control T-47D cells using ActD as transcriptional inhibitor....91
10. Time course of EGF and FAS mRNA decay in MPA-treated T-47D cells using ActD as transcriptional inhibitor....92
11. Measurement of EGF and FAS mRNA half-life in control and MPA-treated cells using ActD as transcriptional inhibitor....93
12. Time course of EGF and FAS mRNA decay in control T-47D cells using DRB as transcriptional inhibitor....94
13. Time course of EGF and FAS mRNA decay in MPA-treated T-47D cells using DRB as transcriptional inhibitor....95
14. Time course of EGF and FAS mRNA decay in CHX + MPA-treated T-47D cells using DRB as transcriptional inhibitor....96
15. Time course of EGF and FAS mRNA decay in cycloheximide-treated T-47D cells using DRB as transcriptional inhibitor....97
16. Measurement of EGF ad FAS mRNA half-life under various treatment using DRB as transcriptional inhibitor....98
17. Restriction fragment analysis of 204-22....102
18. Partial restriction map of EGF 204-22....103
19. Putative regulatory sites in the EGF 5'-flanking region and regions of homology with the mouse EGF gene....105

20. Map of putative regulatory sites in the sequenced portion of the EGF 5'-flanking region (204-22).....108

21. Effects of total DNA concentration and incubation time with $CaPO_4$/DNA ppt. on transient gene expression in T-47D cells.....110

22. Effect of reporter plasmid (TK-CAT 5A) concentration on transient gene expression in T-47D cells.....111

23. Effect of varying protein content in cell extract on CAT activity.....112

24. Effect of acetyl CoA concentration and assay time on the activity of CAT.....113

25. Effect of cell density on transient gene expression in T-47D cells.....115

26. Plasmid map of pGEM-CAT (SalI).....117

27. Plasmid maps of pGEM-CAT/204 and pGEM-revCAT/204.....118

28. Restriction enzyme digestions of pGEM-CAT, pGEM-CAT/204, and pGEM-revCAT/204 to confirm identity.....119

29. Plasmid maps of Basic-CAT/204 and Basic-CAT/3'-204.....121

30. Plasmid maps of Enhancer-CAT/204 and Enhancer-CAT/3'-204.....122

31. Restriction enzyme digestions of Basic-CAT/204 (or 3'-204) and Enhancer-CAT/204 (or 3'-204) to confirm identity.....123

32. Transient transfection analysis of pGEM-CAT activity under the control of EGF 5'-flanking sequences.....125

33. Transient transfection analysis of Basic- and Enhancer-CAT activity under the control of EGF 5'-flanking sequences in T-47D cells.....127

34. Effect of cPR1 (encodes progesterone receptor) concentration on the expression and hormone responsiveness of a co-transfected reporter gene MMTV-CAT in HeLa cells.....130

35. Effect of reporter plasmid (MMTV-CAT) concentration on transient gene expression and hormone responsiveness in HeLa cells.....131

36A. Transient transfection analysis of Basic- and Enhancer-CAT activity under the control of EGF 5'-flanking sequences in HeLa cells.....132

36B. Relative CAT activity of 204-containing reporters in transient transfected HeLa cells.....133

LIST OF TABLES

1. Optimal conditions for $CaPO_4$-mediated transfection and CAT assay.....116

ABBREVIATIONS

Units of measure

cm	= centimeter
min	= minutes
hr	= hour
^{o}C	= degrees centigrade
µCi	= microcurie
µl	= microlitre
ml	= millilitre
l	= litre
ng	= nanogram
µg	= microgram
mg	= milligram
nM	= nanomolar
µM	= micromolar
mM	= millimolar
M	= molar
cpm	= counts per minute
rpm	= revolutions per minute
w/v	= weight per volume
v/v	= volume per volume
vol	= volume
%	= percent
bp	= base pairs
kb	= kilobase
g	= gravitational force i.e. 9.8 m/s^2

Reagents

BSA	= bovine serum albumin
$CHCl_3$	= chloroform
CsCl	= cesium chloride
DTT	= dithiothreitol
ddH_2O	= distilled deionized water
EtBr	= ethidium bromide
EDTA	= ethylenediaminetetraacetic acid
GRB	= gel running buffer
GITC	= guanidinium isothiocyanate
$MgCl_2$	= magnesium chloride
MOPS	= morpholinopropane sulfonic acid
NaCl	= sodium chloride
NaH_2PO_4	= sodium phosphate monobasic
Na_2HPO_4	= sodium phosphate dibasic
NH_4Cl	= ammonium chloride
ONPG	= ortho-nitrophenol galactoside
P	= phosphorous

PAGE = polyacrylamide gel electrophoresis
SDS = sodium dodecyl sulfate
SSPE = standard saline phosphate EDTA
SSC = standard saline citrate
TE = Tris-EDTA
TBE = Tris borate EDTA

Enzymes

RNase = ribonuclease
DNase = deoxyribonuclease
CAT = chloramphenicol acetyltransferase
β-gal = β-galactosidase

Receptors

AR = androgen receptor
ER = estrogen receptor
GR = glucocorticoid receptor
MR = minerocorticoid receptor
PR = progesterone receptor
RAR = retinoic acid receptor
T3R = thyroid hormone receptor
VDR = vitamin D receptor
EGFR = epidermal growth factor receptor

Miscellaneous

ss = single strand
ds = double strand
OD = optical density
UV = ultra violet
dNTP = deoxynucleotide triphosphate
NTP = ribonucleoside triphosphate
G = guanosine
A = adenosine
T = thymidine
C = cytidine
O/N = overnight
DNA = deoxyribonucleic acid
RNA = ribonucleic acid
mRNA = messenger RNA
tRNA = transfer RNA
cDNA = complementary DNA

INTRODUCTION

1. HORMONES, GROWTH FACTORS AND BREAST CANCER

1.1 Epidemiology of Breast Cancer

Breast cancer is the most common malignancy affecting women in western societies. It is estimated that one in ten women will develop this cancer during their lifetime, resulting in more than 110, 000 new cases each year in the United States alone (1, 2). It is therefore not surprising that breast cancer remains the leading cause of death from cancer in women. Past and ongoing effort from epidemiologic studies have and are attempting to define the magnitude of the global epidemic, to identify risk factors and provide clues as to the etiology of human breast cancer with the hope that a more rational approach to breast cancer prevention can one day be implemented. In this section, epidemiologic data will be summarized which pertains to breast cancer risk with emphasis on endocrine factors.

Perhaps the most indirect yet pointing evidence linking the etiology of breast cancer to the female hormone estrogen is the fact that the risk of a female developing breast cancer is 100 times higher than that of the male (1, 2). Also, in women the incidence of developing breast cancer increases sharply from a few years after menarche up to the age of the menopause. Age at menarche is also an important risk factor for breast cancer with early onset being associated with a modest increase (two-fold or less) in risk. In addition, it was found that women who take longer to establish regular menses have a decreased breast cancer risk than those who develop regular cycles sooner (1). Similarly, the age of menopause is also important; menopause before age 45 is associated with a two-fold reduction in risk compared with women where menopause occurrs after age 55 (1, 2). This last

piece of information explains the observation that artificial menopause induced by surgical means is protective against breast cancer.

The large collaborative study in seven countries by MacMahon et al., (3) established the importance of early first full-term pregnancy to breast cancer risk. This study demonstrated that women with a first full-term pregnancy before the age of 20, had one-half the risk of those who had their first child at or after 35 years of age. A late first pregnancy increased the risk above that of nulliparous women. It must be stressed that the protective effect of an early first pregnancy holds only if it goes to term; presumably the full effect of pregnancy-associated hormonal changes act to induce a differentiating effect that is protective to mammary epithelia. More recent studies have also found an additional protective effect of parity (4, 5). On the other hand, some studies have suggested that first trimester abortion, whether induced or spontaneous, is actually associated with a substantial increase in breast cancer risk (1, 2). Abortions after the first full-term pregnancy, however, do not alter the risk. The variety of factors associated with reproductive history altogether infer that endocrinologic events early in life collectively determine the ultimate risk.

There is no doubt that links exist between a family history of breast cancer and the likelihood of a first degree relative developing the disease. In general, the highest risk, estimated to be about nine-fold higher, is seen among first degree relatives of patients with both bilateral and premenopausal breast cancer. Women with a first degree relative who has unilateral and postmenopausal cancer, have a relative increased risk of about two- to three-fold (1, 2). This strong familial association of breast cancer points to genetic predisposition as a strong determinant in breast cancer development. However, the familial trend for the disease can also be explained by shared environmental factors as well such as common dietary

habits. In fact, dietary factors as major contributors to breast cancer risk have received much attention partly because of the increasing awareness that various imbalances in diet can have adverse effects on the general health condition. Most geographic correlation studies have found a positive correlation between certain diet composition (high fat, animal protein intake) and increased breast cancer incidence (1, 2). In support of these data are related studies indicating a positive correlation of risk with relative weight (obesity) in postmenopausal women. These risk factors probably have a hormonal mechanism of action since it is known that obesity and fat consumption are linked to altered steroid metabolism (6).

1.2 Steroid and Peptide Hormones

The growth regulation of both normal and malignant breast tissue is under the influence of a number of steroid and peptide hormones. More recently, increasing data have pointed to the involvement of many other diffusible and locally acting polypeptides, collectively known as growth factors in growth control of breast epithelium. In this section, an attempt will be made to provide an overview of the role of systemic (endocrine) hormones in normal breast development as well as in the pathogenesis of breast cancer. The role of growth factors would be treated in a separate following section.

The seminal work of Beatson (7) about a hundred years ago provided the first evidence that breast cancer is under hormonal control. He found that ovariectomy in premenopausal patients resulted in regression of metastatic breast tumors. Since then estrogens, the main steroids produced by the ovaries, have been intimately linked to the pathogenesis of human breast cancer. Further supportive evidence of a link between estrogen and breast cancer comes from the observation that women with non-functional ovaries have about 1% the frequencyof

breast cancer of those with intact ovaries (8). The most important endogenous estrogen is estradiol (E2), but in postmenopausal women estrone (E1) may also have a significant effect. Estradiol, in conjunction with pituitary factors (prolactin and growth hormone) and possibly one or more growth factors (8, 9, 10), is essential for the ductal phases of mammary gland development during adolescence. Because of the complexity of the system in vivo and the necessity to distinguish between direct and indirect effects, most of the information concerning the role of E2 in breast cancer was obtained from systems in vitro. The principal models were clonal lines of human breast cancer cells. Direct proliferative responses to physiological doses of E2 have been demonstrated in hormone-dependent breast cancer cells (eg. MCF-7, T-47D, ZR-75-1) in culture and grown as tumors in nude mouse model (8, 11, 12). These cell lines respond to treatment with E2 by increased secretion of various growth factors which may then exert their effects through autocrine and paracrine loops (see Introduction 1.3 for definitions). It is now known that one of the effects of the growth inhibitory agent, antiestrogen, is to decrease the secretion of some growth-stimulatory factors such as insulin-like growth factor I (IGF-I) and a 52-kDa cathepsin D-like protein (65, 11).

Estrogens also induce in breast cancer cell lines a variety of enzymes and proteins involved in nucleic acid synthesis, including DNA polymerase, c-myc, thymidine and uridine kinases, thymidylate synthetase, and dihydrofolate reductase (8, 10, 11). Estrogen regulation of thymidine kinase and c-myc mRNA has been shown to occur at the transcriptional level. In addition, estrogens alter the biochemical synthesis and/or secretion of several proteins (laminin receptor, a 52-kDa cathepsin D-like protein, progesterone receptor and pS2) and proteases (plasminogen activators and collagenolytic enzymes) that may contribute to tumor growth and progression. Proteases not only may enhance digestion of basement

membrane but may also facilitate activation of mitogenic growth factor precursors (11).

The effects of estrogens are mediated mainly through the estrogen receptor (ER), the presence of which in breast cancer tissue governs the response to hormonal therapy in breast cancer patients (see Introduction 3). Response rate to hormonal manipulation can be increased from 30% in ER-unselected patients to 55% in ER positive (ER+) cases (Introduction 3 and 60). The response rate is even better in patients with tumors that are positive for both ER and progesterone receptor (PgR). Although the lack of ER confers hormone-independency to breast cancer cells, the converse is not necessarily true. ER+ cells or tumors may become hormone-independent and not responsive to hormonal therapy at all.

Differentiation of the mammary gland during pregnancy is mainly influenced by progesterone and prolactin (9, 10, 13). Progesterone does not appear to be required for ductal development but is necessary for lobuloalveolar cell proliferation. Its potential role in directly mediating growth of human breast epithelium has been investigated using techniques in vitro such as organ and primary cell culture. The results obtained were confusing: progesterone was found to be either growth stimulatory, inhibitory or without effect (13). Similarly, conflicting results were found in human breast cancer cell lines and in experimental tumors in rodents (13).

The roles of other steroid hormones in the normal development of the mammary gland and its transformation into cancerous tissue are far from clear. Glucocorticoids appear to be necessary for maximal ductal growth and androgens seem to be responsible for the partial or complete demise of the male mammary

gland in the mouse (10). Receptors for glucocorticoids and androgens have been found in human breast cancer biopsies (50% and 33%, respectively) (14). Both steroids are used in the endocrine treatment of metastatic breast cancer (see Introduction 3). They have been shown also to exert a direct growth inhibitory effect on breast cancer cells possessing the appropriate receptors (14, 15). Interestingly, case-control sudies measuring urinary testosterone have invariably reported higher levels in breast cancer patients and in women with breast hyperplasia, or with a family history of breast cancer (16). However, the significance of these observations remain unclear. Thyroid hormone does not appear to be essential for breast development or lactation although it has been found to be growth stimulatory in some breast cancer cell lines.

Peptide hormones such as those secreted from the pituitary are also important in breast development and function. In the absence of prolactin and growth hormone, E2 alone is ineffective in inducing ductal growth. As mentioned previously, prolactin along with progesterone stimulates lobuloalveolar development during pregnancy and lactation. Ample evidence exists in the literature to support a role of prolactin in the genesis of mammary tumors in rodents (17); the relationship of prolactin to human breast cancer is, however, largely controversial. Epidemiologic studies so far have produced equivocal and contradictory results concerning the relationship between serum prolactin levels and breast cancer risk. Nevertheless, prolactin receptors have been detected in as many as 70% of all human breast cancer biopsies (18). A number of long-term breast cancer lines, including the ER+ MCF-7 and T-47D cell, also contain prolactin receptors (19). Despite the presence of prolactin receptors, most studies however have failed to demonstrate a mitogenic effect of prolactin on human breast cancer. In summary, the importance of prolactin in human breast tumorigenesis remains to

be established. The recent cloning of the prolactin receptor and the discovery of prolactin-induced proteins in human breast cancer offer new methods to study the relationship of lactogenic hormones and human breast cancer (19, 20).

1.3 Growth Factors

The progression of normal mammary tissue to breast cancer depends on the interaction of multiple elements, including genetic predisposition, mutation, and oncogene activation to name a few. In addition, over the past 10 years evidence has emerged supporting a pathophysiological role for diffusible polypeptide growth factors in the growth and malignant progression of mammary cells. The biology of these factors differs from classical endocrine hormones as neither their sites of synthesis nor sites of action are restricted to defined tissue. The same substances have also been implicated as mediators of the effects of steroid hormones and antihormones during endocrine therapy in responsive cancer cells (8, 11). In vivo, solid breast tumors consist of epithelial cancer cells and a number of different non-malignant stromal cell types in close proximity. Theoretically, all these cells can potentially produce and respond to peptide growth factors through autocrine and/or paracrine pathways if it possesses the appropriate receptors. Autocrine growth control requires a cell to respond to the same peptide that it synthesizes and secretes. Paracrine regulation, on the other hand, implies the involvement of at least 2 distinct cell populations. The donor population secretes a growth factor which can bind to receptors located on the recipient population and thereby cause a biological response. More recently, two other pathways of growth modulation, named intracrine and juxtacrine mechanisms, were described. Intracrine loop does not involve secretion of ligand, and receptor activation occurs intracellularly. Well characterized examples of intracrine activation are binding of the v-*sis* (the oncogene of the simian sarcoma virus) product to the platelet-derived growth factor

receptor (21, 22) and binding of COOH terminus-modified interleukin-3 to its receptor (23). Juxtacrine mechanism, on the other hand, involves a nondiffusible, cell membrane-anchored growth factor precursor protein interacting with its receptors on the surface of adjacent cells. This mode of intercellular communication might be important in supporting cell-cell adhesion during development, in addition to a more traditional role of mitogenic stimulation (24). Examples of juxtacrine growth factors include the precursor forms of transforming growth factor-alpha (proTGFα) (24) and epidermal growth factor (proEGF) (25). Interestingly, a recent report demonstrated the existence of mechanisms that control the switch of TGFα from a juxtacrine (membrane-bound) to a paracrine (soluble) growth factor (26).

A number of growth factors, when applied exogenously, have been found to be mitogenic for human breast cancer cells in culture. Included in this list are transforming growth factor-alpha (TGFα) and epidermal growth factor (EGF) both of which interact with the EGF receptor (27), the insulin-like growth factors (IGF) I and II , which are thought to mediate their growth effect in human breast cancer predominantly via the IGF-I receptor (28). In ER+, hormone-responsive MCF-7, T-47D, and ZR-75-1 cells, stimulation by estrogen is accompanied by an increase in the synthesis and secretion of a TGFα–like factor, which crossreacted with antisera specific to authentic TGFα (8, 12). However, this species has a molecular weight of 30 kDa, significantly larger than the 7 kDa processed form of authentic TGFα (8, 12). TGFα and EGF belong to the same family of growth factors, and share a common receptor (EGF receptor) (27). The EGF receptor is a ligand-inducible tyrosine kinase and has been detected in human mammary tumor biopsies in addition to the above established cell lines. Thus, it is likely that TGFα can act as a auto-stimulatory factor in breast cancer. Estrogen receptor-negative (ER-),

hormone-independent cells are usually more invasive and faster growing. Furthermore in these cells, TGFα-like activity was found to be constitutively expressed and substantially higher than their ER+ counterparts. This finding led to the suggestion that elevated secretion of growth factors such as TGF α, was a mechanism by which breast cancer progressed from a hormone-responsive to unresponsive state, an inevitable and undesirable clinical phenomenon since hormone responsiveness is a prerequisite for endocrine therapy of breast cancer (see Introduction 3). Recently published data, however, do not fully support this hypothesis (29). One such study directly addressed this question by overexpressing TGFα in MCF-7 cells; no significant growth advantage was observed either in vitro and in vivo (30) and more importantly, these cells did not acquire estrogen independent growth. Transgenic mice models have also been used to address the signifcance of TGFα overexpression in the mammary glands (31, 32, 33). All studies showed that the glands were hyperproliferative; in two studies, mammary cancer eventually developed after multiple pregnancies (32, 33). In summary, these transgenic studies all suggested a role of TGFα in the process of mammary carcinogenesis.

The insulin-like growth factors (somatomedins), IGF-I and IGF-II, represent another family of growth factors that is mitogenic for breast cancer cells (8, 11). Original studies by Lippman's group identified IGF-I-related species in all cultured human breast cancer cells. Later studies by the same group, however, suggested that these cells do not make authetic IGF-I. Subsequently, in situ hybridizations studies using human breast cancer biopsies showed that the IGF-I mRNA is expressed in the stromal but not in the malignant epithelial cells (11). Hence, IGF-I seems unlikely to play an autocrine role in human breast cancer growth regulation, although a paracrine function may still operate (34). IGF-II is

similarly expressed in stromal cells, but unlike IGF-I, in breast cancer epithelial cells as well, suggesting a paracrine and/or autocrine mode of action. The mRNA for IGF-II is present in at least two ER+ cell lines (MCF-7 and T-47D). Estrogen treatment increased IGF-II mRNA levels 4-5 fold above control levels (11), raising the possibility that the growth effect of estrogen may be mediated by IGF-II. Both IGF-I and II bind to the IGF-I receptor, a tyrosine kinase that shares strong homology with the insulin receptor. Binding of the factors to IGF-I receptor elicits proliferative effects (28). Interestingly, IGF-II can also bind to another receptor termed the IGF-II receptor which has no tyrosine kinase acitivity and appears to be identical to the mannose-6-phosphate receptor which is thought to be involved in lysosomal enzyme pathways (11). Current thinking suggests that the growth effects of both IGFs are predominantly mediated through the IGF-I receptor whereas IGF-II receptor may primarily be concerned with degradation of IGF-II.

Another newly discovered growth factor termed mammary derived growth factor 1 (MDGF 1) has been purified from human milk (29). Human breast tumors and established cell lines also appear to produce MDGF 1 and possess receptors for it. The factor has growth promoting activity on MCF-7 but not on ZR-75-1 or T-47D, all of which are ER+. Future studies should clarify in more detail the biological significance of MDGF 1 expression in the growth control process of breast cancer cells.

Most if not all breast cancer lines express mRNA for one or more members of the fibroblast growth factor (FGF) family which have potent mitogenic and angiogenic activties (8, 12). Many also secrete a platelet-derived growth factor (PDGF)-like activity (8, 12). Their functions, if any, in the growth control of normal and malignant breast tissue are at present poorly understood.

Whilst growth stimulatory factors undoubtedly have important functions in the proliferation process, it should be emphasized that growth inhibitory factors play an equally essential role in the balance of growth control. The most widely studied inhibitory peptides belong to the TGF β multigene family which comprises of TGF β 1,2,3,4, and 5 (TGF β4 and 5 have not been found in mammals) plus the inhibins, activins and mullerian inhibiting substance (35). Exogenous addition of either TGF β1 or TGF β2 inhibits cell growth of established breast cancer lines (29). The pattern of mRNA expression of TGF β 1, 2, and 3 varies among different cell lines, with T-47D expressing all three while MCF-7 expresses only TGF β1 (36). Most ER- cells express high-affinity TGF β receptors as well as one or more members of the growth factor family suggesting the possibility of an autocrine mechanism of inhibition (11, 37) However, conflicting data exist concerning the expression of TGF β receptors by ER+ lines; so far, T-47D has not been shown to express TGF β receptors, whereas for MCF-7 discrepant data exist (34, 38, 39). The discrepancies may be due to differences in subclones and/or culture conditions used in different laboratories. In hormone-dependent MCF-7 cells, Knabbe et al., (40) reported that estrogen suppressed and antiestrogen induced the expression of TGF β. Furthermore, exogenously added TGF β was growth inhibitory for their MCF-7 cell line. These findings support the hypothesis that antiestrogens mediate their effects, at least in part, via enhanced secretion of auto-inhibitory molecules (40 and section Introduction 3.1). However, cases exist in which ER+ and TGF β receptor-negative breast cancer cells (eg. T-47D) are still sensitive to the growth inhibitory effects of antiestrogens, indicating that mechanisms other than those involving TGF β are also involved (35, 36, 37, 39). Furthermore, treatment of T-47D cells with antiproliferative progestins resulted in a 3-fold reduction of TGF β1 mRNA levels (39). These data are not consistent with TGF β being the only mediator of the growth inhibitory response in human breast

cancer cells. Nevertheless, TGF β is a potent growth inhibitor for certain breast cancer cells, in particular ER- cells (40, 41). More studies in these areas are urgently needed to provide a clearer and unifying picture. TGF βs are not the only known growth inhibitory peptides. At least three other factors with antiproliferative properties have been discovered recently (29). Amphiregulin, a member of the EGF/TGFα family, appears to inhibit breast tumor but not normal mammary epithelial cells (29). The other two, mammastatin and mammary derived growth inhibitor, both inhibit proliferation of normal and malignant mammary epithelial cells in vitro (29).

The vast body of data in the literature undoubtedly demonstrates that breast cancer cells synthesize and secrete a panoply of polypeptide molecules potentially important in the regulation of growth and in the process of tumorigenesis. These findings also have potentially important clinical implications. Blockade of membrane growth factor receptors using monoclonal antibodies could interrupt critical growth regulatory pathways resulting in growth inhibition of breast cancer cells (34), thus offering in the future new treatment strategiesfor this increasingly important disease.

Much has been said about the possible functions of growth factors in mammary carcinogenesis. We should, however, not lose sight of the fact that the same growth factors might also perform critical functions in the nomal development of the gland as well. For instance, normal epithelial cells in culture secrete TGFα, and TGF β and mammastatin (8, 12, 29). The TGFs are also normal constituents of human milk (8, 12). Implants of EGF or TGFα in mammary fat pads of rodents were able to induce lobuloalveolar development, whereas similar studies with TGF β resulted in complete cessation of ductal development (8, 12). Moreover, IGF-I

and II receptors are present in lactational states of normal breast (8, 21). These few examples clearly provide good evidence to support a physiological function for this important class of regulatory peptides in mammary gland development and/or function.

2. EPIDERMAL GROWTH FACTOR AND ITS RECEPTOR

2.1 Biological Effects

Epidermal growth factor (EGF) belongs to a family of peptide growth factors that includes TGFα, amphiregulin, the vaccinia virus growth factor, the myxoma virus and shope growth factor (27, 42, 43). They all share the property of high-affinity binding to the EGF receptor and elicit mitogenic responses in EGF-sensitive cells. EGF itself is a 53-amino acid, 6045 Mr peptide that was first isolated from the submandibular gland of male mice and thereafter from human urine, where it was originally known as urogastrone (27). EGF is synthesized from a large precursor which in the human is 1207 amino acid residues. The mature EGF sequence is located near the C terminus of the precursor at residues 971-1023; the precursor molecule presumably undergoes some processing event (e.g. arginine-specific endopeptidase cleavage) leading to release of the mature 53-amino acid EGF (27). At a cellular and molecular level, the addition of EGF to normal cells elicits a whole range of cellular events including a rapid increase in membrane Na^+/H^+ antiport activity leading to cytoplasmic alkalinization, an increase in intracellular calcium concentration and metabolism such as glucose and amino acid transport, ATP turnover, and increased ornithine decarboxylase activity (43, 44). EGF binding to its receptor evokes phosphorylation of several candidate signal transducer enzymes including casein kinase II, phospholipase Cγ, GTPase activator protein (GAP) and the Raf-1 kinase, stimulates a variety of tyrosine as

well as serine and threonine phosphorylations (42, 43). In addition, the mRNA levels of cellular proto-oncogenes c-fos and c-myc (e.g. in fibroblasts) are induced by EGF (42, 44). Under sterum-starved conditions, these cells respond to EGF with increased growth as well.

EGF is a potent mitogen for a variety of ectodermal and mesodermal cell types. Among the EGF-responsive cells of ectodermal origin are skin keratinocytes, conjunctival and pharyneal tissues which proliferate under its influence leading to enhanced keratinization (27, 44). Among the cells of mesodermal origin are granulosa cells, corneal endothelial cells, vascular smooth muscle, chondrocytes, and fibroblasts. In vivo and in organ culture, EGF promotes proliferation of the basal cell layer of various epithelia. This enhanced proliferation in turn leads to epidermal keratinization, the most prominent consequences of which in the neonate is the stimulation of precocious eyelid opening and tooth eruption (27, 44). This property of being a potent mitogen on epithelia is directly related to the many proposed physiological roles for EGF in adult animals and during neonatal development.

2.2 Physiological Functions

The clarification of the physiological role of EGF has been hampered by the inability to experimentally lower EGF levels in laboratory animals due to the absence of a single site of synthesis. Therefore, most if not all of the proposed functions for EGF are inferred from studies involving its exogenous administration to animals, from cell and organ culture systems and from the normal distribution of EGF and its receptor. EGF is found to be widely distributed in nearly all body fluids. In human, relatively high concentration of EGF is present in prostatic and seminal vesicle fluids, sweat, urine and milk (27, 44). It is also secreted in saliva,

pancreatic juice, bile and tears. Since the main effect of EGF is to promote wound healing by accelerating epithelial cell proliferation, the above data have led to the hypothesis that a major role for EGF in adult animals is to maintain epithelial surfaces including those lining the eyes, gut, and urinary tract. In the gastrointestinal tract, this maintenance is further assisted by the inhibitory effect of EGF on gastric acid secretion minimizing ulceration (45). Hence, EGF derived from milk (mammary gland produces and secretes EGF) has also been suggested to be important in the gut development of the neonate. Removal of the salivary glands (the main source of EGF in the mouse) or administration of EGF antiserum increased spontaneous abortion rate in the pregnant mouse, suggesting an important but undefined role of EGF in the pregnancy process (27, 44). In addition, EGF could also be a fetal hormone important for fetal development since it stimulates lung, gastrointestinal, and pancreatic maturation; promotes palatal development; and stimulates thyroid and adrenal gland growth (44). Moreover, the onset of the endogenous synthesis of EGF in most tissues in the developing mouse occurs during early neonatal period, coinciding with the stages of maximum growth and development (46). As mentioned in section 1.3, EGF has been shown in organ culture to stimulate lobuloalveolar development and epithelial cell proliferation of the mammary gland (9, 47). In ovariectomized mice, implanted EGF also stimulated ductal growth and morphogenesis (48). These two findings lend support to an involvement of EGF in normal mammary tissue development.

2.3 Oncogenic Potential of EGF/EGF Receptor

Knowing that EGF has potent mitogenic activity and is a key component in the growth control pathway of many epithelial cells from which most types of malignancy originate, it would be logical to speculate that EGF can potentially function as a transforming protein. However, to date no oncogene product has

been identified as the homolog of EGF. Nevertheless, several lines of evidence indicate that an EGF-stimulated regulatory system may play a role in carcinogenesis. Firstly, EGF stimulation elicits transformation-associated responses such as the induction of the proto-oncogenes c-fos and c-myc, enhanced secretion of plasminogen activator, and decreased fibronectin synthesis (27, 44). Secondly, EGF can act as a tumor promoter in vivo in tissues like skin, rectum and pancreas and promotes viral transformation in vitro (27, 49). EGF may also indirectly promote cancer growth by suppressing the immune system (43, 49) possibly through suppression of T-cell function and growth inhibition of lymphoid tissues such as the thymus, spleen, and bone marrow. Stern et al., (50) performed transfection studies in an attempt to assess the oncogenic potential of EGF. Constitutive expression of EGF in FR 3T3 and rat-1 fibroblasts conferred a transformed phenotype as assayed by tumor formation in nude mice. Three other studies also showed that in NIH 3T3 cells overexpressing the normal EGF receptor, the presence of the ligand was required to maintain transformation (51, 52, 53).

With regard to mammary tumorigenesis, circumstantial evidence exists to support a role of EGF in the process. Using a mouse strain with intrinsically high susceptibility to spontaneous mammary tumors, it was shown that the rate of tumor formation could be reduced by removal of the submandibular gland which is the main source of EGF in mouse (54). Moreover, reinfusion of EGF into the sialoadenectomized mice could return tumor incidence to the normal level, implicating the growth factor as the active constituent. In human breast cancer biopsies, Dotzlaw et al., (55) demonstrated the expression of EGF mRNA in 83% (50/60) of tumor samples. Interestingly, tumors that were either ER+ or progesterone receptor-positive (PgR+) had an increased incidence of EGF

expression (55). These data along with previous studies confirming the existence of EGF receptors in breast tumors clearly suggest a functional role of EGF in neoplastic mammary tissue.

The membrane receptor for EGF is a 170-kDa glycoprotein with intrinsic protein-tyrosine kinase activity (27, 42-44). It is composed of a large extracellular ligand-binding domain, a single hydrophobic membrane-spanning domain, and a cytoplasmic domain containing the tyrosine kinase activity. The binding of EGF to the extracellular ligand-binding domain activates the cytoplasmic kinase domain, which then undergoes autophosphorylation and subsequently phosphorylates cellular substrates whose functions dictate the ultimate cellular response. Theoretically, qualitative or quantitative alterations of receptor function can lead to transformation by virtue of a continuous mitogenic signal from the receptor. Indeed, the v-erb B oncogene of avian erythroblastosis virus was found to be a truncated form of the EGF receptor, devoid of most of the ligand-binding domain. Moreover, the EGF receptor is frequently overexpressed in various types of tumors including mammary tumors. Several studies have established a marked inverse correlation between estrogen receptor levels and EGF receptor levels in human breast cancer. Also, ER- cells show some 2-10-fold higher expression of EGF receptor than ER+ cells (27, 56). Moreover, EGF receptor overexpression in human breast tumors is associated with a poor overall prognosis and early recurrence (57, 58).

3. ENDOCRINE THERAPY OF BREAST CANCER

The hormonal responsiveness of human breast cancer has been recognized since George Beatson reported in 1896 that bilateral ovariectomy induced tumor regression in some premenopausal patients with advanced disease (7). Since then,

a variety of therapeutic approaches involving hormonal manipulation have been introduced which can essentially be divided into ablative hormonal therapies (surgical ovariectomy, adrenalectomy, hypophysectomy) and additive hormonal therapies (59). The latter involves administration of drugs that are known to inhibit hormone synthesis or to antagonize hormone action in one way or another. Like other cancers, once a breast cancer has spread to a distant or even local metastatic site, the disease is no longer curable by methods such as radical mastectomy. The goals of treatment become palliative with the hope of prolonging the patient's life while at the same time maintaining its quality. Endocrine therapy provides, in addition to cytotoxic chemotherapy, an effective means of achieving the above goals. Furthermore, with the introduction of accurate steroid hormone receptor measurements in the mid-70s, better selection of patients with hormonally responsive tumors became possible. It is now an established fact that the ER and PgR status of a breast cancer is an indicator of response to all types of endocrine therapy (60, 61). While less than 10 percent of receptor-negative tumors respond to any major form of endocrine therapy, response rates can be as high as 70 to 80 percent in tumors which are positive for both ER and PgR (61). The following section will deal only with additive hormonal therapies emphasizing those that are most commonly used in clinical settings.

3.1 Antiestrogen

As discussed in section 1.2 in the Introduction of this thesis, there is now a large body of evidence supporting a role for estrogens in the growth of mammary cancer. Although surgical castration allows removal of primary sites of estrogen production, there is persistence of low levels of estrogen production from peripheral tissues (62). Hence chemical compounds that have the properties of antagonizing the effects of estrogen are commonly employed to further refine the

approach of hormonal therapy. Many different nonsteroidal antiestrogens have been synthesized, however tamoxifen (TAM) is the only clinically relevant agent in the treatment of breast cancer because of its low toxicity and superior efficacy.

Tamoxifen (Nolvadex), a derivative of triphenylethylene is now the treatment of choice for postmenopausal hormonally responsive breast cancer patients, both in the adjuvant and advanced disease settings (63, 64, 65). The mechanism of the antitumor activity of TAM is unknown but probably depends principally on its antiestrogenic properties. The drug is known to compete with estradiol for binding to the nuclear ER resulting in the formation of receptor complexes with altered properties (66). Inactivity of the antiestrogen/ER complexes is not due to the inability to bind to target DNA; rather these complexes are found to be transcriptionally nonproductive (67). Knowing that TAM operates through the ER, the drug should be ineffective in ER negative tumors. Nevertheless, 10-15% of receptor-negative tumors exhibit an objective response to TAM (68) suggesting that non-ER mediated antitumor effects may also exist (66, 69). The blockage of estrogen action by TAM renders the estrogen-dependent tumor cell unable to progress through the cell cycle by arresting cells in the Go/G1 phase of the cell cycle (70, 71). Because of this cytostatic rather than cytotoxic property, endocrine therapy using TAM necessitates long treatment periods to maintain high blood levels of the drug.

One of the major clinical problems associated with TAM treatment is that drug resistance and patient relapse inevitably occur and such therapeutic failure eventually becomes fatal. Known mechanisms by which tumor cells become hormone unresponsive and resistant to TAM include loss of steroid receptors, steroid-independent autocrine/paracrine stimulation of cells by growth factors and

the development of tamoxifen-dependent/stimulated cancer cells that may occur after long periods of adjuvant therapy (66, 72). The latter mechanism may be caused by the weak estrogenic (agonist) activity of TAM, a property that has been well documented. Depending on the target organ, experimental conditions and animal species, the antihormone can also act as a full or partial estrogen (66, 73). The discovery of pure antiestrogens which behave only as an estrogen antagonist thus opened the door to more efficacious endocrine treatment.

Among these new generation antiestrogens that show promising preliminary results, is the steroidal antiestrogen ICI 164,384. The compound binds to ER with substantially higher affinity than TAM (66, 74). Until recently, ICI 164,384 was considered as a full antagonist (pure antiestrogen) exhibiting no agonistic activity in all the models studied. The exception is found in the guinea-pig uterus and vagina isolated cell system where the compound acts as an real agonist with respect to induction of the progesterone receptor (75). The ability of ICI 164,384/ER complexes to interact with target DNA remains controversial. The pure antiestrogen has been reported to form ER complex that is defective in DNA binding (74), whilst another study reported the formation of transcriptionally inactive but DNA-binding complexes (67). Nevertheless, ICI 164,384 has been shown to inhibit tamoxifen-stimulated growth, to more completely block the stimulatory actions of estrogen and is less prone to establishment of drug resistance at least in long-term culture (66, 74). Overall pure antiestrogens such as ICI 164,384 may offer therapeutic advantages over partial agoinsts like tamoxifen.

3.2 Progestin

Progestins have emerged as important agents in the additive hormonal therapy of advanced breast cancer in postmenopausal women. The mechanisms of

its antitumor action are largely unknown. Progestins appear to exert a direct growth inhibitory effect in human breast cancer cell lines (76). Other possible modes of actions include facilitating the conversion of estradiol to the less potent estrogen estrone, suppression of pituitary ACTH secretion leading to estrogen deprivation and metabolic conversion to estrogen (77). Although the major activities of progestins are assumed to be PgR-mediated, the compounds may also bind to the androgen and glucocorticoid receptors, thus opening the possibility of alternative modes of antitumor action (78).

Megestrol acetate (Megace) and medroxyprogesterone acetate (MPA) are the most commonly used progestins in the treatment of advanced breast cancer. Both agents are without significant side effects, except for edema and weight gain associated with high dose treatment (77). When used in conventional low doses (<500 mg daily), MPA was found to be less effective than megestrol acetate which has been shown to be comparable to tamoxifen in terms of therapeutic efficacy (77). However the antitumor action of MPA can be drastically increased by the use of high dose regimens (>500 mg daily). Overall response rates to high dose MPA treatment average 40% in unselected breast cancer patients (79). This is comparable to that observed with conventional doses of megestrol acetate or tamoxifen. Even so, the more general use of high dose MPA in breast cancer is as a second line therapy after relapse following other endocrine therapies. Specifically, it has been shown to induce remissions in 20-30% of patients who developed resistance to tamoxifen (80). The underlying mechanisms of the antitumor activity of MPA are poorly understood. In established human breast cancer cell lines, the effect of progestins is predominantly growth-inhibitory (13). In the few cases demonstrating stimulatory effects of progestins on cell growth, the control cells have been manipulated to grow at very slow rates, and the maximal

increase in cell number is never greater than 2-fold (13). Like other synthetic progestins, MPA has a high affinity for PgR as well as for androgen (AR) and glucocorticoid receptors (GR) in various tissues and in human mammary tumors (78, 81). Accordingly, the effects of MPA on cell proliferation could theoretically result from interactions with up to 3 receptor systems. Using the human breast cancer cell line ZR-75-1, which possesses functional receptors for estrogens, androgens, progestins and glucocorticoids, Poulin et al., (82) showed that MPA (1-100 nM) strongly inhibited cell proliferation mainly through its interaction with the AR, while the drug's glucocorticoid-like activity could play an additional role at concentrations exceeding 100 nM. The contribution of a PgR-mediated inhibition of cell growth in this system was limited, and significant only at subnanomolar concentrations (<1 nM). With these recent findings, the general assumption that the therapeutic efficacy of MPA is due to its PgR-mediated effects might need to be revised. In fact, most studies have failed to show a significant correlation between objective response to progestin therapy and PgR status (83). In addition, in vivo studies have shown that MPA inhibits gonadotropin secretion by direct interaction with pituitary AR (84) whereas the adrenal-suppressing activity of MPA, can most easily be explained by a glucocorticoid-like activity (77). One outcome of the above two actions of MPA is reduction of estrogen synthesis, which may contribute significantly to its antitumor activity. That the data obtained by Labrie's group might indeed be applicable to the clinical settings is supported by the plasma concentration of the progestin measured in patients undergoing high dose MPA therapy. Thus, serum levels of MPA in women treated with the compound are such that its androgenic effect is maximal and at the same time high enough to be within the range of the GR-mediated antiproliferative effects (85).

As with most anticancer drugs, development of drug resistance is a major clinical problem limiting the usefulness of MPA in the treatment of breast cancer. Possible mechanisms whereby cancer cells become resistant include those previously suggested with respect to TAM resistance. Our own data suggest that increased growth factor expression and decreased PgR levels are part of the mechanisms involved in MPA resistance (86). The development of MPA-dependent tumor cells has also been reported both in cell culture (87) and in animal models (88). In fact, many lines of evidence suggest that MPA might even enhance tumorigenesis, a finding that may have significant clinical implications in the use of progestins in breast cancer treatment (13, 89). Nevertheless, the majority of data show an equally good treatment response to high dose MPA as to TAM.

3.3 Aminoglutethimide and others

As already mentioned, estrogen is the major stimulus for the growth of hormone-dependent breast cancer. Although the ovaries are the principal sites of estrogen synthesis, estrogens are also synthesized in multiple other areas such as the adrenal gland and adipose tissue (77). Estrogen production in peripheral tissues accounts for the persistence of its production in postmenopausal women. The female hormones are synthesized by the aromatization of androgens via the enzyme aromatase which catalyzes the rate-limiting step. It is the presence of aromatase in fat, muscle, liver, hair follicles, and even cancer cells that endows these tissues with the ability to produce estrogens from androgens. Consequently, much effort has been devoted to the development of aromatase inhibitors as therapeutic agents for breast cancer.

Aminoglutethimide is a potent inhibitor of adrenal steroidogenesis by inhibition of the enzyme aromatase. The drug effectively blocks androgen conversion to estrogen in adrenal tissue, adipose tissue and breast cancer tissue as well (62). Although clinical responses to aminoglutethimide are similar to those observed in patients receiving tamoxifen, MPA or megestrol acetate, the greater severity of side effects associated with its use compared to the latter agents has made aminoglutethimide at best a third line treatment modality (77). For the same reasons, attempts are now being made to lower aminoglutethimide dosage and to develop new improved aromatase inhibitors.

Tumor regressions can be achieved with other hormone additive therapies such as high dose estrogens, glucocorticoids and androgens (77). Large doses of estrogens were the treatment of choice in postmenopausal patients prior to the introduction of tamoxifen. It is now rarely used since TAM treatment which is equally effective shows less side effects. The mechanism of the antitumor action of androgens and glucocorticoids is largely unknown. Whatever the mechanisms, both androgens and glucocorticoids are less effective than other agents such as TAM and progestins.

4. REGULATION OF EUKARYOTIC GENE EXPRESSION:LEVELS OF CONTROL

4.1 General Overview

Differential gene expression not only underlies the molecular mechanisms of eukaryotic development but also forms the basis of cellular response to extracellular stimuli (e.g.hormones). The importance of regulated gene expression can be easily appreciated considering that any anomaly in one of its critical steps

could result in a deleterious outcome as exemplified by numerous genetic diseases and uncontrolled proliferation during carcinogenesis. Therefore, the many steps leading to the formation of a protein beginning from the genetic blueprint to the actual polymerization of amino acids are controlled in a very precise way (Fig. 1). Not implicated in Fig. 1 is the growing awarness of the importance of subcellular higher-order structure (e.g. chromatin structure) in controlling gene expression (90, 91), an aspect that deserves emphasis but will not be covered here. From the standpoint of a mRNA molecule, its steady-state levels can be modulated at any step in its biogenesis, including transcription initiation and/or elongation, post-transcriptional processing, nuclear export or cytoplasmic degradation of the message (Fig. 1). On the other hand, the activity of a particular protein molecule such as an enzyme could be adjusted by controlling its abundance (transcriptional, post-transcriptional, translational) or by augmenting its activity after its production i.e.post-translationally. In this section attention would be focused on processes involved in controlling the abundance of a gene product, that is, the quantity of either an RNA or a protein molecule. Processes that lead to qualitative changes (eg. alternative splicing, phosphorylation) will be mentioned briefly for the sake of completeness of this discussion. Also, an attempt will be made to limit the discussion to eukaryotic system. Moreover, since this research project is related to steroid hormone-regulated systems, wherever possible, discussion will be constructed around the context of steroid involvement in each of the regulatory level.

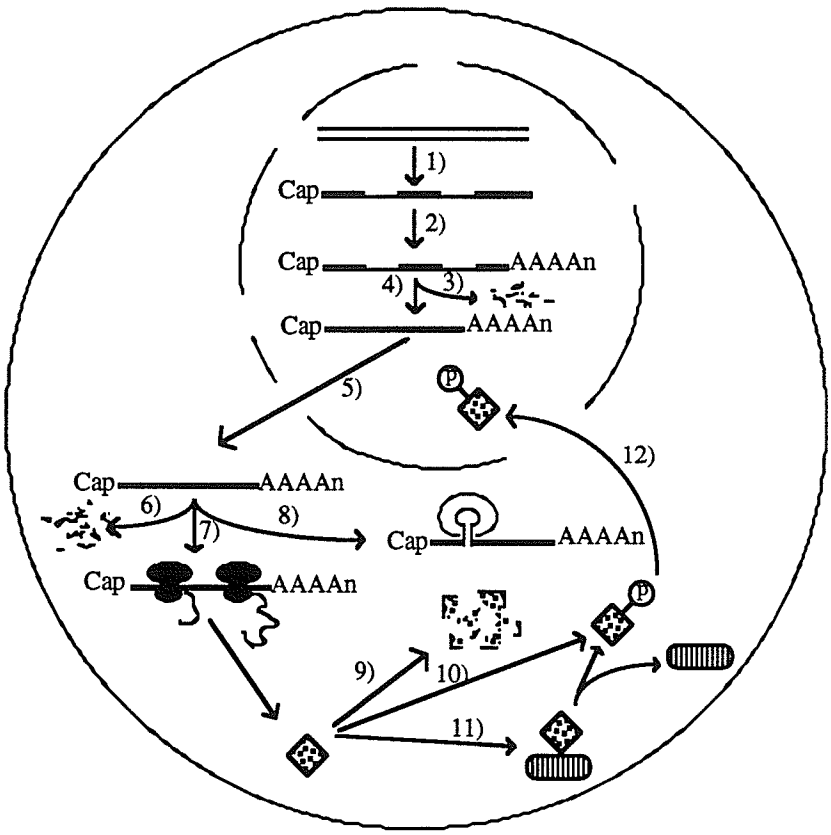


Fig. 1. Control of gene expression in eukaryotic cells

Figure legend:

protein

cytoplasmic inhibitor

Ⓟ post-translational modificaion (e.g.phosphorylation)

1) transcription
2) poly (A) tail processing
3) pre-mRNA turnover
4) splicing
5) nuclear mRNA export
6) mRNA turnover
7) translation
8) translational repression
9) protein turnover
10) post-translational modification (e.g. phosphorylation)
11) protein squestration
12) nuclear protein translocation

4.2 Transcription

4.2.1 Basal Transcription by RNA polymerase II

The past several years have witnessed considerable effort to elucidate the mechanisms that control the transcription of genes by RNA polymerase II (Pol-II). Specifically, the effort has been devoted to two areas: the identification of specific cis-acting DNA elements involved in the regulation of transcription and the isolation, characterization, and cloning of trans-acting protein factors that interact with the control sequences. Trans-acting factors or transcription factors can be grouped into general and regulatory types (transcriptional activators or repressors), the former being involved in the basal transcription process whereas the latter is concerned with achieving an induced or repressed state of transcription.

Regions upstream of mRNA start sites in Pol-II-transcribed genes contain short signal sequences recognized by their cognate transcription factors. Two types of DNA element can be distinguished: promoters are located immediately upstream around the transcription start site and are required for accurate and efficient initiation of transcription (92, 93). Enhancers are traditionally defined as cis-acting regulatory elements that modulate the activity of promoters irrespective of their orientation, position and distance from the start site (92, 93). Enhancers can be situated and function at distances up to thousands of basepairs (bp) upstream or downstream from the start site. They are usually not considered as a basic requirement for basal level transcription although some genes do need enhancers to stimulate transcription to a detectable level. The enhancer elements contain short consensus sequences that are recognition sites for sequence-specific protein factors of the regulatory set, many of them are tissue- or cell-specific (92). Binding of activators to enhancer elements in turn augment the activity of promoters by altering the frequency of transcriptional initiation (94). It is becoming increasingly clear that

the components of promoters and enhancers are very similar entities, having many common properties. Both promoters and enhancers should now be regarded as regions of DNA composed of discrete functional modules consisting of about 7-20 bp of DNA; each module in turn contains one or more recognition sites for transcription factors (92). On the DNA side, binding sites for protein factors in promoter or enhancer region could either be contiguous or overlapping. Functionally, individual modules can act cooperatively or independently to activate transcription. To further blur the distinction between promoter and enhancer, many factors (eg. Sp1) that bind to promoter upstream regions are also capable of binding to classical enhancers and activate long-range gene transcription (92). Thus depending on the gene of interest, there may not be factors specific only to enhancer elements. For the sake of simplicity, enhancers are restricted here to regions of DNA with the ability to stimulate transcription at a distance and regardless of orientation. Promoters must be able to direct initiation of transcription in a particular orientation and are usually located immedately upstream of start sites (93).

Sequence analysis of many eukaryotic Pol-II promoters reveals a common pattern of organization. Many promoters of higher eukaryotic genes contain an AT-rich 'TATA box' (consensus is TATAAA) region at position -25 upstream of transcriptional start site which serves to specify the position of transcription initiation. Further upstream at around -75 is the CAAT box (the core consensus motif is GGCCAAT) which functions to enhance the frequency of transcription (93). Both the TATA and the CAAT boxes are position dependent and operate only in the above-mentioned locations. In addition, a doublet of sequence CA is frequently found with the A at position +1, the transcription start site (93, 111). It should be noted that not all Pol II-transcribed genes contain a TATA-box promoter.

Now, it is clear that TATA box-containing promoters usually belong to the class of genes known as non-housekeeping genes. Housekeeping genes, however, often lack both the TATA and CAAT boxes. In these cases, positioning of start sites is determined by other discrete elements such as the GC box. In view of the large assortment of promoter regulatory elements for Pol-II genes, one can only say that each gene has its own combination and arrangement of cis-acting elements that act in concert to confer a unique pattern of expression.

In recent years, general transcription factors which assist Pol-II in the process of transcription have been isolated and purified to a high degree. This has been due mainly to the development of sequence-specific DNA affinity chromatography and in vitro reconstituted transcription systems. General transcription factors could be classified as transcription initiation factors and elongation factors, each required in separate steps of transcription. The most commonly used nomenclature for general transcription initiation factors is TFIIA, TFIIB, TFIID, and TFIIE (95, 96). Along with Pol-II, these transcription initiation factors are thought to assemble into stable preinitiation complexes in the promoter region prior to actual initiation of transcription. The first step of TATA box- dependent transcription initiation begins with the binding of factor TFIID to the TATA box followed by TFIIA binding to form a template-committed complex. Subsequently, a complete, stable preinitiation complex is formed by the association of TFIIB and Pol-II which then is converted at a later time to a rapid-start complex by association with TFIIE (95, 96). In the presence of ATP and NTPs, the rapid-start complex can initiate RNA synthesis (functional initiation complex) with no appreciable lag. Capping of the first nucleotide by guanyl transferase appears to occur concurrently with the formation of the first two phosphodiester bonds (95). It must be emphasized that the establishment of the preinitiation complex itself is

energy-independent, but activation of transcription initiation requires the hydrolysis of high-energy phosphate bonds. The resulting energy may be used to trigger the melting of the DNA double helix around the start site, a speculation that has gained support from the recent work of Sopta et al., (97) who demonstrated that the RAP30/74 complex (components of a subfraction of TFIIE, designated TFIIF) has an ATP-dependent helicase activity. At least two transcription elongation factors, SII, also called TFIIS and P37, interact with the transcription complex (containing Pol-II) to regulate the process of elongation (95, 96) In vitro kinetic experiments show that SII stimulates elongation by increasing the efficiency with which the RNA polymerase passes through pause sites. Little is known about the sequences or factors required for transcription termination. Since the 3' ends of most Pol-II transcripts are cleaved and processed, mature mRNAs do not contain the termination site (98). It is now believed that termination occurs randomly downstream of the cleavage site (poly (A) addition site) and most frequently occurs in areas rich in poly dT or poly dA.

4.2.2 Regulated Transcription

4.2.2.1 General introduction

Eukaryotic genes are regulated differentially in response to a complex set of environmental and developmental cues. In a multicellular organism, the former would constitute stimuli such from hormones, growth factors, and environmental stresses. Regulation at the transcriptional level is now known to be the most widely used and the primary control point to achieve differential gene expression (99). As in the case of basal transcription, this process is mediated by a plethora of sequence-specific transcriptional regulatory proteins which could either activate or repress transcription by augmenting the activity of the basal transcription apparatus. The cognate cis-acting DNA elements to which this particular category of protein

factors bind, can be dispersed over great distances in the 5'- or 3'-flanking region of a gene, or even be present in introns. In some cases, adjacent cis elements may cooperate to exert a positive synergistic effect, in others, overlapping or superimposed binding sites for different factors can result in different factors competing for the same site leading to negative, inhibitory effects. Furthermore, the same cis element may confer different physiological responses in different cell types, depending on abundance of the factor(s) and their level(s) of activity in different cell types. With such diversity in the type and functionality of transcriptional regulators, along with the aforementioned variation in the layout of control elements, it is possible to develop hypotheses (e.g. combinatorial binding of a variety of transcription factors to different DNA binding sites) to explain how cells can control coordinately the expression of different sets of genes, while at the same time modulating the transcription of an individual member.

Analysis of recently cloned sequence-specific transcription factors has revealed that they consist of discrete structural domains with sequence-specific DNA binding or transcriptional activation functions (99, 100, 101). Four common motifs have been identified to be important for sequence-specific DNA binding: the helix-turn-helix (102), leucine zipper (103, 104), zinc fingers (two types) (105, 106), and the helix-loop-helix (107). The leucine zipper and the helix-loop-helix motifs do not act directly as a DNA-binding domain, rather they act as a dimerization interface, serving to bring into correct positioning the adjacent highly basic regions which form the actual DNA contact surface. At present, three common transcriptional activation domains have been characterized, namely, acidic blobs, glutamine-rich regions, and proline-rich regions (99, 101). In the following section, selected examples of transcription regulators that participate in different

signal transducing pathways are discussed in some detail to illustrate the mode of action of this important class of protein factors.

The products of the protooncogenes, c-fos and c-jun, are nuclear transcription factors containing leucine zipper domains (108, 109, 110). They are two components of a transcription factor historically known as activator protein-I (AP-I) which is now known to consist of a family of related but distinct protein factors which all share the common property of binding to the DNA sequence TGACTCA, termed TPA responsive element (TRE) or AP-I binding site. In vitro assays indicated that the Fos protein by itself is incapable of binding to AP-I sites whereas the Jun protein alone can bind as a homodimer. Dimerization is a prerequisite for Jun-DNA binding. The inability of Fos to achieve DNA binding is due to its inability to form homodimers. However, Fos can dimerize via the leucine zipper with a Jun molecule and this heterodimeric complex is found to be more stable than a Jun-Jun homodimer (108-110). Thus, the prevailing physiological concentrations and/or activity of these two factors in a cell, at a given time, will dictate the binding characteristics of Jun and Fos complexes for the AP-I site.

In most quiescent cells, the level of the Fos protein and its mRNA are undetectable whereas that of c-jun are very low. However, stimulation by hormones, serum, growth factors, or phorbol esters leads to rapid induction of both c-fos and c-jun expression. The heightened levels of the two factors would result in accumulation of active, DNA-binding Jun-Fos heterodimers which in turn cause transcriptional activation of genes (eg.metallothionein IIA, collagenase) containing AP-I sites as one of their regulatory elements (110). But what are the molecular mechanisms underlying the induction of c-fos and c-jun? In the case of c-fos, mutational analysis has identified an enhancer element, around 300 bp upstream of

the start site, that is essential for serum induction of the gene. This enhancer is designated the serum response element (SRE) and contains two modules, the dyad symmetry element (DSE) and an overlapping AP-I site (98, 111). A 67-kDa, serum-induced protein factor, designated the serum response factor is found to bind to the DSE within the SRE and acts as a positive transcription factor for serum-induction of c-fos expression. It is clear that such regulated synthesis of a transcription factor only sets the problem of gene regulation one stage further back, requiring mechanisms to activate the transcription of the gene encoding the transcription factor itself. To prevent an endless futile cycle, it is not suprising that post-transcriptional mechanisms often are involved in modulating the activity of pre-existing factors.

A cell orchestrates its responses to extracellular signals through signal transduction pathways one of them being mediated by cAMP. Most if not all physiological effects of cAMP are mediated by cAMP-dependent protein kinases (protein kinase A or PKA) whose regulated enzymatic activity causes alteration of basic patterns of gene expression (112). Many genes that respond to elevated cAMP levels contain a cAMP response element (CRE), TGACGTCA, in their promoter-regulatory regions. Functional CREs have been identified in genes encoding somatostatin, proenkephalin, α-chorionic gonadotropin, phosphoenolpyruvate carboxykinase, and tyrosine hydroxylase (112, 113). In general, the CRE displays the properties of a classical enhancer, stimulating transcription in a manner that is independent of position and orientation. The response to cAMP requires PKA and is mediated at the nuclear level by a family of transcription factors called cAMP response element binding proteins or CREB, of which there are at least seven related members (114, 115). Like Fos and Jun, the structure of CREB contains a leucine zipper and an adjacent basic DNA binding

domain. A region rich in negatively charged glutamate residues is thought to form the acidic activation domain (113). Phosphorylation of a serine has been shown to be responsible for transcriptional activation. However, phosphorylation does not alter the binding affinity of CREB for the CRE, rather it probably acts to cause a conformational change of the CREB protein, rendering it transcriptionally active (113, 116). The activated CREB can interact with its target protein TFIID, the TATA box binding factor, to facilitate the formation of initiation complex and thus results in increased rate of transcription (113, 116). Recently, several laboratories have reported the formation of heterologous dimers between members of the CREB family and those of the Jun family (114, 115, 117). The ability of one transcription factor to not only heterodimerize with members of the same family but also to different family of transcription factors offers enormous scope for gene regulation in providing the cell with additional flexibility in the control of gene transcription.

Although gene control through transcriptional activation has gained the most attention, a growing number of cases have been described in which selective repression of transcription acts as an important mechanism of transcriptional control. The underlying mechanisms of transcriptional repression can be arbitrarily divided into 3 classes: inhibition of DNA binding, blocking of transcriptional activation, and silencing (118). Blocking of activation can take the form of quenching (the repressor protein binds directly to the activator protein and neutralizes its function) and/or squelching (sequestration of transcription factors via protein-protein interaction) (118, 119). Silencing differs from the others in that it has no requirement for a particular order or arrangement of positive and negative regulatory elements. Analogous to enhancers, silencers act in a position- and orientation-independent manner (118).

A relatively new form of transcriptional control has been demonstrated increasingly in eukaryotes. Termed attenuation, elongation block, or premature termination/pausing, the mechanism offers another level of gene regulation during the elongation phase of transcription (111, 120). It resembles to a certain extent the well-studied prokaryotic counterpart of attenuation except that translation is not involved. To avoid confusion, the term attenuation will not be used here. The first example of such a mechanism was discovered by Bentley and Groudine (121) in the c-myc proto-oncogene. Induced differentiation of human HL60 cells by agents such as dimethyl sulfoxide is associated with a 10-fold reduction of steady-state c-myc RNA. This reduction could be accounted for entirely by a block to transcription elongation at the 3' end of exon 1 (121). Since it is not clear whether this elongation block is true termination with release of transcripts, or the result of premature polymerase pausing or both, the terms premature termination, pausing and elongation block are frequently used interchangeably in the literature. Other examples of elongation control include the genes for c-fos, c-myb, adenosine deaminase, and EGF receptor (122, 123).

4.2.2.2 Steroid Hormone Receptors: prototypes for ligand-regulated transcription factors

Steroid hormones play important roles in cell growth, differentiation, and development of higher eukaryotes. Upon entry into the cell through passive diffusion, steroid hormones bind to high affinity receptors, which belong to the class of ligand-inducible transcription factors. Over the last few years, it has become clear that steroid hormone receptors belong to a large superfamily of receptors whose ligands include steroids, thyroid hormones, several vitamins and xenobiotics (124, 125, 126, 127, 128, 129, 130). On the basis of structure and function, the family can be divided into two groups: one including the

glucocorticoid (GR), progesterone (PR), androgen (AR), mineralocorticoid (MR) and dioxin receptors, and the other including the thyroid hormone (T3R), vitamin D3 (VDR), retinoic acid (RAR) and estrogen receptors (ER). Also belonging to this superfamily are the so-called orphan receptors for as yet unidentified ligands (124, 127, 130). The intracellular localization of the unoccupied receptors differs amongst receptor, with the GR being cytoplasmic. ER and PgR primarily nuclear but loosely bound, whereas VDR and T3R are tightly associated with the nucleus even in the absence of hormone. The primary role of the nuclear receptors is thought to be the regulation of the rate of transcription of responsive genes by binding as a hormone-receptor complex to specific DNA sequences termed hormone responsive elements (HRE) (124-130). This binding process can either stimulate promoter activity (transcriptional activation) or repress gene transcription (124, 127, 128). In addition to augmenting transcriptional rate, steroid receptors can also exert their effects through post-transcriptional processes (see Introduction 4.5.2). Sequence comparisons of cloned receptors demonstrated that they all have a similar modular domain structure. The conserved ligand binding domain (region E) forms the C-terminal portion of the molecule. This region is also required for dimerization of the estrogen receptor (ER) and for nuclear localization of the glucocorticoid receptor (GR). In both receptors, this domain also contains a trans-activation function. The central DNA binding domain (region C) is cysteine-rich. The conformation of this region is such to allow the formation of two zinc finger motifs first identified in the transcription factor TFIIIA. Although both zinc fingers are required for specific DNA binding, the N-terminal finger is crucial for determining sequence specificity while the other one functions in discriminating half-site spacing between HREs (131). The N-terminal regions encompassing regions A and B are extremely variable amongst receptors in size and exhibit neligible sequence similarity. The A/B region is considered to perform a trans-

activation function which may be promoter and cell type specific (132, 133). Before hormone activation, each monomeric steroid receptor molecule (except vitamin D and thyroid receptors) is found to be associated with two molecules of the heat shock protein 90 (hsp90) and other proteins, with the oligomeric complex being unable to bind DNA (125, 129). The role of hormone is to dissociate the receptor from hsp90, activating the receptor by allowing dimerization and DNA binding, and in the case of GR, also enhancing its uptake in to the cell nucleus. Steroid receptors have functional duality: they can either activate or repress gene expression. These two seemingly opposing functions of this family of transcription regulators will be discussed separately below:

1) Activation

The hormone responsive elements that confer hormonal regulation to a gene have enhancer-like properties, are palindromic sequences 13-15 bp in length, and comprise two half-sites that display dyad symmetry. The symmetry of these elements is in accordance with the finding that nuclear receptors bind as dimers, with each molecule recognizing each half-site of the HRE (124, 134, 135). Three classes of HREs for positive transcriptional activation have been identified by gene transfer and receptor-DNA binding studies. Fig 2 shows the consensus sequences for the three classes which include the glucocorticoid (GRE), estrogen (ERE), and the T3 (T3RE) responsive elements (125, 128).

+GRE	GGTACAnnnTGTTCT
ERE	AGGTCAnnnTGACCT
+T3RE	AGGTCA---TGACCT

Fig. 2. Consensus responsive elements for nuclear receptors

GREs have been characterized in promoters including the genes for metallothionein IIA, chicken lysozyme, human growth hormone and rat tyrosine aminotransferase (136). Interestingly, the GRE is also able to mediate induction by progesterone, androgens, and mineralocorticoids (137, 138). Fortunately, nature has evolved mechanisms to ensure specificity of different hormonal responses. These include regulation of particular receptor types, enhanced metabolism of the hormone in unresponsive tissues, regulation (or even exclusion) of receptor levels and of other transcription factors required for the response and subtle contact differences of receptors with similar but nonidentical HREs (125, 135). EREs have been described in the Xenopus vitellogenin A2 and B1 genes, in the chicken vitellogenin II gene and also in the pS2 gene (129). As shown in Figure 2, the ERE is closely related to but distinct from GRE. This similarity is evidenced by the demonstration that an ERE can be converted into a GRE by changing only 1 or 2 bp (139). T3RE, the third class of HRE, have been found in promoters for genes encoding bovine and rat growth hormone, and rat A myosin heavy chain (140). The T3RE is nearly identical to an ERE but lacks the three spacer non-conserved nucleotides. In fact, it has been shown that the T3R can bind to an ERE, but is transcriptionally inactive (128). Retinoic acid and vitamin D can also stimulate through a T3RE (140). Two recent papers have resolved the apparent lack of hormone selectivity associated with T3RE. The groups of Rosenfeld and Evans presented evidence that the orientation and spacing of half-site motifs function as a code to impart selective transcriptional responses to estrogen, retinoic acid, vitamin D and thyroid hormone (141, 142).

2) Repression

Although not as well studied as transcriptional activation, repression by steroid receptors has also been documented in several systems and may soon prove to be as prevalent a mode of regulation as induction. Negative regulation of gene expression by glucocorticoid has been demonstrated for proopiomelanocorticotropin, prolactin, the α-subunit of the glycoprotein hormones, stromelysin, collagen and α-fetoprotein (125, 128, 136) Limited sequence comparison studies identified a preliminary negative GRE (-GRE) which differs from the positive GRE consensus but has some similar features (128). In the case of the α–subunit gene, transcriptional repression by GR is mediated through a 'blocking of DNA binding' type mechanism. In this system, the gene is normally activated by cyclic AMP, and GR-hormone complex was found to bind to a region, overlapping a CRE. Therefore, the GR may interfere with either the binding or the function of the CREB protein (143). A similar competition mechanism has been proposed for proopiomelanocortin, the binding of GR to a site around -60 is thought to prevent the CAAT box binding factor from interacting with its cognate sequence (144). A special type of repression appears to be shared by members of the T3R family. In the absence of hormone, the unoccupied receptor directly binds to T3RE without activating transcription. Hormone binding to bound T3Rs serves to relieve this repression, allowing the receptor protein to adopt a configuration that is transcriptionally stimulating (140). The T3Rs also exhibit another type of repression that is hormone-dependent. This type of repression requires the interaction of the receptors with negatively acting T3RE (-T3RE) which have been found in the promoters of the rat α and β subunit genes for thyroid stimulating hormone. The -T3REs deviate from the positively acting T3REs so far identified and consist of closely spaced direct repeats of the half-site consensus. This type of

pattern was recently demonstrated to endow the responsive element with selective repressive function (141).

4.3 Processing of pre-mRNA

The immediate product of transcription by Pol-II is a polymer of ribonucleotides refered to as the precursor mRNA (pre-mRNA) or the primary transcript. While still in the nucleus, pre-mRNA is modified in a series of steps, which include formation of the 3' end, addition of a poly-A tail, and splicing. The fully processed mRNA is then transported from the nucleus to the cytoplasm, to be translated there to the final gene product. Each of these steps is required to produce a mature, functional mRNA, and each is a potential site for regulation.

A typical eukaryotic mRNA contains of a poly (A) tail of approximately 200 nucleotides at its 3' end. The acquisition of this structure occurs post-transcriptionally in a two-step manner which involves endonucleolytic cleavage of the pre-mRNA at a specific site (cleavage site), followed by addition of the adenosine residues. Analysis of sequences in the pre-mRNA required for processing has shown that the invariant hexamer AAUAAA, located 18-30 nucleotides upstream of the cleavage site, as well as a vaguely defined G+U-rich downstream element are critical for the process (145, 146). In transcription units that contain more than one poly (A) addition signal, different RNAs can be produced depending upon which poly(A) site is chosen (e.g. the secreted and membrane-forms of IgM).

After the formation of a 3' end and the poly (A) tail, the primary transcript undergoes the second stage of processing, namely, splicing to remove its intronic sequences in a precise manner. Pre-mRNA splicing requires an array of trans-

acting splicing factors which associate with the RNA to form a large complex, the spliceosome, in which catalysis occurs. Trans-acting factors that form part of the splicesome include the small nuclear ribonucleoproteins (snRNPs) U1, U2, U4/U6 and U5 (147). The functions of some of these components have been determined. U1 snRNP binds to the 5' splice site whereas U2 snRNP, in the presence of U2 auxillary factor (U2AF) and ATP, binds to the branchpoint sequence of 3' splice site (148). Although much is known about the splicing factors and their constituents, the basic question of how spliceosome promotes RNA catalysis remains unanswered and perhaps controversial. At least two possibilities exist: first, the spliceosome may fold the pre-mRNA to enable the pre-mRNA to catalyse its own splicing; second, the spliceosome may determine the correct conformation required for the expression of catalytic activities, contributed by its snRNA moieties or other proteinaceous components (147).

Many eukaryotic pre-mRNAs contain multiple introns along with numerous cryptic splice sites, some of these can be spliced in more than one way to give rise to multiple protein species. Alternative splicing may or may not be regulated, the latter can occur in a temporal or tissue-specific manner such as that responsible for Drosophila sex determination (149). Another form of splicing control has been identifed in which the concentration of a functional, translatable transcript is regulated through differential removal or retention of one or more introns. The best characterized example is the Drosophila gene encoding suppressor of white apricot, su(wa). Only in the absence of the su(wa) gene product are introns 1 and 2 removed to produce a translatable mature transcript (150). More recently, this type of splicing control was also found in the gene for prostaglandin synthase (PS). In chicken embryo fibroblasts transformed with rous sarcoma virus, the majority of the PS RNA was nonfunctional in nonproliferating cells due to the presence of an

unspliced intron. Upon mitogenic stimulation, this intron was removed, giving rise to functional, fully spliced PS mRNA (151).

4.4 Nucleocytoplasmic mRNA Transport

After processing, mature RNA must be transported from the nucleus to the cytoplasm before translation can occur. Increasing experimental evidence points to nucleocytoplasmic transport of mRNA through the nuclear pore as one important step in gene expression. It is now relatively well established that the transport of at least some mRNAs is an energy-dependent process and the mRNA is structurally bound during transport (152, 153). The nuclear pore complex acts as a molecular sieve through which macromolecules such as proteins, RNAs and ribonucleoprotein particles are transported uni- or bidirectionally. Mechanistically, the process involves first the release of the mature mRNA from the nuclear matrix followed by translocation through a nuclear pore (153). Nuclear retention seems to be largely responsible for the selectivity of transport for mature mRNA species, with immature ones retricted to the nucleus. The other step, namely, pore passage appears to regulate the quantity of the mRNA exported out of the nucleus. At least for poly (A) tail containing mRNA which comprises the majority of the cellular transcripts, the nuclear envelope translocation apparatus appears to consist of at least 4 different components: a nucleotide unspecific nucleoside triphosphatase NTPase, a poly (A) binding p106 polypeptide (the mRNA carrier), two protein kinases, and a phosphoprotein phosphatase (153). The NTPase is assumed to be responsible for providing the energy required for poly (A^+) mRNA transport by hydrolysing ATP or GTP. Its activity is modulated by the p106 mRNA carrier which can undergo phosphorylation and dephosphorylation, in turn these two modifications are modulated by poly (A) binding (153). In the model put forward by Schroder and co-workers, the NTPase is inactive when the mRNA carrier is

phosphorylated by the kinases. Binding of poly (A^+) mRNA to the carrier stimulates its dephosphorylation by the phosphoprotein phosphatase and consequently NTPase becomes activated leading to efflux of mRNA. On the other hand, transport of poly (A^-) mRNA such as that for the histones, is independent of cleavage of high-energy phosphodiester bonds and appears not be mediated by the same system (138). A recent report by the same group has provided evidence that an RNA unwinding activity (RNA helicase; RNA unwindase), which is also associated with the nuclear envelope, may contribute to the nuclear export of poly (A^-) RNAs containing duplex RNA segments such as found in the histone mRNAs (154).

Many physiological perturbations can influence the mRNA transport system. These include different hormonal and nutritional states and various disease states of the cell. The nuclear envelope contains specific receptors for hormones such as insulin, T3 and other growth factors including EGF (153). The responses elicited by insulin and EGF depend on whether the mRNA to be exported contains a poly (A) tail or not. Thus, while the nuclear export of poly (A^+) mRNA (actin or total adenylated mRNA) was enhanced by insulin, the efflux of poly (A^-) histone H4 mRNA was decreased (155). On the other hand, EGF inhibited the transport of adenylated messages, while having no significant effect on poly (A^-) mRNA.

Thus far, there is no known cases in which unequivocal data have been obtained to show any participation of steroid hormones in the regulation of mRNA export. However, dexamethasone has been shown to decrease NTPase activity in skin fibroblasts which correlated with changes in protein synthesis (153) and data have been published which suggest that steroid receptors may interact directly with RNA (156). This latter finding has led Liao et al., (157) to hypothesize that steroid

receptors may influence gene expression through post-transcriptional mechanisms such as mRNA export, in addition to their more recognized effects on transcription.

4.5 mRNA stability

4.5.1 General introduction

Next to transcriptional regulation, the control of steady-state mRNA levels through regulation of cytoplasmic mRNA stability is perhaps the most widely studied and appreciated aspect of gene expression (158). In the literature, there are many examples where cells respond to different conditions by differentially altering the stability of mRNA, for example, mRNAs for tubulin, histone, transferrin receptor and several proto-oncogenes. These well-known examples will be discussed in some detail, even though main portion of this section will be devoted to examples in which the modulating signals are steroid or thyroid hormones.

One of the best characterized systems of regulation of mRNA stability can be found in the synthesis of α and β tubulin, the principle subunits of microtubules. These two types of subunits are constantly in equilibrium between heterodimers and polymerized chains due to the dynamic nature of the cytoskeleton, of which microtubules are the major components. The mRNA for tubulins are apparently destabilized by high levels of their own protein molecules if they exist as unassembled heterodimers (158, 159). Sequences necessary for the autoregulated instability of tubulin mRNAs lie in the first 13 bases to be translated. These sequences encode the tetrapeptide Met-Arg-Glu-Ile (MREI) and it is the peptide sequence rather than the nucleic acid sequence that is the important recognition signal. Cleveland and co-workers (160, 161) postulated that free tubulin heterodimers recognize and interact co-translationally with the nascent tetrapeptide segment, activating a specific ribosome-bound ribonuclease leading to mRNA

degradation. The putative nuclease, however, has yet to be identified before this model can be fully embraced.

A similar system of autoregulated mRNA stability concerns the poly (A^-) histone mRNAs. The steady-state levels of histone transcripts vary between 30- and 50-fold during the cell cycle, rapidly increasing in abundance at the onset of DNA synthesis and declining rapidly at the end of replication. In higher eukaryotes, these fluctuations result from a small transcriptional effect but are largely due to changes in mRNA stability (158, 159). The sequence required for histone mRNA destabilization is at the 3' end of the transcript, comprising a conserved stem-loop (hairpin) structure. As in the tubulin case, autoregulation by histone requires continuing protein synthesis.

Another example of an RNA stem-loop structure acting as a destabilizing determinant can be found in the mRNA encoding the transferrin receptor which mediates the cellular uptake of iron. Iron depletion causes stabilization of the message while high iron levels destabilize it (159, 162). The iron-dependent changes in transferrin receptor mRNA levels is solely post-transcriptional, with no transcriptional component. A domain of 650 bases in the 3' untranslated region is responsible for the iron-dependent regulation. Within this region are five imperfect palindromic sequences referred to as iron response elements (IRE). Interestingly, these IRE are also found in the 5' untranslated region of ferritin mRNA but in this case the IRE confers iron-dependent translational activation (see Introduction 4.6). A 90 kD cytoplasmic protein termed the iron response factor (IRF) has been purified and demonstrated to bind to IREs in the 3' untranslated region of the transferrin receptor mRNA. Such binding stabilizes the transcripts by blocking endonucleolytic attack at the 3' end (159, 162). Increased levels of iron cause

release of bound IRF from IREs, thus exposing the end of the transcript and making it more susceptible to nuclease digestion.

Stability of mRNA plays a significant role in the gene regulation of many well characterized cellular proto-oncogenes, including c-myc and c-fos. Along with many cytokine genes which form a large group of highly unstable messages, c-fos and c-myc share a conserved AU-rich motif (AUUUA) in the 3' untranslated region (111, 159). Using gene transfer techniques, Shaw and Kamen initially showed that the AU-rich domain could destabilize a normally long-lived B globin mRNA (163). However, more recent experiments demonstrated that the AU-rich region is only part of the determinant of RNA instability. Other determinants within the coding region are required for instability of both c-fos and c-myc mRNAs (164, 165) . It should be noted that rapid degradation of these two proto-oncogene mRNAs require ongoing translation and blockade of protein synthesis using cycloheximide results in stabilization of the transcripts. These earlier findings can now be reconciled with the fact that translation is required so as to expose the destabilizing signal located within the protein-coding region. In some systems, the message-stabilizing effects of agents like cylcoheximide are usually intepreted as the result of the decay of a labile protein involved in mRNA turnover but in the light of recent findings, this requirement may reflect instead a need for continuing translation of the protein coding section.

4.5.2 Steroid-regulated mRNA stability

An insightful discovery concerning steroid-regulated mRNA stability came from analysis of the mRNA encoding vitellogenin, a major protein in the eggs of the frog Xenopus laevis. In this case, estrogen treatment led to a 30-fold increase in the stability of the message (159, 166). Since then, many more reports

on steroid-regulation of mRNA stability have appeared, with examples from almost every class of steroids (and thyroid hormones). In addition, examples of both steroid-induced mRNA stabilization and destabilization are now known to exist.

1) mRNA stabilization

In an attempt to elucidate the minimum sequence requirement for estrogen-induced stabilization of vitellogenin mRNA, Shapiro and his colleagues (167) have developed a transfection system and showed that a mini-vitellogenin mRNA lacking 5,100 nucleotides of coding sequence can still be stabilized by estrogen in the presence of transfected estrogen receptors. Another group has identified a 66 kDa estrogen-inducible protein (p66) that binds to the 5' untranslated region of the chicken vitellogenin mRNA and presumably protects it from degradation (168). Another mRNA stabilized by estrogen is that for apo-very low density apolipoprotein II (apoVLDLII). In this case, stabilization is probably the consequence of an estrogen-induced increase in the length of the poly (A) tail, an event that has been correlated with increase in mRNA stability (167). A similar phenomenon was observed in the glucocorticoid stabilization of human growth hormone mRNA (167). Glucocorticoids such as dexamethasone also stabilize the mRNA for fibronectin and phosphoenolpyruvate carboxykinase (PEPCK). Transferring the 3'-terminus of the PEPCK mRNA to the reporter chloramphenicol acetyltransferase (CAT) coding sequence confered glucocorticoid-dependent stabilization upon the hybrid transcript (169). Moreover, this function is only preserved when the sequence is located downstream from the CAT gene, further supporting the presence of a steroid hormone-stabilizing element within the region. The diversity seen in different systems involving regulation of mRNA turnover by

steroid hormones, suggests that there exist a wide array of mechanistic strategies to effect mRNA turnover rate.

2) mRNA destabilization

In addition to regulating gene expression at the post-transcriptional level by stabilizing certain mRNAs, estrogen can exert the opposite effect, namely, selective destabilization of mRNAs. Albumin, another egg protein, is subject to control by estrogen administration which causes an increase in the turnover rate of the mRNA (170). Similarly, glucocorticoids decrease the stability of several mRNAs including that of interleukin-1β (171), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (172), type 1 procollagen (173), and surfactant protein-A (174). An intriguing example of regulated mRNA destabilization was recently reported by Chin and colleagues who demonstrated that thyroid hormone treatment of rat pituitary cells or whole animals decreased the half-life of thyrotropin (thyroid stimulating hormone or TSH) β-subunit mRNA from greater than 24 hr in control cells to 9 hr (175). This change in half-life is associated with a reduction of the poly (A) tail length of the mRNA molecule, an effect also seen with the treatment of cycloheximide Taken together, these data indicated that a labile protein may be involved in maintaining the size and stability of the TSH β mRNA under certain experimental conditions, and that T3 may act at the transcriptional level to inhibit the production of such factor.

4.6 Translational Control

For any structural gene, the translation of its mature mRNA into the final protein product in the cytoplasm is a prerequisite for its functionality. Considering the well established translational control in prokaryotes, it is ironic that only until

recently has the same process been confirmed to exist in eukaryotic systems. In eukaryotic translation, the small ribososmal subunit (part of the 43S pre-initiation complex) contacts the mRNA at or near the 5' end of the transcript. The rate of binding of the pre-initiation complex to the mRNA is enhanced by the presence of a cap structure at the 5' terminus (176). The pre-initiation complex is then thought to migrate along the transcript by a scanning process until it arrives at the initiator AUG codon, at which time the 60S ribosomal subunit will join to initiate translation. According to this 'scanning model', initiation at downstream start codons is generally precluded if the 5' proximal AUG codon is being recognized efficiently. This differential preference in terms of initiation codon usage forms the basis for a novel translational mechanism in regulating the levels of a transcription factor named GCN4.

In response to amino acid starvation, synthesis of the GCN4 protein in yeast is increased, leading to transcriptional activation of genes encoding enzymes involved in amino acid biosynthesis. The novelty of this system is that the elevated synthesis of GCN4 protein is regulated primarily at the translational level. The mRNA for GCN4 contains a long 5' leader with four upstream open reading frames (uORF 1-4, from 5' to 3') which can be translated into short peptides (177, 178). These uORFs allow ribosomes to initiate translation of the GCN4 transcript at the correct downstream AUG codon only when cells are starved of an amino acid. The roles played by each of these 4 uORFs are not the same. The effect of uORF 1 and 2 under starvation conditions is to suppress the inhibitory functions of uORF 3 and 4, presumably by allowing ribosomes to re-initiate translation more efficiently at the correct GCN4 start codon after traversing uORF 4 (177, 178). Under non-starvation conditions the positive effects of uORF 1 and 2 are blocked by negative regulatory factors GCDs (products of *GCD* (**g**eneral **c**ontrol **d**erepressed) genes),

thus the inhibitory actions of uORF 3 and 4 prevail preventing translation of the GCN4 message (177, 178).

The regulated biosynthesis of ferritin molecules represents another excellent example of translational control. When iron is limiting, the cell produces more transferrin receptor and less ferritin. The former allows the cell to extract more iron from the environment and the latter ensures more free iron is readily available for utilization. Conversely, when iron is plentiful, transferrin receptor numbers decrease and ferritin levels rise (162, 179). The increased levels of ferritin ensure that excess iron is sequestered and thus protects the cell from toxic levels of iron. The 5' untranslated region of ferritin mRNA contains a characteristic 28-nucleotide sequence capable of forming an 'iron responsive element' (IRE), whose function is to regulate translation through differential binding of the IRE-binding protein (IRE-BP) the cDNA of which has been cloned recently (180) Under conditions of iron deprivation, IRE-BP acts as a translational repressor by high-affinity binding to the IRE located in the 5' end of the ferritin message and thus physically obstructs translation (162, 179). Conversely, increased iron levels decrease the binding affinity of IRE-BP for IREs so that bound repressors are released, permitting efficient translation to occur.

Another way to control the translation process can be achieved through sequestering the mature mRNA molecules in a form that is not translatable simply due to their inaccessibility to the translation machinery. This type of translational control may be related to that observed with ferritin and is common to many ribosomal protein mRNAs. Under a variety of conditions (e.g. during development of Xenopus and Drosophila, glucocorticoid-induced growth arrest), translation of

ribosomal protein mRNA is repressed due to a shift of mRNA from active polysomes into nonactive messenger ribonucleoprotein complexes (181).

4.7 Post-translational Control

Once a protein molecule is synthesized, it can be subject to modification to modify its activity. For instance, the activity of the enzyme glycogen synthase is regulated by a cascade of phosphorylation and dephosphorylation. Post-translational control can also take the form of regulating protein concentration by altering the rate of degradation and controlling the amount of a specific protein within a particular subcellular compartment. It should be remembered that these processes are not necessarily independent or mutually exclusive, and that the categorization is quite arbitrary.

The localization of nuclear proteins such as histone and transcription factors necessitates the translation of their respective mRNAs in the cytoplasm followed by transportation back into the nucleus. For example, the ligand-regulated glucocorticoid receptor (GR), unlike other members of the steroid receptor family, is largely cytoplasmic in the absence of hormone (124). Upon activation by hormone binding, the receptor rapidly translocates into the nucleus where it effects changes in gene activity. The carboxyl-terminal portion of the receptor contains nuclear localization signals (NLSs) that are ligand-dependent. Only upon receptor transformation through hormone binding do the NLSs become exposed, perhaps by dissociating the normally bound hsp90 protein, and subsequently allowing migration of the receptor into the nucleus (124, 182).

Nuclear factor kapper B (NF-κB) now known to be ubiquitous in mammalian cells was originally identified as a nuclear protein that specifically binds

to a DNA control element termed κB (182, 183). Its activity is regulated at the level of intracellular location. The inactive form of NF-κB is retained in the cytosol by complexing with a protein factor called inhibitor kapper B (IκB) which may act by masking of the NLS. In response to activating agents (e.g. phorbol esters), NF-κB translocates to the nucleus after dissociating from the cytosolic NF-κB/IκB complex, and activates the expression of its target genes. Dissociation requires phosphorylation of IκB by activated cellular kinases (e.g. protein kinase C, protein kinase A) which renders it unable to bind to NF-κB.

The concentration of a protein within a cell is also determined by the balance between its rate of synthesis (transcription and translation) and degradation. In many instances the rates of protein degradation can be regulated in response to changes in physiological and/or biochemical parameters. In general, proteolytic pathways can be grouped into either cytosolic or vesicular, each comprises several individual pathways. The major vesicular pathway utilizes lysosomes which are responsible for the degradation of many long-lived proteins as well as for the enhanced protein degradation seen during starvation (184, 185). Other vesicular degradation systems include those functioning within mitochondria and the endoplasmic reticulum, the latter appears to be responsible for the degradation of newly synthesized abnormal and unassembled proteins (184).

The major non-lysosomal, cytosolic pathway for intracellular proteolysis which has been identified, is the ATP- and ubiquitin-dependent system (186, 187). In this pathway, ubiquitin becomes conjugated to the substrate protein which is then recognized for degradation by an ATP-dependent protease. Thus, ubiquitin conjugation (branched polyubiquitin) serves to mark a protein, usually either short-lived or abnormal, for rapid degradation. Proteins with varying N-termini will be

subject to different rates of degradation according to the 'N-end rule'. Apart from this well-characterized system, there exist pathways that are ubiquitin-independent but either ATP-dependent or ATP-independent; however they are at present only poorly defined (184).

A well known example of regulated protein degradation is the sterol (e.g.cholesterol, mevalonate)-induced degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). The enzyme catalyzes the conversion of HMG-Co A to mevalonic acid, the most critical regulated step in the pathways leading to formation of sterols and isoprenoids (188). With regard to the proteolytic pathway, recent data suggest the participation of endoplasmic reticulum and an essential short-lived protein (189).

RESEARCH OBJECTIVES

The research project described in this thesis lies at the interface of several areas of intense biomedical research activity. Specifically, a major portion of the work was devoted to an investigation of the molecular mechanisms by which a class of steroid hormone (progestins) induced expression of a growth factor (EGF) within the context of human breast cancer biology. This is important to our understanding of the biology of breast cancer as steroid hormones and growth factors have been intimately linked to the development and progression of the disease.

The steady-state levels of EGF mRNA have been shown to be induced by a class of steroid hormones, progestins, in T-47D human breast cancer cell line. By Northern analysis, EGF mRNA was shown to be increased 6-fold over control 12 hr after MPA treatment (190). This observation was extended in this laboratory to more closely map the induction profile using a highly sensitive RNAase protection assay, and at the same time, the protein synthesis requirement of the hormonal effects on EGF gene expression was investigated using cycloheximide to block protein synthesis (191). The mechanism (s) underlying the hormonal response was the subject of this study. In particular, the two levels at which gene expression are mainly controlled, namely, transcription and mRNA stability were investigated. The rate of transcription of the EGF gene was measured by nuclear run-off (= run-on) assay to assess any modification after hormone stimulation. In the analysis of mRNA stability, inhibition of transcription by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) or actinomycin D (ActD) followed by the measurement of the decay rate of the EGF transcripts would allow one to assess if mRNA stabilization plays any role in the observed hormone-induction of steady-state EGF mRNA levels.

Another aspect of this work was to identify putative regulatory sequences in the EGF gene. DNA sequences important for basal level transcription (e.g.promoter) as well as regulated transcription (e.g. enhancer, hormone responsive elements) were investigated by sequence and deletion analysis of the 5'-flanking region. The latter was achieved by constructing plasmids carrying different regions of the EGF 5'-flanking region, followed by measurement of their ability to activate expression of the promoterless chloramphenicol acetyltransferase (CAT) gene in transient transfection assays.

MATERIALS AND METHODS

1. CELL LINES AND CULTURE CONDITIONS

1.1 Cell lines

T-47D is a human breast cancer cell line originally isolated from a pleural effusion obtained from a 54 years old female patient with an infiltrating ductal carcinoma. The cells were found to contain receptors for steroid hormones including estrogens, progestins, glucocorticoids and androgens (192, 193). The stock used in this study was obtained from Dr. R. L. Shiu (Department of Physiology, University of Manitoba, Winnipeg, Canada).

The HeLa cell line was isolated from a cervical carcinoma of a 31 year-old black female, Henrietta Lacks in February, 1951. It has been re-examined by Jones et al. to be an adenocarcinoma (194). The cells are devoid of receptors for steroid hormones such as progestins and estrogens, but do contain receptors for glucocorticoids. It was obtained from Dr. R. L. Shiu (Department of Physiology, University of Manitoba, Winnipeg, Canada) who originally purchased it from the American Type Culture Collection (ATCC CCL 2 HeLa).

1.2 Culture conditions

All cell lines were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % (v/v) fetal calf serum (FCS), penicillin (50 units/ml), streptomycin (50 μg/ml), glutamine (4mM), and glucose (4.5g/l). The two antibiotics may be replaced with 20 μg/ml gentimycin. This formulation is referred to as complete medium minus insulin (CM-I). Cells were incubated at 37°C in an incubator kept at 5% CO_2 and humidity of 95%. Tissue culture

containers used were polystyrene culture flasks (150 cm^2) and small tissue culture dishes (100 X 20 mm), both from Corning Glass Works, Corning, New York, USA. Large tissue culture dishes (150 X 20 mm) were from GIBCO, Grand Island, NY. Other cell culture reagents were supplied by FLOW and GIBCO.

1.3 Routine culture procedures

1.3.1 Cell removal by trypsin

To remove cells from culture flasks for routine maintenance or setting up experiments, the growth medium was aspirated and the flask surface rinsed once with 5 ml of sterile trypsin/EDTA solution containing 0.05% trypsin and 0.02% EDTA (Flow Laboratories Inc., Rockville, MD). Five ml of fresh trypsin/EDTA was added to the flask, which was then incubated for 5 min at 37°C. An equal amount of CM-I was then added to the flask to neutralize the effects of trypsin. Cells were pipetted into new flasks containing new medium or into culture dishes at a pre-determined number of cells.

1.3.2 Cell counting

A 1 ml aliquot of cells treated as described above, was removed and passaged through a 21-gauge needle three times to prevent clumping. Half a ml of this aliquot was diluted with 19.5 ml of isoton II™ (Coulter Electronics Ltd., Ont., Canada) and counted with a Coulter Counter (Coulter Electronics Ltd.). Cell density was determined using the known dilution factor. A pre-determined number of cells was dispensed into tissue culture dishes with the aid of 50 ml plug-seal cap centrifuge tubes (Canlab, Mississauga, Ont.). Occasionally, cell numbers were counted using a hemacytometer.

2.ROUTINE EXPERIMENTAL MANIPULATIONS AND ANALYSES

2.1 Treatments with hormones and various reagents

Medroxyprogesterone acetate (MPA), cycloheximide (CHX), actinomycin D (Act D), and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Stock solutions of MPA (10 μM) was prepared in 100% ethanol. Stock CHX (5 mM) was prepared in sterile H_2O whereas stock solutions of Act D (5 mg/ml) and DRB (25 mg/ml) were prepared in dimethyl sulfoxide. All the above stock solutions except that of CHX were 1000X (CHX was 100X that of the final concentration to be used). The above reagents were added directly to culture dishes to achieve the final concentration of 1X.

2.2 Measurement of mRNA half-life

There are in general two common experimental approaches to measure the decay rates of individual mRNAs. One involves in vivo labeling using a radioactive marker followed by monitoring the disappearance with time of specific mRNAs after a chase with radiolabeled NTPs. Alternatively, transcription is inhibited and the decay rate derived by determining the abundance of the mRNA of interest at various times after such inhibition. This latter approach, because of its wide application and relative ease was adopted in this work to analyse the stability of EGF mRNA.

1.5×10^6 cells/dish were seeded onto 150 mm dish under logarithmically growth conditions. Three to four days later, the cells were treated with or without (i.e. control) various inducers for 24 hr to stimulate EGF expression, following

which, either Act D or DRB was added to inhibit transcription. At various time points after the addition of transcription inhibitors, cells were harvested into 50 ml tubes, centrifuged, and the cell pellet frozen and stored at -70°C until RNA preparation (see below).

The concentration of mRNA at any time is expressed as described in the following equation: $\ln ([mRNA]_t / [mRNA]_{0)} = -kt$; where $[mRNA]_0$ is the mRNA concentration at time zero' $[mRNA]_t$ is the mRNA concentration at time t; and k represents the rate constant for decay. Messenger RNA half-life can be calculated from the slope of the line of best-fit obtained by plotting the log of the percentage of RNA remaining relative to time zero against time and using the formula $t_{1/2} = \ln 2/k$, where -k is the slope of the line and $t_{1/2}$ is the mRNA half-life.

3. SOUTHERN AND NORTHERN ANALYSIS

3.1 RNA isolation and Northern blot analysis

Cells were cultured in 150 mm dish under logarithmically growth conditions, harvested at appropriate time into 50 ml centrifuge tubes, centrifuged, the pellet frozen and stored at -70°C until RNA preparation. Total cellular RNA was isolated from frozen cell pellet using the guanidinium thiocyanate/cesium chloride method (195). Briefly, the frozen pellet was thawed in 8 ml of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.5), 0.5% N-lauroylsarcosine, 0.007% β-mercaptoethanol. The resulting solution was vortexed until total lysis and dissolution of cells occurred, followed by passaging through a 21-gauge needle for 6-10 times to shear the DNA and reduce viscosity. The lysate was then layered on top of 4 ml of 5.7 M CsCl and 0.1 M EDTA (pH 7.0) in a

Beckman polyallomer Quick-Seal™ tube. The suspension was then centrifuged at 22°C in a Ti 70.1 Beckman rotor at 28,000 rpm for 18 hr. A 16-gauge needle connected to a 10 ml syringe was inserted at about half-way down the tube, and the solution mixture above the insertion point was removed. The tube was then cut into halves with a red-hot scalpel followed by removal of the remaining solution contaminated with DNA fragments using a sterile pasteur pipette. The halved tube was further cut slightly above the pellet to avoid excess salt contamination. The pellet was drained dry, dissolved in 1 ml TE buffer (TE or Tris-EDTA buffer contains 10 mM Tris-Cl, pH 7.5 and 1 mM EDTA, pH 8.0) (heated at 65°C if necessary) and transferred to a sterile 13-ml Falcon tube. Two volumes of absolute ethanol and 267 µl of 3 M sodium acetate (pH 5.5) were added to the tube to precipitate the RNA by incubating overnight at -20°C or at -70°C for 30 min. The precipitated RNA was recovered by centrifuging in a SS34 rotor at 11,000 rpm for 20 min, briefly dried in vacuum, and resuspended in a pre-determined amount of TE buffer to give approximately an RNA concentration of 5 µg/µl . Long-term storage of total RNA was at -70°C.

The exact concentration of RNA in a sample was determined by measuring the absorbance of a diluted RNA sample at 260 nm using the formula: 1 OD_{260} = 40 µg/ml RNA (196). Preparation of RNA for gel electrophoresis is as follows (197): to 10 µl RNA sample containing 40 µg of total RNA was added 4 µl 5 X GRB buffer (1 X GRB contains 40 mM morpholinopropanesulfonic acid, pH 7.0, 10 mM sodium acetate (pH 5.5) and 1 mM EDTA), 7 µl formaldehyde and 20 µl deionized formamide. The sample was then incubated at 65°C for 30 min to denature any secondary structure, after which it was cooled rapidly in ice-water to prevent reannealing. Four µl of 10 X gel loading buffer containing 50 % glycerol, 0.1% bromophenol blue and 0.1% xylene cyanol was added. The sample was then

loaded onto a 1% (w/v) agarose gel containing 2.2 M formaldehyde, 1 X GRB and 7 µl of 10 mg/ml ethidium bromide. The sample was run into the gel dry (gel was not submerged in 1 X GRB) for 25 min at 120 volts, following which 1 X GRB was added to totally cover the gel, which was then electrophoresed overnight at 30 volts. Fluorescence-photography was performed of the gel before transfer with the help of a UV-light box. The RNA was then blotted by capillary transfer onto NitroPlus transfer membrane (Micron Separations Inc., Westboro, MA) using 20 X SSC (1 X SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) buffer. The membrane was first placed next to the gel followed in order by 2 pieces of Whatman filter paper, a stack of paper towels, a glass plate and a weight to ensure even contact of the membrane and filters with the gel and therefore even transfer of RNA onto the nitrocellulose filter . The next day the blotted membrane was peeled off from the gel, air-dried, and baked for 2 hr at 80°C under vacuum. The blots were first prehybridized for at least 3 hr at 42°C in the presence of 50% (v/v) deionized formamide, 5 X Denhardt's solution (1 X Denhardt's = 0.02% w/v each of BSA, Ficoll and polyvinylpyrrolidine), 5 X SSPE (1 X SSPE = 1.15 M NaCl, 0.01 M NaH_2PO_4 and 1 mM EDTA), 250 µg/ml denatured salmon sperm DNA and 0.1% SDS. Hybridization was performed in the same solution for 2 days with ^{32}P-labeled nick-translated cDNA probe (specific activity of approximately 10^8 cpm/µg). At the end of the hybridization period the blots were washed twice in 2 X SSC, 0.1% SDS for 15-30 min at room temperature, followed by three 20 min washes in 0.1 X SSC, 0.1% SDS at 65°C. Filters were exposed to Kodak XAR film at -70°C with an intensifying screen for a period of 1-4 days. Quantitation was achieved by densitometric scanning of various exposures of the autoradiograms using the Macintosh-compatible Image 1.37 image processing and analysis program.

3.2 Southern blot analysis of restriction-cut DNA fragments

Plasmids or DNA inserts were first digested with the appropriate restriction enzymes and the fragments resolved electrophoretically on 1% (w/v) agarose gel . The gel was then blotted onto Nitroplus 2000 transfer membrane as described for RNA (Northern blotting) except for the following steps: 1) Before blotting, the gel was washed twice in 1.5 M NaCl, 0.5 M NaOH solution, each for 20 min. This step would allow denaturation of the double-stranded DNA into a single-stranded form. 2) The gel was then neutralized in 1 M ammonium acetate, 0.02 M NaOH buffer by soaking in the solution twice for 30 min at room temperature. The same buffer was also used as transfer buffer during the blotting procedure. The preparation of membrane for hybridization and the hybridization itself were as described for Northern analysis except that the amount of cDNA nick-translated was 50 ng only.

3.3 Labeling of cDNA probes with ^{32}P-dCTP

All nick translation reactions were performed using a nick translation kit purchased from Amersham. α-^{32}P-dCTP (specific activity 3000 Ci/mmol) was purchased from New England Nuclear (Lachine, Quebec). The amount of DNA used in each nick translation ranged from 50 to 200 ng. Reactions were performed according to the kit instruction, with minor modifications. A standard reaction consisted of 4 µl of a nucleotide mixture (100 µM dATP, 100 µM dTTP, 100 µM dGTP in a Tris-Cl/$MgCl_2$/β-mercaptoethanol buffer), 2 µl of an enzyme mixture (0.5 U/µl DNA polymerase, 20 pg/µl DNase I in a Tris-buffered solution containing $MgCl_2$, glycerol and BSA), 7 µl of α-^{32}P-dCTP, and the DNA in a reaction made up to 20 µl with double-distilled water (ddH_2O). The reaction was carried out at 15°C for 90 min, then terminated by adding 25 µl stop buffer (20 mM

EDTA, 0.2% (w/v) SDS, 2 mg/ml salmon sperm DNA). Labelled DNA was separated from the unincorporated free nucleotide by passing through a Sephadex G-50 column equilibrated with TE (pH 7.5) buffer. The average specific activity of each labeled probe was about 10^8 cpm/μg. Before hybridization, probes were boiled for a minimum of 5 minutes to separate the two DNA strands, followed by rapid cooling to prevent renaturation.

4. ISOLATION AND PURIFICATION OF DNA FRAGMENTS AND PLASMIDS

4.1 Sources of cDNA probes and plasmids

The following plasmids were purchased from Promega: SP72, pGEM, Basic-CAT, Enhancer-CAT. TK-CAT 5A and MMTV-CAT were kindly provided by Dr. M. Beato (198). cPR1 progesterone expression vector were a gift of Dr. P. Chambon (199). cDNA for fatty acid synthetase (FAS) and genomic DNA of EGF 5'-flanking region (204-22) were kindly provided by H Rochefort (200) and Dr. G. I. Bell, respectively (201).

4.2 Large scale plasmid preparation

Large scale preparations of plasmid DNA were performed according to the procedures of Maniatis et al (196). A single colony of DH5α E. coli transformed with the appropriate plasmid was inoculated into 5 ml LB medium (Luria-Bertani or LB medium contains 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1% (w/v) NaCl, pH 7.0) with 50 μg/ml ampicillin and incubated at 37°C overnight. The entire bacterial culture was then inoculated into 500 ml LB medium and incubated at 37°C until an OD_{600} value of 0.6 was reached. This usually

requried about 3.5 hr. At this point, 2.5 ml of 4 mg/ml chloramphenicol in ethanol was added and the cells were incubated overnight in a rotary shaker/incubator at 300 rpm. The cell suspension was transferred to a 500 ml Beckman centrifuge bottle and spun in a GS-3 rotor at 4000 X g for 15 min at 4°C. The supernatant was discarded while the pellet was drained and resuspended in 10 ml of a ice-cold solution of 23 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and 0.9% (w/v) glucose. To the mixture was added 0.5 ml of lysozyme solution (20 mg/ml) followed by thorough mixing by inversion. The mixture was incubated on ice for 10 min with gentle shaking and then 10 ml of 0.2 M NaOH, 0.2% (w/v) SDS was added and mixed. The whole suspension was then shaken gently on ice for at least 10 min until the lysate became clear. Following this, 10 ml of ice-cold 3 M sodium acetate (pH 5.5) was added and incubated on ice with shaking for another 30 min. The sample was then centrifuged for 30 min at 15,000 rpm at 4°C in an SS34 rotor to spin down bacterial debris and precipitated high molecular weight DNA. The supernatant was saved and equal volumes transferred into two 30 ml sterile Corex tubes. To each tube was added 0.6 volumes of iso-propanol followed by mixing and incubation at room temperature for 30 min-60 min until a clearly visible white precipitate was observed. The sample was centrifuged in an SS34 rotor at 7,500 rpm for 30 min at room temperature. The supernatant was then discarded and the pellet dessicated for 10 min under vacuum. To each pellet (there were two per 500 ml bacterial culture) was added 3 ml TE (pH 8.0) and dissolution occurred by heating at 65°C for 10 min. The two samples were now pooled into one tube (total volume now 6 ml), and 1.1 g of solid CsCl per ml was added, dissolved with heating at 65°C. The whole mixture was then placed into a Quick-Seal™ centrifuge tube and 0.5 ml of a 10 mg/ml ethidium bromide solution was layered on top using a 3 ml syringe. The tube was filled close to the top with mineral oil, balanced, and then sealed with a Beckman heat sealer. The CsCl and ethidium bromide solutions

were now mixed by inversion. Immediately afterwards, the tube was placed in a Beckman Ti 70.1 rotor and centrifuged at 55,000 rpm for 18 hr at 22°C, following which the speed was reduced to 45,000 rpm for a further 1 hr. Two bands were usually visible under ultraviolet light, the lower band being closed circular plasmid DNA, whereas the top one consisted of nicked plasmid which was later discarded. The desirable band (the bottom one) was removed to a 13-ml Falcon tube by puncturing the side of the centrifuge tube with a 21-gauge needle (fitted to a 5 ml syringe) at a position just below the band. The eithidium bromide in the plasmid solution was removed by extracting 5 to 7 times with approximately 3 volumes of iso-amyl alcohol. Following the extraction, the aqueous bottom layer containing the plasmid was transferred to a fresh tube and the volume made up to 4 ml with ddH_2O. To precipitate the plasmid, 268 µl of 3M sodium acetate (pH 5.5) and 8.5 ml icecold absolute ethanol were added and mixed. Precipitation was allowed to continue at -20°C overnight. The next day the precipitated plasmid was recovered by centrifugation at 11,000 rpm for 30 min at 4°C in an SS34 rotor. The pellet was dried under vacuum and dissolved in 100-200 µl TE buffer (pH 8.0). The plasmid sample was stored at -20°C. The concentration of the plasmid DNA solution was determined by measuring the absorbance of a diluted sample at 260 nm using the formula: 1 OD_{260} = 50 µg/ml DNA.

4.3 Isolation and electrophoresis of DNA fragments

Agarose gel electrophoresis was routinely used to separate and monitor restriction-digested DNA fragments. 'Electroelution' procedure was used, after size fractionation, to separate and purify DNA fragments including cDNA inserts, cut plasmids and fragments of EGF 5'-flanking region. For gel electrophoresis, the percentage of agarose used varies depending on the size of the fragments to be

separated, but usually 1% (w/v) agarose was used. Ultra-pure agarose (BRL) was placed in water and melted by heating in a microwave oven. An appropriate volume of 10 X TBE (Tris-borate/EDTA) buffer (10 X TBE = 108 g Tris base, 55 g boric acid, 40 ml 0.5 M EDTA, pH 8.0 per liter) was then added to give a final 1 X concentration. The whole mixture was stirred to mix and cool, and then poured onto the appropriate gel casting platform. Once the gel was set, it was mounted in the electrophoresis tank (Bio-Rad). Enough 1 X TBE buffer was added to totally submerge the gel. The DNA samples and markers to be fractionated were made 1 X with the addition of an appropriate volume of 10 X gel loading buffer. The voltage applied depends on the length of the run. Usually, for a rapid run of about 1.5 hr, 110-120 volts was used and for overnight runs, 30 volts was used. If the gel did not contain ethidium bromide, it would be visualized by first staining in 0.5 μg/ml of ethidium bromide solution, followed by illumination with UV light. If necessary, photography would be performed using Polaroid type-57 films.

To isolate a particular piece of DNA fragment by electroelution, the plasmid was first cut with the appropriate restriction endonucleases (BRL or Pharmacia) at 37^{o}C for at least 3 hr or overnight using approximately 1.5 units/μgDNA. Following digestion the mixture containing the cut fragments was size fractionated on 1% (w/v) agarose gels in 1 X TBE buffer containing ethidium bromide (7 μl of 10mg/ml solution per 150 ml gel). After running the samples overnight at 30 volts, the gel was visualized by UV and the band of interest cut out from the gel. The gel slice containing the band was then placed in dialysis tubing containing 1 ml of 1 X TBE and the insert isolated from the gel by electrophoresis using 200 volts for 15 to 20 min. After all the insert had moved out of the gel as monitored by UV-induced fluorescence, the current polarity was reversed, and electrophoresis was continued for 2 min to release any DNA that might have stuck to the dialysis tubing.

The solution containing the DNA fragments was recovered and transferred to a 13-ml Falcon tube. The dialysis tubing was rinsed once with 0.5 ml of TBE and this rinse was placed into the same tube. The pooled DNA solution was then extracted 3 times with 3 volumes of iso-amyl alcohol to remove ethidium bromide. The precipitation, recovery and dissolution procedures were as described in section 4.2 (large scale plasmid preparation).

5. RIBONUCLEASE PROTECTION ASSAY

The procedures are schematically outlined in Fig. 3 and arbitrarily separated into three individual parts as follows.

5.1 In vitro synthesis of riboprobe

The HindIII-PstI fragment of the EGF 116 cDNA (kindly provided by Dr. G. I. Bell) was subcloned into the vector pSP73 (Promega/Biocan, Mississauga, Ontario, Canada). It extends from nucleotides 3925-4240 of the EGF precursor protein mRNA and codes for the terminal 44 amino acids of the EGF precursor protein and contains 183 base pairs of 3'-untranslated sequence. This plasmid construct was later used as a template for in vitro synthesis of labeled single-stranded antisense RNA molecules (riboprobe). Before in vitro RNA synthesis, the plasmid was linearized by digestion with the HindIII restriction enzyme, so that run-off transcripts would result from disengaging polymerases. Antisense RNAs labeled with ^{32}P-CTP were synthesized by SP6 RNA polymerase using the following reaction conditions: 40 mM Tris/Cl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 400 µM each of ATP, GTP, and UTP, 6.25 uM α-^{32}P-CTP (800 Ci/mmol, NEN, Lachire, Quebec, Canada), 1 µg linearized template DNA, 40 units RNAsin (Promega/Biocan), and 10 units SP6 RNA polymerase (Pharmacia, Baie

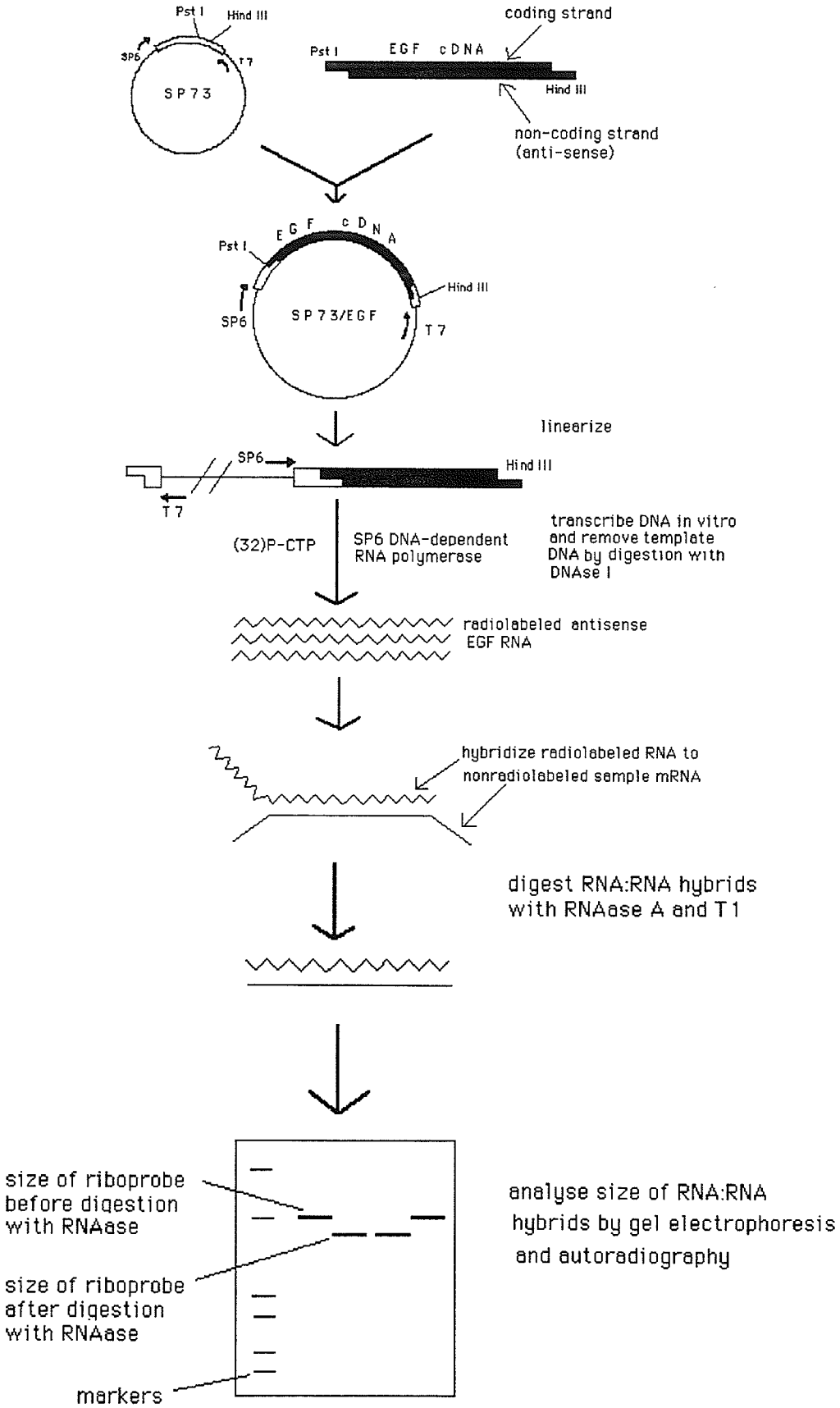


Fig. 3. Schematic representation of RNAase Protection Assay.

d'Urfe, Quebec, Canada) in a final volume of 20 µl. After 45 min of incubation at 37°C, the reaction was terminated by the addition of 10 units RNase-free DNase (Promega/Biocan) and incubated at 37°C for an additional 15 min. Twenty µg of yeast tRNA and water to bring the volume to 50 µl were then added, and the mixture was extracted once with 50 µl phenol/chloroform/iso-amyl alcohol (25:24:1). Unincorporated nucleotides were removed by adding 200 µl 2.5 M ammonium acetate and 750 µl ethanol, followed by incubation at -80°C for 15 min,and centrifugation for 15 min at 4°C. This precipitation step was repeated three more times to further purify the probe. After a rinse in 0.1 M sodium acetate (pH 5.5) / ethanol (25%/75% v/v), the probes were dessicated and dissolved in 100 µl hybridization buffer containing 40 mM 1,4-piperazinediethanesulfonic acid (PIPES) (pH 6.4), 0.4 M NaCl, 1 mM EDTA, and 80% (v/v) deionized formamide. The specific activity of the riboprobe was determined by scintillation counting of a 1 µl sample.

5.2 Liquid hybridization of total RNA with riboprobe

Sample RNA was added to sterile ddH_2O to a final volume of 100ul, precipitated by the addition of 3 M sodium acetate (pH 5.5) to give a final concentration of 0.1 M and 2.2 volumes ethanol, followed by incubation at -80°C for 15 min and centrifugation for 15 min at 4°C. Sample RNA pellets were then dessicated and dissolved in 29 µl hybridization buffer. One µl of ^{32}P-labeled antisense RNA probe containing 2.5 X 10^5 - 1 X 10^6 cpm was added to each sample prior to denaturation at 80°C for 5 min and followed by immediate transfer to the hybridization temperature (55°C). Controls containing 10 µg yeast tRNA were included in each assay. Hybridization was allowed to proceed for 12-16 hr, after which 350 µl 10 mM Tris-Cl (pH 7.5), 0.3 M NaCl, 5 mM EDTA containing

40 µg/ml RNase A and 2 µg/ml RNase T1 was added to each sample, and the incubation continued for 1 hr at 37°C. Ten µl 20% (w/v) SDS and 2.5 µl proteinase K (20 mg/ml) were added and the incubation continued for an additional 15 min at 37°C. Samples were then extracted once with 400 µl phenol/chloroform/iso-amyl alcohol (25:24:1) and the aqueous phase transferred to a microfuge tube containing 25 µg yeast tRNA. The product was precipitated by the addition of 1 ml ethanol (15 min at -80°C) followed by centrifugation at 4°C for 15 min. The pellets were dessicated and dissolved in 20 µl 80% (v/v) formamide, 1 mM EDTA (pH 8.0), 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol. Samples were denatured for 5 min at 80°C, after which they were ready for electrophoresis.

5.3 Polyacrylamide gel electrophoresis

Electrophoresis of samples was carried out in 5% (w/v) polyacrylamide, 7 M urea-sequencing gel using a Bio-Rad sequencing-gel apparatus. Firstly, a solution containing 21 g urea, 5 ml 10 X TBE, and 8.3 ml 29:1(w/w) of acrylamide:bisacrylamide was heated with stirring until the urea was dissolved. The volume of the mixture was made up to 50 ml with ddH_2O and allowed to cool to room temperature. Then, the cooled solution was degassed for 15 sec using a vacuum pump. To the solution was added 70 µl 25% (w/w) ammonium persulfate and 50 µl N, N, N', N'-tetramethylethylenediamine (TEMED) to initiate polymerization of the gel which was poured immediately between the gel plates. The appropriate well-forming comb was then inserted down 0.25 inches into the gel and the two plates clamped tightly using binder clamps. The gel was left at room temperature to polymerize (1.5 hr). After polymerization, the gel was pre-warmed to a temperature of 55°C in 1 X TBE using 1800 volts. The voltage was reduced to

1600 once the desired temperature was reached. Electrophoresis of the denatured samples was performed at 1600 volts for 1.5 hr, following which the gel was removed onto 3 MM Whatman paper, dried at 80°C for 40 min, and exposed to X-ray film (Kodak) with an intensifying screen.

6. NUCLEAR RUN-ON ASSAY

The procedures are schematically outlined in Fig. 4 and consist of 3 parts as described in the following.

6.1 Isolation of active nuclei

T-47D cells from ten 150 X 20 mm dishes/ time point were washed twice with ice-cold 1 X phosphate-buffered saline (10 X PBS or phosphate-buffered saline = 2 g KCl, 2 g KH_2PO_4, 8 g NaCl, and 11.5 g Na_2HPO_4 per liter, pH 7.1), scaped into a 50 ml plug-seal tube, and collected by centrifugation for 5 min in a HN-SII bench-top centrifuge at 4°C. The cell pellet was then resuspended in approximately 4 ml of sterile lysis buffer (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.5), 0.32 M RNase-free sucrose, 14 mM β-mercaptoethanol, 1 mM spermidine, 1 mM EGTA, 2 mM EDTA, and 0.1% (v/v) NP-40, adjusted to pH 7.4) to give a final volume of 6 ml (202). After gentle vortexing, the suspension was homogenized to disrupt cell membranes in a Dounce homogenizer with 15 strokes using a tight-fitting B pestle. To this nuclei homogenate was added 13 ml of a solution (Buffer B) identical to the lysis buffer except that the final sucrose concentration was 1.9 M and NP-40 was omitted. The resulting mixture, now 1.3 M in sucrose, was divided into 2 halves, each (9.5 ml) layered on top of 3.5 ml of a buffer similar to Buffer B except that the sucrose

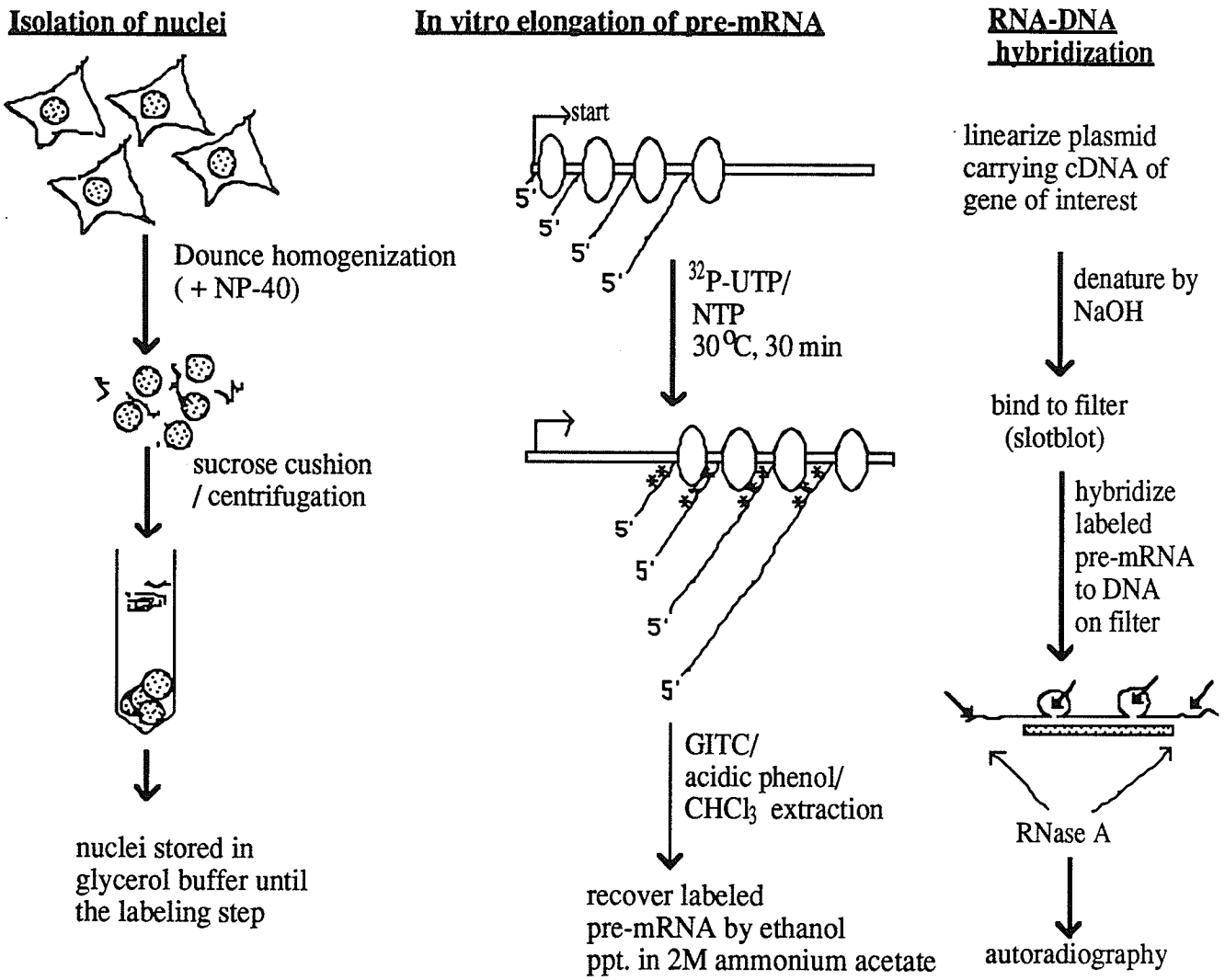


Fig. 4. **Schematic representation of nuclear run-off assay**

concentration was 1.5 M. This concentration of sucrose in the cushion (bottom layer) had been determined empirically to permit T-47D nuclei to pass through. The samples were then centrifuged in a SW 40 rotor at 22,500 rpm for 30 min at 4°C to pellet the nuclei. The supernatant containing membrane fragments and other organelles was withdrawn with a pipette and discarded. Afterwards, 0.5 ml of glycerol storage buffer (20 mM Tris-Cl (pH 7.9), 75 mM NaCl, 0.5 mM EDTA,0.85 mM DTT, 0.125 mM phenylmethylsulfonylfluoride (PMSF), 50% (v/v) glycerol, adjusted to pH 7.4) was added to each nuclei pellet which was then resuspended by gentle pipetting using 1 ml pipette tips with the tip portion cut-off to widen the bore. The resuspended nuclei from the two separate tubes were pooled, pelleted in a microcentrifuge for 4 min at 4°C, and resuspended in 100-150 µl fresh storage buffer. The suspended nuclei were frozen immediately at -70°C until the in-vitro elongation step, but stored for no longer than 1 wk to ensure optimal activity.

6.2 In-vitro elongation and isolation of hnRNA

Frozen nuclei was thawed on ice and approximately 9-12 X 10^6 nuclei was transferred to a 13-ml tube, followed by addition of nuclei storage buffer to 150 µl. An equal volume of 2 X reaction buffer (2 X reaction buffer = 300 mM KCl, 10 mM $MgCl_2$, 40 mM Tris-Cl, pH 7.9, 1.15 mM DTT, 0.1 mM S-adenosylmethionine, 20 mM creatine phosphate, 4 ng/ul phosphocreatine kinase, 260 U/ml RNasin, 1 mM each of ATP, GTP, and CTP, and 1 µCi/µl α-^{32}P-UTP (800 Ci/mmol, 10 mCi/ml)) was added to initiate the labeling reaction. The in-vitro elongation step was allowed to proceed for 30 min at 30°Cwith gentle shaking.

An acidic phenol/guanidinium thiocyanate method (203, 204) was used in the isolation of nuclear RNA. After the labelling step, 1.2 ml of GITC buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.5), 0.5% N-lauroysarcosine, 0.007% β-mercaptoethanol) was added to each 300 µl reaction mixture to stop the reaction and lyse the nuclei. The contents of all three tubes from the same time point were pooled together with a 3 ml syringe attached to a 21-gauge needle and passaged through the needle 10 times to shear DNA. To this 4.5 ml mixture was sequentially added 450 µl 2 M sodium acetate (pH 4.0), 4.5 ml phenol (H_2O-saturated), and 0.9 ml chloroform/iso-amyl alcohol (49:1) with vigorous vortexing after each addition. The resulting mixture was cooled on ice for 15 min and then centrifuged at 9500 rpm, 4°C in a SS34 rotor (10,000 g). The top aqueous phase (4.5 ml) containing labeled nuclear RNA was transferred to a fresh tube. To ensure precipitation of small quantities of nuclear RNA, 300-400 µg of yeast tRNA was then added followed by 4.5 ml of isopropanol. The samples were then stored at -20°C overnight and RNA recovered by centrifuging at 10,000 g for 20 min. The radioactive supernatant was decanted followed by addition of 1 ml each of GITC and isopropanol. Precipitation was again performed at -20°C for several hr or at -70°C for 0.5 hr, after which RNA was recovered as above, and dried briefly under vacuum. The pellet was further purified with the addition of 300 µl of 2 M ammonium acetate, then transferred to a microfuge tube, and precipitated with 750 µl ethanol at -70°C for 0.5 hr. Final recovery was achieved by centrifugation for 15 min at 4°C. RNA was dissolved in 150 µl of 50% (v/v) formamide/ddH_2O. The radioactivity in 1 µl of the RNA sample was determined by scintillation counting.

6.3 Filter strip hybridization

Five μg of each plasmid were linearized using the appropriate restriction enzymes and denatured by incubation with NaOH to a final concentration of 0.4 M for 30 min at room temperature. The mixture was then neutralized with an equal volume of 2 M ammonium acetate. Plasmid DNA was spotted onto Nitroplus 2000 using a slotblot apparatus (Bio-Dot^R, Bio-Rad) and rinsed with 300 μl of 1M ammonium acetate. The filter was air-dried, then baked at 80^{o}C for 2 hr under vacuum. Filter strips were prehybridized for at least 2 hr at 50^{o}C in a solution containing 65% (v/v) deionized formamide, 20 mM PIPES, pH 6.4, 0.4 M NaCl and 100 μg/ml yeast tRNA (205). Prior to hybridization ^{32}P-labeled RNA was heated for 3 min in a boiling water bath, cooled quickly and then added to fresh hybridization solution. Hybridization was carried out at 50^{o}C for 2 days. The filters were then washed 4 times in 2X SSC, 0.1% SDS (w/v) for 5 min at room temperature and then 2 times in 1X SSC, 0.1% (w/v) SDS for 30 min at 65^{o}C. Following this the filters were treated with 10 μg/ml RNase A in 0.3 M NaCl for 1-2 min, followed by 10 min in 0.3 M NaCl at room temperature The filters were air dried and then exposed to X-ray film at -70^{o}C.

7. CONSTRUCTION AND MANIPULATION OF PLASMIDS

7.1 Modifications of restriction enzyme-digested DNA fragments

Dephosphorylation of plasmid ends were achieved by treatment with calf-intestine alkaline phosphatase (Boehringer Mannheim, Laval, Quebec). 1U/10 ug DNA was added directly to the reaction mixture following restriction enzyme digestion and incubated for 1 hr at 37^{o}C.

The blunt-ending reaction was carried using the Klenow fragment of DNA polymerase (Pharmacia). To 10 µl DNA solution was added 2 µl 10X Klenow buffer (0.5M Tris/Cl pH7.5, 0.1 M $MgCl_2$, 10 mM DTT), 1µl of a nucleotide mixture containing 1mM each of dATP, dCTP, dTTP, dGTP, 6.5 µl H_2O and 3 U DNA polymerase (Klenow fragment). The reaction was incubated at 37°C for 30 min.

7.2 Construction of reporter plasmids pGEM-CAT(S), pGEM-CAT/204, and pGEM-revCAT/204

CAT plasmids based on the blank vector pGEM were constructed by ligating the CAT coding region (flanked by either HindIII: pGEM-CAT (H), or SalI: pGEM-CAT (S), restriction sites) into pGEM cut with the same enzymes (Fig. 5). The orientation of the CAT insert was determined by asymmetric restriction enzyme digestions (see Results 4.2.2). A chimeric CAT plasmid containing 3 kbp of the human EGF 5'-flanking region plus 370 bp of exon 1 (i.e. the 3.3 kbp EcoRI-SmaI fragment of 204-22, see Result 4 and Fig. 18 for description) was made starting with the blank vector pGEM (Fig. 5). pGEM was first digested with SmaI, then EcoRI, both restriction sites were in the multiple cloning site. The double-digested pGEM was dephosphorylated by phosphatase and subsequently ligated to the 3.3 kbp subfragment of 204-22 cut also by SmaI and EcoRI. This allowed uni-directional ligation of the insert. The final plasmid was completed by inserting a promoterless CAT gene into the SalI site of the intermediate pGEM/204, placing the CAT cartridge immediately downstream of the 204 fragment. Since the CAT insert could be ligated in either of two directions, plasmids containing both types of CAT insert were prepared. The plasmid containing the CAT gene and the 204 fragment in opposing direction was named pGEM-revCAT/204.

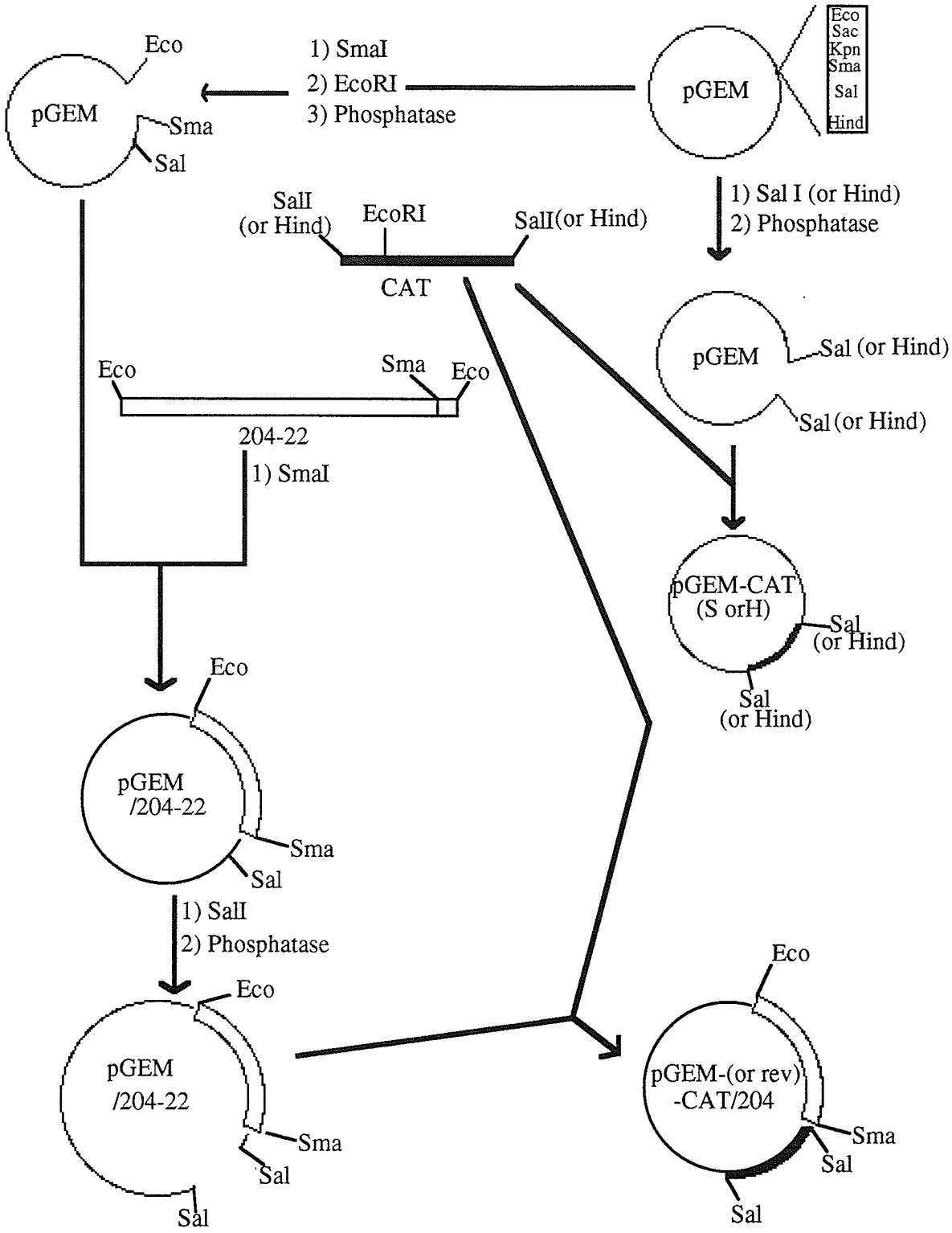


Fig. 5. Construction scheme of plasmids pGEM-CAT(Sal or Hind), pGEM-CAT/204 , and pGEM-rev-CAT/204

7.3 Construction of reporter plasmids Basic and Enhancer-CAT/204 or 3'-204

To construct Basic-CAT/204 and Enhancer-CAT/3'-204, full-length 3.5 kbp 204-22 fragment (3 kbp 5'-flanking region plus approximately 500 bp of exon 1) was first treated with the Klenow fragment of DNA polymerase to blunt the EcoRI restriction sites at each end. The fragment was then cloned into the blunted SalI site of the promoterless CAT vectors (Fig. 6).

To construct Basic-CAT/3'-204 and Enhancer-CAT/3'-204, the 204-22 fragment was first blunted at the EcoRI ends with Klenow, then cut with HindIII to yield two fragments of approximate sizes of 1.65 and 1.95 kbp (Fig. 6 and 18). The resulting fragments have one blunted EcoRI end and a cohesive HindIII end. The vectors into which the 204 subfragments were subcloned, were first cut with SalI, then treated with Klenow to create a blunt end, followed by treatment with HindIII to expose the cohesive site. The vectors were then ligated to the 204-22 fragments that were now equipped with compatible ends. The correct constructs, those containing the 1.95 kbp (3'-204) fragment were identified by various restriction cuts (4.2.2).

7.4 Transformation of DH5α or RR1 strains of E. coli

Transformation competent bacterial cells (RR1 or DH5α) were prepared by inoculating 1 ml of fresh overnight culture in 50 ml LB broth and incubating at 37°C with shaking until OD_{600} was about 0.6. The cells were chilled in ice-water and collected by centrifugation. The cell pellet was resuspended in 20 ml 50 mM $CaCl_2$ for 20 min in ice-water, centrifuged to pellet at 4°C, and resuspended again

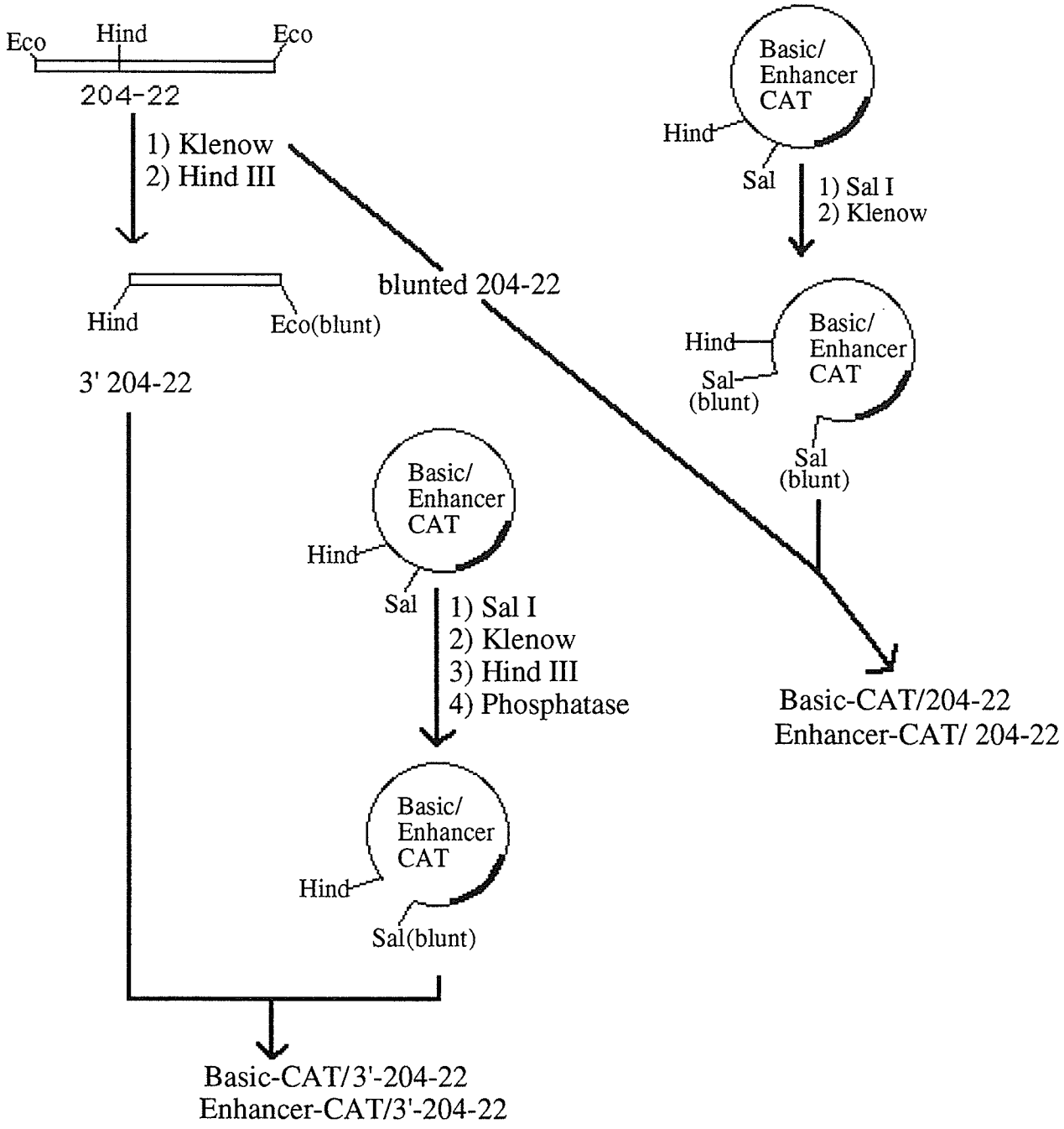


Figure 6. Construction of plasmids Basic Enhancer CAT/ 204-22 3'-204-22

in 3.7 ml 50 mM $CaCl_2$ and left at 4°C overnight. For transformation, 200 µl of competent cells were incubated with the appropriate plasmid or ligation product for 40 min on ice, followed by heat-shocking at 42°C for 3 min. Then 200 µl of LB broth was added to each sample and incubated at 37°C for 1 hr to allow expression of the antibiotic-resistance gene. The entire 400 µl were plated out onto antibiotic containing LB agar (1.5 % w/v) plates and incubated overnight.

7.5 Mini plasmid preparation

Isolated colonies from antibiotic plates were picked, inoculated in 2 ml of LB plus the appropriate antibiotic and incubated overnight at 37°C with shaking. One and half ml of the culture was transferred to a microfuge and centrifuged in a bench-top microcentrifuge for 1 min at room temperature. After removal of the medium, 100 µl of ice-cold GETL buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris/Cl pH 8, 1 mg/ml lysozyme) was added to the tube followed by vigorous vortexing. After incubation at 37°C for 5 min, 200 µl of Lysis Solution (0.2 M NaOH, 1% (w/v) SDS) was added, gently mixed by inversion, and then incubated on ice for 5 min. To each tube was then added 150 µl ice-cold 3M potassium acetate pH 4.8 with gentle vortexing for 10 sec followed by incubation on ice for 5 min and then centrifugation at 4°C for 5 min. Three hundred and fifty µl of supernatant was transferred to a microfuge tube and extracted with the same volume of phenol/$CHCl_3$/iso-amyl alcohol (25:24:1) by vortexing for 5 sec. After centrifugation for 2 min at room temperature, 250 µl of supernatant was transferred to another microfuge tube followed by the addition of 250 µl of $CHCL_3$/iso-amyl alcohol (24:1). After vortexing and centrifugation as above, 150 µl of supernatant was removed to a clean tube with the addition of 300 µl absolute ethanol. The solution was left at room temperature for 10 min and then centrifuged for 10 min at 4°C. The supernatant was drained and the DNA pellet rinsed in 1 ml of 70% (v/v)

ethanol in water. The plasmid DNA was finally recovered by centrifuging for 10 min at 4°C and the pellet dessicated for 10 min. Contaminating RNA was eliminated by treating the sample with 25 µl 0.2 mg/ml RNAse A. The sample was now ready for restriction enzyme analysis using 5-10 µl sample for a typical reaction.

8. TRANSIENT TRANSFECTION ANALYSIS OF REPORTER GENE ACTIVITY

8.1 Transfection by the $CaPO_4$/DNA precipitate method

The methodology used was as described in reference (206) with modifications. Cells to be transfected were split into 100 X 20 mm dishes the day before transfection. The next day the medium was replaced with 9.5 ml of fresh medium 2 to 4 hr prior to addition of the $CaPO_4$/DNA precipitate. To prepare the precipitate, a 0.5 ml mixture containing 0.25 M $CaCl_2$ and plasmid DNAs (amount depending on experiments) was added dropwise to 0.5 ml 2 X HEPES buffer (0.28 M NaCl, 50 mM HEPES sodium salt, 1.5 mM Na_2HPO_4, pH 7.05 $\pm$ 0.05) contained in a 15 ml tube and mixed by bubbling air through a continous stream of filter (0.22 µM). The resulting mixture was shaken for 5 sec and was left to sit at room temperature for 30 to 45 min to allow formation of the $CaPO_4$ precipitate to which DNA would be bound. The solution was shaken, then added evenly onto the cell surface with gentle agitation of the dish to ensure immediate neutralization of the acidic precipitate solution. The cells were incubated with the precipitate for 6 hr and then the medium removed. After that, the cells were 'shocked' by adding 5 ml of a sterile 10-20% (v/v) glycerol/DMEM solution for 3-5 min at room temperature. This step allowed the cells to efficiently take up the DNA/$CaPO_4$ precipitate which was now attached to the cell surface. The glycerol solution was first diluted by adding copious amount of 1 X PBS and removed by aspiration.

The cells were then rinsed twice with copious amount of 1 X PBS, then fed with fresh medium. If required, various drugs (e.g. hormones, sodium butyrate) would be added at this point. If cells were treated with sodium butyrate (6mM) to enhance gene expression, the drug would be removed 14 hr later by aspirating and rinsing with 1 X PBS. The cells would be replenished with fresh medium and hormones, if previously included. Forty-eight hr after glycerol-shock, the cells were harvested by first rinsing 2 times with 1 X PBS, then incubating on ice in the presence of 1 ml TEN solution (TEN or Tris/EDTA/NaCl solution contains 40 mM Tris-Cl, pH7.5, 1 mM EDTA, pH 8.0, and 150 mM NaCl) for 5 min. Cells were scraped into microfuge tube, centrifuged for 1 min in a microcentrifuge, and the supernatant aspirated off. The cell pellet was resuspended in 100-150 µl (depending on pellet size) ice-cold 0.25 M Tris-Cl, pH 7.5 and immediately frozen at -70°C in a CryoCool refrigeration unit for 5 min, followed by thawing for 5 min at 37°C. This cycle of freezing and thawing was repeated twice more to ensure disruption of cell memebrane. Then the cell lysate was cooled on ice and spun for 5 min at 4°C in a microcentrifuge. The clear supernatant (containing CAT and β-galactosidase (β-gal) enzymes) was saved and stored at -20°C.

8.2 Protein determination

The method of Lowry was used (207). Briefly, 5 or 10 µl of cell extract was made 0.67 N NaOH by the addition of 2 volumes of 1N NaOH, and the final volume made to 500 µl using 0.67 N NaOH. The standard curve was prepared using 0, 10, 30, and 50 µg of BSA. To each tube was added 2.5 ml of a copper tartrate/Na_2CO_3 solution made by adding 1 ml of copper reagent (0.02% (w/v) sodium potassium tartrate, 0.01% (w/v) copper pentahydrate, 0.025 N NaOH) to 50 ml 2% (w/v) Na_2CO_3. The tubes were incubated at room temperature for at least 10 min, after which 250 µl of 1:1 (vol/vol) Phenol Reagent : water was added

with mixing. At least 30 min was allowed for color development and OD was measured at 625 nm wavelength.

8.3 Chloramphenicol acetyltransferase assay

The method of Kingston (208) was followed with modifications. If β-galactosidase was to be determined (i.e. pCH110 plasmid was transfected), a small portion (30%) of the lysate was aliquoted into a separate tube for the later determination of enzyme activity. The remaining 70% of the extract was then subjected to heat treatment (60°C for 10 min) to eliminate any potential interference by deacetylase activity. The CAT enzyme is stable to this treatment. The tubes were then spun for 1 min to pellet denatured, precipitated proteins. Ten µl of the heat-treated supernatant was used for the determination of protein content by the Lowry assay (see section 8.2). An aliquot of the heated cell lysate containing 40 µg protein was made to 150 ul with ddH_2O. To this was added 54.5 µl of reaction cocktail which contained 2 µl ^{14}C-chloramphenicol (200 µCi/ml, NEN), 20 µl 4 mM acetyl-CoA (Sigma) and 32.5 µl 1M Tris-Cl, pH 7.5. The mixture was incubated for 1.5 hr at 37°C, followed by extraction with 1 ml ethyl acetate and centrifugation for 1 min in a microcentrifuge. The top organic phase containing both unreacted and acetylated chloramphenicol was removed into a new tube and the ethyl acetate was evaporated under vacuum for 45 min . The dried sample was then resuspended in 30 µl ethyl acetate, spotted onto a thin layer chromatography (TLC) plate (Baker-flex silica gel 1B, John's Scientific, Toronto, Ont.). The TLC plate was developed for 2 hr in a chromatography tank previously equilibrated with 200 ml of 19:1 (v/v) chloroform: methanol. The TLC was removed, air dried, and sprayed 3 times with EN^{3}HANCE™ SPRAY (Du Pont, Mississauga, Ont.) to enhance the intensity of radio-signals. Autoradiography was for 3-5 days using Kodak XAR film. Quantitation was achieved by aligning the TLC plate with its

autoradiogram, outlining each spot on the TLC, cutting out each spot which was then placed in a scintillant (Aquosol, Du pont) and counted in a Beckman C53 801 scintillation counter . The activity of CAT was calculated as follows: % acetylated = 100 X (counts in acetylated species)/(counts in acetylated species+counts in unacetylated species of chloramphenicol).

8.4 β-galactosidase assay

The protein content of the unheated portion of cell lysate was first determined by the method of Lowry. The procedures for the β-gal assay was as described in reference (209). An aliquot of cell extract equivalent to 30 μg protein was incubated with 3 μl 100 X magnesium buffer (100 mM $MgCl_2$, 5 M β-mercaptoethanol), 66 μl o-nitrophenyl-β-D-galactopyranoside (Sigma) solution (4 mg/ml in sodium phosphate buffer) and sodium phosphate buffer (0.1 M Na_2HPO_4 adjusted to pH 7.3 using 0.1 M NaH_2PO_4) to a final volume of 300 μl. The reaction mixture was incubated at 37°C until a yellow color was visible (usually 3 to 4 hr). The reaction was then stopped by adding 0.5 ml of a 1 M Na_2CO_3 solution, and the OD measured at 410 nm.

RESULTS

1. ACCUMULATION OF EGF mRNA AFTER TREATMENT WITH MPA, CHX AND MPA + CHX

Murphy et al., (55, 191) demonstrated for the first time that the gene for EGF was expressed in human breast cancer biopsies and in the established human breast cancer lines T-47D and ZR-75-1. Using Northern blot analysis, they had been able to show unequivocally that in T-47D cells progestins including the synthetic analog medroxyprogesterone acetate (MPA) caused a maximum 6-fold increase in steady-state EGF mRNA levels. The first observable increase was registered at 6 hr after hormone treatment. To further examine this induction of EGF gene expression more fully, a more sensitive RNAase protection assay was employed to measure EGF mRNA levels after various treatments and times. As can be seen on Fig. 7A and C, with this assay, treatment of T-47D cells with 10 nM MPA led to a rapid increase in EGF mRNA levels as early as 3 hr after stimulation, 9 hr earlier than that observed by Northern analysis. The induction of EGF reached maximal levels (11-fold increase) by 24 hr of exposure to the hormone, after which time the levels declined gradually. Once again the observed large increase in EGF mRNA levels demonstrated the usefulness and sensitivity of the protection assay in detecting the EGF mRNA which previously was only detected by Northern analysis of poly (A^+) enriched RNA.

To determine if ongoing protein synthesis was required for the effect of MPA on EGF gene expression, T-47D cells were treated with 50 uM cycloheximide 2 hr before and during MPA treatment to block protein synthesis. Total RNA was then isolated at the indicated times after treatment and analysed by RNAase protection. Fig. 7A and C show that in the presence of cycloheximide, MPA still

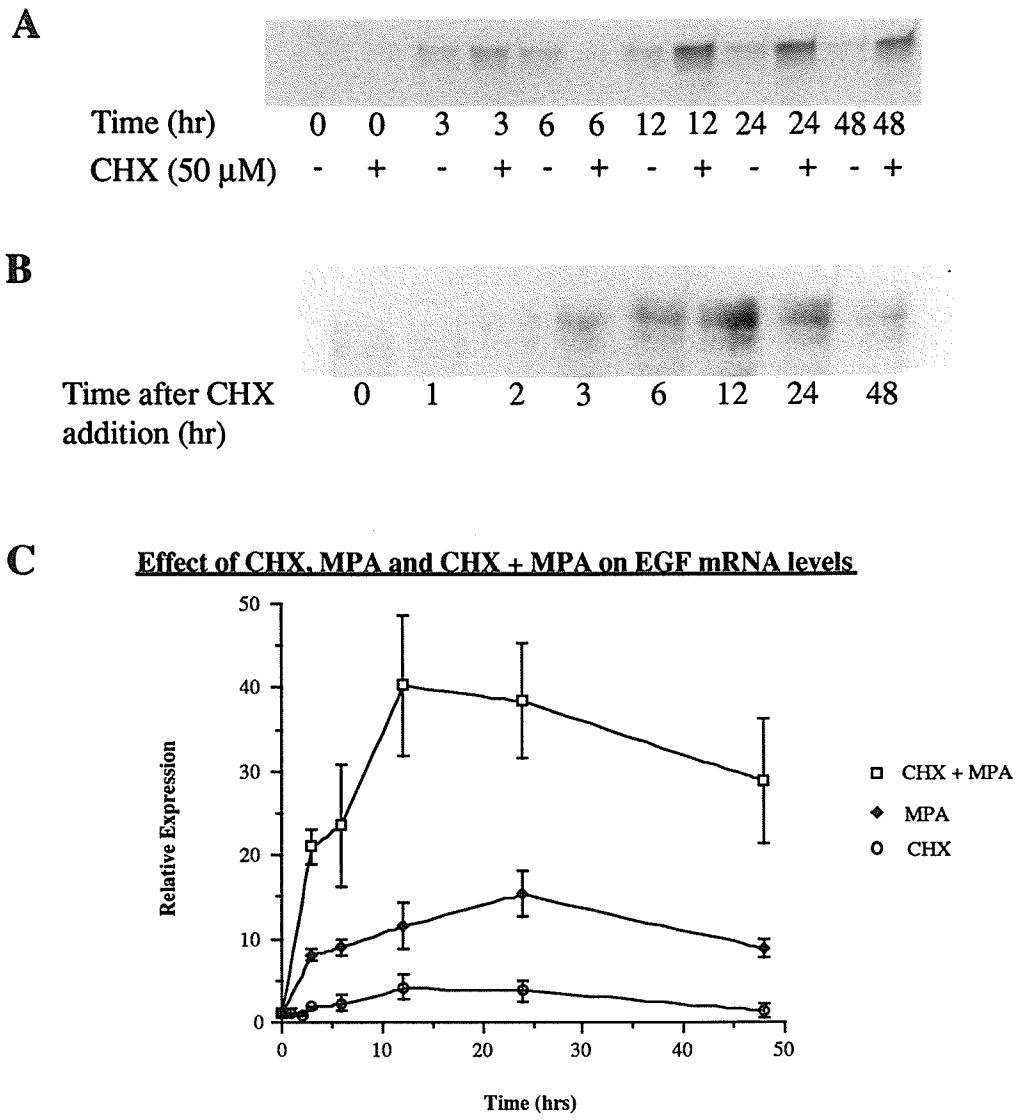


Fig.7. Time course of the effects of MPA, MPA+CHX and CHX alone on EGF mRNA levels.

A. T-47D cells were treated with MPA alone (10 nM) or pretreated with 50 µM cycloheximide (CHX) for 2 hr before MPA stimulation. At the indicated times after MPA addition, cells were harvested and total RNA prepared and analysed by RNAase protection assay (10 µg/lane). An autoradiogram(exposed overnight) representative of 3 separate experiments is shown (data obtained by H. Dotzlaw and L. Murphy).

B. T-47D cells were treated with CHX (50 µM) and harvested at the indicated times. Total RNA was analysed as in A. An autoradiogram(exposedfor 2 days) representative of 3 experiments is shown.

C. Graphic presentation of the same data in A and B after quantitating autoradiograms by densitometric scanning. Mean +/- SEM of 3 experiments is shown.

resulted in increased levels of EGF mRNA. Moreover, the combined treatment of cycloheximide and MPA caused a "super-induction" of EGF mRNA, at 12 hr after MPA treatment the levels were 37-fold greater than in untreated control cells. This value was more than 3 times the highest value observed for MPA treatment alone. Cycloheximide treatment by itself had a small but significant effect on EGF mRNA levels. A 4-fold induction was apparent at between 12 to 24 hr after treatment (Fig 7B and C). These results demonstrated superinduction, that is, the MPA-induced EGF mRNA levels in the absence of on-going protein synthesis exceeded those in the presence of on-going protein synthesis, and thus de novo protein synthesis is not a requirement for the effect of MPA on EGF gene expression.

2. EFFECT OF MPA ON EGF GENE TRANSCRIPTION

Since mechanisms responsible for elevations in steady-state RNA levels include increases in transcription, mRNA processing, mRNA stability and export from the nucleus (see Introduction 4), it was decided that the contribution of two of these mechanisms, namely, transcription and mRNA stability to the increased EGF mRNA expression would be examined in the first instance. These two levels of gene regulation were chosen mainly because they are the most studied and understood and are also the most amendable to experimental manipulations and analyses. Also, since steroids usually affect gene expression mainly through gene transcription and message stability, it was only logical to limit the investigation to these two processes.

We first determined if transcriptional mechanisms were responsible for the changes. For this purpose, nuclear run-off assays were performed to measure the rate of nascent EGF transcripts production in isolated nuclei from control and

MPA-treated T-47D cells. T-47D nuclei were isolated at 1, 3, 6, and 9 hr after addition of MPA and in-vitro elongation of transcripts was allowed to continue in the presence of α-^{32}P-UTP. Labeled nuclear RNA was isolated and the extent of radioactive UTP incorporation in EGF transcripts determined by filter hybridization to an excess of EGF cDNA immobilized on nitrocellulose strips. It is important to note that under the assay conditions, initiation of transcription in isolated nuclei does not occur so that amount of radioactive incorporation is the result of elongation of nascent chains already initiated at the time of nuclei isolation. Thus it allows a measure of alteration in the rate of transcriptional initiation, if any, as a consequence of some input of stimuli such as that of hormones and second messengers.

Fig. 8 presents some preliminary evidence that the transcription rate of the EGF gene is enhanced by MPA. In untreated T-47D cells, EGF gene transcription was undetectable, an observation consistently obtained under the conditions described here. An increase was observed after 1 hr of MPA treatment, which reached a maximum at 3 hr. Thereafter the signal decreases only slightly for the remaining time points examined (6 and 9 hr). In contrast, β-actin gene transcription which served as a negative control was unaffected by MPA, whereas background nonspecific hybridization as detected by the blank plasmid pSP72 was minimal (Fig. 8). Fatty acid synthetase (FAS) has been shown to be transcriptionally regulated by progestins in T-47D cells (200) and thus served as a positive control. Its transcription rate was increased at 3 hr after hormone treatment (Fig. 8). Consistent with the low steady-state EGF mRNA levels detected by Northern and RNAase protection analyses, the basal transcription rate of EGF was found also to be extremely low, especially when it is taken into account that in the particular experiment shown 30×10^6 nuclei and 35×10^6 cpm of radioactivity per time

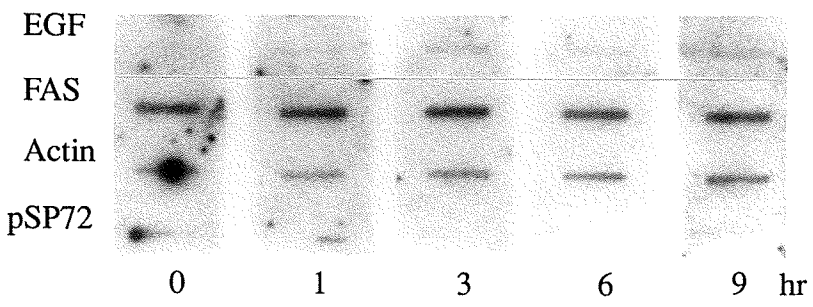


Fig. 8. Effect of MPA on transcription of the EGF gene. Nuclei (30 X 10^6 nuclei/time point) were isolated from T-47D cells after MPA (10nM) stimulation for the indicated periods of time, Nuclear run-off transcription assay was performed as described in "Materials and Methods". Equal counts of radiolabeled run-off transcripts were hybridized to 5 μg of linearized plasmids carrying EGF, FAS or Actin cDNA inserts. The pSP72 plasmid was a blank vector used as negative control. Shown here is the autoradiogram after 1 wk exposure with 2 intensifying screens.

point were used. This presented great technical difficulty, preventing the reproducible demonstration of transcriptional activation as the mechanism of MPA-induced EGF gene expression. The inability to detect a transcription signal in untreated cells precluded the accurate quantitation of the radioactive signal obtained at maximum transcription rate induction. Hence, fold-increase of transcription by MPA is not given. The increase from undetectable to detectable would argue for a significant induction, which would account at least in part for the 11-fold maximum induction at 11 hr after MPA stimulation, as determined by RNAase protection analysis (see Fig. 7C).

3. MEASUREMENT OF mRNA HALF-LIFE IN T-47D CELLS

Since the analysis of transcription by run-off assay did not provide an unequivocal result, we next investigated possible effects of progestins on EGFmRNA stability. To do this the rate of decay of EGF mRNA levels after actinomycin D treatment in control T-47D cells (Fig. 9) and in MPA-treated cells (Fig.10) was measured. Actinomycin D (ActD) was added to the cells 24 hr after MPA treatment to block all transcriptonal activity. In mammalian cells, ribosomal RNA (rRNA) synthesis is known to be up to 100-fold more sensitive to inhibition by ActD than is mRNA (210, 211, 212, 213). Low concentrations of ActD (10^{-7} to 10^{-8} M) interfere primarily with the synthesis of pre-rRNA, but higher concentrations (> 10^{-6} M) effectively inhibits all classes of RNA synthesis (211, 212). Therefore, an ActD concentration of 5 μg/ml (4 X 10^{-6} M), commonly adopted in mRNA stability analysis, was used in these studies. Total RNA was extracted and analysed by Northern to probe for FAS mRNA (stabilized by progestins) (200) or RNAase protection analysis to detect EGF transcripts. Since blockade of transcription would affect all mRNAs leading to their eventual decay, even loading of RNA samples during Northern analysis cannot be controlled by

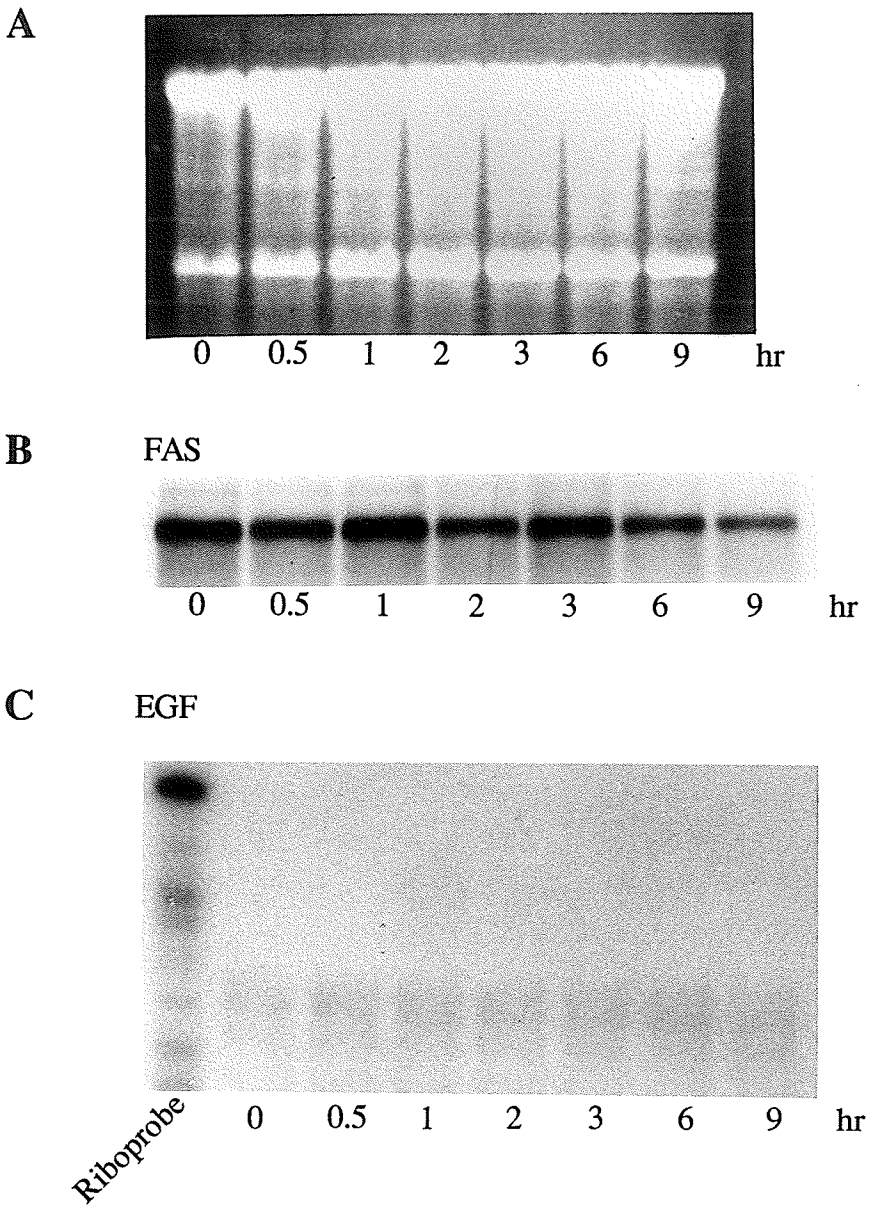


Fig. 9. Time course of EGF and FAS mRNA decay in control T-47D cells using ActD as transcriptional inhibitor.

T-47D cells were harvested at the indicated times after actinomycin D (ActD) treatment (5μg/ml). Total RNA was isolated and either subjected to Northern blot analyses (40 μg/lane) to probe for fatty acid synthetase (FAS), or analyzed by RNase protection assay (30 μg/lane) to detect EGF mRNA.

A. An ethidium bromide (EtBr) - stained Northern gel representative of 3 separate experiments.

B. Autoradiogram obtained after hybridizing Northern blot (from A) with ^{32}P-labeled FAS cDNA, and exposed to X-ray film for 2 days.

C. A representative autoradiogram (exposed for 4-5 days) of RNAase protection assay showing the EGF mRNA decay.

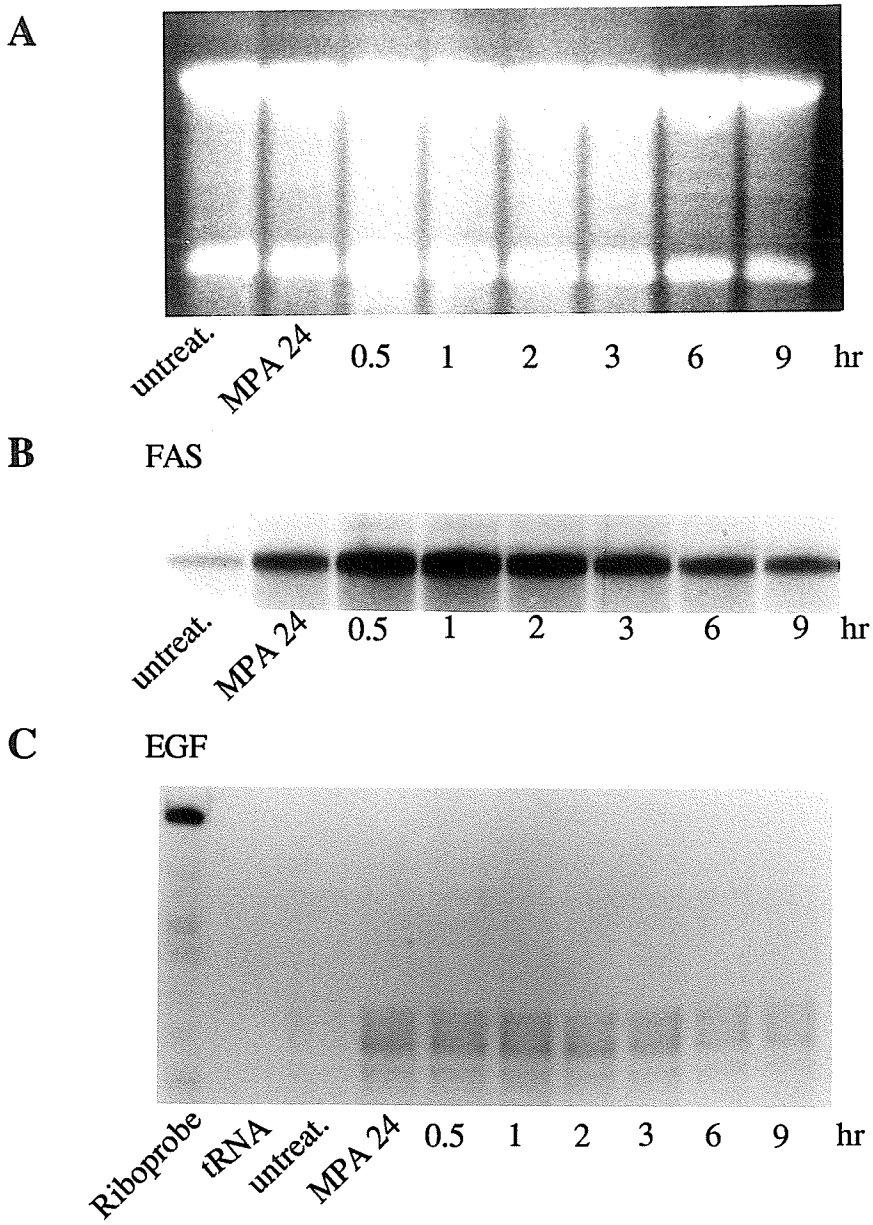


Fig. 10. Time course of EGF and FAS mRNA decay in MPA-treated T-47D cells using ActD as transcriptional inhibitor.

T-47D cells were treated with 10 nM MPA followed by ActD. Cells were harvested at the indicated times after ActD addition. Total RNA was isolated and subjected to Northern blot analysis (40 µg/lane) to probe for FAS, or analyzed by RNAase protection assay (10 µg/lane) to detect EGF mRNA.

A. An EtBr - stained Northern gel representative of 3 separate experiments.
B. Autoradiogram obtained after hybridizing Northern blot (from A) with ^{32}P-labeled FAS cDNA, and exposing for 2 days to X-ray film.
C. A representative autoradiogram of RNAase protection assay showing EGF mRNA decay. Autoradiography was for 2-4 days.

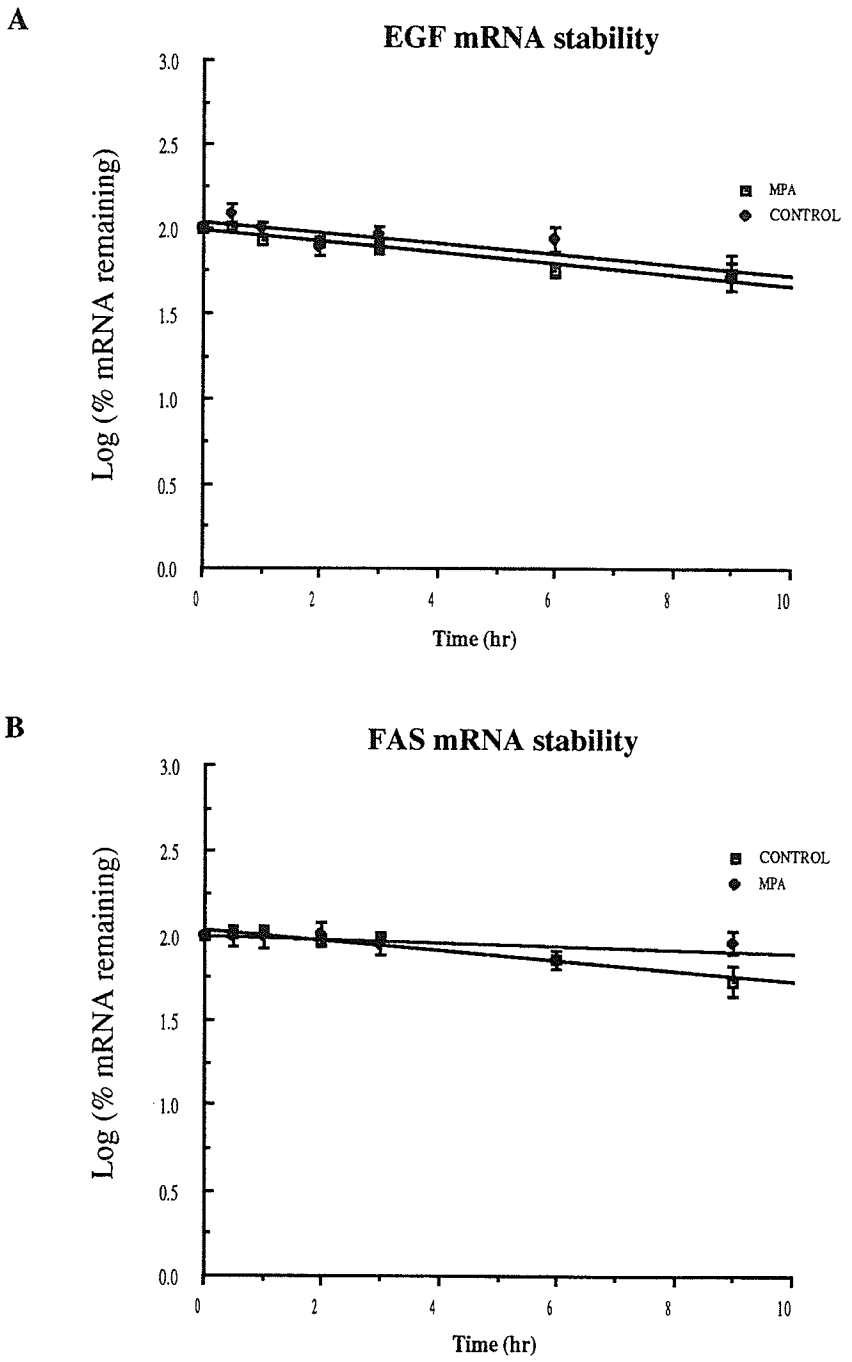


Fig. 11. Measurement of EGF and FAS mRNA half-life in control and MPA-treated cells using ActD as transcriptional inhibitor. The same data in Fig. 9 and 10 were plotted as mRNA dacay curves.

A. EGF mRNA decay curves. Mean +/-SEM was shown, n=3 and 2 for MPA and Control, respectively.

B. FAS mRNA decay curves. Mean +/-SEM was shown, n=3 for both Control and MPA.

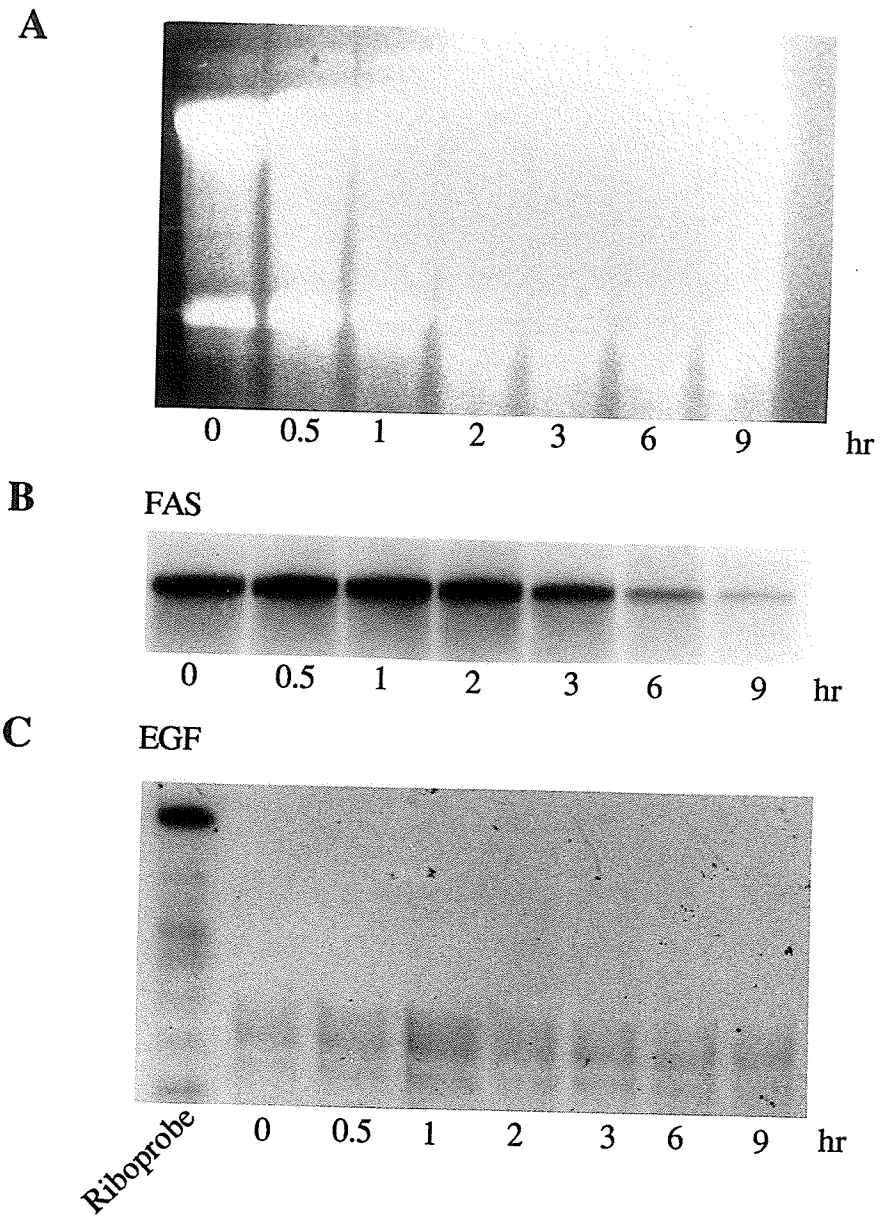


Fig. 12. Time course of EGF and FAS mRNA decay in control T-47D cells using DRB as transcriptional inhibitor.

T-47D cells were harvested at the indicated times after DRB treatment (25μg/ml). Total RNA was isolated and subjected to Northern blot analysis (40 μg/lane) to probe for fatty acid synthetase (FAS), or analyzed by RNAase protection assay (30 μg/lane) to detect EGF mRNA.

A. An ethidium bromide (EtBr) - stained Northern gel representative of 3 separate experiments.

B. Autoradiogram obtained after hybridizing Northern blot (from A) with ^{32}P-labeled FAS cDNA, and exposing to X-ray film for 2 days.

C. A representative autoradiogram of RNAase protection assay showing EGF mRNA decay. Autoradiography was for 4-5 days.

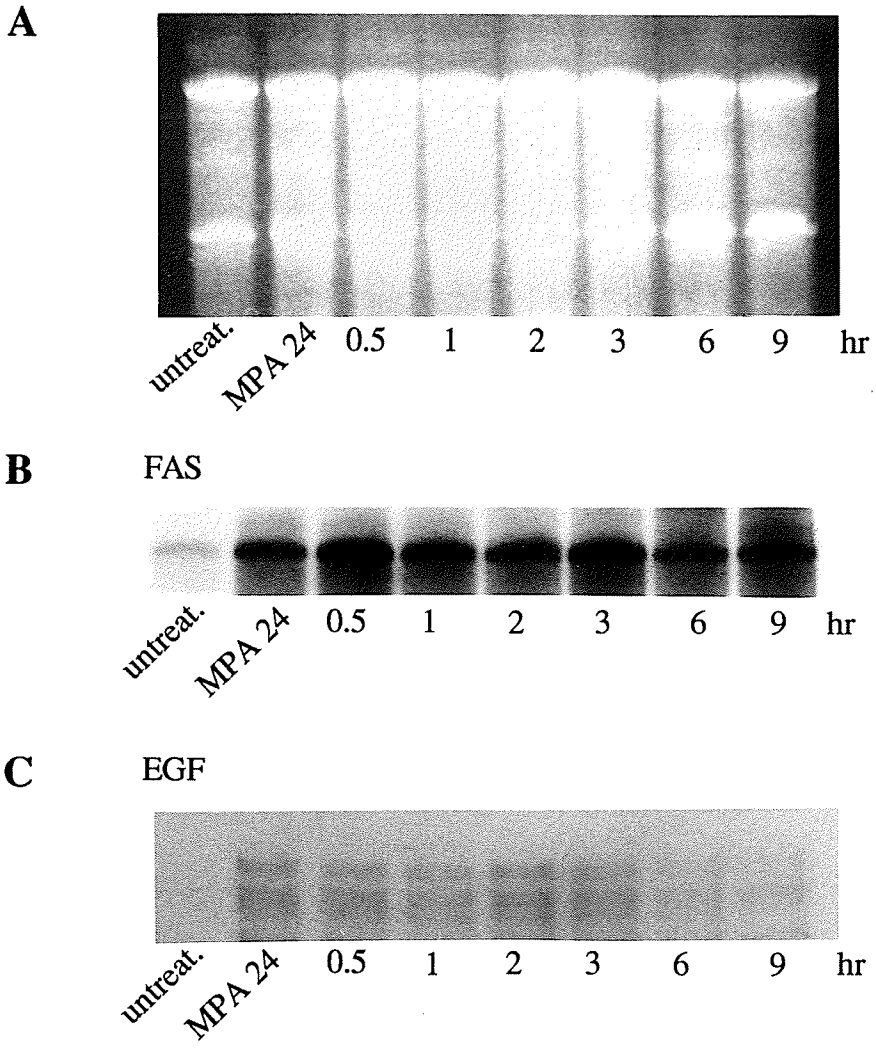


Fig. 13. Time course of EGF and FAS mRNA decay in MPA-treated T-47D cells using DRB as transcriptional inhibitor.

T-47D cells were treated with 10 nM MPA for 24 hr followed by DRB (25 ug/ml). Cells were harvested at the indicated times after DRB treatment. Total RNA was isolated and subjected to Northern blot analysis (40 µg/lane) to probe for FAS mRNA, or analyzed by RNAase protection assay (10 µg/lane) to detect EGF mRNA.

A. An EtBr - stained Northern gel representative of 3 separate experiments.

B. Autoradiogram obtained after hybridizing Northern blot (from A) with ^{32}P-labeled FAS cDNA, and exposing to X-ray film for 2 days.

C. A representative autoradiogram of RNAase protection assay showing EGF mRNA decay. Autoradiography was for 3-4 days.

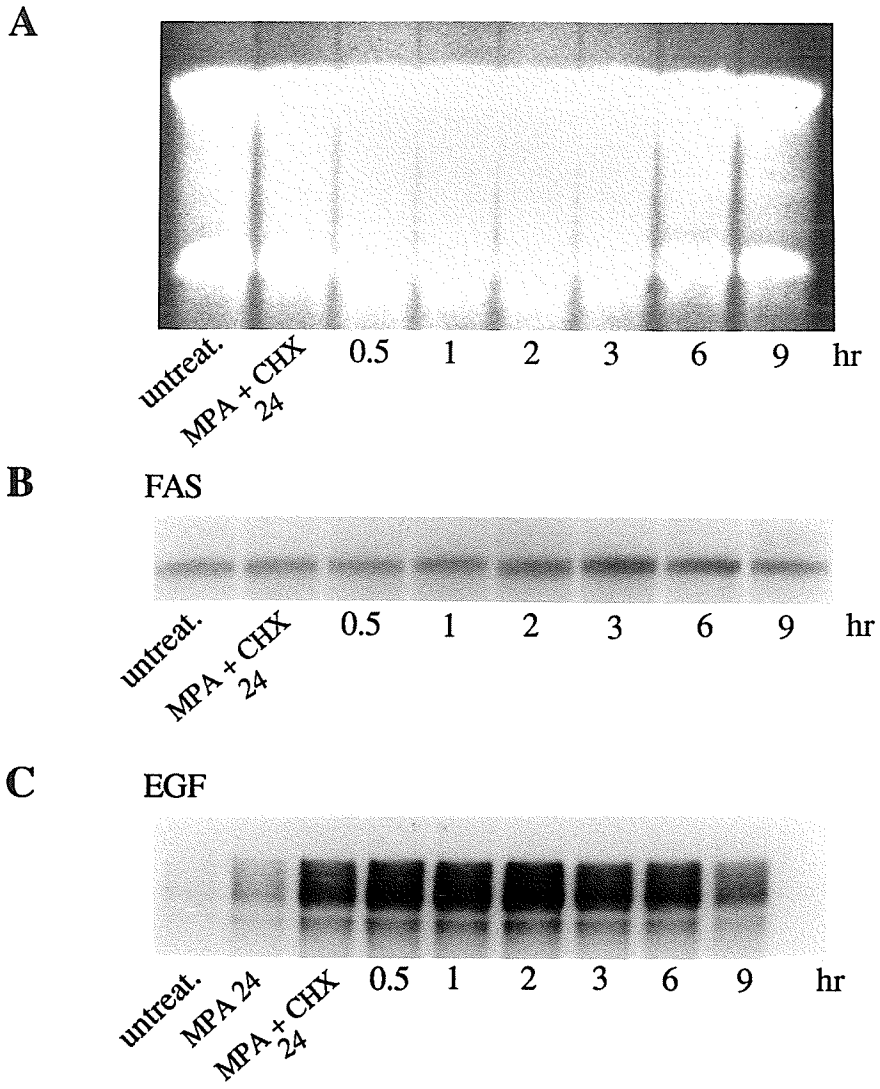


Fig. 14. Time course of EGF and FAS mRNA decay in CHX + MPA-treated T-47D cells using DRB as transcriptional inhibitor.

T-47D cells were pretreated with 50 μ M cycloheximide (CHX) for 2 hr before addition of MPA. DRB (25 ug/ml) was then added 24 hr after MPA treatment. At the indicated times after addition of DRB. Cells were harvested and total RNA isolated was subjected to Northern blot analysis (40 μg/lane) to probe for FAS mRNA, or analyzed by RNAase protection assay (10 μg/lane) to detect EGF mRNA.

A. An EtBr - stained Northern gel representative of 3 separate experiments.

B. Autoradiogram obtained after hybridizing Northern blot (from A) with ^{32}P-labeled FAS cDNA, and exposing to X-ray film overnight.

C. A representative autoradiogram of RNAase protection assay showing EGF mRNA decay. Autoradiography was for 2 days.

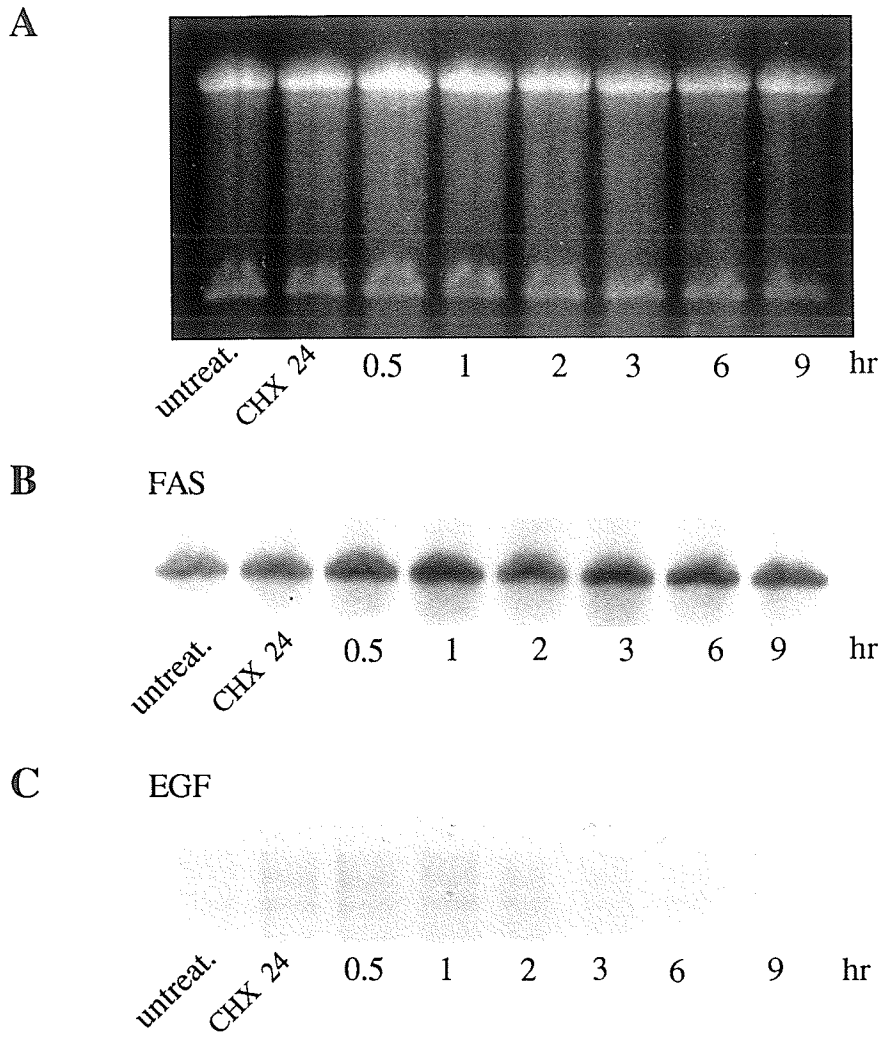


Fig. 15. Time course of EGF and FAS mRNA decay in cycloheximide-treated T-47D cells using DRB as transcriptional inhibitor.

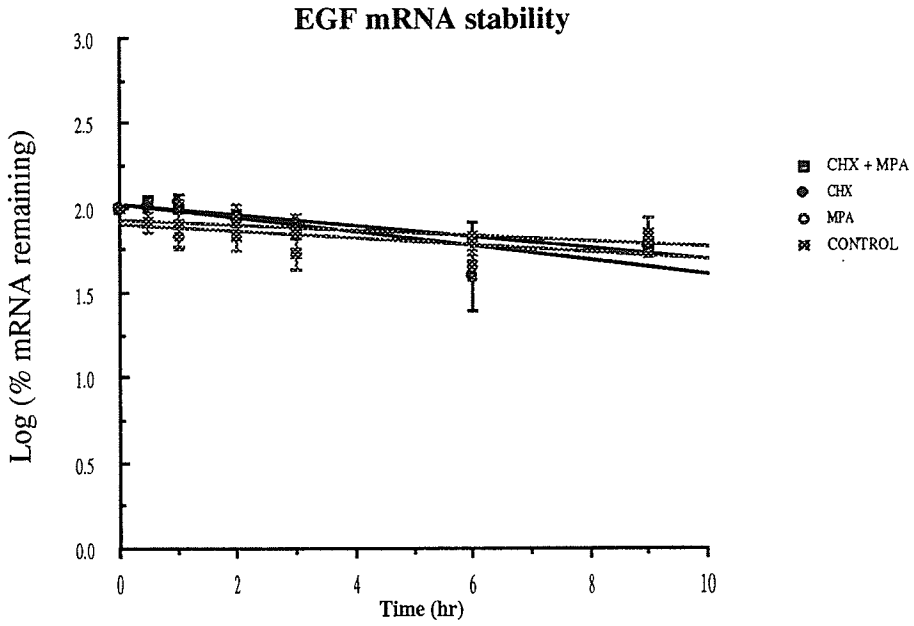
T-47D cells were treated with 50 μM cycloheximide (CHX) for 24 hr followed by DRB (25 ug/ml). Cells were harvested at the indicated times after DRB treatment. Total RNA was isolated and subjected to Northern blot analysis (40 μg/lane) to probe for FAS mRNA, or analyzed by RNAase protection assay (20 μg/lane) to detect EGF mRNA.

A. An EtBr - stained Northern gel representative of 3 separate experiments.

B. Autoradiogram obtained after hybridizing Northern blot (from A) with ^{32}P-labeled FAS cDNA, and exposing to X-ray film for 2 days.

C. A representative autoradiogram of RNAase protection assay showing EGF mRNA decay. Autoradiopgraphy was for 4 days.

A



B

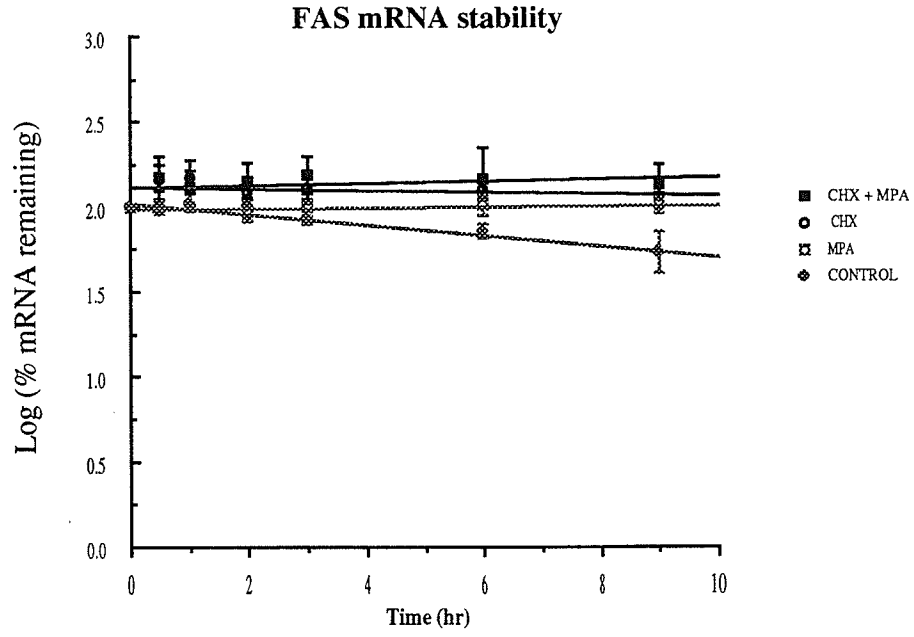


Fig. 16. Measurement of EGF and FAS mRNA half-life under various treatment using DRB as transcriptional inhibitor. The same data in Fig. 12-15 were plotted as mRNA dacay curves.

A. EGF mRNA decay curves. Mean +/-SEM was shown, n=3 for CHX + MPA; n=2 for CHX; n=5 for MPA and Control with the 9 hr time pt. taken in 2 expts.

B. FAS mRNA decay curves. Mean +/-SEM was shown, n=3 for all treatments.

normalization with respect to another mRNA species. Therefore, ethidium bromide-stained gel was used to visually justify approximate equal loading (Panel A of Fig. 9, 10, 12-15). As expected, FAS mRNA was stabilized by progestin, its estimated half-life increased from around 9.8 hr to 31.8 hr (Fig 11B), in close agreement with previously reported figures (6-9 hr in untreated cells to 24-33 hr in progestin-treated cells) (200). On the other hand, it is obvious from the essentially identical mRNA decay curves for EGF that progestin (MPA) has no effect on EGF mRNA stability (Fig. 11A). In order to eliminate possible nonspecific effects of ActD including those on DNA synthesis (214) and translation (215, 216), similar studies were undertaken using 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a purine nucleoside analog that selectively inhibits heterogeneous nuclear RNA synthesis by RNA polymerase II (217, 218, 219). The drug is thought to act via a completely different mechanism to that of ActD which acts by intercalating tightly between GC base pairs in a pre-melted DNA conformation present within the transcriptional complex, stabilizing the complex, and blocking transcriptional elongation (220). With DRB, the mechanisms are not well understood, both inhibition at the levels of transcriptional initiation (221, 222, 223, 224) and elongation have been reported (225, 226, 227, 228 229). More recent data from the laboratory of P. Sharp suggested inhibition of transcriptional elongation, by enhancing transcriptional pausing and/or premature termination, as the mechanism of DRB action (230). In any case, it was generally agreed that the target site of DRB is likely to be a protein factor of the transcriptional machinery other than polymerase II itself (230, 231). The concentration of 25 μg/ml has been used sucessfully in the analysis of mRNA stability. Under these conditions, the half-life of EGF mRNA was analysed in control cells and in cells treated with MPA (Fig. 12 and 13). The effects of CHX + MPA (super-inducing conditions) (Fig. 14) and CHX (Fig. 15) on the stability of EGF mRNA were also investigated using

DRB as transcriptional inhibitor. The first order decay curves derived from quantitating the EGF mRNA levels during the time course were shown in Fig. 16. By inspection, the decay curves for all three treatments are essentially overlapping with that for the controls, no mRNA stabilization was apparent (Fig. 16A). Statistical analysis was then performed to further substantiate the above observation. Calculated mean half-lives (in hours) ± SEM for control, MPA-treated, CHX + MPA-treated, and CHX-treated T-47D cells are respectively, 7.3 ± 1.58, n=5; 7.4 ± 0.26, n=5; 13.4 ± 3.58, n=3; and 9.0 ± 2.52, n=2. From these figures, it was concluded that MPA and CHX had no effect on EGF mRNA stability, whereas CHX + MPA did show a statistically significant stabilizing effect ($p=0.05$ with Student's t test), although the significance of it is marginal and small (less than 2-fold). The stabilizing effect is also too minimal to account for the 37-fold maximal increase in EGF steady-state mRNA levels the treatment induces. It should be noted that this lack of effects of all treatments on EGF mRNA half-life is not a general phenomenon in these cells, since under the same conditions FAS mRNA was stabilized by MPA, CHX and CHX + MPA (Fig. 16B), as has been previously reported (200, 232 and Chalbos D et al., unpublished data). In fact, the large stabilizing effects on FAS mRNA under these conditions (Fig. 16B) gave rise to nearly horizontal decay curves, and hence the half-life were not estimated. In summary, the effects of MPA, CHX and possibly CHX + MPA on the EGF mRNA accumulation are not the result of enhanced mRNA stability. Either transcriptional activation and/or other post-transcriptional effects probably play an important role in the observed changes (see Discussion 2).

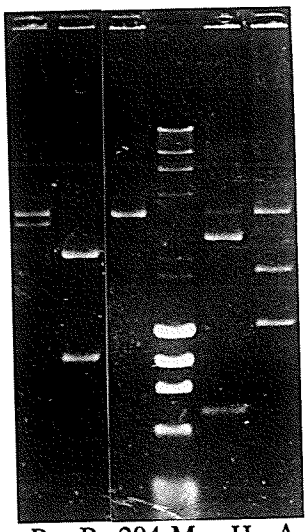
4. ANALYSIS OF 5'-FLANKING REGION OF THE EGF GENE

Genomic DNA clones representing the entire EGF precursor gene and its 5'- and 3'-flanking regions have been isolated by Bell et al (201). The clone hEGF204 was cloned into 4 fragments, each of them flanked by EcoR1 sites. It contains, in addition to all of the first exon and 4 kbp of sequences from the first intron, approximately 6.5 kbp of 5'-flanking sequences. One of the 4 clones of clone 204, designated 204-22, contains approximately 3 kbp of 5'-flanking sequences (part of it sequenced) joined to a portion of the first exon (approximately 500 bp) is particularly interesting since it would most likely carry transcription regulatory sequences such as the promoter and enhancers. Therefore, the 5'-flanking sequences in 204-22 were chosen to be further characterized as described in this work. For simplicity purposes, the designations 204-22 and 204 will be used interchangeably in this thesis, but refers only to the actual nucleotide sequences contained in the former.

4.1 Structural analysis of 204-22

The fragment 204-22 was subcloned into pGEM vector for large scale preparation. The 3.5 kbp insert was isolated from the purified plasmid by electroelution and then subjected to restriction enzyme analysis followed by DNA hybridization. Fig. 17 shows a representative pattern of cleavage by several enzymes that give manageable numbers of restriction fragments. The same gel was blotted onto nitrocellulose filter and hybridized to a 172 bp SmaI-EcoRI cDNA fragment corresponding to the most 3' region of 204-22 (the 0.17 kbp SmaI-EcoRI fragment shown in Fig. 18). This allowed the identification of restriction fragments corresponding to the 3' portion of 204-22, and thus permits the orientation and mapping of the restriction sites identified. For instance, the BglII digests consist of two restriction fragments of 1.1 and 2.4 kbp in size. However, only the 2.4 kbp fragment hybridized to the cDNA probe, indicating that its location within the full

A



B

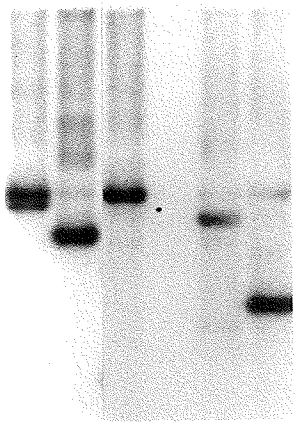


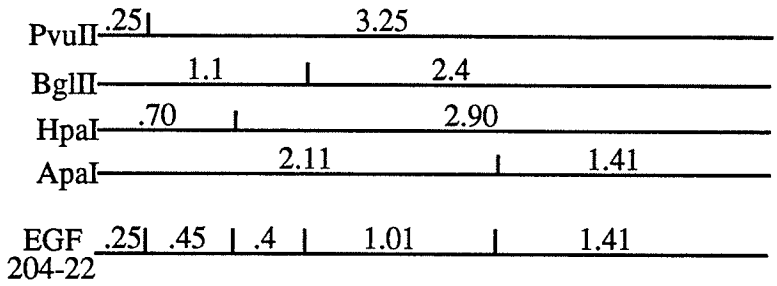
Fig. 17. Restriction fragment analysis of 204-22. 204-22 DNA insert (400 ng) was digested with various restriction enzymes and the products resolved electrophoretically on a 1% agarose gel.

A. The EtBr-stained gel showing the restriction pattern of 204-22 after digestions.

B. The same gel in A. was blotted onto nitrocellulose and probed with the 172 bp SmaI-EcoRI fragment of 204-22 (see Result 4.1). Hybridization and washing conditions were as described in Materials and Methods 3.2. The filter was exposed to Kodak XAR film for 5 hr.

P, PvuII; B, BglII; M, Markers (sizes from top to bottom: 23, 9.5, 6.6, 4.4, 2.3, 2.0, 1.4, 1.1, 0.9, 0.6 kbp); H, HpaI; A, ApaI.

Restriction enzyme	Fragment size (kbp)
PvuII	3.25
	0.25
BglII	2.48
	1.10
HpaI	2.90
	0.70
ApaI	2.11
	1.41



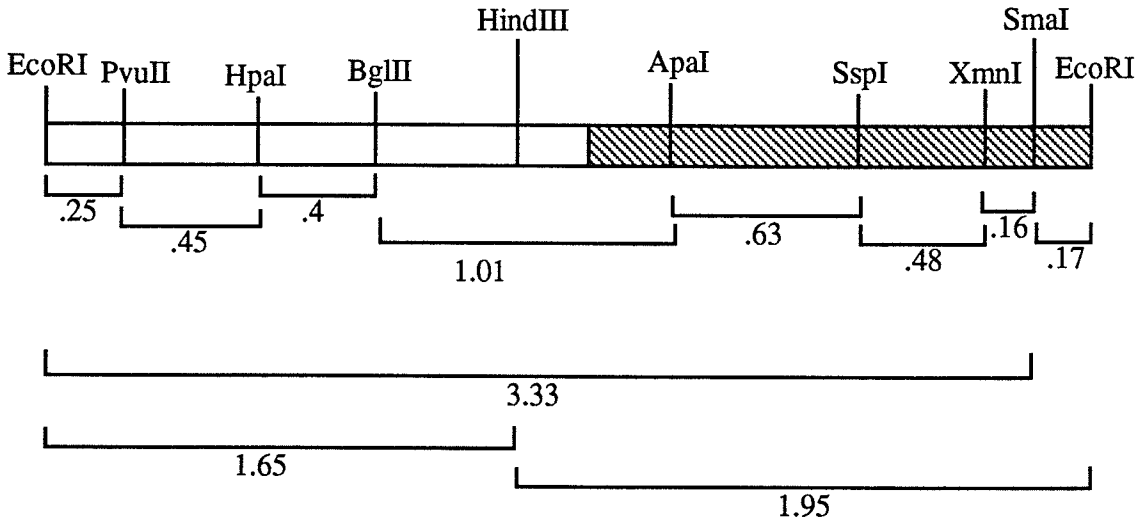
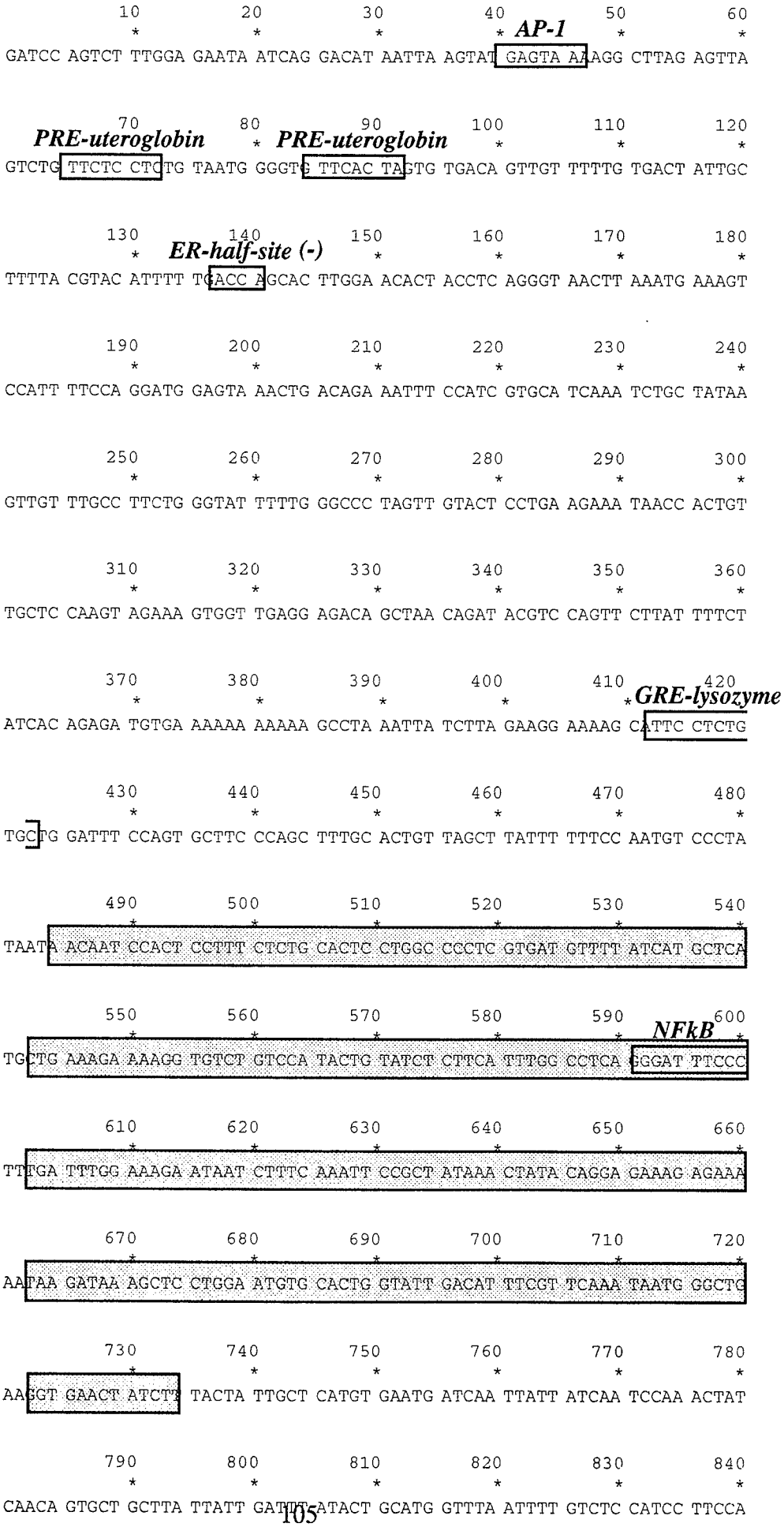


Fig. 18. Partial restriction map of EGF 204-22. Restriction sites shown were derived mainly from analysis of Fig.17., or are known sites. Other sites depicted (e.g. HindIII) were determined by alternative methods such as multiple restriction digestions. ▨ shows the region of known sequence (Bell et al., unpublished data).

length insert is at the 3' portion. Fig. 18 shows a partial restriction map of 204-22. Included in this map are several known restriction sites (201 and Bell et al., unpublished data) and others that were mapped using the common approach of double restriction digestions. None of the following restriction sites were found: BamHI, SalI, SacI, ClaI, NdeII, XhoI.

That portion of 204-22 for which the nucleotide sequence is known (see Fig. 18) was analysed in an attempt to identify consensus binding sites for transcription factors of both the general and regulatory types (see Introduction 4.2.1 and 4.2.2). Sequence analyses were performed using the MacVector program. Shown in Fig. 19 is the sequenced segment of 204-22 excluding 111 bp of EGF precursor coding region. Only 900 bp of the mouse EGF 5'-flanking sequences have been published (233), but a comparison with the available mouse sequences revealed a high degree of similarity. The similarity was particularly high in 2 sub-regions, including one directly proximal to the putative transcription start site at position 1099 from the 5' end of the human gene (49 bp upstream of the 5'-end of the human EGF cDNA sequence Fig. 19). The presumptive site of transcriptional initiation was deduced (201) from an analysis of consensus sequences flanking functional CAP sites (234). In the EGF gene, the potential CAP site is located at the sequence CCAC (positions 1097-1100), with nucleotide A corresponding to the mRNA cap site (transcription start site). Two elements resembling a TATA and CAAT box were identified 26 and 65 bp upstream of the putative mRNA start site, respectively (201). While the CAAT-like sequence (CAAGT) differs from the consensus in having an additional G, the TATA-like sequence (TTTAAA) is more distantly related to the conserved consensus in that it does not spell TATA which is the main hallmark of known TATA boxes. In addition to binding sites for general transcription factors, consensus binding sites for other transcriptional activators that usually are involved in regulating gene expression in response to environmental or


AP-1
PRE-uteroglobin
PRE-uteroglobin
ER-half-site (-)
GRE-lysozyme
NFkB

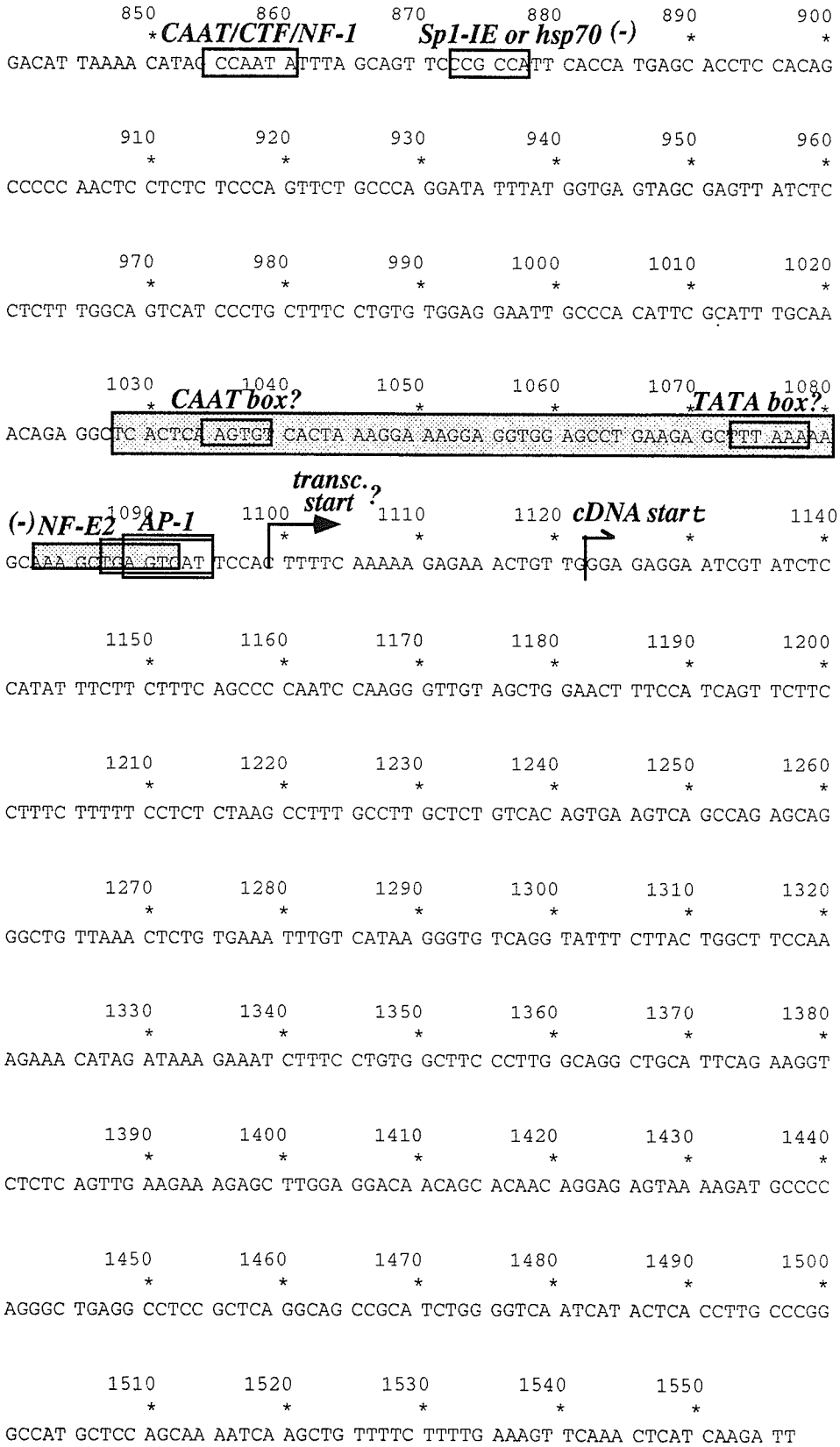


Fig. 19. Putative regulatory sites ( ) in the EGF 5' flanking region and regions of homology with the mouse EGF gene ( ).

(-) indicates sites on opposite strand.

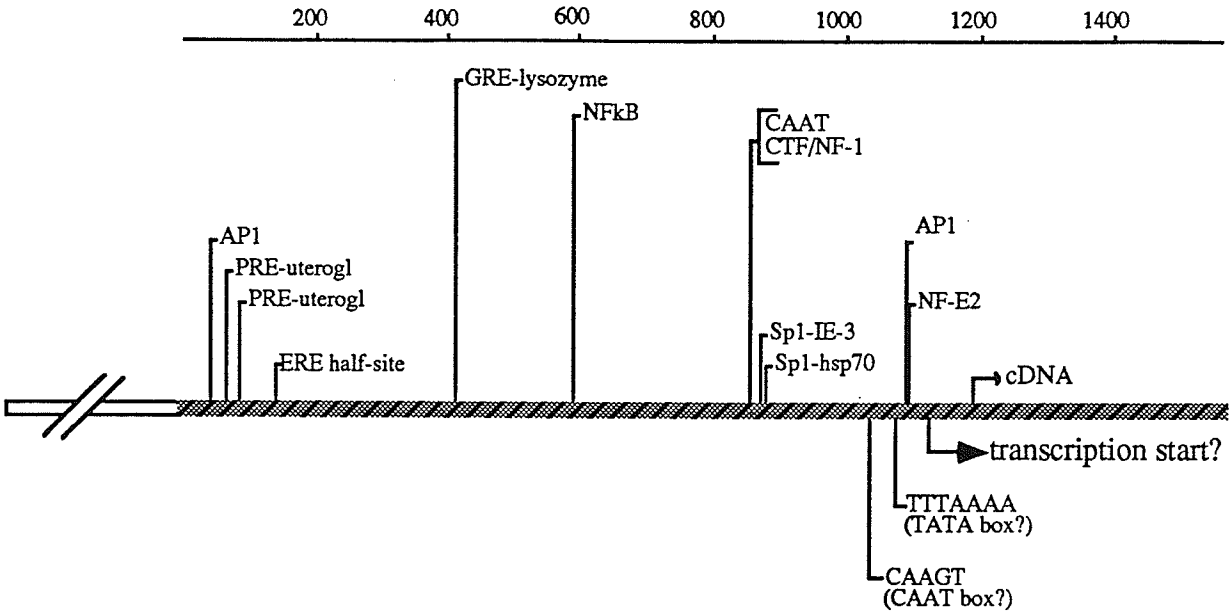
This sequence information was generously provided by Dr G. I. Bell.

developmental stimuli were also identified in this search. These are shown in Fig. 19 also. The number of common consensus binding sites entered in the computer search was approximately 100, and only sites that showed perfect homology to consensus elements are shown. Approximately 10 bp immediately downstream of the putative TATA box are two overlapping binding sites for the transcription factors AP-I and NF-E2, their positions place them even closer to the putative start site than the TATA-like element (Fig. 19, 20). Another AP-I site is located far upstream close to the 5' end of the sequenced segment. Two binding sites each for the progesterone receptor (PRE) and the Sp1 factor were also identified. Interestingly, both copies of each of these sites are situated close to each other. Other sites identified included a consensus CAAT box, an NFκB binding site, an ERE- half site, and a GRE from the lysozyme gene (Fig. 19, 20).

4.2 Functional analysis of 204-22

4.2.1 Optimization of CAT assay

A given cell type has a unique set of conditions within which the artificial introduction of exogenous DNA will occur with maximum efficiency. Because of the variation of transfection conditions across different cell types, even for closely related ones, a variety of conditions were screened to identify combinations that were optimal for T-47D cells. Transfection conditions were optimized using the recombinant plasmid TK-CAT 5A (kindly provided by Dr. M. Beato and ref. 198), in which the CAT gene is driven by the constitutive thymidine kinase (TK) promoter, which in turned is linked to a subregion of mouse mammary tumor virus long terminal repeat (MMTV-LTR) consisting of sequence elements which confer transcriptional responsiveness to several steroid hormones including progestins. Therefore, in the presence of appropriate hormones, TK-CAT 5A would express

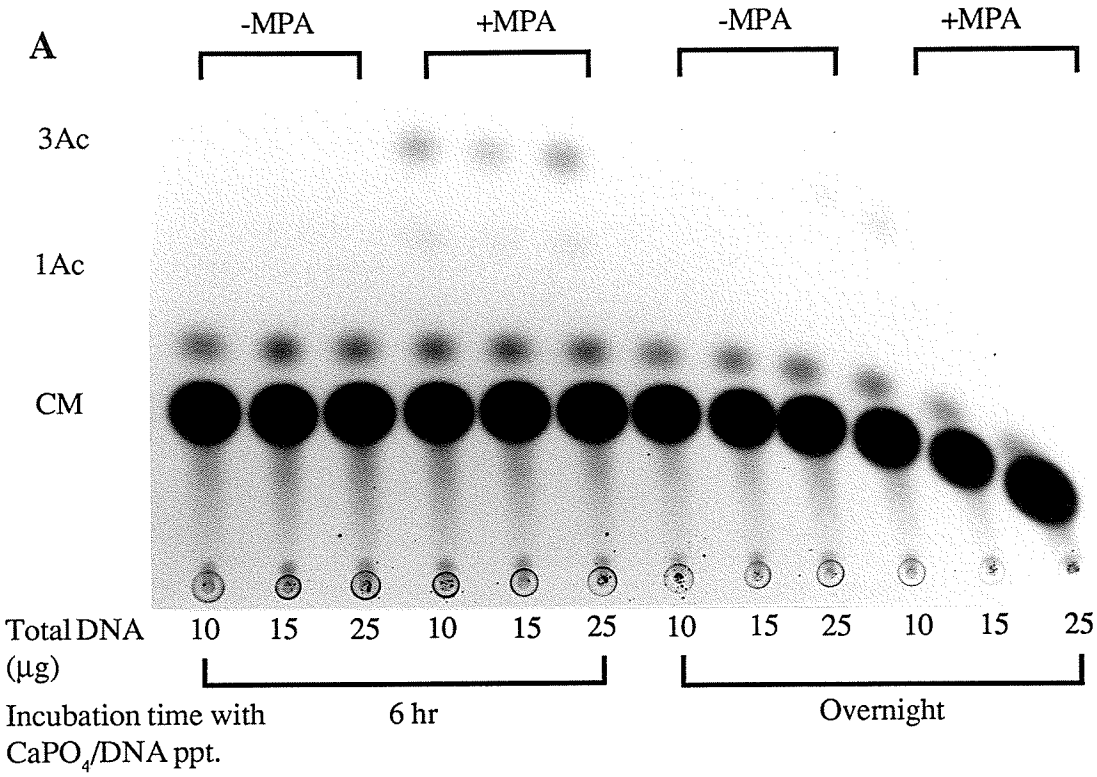


Regulatory site	recognition sequence	Reference
AP1	TGANTMA	Cell 49: 741-52, 87
CAAT	AGCCAAT	Cell 53: 11-24, 88
CTF/NF-1	AGCCAAT	Cell 48: 79-89, 87
ER-half-site	GGTCA	PNAS 87: 3047-3051, 90
GRE-lysozyme	ATTCCTCTGT	PNAS 83: 2817-21, 86
NF-E2	TGACTCAGC	PNAS 86: 6548-52, 89
NFkB	GGGRHTYYHC	Cell 58: 227-229, 89
PRE-uteroglobin	TGTTCACT	NAR 15: 4535-52, 87
PRE-uteroglobin	TGTTCTCC	NAR 15: 4535-52, 87
Sp1-hsp70	GGCGGG	Mol. Cell. Biol. 7: 3646-55, 87
Sp1-IE-3	CCCGCC	Nature 317: 179-82, 85

Fig. 20. Map of putative regulatory sites in the sequenced portion of the EGF 5'-flanking region (204-22)
(note: see Appendix for IUPAC-IUB codes for nucleotides)

elevated amounts of CAT enzyme above that seen for the uninduced basal level. This would allow the sensitive detection of differences in transfection efficiency.

Initially, the total amount of DNA used per dish in the formation of the $CaPO_4$/DNA precipitate was varied to determine its effect on transient transfection. The experiment presented in Fig. 21 demonstrates that the tested range of 10-25 μg total DNA did not differ very much in terms of the level of expression of the CAT enzyme. For economical and practical purposes, 10 μg total DNA was arbitrarily chosen as the 'optimal' working condition. However, from the same experiment, the duration of exposure to the $CaPO_4$/DNA precipitate was found to have a marked effect on transfection efficiency and thus reporter gene expression. Overnight incubation in the presence of the precipitate resulted in decreased absolute CAT activity and also its hormone responsiveness as indicated in the 'relative fold induction' by hormone (Fig 21). The shorter exposure time of 6 hr resulted in much higher CAT activity and also hormone responsiveness of the reporter plasmid (Fig. 21). The next parameter subject to optimization was the amount of reporter plasmid within the constant total amount of DNA (10 μg) transfected. Such a dose response analysis, varying the amount of reporter plasmid allows one to know the smallest amount of the reporter that will give a detectable signal. The data from the described experiment are shown in Fig 22. As can be seen, increasing the reporter concentration led to higher CAT expression until a point beyond which expression level actually declined. The reporter concentration at the peak of the bell-shaped profile of CAT acitivity is 6 μg. At this stage, we optimized the CAT assay itself which is critical for monitoring the level of transient gene expression. Several parameters were varied in different experiments including the amount of extract used (Fig.23), acetyl CoA concentration used (Fig. 24A) and incubation time (Fig. 24B). The optimized conditions for $CaPO_4$-mediated transfection in T-47D cells



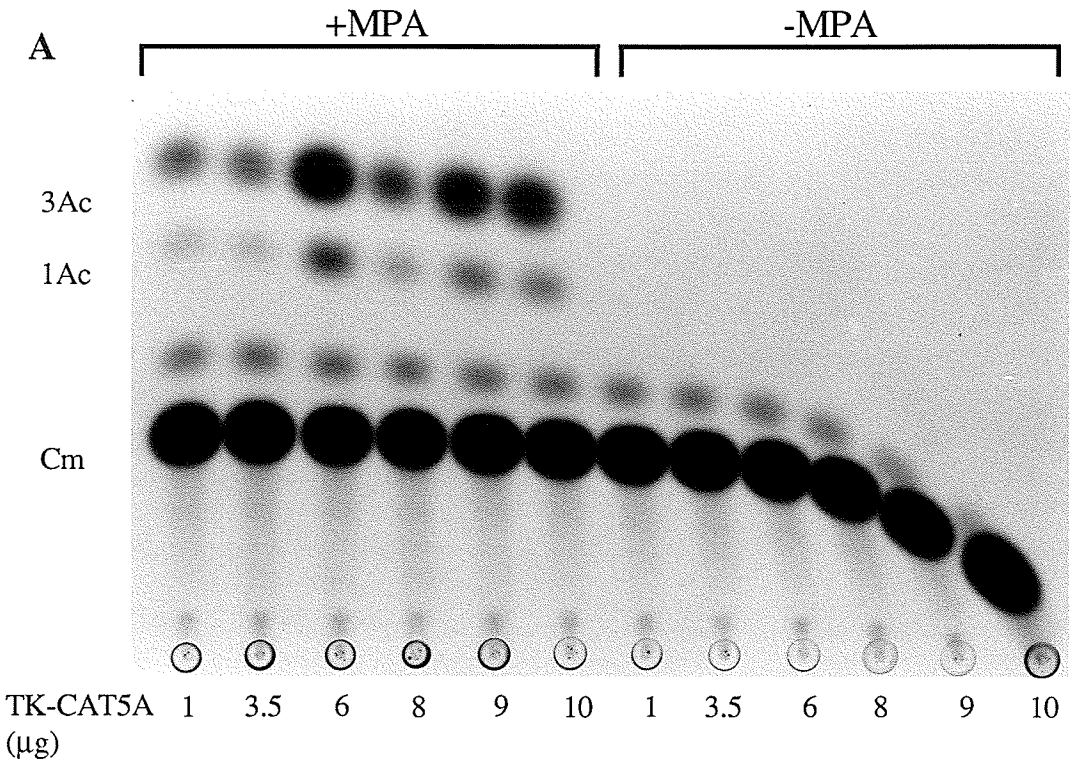
B

Total DNA (μg)	Incubation Time with $CaPO_4$/DNA ppt.	% acetylation (+/- 10 nM MPA) -	% acetylation (+/- 10 nM MPA) +	Relative fold induction
10	6 hr	0.10	1.33	13.3
15		0.13	1.29	9.9
25		0.14	1.89	13.5
10	overnight	0.09	0.41	4.6
15		0.08	0.92	11.5
25		0.09	0.24	2.7

Fig. 21. Effects of total DNA concentration and incubation time with $CaPO_4$/DNA ppt. on transient gene expression in T-47D cells. T-47D cells (plated at $6X10^5$ cells/dish) were transfected with various total amounts of plasmid DNA with TK-CAT5A kept constant at 5 μg. Carrier DNA used was pGEM. Exposure time to $CaPO_4$/DNA ppt. was 6 hr or overnight. Cell harvesting, extract preparation and CAT assay were performed as described except that assay time for CAT was 1 hr.

A. Autoradiogram of the CAT assay obtained after an exposure time of 4 days. CM, chloramphenicol; 1Ac, 1-acetate-chloramphenicol; 3Ac, 3-acetate-chloramphenicol.

B. Quantification of CAT activity in A.



B

TK-CAT 5A (μg/dish)	% acetylation (+/- 10 nM MPA) +	-	Relative fold induction
1	3.33	0.30	11.1
3.5	3.12	0.14	22.3
6	18.44	0.18	102.4
8	5.20	0.12	43.3
9	11.50	0.13	88.5
10	10.28	0.13	79.1

Fig. 22. Effect of reporter plasmid (TK-CAT5A) concentration on transient gene expression in T-47D cells. T-47D cells (plated at $6X10^5$ cells/dish) were transfected with various quantities of TK-CAT5A plasmid made up to 10 μg with pGEM. Cells were harvested and CAT activity determined as described except that incubation with ^{14}C-chloramphenicol was for 1 hr.

A. Autoradiogram of the CAT assay obtained after an exposure time of 5 days.

B. Quantification of CAT activity in A.

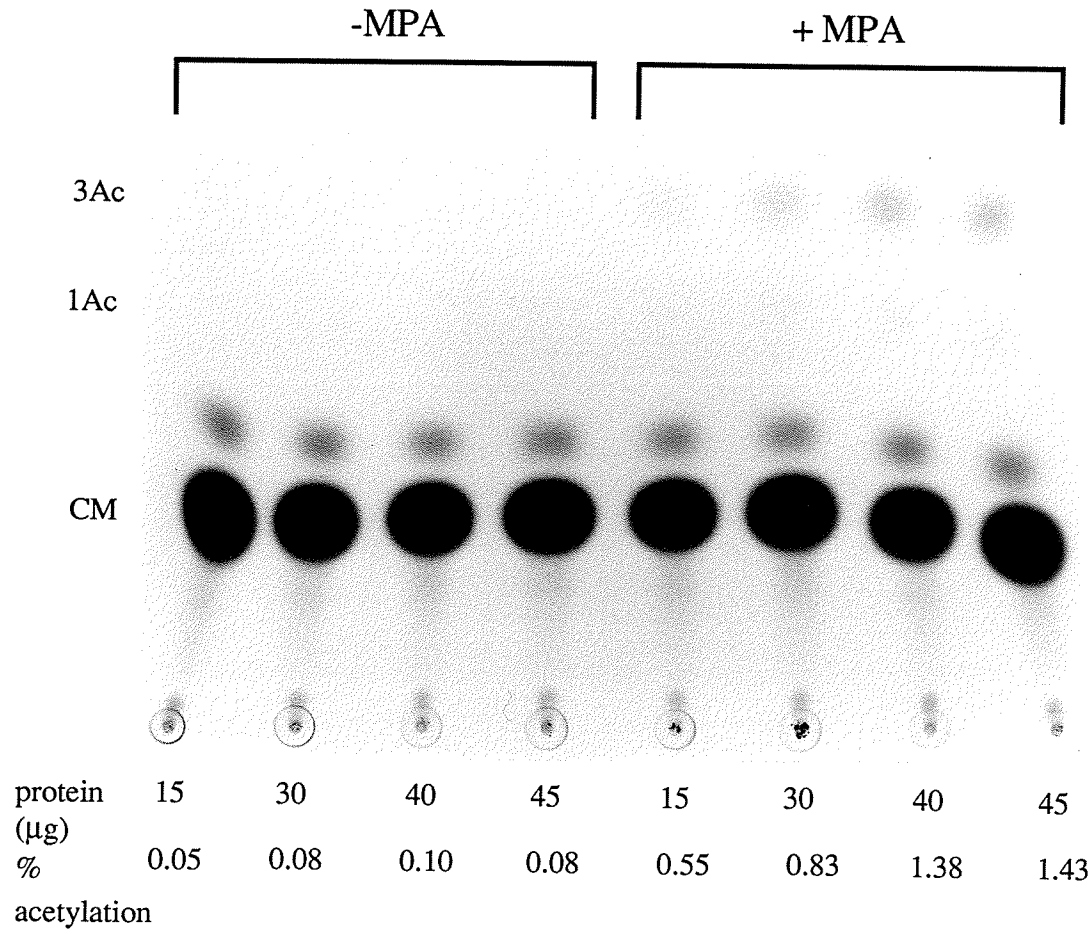


Fig. 23. Effect of varying protein content of cell extract on CAT activity. T-47D cells (plated at $6X10^5$ cells/dish) were transfected with 5 μg of TK-CAT5A reporter plasmid plus 5 μg of pGEM as carrier. Cells were harvested and extracts prepared as described. Samples containing varying amounts of protein from the same extract were assayed for CAT activity by incubating with ^{14}C-chloramphenicol for 1 hr. The autoradiogram was obtained after an exposure time of 4 days.

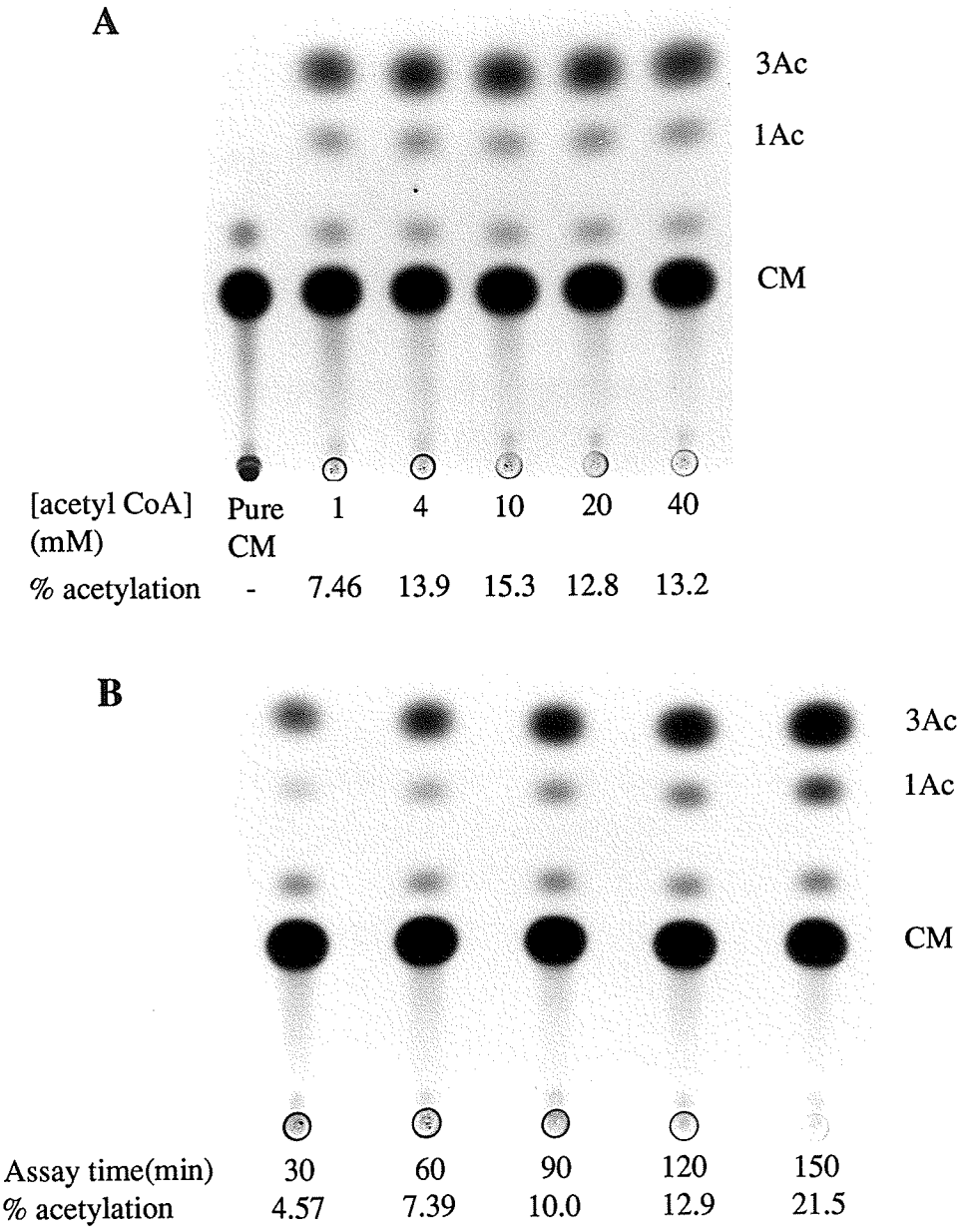


Fig. 24. Effect of acetyl CoA concentration (A) and assay time (B) on the activity of CAT.

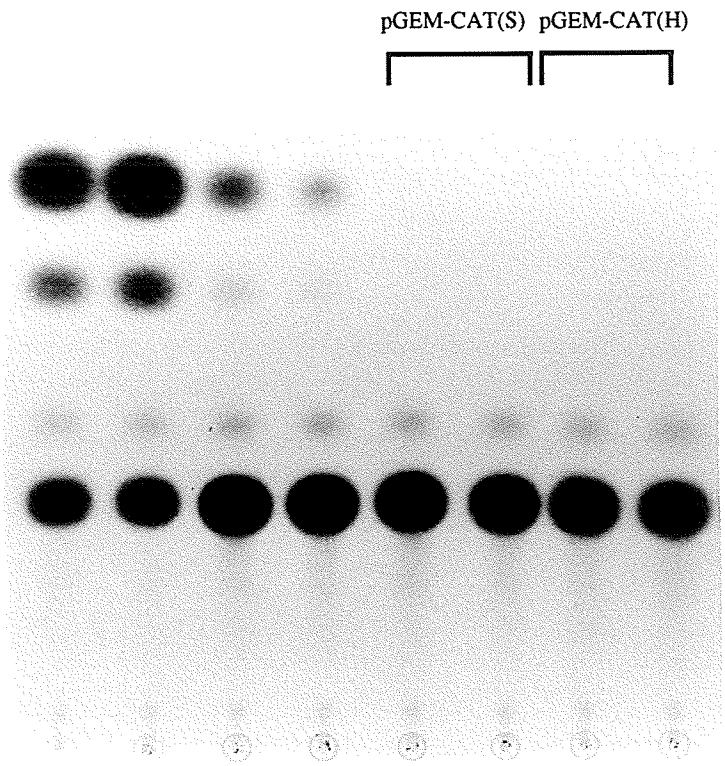
A. T-47D cells ($6X10^5$ cells/dish) were transfected with TK-CAT5A and carrier plasmid in the presence of 10 nM MPA, harvested 48 hr later, and CAT activity was measured using different concentrations of acetyl CoA. The TLC plate was subjected to autoradiography for 4 days.

B. Transfection, cell harvesting, and extract preparation were as in A except that CAT assay incubation time varied with samples as shown. Autoradiography was for 4 days.

and for the CAT assay are summarized in Table 1. The final parameter tested in this work was the density of cells at the time of plating, which turned out to have a significant effect on the expression of the transfected gene. Fig. 25 demonstrates that cells transfected with TK-CAT 5A at high cell densities (3 or 4 X 10^6 cells/dish) express much lower CAT activities than those transfected at lower densities (1 or 2 X 10^6 cells/dish) (Fig. 25). The CAT activity of T-47D cells transfected at 2 X 10^6 cells/dish was approximately 20-fold higher than that at 4 X 10^6 cells/dish (Fig. 25, Table 1).

4.2.2. Construction of CAT reporter plasmids carrying EGF 5'-flanking sequences

Chimeric 204-22-CAT reporter plasmids were constructed in order to test for promoter activity and to identify other regulatory sequences such as elements that might confer hormone responsiveness to progestins (PRE). Initially we fused the bacterial CAT coding sequence to the blank vector pGEM to ensure the promoterless pGEM/CAT constructs did not result in high background CAT activity. Two such test promoterless CAT reporters were made which differ in the restriction sites that flanked the CAT gene. One of them was flanked by SalI sites, the other by HindIII. Both coding sequences were ligated to pGEM after cutting the plasmid with the appropriate enzyme. The pGEM/CAT constructs were analyzed by restriction enzyme digestion to confirm their identities and to determine the correct orientation of the CAT insert (5' to 3' w.r.t. EcoRI or SmaI) (Fig. 26 and 27). Restriction patterns for pGEM/CAT-Sal (or pGEM/CAT(S)) are shown in Fig. 28. As can be seen, the correct size of the linearized plasmid (3.5 kbp, EcoRI cut), the presence of the CAT insert (0.77 kbp, SalI cut), and of a 0.25 kbp fragment which indicates the correct orientation are in total agreement with the plasmid presumed identity (compare Fig. 26 and 28). The two promoterless CAT constructs were then transfected into T-47D cells to test for background CAT



Cell no./dish (X10^6)	1	2	3	4	1	1	1	1
% acetylation	65.5	74.3	9.01	3.51	0.14	0.17	0.33	0.25

Fig. 25. Effect of cell density on transient gene expression in T-47D cells. T-47D cells were plated at different density (1-4 X 10^6 cells/dish) the day before transfection. Transfection (6 μg reporter, 4 μg carrier), extract preparation, and CAT assay were performed as described except that all the samples were treated with MPA (10 nM). Autoradiography was for overnight. The two promoterless CAT constructs, pGEM-CAT(S) and pGEM-CAT(H) were included in the same experiment to determine background CAT activity.

For Transfection

Parameter	Optimal condition
Total plasmid DNA	10 ug/dish
Reporter plasmid DNA	6 ug
Carrier plasmid DNA	4 ug
Exposure time to $CaPO_4$/DNA ppt.	6 hours
Cell density	$2X10^6$ cells/dish

For CAT assay

Amount of protein/assay	40 ug
Assay time in ^{14}C-chloramphenicol	1.5 hours
Final concentration of acetyl CoA	4 mM

Table 1. Optimal conditions for $CaPO_4$ -mediated transfection and CAT assay

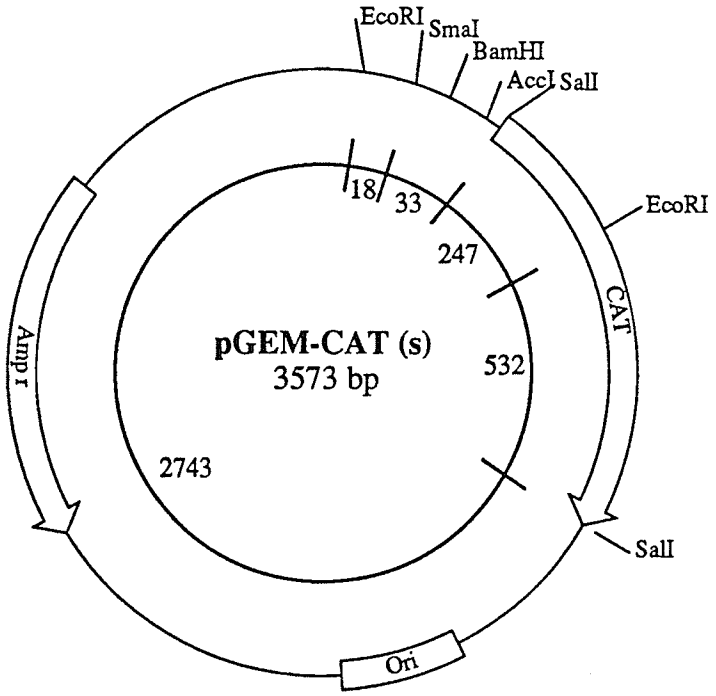
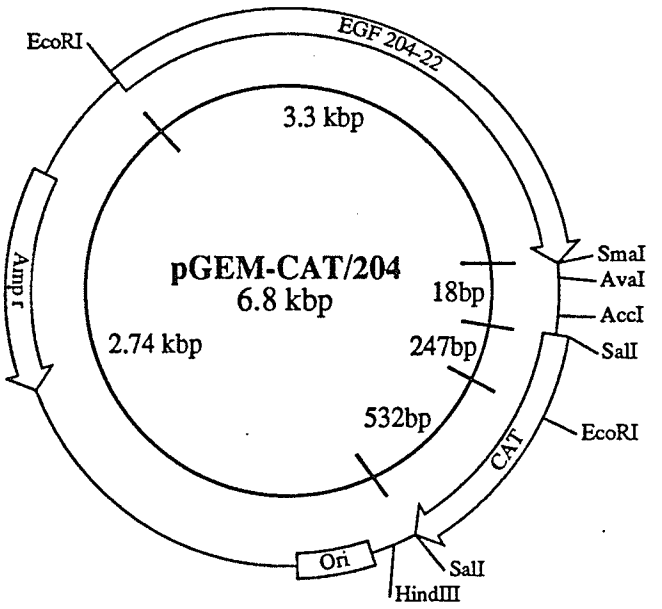


Fig. 26. Plasmid map of pGEM-CAT (SalI)

A



B

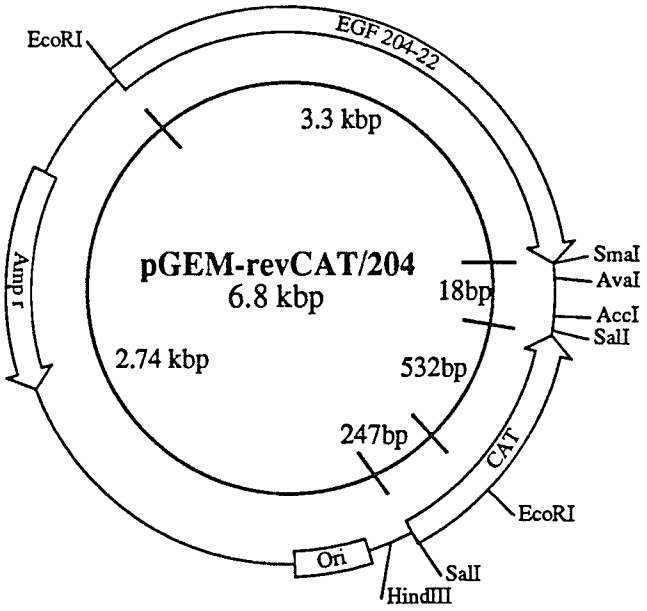


Fig. 27. Plasmid maps of pGEM-CAT/204 (A) and pGEM-revCAT/204 (B).

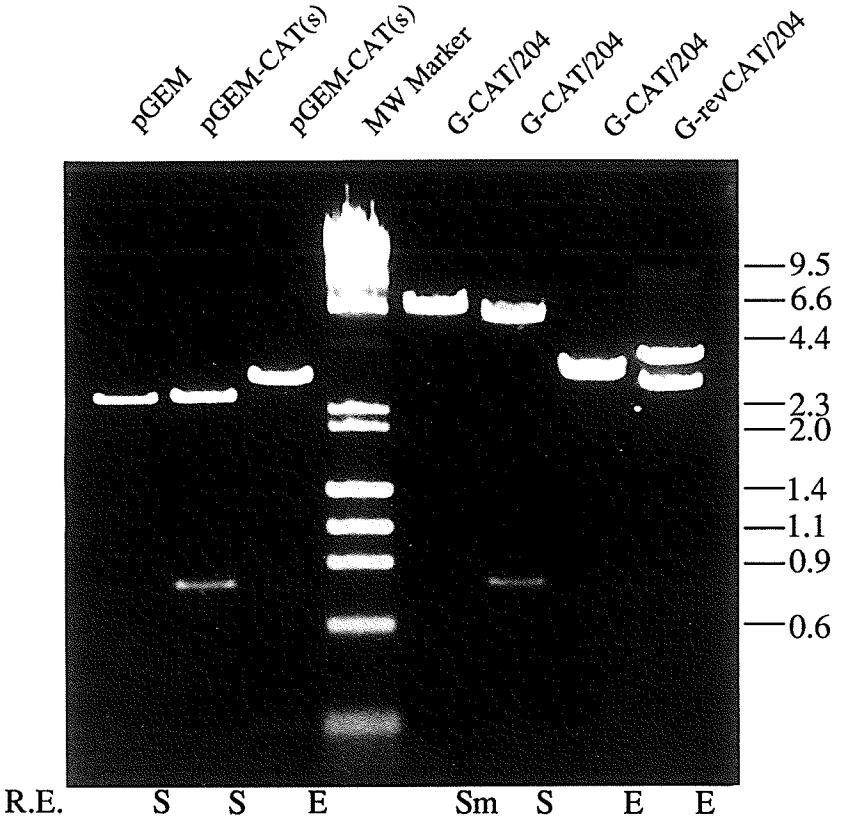


Fig. 28. Restriction enzyme digestions of pGEM-CAT, pGEM-CAT/204, and pGEM-revCAT/204 to confirm identity.

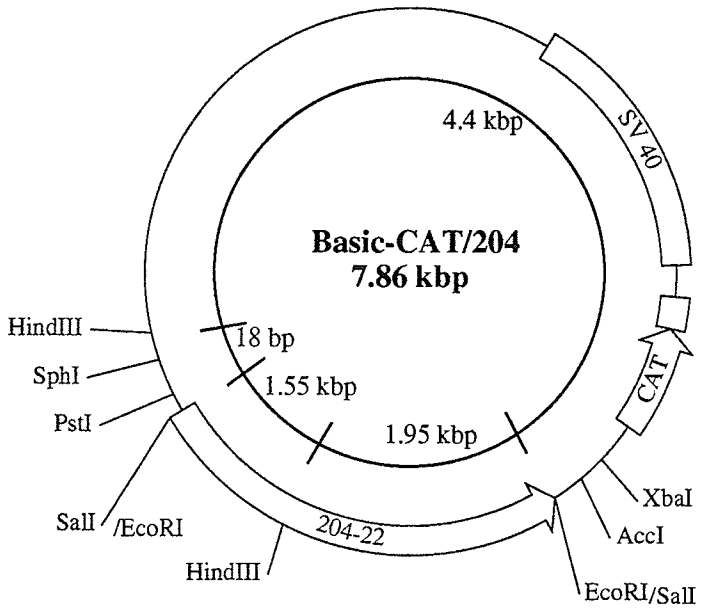
Approximately 0.4 μg plasmid DNA was digested with various enzymes and the products resolved electrophoretically on a 1% agarose gel.

R.E., Restriction Enzyme; S, Sal I; E, Eco RI; SM, Sma I.

activity (Fig. 25). Both constructs showed little relative CAT expression, and the one flanked by SalI sites was chosen as the framework for future construction of reporter plasmids carrying EGF 5'-flanking sequences. Two such plasmids named pGEM-CAT/204 and pGEM-revCAT/204 (Fig. 27) were constructed using a directional cloning approach as already described under Materials and Methods. Their identities were checked by multiple restriction digestions (Fig. 28). For example, in the case of pGEM-CAT/204, the correct size of 6.8 kbp of the linearized vector was obtained after SmaI digestion, whereas two fragments of 3.57 and 3.27 kbp were seen upon EcoRI digestion, a pattern expected only if the 204 and the CAT inserts are in the correct orientation with respect to each other (compare Fig. 27A and 28). The pGEM-revCAT/204 contains the CAT sequence in the opposite direction to the 204 fragment, EcoRI digestion yielded two more distantly associated bands of the expected sizes 3.9 and 3.3 kbp (Fig. 27B and 28).

For reasons that will be described later, another series of chimeric CAT reporter plasmids were constructed based on Promega's pCAT-Basic and pCAT-Enhancer (renamed as Basic-CAT and Enhancer-CAT in this thesis) promoterless vectors. The major differences in this series of vectors as compared to the pGEM series are that the former contains the CAT gene followed by a polyadenylation signal sequence from the SV40 virus. Altogether 4 constructs were made: two of them contain the full length 204-22, the other two the 3' HindIII-EcoRI fragment (see restriction map). The difference between the Basic-CAT (Fig. 29) and Enhancer-CAT (Fig. 30) carrying identical EGF 5'-flanking sequences lies in the parent CAT vector. The Enhancer-CAT constructs (Fig. 30) contain an additional enhancer sequence from SV40. Fig. 31 shows the restriction enzyme patterns intended to confirm their identities. Using Basic-CAT/204 and 3'-204 (Fig. 29) as examples, the former is 7.8 kbp in size (HindIII cut), contains the 204 insert of 3.5 kbp (PstI and XbaI cut, EcoRI sites were blunted and fused with SalI) and released a fragment of 1.5 kbp upon HindIII digestion indicative of a

A



B

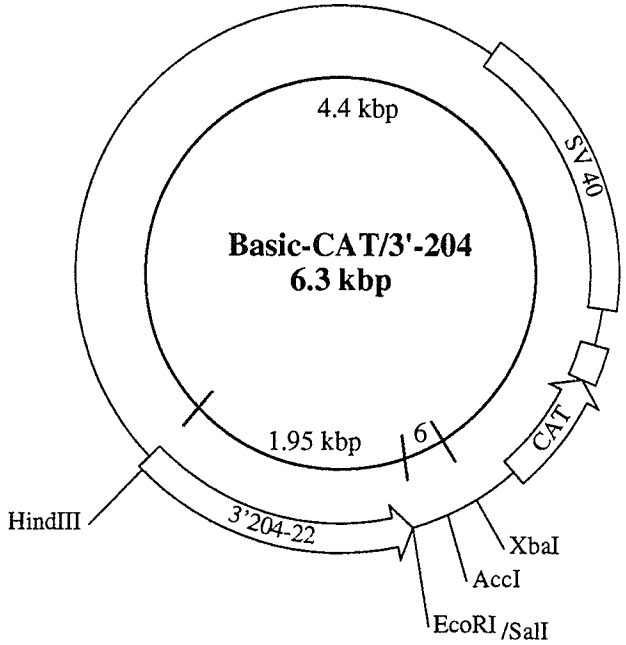
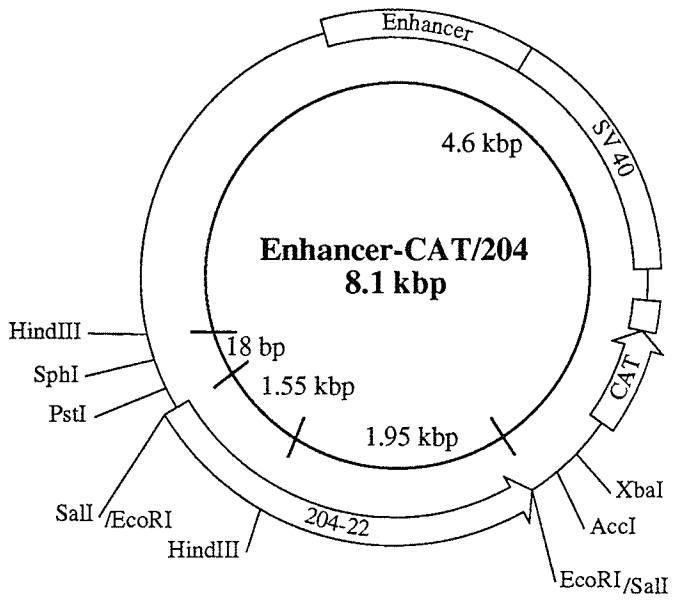


Fig. 29. Plasmid maps of Basic-CAT/204 (A) and Basic-CAT/3' 204 (B)

A



B

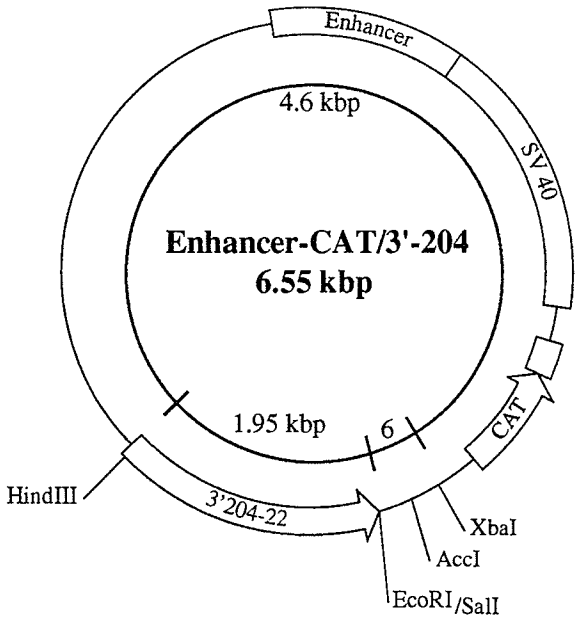


Fig. 30. Plasmid maps of Enhacner-CAT/204 (A) andEnhancer-CAT/3'-204 (B)

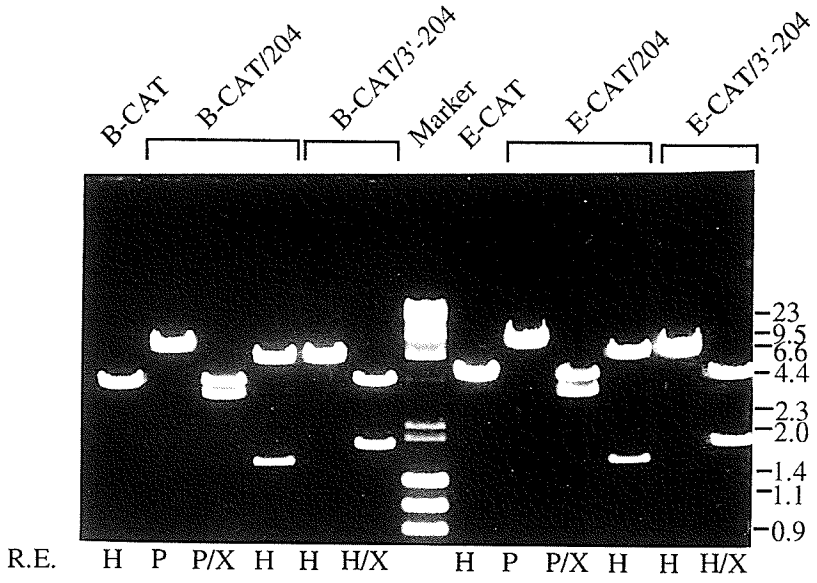


Fig. 31. Restriction enzyme digestions of Basic-CAT/204 (or 3'-204) and Enhancer-CAT/204 (or 3'-204) to confirm identity. 0.4 μg of various plasmids were digested with the indicated enzymes and sized on a 1% agarose gel.

R.E., restriction enzymes; P, Pst I; X, Xba I; B-CAT, Basic-CAT; E-CAT, Enhancer-CAT.

correct orientation (compare Fig. 29A and 31). The smaller Basic-CAT/3'-204 was constructed by directionally ligating the HindIII-EcoRI fragment of 204-22 into HindIII and SalI-digested Basic-CAT vector after blunting the EcoRI and SalI sites. Double digestions by HindIII and XbaI thus released the cloned fragment of 1.95 kbp plus the Basic-CAT portion of 4.4 kbp (Fig. 29B and 31). The same pattern of restriction digestions was observed for Enhancer-CAT based constructs except that fragments corresponding to the parent vector were slightly larger in size because of the additional SV40 enhancer sequence (Fig. 30).

4.2.3 Promoter activity in transient transfected T-47D cells

The 204-CAT chimeric constructs of the pGEM series were transfected into the T-47D cell line in which the endogenous EGF gene has been shown to be expressed and regulation by progestins. Cells were harvested 48 hr post-transfection and assayed for CAT enzyme activity. As shown in Fig.32, both the promoterless pGEM/CAT (S) and pGEM-revCAT/204 exhibited very small yet detectable amounts (approximately 1% acetylation) of CAT activity. This background CAT activity has been observed by others and probably represents readthrough transcripts originating in the prokaryotic part of the vector. This background problem may be circumvented by inserting an UMS (mouse upstream sequence) sequence upstream of the CAT gene (235). The UMS sequence was derived from the mouse c-mos gene and has been shown to function as a potent transcription terminator.

The construct containing 3.3 kbp of 204-22 fragment, pGEM-CAT/204, reproducibly showed a small increase in CAT activity over and above that of the background seen for the promoterless pGEM/CAT (S) (compare lanes 7, 8 to lanes 3, 4, Fig. 32). However, this increase was not statistically significant when compared to the background exhibited by pGEM-revCAT/204 which had the CAT coding sequence and 204 in opposite directions (compare lanes 7, 8 to lanes 5, 6, Fig. 32). In addition, increasing amounts of pGEM-CAT/204 for transfection

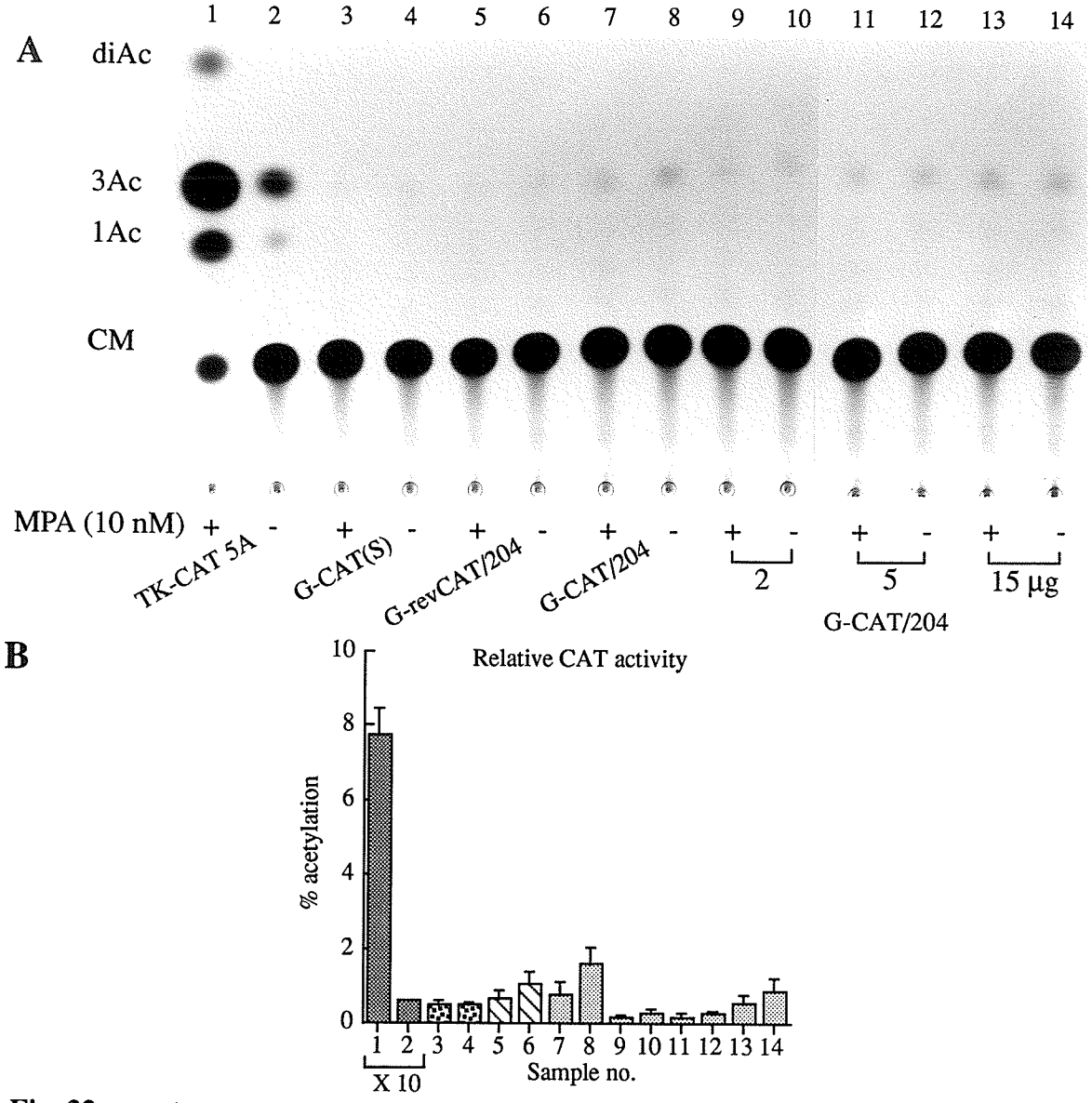


Fig. 32. Transient transfection analysis of pGEM-CAT activity under the control of EGF 5'-flanking sequences in T-47D cells. T-47D cells ($2X10^6$ cells/dish) were transfected with various constructs in equimolar amounts to that of 5 µg G-CAT(S), along with 3 µg pCH110 and carrier DNA to 10 µg of total DNA. Dishes 9-14 were transfected with increasing amounts of G-CAT/204 as indicated. Cells were subjected to NaBu treatment as described in "Materials and Methods". CAT assay was performed using 20 mM acetyl CoA and incubated for 14 hr with ^{14}C-chloramphenicol.

A. An autoradiogram representative of 3 separate experiments. Autoradiography was for 3-4 days. G-CAT(S), pGEM-CAT(SalI).

B. CAT activities were quantitated, normalized using β-gal activities and depicted in histographic form showing mean+/- SEM, n=3.

resulted in a linear increase in CAT acitivity but even at 15 μg/dish, the absolute amount of acetylation was still around 1-2 %, no drastic improvement was observed (lanes 9-14, Fig. 32). It should be noted that in this experiment, cells had already been treated with 6 mM sodium butyrate (NaBu) for 14 hr to enhance the expression of the transfected gene (236). Furthermore, CAT activity was assayed for 14 hr in the presence of radiolabeled chloramphenicol and 20 mM acetyl CoA. These two modifications from the 'optimized procedures' were adopted in order to detect the weak but observable EGF promoter activity residing in the 204 fragment. Another important observation was that none of the samples showed a differential increase in CAT activity upon progestin treatment, arguing against the existence of a functional PRE in this fragment of the 5'-flanking region.

A possible shortcoming of the experiment described above was that all the constructs used were devoid of a functional poly (A) signal, usually incorporated into eukaryotic expression vectors to ensure efficient polyadenylation of resulting transcripts. This defficiency may result in low CAT activtiy associated with the pGEM-based constructs. The poly (A) tail has been described as essential to the stability of mRNA and for efficient gene expression (237). Others however reported a stable population of RNA lacking poly (A) tail (238). Therefore another series of CAT constructs were made basing on the promoterless parental vectors, Basic-CAT and Enhancer-CAT which all contain a poly (A) signal sequence. The difference between them is that the latter has an additional constitutive SV40 enhancer which gives a higher level of CAT expression under the control of a heterologous promoter, in this case the putative EGF promoter. The existence of a constitutive enhancer in Enhancer-CAT might enhance the weak EGF promoter activity.

As shown in Fig. 33, transfection of T-47D cells with Basic-CAT and Enhancer-CAT containing sequences from the 204 fragment gave rise to CAT

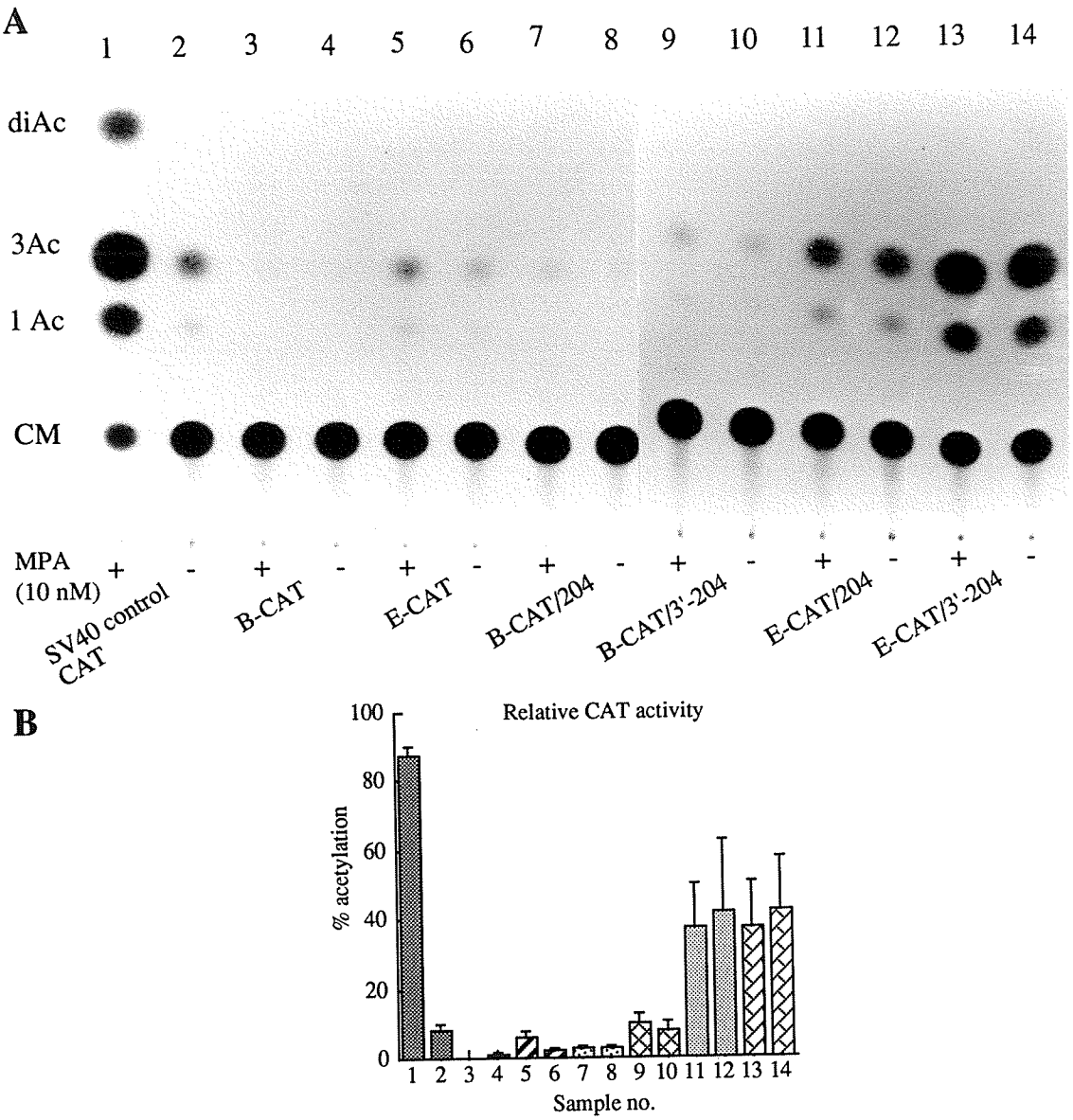


Fig. 33. Transient transfection analysis of Basic- and Enhancer-CAT activity under the control of EGF 5'-flanking sequences in T-47D cells. T-47D cells ($2X10^6$ cells/dish) were transfected with equimolar amounts of various constructs, and 3 μg pCH110 in a total of 10 μg of DNA. NaBu treatment was for 14 hours after which cells were refed with fresh medium plus hormone. CAT assay was performed by incubating with ^{14}C-chloramphenicol for 14 hr in the presence of 20 mM acetyl CoA.

A. An autoradiogram representative of 3 separate experiments. Autoradiography was for 3-4 days.

B. CAT activities were quantitated and normalized for β-gal activities. The histograms show mean +/- SEM of the 3 experiments. B-CAT, Basic-CAT; E-CAT, Enhancer-CAT.

expression that were obviously much higher than that of the background (compare lanes 7-10 to lanes 3,4; and lanes 11-14 to lanes 5, 6, Fig. 33). More importantly, Enhancer-CAT/204 or 3'-204 showed acetylation levels of around 40 % whereas the parent vector Enhancer-CAT only showed a background CAT acitvity of less than 5 % (Fig. 33 B). The effect of the putative promoter activity in fragment 204 on the expression of Basic-CAT was similar although less dramatic due to the absence of an enhancer. These data demonstrate convincingly the existence of a weak promoter activity in the 204 EGF 5'-flanking fragment, extending the findings obtained with the previous set of plasmids. Again, hormonal treatment did not result in an increased level of CAT activity in any of the constructs, further supporting the previous observation of the absence of a functional hormone responsive element. Moreover, 5' deletion of 1.65 kbp from the 204 fragment (Basic-CAT/3'-204 and Enhancer-CAT/3'-204) did not alter the hormone responsiveness of CAT expression as compared to the full-length sequence. The heightened CAT levels seen in Enhancer-CAT/3'-204 relative to that of Enhancer-CAT/204 was an experimental artifact (compare lanes 11, 12 to lanes 13, 14, Fig. 33A). After correction for differences in transfection efficiency using β-gal activities, the normalized CAT activities did not show any significant differences (Fig. 33B). Nevertheless, the observation was interesting as it was highly reproducible, suggesting that the deletion imparted certain structural differences to the plasmids that would result in a more efficient uptake by the transfected cells. However, Basic-CAT/3'-204 did show a satistically significant increase in basal CAT activity relative to that of Basic-CAT/204. The reason for the discrepancy between the effects of the 204 deletion on Basic-CAT and Enhancer-CAT expression is unknown.

4.2.4 Promoter activity in transient transfected HeLa cells

Because of the inability to observe a progestin inducible response in T-47D cells with the EGF 5'-flanking-CAT constructs, it was decided to test them in another cell line that normally did not express the progesterone receptor. HeLa cells

were chosen and were transfected with the 204-containing Basic-CAT and Enhancer-CAT constructs along with an expression vector cPR1 which encoded chicken progesterone receptor (kindly provided by Dr. P. Chambon, ref.). The ability of artificially expressed progesterone receptors to activate in a hormone-dependent fashion the EGF promoter region was tested. Before testing the constructs, the optimal concentration of cPR1 for transfection was determined. Various amounts of cPR1 along with a fixed amount of a reporter CAT construct, in this case, MMTV-CAT (similar to TK-CAT 5A except that the promoter was from MMTV instead of TK, ref. 198) were tested. Fig. 34 shows the result of one such optimization experiment. An optimal concentration of cPR1 was set at 0.1 μg since this particular concentration showed the best differential response to progestin (4-fold induction upon hormone stimulation, Fig. 34). In fact, this experiment showed that overexpressing progesterone receptor by increasing cPR1 concentration during transfection gradually resulted in the loss of hormone responsiveness (see 0.75, 1, and 3 μg cPR1, Fig. 34). A similar experiment was performed using a fixed amount of cPR1 (0.1 μg) but with varying amounts of the reporter concentration. Again, an optimal reporter concentration was observed at around 3.5 - 5 μg. Too little or too much MMTV-CAT reporter during transfection resulted in decreased hormone responsiveness (Fig. 35). These findings are in agreement with other reports where overexpression of steroid hormone receptors lead to abnormal subcellular distribution (239).

Shown in Fig. 36A and B are the data from transfecting 204-containing Basic-CAT and Enhancer-CAT into HeLa cells transiently expressing progesterone receptors. Unfortunately, the two parent promoterless vectors Basic-CAT and Enhancer-CAT both showed detectable background CAT activity as high as 5 % in some cases. Both Basic-CAT/204 and 3'-204 showed only a very small if any

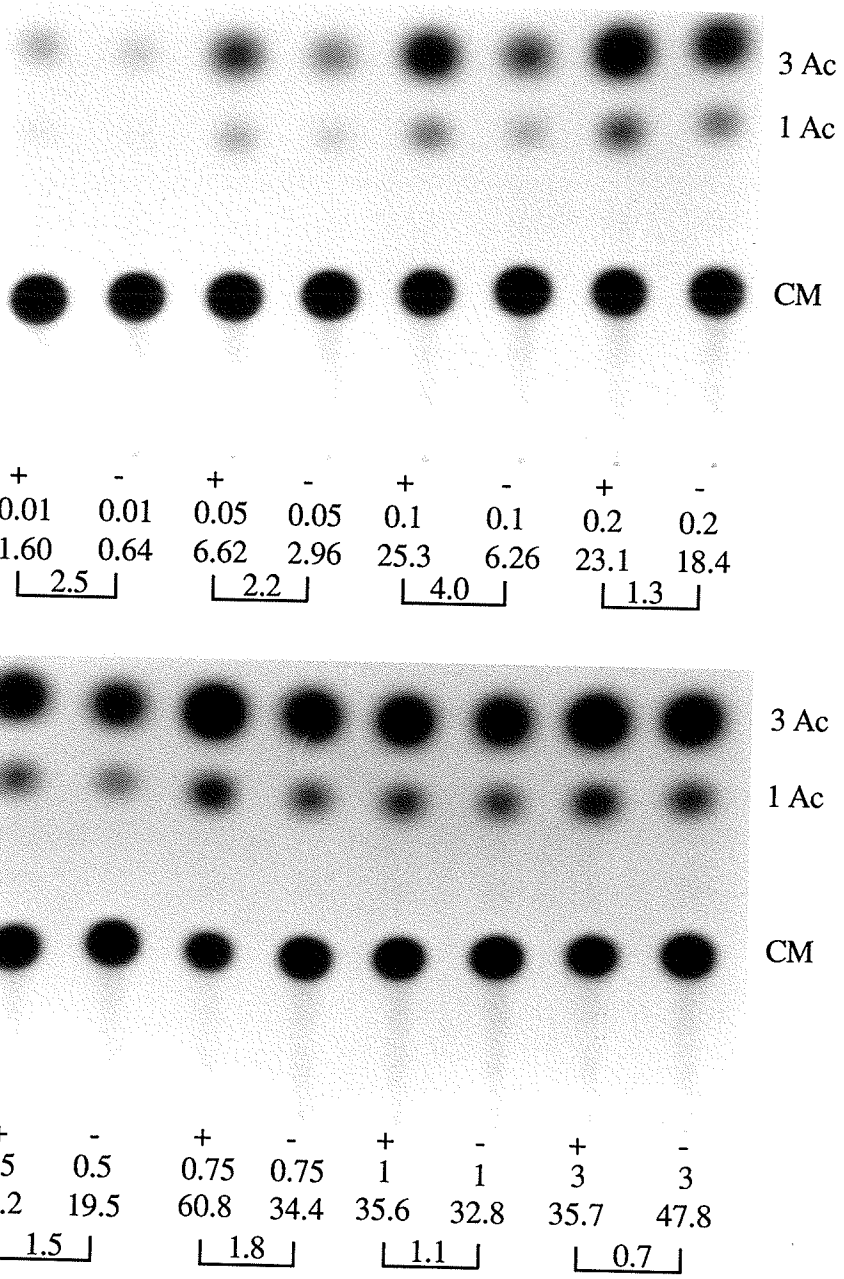


Fig. 34. Effect of cPR1 (encodes progesterone receptor) concentration on the expression and hormone responsiveness of a co-transfected reporter gene MMTV-CAT in HeLa cells. HeLa cells ($1X10^6$ cells/dish) were transfected with 5 μg MMTV-CAT, 3 μg pCH110, and varying amounts of cPR1 vector. Total amount of DNA was held constant at 10 μg with SP72. CAT activity in cell extracts was measured according to established procedures. % acetylation was expressed after normalization of β-gal activities to correct for transfection efficiency.

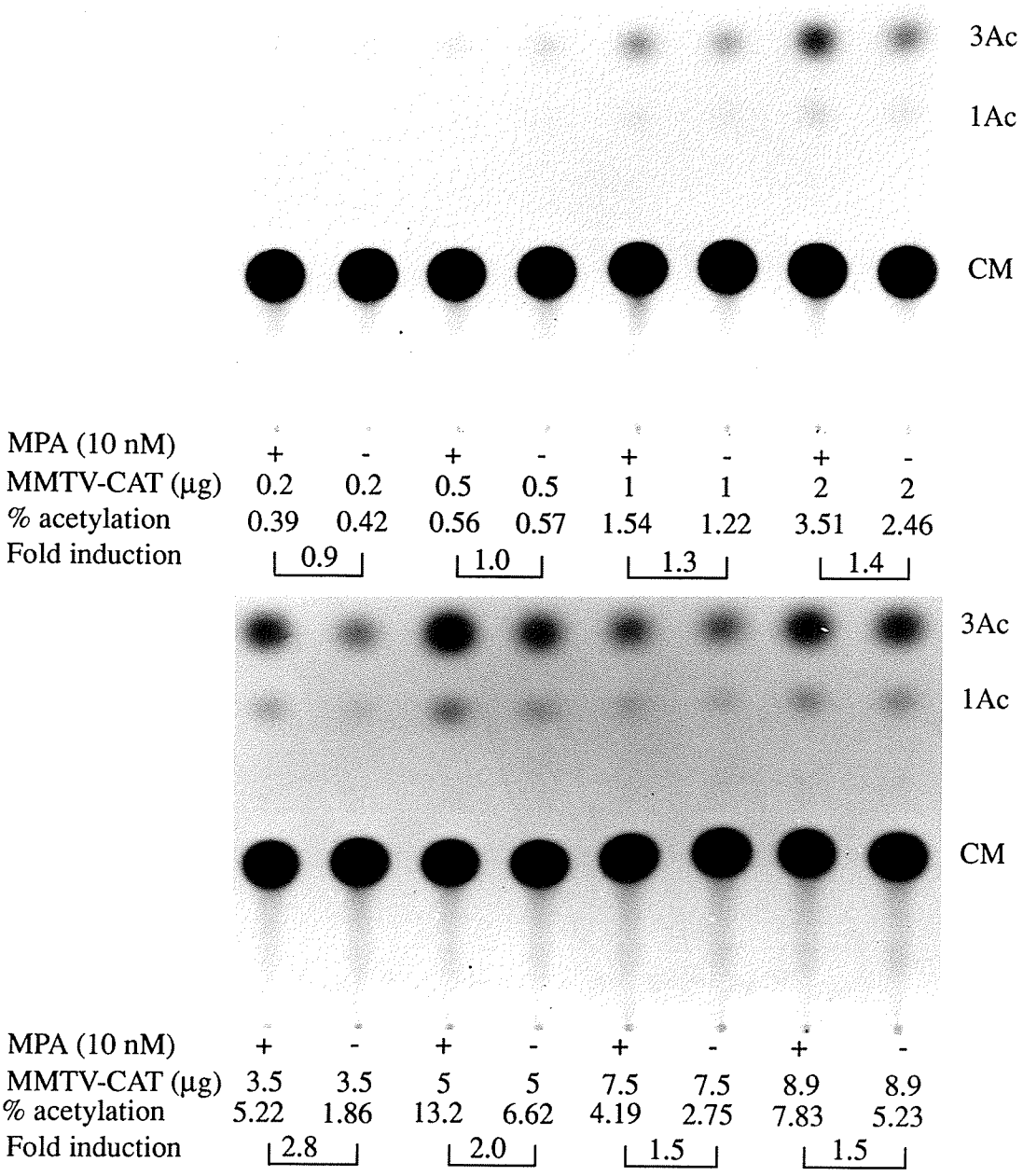


Fig. 35. Effect of reporter plasmid (MMTV-CAT) concentration on transient gene expression and hormone responsiveness in HeLa cells. HeLa cells ($1X10^6$ cells/dish) were transfected with 3 μg pCH110, 0.1 μg cPR1 and varying amounts of MMTV-CAT reporter plasmid. Total DNA was kept at 10 μg with pSP72. CAT activity was measured according to established procedures and was expressed after normalization for β-gal activities. Autoradiography was for 2 days.

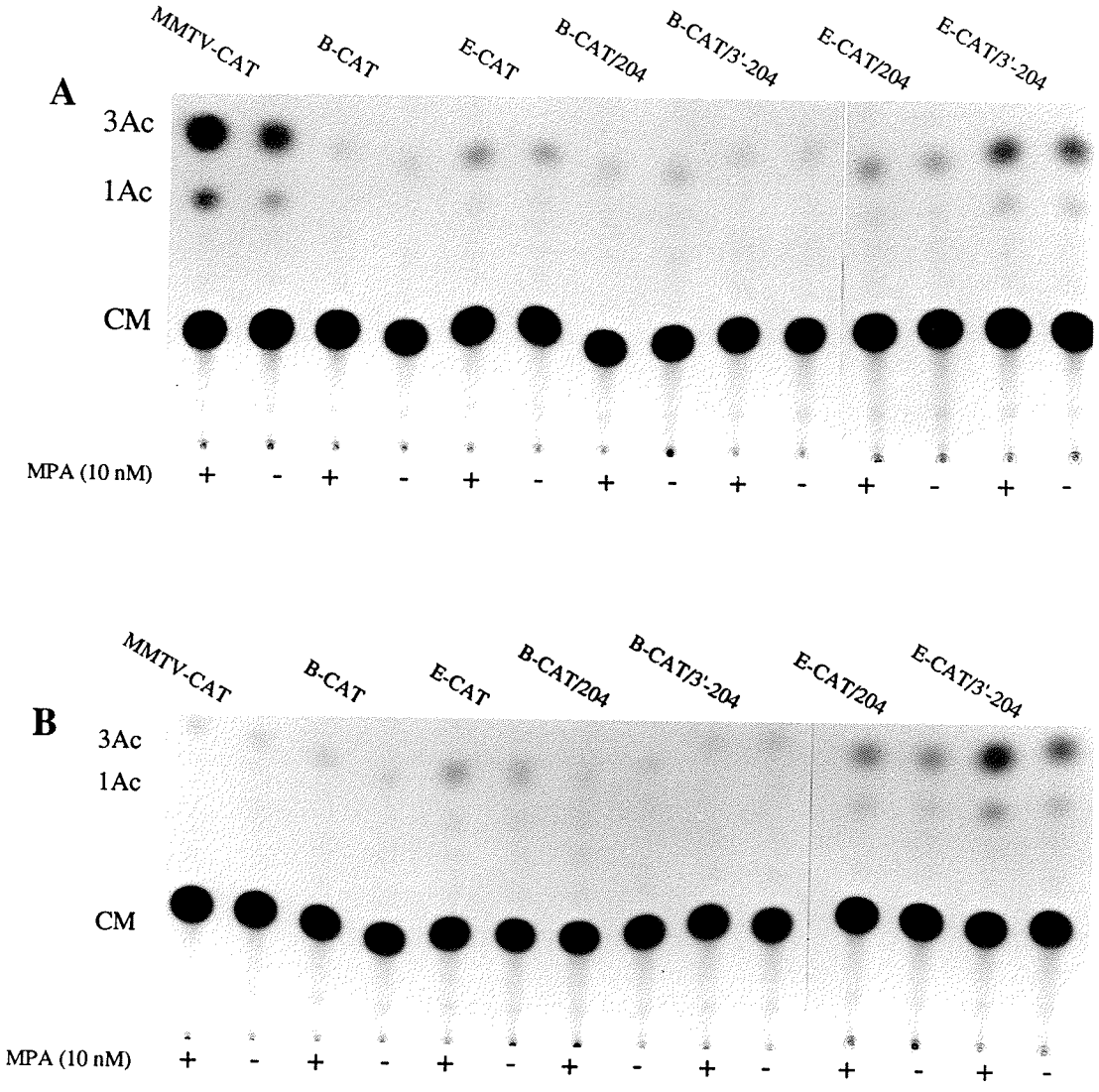


Fig. 36A. Transient transfection analysis of Basic- and Enhancer-CAT activity under the control of EGF 5'-flanking sequences in HeLa cells. HeLa cells ($1X10^6$/dish) were co-transfected with (or without) 0.1 μg cPR1, 3 μg pCH110 plus various reporters in equimolar amounts in a total of 10 μg of DNA. TLC plates were run as described and autoradiography was for 2-3 days (see Fig. 36B for quantitiative measurement). Abbreviations for plasmids are the same as in Fig. 34.

A. With cPR1

B. Without cPR1

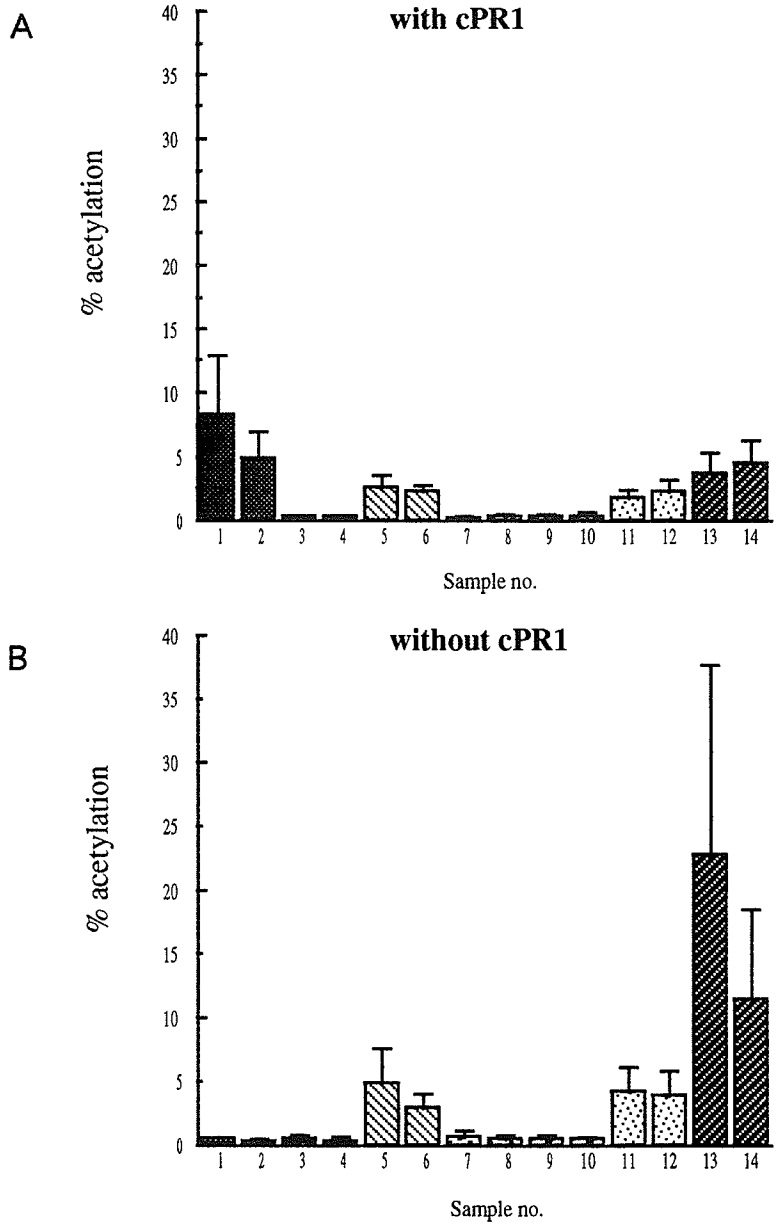


Fig. 36B. Relative CAT activity of 204-containing reporters in transient transfected HeLa cells. The same TLC plates in Fig. 36A were used to quantitate CAT activity. After normalization using β-gal activities., relative CAT expression were depicted in histographic form showing mean +/- SEM, n=2. Lanes 1 and 2, MMTV-CAT; lanes 3 and 4, Basic-CAT; lanes 5 and 6, Enhancer-CAT; lanes 7 and 8, Basic-CAT/204; lanes 9 and 10, Basic-CAT/3'-204; lanes 11 and 12, Enhancer-CAT/204; lanes 13 and 14, Enhancer-CAT/3'-204.

A. with cPR1

B. without cPR1

significant further increase in CAT activity over the background (Fig. 36A and 36B). Enhancer-CAT/3'-204 yielded more definitive data indicative of a promoter function whereas Enhancer-CAT/204 showed very little significant increase than the background (Fig. 36B). Similar to the T-47D line, hormone stimulation of the progesterone receptor-expressing HeLa cells did not alter the CAT gene expression as driven by EGF 5'-regulatory regions (Fig. 36B). In other words, no differential increase in CAT activity was observed upon hormone treatment, once again pointing to the absence of a functional progeseterone responsive element in the 204 fragment.

DISCUSSION

1. EXPRESSION OF EGF mRNA IN T-47D CELLS: SIGNIFICANCE AND PROGESTIN REGULATION

A growing body of evidence suggests that mammary gland growth and development is regulated not only by the classical sex hormones, but also by the participation of locally synthesized polypeptide growth factors such as EGF. Aberrant expression of the same hormones or growth factors may also contribute to the development of mammary tumors. Human breast tumors frequently bear receptors for estrogen and/or progesterone. There is a high correlation of the presence of estrogen and progesterone receptors in a tumor with a hormone responsive phenotype. This means that the tumor will respond to some form of hormonal therapy, e.g. antiestrogen treatment, high dose estrogen or progestin treatment (60, 61 and Introduction 3). Receptors for other growth factors have also been identified in breast tumors. In particular, receptors for EGF have been shown to be expressed and elevated in levels in some mammary tumors. More importantly, elevated levels of EGF receptors were found to be correlated with poor prognosis and early recurrence in some breast cancer patients (57, 58), strongly supporting a role of EGF and/or TGFα in the etiology or progression of the disease. TGFα is also a ligand for the EGF receptor (27) and TGFα has also been shown to be expressed by human breast cancer cell lines and some biopsy samples (240, 241). Evidence has been provided by Murphy et al., that the two apparently independent pathways, one concerning steroid hormones, the other growth factors such as EGF, may act in concert to promote breast cancer development and/or progression. This group reported that progestins specifically upregulated the expression of the EGF receptor gene in hormone-responsive mammary carcinoma cells including T-47D (242, 243). In a later report, the same group also reported unequivocally for the first time the expression of EGF, one of the two knwon

ligands for the EGF receptor, in T-47D and ZR 75 cell lines (190). Furthermore, the EGF mRNA levels in these cell lines can be increased by treatment with progestins to approximately 6-fold above untreated control in T-47D cells when analysed by Northern blotting. When analysed by a more sensitive RNAase protection assay, the induction of EGF mRNA by progestins was found to be higher in magnitude, ie. 11-fold above control after 24 hr of treatment, and occurring apparently earlier at 3 hr after stimulation (Fig. 7A and C). These findings suggest a possible autocrine growth loop for EGF that may give the cells a growth advantage in the presence of progestins. However, progestin treatment of breast cancer cells under these conditions was actually antiproliferative, the inhibition of cell growth being time and dose dependent. This is in contrast to the usual growth-promoting properties attributed to EGF which has also been demonstrated in breast cancer cells (244, 245). To reconcile with these two apparently contradictory findings, an hypothesis has been put forward by Murphy et al., who suggested that the expression of the EGF gene and its induction by progestins could represent a compensatory response to growth inhibition by progestins. Two other observations support this model of EGF action: 1) an EGF nonexpressing variant of T-47D cells is found to be much more sensitive to the antiproliferative effects of progestins than the expressing parent line (246). 2) the growth inhibitory effects of both progestins and antiestrogens in T-47D cells can be reversed by the exogenous addition of the 53-amino acid EGF peptide (246, 247, 248, 249). One should be reminded that progestational agents such as MPA is widely used in hormonal therapy of breast cancer (Introduction 3.2), and one of the commonly encountered problem associated with its clinical use is the development of drug resistance. The EGF-inducing effect of MPA in T-47D cells is consistent with the above hypothesis, a corollary of which is that increased expression of growth factors might contribute to hormone resistance by providing alternative

growth pathways to oppose the growth inhibitory effects of progestins and/or antiestrogens. Additional data consistent with this hypothesis was offered from a recent report of this laboratory. It was found that increased expression of TGFα (which also binds EGF receptor) and the EGF receptor genes is assoicated with resistance to growth inhibition by progestins in a cell line derived from T-47D-5 cells by stepwise selection in increasing concentrations of MPA (86).

2. MOLECULAR MECHANISMS OF EGF mRNA INDUCTION BY PROGESTINS, CYCLOHEXIMIDE AND PROGESTIN PLUS CYCLOHEXIMIDE

Since CHX treatment did not inhibit progestin-induction of EGF mRNA accumulation, the effect was considered as direct and did not require de novo protein synthesis. This is in agreement with our hypothesis that the effect of progestin was at the transcriptional level. Nuclear run-off experiments provided some evidence that the transcriptional rate of the EGF gene was indeed altered upon MPA stimulation and induction was apparent as early as 3 hr. However, the low abundance of the EGF primary transcripts (reflecting a low basal transcription rate) precluded further detail analysis. Future attempt to measure transcription rate of EGF gene would likely require more sensitive assay such as that based on polymerase chain reaction (PCR) technology (250).

When compared with untreated control cells, MPA and CHX were found to have no effect on the EGF message half-life, whereas treatment with CHX + MPA resulted in a very small increase in EGF mRNA stability. These data along with the demonstration of a transcriptional effect by MPA suggested that the majority of the effects mediated by CHX and MPA + CHX are probably also transcriptional. This would imply normally the EGF gene is under negative control through a labile

repressor protein. CHX treatment lead to decay and removal of this repressor from its binding site (s) allowing a higher levels of gene transcription to be reached. Interestingly, CHX was known to superinduce the c-fos gene; deletion analysis has identified the serum response element in the promoter of c-fos as the determinant mediating the super-inducing effects of CHX (251). In addition, this element by itself is able to confer an inductive response to CHX alone (251). Hence, the c-fos system has close similarity to the EGF system in terms of response to inhibition of protein synthesis by cycloheximide. A similar system in which superinduction has been investigated in detail involved the regulation of the urokinase gene. In this case, a DNA element similar to the the binding site for the transcription factor NFκB was found to confer CHX-mediated superinducibility by agents such as phorbol esters (252). In both the cases of c-fos and urokinase, the putative labile repressor has not yet been identified. The only known system in which a candidate repressor mediating the effect of CHX, was isolated is found in the gene for the glycoprotein hormone α-subunit whose transcription rate could be increased 2.6-fold by CHX (253). Furthermore, a nuclear protein p50 was identified and shown to bind to a DNA fragment corresponding to 5'-flanking sequences immediately upstream of the α-subunit gene transcription start site (253). More importantly, the p50 DNA binding activity decreases in the presence of CHX at a rate similar to that at which α-subunit mRNA increases, strongly suggesting it as the labile repressor. From the above considerations, it would be of interest to see if similar CHX responsive DNA elements exist in the EGF gene which act as binding sites for certain negative regulatory factor. To this end, the existence of a conserved NFκB binding site in the 204-22 fragment (Fig. 19 and 20) is intriguing. Further transient transfection and deletion analyses will be needed to test the above hypotheses.

3. CHARACTERIZATION OF 5'-FLANKING REGION OF THE EGF GENE

Presented in this thesis (see Result 4.1) is an analysis of the known nucleotide sequence of the 204-22 fragment of the EGF 5'-flanking region for putative protein-factor binding sites. Computer-assisted analysis demonstrated the existence of a potential TATA and CAAT box at positions 26 and 65 upstream of the putative transcription startsite which is consistent with previous predictions (201). The presence of both TATA and CAAT boxes is a common feature to many Pol-II gene promoters although there is a large subclass of genes that lack both motifs. Examples of the latter include several house-keeping genes (e.g. dihydrofolate reductase (254), adenosine deaminase (255)) as well as non-housekeeping genes (e.g. TGFα (256), EGF receptor (257)). However, close inspection of the two motifs in the EGF gene revealed their dissimilarity to the consensus sequence. The human EGF 'TATA' box has the sequence TTTAAA whereas that of the 'CAAT' box is CAAGT. It should be emphasized that there are a large number of genes whose promoters contain non-consensus TATA boxes, and that the same TATA-binding factor, TFIID, appears to bind to these nonconsensus sites and promote transcription (258). Furthermore, when compared with the corresponding region of the mouse EGF gene, we found the exact same motifs at almost identical locations with respect to the known startsite (233). This strongly suggests that the same motif in the human EGF gene is also functional and specifies transcriptional initiation at the putative startsite mapped only by sequence comparison at this stage (201). At least 5 additional findings lend credence to the functional significance of the putative CAAT and TATA-like sequences: 1) They all reside in a stretch of DNA that bears high sequence similarity to the corresponding region in the mouse gene indicating an evolutionary conservation probably as a result of a functionally important selection pressure. 2) The relative locations of the

two motifs with respect to the putative startsite are in agreement with known functional TATA and CAAT boxes (80). 3) Preliminary data from our laboratory using DNase I hypersensitive site analysis shows the existence of a hypersensitive site that mapped to the region containing the TTTAAA sequence, suggesting a more open chromatin structure around that area which in turn reflects functional activity (259). 4) The TTTAAA sequence is also identical to the TATA box sequence of the genes for the α and γ subunits of mouse nerve growth factor (NGF) (260) and similar to that of the β subunit gene (TTAAA) (261). Like EGF, these NGF genes are transcribed at high levels in the mouse submandibular gland. 5) When linked to the promoterless CAT gene, the 204 EGF 5' fragment directed a weak and constitutive activity as best demonstrated by the 204-containing Enhancer-CAT constructs in T-47D cells (Result 4.2.3 and Fig. 33). This provided functional proof for the existence of a functional promoter in the fragment.

The functional significance of other putative protein factor binding sites in the EGF 5'-flanking region is unknown. The AP-I site is the binding site for the Fos/Jun complexes, whereas the two juxtaposed Sp1 sites (the GC box) bind the transcription factor Sp1. Multiple GC boxes are common in promoters lacking TATA and CAAT box (254, 255). In these cases multiple GC boxes replace the function performed by the TATA sequence in activating transcription initiation. Nevertheless, it is obvious that not all the identified regulatory elements in the 204 fragment are actual functional in T-47D cells. The endogenous EGF gene was shown to be regulated by progestins but not other steroids in T-47D cells. The specificity of progestins was also confirmed by the use of antiprogestin which blocked progestin induction of EGF mRNA (190). Moreover, in contrast to ZR 75-1 cells in which the action of MPA was mainly through the androgen and glucocorticoid receptors, these two hormones were shown to be ineffective in T-

47D cells in stimulating EGF gene expression. This discrepancy is probably due to the special properties associated with T-47D cells which have unusually high levels of progesterone receptors (PgR) (13, 262, 263), thereby acting as the principle receptor through which progestins would act. Using these arguments, the glucocorticoid responsive element (GRE) and estrogen half-site are unlikely to be active in T-47D cells. The possibility that the GRE might interact with PgR, as has been shown for MMTV-LTR (137), was excluded on the basis that progestin treatment had no additional effect on the ability of 204-22 to drive CAT expression (see below). Although two progesterone responsive elements (PRE) identical in sequence to functionally identified PREs in the uteroglobin gene, were identified in the 204-22 fragment, MPA treatment was found not to alter levels of CAT expression when EGF 5'-flanking sequences containing these sites were placed upstream of the CAT gene in transient transfection assays. These functional analyses, using both T-47D and HeLa cells, clearly demonstrated the absence of a functional PRE in the tested DNA fragment (204-22) (Results 4.2.3 and 4.2.4). Therefore, we concluded that the two PREs, like the other two steroid responsive elements, were not functional under the conditions we have used. These studies led to the conclusion that the hormonal response seen with the endogenous gene is unlikely to be mediated by these elements. The question that remains to be answered is: where is the functional PRE located if not in the 3.3 kbp 5'-flanking region tested in this study? Previous data in the literature suggest that it may reside in regions of the gene anywhere from further upstream than the 204 sequence to 3'-flanking region of the EGF gene, or even in intronic sequences. Enhancers or HRE have been identified in far upstream regions, examples of which include the cAMP responsive element and glucocorticoid responsive element in the tyrosine aminotransferase gene (264, 265). An extreme case is provided by the human α-globin gene cluster in which a positive regulatory element was identified in a region

30-50 kbp upstream of the α-globin genes (266). Known examples of enhancers residing in the 3'-flanking regions (ie. downstream of poly (A) site) include the rat myosin light chain (MLC) 1/3 locus (267) and the human A-gamma globin gene (268). The former enhancer region contains binding sites for muscle-specific helix-loop-helix protein factors, whereas erythroid-specific GATA-1 binding sites are found in the latter. Several hormone responsive elements have also been identified in intronic sequences of genes such as the rat and the human growth hormone genes. Specifically, a glucocorticoid responsive element was located in the first intron of the rat gene (269), whereas the human gene contains a thyroid responsive element in the third intron (270). In view of the variability of possible locations for enhancers and HREs, it is worth pointing out that most functional studies regarding regulatory sequences usually use, as a starting point, 5'-flanking regions of the gene of interest. Such an analysis would of course fail to detect any regulatory elements that may reside downstream of the cap site. In order to circumvent this problem, including an analysis of the EGF gene, three experimental approaches can be adopted: 1) Before proceeding with deletion analysis, one can utilize DNase I hypersensitive site assays, which will crudely map the location of any putative HRE. It is known that activation of steroid-dependent enhancer elements is often associated with changes in chromatin structure (265, 271, 272). A hormone-modulated hypersensitive site would be indicative of the presence of a HRE around that region. 2) The study in which a thyroid hormone responsive element was identified in the third intron of the rat growth hormone gene provided an ingenious method to locate and isolate HREs regardless of their natural locations within the gene. In this approach, the gene of interest was first cleaved by a set of restriction enzymes, and the resulting mixture of fragments end-labeled by Klenow. Then extracts containing enriched amounts of a particular steroid receptor were allowed to incubate with the DNA fragment mixtures. Receptor-HRE interaction presumably

would occur under proper conditions. Subsequently, fragments containing binding sites are retrieved by immunoprecipitation with anti-receptor antibodies. 3) A third approach is designed to simultaneously analyse the 5'- and 3'-flanking regions of a particular gene for regulatory sequences. A series of chimeric minigenes consisting of different 5'-flanking sequences linked to the gene's coding region (with or without some of the introns) or to a reporter gene (e.g. CAT, globin) and 3'-flanking sequences are constructed. The activities of these constructs are then determined by transfecting them into cultured cells followed by measuring the levels of mRNA or enzyme derived from the chimeric gene with or without hormonal stimulation. This strategy essentially cuts down the time involved in analysing regulatory regions separately. Using this approach, Li et al., established that downstream sequences of the mouse thymidylate synthase gene are required for normal growth regulation (273).

A logical continuance of this work would be to finely map the cis-acting elements located in the functional promoter described in this thesis, firstly by deletion studies followed by DNA footprinting analysis. Also, the putative transcription startsite required confirmation using S1 nuclease and primer extension analyses. Although the location of the element responsible for conferring a progestin response to the EGF gene remains undefined, the availability of novel approaches such as those discussed above, would help to ensure its identification or at the very least to narrow down the regions need to be tested in a more refined ways.

REFERENCES

1. David M and Vessey MP. 1991. Epidemiology and primary prevention of breast cancer. In: The Breast: Comprehensive management of benign and malignant disease, Bland KI and Copeland III EM (eds), W. B. Saunders Company, New York, pp. 235-246.

2. Thomas DB. 1986. Hormones and hormone receptors in the etiology of breast cancer. Breast Cancer Res Treat 7(suppl): 11-22

3. MacMahon B, Cole P and Lin TM, et al. 1970. Age at first birth and breast cancer risk. Bull WHO 43: 209-212.

4. Layde PM, Webster LA, Baugham AL, Wingo PA, Rubin GL, and Ory HW. 1989. The independent associations of parity, age at first full-term pregnancy, and duration of breast-feeding with the risk of breast cancer. J Clin Epidemiol 42: 963-973.

5. Pathak DR, Speizer FE, Willett WC, Rosner B, and Lipnick RJ. 1986. Parity and breast-cancer risk: possible effect on age at diagnosis. Int J Cancer 37: 21-25.

6. Boyle P and Leake R. 1988. Progress in understanding breast cancer: epidemiological and biological interactions. Breast Cancer Res Treat 11: 91-112.

7. Beatson GW. 1896. On the treatment of inoperable cases of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet ii: 104-162.

8. Lippman ME and Dickson RB. 1989. Mechanisms of growth control in normal and malignant breast epithelium. Recent Prog Horm Res 45: 383-435.

9. Vonderhaar BK. 1984. Hormones and growth factors in mammary gland development. In: Control of cell growth and proliferation, Veneziale CM, Van Nostrand-Reinhold (eds), New York, pp. 11-33.

10. Topper YJ and Freeman CS. 1980. Multiple hormone interactions in the developmental biology of the mammary gland. Physiol Rev 60: 1049-1106.

11. Cullen KJ and Lippman ME. 1989. Estrogenic regulation of protein synthesis and cell growth in human breast carcinoma. Vitamins and Hormones 45: 127-172.

12. Dickson RB and Lippman ME. 1991. Growth regulation of normal and malignant breast epithelium. In: The Breast: Comprehensive management of benign and malignant disease, Bland KI and Copeland III EM (eds), W. B. Saunders Company, pp. 363-394.

13. Clarke CL and Sutherland RL. 1990. Progestin regulation of cellular proliferation. Endocrine Rev 11(2): 266-301.

14. Lippman ME. 1981. Hormonal regulation of human breast cancer cells in vitro. In: Banbury Report: Hormones and Breast Cancer, Pike MC, Siiteri PK, Welsch CW (eds), Cold Spring Harbour Laboratory USA, pp. 171-184.

15. Osborne CK, Monaco ME, Kahn CR, Huff K, Bronzert D, and Lippman ME. 1979. Direct inhibition of growth and antagonism of insulin action by glucocorticoids in human breast cancer in culture. Cancer Res 39: 2422-2428.

16. Moore JW, Thomas BS, and Wang DY. 1986. Endocrine status and the epidemiology and clinical course of breast cancer. Cancer Surv 5 (3): 537-559.

17. Welsch CW. 1985. Host factors affecting the growth of carcinogen induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. Cancer Res 45: 3415-3443.

18. Pearson OH, Manni A, Chambers M, Brodkey J, and Marshall JS. 1978. Role of pituitary hormones in the growth of human breast cancer. Cancer Res 38: 4323-4326.

19. Shiu RPC, Murphy LC, Tsuyuki D, Myal Y, Lee-Wing M, and Iwasiow B. 1987. Biological actions of prolactin in human breast cancer. Recent Prog Horm Res 43: 277-303.

20. Boutin JM, Edery M, Shitora C, Jolicoeur C, Lesueur L, Ali S, Gould D, Djiane J, and Kelly PA. 1989. Identification of cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells. Molec Endocr 3: 1455-1461.

21. Bejcek BE, Li DY, and Deuel TF. 1989. Transformation by v-*sis* occurs by an internal autoactivation mechanisms. Science 245: 1496-1499.

22. Keating MT, and Willimans LT. 1988. Autocrine stimulation of intracellular PDGF receptors in v-*sis* -transformed cells. Science 239: 914-916.

23. Dunbar CE, Breowder TM, Abrams JS, and Nienhuis AW. 1989. COOH-terminal-modified interleukin-3 is retained intracellularly and stimulates autocrine growth. Science 245: 1493-1496

24. Anklesaria P, Teixido J, Laiho M, Pierce JH, Greenberger JS, and Massague J. 1990. Cell-cell adhesion mediated by binding of membrane-anchored transforming growth factor α to epidermal growth factor receptors promotes cell proliferation. Proc Natl Acad Sci USA 87: 3289-3293.

25. Mroczkowski B, Reich M, Chen K, Bell GI, and Cohen S. 1989. Recombinant human epidermal growth factor precursor is a glycosylated membrane protein with biological activity. Mol Cell Biol 9: 2771-2778.

26. Pandiella A and Massague J. 1991. Cleavage of the membrane precursor for transforming growth factor α is a regulated process. Proc Natl Acad Sci USA 88: 1726-1730.

27. Carpenter G and Wahl MI. 1990. The epidermal growth factor family. Handb Exp Pharmacol 95 (1): 69-171.

28. Osborne CK, Clemmons DR and Arteaga CL. 1990. Regulation of breast cancer growth by insulin-like growth factors. J Steroid Biochem Molec Biol 37 (6): 805-809.

29. Dickson RB. 1990. Stimulatory and inhibitory growth factors and breast cancer. J Steroid Biochem Molec Biol 37 (6): 795-803.

30. Clarke R, Brunner N, Katz D, Glenz P, Dickson RB, lippman ME, and Kern F. 1989. The effects of a constitiutive production of TGFα on the growth of MCF-7 human breast cancer cells in vitro and in vivo. Mol Endocrinol 3: 372-380.

31 Jhappan C, Stahle C, Harkins RN, Fauston N, Smith GH, and Merlino GT. 1990. TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 61: 1137-1146.

32. Sandgren EP, Luetteke NC, Palmiter RD, Brinsten RL, and Lee DC. 1990. Overexpression of TGFα in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia and carcinoma of the breast. Cell 61: 1121-1135.

33. Matsui Y, Halter SA, Holt JT, Hogan BLM, and Coffey R. 1990. Development of mammary hyperplasia and neoplasia in MMTV-TGFα transgenic mice. Cell 61: 1147-1155.

34. Yee D, Paik S, Lebovic GS, Marcus RR, Favoni E, Cullen KJ, Lippman ME, and Rosen N. 1989. Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. Mol Endocrinol 3 (3): 509-517.

35. Massague J. 1990. The transforming growth factor-β family. Annu Rev Cell Biol 6: 597-641.

36. Arrick BA, Korc M, and Derynck R. 1990. Differential regulation of expression of three transforming growth factor-β species in human breast cancer cell lines by estradiol. Cancer Res 50: 299-303.

37. Osborne CK and Arteaga CL. 1990. Autocrine and paracrine growth regulation of breast cancer: clinical implications. Breast Cancer Res Treat 15: 3-11.

38. Brunner N. 1990. Human breast-cancer growth and progression: role of secreted polypeptide growth factors. Int J Cancer (Supplement) 5: 62-66.

39. Murphy LC and Dotzlaw H. 1989. Regulation of transforming growth factor α and transforming growth factor β messenger ribonucleic acid abundance in T-47D human breast cancer cells. Mol Endocrinol 3: 611-617.

40. Knabbe C, Lippman ME, Wakefield L, Flanders K, Kasid aaA, Derynck R, and Dickson RB. 1987. Evidence that TGF-beta is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48: 417-428.

41. Arteaga CL, Tandon AK, VonHuff D, and Osborne CK. 1988. Transforming growth factor β: potential autocrine growth inhibitor of estrogen receptor-negative human breast cancer cells. Cancer Res 48: 3898-3904.

42. Carpenter G. 1987. Receptors for epidermal growth factor and other polypeptide mitogens. Ann Rev Biochem 56: 881-914.

43. Carpenter G and Cohen S. 1990. Epidermal growth factor. J Biol Chem 265 (14): 7709-7712.

44. Fisher DA and Lakshmanan J. 1990. Metabolism and effects of epidermal growth factor and related growth factors in mammals. Endocrine Rev 11 (3): 418-361.

45. Schultz G, Rotatori DS, and Clark W. 1991. EGF and TGF-a in wound healing and repair. J Cellular Biochem 45: 346-352.

46. Popliker M, Shatz A, Avivi A, Ullrich A, Schlessinger J, and Webb CG. 1987. Onset of endogenous synthesis of EGF in neonatal mice. Developmental Biology 119: 38-44.

47. Tonelli QJ and Sorof S. 1980. Epidermal growth factor requirement for the development of cultured mammary gland. Nature 285: 250-252.

48. Coleman S, Silberstein GB and Daniel CW. 1988. Ductal morphogenesis in the mouse mammary gland: evidence supporting a role for epidermal growth factor. Dev Biol 127: 304-315.

49. Stoscheck CM and King J LE. 1986. Role of epidermal growth factor in carcinogensis. Cancer Res 46: 1030-1037.

50. Stern DF, Hare DL, Cecchini MA, and Weinberg RA. 1987. Construction of a novel oncogene based on synthetic sequences encoding epidermal growth factor. Science 235: 321-324.

51. DiFiore PP, Pierce JH, Fleming TP, Hazan R, Ullrich A, Kling CR, Schlessinger J, and Aaronson SA. 1987. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH3T3 cells. Cell 51: 1063-1070.

52. Riedel H, Massoglia S, Schlessinger J, and Ullrich A. 1988. Ligand activation of overexpressed epidermal growth factor receptors transforms NIH 3T3 mouse fibroblasts. Proc Natl Acad Sci USA 85: 1477-1481.

53. Velu TJ, Beguinot L, Vass WC, Willingham MC, Merlino GT, Pastan I, and Lowy DR. 1987. Epidermal growth factor-dependent transformation by a human EGF receptor proto-oncogene. Science 238: 1408-1410.

54. Oka T, Tsutsumi O, Kurachi H, and Okamoto S. 1988. The role of epidermal growth factor in normal and neoplastic growth of mouse mammary cells. In: Breast Cancer: Cellular and Molecular Biology, Lippman ME, Dickson RB (eds), Kluwer Academic Press, Norwell, MA. pp. 343-362.

55. Dotzlaw H, Miller T, Karvelas J, and Murphy LC. 1990. Epidermal growth factor gene expression in human breast cancer biopsy samples: relationship to estrogen and progesterone receptor gene expression. Cancer Res 50: 4204-4208.

56. Sainsbury JRC, Farndon JR, Sherbet GV, and Harris AL. 1985. Epidermal growth factor receptors and estrogen receptors in human breast cancer. Lancet i: 364-366.

57. Sainsbury JRC, Malcolm AJ, Appletion DR, Farnadon JR, and Harris AL. 1985. Presence of epidermal growth factor receptor as an indicator of poor prognosis in patients with breast cancer. J Clin Pathol 38: 1225-1228.

58. Sainsbury JRC, Farndon JR, Needham GK, Malcolm AJ, and Harris AL. 1987. Epidermal growth factor receptor status as predictor of early recurrence of, and death from, breast cancer. Lancet i: 1398-1402.

59. Sheth SP and Allegra JC. 1991. Endocrine therapy of breast cancer. In: The Breast: Comprehensive management of benign and malignant disease, Bland KI and Copeland III EM (ed), W. B. Saunders Company. pp. 937-947.

60. Osborne CK, Yochmorwitz MG, and Knight WA. 1980. The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer 46: 2884-2888.

61. Wittliff JL. 1984. Steroid-hormone receptors in breast cancer. Cancer 53: 630-643.

62. Siiteri PK. 1982. Review on studies of estrogen biosynthesis in the human. Cancer Res (Suppl). 42: 3269-3273.

63. Furr BJA and Jordan VC. 1984. The pharmacology and clinical uses of tamoxifen. Pharmacol Ther 25: 127-205.

64. Baum M et al. 1985. Controlled trial of tamoxifen as single adjuvant agent in management of early breast cancer. Lancet i: 127-205.

65. Fisher B et al. 1987. Prolonging tamoxifen therapy for primary breast cancer. Ann Int Med 106: 649-654.

66. Jordan VC and Murphy CS. 1990. Endocrine pharmacology of antiestrogens as antitumor agents. Endocrine Rev 11 (4): 578-610.

67. Pham TA, Elliston JF, Nasaz Z, McDonnell DP, Tsai M-J, and O'Malley BW. 1991. Antiestrogen can establish nonproductive receptor complexes and alter chromatin structure at target enhancers. Proc Natl Acad Sci USA 88: 3125-3129.

68. Patterson JS. 1981. 'Nolvadex' (tamoxifen) as an anti-cancer agent in humans. In: Non-steroidal Antiestrogens, Sutherland RL and Jordan VC (eds), Academic Press, Sydney. pp. 143-163.

69. Kon OL. 1989. Antiestrogens and cell proliferation. Bioessays 10 (6): 210-214.

70. Sutherland RL, Green MD, Hall RE, Reddel RR, and Taylor IW. 1983. Tamoxifen induces accumulation of MCF-7 human mammary carcinoma cells in the Go/G1 phase of the cell cycle. Eur J Cancer Oncol 19: 615-621.

71. Osborne CK, Boldt DH, Clark GM. et al. 1983. Effects of tamoxifen on human breast cancer cell cylce kinetics: accumulation of cells in early G1 phase. Cancer Res 43: 3583.

72. Jordan VC. 1990. Long-term adjuvant tamoxifen therapy for breast cancer. Breast Cancer Res Treat 15: 125-136.

73. Pasqualini JR, Sumida C, and Giambiagi N. 1988. Pharmacodynamic and biological effects of antiestrogens in different models. J Steroid Biochem Molec Biol 31: 613-643.

74. Wakeling AE. 1990. Therapeutic potential of pure antiestrogens in the treatment of breast cancer . J Steroid Biochem Molec Biol 37 (6): 771-775.

75. Pasqualini JR, Giambiagi N, Gelly C, and Chetrite G. 1990. Antiestrogen action in mammary cancer and in fetal cells. J Steroid Biochem Molec Biol 37 (3): 343-348.

76. Allegra JC and Kiefer SM. 1985. Mechanisms of action of progestational agents. Semin Oncol (Suppl 1) 12: 3.

77. Santen RJ, Manni A, Harvey H, and Redmond C. 1990. Endocrine treatment of breast cancer in women. Endocrine Rev 11 (2): 221-265.

78. Teulings FAG, Van Gielse HA, Henkelman MS, Portengen H, and Alexieva-Figush J. 1980. Estrogen, androgen, glucocorticoid, and progesterone receptors in progestin-induced regression of human breast cancer. Cancer Res 40: 2557-2561.

79. Horwitz KB. 1987. The structure and function of progesterone receptors in breast cancer. J Steroid Biochem 27: 447-457.

80. Cavalli F, Goldhirsch A, Jungi F, Martz G, Mermillod B, Schafer P, and Alberto P. 1984. Randomized trial of low-versus-high dose medroxyprogesterone acetate in the treatment of postmenopausal patients with advanced breast cancer. In: Role of Medroxyprogesterone acetate in Endocrine-Related Tumors, Pellegrini A, Robustelli Della Cuna G, Pannuti F, Pouillart P, Jonat W (eds), Vol III. Raven Press, New York, pp. 79-89.

81. Young PCM, Keen FK, Einhorn LH, Stanick BM, Ehrlich CE, and Cleary RE. 1980. Binding of medroxyprogesterone acetate in human breast cancer. Am J Obstet Gynecol 137: 284-292.

82. Poulin R, Baker D, Poirier D, and Labrie F. 1989. Androgen and glucocorticoid receptor-mediated inhibition of cell proliferation by medroxyprogesterone acetate in ZR-75-1 human breast cancer cells. Breast Cancer Res Treat 13: 161-172.

83. Haller DG and Glick JH. 1986. Progestational agents in advanced breast cancer: an overview. Semin Oncol 13 (suppl): 2-8.

84. Perez-Palacios G, Chavez B, Vilchis F, Escobar N, Larrea F, and Perez AE. 1983. Interaction of medroxyprogesterone acetate with cytosol androgen receptors in the rat hypothalamus and pituitary. J Steroid Biochem 19: 1729- 1735.

85. Lundgren S, Kvinnsland S, and Utaaker E. 1989. Oral high-dose progestins as treatment for advanced breast cancer. Acta Oncol 28: 811-816.

86. Murphy LC, Dotzlaw H, Wong MSJ, Miller T, and Murphy LJ. 1991. Mechanisms involved in the evolution of progestin resistance in human breast cancer cells. Cancer Res 51: 2051-2057.

87. Braunsberg H, Coldham NG, leake RE, Cowan SK, and Wong W. 1987. Actions of a progestogen on human breast cancer cells: mechanisms of growth stimulation and inhibition. Eur J Cancer Clin Oncol 23: 563-571.

88. Kordon E, Lanari C, Meiss R, Elizalde P, Charreau E, and Pasqualini CD. 1990. Hormone dependence of a mouse mammary tumor line induced in vivo by medroxyprogesterone acetate. Breast Cancer Res Treat 17: 33-43.

89. Nagasawa H, Aoki M, Sakagami N, Ishida M. 1988. Medroxyprogesterone acetate enhances spontaneous mammary tumorigenesis and uterine adenomyosis in mice. Breast Cancer Res Treat 12: 59-66.

90. Jackson DA. 1991. Structure-function relationships in eukaryotic nuclei. BioEssays 13 (1): 1-10.

91. Grunstein M. 1990. Histone function in transcription. Ann Rev Cell Biol 6: 643-78.

92. Dynan, WS. 1989. Modularity in promoters and enhancers. Cell 58: 1-4.

93. Lewin B. 1987. RNA polymerase-promoter interactions control transcription. In: Genes III, John Wiley and Sons, Toronto. pp. 183-218.

94. Ptashne M. 1988. How eukaryotic transcriptional activators work. 1988. Nature 335: 683-689.

95. Sawadogo M and Sentenac A. 1990. RNA polymerase B (II) and general transcription factors. Annu Rev Biochem 59: 711-754.

96. Saltzman AG and Weinmann R. 1989. Promoter specificity and modulation of RNA polymerase II transcription. FASEB J 3: 1723-1733.

97. Sopta M, Burton ZF, and Greenblatt J. 1989. Structure and associated DNA-helicase activity of a general transcription initiation factor that binds to RNA polymerase II. Nature 341: 410-414.

98. Proudfoot NJ. 1989. How RNA polymerase II terminates transcription in higher eukaryotes. TIBS 14: 105-110.

99. Mitchell PJ and Tjian R. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245: 371-378.

100. Johnson PF and McKnight SL. 1989. Eukaryotic transcriptional regulatory proteins. Annu Rev Biochem 58: 799-839.

101. Latchman DS. 1990. Eukaryotic transcription factors. Biochem J 270: 281-289.

102. Pabo CO and Sauer RT. 1984. Protein-DNA recognition. Annu Rev Biochem 53: 293-321.

103. Bush SJ and Sassone-Corsi P. 1990. Dimers, leucine zippers and DNA-binding domains. TIG 6 (2): 36-40.

104. McKnight SL. 1991. Molecular zippers in gene regulation. Scientific American April: 54-64.

105. Berg JM. 1990. Zinc finger domains: hypotheses and current knowledge. Annu Rev Biophys Chem 19: 405-421.

106. Berg JM. 1990. Zinc fingers and other metal-binding domains. J Biol Chem 265 (12): 6513-6516.

107. Murre C, McCaw PS, and Baltimore D. 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell 56: 777-783.

108. Ransone LJ and Verma IM. 1990. Nuclear proto-oncogenes Fos and Jun. Annu Rev Cell Biol 6: 539-557.

109. Distel RJ and Spiegelman BM. 1990. Protooncogene c-fos as a transcription factor. Adv in Cancer Res 55: 37-55.

110. Vogt PK and Bos TJ. 1990. jun: oncogene and transcription factor. Adv in Cancer Res 55: 1-35.

111. Reeves R and Magnuson NS. 1990. Mechanisms regulating transient expression of mammalian cytokine genes and cellular oncogenes. Prog Nucl Acid Res Molec Biol 38: 241-282.

112. Roesler WJ, Vandenbark GR, and Hanson RW. 1988. Cyclic AMP and the induction of eukaryotic gene transcription. J Biol Chem 263 (19): 9063-9066.

113. Montminy MR, Gonzalez GA, and Yamamoto KK. 1990. Characteristics of the cAMP response unit. Recent Prog Horm Res 46: 219-230.

114. Ziff EB. 1990. Transcription factors: a new family gathers at the cAMP response site. TIG 6 (3): 69-72.

115. Habener JF. 1990. Cyclic AMP response element binding proteins: a cornucopia of transcription factors. Mol Endocrinol 4 (8): 1087-1094.

116. Andrisani OM and Dixon JE. 1990. Somatostatin gene regulation. Annu Rev Physiol 52: 793-806.

117. Jones N. 1990. Transcriptional regulation by dimerization: two sides to an incestuous relationship. Cell 61: 9-11

118. Renkawitz R. 1990. Transcriptional repression in eukaryotes. TIG 6 (6): 192-197.

119. Levine M and Manley JL. 1989. Transcriptional repression of eukaryotic promoters. Cell 59: 405-408.

120. Spencer CA and Groudine M. 1991. Control of c-myc regulation in normal and neoplastic cells. Adv Cancer Res 56: 1-48.

121. Bentley DL and Groudine M. 1986. A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. Nature 321: 702-706.

122. Spencer CA and Groudine M. 1990. Transcription elongation and eukaryotic gene regulation. Oncogene: 4: 1503-1508.

123. Haley JD and Waterfield MD. 1991. Contributory effects of de novo transcription and premature transcript termination in the regulation of human epidermal growth factor receptor proto-oncogene RNA synthesis. J Biol Chem 266 (3): 1746-1753.

124. Wahli W, and Martinez E. 1991. Superfamily of steroid nuclear receptors: positive and negative regulators of gene expression. FASEB J 5: 2243-2249.

125. Carson-Jurica MA, Schrader WT, and O'Malley BW. 1990. Steroid receptor family: structure and functions. Endocrine Rev 11 (2): 201-220.

126. O'Malley BW. 1990. The steroid receptor superfamily: more excitement predicted for the future. Mol Endocrinol 4 (3): 363-369.

127. Weinberger C and Bradley DJ. 1990. Gene regulation by receptors binding lipid-soluble substances. Annu Rev Physiol 52: 823-840.

128. Beato M. 1989. Gene regulation by steroid hormones. Cell 56: 335-344.

129. Green S and Chambon P. 1988. Nuclear receptors enhance our understanding of transcription regulation. TIG 4 (11): 309-314.

130. Evans RM. 1988. The steroid and thyroid hormone receptor superfamily. Science 240: 889-895.

131. Berg JM. 1989. DNA binding specificity of steroid receptors. Cell 57: 1065-1068.

132. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, and Chambon P. 1990. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 9: 1603-1614.

133. Bocquel MT, Kumar V, Stricker C, Chambon P, and Gronemeyer H. 1989. The contribution of the N- and C-terminal regions of steroid receptors to activation of transcription is both receptor and cell-specific. Nuc Acid Res 17 (7): 2580-2595.

134. Forman BM and Samuels HH. 1990. Dimerization among nuclear hormone receptors. New Biologist 2: 587-594.

135. Forman BM and Samuels HH. 1990. Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. Mol Endocrinol 4 (9): 1293-1301.

136. Miesfeld RL. 1989. The structure and function of steroid receptor proteins. Crit Rev Biochem Molec Biol 24 (2): 101-117.

137. Ham J, Thomson A, Neddham M, Webb P, and Parker M. 1988. Characterization of response elements for androgens, glucocorticoids and progestins in mouse mammary tumor virus. Nuc Acids Res 16: 5263-5277.

138. Strahle U, Klock G, and Schutz G. 1987. A DNA sequence of 15 base pairs is sufficient to mediate both glucocorticoid and progesterone induction of gene expression. Proc Natl Acad Sci USA 84: 7871-7875.

139. Klock G, Stahle U, and Schutz G. 1987. Estrogen and glucocorticoid responsive elements are closely related but distinct. Nature 329: 734-736.

140. Brent GA, Moore DD, and Larsen PR. 1991. Thyroid hormone regulation of gene expression. Annu Rev Physiol 53: 17-35.

141. Naar AM, Boutin J-M, Lipkin SM, Yu VC, Holloway JM, Glass CK, and Rosenfeld MG. 1991. The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell 65: 1267-1279.

142. Umesono K, Murakami KK, Thompson CC, and Evans RM. 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid and vitamin D3 receptors. Cell 65: 1255-1266.

143. Akerblom IW, Slater EP, Beato M, Baxter JD, and Mellon PL. 1988. Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. Science 241: 350-353.

144. Drouin J, Charron J, Gagner JP, Jeannottee L, Nemer M, Plante RK, and Wrange O. 1987. The pro-opiomelanocortin gene: a model for negative regulation of transcription by glucocorticoids. J Cell Biochem 35: 293-304.

145. Proudfoot N. 1991. Poly (A) signals. Cell 64: 671-674.

146. Manley JL. 1988. Polyadenylation of mRNA precursors. Biochim Biophy Acta 950: 1-12.

147. Maniatis T and Reed R. 1987. The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing. Nature 325: 673-678.

148. Maniatis T. 1991. Mechanisms of alternative pre-mRNA splicing.Science 251: 33-34.

149. Mattaj IW and Hamm J. 1989. Regulated splicing in early development and stage-specific U snRNPs. Development 105: 183-189.

150. Zachar Z, Chou T-B and Bingham PM. 1987. Evidence that a regulatory gene autoregulates splicing of its transcript. EMBO J 6: 4105-4111.

151. Xie W, Chipman JG, Robertson DL, Erikson RL, and Simmons DL. 1991. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci USA 88: 2692-2696.

152. Agutter PS. 1988. Nucleocytoplasmic transport of mRNA: its relationship to RNA metabolism, subcellular structure and other nucleocytoplasmic exchanges. Prog Mol Subcell Biol 10: 15-96.

153. Schroder HC, Bachmann M, Diehl-Seifert B, and Muller WEG. 1987. Transport of mRNA from nucleus to cytoplasm. Prog Nucl Acid Res Molec Biol 34: 89-142.

154. Schroder HC, Ugarkovic D, Langen P, Bachmann M, Dorn A, Kuchino Y, and Muller W. 1990. Evidence for involvement of a nuclear envelope-associated RNA helicase activity in nucleocytoplasmic RNA transport. J Cell Physio 145: 136-146.

155. Schroder HC, Wenger R, Ugarkovic D, Friese K, Bachmann M, and Muller WEG. 1990. Differential effect of insulin and epidermal growth factor on the mRNA translocation system and transport of specific poly (A+) mRNA and poly (A-) mRNA in isolated nuclei. Biochem 29: 2368-2378.

156. Webb ML and Litwack G. 1986. Association of RNA with the glucocorticoid receptor and possible role in activation. In: Biochemical Actions of Hormones, Litwack G (ed), Academic press, New York. Vol 13. pp. 379-403.

157. Liao S, Kokontis J, Sai T and Hiipakka RA. 1989. Androgen receptors: structures, mutations, anitbodies and cellular dynamics. J Steroid Biochem 34: 41-51.

158. Ross J. 1989. The turnover of messenger RNA. Scientificic American April: 48-55.

159. Atwater JA, Wisdom R and Verma IM. 1990. Regulated mRNA stability. Annu Rev Genet 24: 519-541.

160. Yen TJ, Gay DA, Pachter JS, and Cleveland DW. 1988. Autoregulated changes in stability of polyribosome-bound β-tubulin mRNAs are specified by the first 13 translated nucleotides. Mol Cell Biol 8: 1224-1235.

161. Yen TJ, Machlin PS, and Cleveland DW. 1988. Autoregulated instability of β-tubulin mRNAs by recognition of the nascent amino terminus of β-tubulin. Nature 334: 580-585.

162. Klausner RD and Harford JB. 1989. Cis-trans models for post-transcriptional gene regulation. Science 246: 870-872.

163. Shaw G and Kamen R. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46: 659-667.

164. Shyu A-B, Belasco JG, and Greenberg ME. 1991. Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay. Genes & Dev 5: 221-231.

165. Wisdom R and Lee W. 1991. The protein-coding region of c-myc mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. 5: 232-243.

166. Shapiro DJ, Barton MC, McKearin DM, Chang T-C, Lew D, Blume J, Nielsen DA, and Gould L. 1989. Estrogen regulation of gene transcription and mRNA stability. Recent Prog Horm Res 45: 29-60.

167. Nielsen DA and Shapiro DJ. 1990. Insights into hormonal control of messenger RNA stability. Mol Endocrinol 4 (7): 953-957.

168. Liang H and Jost J-P. 1991. An estorgen-dependent polysomal protein binds to the 5' untranslated region of the chicken vitellogenin mRNA. Nuc Acid Res 19 (9): 2289-2294.

169. Petersen DD, Koch SR, and Granner DK. 1989. 3' noncoding region of phosphoenolpyruvate carboxykinase mRNA contains a glucocorticoid-responsive mRNA stabilizing element. Proc Natl Acad Sci USA 86: 7800-7804.

170. Wolffe AP, Glover JF, Martin SC, Teniswood MPR, Williams JL, and Tata JR. 1985. Deinduction of transcription of Xenopus 74-kDA albumin genes and destabilization of mRNA in vivo and in hepatocyte cultures. Eur J Biochem 146: 489-496.

171. Lee SW, Tsov A-P, Chan H, Thomas J, Petrie K, Eugni EM, and Allison AC. 1988. Glucocorticoids selectively inhibit the transcription of the interleukin 1β gene and decrease the stability of interleukin 1β mRNA. Proc Natl Acad Sci USA 85: 1204-1208.

172. Simonet WS and Ness GC. 1989. Post-transcriptional regulation of 3-hydroxy-3-methyglutaryl-CoA reductase mRNA in rat liver. Glucocorticoids block the stabilization caused by thyroid hormones. J Biol Chem 264: 569-573.

173. Raghow R, Gossage DL, and Kang AN 1986. Pretranslational regulation of type I collagen, fibronectin, and a 50-kildalton noncollagenous extracellular protein by dexamethasone in rat fibroblasts. J Biol Chem 261: 4677-4684.

174. Boggaram V, Smith ME, and Mendelson CR. 1991. Posttranscriptional regulation of surfactant protein-A messenger RNA in human fetal lung in vitro by glucocorticoids. Mol Endocrinol 5 (3): 414-423.

175. Krane IM, Spindel ER, and Chin WW. 1991. Thyroid hormone decreases the stability and the poly (A) tract length of rat thyrotropin β-subunit messenger RNA. Mol Endocrinol 5 (4): 469-475.

176. Shatkin AJ. 1985. mRNA cap binding proteins: essential factors for initiating translation. Cell 40: 223-224.

177. Hinnebusch AG. 1988. Novel mechanisms of translational control in Saccharomyces cerevisiae. TIG 4 (6): 169-174.

178. Hinnebusch AG. 1990. Transcriptional and translational regulation of gene expression in the general control of amino acid biosynthesis in Saccharomyces cerevisiae. Prog Nuc Acid Res Mole Biol 38:195-240.

179. Munro HN. 1990. Iron regulation of ferritin gene expression. Journal of Cellular Biochemistry 44: 107-115.

180. Rouault TA, Stout CD, Kaptain S, Harford JB, and Klausner RD. 1991. Structural relationship between an iron-regulated RNA-binding protein and aconitase: functional implications. Cell 64: 881-883.

181. Mager WH. 1988. Control of ribosomal protein gene expression. Biochim Biophy Acta 949: 1-15.

182. Nigg EA. 1990. Mechanisms of signal transduction to the cell nucleus. Adv Cancer Res. 55: 271-310.

183. Lewin B. 1991. Oncogenic conversion by regulatory changes in transcription factors. Cell 64: 303-312.

184. Dice JF. 1987. Molecular determinants of protein half-lives in eukaryotic cells. FASEB J 1: 349-357.

185. Dice JF. 1990. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. TIBS 15: 305-309.

186. Jentsch S, Seufert W, Sommer T and Reins H-A. 1990. Ubiquitin-conjugating enzymes: novel regulators of eukaryotic cells. TIBS 15: 195-198.

187. Ciechanover A and Schwartz AL. 1989. How are substrates recognized by the ubiquitin-mediated proteolytic system. TIBS14: 483-488.

188. Luskey KL. 1988. Regulation of cholesterol synthesis: mechanism for control of HMG CoA Reductase. Recent Prog Horm Res. 44: 35-48.

189. Chun KT, Bar-Nun S, and Simoni RD. 1990. The regulated degradation of 3-hydroxy-3-methylglutaryl-CoA reductase requires a short-lived protein and occurs in the endoplasmic reticulum. J Biol Chem 265 (35): 22004-22010.

190. Murphy LC, Murphy LJ, Dubik D, Bell GI, and Shiu RPC. 1988. Epidermal Growth Factor gene expression in human breast cancer cells: regulation of expression by progestins. Cancer Res 48: 4555-4560.

191. Murphy LC, Dotzlaw H, Wong MSJ, Miller T, Mrockowski B, Gong Y, and Murphy LJ. 1990. Epidermal growth factor: receptor and ligand expression in human breast cancer. Semin Cancer Biol 1: 305-315.

192. Keydar I, Chen L, Karby S, Weiss FR, Delarea J, Radu M, Chaitcik S, and Brenner HJ. 1979. Establishment and characterization of a cell line of human breast carcinoma origin. Eur J Cancer 15: 659-670.

193. Freake HC, Marcocci C, Iwasaki J, and MacIntyre I. 1981. 1, 25-dihydroxyvitamin D_3 specifically binds to a human breast cancer cell line (T-47D) and stimulates growth. Biochem Biophys Res Commun 101: 1131-1138.

194. Jones Jr HW, McKusick VA, Harper PS, and Wuu Kuang-Dong. 1971. The HeLa cell and a reappraisal of its origin. Obstet Gynecol 38: 945-949.

195. Chirgwin J, Pryzbyla A, MacDonald R, and Rutter W. 1979. Isolation of biologically active ribonucleic acid from sources enriched for ribonuclease. Biochemistry 18: 5294-5298.

196. Sambrook J, Fritsch EF, and Maniatis T. 1989. Molecular cloning: A laboratory manual, 2 nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

197. Thomas PS. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci USA 77: 5201-5205.

198. Cato ACB, Miksicek R, Schutz G, Arnemann J, and Beato M. 1986. The hormone regulatory element of mouse mammary tumor virus mediates progesterone induction. EMBO J 5: 2231-2240.

199. Gronemeyer H, Turcotte B, Quirin-Stricker C, Bocquel MT, Meyer ME, Krozowski Z, Jeltsch JM, Lerouge T, Garnier JM, and Chambon P. 1987. The chicken progesterone receptor: sequence, expression and functional analysis. EMBO J 6 (13): 3985-3994.

200. Joyeux C, Rochefort H, and Chalbos D. 1989. Progestin increases gene transcription and messenger ribonucleic acid stability of fatty acid synthetase in breast cancer cells. Mol Endocrinol 4: 681-686.

201. Bell GI, Fong NM, Stempien MM, Wormsted MA, Caput D, Lailing K, Urdea MS, Rall LB, and Samchez-Pescador R. 1986. Human epidermal growth factor precursor: cDNA sequence, expression in vitro and gene organization. Nuc Acid Res 14: 8427-8446.

202. Marzluff WF. 1990. Preparation of active nuclei. Methods Enzymol 181: 30-36.

203. Chomczynski P and Sacchi N. 1987. Single-step method of RNA isolation by acidic guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159.

204. Chauhan J and Dakshinamurti K. 1991. Transcriptional regulation of the glucokinase gene by biotin. J Biol Chem 266 (16): 10035-10038.

205. Murphy LC, Tsuyuki D, Myal Y, and Shiu RPC. 1987. Isolation and sequencing of a cDNA clone for a prolactin-inducible protein (PIP). J Biol Chem 262: 15236-15241.

206. Kingston RE. 1989. Calcium phosphate transfection. In: Current Protocols in Molecular Biology Vol 1, Ausubel FM, Brent R, Kingston RE, Moore DD, and Seidman JG. (eds), Wiley Interscience, New York. pp. 903-919.

207. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275.

208. Kingston RE. 1989. Harvest and assay for chloramphenicol acetyltransferase. In: Current Protocols in Molecular Biology Vol 1, Ausubel FM, Brent R, Kingston RE, Moore DD, and Seidman JG. (eds), Wiley Interscience, New York. pp. 963-966.

209. Rosenthal N. 1987. Identification of regulating elements of cloned genes with functional assay. Methods Enzymol 152: 704-720.

210. Miller MJ. 1987. Sensitivity of RNA synthesis to actinomycin D inhibition is dependent on the frequency of transcription: a mathematical model. J Theor Biol 129: 289-299.

211. Perry RP. 1963. Selective effects of actinomycin D on the intracellular distribution of RNA synthesis in tissue culture cells. Exp Cell Res 29: 400-406.

212. Perry RP and Kelley DE. 1970. Inhibition of RNA synthesis by actinomycin D: characteristic dose-response of different RNA species. J Cell Physiol 76: 127-140.

213. Goldberg IH and Friedman PA. 1971. Antibiotics and nucleic acids. Ann Rev Biochem 40: 775-810.

214. Hyman RW and Davidson N. 1970. Kinetics of the in vitro inhibition of transcription by actinomycin. J Mol Biol 50: 421-438.

215. Leinwand L and Ruddle FH. 1977. Stimulation of in vitro translation of messenger RNA by actinomycin D and cordycepin. Science 197: 381-383.

216. Cooper HL and Braverman R. 1977. The mechanism by which actinomycin D inhibits protein synthesis in animal cells. Nature 269: 527-529.

217. Seghal PB, Derman E, Molloy GR, TammI, and Darnell JE. 1976. 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole inhibits initiation of nuclear heterogeneous RNA chains in HeLa cells. Science 194: 431-433.

218. Tamm I. 1977. Definition of subclasses of nucleoplasmic RNA. Proc Natl Acad Sci USA 74: 5011-5015.

219. Harlow P and Molloy G. 1980. Effect of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole on ribonucleotide metabolism and accumulation of mitochondrial RNA and low-molecular-weight cytoplasmic RNA in HeLa cells. Arch Biochem Biophys 203: 764-773.

220. Sobell HM. 1985. Actinomycin and DNA transcription. Proc Natl Acad Sci USA 82: 5328-5331.

221. Egyhazi E. 1975. Inhibition of Balbiani ring RNA synthesis at the initiation level. Proc Natl Acad Sci USA 73: 947-950.

222. Sehgal PB and Tamm I. 1978. Halogenated benzimidazole ribosides. Novel inhibitors of RNA synthesis. Biochem Pharmacol 27: 2475-2485.

223. Zandomeni R, Bunick D, Ackerman S, Mittleman B, and Weinmann R. 1983. Mechanism of action of DRB. Effect on specific in vitro initiation of transcription. J Mol Biol 167: 561-574.

224. Mukherjee R and Molloy GR. 1987. 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole inhibits transcription of the β-hemoglobin gene in vivo at initiation. J Biol Chem 262 (28): 13697-13705.

225. Tweeten KA and Molloy GR. 1981. Induction of premature termination of transcription of the mouse β-globin gene by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). Nuc Acid Res 9: 3307-3319.

226. Tamm I, Kikuchi T, Darnell JE, and Salditt-Georgieff M. 1980. Short capped hnRNA precursor chains in HeLa cells: continued synthesis in the presence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Biochemistry 19: 2743-2748.

227. Laub O, Jakobovits EB, and Aloni Y. 1980. 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole enhances premature termination of late transcripion of simian virus 40 DNA. Proc Natl Acad Sci USA 77: 3297-3301.

228. Tamm I and Kikuchi T. 1979. Early termination of heterogeneous nuclear RNA transcripts in mammalian cells: accentuation by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Proc Natl Acad Sci USA 76: 5750-5754.

229. Seghal PB, Fraser NW, and Darnell JE. 1978. DRB-induced premature termination of late adenovirus transcription. Nature 272: 590-593.

230. Chodosh LA, Fire A, Samuels M, and Sharp PA. 1989. 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole inhibits transcription elongation by RNA polymerase II in vitro. J Biol Chem 264 (4): 2250-2257.

231. Zandonmeni R, Zandonmeni MC, Shugar D, and Weinmann R.1986. Casein kinase type II is involved in the inhibition by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole of specific RNA polymerase II transcription. J Biol Chem 261 (7): 3414-3419.

232. Chalbos D, Galtier F, Emiliani S, and Rochefort H. 1991. The anti-progestin RU486 stabilizes the progestin-induced fatty acid synthetase mRNA but does not stimulate its transcription. J Biol Chem 266 (13): 8220-8224.

233. Pascall JC and Brown KD. 1988. Structural analysis of the 5'-flanking sequence of the mouse epidermal growth factor gene. J Mol Endocr: 1: 5-11.

234. Breathnach B, Chambon P. 1981. Organization and expression of eukaryotic split genes coding for proteins. Annu Rev Biochem 50: 349-383.

235. Heard J-M, Herbomel P, Ott M-O, Mouttura-Rollier A, Weiss M, and Yaniv M. 1987. Determinants of rat albumin promoter tissue specificity analysed by an improved transient expression system. Mole Cell Biol 7 (7): 2425-2434.

236. Gorman CM and Howard BH. 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. Nuc Acid Res 11 (21): 7631-7648.

237. Pfarr DS, Rieser LA, Woychik RP, Rottman FM, Rosenberg M, and Reef ME. 1986. Differential effects of polyadenylation regions on gene expression in mammalian cells. DNA 5 (2): 115-122.

238. Swartwout SG, Preisler H, Guan W, and Kinniburgh AJ. 1987. Relatively stable population of c-myc RNA that lacks long poly (A). Mole Cell Biol 7 (6): 2052-2058.

239. Martins VR, Pratt WB, Terracio L, Hirst MA, Ringold GM, and Housley PR. 1991. Demonstration by confocal microscopy that unliganded overexpressed glucocorticoid receptors are distributed in a nonrandom manner throughout all planes of the nucleus. Mol Endocrinol 5: 217-225.

240. Bates SE, Davidson NE, Valverius EM, Freter CE, Dickson RB, Tam JP, Kudlow JE, Lippman ME, and Salomom DS. 1988. Expression of transforming growth factor alpha and its messenger ribonucleic acid in human breast ccancer: its regulation by estrogen and its possible functional significance. Mol Endocrinol 2: 543-555.

241. Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS, and Berger WH. 1987. Synthesis of messenger RNAs for transforming growth factors alpha and beta and the epidermal growth factor receptor by human tumors. Cancer Res 47: 707-712.

242. Murphy LJ, Sutherland RL, Stead B, Murphy LC, and Lazarus L. 1986. Progestin regulation of epidermal growth factor receptor in human mammary carcinoma cells. Cancer Res 46: 728-734.

243. Murphy LC, Murphy LJ, and Shiu RPC. 1988. Progestin regulation of EGF receptor mRNA accumulation in T-47D human breast cancer cells. Biochem Biophys Res Commun 150: 192-196.

244. Imai Y, Leung CKH, Friesen HG, and Shiu RPC. 1982. Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long term tissue culture. Cancer Res 42: 4394-4398.

245. Osborne CK, Hamilton B, Titus G, and Livingston RB. 1980. Epidermal growth factor stimulation of human breast cancer cells in culture. Cancer Res 40: 2361-2366.

246. Murphy LC, and Dotzlaw H. 1989. Endogenous growth factor expression in T-47D human breast cancer cells, associated with reduced sensitivity to the antiproliferative effects of progestins and antiestrogens. Cancer Res 49: 599-604.

247. Sarup JC, Rao KVS, and Fox CF. 1988. Decreased progesterone binding and attentuated progesterone action in cultured human breast carcinoma cells treated with epidermal growth factor. Cancer Res 48: 5071-5078.

248. Koga M, Musgrove EA, and Sutherland RL. 1989. Modulation of the growth-inhibitory effects of progestins and the antiestrogen hydroxyclomiphene on human breast cancer cells by epidermal growth factor. Cancer Res 49: 112-116.

249. Koga M and Sutherland RL. 1987. Epidermal growth factor partially reverses the inhibitory effects of antiestrogens on T-47D human breast cancer cell growth. Biochem Biophys Res Commun 146: 739-745.

250. Lipson KE and Baserga R. 1989. Transcriptional activity of the human thymidine kinase gene determined by a method using the polymerase chain reaction and an intron-specific probe. Proc Natl Acad Sci USA 86: 9774-9777.

251. Subramaniam M, Schmidt LJ, Crutchfield III CE, and Getz MJ. 1989. Negative regulation of serum-responsive enhancer elements. Nature 340: 64-66.

252. Novak U, Cocks BG, and Hamilton JA. 1991. A labile repressor acts through the NFκB-like binding sites of the human urokinase gene. Nuc Acid Res 19 (12): 3389-3393.

253. Cox GS, Cosgrove DE, Sullivan TT, and Haas MJ. 1990. Induction by cycloheximide of the glycoprotein α-subunit gene in human tumor cell lines and identification of a possible negative regulatory factor. J Biol Chem 265 (22) 13190-13197.

254. Azizkhan JC, Vaughn J, Christy RJ, and Hamlin JH. 1986. Nucleotide sequence and nuclease hypersensitivity of the Chinese hamster dihydrofolate reductase gene promoter region. Biochem 25: 6228-6236.

255. Valerio D, Duyvesteyn GC, Dekker BMM, Weeda G, Berkvens TM, VanderVoorn L, van Ormondt H, and van der Eb AJ. 1985. Adenosine deaminase: characterization and expression of a gene with a remarkable promoter. EMBO J 4: 437-443.

256. Blasband AJ, Rogers KT, Chen X, Azizkhan JC, and Lee DC. 1990. Characterization of the rat transforming growth factor a gene and identification of promoter sequences. Mol Cell Biol 9: 4994-5002.

257. Ishii S, Xu Y-H, Stratton RT, Roe BA, Merlino GT, and Pastan I. 1985. Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. Proc Natl Acad Sci USA 82:4920-4924.

258. Hahn S, Buratowski S, Sharp PA, Guarente L. 1989. Yeast TATA-binding protein TFIID binds to TATA elements with both consensus and nonconsensus DNA sequences. Proc Natl Acad Sci USA 86: 5718-5722.

259. Svaren J and Chalkley R. 1990. The structure and assembly of active chromatin. TIG 6 (2): 52-56.

260. Evans BA and Richards RI. 1985. Genes for the α and β subunits of mouse nerve growth factor are contiguous. EMBO J 4: 133-138.

261. Selby MJ, Edwards R, Sharp F and Rutter WJ. 1987. Mouse nerve growth factor gene: structure and expression. Mol Cell Biol 7: 3057-3064.

262. Vignon F, Bardon F, Chalbos D, and Rocherfort H. 1983. Antiestrogenic effect of R5020, a synthetic progestin in human breast cancer cells in culture. J Clin Endocrinol Metab 56: 1124-1130.

263. Horwitz KB, Mockus MB, and Lessey BA. 1982. Variant T47D human breast cancer cells with high progesterone-receptor levels despite estrogen and antiestrogen resistance. Cell 28: 633-642.

264. Weih F, Stewart AF, Boshart M, Nitsch D, and Schutz G. 1990. In vivo monitoring of a cAMP-stimulated DNA-binding activity. Genes Dev 4: 1437-1449.

265. Jantzen H-M, Strahle U, Gloss B, Stewart F, Schmid W, Boshart M, Miksicek R, and Schutz G. 1987. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell 49: 29-38.

266. Higgs DR, Wood WG, Jarman AP, Sharpe J, Lida J, Pretorius I-M, and Ayyub H. 1990. A major positive regulatory region located far upstream of the human α–globin gene locus. Genes Dev 4: 1588-1601.

267. Rosenthal N, Berglund EB, Wentworth BM, Donoghue M, Winter B, Bober E, Braun T, and Arnold H-H. 1990. A highly conserved enhancer downstream of the human MLC1/3 locus is a target for multiple myogenic determination factors. Nuc Acid Res 18 (21): 6239-6246.

268. Purucker M, Bodine D, Lin H, McDonagh K, and Nienhuis AW. 1990. Structure and function of the enhancer 3' to the human $^{A}\gamma$ globin gene. Nuc Acid Res 18 (24): 7407-7415.

269. Slater EP, Rabenau O, Karin M, Baxter JD, and Beato M. 1985. Glucocorticoid receptor binding and activation of a heterologous promoter by dexamethasone by the first intron of the human growth hormone gene. Mol Cell Biol 5 (11): 2984-2992.

270. Sap J, de Magistris L, Stunnenberg H, and Vennstrom B. 1990. A major thyroid hormone response element in the third intron of the rat growth hormone gene. EMBO J 9 (3): 887-896.

271. Zaret K and Yamamoto KR. 1984. Reversible and persistent changes in chromatin structure accompany activation of a glucocorticoid-dependent enhancer element. Cell 38: 29-38.

272. Burch JBE abd Weintraub H. 1983. Temporal order of chromatin structural changes associated with activation of the major chicken vitellogenin gene. Cell 33: 65-76.

273. Li Y, Li D, Osborn K, and Hounson LF. 1991. The 5'-flanking region of the mouse thymidylate synthase gene is necessary but not sufficient for normal regulation in growth-stimulated cells. Mol Cell Biol 11 (2): 1023-1029.

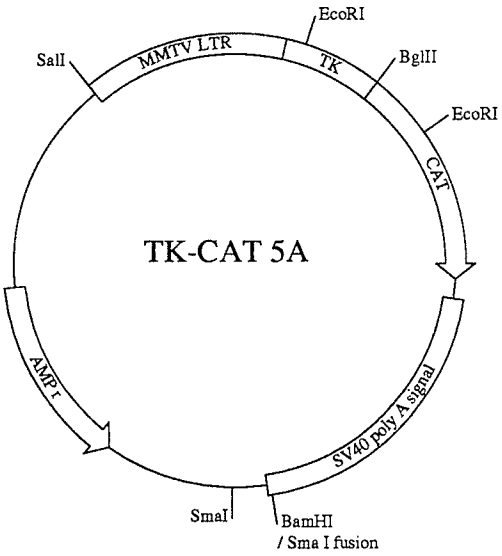
APPENDIX

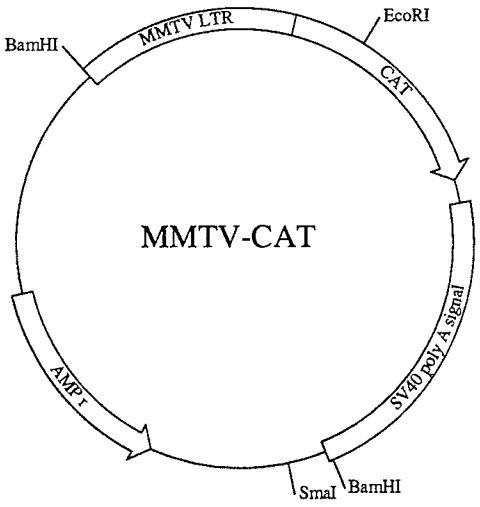
Nucleic Acids:

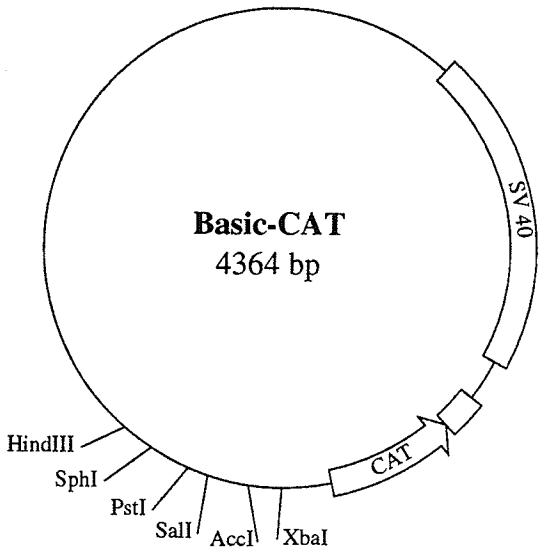
A	Adenine	
C	Cytosine	
G	Guanine	
T	Thymine	
U	Uracil	
R	puRine	(A or G)
Y	pYrimidine	(C or T/U)
K	Keto	(G or T/U)
M	aMino	(A or C)
S	Strong	(C or G)
W	Weak	(A or T)
B	not A	(C or G or T/U)
D	not C	(A or G or T/U)
H	not G	(A or C or T/U)
V	not T/U	(A or C or G)
N	aNy	(A or C or G or T/U)

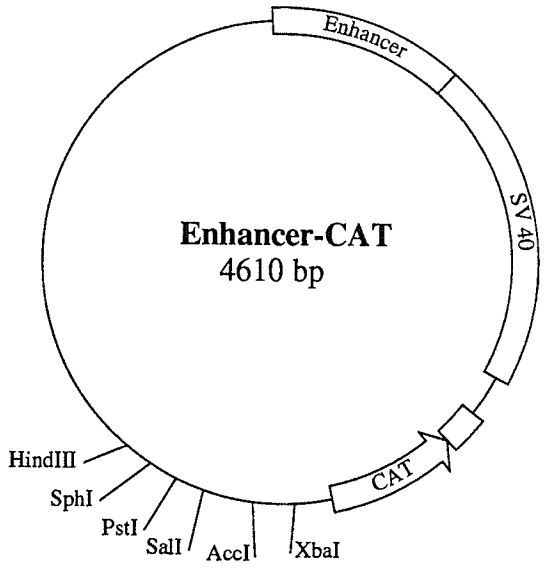
Amino Acids:

A	Ala	Alanine
B	Asx	Asparagine or aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
X	Xxx	Any amino acid, unknown
Y	Tyr	Tyrosine
Z	Glx	Glutamine or glutamic acid


EcoRI
SalI
MMTV LTR
TK
BglII
EcoRI
CAT
TK-CAT 5A
AMP r
SV40 poly A signal
SmaI
BamHI
/ Sma I fusion


BamHI
MMTV LTR
EcoRI
CAT
MMTV-CAT
AMP r
SV40 poly A signal
SmaI
BamHI


Basic-CAT
4364 bp
SV 40
CAT
HindIII
SphI
PstI
SalI
AccI
XbaI


Enhancer-CAT
4610 bp
Enhancer
SV 40
CAT
HindIII
SphI
PstI
SalI
AccI
XbaI