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STUDIES ON THE MECHANISM OF 1,25-DIHYDROXYVITAMIN D_3 ACTION ON KERATINOCYTES AS THEY PROGRESS FROM THE NORMAL TO THE MALIGNANT PHENOTYPE

by

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A thesis submitted to the faculty of Graduate Studies and Research, McGill University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

1,25-dihydroxyvitaminD$_3$ (1,25(OH)$_2$D$_3$) is anti-proliferative and pro-differentiative in a variety of cell types, including human keratinocytes. Exposure of cultured normal human keratinocytes to 1,25(OH)$_2$D$_3$ markedly reduces [^{3}H] thymidine incorporation and cell number and arrests these cells in the G1/G0 phase of the cell cycle. Calcium and 1,25(OH)$_2$D$_3$ act in concert to modulate the expression of two important cell-cycle associated genes, c-fos and p53, and of markers of keratinocyte differentiation. In contrast, inhibition of cell growth and of the cell cycle associated oncogene, c-*myc*, in the malignant keratinocyte cell line, HPK1A*ras*, requires 10 to 100 fold higher concentrations of 1,25(OH)$_2$D$_3$ than do the immortal non-malignant human keratinocyte cell line, HPK1A. Cell cycle analysis also reveals that 10-100 fold higher concentrations of 1,25(OH)$_2$D$_3$ are required to induce cell cycle arrest in HPK1A*ras* cells as compared to HPK1A cells. Analysis of the vitamin D receptor (VDR) from these two cell lines reveals identical sizes, numbers, and ligand binding characteristics. Furthermore, the sequence of the DNA binding domain of this receptor is unchanged in the vitamin D resistant HPK1A*ras* cells. Gel mobility shift assays using extracts from both cell lines reveals that the complexes formed by HPK1A*ras* nuclear extracts in the presence of a vitamin D DNA response element (VDRE) probe contain VDR but not its dimerization partner, the retinoic acid X receptor (RXR). In contrast, HPK1A nuclear extracts form complexes that contain both VDR and RXR. Overexpressing wild type RXRα in HPK1Aras cells results in VDRE-binding complexes containing VDR/RXR heterodimers. However, the sequence of the RXRα is unchanged in HPK1A*ras* cells and its expression in both cell lines is the same. Western blot analysis of RXRα from *ras* transformed keratinocytes suggests that its post-translational modification is different when compared to the RXRα from control cells. Consequently, these results suggest that VDR/RXR heterodimer disruption occurs

in *ras*-transformed keratinocyte and may at least in part explain the mechanism(s) by which *ras* transformed keratinocytes escape growth inhibitory influences.

RESUME

La 1,25-dihydroxyvitamine D_3 ($1,25(OH)_2D_3$) inhibe la prolifération et stimule la différenciation d'une variété de cellules, incluant les kératinocytes humains. Le traitement des kératinocytes par la $1,25(OH)_2D_3$ entraîne un arrêt des cellules en phase G1/G0 du cycle cellulaire, provoque une diminution de l'incorporation de thymidine tritiée et diminue le nombre de cellules. Le calcium et la $1,25(OH)_2D_3$ agissent ensemble pour moduler l'expression de deux gènes associés au cycle cellulaire, le c-fos et le p53, ainsi que de nombreux marqueurs de la différenciation cellulaire. Dans la lignée cellulaire maligne, HPK1A*ras*, l'inhibition de la croissance cellulaire ainsi que l'expression de l'oncogène associé au cycle cellulaire, c-*myc*, nécessitent des concentrations de $1,25(OH)_2D_3$ 10 à 100 fois plus élevées que dans la lignée cellulaire de kératinocyte humain immortalisée et non-tumorale, HPK1A. L'analyse du cycle cellulaire révèle aussi que les concentrations de $1,25(OH)_2D_3$ nécessaires pour causer un arrêt du cycle cellulaire sont de 10 à 100 plus élevées dans les cellules HPK1A*ras* que dans les cellules HPK1A. Malgré cette résistance, les cellules HPK1A*ras* ont le même nombre de récepteurs à la vitamine D (VDR) que les cellules HPK1A. L'analyse de ces récepteurs révèle qu'ils ont la même taille et les mêmes caractéristiques de fixation du ligand. De plus, la séquence du domaine de fixation de l'ADN du VDR demeure inchangée dans la lignée cellulaire résistante à la vitamine D, HPK1A*ras*. Dans les extraits nucléaires HPK1A*ras*, les complexes protéine-ADN formés en présence de l'oligonucléotide codant pour un élément de réponse de la vitamine D (VDRE), comprennent le VDR mais pas son partenaire de dimérisation, le récepteur X de l'acide rétinoïque (RXR). Les extraits nucléaires de cellules HPK1A forment des complexes qui, au contraire, contiennent le VDR et le RXR. Les extraits nucléaires de cellules HPK1A*ras* transfectées avec un plasmide permettant la sur-expression du RXRα forment des complexes avec le VDRE constitués d'hétérodimers

VDR/RXR. La séquence du domaine carboxy-terminal du RXRα des cellules HPK1A*ras* demeure inchangée. De plus le niveau d'expression du RXRα est identique dans les deux lignées cellulaires. L'analyse par "Western blot" suggère que les modifications post-traductionnelles du RXRα dans les cellules HPK1A*ras* sont différentes de celles des cellules-contrôles. Ces résultats démontrent l'absence d'un hétérodimer VDR/RXR lié à l'ADN dans les cellules HPK1A*ras*. Ceci pourrait expliquer le mécanisme par lequel les kératinocytes transformés par le *ras* échappent au contrôle de la croissance cellulaire.

FOREWORD

The following excerpt is taken from the Guidelines Concerning Thesis Preparation, Faculty of Graduate Studies and Research, McGill University, and applies to this thesis:

Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory**. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**

PREFACE

The work described in chapters 2-4 of this thesis has been puslished in the following journals:

Chapter 2: Sebag, M., Gulliver, W., and Kremer, R., 1994. J. Invest. Dermatol. 103:323-329

Chapter 3: Sebag, M., Henderson, J., Rhim, J., and Kremer, R., 1992. J. Biol. Chem. 267:12162-12167

Chapter 4: Sebag, M., Rhim, J., and Kremer, R., 1996. J. Clin. Invest., submitted

The work presented in chapters 2-4 is my own, except for the contributions of my coworkers and co-authors as follows: in chapter 2, Janet Henderson provided figure 1b,c and Richard Kremer provided figure 4. In chapter 3, Wayne Gulliver provided figure 1. In chapters 2 and 4, Johng Rhim provided the HPK1A and HPK1A*ras* cell lines.

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I dedicate this work to my family for all the love, support, and encouragement they bestowed upon me over the years.

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LIST OF ABBREVIATIONS

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
Ab	antibody
BPE	bovine pituitary extracts
CAT	chloramphenicol acetyl transferase
cDNA	cloned deoxyribonucleic acid
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
GF	growth factors
GTP	guanosine triphosphate
HPV	human papilloma virus
kb	kilobase
KBM	keratinocyte basal medium
Kd	dissociation constant
kDa	kilodalton
KGM	keratinocyte growth medium
MAPK	membrane associated protein kinase
MEM	minimal essential medium
mRNA	messenger ribonucleic acid
OC	osteocalcin
OP	osteopontin
PAGE	polyacrylamide gel electrophoresis

PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
RAR	retinoic acid receptor
RT	reverse trascriptase
RXR	retinoic acid X receptor
SDS	sodium dodecyl sulphate
TR	thyroid hormone receptor
TRE	thyroid hormone responsive element
VDR	vitamin D receptor
VDRE	vitamin D-responsive element

CHAPTER 1

INTRODUCTION: CONTROL OF GROWTH AND DIFFERENTIATION OF KERATINOCYTES AS THEY PROGRESS FROM THE NORMAL TO THE MALIGNANT PHENOTYPE

1.1 General Introduction

The epidermis consists of stratified squamous keratinized epithelial cells which forms the skin. Five layers of keratin-producing cells called keratinocytes make up the epidermis. The basal layer of skin is the only layer that is able to replicate. As keratinocytes move up towards the surface layers of the skin they not only lose the ability to replicate, but also begin a process of terminal differentiation. This process can take 2 to 4 weeks, and results in enucleated, metabolically inactive keratinocytes that are toughened by the presence of cornified keratinized envelopes. Calcium and 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active metabolite of vitamin D, are major players in the induction and modulation of these processes. $1,25(OH)_2D_3$, which normally has a role in calcium homeostasis, specifically influences the cell cycle to arrest keratinocytes within the G1/G0 phase. The mechanisms by which this is achieved involve the transcriptional modulation of cell cycle controlling proteins as well as of cellular oncogenes that play a role in cell growth and will be addressed in this review.

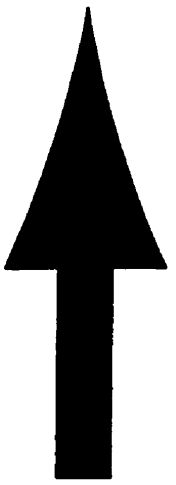
Carcinogenesis is a multistep process involving the co-ordination of a multitude of factors and cellular processes. As keratinocytes go from the normal, to the established and onto the fully malignant phenotype, their morphology, biochemistry and their response to external controls change drastically. Various factors that can contribute to this process will also be discussed in part of this introduction.

1.2 Epidermal Differentiation

The epidermis consists mainly of a stratified squamous keratinized epithelium and forms the protective covering of the skin. From the dermis outward the epidermis

consists of five layers of keratin-producing cells called keratinocytes. These five layers include: the stratum basale, the stratum spinosum, the stratum granulosum, the stratum lucidum and the

Fig 1.1 Epidermal Differentiation



Stratum Corneum
(terminally differentiated)

Stratum Basale
(undifferentiated)

Ca^{++}
$1,25(OH)_2D_3$

G0/G1

$1,25(OH)_2D_3$

S

Fig 1.1 **Epidermal Differentiation**

stratum corneum. Only the cells of the basal layer have the ability to synthesize DNA and therefore replicate. As keratinocytes terminally differentiate they travel upwards towards the skin surface, a process which can take approximately 2 to 4 weeks. En route they undergo a number or biochemical and morphological changes which result in layers of mitotically inactive, enucleated, flattened cells which are continuously sloughed from the surface as they are being replaced with basal cells differentiating outward (Ross, 1995).

The stratum basale consists of a single layer of columnar or cuboidal cells resting on the junction separating the dermis from the epidermis. The keratinocytes found here are mitotically active, and are responsible for the renewal of skin which occurs every 10-30 days. This layer of cells adheres to the basement membrane through calcium activated adhesion structures, called hemidesmosomes which contain anchoring proteins such as the α6ß4 integrins (Jones and Green, 1991). Basal keratinocytes attach to their neighbours via calcium-activated membranous plaques, called desmosomes (Jones and Green, 1991). Within basal cells, hemidesmosomes and desmosomes are attached to a cytoskeletal network of keratin filaments. All cells in the stratum basale contain filaments about 10nm in diameter which make up their cytoskeleton. Filaments are principally heterodimeric composed of one member of the acidic and one of the basic subfamily of keratins. In the basal cell this pair is formed by the type II keratin 58kDa K5 and the type I 50kDa K14 (K5/K14) (Roop et al., 1987). Keratins 8 and 18 (K8/K18) can also be found the basal keratinocytes of simple non-stratified epithelia, albeit at a much lower concentration than K5 and K14 (Leigh et al., 1993). Together these keratins constitute approximately 20% of a basal cell's total protein. As basal keratinocytes differentiate they slow down their production of K5 and K14 and up-regulate the expression of a different set of keratins (Lynch, 1994).

In the second layer of skin, the stratum spinosum, keratinocytes appear slightly

flattened and the filaments found in basal cells are now organized in thicker bundles, called tonofibrils. These bundles converge and end at small cellular extensions producing the spiny projections which are characteristic of this layer of skin. The keratins found in the lower layer are replaced by the 67 kDa K1 and the 56.5kDa K10 (K1/K10) as well as the 65 kDa K2. K1 and K10 filaments bundle together to form tonofibrils (Fuchs, 1993). Although mitotically inactive, spinous keratinocytes are metabolically very active. They devote much of their cellular processes to synthesizing these differentiation specific keratins. Eventually keratins account for more than 85% of the total protein of a suprabasal keratinocyte. The increase in keratin production results in a tougher more resistant keratinocyte, able to withstand the sheering forces of abrasion. The functional purpose of the preferential expression of certain keratins over others during differentiation has not yet been elucidated.

The stratum granulosum is characterized by three to five layers of flattened polygonal cells whose tonofibrils are gathered as large 'cable' like structures. A histidine-rich, basic protein, called filaggrin is expressed and may be responsible for the organization of these tonofibril cables (Harding, and Scott, 1983). Stratum granulosum keratinocytes are filled with coarse basophilic granules called keratohyalin granules as well as membrane-coated ones called lamellar granules. The lamellar granules fuse with the cell membrane and release their contents into the intercellular spaces. The substances released are lipid rich and act as 'water-proof cement' preventing penetration by foreign materials. Keratohyalin granules contain phosphorylated histidine-, glutamine and lysine-rich proteins which are deposited on the inner surface of the plasma membrane (Rice and Green, 1979). These envelope proteins include involucrin and loricrin (Simon, and Green, 1984). Keratinocyte transglutaminase, activated by differentiation, catalyzes the formation of ε-(γ–glutamyl) lysine isopeptide bonds which cross link envelope proteins into a

structure containing keratin fibres (Simon and Green, 1985). The final differentiation step is heralded by the intracellular release of lytic enzymes which end all metabolic activities. Keratinocytes at the top-most layer of skin, the stratum corneum, are organized into squames, sealed together by lipids and toughened by their cornified envelope. These cells are continuously shed at the surface of the stratum corneum (Ross, 1995).

1.2.1 Control of Keratinocyte Differentiation

A number of factors have been identified which promote the differentiation of keratinocytes (see figure 1.1). Some factors are produced endogenously and act in an autocrine or paracrine fashion, while others are exogenous. Factors which promote the differentiation of cells are often also responsible for anti-mitogenic effects. While this is certainly true for many factors, there do exist factors which only exert one or the other effects on keratinocytes. In any case, the differentiating effects of certain factors can be at least mechanistically uncoupled from their mitogenic effects. Four factors which promote the differentiation of keratinocytes will be discussed, these include: Ca^{++}, 1,25 dihydroxyvitamin D_3, the keratinocyte growth factors and the retinoids. The mitogenic or anti-mitogenic potentials of these factors will be discussed later on.

(a) Calcium

Calcium has been shown to play an important role in keratinocyte differentiation both *in vivo* and *in vitro*. Keratinocytes cultured in medium containing low concentration of Ca^{++}, $\leq$0.15nM, do not differentiate and do not form desmosomes. When the calcium concentration is increased to greater than 0.15nM, desmosomes are formed and the cells

begin to stratify. After several days in culture in the presence of high calcium concentrations, keratinocyte transglutaminase is produced and cornified envelopes are seen (Hennings et al., 1980). The importance of calcium is emphasized by the finding that certain calcium ionophores have been shown to quicken epidermal differentiation *in vivo* (Jaken and Yuspa, 1988)

Calcium can act to control the transcription of certain differentiation markers including K1 and K10 (Yuspa et al, 1991) and loricrin (Hohl et al., 1991). Transcription of transforming growth factor ß2 (TGF-ß2) is also upregulated after an increase in extracellular calcium. TGF-ß is thought to act rapidly in preventing the proliferation of keratinocytes and in regulating certain differentiation events (Glick, 1990). Raising extracellular calcium concentrations to 1.2mM also stimulates the transcription of involucrin and keratinocyte transglutaminase (Sebag et al., 1994; Gibson et al, 1996). Much of calcium's influences on differentiation, however, are thought to involve non-genomic mechanisms. That is, calcium can act specifically on changing the cellular architecture towards a more differentiated state. Desmosomal proteins are synthesized in both high and low calcium conditions, however only the higher calcium concentrations enable their assembly (Duden and Franke, 1988). As well, while calcium does not have an effect on the transcription of the fillaggrin gene, it does have an effect on the post-translational processing of that protein (Presland et al., 1992). Calcium's effects are therefore diverse and its mechanism of action only partly understood.

An increase in extracellular calcium (Cao) above 0.15mM has been shown to cause a rapid increase in the keratinocyte's intracellular calcium (Cai). This increase in Cai is apparent after Cao increases by as little as 0.02mM, demonstrating the keratinocyte's extreme sensitivity to external calcium concentrations. The extracellular calcium concentration range at which the keratinocytes are optimally sensitive (0.1mM-0.2mM) is

the same range that can trigger the differentiation cascade (Kruszewski et al, 1991, Yuspa et al, 1989). Lanthanum has also been shown to block the increase in Cai following an increase in Cao and thereby prevent the triggering of differentiation (Pillai and Bikle, 1992). These findings all point to the possibility that a rise in intracellular calcium following a rise in extracellular calcium is the key to calcium induced keratinocyte differentiation.

The means by which keratinocytes sense extracellular calcium concentrations has recently been elucidated. It was noted that an acute rise in Cai followed by a sustained increase in Cai always followed an increase in Cao. This phenomenon was thought to be similar to the calcium response seen in parathyroid cells, in which a calcium sensitive receptor (CaR) has been identified and cloned (Brown et al, 1993). An almost identical calcium receptor has been identified and cloned from normal human keratinocytes (Bikle et al, 1996). Once this receptor senses an increase in Cao, it can activate the phospholipase C (PLC) pathway leading to an increase in inositol triphosphate (IP3) levels as well as activate voltage independent nonspecific cation channels permeable to calcium. These events both lead to an increase in Cai, either through the release of Ca^{++} through intracellular stores or through an increase in transmembrane calcium flux.

(b) 1,25 dihydroxyvitamin D_3

The skin is a source of the precursor for a family of vitamin D metabolites whose best understood, but not necessarily most important, role is in the regulation of bone and mineral homeostasis. The first step in the synthesis of vitamin D, is the cleavage of the B ring of 7-dehydrocholesterol (Holick, 1981). This step occurs in the skin with ultraviolet irradiation being the catalyst. Vitamin D_3 is then released into the body where it is first

metabolized in the liver into 25-OHD_3. Final activation occurs in a number of tissues where a hydroxylation at the 1α position produces the most active metabolite, 1,25$(OH)_2D_3$ metabolite. Through its intracellular receptor vitamin D is thought to have both genomic and non-genomic effects (Barsony and Marx, 1990; Nemere et al., 1993). The slower, genomic, cyclohexamide sensitive effects of vitamin D are by far the most abudant and best characterized.

In addition to producing the precursor, vitamin D, keratinocytes make 1,25$(OH)_2D_3$ and contain 1,25$(OH)_2D_3$ receptors. Keratinocytes respond to 1,25$(OH)_2D_3$ by changes in proliferation and differentiation. 1,25$(OH)_2D_3$ has been shown to upregulate levels of transglutaminase and involucrin in a dose- and time-dependent manner (Smith et al., 1986, Su et al., 1994; Sebag et al, 1993). Cultured normal keratinocytes respond to doses of 1,25$(OH)_2D_3$ as little as 10^{-10}M, a concentration that is physiologically relevant.

(c) Growth Factors

In order to enable differentiation keratinocytes must first halt their progression through the cell cycle. The means by which this is achieved will be discussed later on. The most important and potent growth arrestor of keratinocytes is TGF-ß. The growth arrest induced by TGF-ß has been demonstrated to be completely reversible upon removal of this protein. As mentioned, keratinocytes can produce their own TGF-ß and do so when they have differentiated past the basal state (Pelton 1989). It has been shown that at high concentrations TGF-ß can inhibit the transcription of certain markers of differentiation such as K1, K10 and fillaggrin (Choi and Fuchs, 1990). It also induces the expression of other keratins that are normally associated with wound healing or the hyperproliferation of

suprabasal keratinocytes (Mannbridge and Knapp, 1987, Stoler et al., 1988). The reversible nature of TGF-ß's growth arrest coupled to its promotion of abnormal differentiation, almost de-differentiation, suggests that it is a withdrawal of the TGF-ß signal that can act as a signal to trigger differentiation.

(d) Retinoids

The intracellular receptors for retinoic acids have been identified in skin (Elder et al., 1992). *In vitro* the addition of 10^{-6}M all-trans retinoic acid to keratinocyte cultures results in the suppression of K1 and K10 expression (Kopan and Fuchs, 1989), as well as the suppression of both fillaggrin expression (Fleckman et al., 1985) and cornified envelope formation (Yuspa and Harris, 1974). It would therefore seem that retinoic acids inhibit the differentiation of keratinocytes. The *in vitro* findings sharply contrast a recent *in vivo* experiment in which action of the intracellular receptor for retinoic acid, the RAR, was disrupted. To study the effects of retinoic acid on skin development, the group of Saitou et al (Saitou et al., 1995) chose to target the expression of a dominant-negative retinoic acid receptor instead of simply disrupting, or 'knocking out' the gene in every tissue. Under the control of a keratin 14 promoter, their dominant negative RAR was expressed exclusively in the basal layers of mouse epidermis. They noted that not only was the expression of K10 inhibited in targeted mice, but that the expression of the basal cell keratin K5 was enhanced. The switch over from expression of K5/K14 to K1/K10 as cells normally differentiate outwards, did not occur where the dominant negative RAR was present. RA is therefore absolutely required for normal skin differentiation. It would seem as if retinoic acid could both inhibit as well as enhance the differentiation of keratinocytes. This dichotomy is consistent with the effects observed during treatment of

certain skin disorders with retinoids (Saitou et al., 1995).

1.3 Control of Keratinocyte Proliferation

Normal keratinocyte proliferation is influenced by a number of factors, both endogenous and exogenous. For instance, keratinocytes produce TGF-ß and 1,25 dihydroxyvitamin D_3 which both prevent these cells from progressing through the cell cycle. Positive regulators of keratinocyte proliferation include EGF and the induction of certain cellular proto-oncogenes which have powerful roles in promoting a progression through the cell cycle. Before attempting to understand the effects of these factors, one must first have a comprehension of the mechanisms by which the cell regulates its growth.

Most growing mammalian cells take from 12-20 hours to double in number, although some can take even longer (Darnell et al, 1990). As cells replicate they go through distinct periods of activity known as cell cycle phases. In the S phase the cell synthesizes new strands of DNA as well as histones and other proteins that make up chromosomes. At the end of the S phase, the cell possesses two identical sets of chromosomes. The S phase is followed by a gap period or interphase called the G2 phase. During this phase, no DNA replication occurs, but the cell does continue its cellular growth and synthesis of proteins and other molecules needed for cell division. The cell divides (mitosis) during the M phase of the cell cycle, evenly distributing its duplicated genetic material to its two daughter cells. The period in which cells prepare for another round of DNA synthesis and which follows the M phase is called the G1 phase. Most mammalian cells spend roughly the same amount of time in the S, G2 and M phases of the cell cycle, roughly 7h, 3h and 1h, respectively. The variability in cell division time among cells is largely attributable to the G1 phase, which can last anywhere from 2h to 3h

to several days (Darnell et al., 1990). Cultured cells which have either depleted their nutritional resources or which have become non-replicating spend their time in a subset of G1 called G0. In this phase, unlike G1, the cells are not preparing to synthesize DNA.

1.3.1 The Cyclins and Cell Cycle Progression

The rate at which cells proliferate largely depends on events that occur during the G1/G0 phases of the cell cycle. Therefore much work has been done elucidating the mechanisms controlling G1 to S progression and how cells combine these mechanisms with mitogenic and growth-suppressive signals. Cells respond to stimuli until they reach a restriction point in late G1 (the START checkpoint), following which they can choose to divide if all the necessary factors are in place to support this option.

(a) Cyclin D

The major players in G1 progression include three D-type cyclins, D1, D2 and D3, which associate with their cyclin dependent kinases, cdk4 and cdk6 and cyclin E which combines with cdk2 (Sherr, 1993, Sherr 1994). The importance of the D and E type cyclins was demonstrated by transfecting cells with inducible transcription vectors containing these cyclins (Resnitzky et al, 1994). When overexpressed, the cyclins cause the cell to progress through the cell cycle more rapidly and to divide in absence of external mitogenic stimuli. The D type cyclins are produced as long as growth factor stimulation is present, their production begins at the start of G1 and their peak concentrations are achieved at the G1 to S boundary. When the mitogenic stimulus is withdrawn, the D type cyclins are no longer synthesized and their presence diminishes thanks to PEST sequences

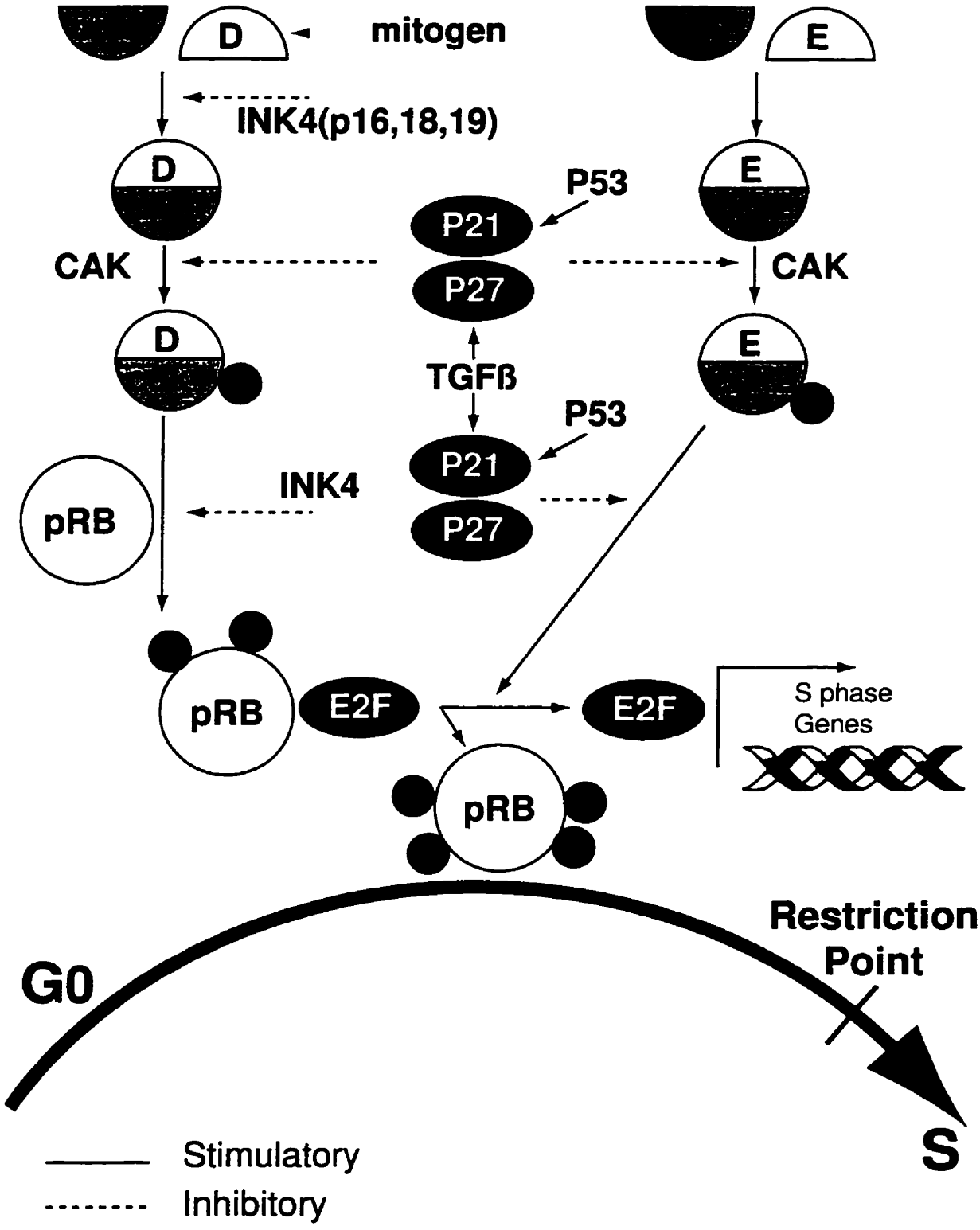


Figure 1.1 **Positive and negative regulators of G1 phase progression.**

which targets them for rapid degradation (Salama et al., 1994). Proof that despite its constitutive presence cyclin D1 is only needed in the middle of G1, came from experiments using microinjected antibodies targeted against this cyclin. Injected at mid G1 these antibodies prevented the progression of growing cells to the S phase. When injected later on at the boundary between G1 and S, the antibodies did not halt the cell cycle (Baldin et al., 1993). The E type cyclin, however, is made periodically, only when it is needed. It is synthesized and used during the latter part of G1 and then it is degraded in the S phase by the same mechanism that destroys the D type cyclins (Salama et al., 1994).

The cyclins assemble with cdk4 and cdk6 only when a mitogenic stimulus is present (Matsushime et al. 1994). Once cyclin D is bound to cdk4 or 6 this complex must first be activated by one single threonine phosphorylation. A cdk-activating Kinase (CAK) is responsible for the threonine phosphorylation of the cyclin bound cdk's (Matsuoka et al. 1994). CAK is composed of a cyclin H bound to a kinase, cdk7, and is constitutively active, it does not respond to mitogen stimulus (Kato et al., 1994b). Once activated the phosphorylated cyclin D complexes are free to phosphorylate their target, the retinoblastoma gene product, pRB (Dowdy et al., 1993; Ewen et al., 1993). Cyclin D has been shown to bind directly to pRB. Once bound, cdk4 or cdk6 can then phosphorylate pRB. In its hypophosphorylated state, pRB normally binds to and inhibits the function of transcriptional factors such as E2F. E2F is normally required to initiate the synthesis of proteins that are necessary for DNA synthesis and therefore S phase entry (reviewed in Hinds and Weinberg, 1994). Phosphorylation of pRB disables its binding affinity for E2F, thereby releasing it and enabling S phase entry (see figure 1.1).

(b) Cyclin E

In contrast to cyclin D, cyclin E's role in G1 progression is a little more nebulous. Like cyclin D, it binds to a cdk, cdk2, to form a holoenzyme that is also activated by CAK phosphorylation (Koff et al, 1992; Dulic et al., 1992). Cyclin E might also associate with and activate another kinase, cdk3, a structural homologue of cdk2 that plays a part in G1 exit (Harper et al. 1995). In cells that lack functional pRB, cyclin E appears to be necessary for entry into S phase rather than cyclin D (Ohtsubo et al. 1995). However, unlike cyclin D, cyclin E does not induce pRB phosphorylation (Resnitzky and Reed 1995). Activated cyclin E complexes may enable cyclin D to phosphorylate pRB or it may phosphorylate other unknown substrates that are key to DNA replication and S phase entry (Hatakeyama et al., 1994)

Fig 1.2 Positive and Negative Regulators of G1 Phase Progression

1.3.2 The cyclin inhibitors

Positive regulation of the cell cycle through cyclins is only one part of mechanism that controls cell division. Negative regulation plays an important role in keeping activators from constitutively stimulating cell cycle progression. Many proteins have been identified that co-immunoprecipitate with cyclin complexes when these are in an inactive state. Once the inhibitors 'release' their targets, these latter are free to perform their duties as S phase initiators. Several negative regulators, or cdk inhibitors, will be discussed.

(a) p21

P21, also known as Cip1 (cdk interacting protein 1), is a 164 amino acid long, 21kDa protein that was found in complexes that also contain a cyclin, a cdk and the proliferating cell nuclear antigen (PCNA) (Xiong et al., 1992). The PCNA is a subunit and activator of DNA polymerase-δ, one of the enzyme complexes responsible for DNA duplication. PCNA is also required for nucleotide excision repair of DNA (reviewed in Ma et al., 1995). *In vitro*, p21 has been shown to directly inhibit PCNA dependent DNA replication, without involving the cyclins (Waga et al. 1994). *In vivo*, p21's effects are a little more complicated, and probably do involve the cyclins. Overexpression of p21 in cells results in the marked reduction of cdk activity and the inability of these cells to leave the G1 phase of the cell cycle (Harper et al., 1993; Harper et al., 1995; El-Deiry et al., 1993). P21 appears to be a universal inhibitor of cdk's as it binds to and downregulates the activities of active complexes formed by cyclin D-cdk4, cyclin E-cdk2 as well as cyclin A-cdk2, a complex which may be responsible for S phase progression (Li et al., 1994). It

appears that only the first 71 amino acids are required for p21's cdk and inhibition of DNA synthesis (Nakanishi et al., 1995). When only one p21 molecule has been found associated with cyclin-cdk complexes, these complexes were demonstrated to be active. Multiple p21's must bind to cyclin-cdk complexes in order to inactivate them (Zhang et al., 1994). CAK has been shown to be incapable of phosphorylating cdk when cyclin-cdk complexes are bound to several p21 molecules (Zhang et al., 1994). In addition to preventing CAK phosphorylation, multiple p21 binding also inhibits active/phosphorylated cyclin-cdk from associating with pRB. Because of its actions, p21 can be termed an outright growth suppressor.

It is not yet fully understood how p21 activity is controlled. It is known that p21 transcription is directly upregulated by the tumor suppressor/growth suppressor p53 (El-Deiry et al., 1994). P21 expression is also stimulated by a number of other growth suppressors, such as TPA (a phorbol ester) (Zhang et al., 1995a), 1,25 dihydroxyvitamin D_3 (Jiang et al., 1994) and retinoic acid (Zhang et al., 1995a) as well as certain mitogens such as serum, the epidermal growth factor (EGF), the platelet derived growth factor (PDGF) and the fibroblast growth factor (FGF) (Michieli, 1994). If the stimulation of p21 production by growth inhibitors such as vitamin D seems logical, the same stimulation by mitogens like EGF would appear paradoxical. It has been proposed that the cell can determine its proliferative fate by varying the ratio of p21 to cyclin-cdk (Sherr and Roberts, 1995). By producing more active cyclin-cdk complexes, the cell increases the likelihood that fewer p21 molecules will associate with these complexes, thereby allowing them to react with their targets.

(b) p16

P16, is another protein that has a role in inhibiting cyclin-cdk mediated cell cycle progression. This 16kDa protein is part of a family of proteins called the INK4 and was discovered, much like p21, to associate with a cyclin dependent kinase, namely cdk4 (Serrano et al., 1993). It was cloned using a two-hybrid protein-protein interaction/detection system using cdk4 as bait (Serrano et al., 1993), and was found to be 148 amino acids in length composed of repeated ankyrin domains. P16 binds exclusively to cdk4 and cdk6, it does not recognize the cyclins or other cdks. Once bound to cdk4, for instance, it inhibits that kinase from associating with its cyclin, and renders the cdk inactive (Parry et al., 1995). Because of its inhibitory function and its specificity for cdk4 this protein is also sometimes called INK4a. P16 is a good candidate to be a growth suppressor as experiments designed to overexpress p16 in target cells result in G1 arrest (Guan et al., 1994; Serrano et al., 1995).

The importance of p16 in the normal cell is underscored by its role as a tumor suppressor. The p16 gene has been mapped to the small arm of chromosome 9, specifically 9p21 (Cairns et al., 1994). Several groups have reported that either p16 mutations or p16 deletions were evident in several cancer cell lines deriving from brain, breast, bone, hematological cells, skin, ovary, lung and bone (Nobori et al., 1994). Finally, a form of familial melanoma has been linked to a missense mutation in the gene encoding p16, the same mutation is also sometimes found in sporadic cases of the same cancer (Ranade et al., 1995).

To date, p16's only known function is to bind and inhibit cdk4 and 6. The downstream effect of an inhibited cdk4, is a hyperphosphorylated pRB which remains associated with E2F, thereby preventing S phase entry. Unlike p21, this is the only way in which p16 is known to function. One would therefore predict that in absence of pRB, p16 expression or overexpression would be ineffective in cell-cycle arrest. Indeed,

experiments using pRB deficient fibroblasts show that even if p16 is overexpressed the cells progress through the cell cycle without pausing at G1 (Lukas et al., 1995). Furthermore, in cells immortalized with the adenovirus E1A gene, p16 expression is inneffective (Serrano et al., 1995). It has been shown that the E1A gene product binds pRB and inactivates it (Egan et al., 1989). By contrast, p16 is able to suppress the growth of cell lines that have been established by means other than those involving the inactivation of pRB (Serrano et al., 1995).

Other proteins have been identified that bear significant similarities with p16. They all bind to cdk4 or 6 and have been designated as p15 (INK4b), p18 (INK4c) and p19 (INK4d) (Hannon and Beach, 1994; Hirai et al., 1995; Chan et al., 1995). The mechanism of action of these proteins seems to be very similar to that of p16. In fact even the p18 growth suppressive abilities were shown to be dependent on the presence of pRB (Guan et al., 1994). In addition, p15 was shown to be induced in keratinocytes treated with TGFß (Hannon and Beach, 1994). Quite by accident, a link between nuclear receptors and the cyclin inhibitors was discovered during the cloning of p19. Interested in elucidating a function for the orphan nuclear receptor NGFI-B, Chan and colleagues used a two hybrid system to fish out proteins that could associate with this receptor (Chan et al., 1995). To their surprise, p19, INK4d was isolated. The implications of this link are still under investigation.

(c) p27

P27 or KIP1, is yet another cdk inhibitor, cloned by virtue of its association with the cyclin kinases (Toyoshima and Hunter, 1994; Slingerland et al., 1994). Its N-terminus bears an almost 50% homology with p21's, and was initially identified because of its

ability to bind in affinity chromatography assays to cyclinE-cdk2 matrices (Polyak et al., 1994a). P27 seems to bind effectively to cyclinE-cdk2 as well as to cyclinD-cdk4 (Hengst et al., 1994; Toyoshima and Hunter, 1994) (see figure 1.1). It has been proposed that it functions by sequestering cyclin-cdk complexes as they are formed until a certain threshold is reached (Sherr, 1994). Although sequestration of cyclin-cdk complexes implies that p27 growth inhibtion is pRB dependent, a pRB independent mechanism has also been ascribed to p27. In cells that are pRB defficient, p27 was still able to induce growth arrest (Toyoshima and Hunter, 1984).

Unlike p21, p27 mRNA levels are not upregulated by the presence of mitogens, and do not vary much during the different phases of the cell cycle (Polyak et al., 1994b). While p21 levels were seen to be increased by mitogenic stimuli (Michieli, 1994), p27 levels were actually observed to decrease with mitogen (Polyak et al., 1994b). In contrast, p27 mRNA levels are stimulated in keratinocytes that are growth arrested by TGFß (Reynisdottir et al., 1995). These facts support the possibility that with mitogen, cycling cells have to overcome the inhibitory threshold set up by p21, while in resting cells, p27 is the main cyclin-cdk binding inhibitor.

1.3.3 c-myc

Regulators of the cell cyle do not act in isolation, they are responsive to external as well as internal demands. The cellular proto-oncogene c-*myc* appears to play an important role as an intermediate between extracellular signals and cell cycle changes (Kato and Dang, 1992). In this section I will attempt to elucidate a link between c-*myc* and the cell cycle machinery itself.

C-*myc* was discovered as the cellular homologue of avian retrovirus v-*myc* genes

(Vennstrom et al., 1982). Since then a large number of *myc*-related genes and proteins have been identified viruses as well as humans and other species (Ingvarsson et al., 1988). Initially identified in viruses as an oncogene, *myc* has been found in normal, rapidly dividing cells suggesting a role for this proto-oncogene in cell proliferation. It has been shown that c-*myc* expression is higher in proliferating cells than in terminally differentiated cells (Lachman and Skoultchi, 1984). In fact it has been suggested that *myc* is one of the key players in the control of cell proliferation (Meichle et al., 1992). Addition of growth factors to quiescent cells in culture, results in the immediate induction of c-*myc* mRNA (Almendral, et al., 1988), peaking at about 2 to 3 hours post induction (Müller et al., 1982).

C-*myc* encodes a 439-453 amino acid nuclear phosphoprotein depending on which translational start site is used (Hann et al., 1988). The structural elements of Myc were determined by sequence homology to elements found in other proteins. Two structural regions stand out in particular, the first, a leucine-zipper, is responsible for protein-protein interactions, and the second, a helix-loop-helix, participates in DNA recognition and binding. Because of these elements, Myc greatly resembles a transcriptional factor.

The leucine-zipper domain of Myc resembles similar domains found in various transcriptional factors (Lamb et. al., 1991). In general these domains are responsible for homo- and hetero-dimerization of these factors. Mutations of the leucine-zipper region of *myc* greatly inhibits its oncogenic potential and therefore its biological activity (Smith et al., 1990). *In vitro,* Myc has been shown to homo-dimerize, but only at very high protein concentrations (Dang et al., 1989). The concentrations observed *in vivo* do not support homodimerization (Dang et al., 1991). It is now believed that *myc* specifically dimerizes with a smaller protein known as Max (Prendergrast et al., 1991).

Max is a phosphoprotein which shares significant sequence and structural

homology with Myc. Although much smaller than Myc, it nevertheless contains both a helix-loop-helix domain and a leucine zipper domain (Blackwood et al., 1991). The Myc-Max dimerization occurs through the leucine-zipper domain of both proteins.

All loop-helix-loop containing proteins appear to bind to a common consensus sequence, (Kerkhoff and Blister, 1991). It has been shown that Myc can specifically bind to this consensus sequence but that optimal binding occurs with a perfect 12 nucleotide long palindrome: GACCACGTGGTC (Halazonetis and Kandil, 1991). Max appears to have the same DNA binding affinities (Kato et al., 1992). Gel mobility shift assays using antibodies specific for Myc or Max have demonstrated that DNA binding to this palindrome required the dimerization of Myc and Max (Kato et al., 1992). Control of DNA binding is achieved through sequence specificity and post-translational modification. Undimerized Max, when phosphorylated by casein kinase II, has a lower affinity for DNA, whereas already formed Myc/Max dimers are unaffected by casein kinase II (Berberich and Cole, 1992).

In addition to a DNA binding domain, a putative transcription factor must also have domain(s) that interact with the transcriptional machinery. In experiments which fused the DNA binding domain of the yeast GAL4 protein to various Myc segments, three distinct regions were localized which can activate transcription (Kato et al., 1990). The transcriptional properties of Myc appear to be also regulated by phosphorylation (Seth et al., 1991). Unlike DNA binding, phosphorylation at serine 62 enhances transcriptional activation, whereas deliberate mutational substitutions of this site result in the inability to transactivate (Seth et al., 1991). Phosphorylation of the transactivation domain of Myc is controlled by a serine/threonine kinase known as the mitogen activated protein-kinase, or MAPK. Significant are the facts that MAPK is activated by mitogenic stimuli and that MAPK activation could in turn modulate the transcriptional function of Myc. Hence, the

cell possesses two ways to upregulate *myc* when it is presented with a mitogen. The first involves the rapid induction of *myc* mRNA, and the second the activation of Myc through MAPK phosphorylation.

In addition to early upregulation following mitogen stimulation, the importance of *myc* in cell proliferation is underscored by the effects of its overexpression in certain cells. Cell lines that constitutively express Myc can proliferate in the absence of any external stimulus (Armelin et al., 1984). Further evidence of *myc* participation in cell cycle progression came with pivotal experiments in which either antisense *myc* sequences or anti *myc*-protein antibodies were introduced into cells (Sklar et al., 1991; Iguchi-Ariga et al., 1987, respectively). Induction of the antisense *myc* had the same result as injection of antibodies, principally, Myc protein depletion. Once depleted of Myc, cells could no longer leave the G1 phase of the cell cycle. These results merely indicate that *myc* could have a role as an intermediate between external stimulus and the cell cycle machinery. This role has proved elusive until recently.

A direct link between the Myc protein and the expression of G1 cell cycle proteins has been established. Disruption of *myc* by the targeted homologous recombination of one allele results in a two fold reduction in Myc expression (Hanson et al., 1994). This reduction in the normal levels of *myc* protein resulted in longer cell cycle progression times and a therefore slower cellular growth rate. Specifically, the G0 to S phase transition was significantly longer (Hanson et al., 1994). It was noted that when cells express less Myc the induction of cyclin E, cyclin A and Rb phosphorylation were delayed in response to mitogenic stimulus. Cyclin D levels were not affected by the continuous underexpression of c-*myc*. When Myc is constitutively and continuously overexpressed in fibroblasts, the expression of cyclin D is chronically depressed (Philipp et al., 1994). It was determined in the latter study that Myc protein directly represses the cyclin D1 promoter by binding to

specific core promoter elements and displacing stronger transactivators that are normally present in that region. Myc is not normally constitutively expressed, in fact *myc* protein levels rise sharply hours after mitogen stimulation and then fall back down following prompt degradation. To take into account the temporal expression of Myc, an inducible Myc system was used (Daksis et al., 1994). The mitogen-free induction of Myc in this system, resulted in a rapid 5 fold induction of endogenous cyclin D1 mRNA. These results indicate that the short term effect of Myc induction is cyclin D stimulation, while protracted Myc levels lead to a decrease in cyclin D1 synthesis. One can speculate that chronic stimulation of cyclin D1 expression by c-*myc* leads to a desensitization of that pathway and that the resultant decrease in cyclin D could be a normal response of the cell to slow down its *myc* induced growth. Finally, Myc may have yet another tie to the cell cycle machinery. Overexpression of Myc was found to stimulate the induction of a heat-labile protein that binds to and inhibits p21 (Hermeking et al., 1995). Inhibition of the normally growth supressive p21 seems a plausible mechanism for *myc* growth induction.

1.3.4 p53

In contrast to c-*myc* and its ability to increase cell growth when expressed, p53 is notable for suppressing cellular division. P53 was initially discovered as a host protein that bound to SV40 T-antigen (Lane and Crawford, 1979). It was quickly realized that the form of p53 overexpressed in cancer cells was a mutated one (Hinds et al., 1989). The wild type form of p53 expressed in normal cells was capable of actually restraining the cancerous phenotype (Baker et al., 1990). Since these properties of p53 were discovered, a flury of activity ensued attempting to link either loss or mutation of p53 to human neoplasms. To that end, it was determined that patients with certain hereditable

susceptibilities to developing cancer, such as the Li-Fraumeni syndrome, did have p53 mutations. Transgenic mice in which the p53 gene has been knocked out by homologous recombination, demonstrate a distinctly higher rate of developing tumours (Donehower et al., 1992). Furthermore, fibroblasts harvested from these mice exhibit much higher proliferation rates *in vitro* (Harvey et al., 1993). In the normal cell, p53 is induced by DNA strand breaks (Ewen et al., 1993). Following DNA damage, the cell must stop its growth and repair the damage. If this damage is irreparable, the cell must die. It is believed that in the normal cell p53 acts to induce G1 arrest or programmed cell death (apoptosis) following DNA damage.

P53 is a nuclear phosphoprotein with DNA binding and transcriptional activating properties (Fields and Jang, 1990). P53 has essentially three functional domains: an amino terminal transactivation domain, a mid-region, DNA binding domain and a carboxy terminal tetramerization domain (Arrowsmith and Morin, 1996). Four molecules of p53 are required in order to efficiently and specifically bind to DNA sites found in the promoter region of target genes (El-Deiry et al., 1992). The tetrameric p53 binds to four repeats of a consensus DNA site consisting of 5'-PuPuPuC(A/T)-3', arranged as two consecutive inverted repeats (El-Deiry et al., 1992). Once bound to DNA, p53 activates the transcription of downstream genes through its ability to interact with two subunits of the transcription factor TFIID complex, TAFII40 and TAFII60 (Thut et al., 1995). A number of genes have been identified as definitive targets of p53 induced activation including, p21, GADD45 and mdm2 (Oliner et al., 1992).

The most important part p53 plays in the control of the cycle is its role in the induction of p21 production. Levels of p21 increase as a direct consequence of p53 induction following ionizing radiation provoked DNA damage (El-Deiry et al., 1993). p21 induction leads to the inhibition of PCNA dependent DNA replication as well as to the

repression of cyclin-cdk inactivation of pRB (see above). P53 also transactivates the expression of a PCNA associated protein GADD45 (Smith et al., 1994). It has been shown that GADD45 is implicated in DNA repair and in the inhibition of DNA synthesis (Smith et al., 1994). Finally, p53 also induces the mouse double-minute 2 gene (mdm-2) (Perry et al., 1993, Barak et al., 1993)). mdm-2 has been shown to specifically bind to and inhibit p53 function (Chen et al., 1994). The inhibition of p53 by a gene that it itself induces would therefore represent negative feed back loop control of p53 function.

1.4 Keratinocyte Carcinogenesis

Carcinogenesis is the progression from a normal cell to a quasi-abnormal, hyperplastic variant (immortalized cell or benign tumor),and finally to a frankly neoplastic, invasive cancer. This process occurs in skin relatively frequently. In fact, almost one third of all cancers in North America are said to be non-melanoma skin cancers (Leigh et al., 1993). Of these skin cancers, most are basal cell carcinomas and the remainder are squamous cell carcinomas. The factors that contribute to causing non-melanoma skin cancers include exposure to ultraviolet light (sunburns), chemical carcinogens, long outdoor/wind exposure, cigarette smoke and human papilloma virus (HPV) infections (Leigh et al., 1993).

In the classical mouse skin carcinogenesis model, a chemical carcinogen is applied to the skin of animals leading to the formation of benign growths (papillomas) (Boutwell, 1974). This initiating event often leads to the induction of an activating point mutation in the mouse cellular Harvey-*ras* (Ha-*ras*) proto-oncogene (Cheng et al., 1988; Brown et al., 1993b). Analyses of human squamous cell carcinomas have revealed a high number of mutations of *ras*, as well as of growth suppressors such as p53 (Pelisson et al., 1992;

Daya-Grosjean et al., 1993).

1.4.1 Keratinocyte Differentiation During Carcinogenesis

Suprabasal keratinocytes are enucleated and unable to replicate. It is the basal keratinocytes that are therefore susceptible to undertake a neoplastic transformation. As basal keratinocytes become cancerous, their differentiative processes are invariably perturbed. It has been proposed that pre-cancerous cells grow faster than normal cells once they become resistant to inducers of differentiation (Rheinwald and Beckett, 1980; Wille et al., 1982). As a result one may conclude that increased proliferation is tied to the disruption of differentiation. However it must be noted that the induction of abnormal differentiation is insufficient to be tumorigenic (Fusenig et al., 1995)

Normal skin is characterized by a small actively proliferating basal layer, and larger more differentiated but mitotically inert upper layers. Undifferentiated, basal keratinocytes express exclusively the K5/K14 pair of keratins (see above). As they differentiate, K5/K14 synthesis decreases and is replaced by the K1/K10 pair (Roop et al., 1987). *In vivo*, benign tumours are distinguished from normal tissue by having a much larger number of proliferating keratinocytes. As such, the expression of the K5/K14 pair extends beyond the single basal layer of cells and into the suprabasal compartment (Roop et al., 1988). The total amount of suprabasal expression of differentiation associated K1/K10 pair is markedly reduced (Roop et al., 1988). Keratins that are normally found in simple, nonstratified epithelia can ectopically be expressed in skin tumours. These ectopically produced keratins include K8, K18 and K19 (Bowden et al., 1987).

Other markers of differentiation can be absent or aberrent in neoplastic keratinocytes. The formation of a cornified envelope and production of one of its major

components, involucrin, are delayed. Malignant keratinocytes consistently exhibit a reluctance to terminally differentiate (Rheinwald and Beckett, 1980). Squamous cell carcinomas also express substantially lower amounts of involucrin than do their normal counterparts (Levitt et al., 1990).

1.4.2 Human Papillomavirus

Infections by the human papilloma virus (HPV) have been associated with cancers of the cervix, genitals and epidermis. HPV alone is insufficient to cause frank malignancy. The presence of additional factors have always been implicated in the genesis of cancerous lesions. It is estimated that HPV infections cause benign papillomas (warts) in 90% immunosuppressed renal transplant patients. Of these many are 'atypical' or dysplastic when present in sun-exposed sites (Leigh and Glover, 1995).

There are over 60 different human papilloma viruses associated with a variety of surface lesions (Deviliers, 1989). Among these only a small number, including HPV16,18,31,33,35, have been found to be associated with cancer (Lorincz et al., 1987). These HPV types are invariably found in the vast majority of anogenital squamous cell dysplasias. These cancerous viruses can be used to immortalize cultured human keratinocytes (Durst et al., 1989). Keratinocytes immortalized with oncogenic HPVs, not only grow indefinately in culture, but are also defective in their onset and execution of terminal differentiation (Woodworth et al., 1992)

In cervical cancers, the HPV E6 and E7 genes are selectively retained and expressed. The importance of these genes for HPV immortalization is underscored by the finding that transfection of E6 and E7 alone is sufficient to induce cell immortality (Pirisi et al., 1987; Woodworth et al., 1990).

E7 proteins encoded by HPV16 and 18, as well as the adenovirus E1A, and the SV40 large T antigen all share pRB (retinoblastoma gene product) binding domains (Pietenpol et al, 1990). E7 binding to pRB presumably blocks its growth suppressive abilities by preventing it from associating with E2F thereby allowing the synthesis of genes necessary for the S phase of the cell cycle. Keratinocytes are capable of suppressing their own growth through the secretion of TGF-ß. However, even this growth suppressive measure is abrogated by the presence of HPV E7, as TGF-ß activity is ultimately dependent on a functional pRB downstream (Moses, 1992). Recently, it has been demonstrated that E7 proteins can dimerize *in vitro* and that this dimer posses intrinsic transcriptional activating ability (Clemens et al., 1995). The targets of this transactivation remain unknown.

The mechanism of E6 function is somewhat different from that of E7. E6 is able to bind to p53 and cause its degradation via a ubiquitin-dependent protease system (Scheffner et al., 1990). The first 160 amino acids of p53 are necessary for ubiquitin conjugation and therefore degradation. Amino acids 160-345 of p53 were shown to be required for complex formation with E6 (Mansur, et al., 1995). E6 expression ultimately leads to a reduction in p21 and the loss of inhibition of DNA synthesis and S phase entry.

1.4.3 *ras* and carcinogenesis

The *ras* oncogene has long been implicated in carcinogenesis. Its role in the normal cell is in the molecular transduction of a mitogenic signal from the cell surface to nuclear effectors. Transgenic mice expressing activated *ras* oncogenes driven by keratinocyte-keratin (K10) specific promoters are highly susceptible to developing squamous cell carcinomas (Bailleul et al., 1990). Immortalized cultured keratinocytes can

be induced to express a more malignant phenotype when transfected with an activated *ras* oncogene (Durst et al., 1989). These same cells exhibit shorter cell doubling times than their non-*ras* transfected counterparts. Until recently it was not known if the faster proliferative rates observed in *ras* transformed cells were due to their malignant phenotype or to *ras* control of the cell cycle. Recent experiments have shown that *ras* and its downstream effectors modulate key players of cell cycle progression.

(a) *ras* signalling pathway

The pathway begins when a peptide growth factor (GF) binds to its cognate cell surface membrane spanning growth factor receptor (GFR). Hormone induces receptor activation and cross phosphorylation of individual subunits by endogenous protein tyrosine kinase activity of the receptor. Autophosphorylated tyrosine residues on the cytoplasmic tail of the receptor form specific, high affinity binding sites for the Src-homology 2 (SH2) domains of downstream signaling molecules. A prominent GFR-associated protein is Grb2. Grb2 acts as an adapter molecule, which binds the phosphorylated receptor via its SH2 domain (Schlessinger, 1993). In addition to its SH2 domain, Grb2 has two SH3 domains. SH3 domains bind to regions in proteins which are rich in proline and hydrophobic amino acids. In the *ras* pathway, Grb2 interacts with Sos, a mammalian protein which shares extensive homology with the Drosophila Son of Sevenless gene, via its SH3 domains. This SH3 binding event serves to translocate Sos from the cytoplasm to the plasma membrane, where it becomes noncovalently coupled to the activated receptor by the Grb2 (Schlessinger, 1993). *Ras* is membrane bound by virtue of its C-terminal farnesylation, and is a member of a family of small GDP-binding proteins which are activated when bound to GTP. Membrane-localized Sos activates GDP-

bound *ras* through its guanidine nucleotide exchange factor function. Exchange of GDP for GTP activates *ras* and allows for further downstream signaling events to occur. Following activation, *ras* stimulates several downstream targets, including the raf Ser/Thr-kinase. Activated raf then phosphorylates the MEK (also known as MAP kinase kinase or MAPKK) protein kinase on serine which results in its activation (Kyriakis et al., 1992). MEK is a serine/threonine kinase whose target is MAP kinase. Cells transfected with constitutively activated MEK have the same phenotype as cells that have been transfected with an activated *ras*, illustrating their central role in the *ras* signalling pathway (Mansour et al., 1994) The MAP kinases are a family of Ser/Thr kinases that phosphorylate many proteins associated with cell cycle progression, including several transcription factors such as c-fos and c-myc (for review see Campbell et al, 1995).

Ras has also been shown to associate with phosphatidylinositol 3-kinase (PI3K) (Rodriguez-Viciana et al., 1994). Specifically, GTP-bound *ras* can bind to the catalytic subunit of this enzyme, thereby activating it. PI3K contains SH2 and SH3 domains that are normally recognized and activated by GFRs. Activated PI3K is responsible for the phosphorylation of inositol diphosphate into the second messenger inositol triphosphate (IP3). IP3 appears to directly activate protein kinase C, an important player in mitogenic signalling (Nakanishi et al., 1993).

Termination of the *ras* signal involves hydrolysis of bound GTP to GDP, a reaction that *ras* can perform intrinsically, but which is catalyzed very slowly. The GTPase reaction can be greatly accelerated by association of *ras* with GTPase-activating protein (p120GAP) (Bollag et al, 1991). Structurally, GAP contains a C-terminal catalytic domain, two SH2 domains and an SH3 domain. Activation of most receptor tyrosine kinases leads to association with GAP via its SH2 domains. Additionally, proline rich regions in the N-terminal domain of GAP have been shown to function as an SH3 target domain for

several Src-kinase family members (Briggs et al, 1995). Recruitment of GAP is thought to be crucial for termination of the *ras* signal, although additional evidence implicates GAP as a downstream effector of *ras* in cytoskeletal signal transmission (McGlade et al, 1993).

(b) *ras* and the cell cycle

Several mechanisms have recently been proposed through which the *ras* signalling pathway can interact with the machinery controlling cell cycle progression. The inducible expression of an activated *ras* oncogene in mouse fibroblasts shortened the duration of the G1 phase as well as abolished the need for platelet-derived growth factor for proliferation (Winston et al., 1996). In human keratinocytes, transfection of Ha-*ras* results in a 70% decrease in TGFß mRNA expression (Chen et al., 1993) The induction of mutated *ras* expression also dramatically increases cyclin D1 expression (Winston et al., 1996; Filmus et al., 1994). Antisense cyclin D1 oligonucleotides abrogate the increase in the rate of cell proliferation normally observed following mutant-*ras* expression This illustrates a possible role for cyclins in *ras* mediated cell division (Filmus et al., 1994). A specific region of the cyclin D1 gene promoter was identified to be responsible for *ras* induction. If position -954 of the cyclin D1 promoter is mutated, *ras* inducibility is lost. EGF stimulation of cyclin D1 is dependent upon that same site. Furthermore, transfection of a dominant negative form of MAPK inhibits both the expected *ras* stimulation of cyclin D1 but also the stimulation caused by EGF (Albanese et al., 1995). Mitogenic stimulation of cyclin D leading to G1 progression, utilizes, at least in part, the *ras* signalling pathway.

(c) cancer and the *ras*-MAPK pathway

The *ras*-MAPK pathway is activated by several external stimuli. The complexity and high degree of regulation required to keep the pathway functioning smoothly dictates that aberrations within the cascade may be responsible for the loss of cellular regulation in tumor progression. Mutations in *ras* have frequently been associated with the onset of a wide variety of tumors in humans as well as experimental animal models and cell lines. Mutations in *ras*-activators, such as GFRs, Grb2 and Sos have been associated with stimulating the *ras*/MAPK pathway and allowing hyperproliferation and dedifferentiation of cells. Finally, overexpression or constitutive activation of downstream effectors of *ras*, such as MAPK has also been associated with neoplastic transformation (Clark and Der, 1995).

Alterations in the structure and expression of members of the *ras* gene family have been implicated in the development of neoplasias in many systems. Several mutations within the coding regions of *ras* exons have been identified which impart a proliferative phenotype on cells harboring the mutation. In particular, mutations at codons 12, 13, 59, 69 and 117 have been identified as those associated with the aggressive growth and metastatic properties of squamous cell carcinomas (Kiaris et al., 1995; Spencer et al., 1995). Roughly 10-20% of all human tumors and approximately 30-40% of some colorectal cancers and leukemias contain mutant *ras* genes. This figure can be as high as 90% in the case of some pancreatic tumors. Other cancers, such as human breast carcinomas, have a very low incidence (less than 5%) of *ras* mutation. Instead, overexpression of normal p21ras, particularly c-Ha-*ras*, is observed in roughly 66% of human breast carcinomas, as determined by RNA analysis, immunoblotting and immunocytochemistry (Slamon et al, 1984; Hand et al, 1984). This therefore implicates *ras* transcriptional regulation in the carcinogenic process of some tumors.

The levels of c-Ha-*ras* mRNA and the resulting protein have been directly

correlated with the metastatic behaviour of some mouse mammary tumor subpopulations. The metastatic sublines 66c14 and 4T1 (Shekhar et al, 1995) express a 6-8 fold higher level of c-Ha-*ras* message and protein than nonmetastatic sublines, suggesting a crucial role for p21ras in dictating malignant phenotype.

In addition to gene regulation, mutations within *ras* exons have been reported to render *ras* insensitive to GAP-inhibition. Such oncogenic *ras* proteins are thus constitutively bound to GTP and keep the pathway in an activated state (Clark and Der, 1995).

Genetic analysis of c-Ha-*ras* in the most highly metastatic 4T1 subline indicated the presence of a single novel point mutation within intron 1 of the gene (Shekhar et al., 1995). The resulting A*rG mutation is located at the 3' end of a palindromic TGATCCATCA* sequence. It has been suggested that this mutation switches expression of Ha-*ras* to a constitutive mode by rendering the Ha-*ras* gene less responsive to steroid hormones. This hypothesis is indirectly supported by the presence of an extra band in malignant 4T1 cells as seen by gel-shift analysis. Alternatively, this mutation may induce binding of an as yet unidentified transcription factor, or alter the mRNA stability for c-Ha-*ras*.

In a study of 25 human renal cell carcinomas, close to 50% were found to have constitutively activated MAPK. As well, these same tumors were seen to have overexpressed MAPKK (MEK) levels as seen by Western blot analysis (Oka et al., 1995). Similar results are obtained in cultured fibroblasts that have been transfected with MAPKK constitutively active mutants. These fibroblasts were no longer dependent on growth factors to initiate cell division (Brunet et al., 1994). These findings constitute proof that abnormally high levels of MEK invariably lead to a constitutively active MAPK, leading to increased cell proliferation.

(d) *ras*-related proteins

Ras is a member of a large superfamily of *ras*-related proteins (Macara et al., 1996; Khosravi-Far et al., 1994), which share significant biochemical (GTP/GDP binding) and sequence identity (30-55%). It has therefore been suggested that other members of the family may be candidate oncogenes. Although mutations in the Rab, Rho and Ran families of *ras*-like proteins, involved in intracellular vescicular transport, cytoskeletal function and cell cycling, respectively, have been shown to induce transformation, the malignant phenotypes of these tumors are different from those associated with oncogenic *ras*. Recent studies however have identified two *ras*-related proteins with a significant degree of transforming potential similar to *ras* (Graham et al, 1994; Cox et al, 1994). TC21/R-Ras2 exhibits a 55% homology with *ras* proteins on the amino acid level. Furthermore, introduction of the mutations analogous to the classical activating mutations of *ras* will also activate TC21/R-Ras2 transforming potential. Cells overexpressing the normal TC21/R-Ras2 will also produce tumors when injected into athymic nude mice. It has been shown that both normal and oncogenic TC21/R-Ras2 are able to activate MAPK, as well as other downstream *ras* effector molecules. This represents the first *ras*-related protein with similar oncogenic potential. TC21/R-Ras2 has shown to be frequently overexpressed in breast cancer cell lines (Clark and Der, 1995). Furthermore, mutations within TC21/R-Ras2 have been reported to augment its transformational potential, suggesting a role for a mutant TC21 in tumor cell biology (Saez et al., 1994; Huang et al., 1995).

The second Ras-like oncogenic protein identified is R-*Ras*, which also has a 55% amino acid identity to *ras*, and a 70% identity to TC21/R-Ras2. Introduction of oncogenic mutations into R-*Ras* fail to induce the predicted transformation of NIH3T3 cells, as was

shown with both *ras* and TC21/R-Ras2. R-Ras-transfected cells however, are able to form colonies in soft agar, and initiate tumor growth when injected into mice, providing evidence for its tumorigenic capabilities in vivo (Clark et al, 1995). No mechanism of action for R-*Ras* has yet been proposed, and it is likely that R-*Ras* has a very distinct mode of action from both *ras* and TC21/R-Ras2. It has recently been shown that R-*Ras* can interact with the apoptosis-inhibiting protein bcl-2, suggesting a role for this protein in the regulation of apoptosis (Fernandez-Sarabia and Bishoff, 1993).

Another group of *ras*-related proteins, the Rho1 family, is responsive to activation of the epidermal growth factor receptor (EGFR), which is tightly coupled to the *ras*-MAPK signaling pathway. Cumulative data suggest a role for Rho1 proteins in the maintenance of cytoskeletal structure and in the interaction of membranes with the cytoskeleton (DeCremoux et al., 1994). In particular, it has been described that the rhoB form of Rho1 is an immediate early gene induced by EGF or PDGF in cultured rat fibroblasts. While rhoB is rapidly and transiently expressed in normal epithelia cells treated with EGF, it displays a non-uniform expression pattern in a variety of similarly treated cancer cell lines. These experiments demonstrate that a breakdown in the signal pathway may be occurring in certain types of tumors, and that the resulting constitutive expression of rhoB may play an important role in certain malignancies. Additionally, this study suggests that other Ras-related proteins which are coupled to the *Ras*/MAPK pathway may have oncogenic properties.

(e) the role of other signal transduction components

In addition to *ras* and ras-related proteins, several other member of the *ras*/MAPK pathway have been implicated as oncogenes. The erbB-2 (HER2/neu) gene product is a

185 kDa transmembrane protein which is highly homologous to the EGF receptor. Overexpression of erbB-2 has been shown to be tumorigenic in cultured fibroblast and breast epithelium. Clinically, overexpression of erbB-2 is seen in breast, gastric and prostrate cancers of epithelial origins.

Cancer cells which overexpress erbB-2, the receptor were found to have a ligand-independent constitutive phosphorylation. Western Blotting of Grb2 and Sos immunoprecipitates from these cells revealed that erbB-2 coprecipitates with these two proteins, indicating that an erbB-2/Grb2/Sos complex forms in vivo. Additionally, MAPK activity was shown to be markedly increased in these cell lines. These experiments indicate that GFRs which harbor mutations that render them hyperactive are strongly oncogenic, and act directly to overstimulate the ras-MAPK pathway (Jane et al., 1994).

Grb2 protein and mRNA overexpression is accompanied by a small gene amplification event which occurred at the Grb2 locus. Coprecipitation studies showed an increased level of complex formation between overexpressed Grb2 and Sos in vivo, suggesting that the *ras*/MAPK pathway is overstimulated in these cell lines and is providing a link between oncogenic Grb2 and tumorigenicity in some cancers. The level of activity of MAPK was not assayed in these studies, leaving open the possibility that Grb2/Sos may be acting through an alternate kinase (Daly et al., 1994).

1.5 1,25 dihydroxyvitamin D_3

It was originally believed that the active form of vitamin D_3 was exclusively made in the liver and the kidney generating the secosteroid $1,25(OH)_2D_3$ (Norman, 1979). Since then there has been an explosive growth in our understanding of this hormone. It is

now believed that $1,25(OH)_2D_3$ is produced both by the kidneys and locally by activated macrophages and keratinocytes to act as an endocrine, paracrine and an autocrine factor (Reichel et al., 1989). Its biological effects are wider and more varied than what was originally a role as an effector in the regulatory mechanism of calcium homeostasis. In the keratinocyte, $1,25(OH)_2D_3$ is an important modulator of both differentiation (see above) and proliferation. $1,25(OH)_2D_3$ exerts its biological effects through genomic and non-genomic mechanisms (Ozono et al., 1991; Nemere et al., 1984).

1.5.1 Metabolism of $1,25(OH)_2D_3$

(a) Synthesis

In 1932 it was determined that vitamin D_3 was not actually a nutritionally derived vitamin, but a steroid with one of of its cyclopentano-like rings broken, a seco-steroid. Vitamin D begins as 7-dehydrocholesterol in the epidermis when light (UV-irradiation), through sunlight exposure, will cause breakage of the B-ring to form previtamin D_3 (Norman, 1979). Previtamin D_3 leaves the skin and travels to the liver through the circulation bound to a vitamin D binding protein, transcalciferin (Bouillon et al., 1976). A liver microsomal enzyme, 25-hydroxylase, converts previtamin D_3 into 25-hydroxyvitamin D_3 (Bouillon et al., 1976). 25-hydroxyvitamin D_3 goes through one more hydroxylation step at the 1-alpha position before becoming the most potent vitamin D metabolite, $1\alpha,25(OH)_2D_3$. This hydroxylation was originally thought to occur exclusively in the mitochondria of renal proximal tubule cells by the cytochrome P-450 enzyme 1α-hydroxylase (Lawson et al., 1971). However, it is now believed that a number of other tissues and cells types, such as keratinocytes and activated macrophages,

are capable of accomplishing the final activation step of vitamin D (Reichel et al., 1989). The 1-alpha hydroxylation of 25 $(OH)D_3$ is stimulated by the presence of parathyroid hormone (PTH), but inhibited by both high concentration of $1,25(OH)_2D_3$ and plasma phosphate (Reichel et al., 1989). There also exist hundreds of chemically synthesized analogues of $1,25(OH)_2D_3$ and $25(OH)D_3$ which not only have varying degrees of biological potency, but can also preferentially stimulate one effect of vitaminD over all others (for exhaustive review see Bouillon et al., 1995)

(b) Metabolism

$1,25(OH)_2D_3$ is actively metabolized into a number of inactive side-chain shortened metabolites. $1,25(OH)_2D_3$ is converted by a 5-step pathway to the smaller, inactive, calcitroic acid (Makin et al., 1989). Vitamin D_3 metabolism is dependent on the cytocrome P450 24-hydroxylase enzyme which was recently cloned (Chen et al., 1993), and found to be upregulated by $1,25(OH)_2D_3$. The product of this specific hydroxylation is $1,24,25,(OH)_3D_3$. Other hydroxylation products include 24,25-; 25, 26-; $1,25,26-(OH)_xD_3$ (Chen et al., 1993a)

1.5.2. Biological Effects of $1,25(OH)_2D_3$

(a) Non-genomic effects of $1,25(OH)_2D_3$

The first function ascribed to $1,25(OH)_2D_3$ was that of a regulator of calcium homeostasis. In response to low plasma calcium concentrations and high PTH, the rate of $1,25(OH)_2D_3$ synthesis increases. $1,25(OH)_2D_3$ radically augments the rate of calcium

absorption from the intestine. $1,25(OH)_2D_3$ also causes increased plasma calcium concentrations by increasing bone turnover and, to a lesser extent, augmenting the reabsorption of filtered calcium in the kidney. In regulating the capacity of the intestine to absorb calcium, $1,25(OH)_2D_3$ uses a genomic as well as non-genomic mechanism of action.

A hormone is said to have a non-genomic effect if a physiological response can be observed within seconds or minutes following the addition of the hormone. Non-genomic mechanisms of action have already been ascribed to glucocorticoids, estrogen and progesterone, steroid hormones which normally function by modulating gene transcription (for review see Nemere et al., 1993). In the perfused duodenal loop of vitamin D deficient chicks, the addition of $1,25(OH)_2D_3$ causes a rapid increase in calcium transport from the lumen to the venous side of the duodenum (Yoshimoto and Norman, 1986). Certain analogs of $1,25(OH)_2D_3$ are better than $1,25(OH)_2D_3$ itself at inducing this calcium transport called transcalthachia. Many of these same analogs are unable to mediate the genomic effects of $1,25(OH)_2D_3$ (Norman et al., 1993). It was observed that transcaltachia was inducible only when $1,25(OH)_2D_3$ was presented to the basal-lateral membrane, but not to the lumenal surface (Nemer et al., 1994). This finding suggests the possible existence of a membrane bound $1,25(OH)_2D_3$ receptor that preferentially localizes to the only side of the cell that can come into contact with blood borne hormones. This receptor has not yet been identified nor cloned.

(b) Genomic Effects of $1,25(OH)_2D_3$

Most of the effects that are induced by $1,25(OH)_2D_3$ are mediated via a genomic pathway. $1,25(OH)_2D_3$ appears to have a role not only in calcium homeostasis, but also

cell growth, cell differentiation, immune-modulation. In epithelial cells of tissues such as skin, breast and prostate, $1,25(OH)_2D_3$ inhibits proliferation and promotes differentiation. The anti-proliferative capacities of $1,25(OH)_2D_3$ are thought to be linked to the inhibition of c-*myc* synthesis. The addition of $1,25(OH)_2D_3$ to mitogenically stimulated cultured human keratinocytes results in a net inhibition of c-*myc* mRNA production (Sebag et al., 1992). $1,25(OH)_2D_3$ has also been shown to modulate cell growth by inducing a key cell-cycle progression inhibitor, p21, in keratinocytes (Liu et al., 1996a; Hannon and Beach, 1994). TGFß stimulation is another mechanism by which $1,25(OH)_2D_3$ can inhibit cell growth (Danielpour, 1996; Koli and Keski-Oja, 1995).

1.5.3 Nuclear Receptor Superfamily

Lipophillic hormones are said to play a key role in cellular differentiation, development and growth (Mangelsdorf et al., 1995). These hormones function by easily and passively traversing the cell membrane and binding to intracellular nuclear receptors, examples of which are found throughout evolution. Ligand bound receptors bind to specific promoter elements found in the 5' region of target genes thereby modulating their transcription (Evans, 1988; Beato 1989). The promoter elements recognized by nuclear receptors are short segments of DNA often located within 1kb of the transcriptional start sites of target genes. These hormone responsive elements comprise precise sequences of DNA that are organized into specific patterns.

Nuclear receptors are part of a superfamily all sharing homologous regions that have distinct functions. Ten years following the isolation and cloning of the first nuclear receptors, estrogen and glucocorticoid, some 41 members of this family exist today (Mangelsdorf et al., 1995). These include receptors for steroids, vitamin D_3, farnesoids,

retinoids, eicosanoids and other lipophillic molecules. As well, certain members of this family exist in different isoforms, products of alternate mRNA splicing or alternate translational start sites. The ligands and functions of some of the newer members of this family have not even yet been discovered.

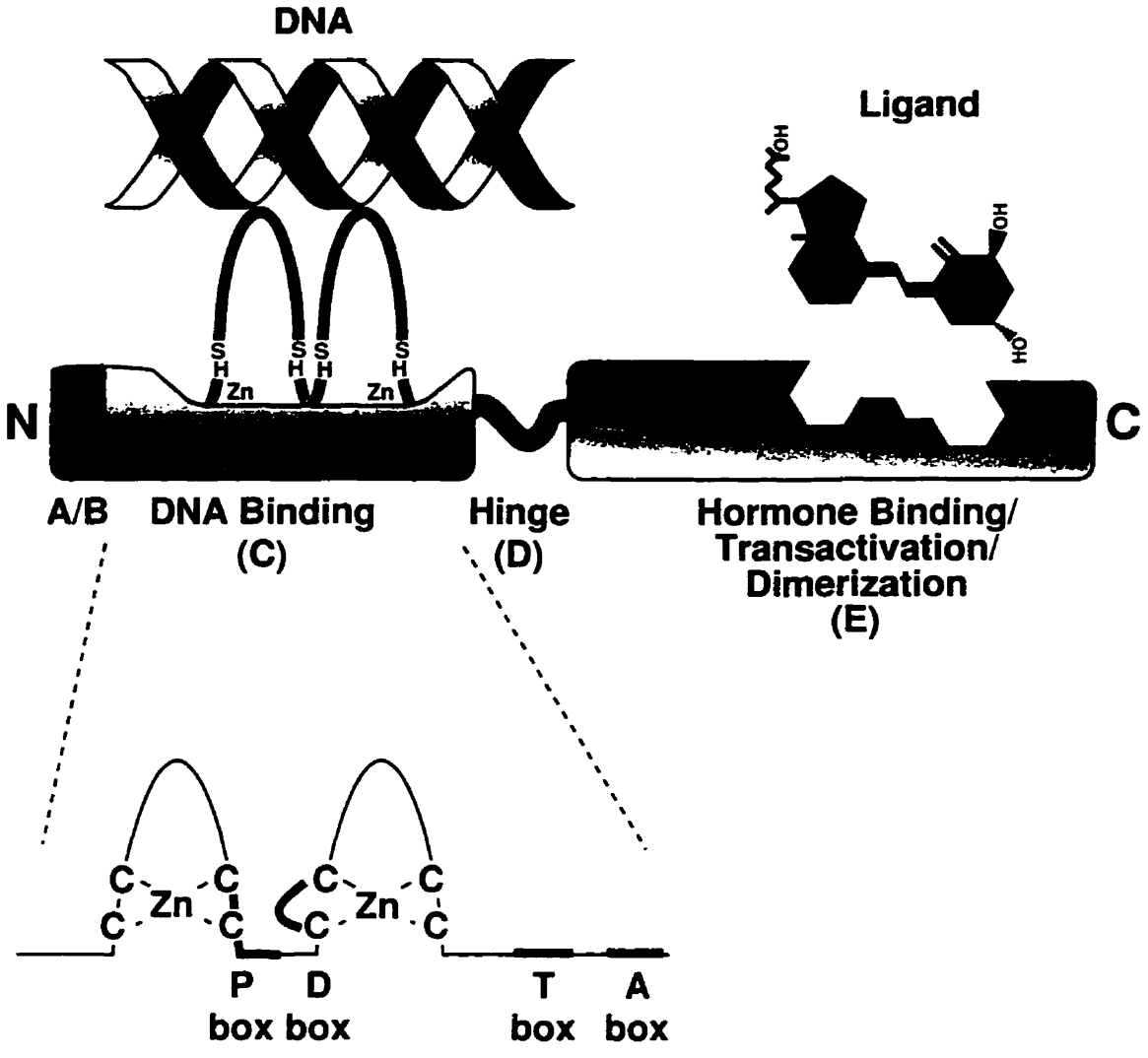


Figure 1.2 **Organization of nuclear receptors.**
Ligand is $1,25(OH)_2D_3$, shown here as an example.

Fig 1.3 Nuclear Receptor Organization

(a) Structure and Function of Nuclear Receptors

Nuclear receptors are organized as sequential segments of conserved regions (Green and Chambon, 1988, Mangelsdorf et al., 1995). The amino terminal, or A/B region, shows much variability among nuclear receptors. Although its function is not yet clear, it often harbours transactivation abilities through a region known as the AF-1 (Evans, 1988; Green and Chambon, 1988; Beato 1989). The C region is responsible for DNA recognition and binding and is the most highly conserved region among nuclear receptors. DNA binding occurs through a 66-69 amino acid long conserved region arranged into two zinc fingers, each consisting of 4 cysteines coordinated around one zinc molecule (Evans 1988; Green and Chambon, 1988; Umesono and Evans, 1989). Next to the C domain is the D or hinge region, notable for its variability between different nuclear receptors. The carboxy terminal domain is known as the E region and is responsible for ligand binding, dimerization and transactivation (Green and Chambon, 1988; Mangelsdorf, 1995). Although this region is largely conserved between nuclear factors, the variability observed is responsible for conferring ligand specificity. The E region also contains heptad repeats of hydrophobic amino acids which are well conserved and said to be responsible for dimerization. The amino terminal end of most nuclear receptors contains a hypervariable F region whose function has not yet been determined (Wahli and Martinez, 1991).

All ligands of nuclear factors are capable of passively crossing the cell membrane by virtue of their lipophilicity (Mangelsdorf et al., 1995). They reach the nucleoplasm where they bind to the E region of their receptors (Green and Chambon, 1988). This binding can be thought of as ligand-induced conformational switch, that modulate the transcriptional activities of the receptor (Mangelsdorf et al., 1995). Most nuclear receptors

bind to DNA once they dimerize, either to themselves or to other nuclear receptors (Stunnenberg, 1993). Bound to DNA, activated nuclear receptors can then influence the transcriptional machinery of the cell (Blanco et al., 1995).

(b) Response element specificity

The specificity of DNA target recognition and binding by nuclear receptors is determined by two domains, one that mediates protein-DNA interactions and the other that mediates protein-protein interactions (Glass, 1994). The protein-protein interaction is necessary to form homo- or heterodimers prior to DNA binding. Nuclear factors recognize DNA elements, the smallest of which is a 6 base pair sequence known as a core recognition motif (Glass, 1994). Receptors that bind as monomers, such as the NGFI-B (Hirata et al., 1993) recognize one copy of this motif, while receptors that must first form homo- or heterodimers recognize 2 repeats of this motif.

Estrogen and glucocorticoid response elements were the first to be characterized (Kumar and Chambon, 1988; Umesomo and Evans, 1989; Tsai et al., 1988). The classical estrogen response element (ERE) consists of two core recognition motifs (PuGGTCA) arranged as inverted repeats and separated by three nucleotides (5'-PuGGTCAnnnTGACCT-3') making a pseudopalindrome (Kumar and Chambon, 1988). Although the core motif is slightly different in the glucocorticoid response element, it is arranged in exactly the same fashion as the ERE (Tsai et al., 1988). By consensus, an idealized, synthetic, steroid response element has been determined to consist of AGAACA core motifs. Naturally occuring response elements generally consist of variations of this motif (Glass, 1994).

Most steroid hormone response elements are arranged as pseudopalindromic

repeats. Recent results suggest that there are exceptions to this rule. In vitro, the ER and GR can bind to two direct repeats (DR) of the core motif separated by 5 to 9 nucleotides (DR5/DR9) (Kato et al., 1995; Aumais et al., 1996).

Steroid receptors bind as homodimers and X-ray crystallography has demonstrated that the DNA binding domain of each receptor makes contact with one motif (Luisi, 1991).

The structural similarity of the different steroid response elements indicates that small variations in motif sequence must be responsible for receptor specificity. As the ERE and GRE differ by only 2 base pairs in each half-site, it was determined that subtle differences in crucial regions of the ER and GR account for discriminatory recognition (Mader et al., 1989; Umesono and Evans, 1989; Green et al., 1988; Danielsen et al., 1989). The differences between the GR and ER gave rise to the identification of sub-regions within the DNA binding domain of nuclear factors. Structurally, the C-domain contains two helices which separate the two Zn finger domains. In between these two helices and within the second zinc finger is the D box It is reponsible for steroid receptor dimerization only once these have bound to palindromic hormone response elements (Umesono and Evans, 1989). The P-box, located within the first helix, is responsible for differentiating between the sequences of GRE and ERE, while the more distal D box discriminates the spacing of core motifs (Umesome and Evans, 1989; Glass, 1994). The exchange of just three amino acids within the P box is sufficient to confer ER GRE specificity and GR ERE specificity (Mader et al., 1989).

Dimer formation with RXR has been found to be crucial for the response element recognition and transactivation function of several nuclear receptors. These receptors include the vitamin D receptor (VDR), thyroid hormone receptor (TR), all trans-retinoic acid receptor (RAR), 9-cis-retinoic acid receptor (RXR), chicken ovalbumin upstream

promoter-transcription factor (COUP-TF), peroxisome proliferator activating receptor (PPAR) as well as orphan receptors such as liver X receptor (LXR) (Willy et al., 1995; Chan et al., 1992). In contrast to steroid responsive elements which bind mainly to core motifs arranged as pseudopalindromic inverted repeats, heterodimeric nuclear receptors can bind to core motifs arranged as either direct repeats, palindromes and inverted palindromes (Glass, 1994). Analysis of all known core motifs reveals a consensus sequence (AGGTCA) that can specifically bind any known RXR-heterodimers when repeated in a manner preferred by RXR's dimerization partner. For instance, direct repeats of this sequence separated by either 1, 2-5, 3, or 4 nucleotides can confer binding specificity to RXR-RXR, RXR-RAR, RXR-VDR and RXR-TR respectively (Mangelsdorf et al., 1991; Smith et al., 1991; Noda et al., 1990; Hoffmann et al., 1990).

The spatial configuration of heterodimeric DNA binding has also been determined. Protein cross-linking experiments indicate that RXR is always 5' to its dimerization partners, giving RXR access to the upstream recognition motif (Kurokawa et al., 1993; Zechel et al., 1994)

If response element specificity among different nuclear receptors can be attained by specifying half-site spacing and RXR is the preferred dimerization partner, then the responsibility of response element specificity cannot lie with RXR. Variations in amino acid sequence among the nuclear factors that dimerize to RXR must account for response element discrimination. Just distal to the second zinc finger are two regions that are highly conserved among isoforms of nuclear receptors (eg., RARα, RARβ, RARγ), but not among the different nuclear receptors themselves (ie.: RAR vs VDR). These 'A' and 'T' boxes are crucial for high affinity binding exhibited by the non-steroidal nuclear receptors (Wilson et al., 1992; Lee et al., 1994) (see figure 1.2). Mutations within the T box of TR were able to completely prevent TR DNA binding (Katz and Koenig, 1993). RAR-RXR

heterodimer specificity for direct repeats separated by 2 nucleotides (DR2) is largely determined by amino acids found within the T box of RAR and the interface this box forms with the second zinc finger of RXR (Zechel et al., 1994).

1.5.4 The Vitamin D Receptor (VDR)

(a) Structure of the VDR

The human vitamin D receptor (VDR) is a 427 amino acid, 49kDa protein organized in much the same manner as the previously described nuclear receptors (Beato, 1990). The A/B domain of the VDR is only 24 amino acids long. The DNA binding domain is composed of 66 amino acids and contains two zinc fingers, like all other nuclear receptors (Evans, 1988). The C-terminus contains the ligand binding domain and is also responsible for dimerization and transactivation.

(b) Dimerization

The VDR has been shown to require the help of an accessory factor in order to effectively bind DNA and activate transcription (Sone et al., 1991). This 55kDa accessory factor is in all likelyhood the 9-cis-retinoic acid receptor, RXR (Zhang et al., 1992). RXRs have also been shown to be present in virtually all tissues where VDR can be found.

The C-terminus, or E domain, contains a series of nine heptad repeats of hydrophobic amino acids (Forman and Samuels, 1990) crucial for dimerization. Another crucial dimerization structure is the E1 region, located between residues 244 and 263 and first identified by Lee et al. (Lee et al., 1992). This E1 region is conserved among other

nuclear factors and participates in the interaction of steroid receptors with heat shock protein (hsp90). Unlike steroid receptors hVDR does not bind to hsp90, instead, the E1 region participates in dimerization and transactivation (Whitfield et al., 1995). Within the E1, mutation of the glutamine 259 impaired transactivation, while lysine 246 mutants were unable to dimerize (Whitfield, et al., 1995). As well, mutations within the fourth (leucine 325 to leucine 332) and ninth heptads (lysine 392 to arginine 402) were shown to abrogate VDR dimerization to RXR (Nakajima et al., 1994).

Three different forms of RXR have been identified, RXRα, RXRß, RXRγ (Mangelsdorf, 1992). Isotypes of each isoforms also exist (eg.: RXRα1, RXRß2) and are the result of differential splicing or promoter usage (Mangelsdorf, 1992). Certain RXR isoforms and isotypes can be present in a particular cell or tissue type, while absent in another. VDR has been shown to bind to any of the three different forms of RXR, and that the heterodimers formed are capable of binding vitamin D responsive elements (VDRE). However, only RXRα and RXRγ are capable of enabling VDR stimulation of transcription once bound to a VDRE (Kephart et al., 1996).

It must be mentioned that a few groups have proposed that VDR can bind as a homodimer to certain DNA response elements, including that of the PTH gene (Freedman et al., 1994; Carlberg et al., 1993; Mackey et al., 1996). In addition it has been postulated that VDR can heterodimerize with RAR, and that this complex is transcriptionally active (Carlberg et al., 1993). These findings obviously conflict with the report that yeast expressed and purified human VDR has an absolute requirement for RXR in order to bind to DR3 response elements (Jin and Pike, 1996).

(c) DNA binding and Transactivation

As with all known nuclear factors, VDR binds to its HRE, the vitamin D response element (VDRE) through its C-domain. VDREs identified to date include those found in the genes of: the rat osteocalcin (Terpening et al., 1991), the human osteocalcin (Ozono et al., 1990), the mouse osteopontin (Noda et al., 1990), the rat 24-hydroxylase (Ohyama et al., 1993), the mouse calbindin-28K (Gill and Christakos, 1993), the intestinal calbindin 9K (Nishikawa et al., 1993), the chicken carbonic anhydrase-II (Quelo et al., 1994), the human PTH (Mackey et al., 1996), the chicken PTH (Liu et al., 1996b), the rat PTHrP gene (Kremer et al., 1996), the c-fos gene (Candeliere et al., 1996) and the atrial natriuretic peptide (Kahlen and Carlberg 1996). Most of these sequences consist of direct repeats of a core recognition motif separated by 3 base pairs (DR3) which appear to bind with high specificity to VDR/RXR heterodimers.

Sites within the DNA binding domain of the VDR appear to be crucial for DNA recognition and binding. In vitro mutagenesis of sites within the P box of VDR indicate that this box plays no role in DNA binding domain or ligand binding domain heterodimerization (Hsieh et al., 1995). It does, however, play a role in DNA recognition. The T box, found just past the second Zn finger, plays a much more important role. Mutations within this region severely attenuated VDRE binding (Hsieh et al., 1995).

Ligand occupancy does not seem to be required for a VDR/RXR heterodimer to bind to a VDRE (Kephart et al., 1996). Neither 1,25-dihydroxyvitamin D_3 nor 9-cis-retinoic acid are necessary, in vitro experiments, to induce VDR/RXR binding to a VDRE. However 1,25-dihydroxyvitamin D_3 alone is necessary to induce the transactivation function of the VDR (Kephart et al., 1996; Dr. David Mangelsdorf personal communication).

The active VDR/RXR complex, once bound to a VDRE, must signal its presence to the transcriptional machinery in order to modulate its activity. The mechanism for this

interaction is not yet clear. Removal of the last 24 amino acids of VDR results in unimpeded ligand binding, normal heterodimerization and DNA binding, but no transactivation (Nakajima et al., 1994). Recent evidence suggests that VDR can form specific protein-protein interactions with the basal transcription factor TFIIB (Blanco et al., 1995). Mutational analysis reveals that these interactions appear to be mediated through the carboxyterminal domain of VDR (MacDonald et al., 1995).

The post-translational modifications of VDR could play an important role in controlling both DNA binding and transactivation. VDR has been definitively shown to be phosphorylated in at least two positions, serine-51 and serine-208. Phosphorylation by protein kinase C at serine-51 appears to reduce DNA binding (Hsieh et al., 1993), while casein kinase II phosphorylation of serine-208 appears to enhance transcriptional activation (Jurutka et al., 1993).

1.6 Objectives of Thesis

1. To study the molecular actions of 1,25-dihydroxyvitamin D_3 on the growth and differentiation of normal human keratinocytes.

2. To study the effects of 1,25-dihydroxyvitamin D_3 on the growth of human keratinocytes as they progress from the normal to the malignant phenotype.

3. To characterize and identify the mechanism of 1,25-dihydroxyvitamin D_3 resistance in *ras* transformed keratinocytes.

CHAPTER 2

EFFECTS OF 1,25 DIHYDROXYVITAMIN D_3 AND CALCIUM ON GROWTH AND DIFFERENTIATION AND ON C-FOS AND P53 GENE EXPRESSION IN NORMAL HUMAN KERATINOCYTES.

ABSTRACT

Calcium enhances keratinocyte differentiation, and 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is both antiproliferative and pro-differentiative in many cell types, including normal human keratinocytes. In the present study we examined the combined effects of calcium and $1,25(OH)_2D_3$ on parameters of growth and differentiation and on c-*fos* and p53 gene expression in normal human keratinocytes. Exposure of normal human keratinocytes to $1,25\ (OH)_2D_3$ markedly reduced [^{3}H] thymidine incorporation and cell number at low and high medium Ca^{++} concentrations. Simultaneously, cells in the G_0/G_1 phase of the cell cycle increased significantly and those in S phase fell precipitously. $1,25(OH)_2D_3$ and calcium also induced keratinocyte differentiation independently, as assessed by immunocytochemistry and by induction of involucrin mRNA. Both Ca^{++} and $1,25(OH)_2D_3$ were shown, by nuclear run-on assays, to increase involucrin gene transcription. A rapid, transient elevation in c-*fos* proto-oncogene expression preceded these effects when epidermal growth factor (EGF) was present alone. When $1,25\ (OH)_2D_3$ was added to quiescent keratinocytes, there was a marked augmentation of c-*fos* mRNA accumulation at low and high medium Ca^{++} concentrations. Varying medium Ca^{++} concentrations had no effect on c-*fos* mRNA levels. Increasing medium Ca^{++} concentrations from 0.15 to 2.0mM produced marked elevations of p53 mRNA accumulation and of the rate of p53 gene transcription, while $1,25(OH)_2D_3$ had no effect.

These results therefore suggest that $1,25(OH)_2D_3$ and calcium act in consort to modulate the expression of two important cell-cycle associated genes, which may be important components in the initial programming of growth and differentiation of normal human keratinocytes.

INTRODUCTION

1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is believed to exert its action through an interaction with its hormone-receptor which complexes with DNA and concentrates the hormone in the nucleus (Hausser, 1986), similar to other steroid hormones (O'Malley, 1984). Several target genes, such as the proto-oncogenes c-myc and c-*fos* probably play an important role in the regulation of growth and differentiation of several cell types (Shilo and Weinberg, 1981). Activation of c-*myc* is associated with cell proliferation (Müller et al., 1982), whereas activation of c-*fos* is thought to occur in association with cellular differentiation (Milbrandt, 1986; Kouzarides and Ziff, 1987). Elevation of medium calcium concentrations to 1.0mM or more inhibits growth strongly and promotes differentiation of keratinocytes in culture (Hennings et al., 1980); $1,25(OH)_2D_3$ potentiates these effects (Hosomi et al., 1983; Smith et al., 1986). Recent evidence shows that the inhibition of the growth of normal human keratinocytes by $1,25(OH)_2D_3$, is preceded by marked inhibition of c-*myc* mRNA (Matsumoto et al., 1990). In addition to proto-oncogenes, tumor suppressor genes such as p53 can also alter cell growth (Baker et al., 1990). Growth inhibition is observed when p53 is stably transfected into cancer cell lines. Mutations along the p53 gene, which result in its decreased suppressor activity, are found in a wide variety of cancers (Marshall, 1991). p53 is also expressed in normal cells and is thought to play a role in normal cell growth (Milner, 1984; Mercer et al., 1982; Reich and Levine, 1984).

At present we do not know whether the effect of calcium and $1,25(OH)_2D_3$ on keratinocyte growth and differentiation is accompanied by changes in the expression of the cell-cycle associated genes, c-*fos* and p53.

We therefore analyzed the effect of calcium and $1,25(OH)_2D_3$ alone and in

combination on parameters of cell growth and differentiation and on expression of c-*fos* and p53 genes.

MATERIALS AND METHODS

1) Culture of Normal Human Keratinocytes.

We isolated normal human keratinocytes from skin tissue removed during breast reduction, according to the method of Boyce and Ham (Boyce and Ham, 1985). In brief, epidermal tissue was separated from dermal tissue following a 3 hr collagenase digestion. Single cells were released from the epidermis using trypsin and were suspended in KGM, the complete medium for clonal growth of keratinocytes. KGM consists of keratinocyte basal medium (KBM Clonetics Corp., San Diego CA) containing 0.15mM calcium (Ca^{++}) supplemented with the following growth factors (GFs): 10ng/ml epidermal growth factor (EGF, Sigma), 5μg/ml insulin (Sigma), 0.5μg/ml hydrocortisone (Clonetics), 0.4% (W/V) and bovine pituitary extract (BPE, Clonetics). It provided maximal proliferation without differentiation. Cells grown in these conditions were then seeded at a density of 2.5×10^4 cells/well in 6-well cluster plates in KGM for 24h. Following a 24h incubation in KBM and 0.15mM Ca^{++} (basal conditions), the medium was replaced at time 0 with KGM containing EGF (10 ng/ml) in which Ca^{++} concentrations varied between 0.15 and 2.0mM, verified by direct measurement by atomic absorption spectrophotometry. Incubations were then continued for up to 96h with or without 10^{-8}M $1,25(OH)_2D_3$ and medium collected at timed intervals.

2) Cell counts

Wells were rinsed with PBS, to discard any floating debris, gently trypsinized using 0.025% trypsin in complete medium (KGM), dispersed and an aliquot was counted in a Coulter counter (Coulter Electronics, Beds, U.K.) at timed intervals up to 96h after medium change. Remaining cells were centrifuged at low speed (600g), rinsed with

phosphate-buffered saline and lysed with a mixture of 4 M guanidium thiocyanate, 25 mM trisodium citrate, 1 mM EDTA, and 0.1 M ß-mercaptoethanol (GTC mixture). GTC extracts were stored at -70°C for later RNA analysis by Northern blot hybridization.

3) [^{3}H]Thymidine incorporation into DNA

We replaced the medium at 22h and at 94h with calcium free MEM (Gibco, Grand Island NY) supplemented with either 0.15, 0.5 or 2.0mM Ca^{++} concentration and 1 µCi/ml of [^{3}H]thymidine (New England Nuclear, Boston MA). Following a 2h incubation at 37°C, the medium was aspirated, cells were washed twice with cold Hank's Balanced Salt Solution and incubated 15 min with 1ml of cold 10% trichloroacetic acid (TCA) to precipitate protein. After aspirating the TCA, protein precipitable material was dissolved in 1 ml of 1 N NaOH and the ^{3}H content in an aliquot was determined by liquid scintillation spectrometry in an LKB ß radiation counter. Cell numbers were counted and the [^{3}H] thymidine counts/min. (cpm) were corrected for cell number, with final values expressed as cpm/10^4 cells.

4) Immunocytochemistry.

The cellular content of specific keratins was determined by immunocytochemistry, using a polyclonal antiserum to keratins, predominantly of MW 56.5kD and 65-67kD (Dimension Labs, Mississauga, Ont. Canada). These keratins have been immunolocalized to the suprabasal layers of human epidermis and have been characterized as markers for skin-type differentiation (Woodcock-Mitchell et al., 1982; Moll et al., 1982). Cells seeded in 4 chamber glass slides (GIBCO) in KGM were grown to approximately 30% confluency. The medium was then changed to KBM (basal conditions). After 24h in basal

conditions, the medium was replaced with KBM containing 10ng/ml EGF and either 0.15 or 2.0mM Ca^{++}, with or without 10^{-8}M $1,25(OH)_2D_3$. Incubation continued for 5 days with a medium change after 3 days. Cells were then fixed in 95% ethanol for 5 min, rinsed with distilled water and stained by a modification of the three-layer peroxidase-antiperoxidase technique (Sternberger and Hardy, 1970).

5) Flow cytometry

Cells were grown in KGM until they reached 50% confluency. Following a 24h incubation in KBM, the medium was replaced with KBM containing EGF (10ng/ml) at low (0.15mM) or high (2.0mM) Ca^{++} concentration and were incubated in the presence or absence of 10^{-8}M $1,25(OH)_2D_3$. 24 hours later they were analyzed by flow cytometry. Following trypsinization cells were centrifuged at low speed (600g), rinsed once with phosphate buffered saline (PBS) and stained according to the technique of Vindeløv (Vindeløv, 1977). Briefly, the pellet was resuspended in 1ml of 3.5mM Tris, 7.5μM propidium iodide (Calbiochem), 0.1% nodinet P40 (Sigma, St-Louis, MO), 700 u/L RNAse (Boehringer Mannheim, Canada) and 10mM NaCl. The solution was added dropwise while vortexing. After standing at least 10min on ice, the nuclei were analyzed in a FACScan (Becton Dickinson Inc., Oxnard, CA), which sorts and plots the number of cells against the relative fluorescence intensity. The calculation of the percentage distribution in various phases of the cell cycle was performed with Cell Fit software (Becton Dickinson Inc.), using a sum of broadened rectangles fit.

6) RNA analysis

For Northern Blot analysis, we purified GTC extracts by cesium chloride gradient

centrifugation (Chirgwin et al., 1979) and electrophoresed 10μg of total RNA on a 1.1% agarose-formaldehyde gel. RNA was transferred by capillary blotting onto a nylon membrane (Nytran). The filters were air-dried, baked at 80°C for 2h, and then hybridized with probes labeled with ^{32}P dCTP (IC Biomedical Canada LTD) by the random primer method (Amersham Canada LTD, Ontario, Canada). After incubation at 42°C for 24h, filters were washed successively in 1 x SSC, 1% SDS for 15min at room temperature, and 0.1 x SSC, 0.1 % SDS twice for 30min at 55°C (1 x SSC is 0.15 M sodium chloride, 0.015M trisodium citrate). Autoradiography of filters was carried out at -70°C using Kodak XAR films (Eastman Kodak Co. Rochester, NY) and two intensifying screens. The intensities of the observed bands were analyzed by laser densitometry (Ultroscan XL, LKB).

7) DNA probes

a) Involucrin was used as differentiation marker of keratinocytes. Involucrin is produced abundantly during terminal differentiation of keratinocytes and works as a precursor of a cross-linked envelope during terminal differentiation (Rice and Green, 1977; Rice and Green, 1979; Simon and Green, 1984; Simon and Green, 1985). A 2.1 kb fragment of the human involucrin gene, containing the entire coding region, was used as a probe (Tseng and Green, 1988).

b) c-*fos*: the EcoR1-SalI restriction fragment encoding c-*fos* released from the plasmid p-*fos* BS (Curran et al., 1982) was used as a probe.

c) p53: A 2.0 KB Bam H1 restriction fragment of p53 cDNA was used (Givol et al., 1985).

d) Cyclophilin : Filters were also probed with a 800 bp BamH1, restriction fragment of

rat cyclophilin (Danielson et al., 1988) as a control for the amount of RNA loaded.

e) GAPDH: a rat Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was prepared from a PstI digested pRGAPDH13 plasmid (Piechaczyk et al., 1984).

8) *In vitro* nuclear run-on assays

Relative transcription rates of involucrin, p53 and cyclophilin genes were measured using a nuclear run-on assay (McKnight et al., 1979; Dufort et al., 1993). Nuclei were prepared from 10-20 x 10^6 cells prepared as described in paragraph 1 in the presence or absence of EGF, $1,25(OH)_2D_3$ and in low (0.15 mM) or high (2.0mM) Ca^{++} concentrations. Cells were scraped into ice-cold PBS, pH 7.4, pelleted at 4°C and lysed with Nodinet P40 lysis buffer [0.3M sucrose, 60mM KCl, 15mM NaCl, 15mM HEPES (pH 7.5), 2mM EDTA, 0.5mM EGTA, 0.15mM spermine, 0.5mM spermidine, 14mM ß-mercapto-ethanol and 0.2% Nodinet P40]. After 8 min on ice, nuclei were pelleted at 800x g. They were rinsed once with 1 ml nuclei storage buffer [50% glycerol, 20mM Tris (pH 7.9), 75mM NaCl, 0.5mM EDTA, 0.85mM DTT, and 0.125mM PMSF], snap frozen in liquid nitrogen and stored at -80°C until assay. Run-on reactions were carried out at 30°C in 300mM $NH_4(SO_4)_2$, 100mM Tris HCl (pH 7.9), 4mM $MgCl_2$, 4mM $MnCl_2$, 50mM NaCl, 0.4mM EDTA, 1.2µM DTT, 0.1mM PMSF, 10mM creatine phosphate, 29% glycerol, 150 µCi [^{32}P] UTP, 650 Ci/mmol (ICN, Mississauga, Ontario, Canada) and 1.5mM each of CTP, ATP, and GTP (Boehringer Mannheim Canada) for 45 min. Reactions were quenched with tRNA and treated with (RNAse-free) DNAse and proteinase-K and phenol-chloroform-isoamyl alcohol extracted. ^{32}P labeled transcripts were spun, column chromotographed through sephadex G50, TCA precipitated, NaOH treated and ethanol precipitated. DNA inserts (0.15µg) of involucrin, p53 and GAPDH prepared as described in paragraph 7 were NaOH denatured, slot blotted (Hybrislot,

Gibco/BRL, Burlington, Ontario) and hybridized with 2 x 10^7 cpm ^{32}P labeled transcripts in 50% formamide, 50mM HEPES (pH 7.3), 0.75M NaCl, 2mM EDTA, 0.5% SDS, 10x Denhardt's and 200μg/ml salmon sperm DNA for a minimum of 40h. In any single experiment, equal number of counts were used for all conditions. Nytran filters were exposed to autoradiographic film and quantitation of the bands was done by laser densitometry.

9) Statistical Analysis

Results are expressed as the mean ± SE of replicate (at least triplicate) determinations and statistical comparisons are based on ANOVA or the Student's t test. A probability value of 0.05 was considered significant.

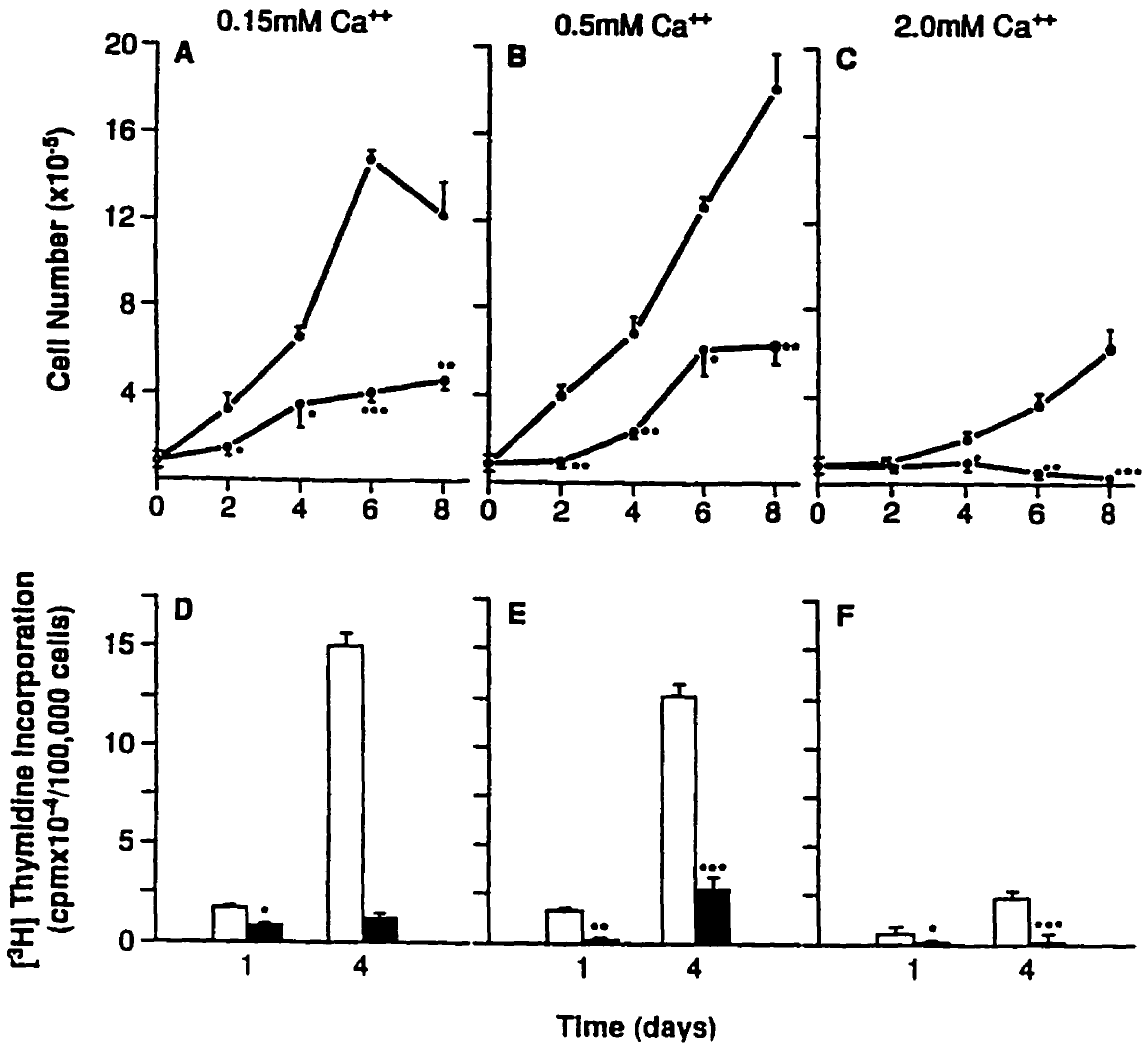

0.15mM Ca++
0.5mM Ca++
2.0mM Ca++
A
B
C
D
E
F
Cell Number (x10-5)
[3H] Thymidine Incorporation (cpmx10-4/100,000 cells)
Time (days)

Figure 2.1

Effect of Ca^{++} and 1,25 dihydroxyvitamin D_3 on cell number and [^{3}H] thymidine uptake in quiescent keratinocytes stimulated with EGF supplemented KBM. EGF alone (,) or with 10^{-8}M $1,25(OH)_2D_3$ (,). EGF in 0.15mM Ca^{++} (A and D), in 0.5 mM Ca^{++} (B and E), or in 2.0mM Ca^{++} (C and F). Cells were counted at the times indicated and [^{3}H] thymidine uptake was measured as described in materials and methods. Results are expressed as the mean ± SE of six determinations. Asterisks represent significant differences from corresponding incubations performed in the absence of $1,25(OH)_2D_3$. *$p<0.01$ **$p<0.005$ ***$p<0.001$.

Figure 2.2

Effect of Ca^{++} and 1,25 dihydroxyvitamin D_3 on keratinocyte differentiation using immunocytochemical stain for the 56.5kD/65-67kD keratin pair. Keratinocytes were fixed, permeabilized and processed for immunocytochemistry as described in materials and methods. Cells were cultured in (A) 0.15mM Ca^{++}; (B) 0.15mM Ca^{++} and $1,25(OH)_2D_3$; (C)2.0mM Ca^{++} and $1,25(OH)_2D_3$. Magnification x 100.

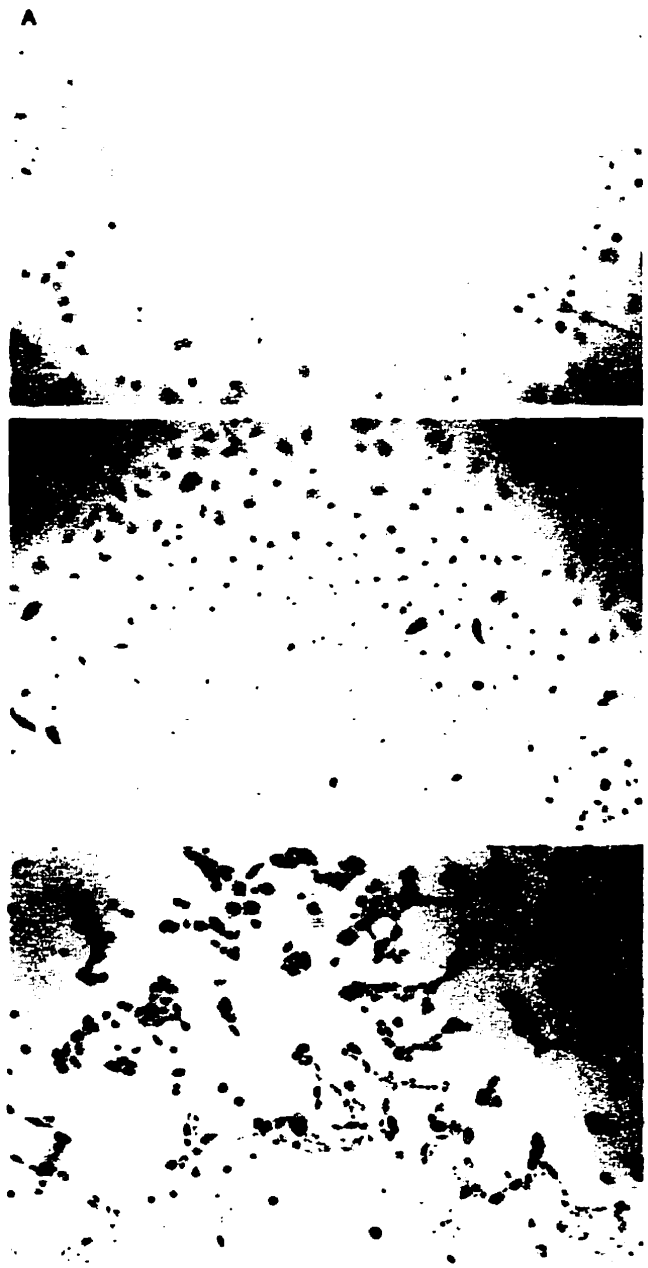

A

Figure 2.3

Northern blot analysis of involucrin mRNA in normal human keratinocyte extracts. Filters were hybridized as described in materials and methods with a ^{32}P-labeled involucrin probe and with a ^{32}P-labeled cyclophilin probe as a control for RNA loading. The upper panels represent the densitometric ratios of involucrin/cyclophilin mRNA seen in lower panels, except for panel C which represents the densitometric ratios seen in panels A and B in the absence of 1,25 $(OH)_2D_3$ at 0.15mM and 2.0mM Ca^{++}. The arrows represent the 2.1kb involucrin message and the 0.8kb cyclophilin message. Influence of 1,25$(OH)_2D_3$ in 0.15mM Ca^{++} (A) or 2.0mM Ca^{++} (B), and the influence of Ca^{++} (C)on the time course of involucrin mRNA. Each lane contained 10μg of total cellular RNA. The ratios are expressed as mean ±SEM of three different experiments. Asteriks (*) represent significant differences from corresponding incubations in absence of 1,25$(OH)_2D_3$ and circles (°) in the presence of 2.0mM calcium.

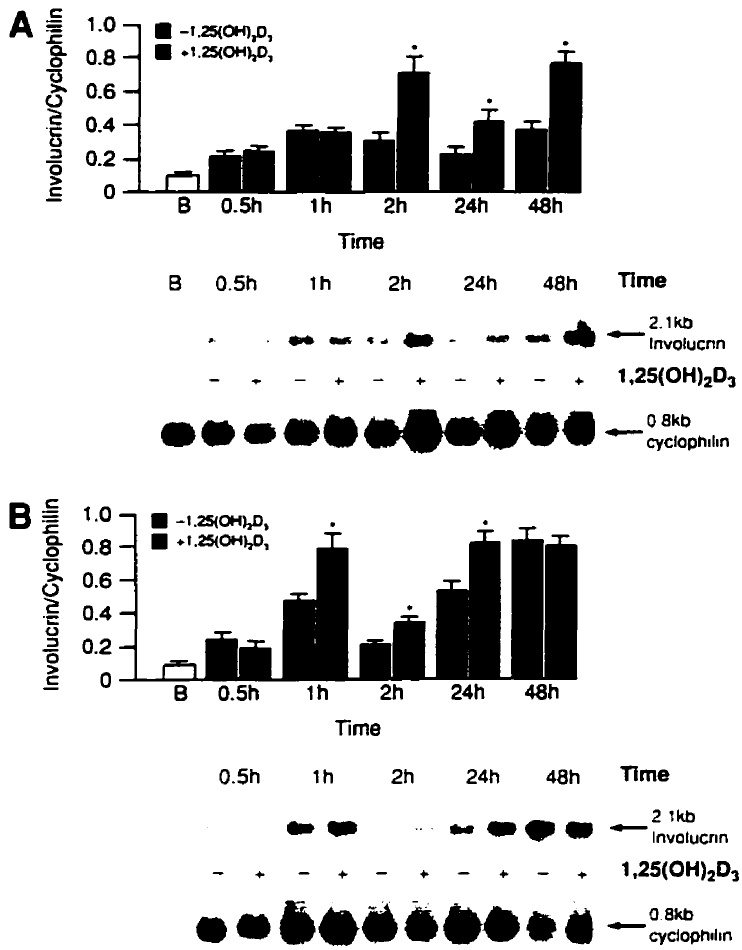

A
Involucrin/Cyclophilin
1.0
0.8
0.6
0.4
0.2
0
−1.25(OH)2D3
+1.25(OH)2D3
B
0.5h
1h
2h
24h
48h
Time
2.1kb
Involucrin
1,25(OH)2D3
0.8kb
cyclophilin
B
Involucrin/Cyclophilin
1.0
0.8
0.6
0.4
0.2
0
−1.25(OH)2D3
+1.25(OH)2D3
B
0.5h
1h
2h
24h
48h
Time
0.5h
1h
2h
24h
48h
Time
2.1kb
Involucrin
1,25(OH)2D3
0.8kb
cyclophilin
C
Involucrin/Cyclophilin
1.0
0.8
0.6
0.4
0.2
0
0.15mM Ca++
2.0 mM Ca++
B
0.5h
1h
2h
24h
48h
Time

Figure 2.4

Northern blot analysis of c-*fos* mRNA in normal human keratinocyte extracts. Filters were hybridized as described in materials and methods with a ^{32}P-labeled c-*fos* probe and a ^{32}P-labeled cyclophilin probe as a control for RNA loading. The upper panels represent the densitometric ratios of c-*fos*/cyclophilin mRNA seen in lower panels, except for panel C which represents the densitometric ratios seen in panels A and B in the absence of 1,25 $(OH)_2D_3$ at 0.15mM and 2.0mM Ca^{++}. The arrows represent the 2.2kb c-*fos* message and the 0.8kb cyclophilin message. Influence of 1,25$(OH)_2D_3$ in 0.15mM Ca^{++} (A) or 2.0mM Ca^{++} (B), and the influence of Ca^{++} (C) on the time course of c-*fos* mRNA. Each lane contained 10μg of total cellular RNA. The ratios are expressed as mean ±SEM of three different experiments. Asteriks (*) represent significant differences from corresponding incubations in absence of 1,25$(OH)_2D_3$ and circles (°) in the presence of 2.0mM calcium.

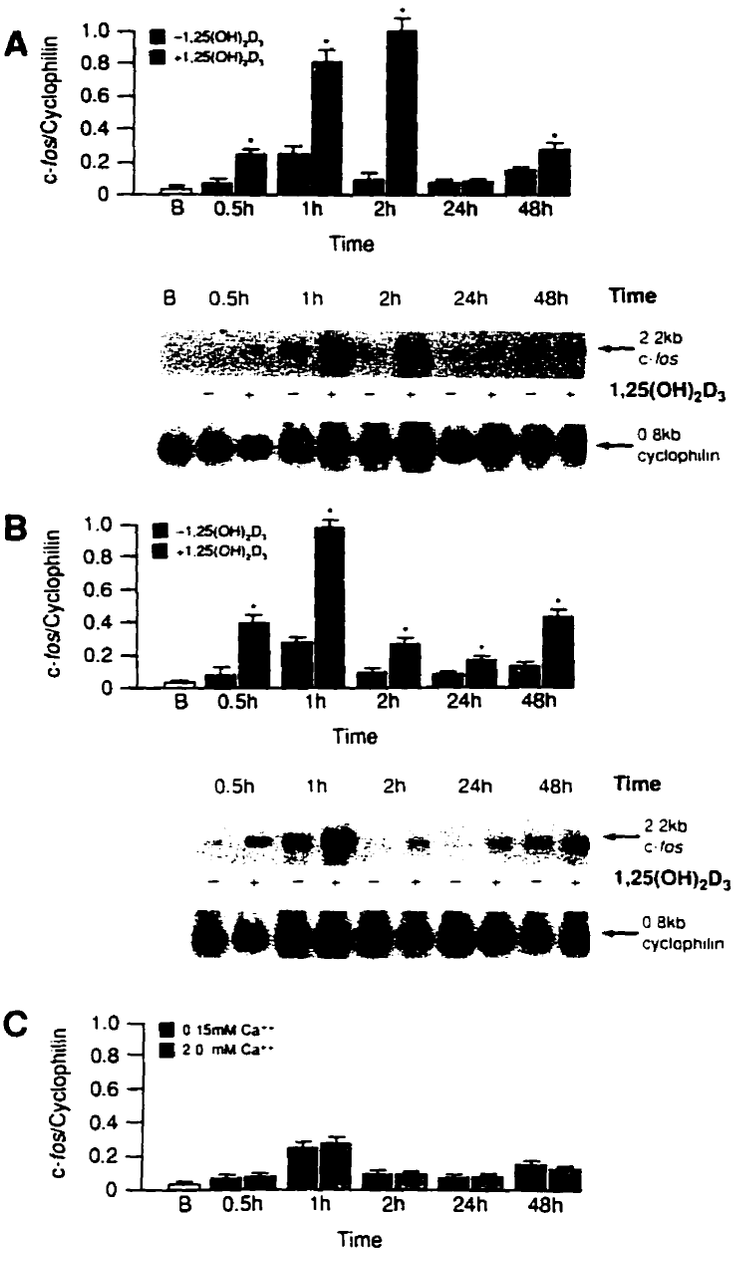

A
c-fos/Cyclophilin
1.0
0.8
0.6
0.4
0.2
0
−1,25(OH)2D3
+1,25(OH)2D3
B 0.5h 1h 2h 24h 48h
Time
B 0.5h 1h 2h 24h 48h Time
2.2kb c-fos
− + − + − + − + − +
1,25(OH)2D3
0.8kb cyclophilin
B
c-fos/Cyclophilin
1.0
0.8
0.6
0.4
0.2
0
−1,25(OH)2D3
+1,25(OH)2D3
B 0.5h 1h 2h 24h 48h
Time
0.5h 1h 2h 24h 48h Time
2.2kb c-fos
− + − + − + − + − +
1,25(OH)2D3
0.8kb cyclophilin
C
c-fos/Cyclophilin
1.0
0.8
0.6
0.4
0.2
0
0.15mM Ca++
2.0 mM Ca++
B 0.5h 1h 2h 24h 48h
Time

Figure 2.5

Northern blot analysis of p53 mRNA in normal human keratinocyte extracts. Filters were hybridized as described in materials and methods with a ^{32}P-labeled p53 probe and a ^{32}P-labeled cyclophilin probe as a control for RNA loading. The upper panels represent the densitometric ratios of p53/cyclophilin mRNA seen in lower panels, except for panel C which represents the densitometric ratios seen in panels A and B in the absence of 1,25 $(OH)_2D_3$ at 0.15mM and 2.0mM Ca^{++}. The arrows represent the 3.1kb p53 message and the 0.8kb cyclophilin message. Influence of 1,25$(OH)_2D_3$ in 0.15mM Ca^{++} (A) or 2.0mM Ca^{++} (B), and the influence of Ca^{++} (C) on the time course of p53 mRNA. Each lane contained 10μg of total cellular RNA. The ratios are expressed as mean ±SEM of three different experiments. Asteriks (*) represent significant differences from corresponding incubations in absence of 1,25$(OH)_2D_3$ and circles (°) in the presence of 2.0mM calcium.

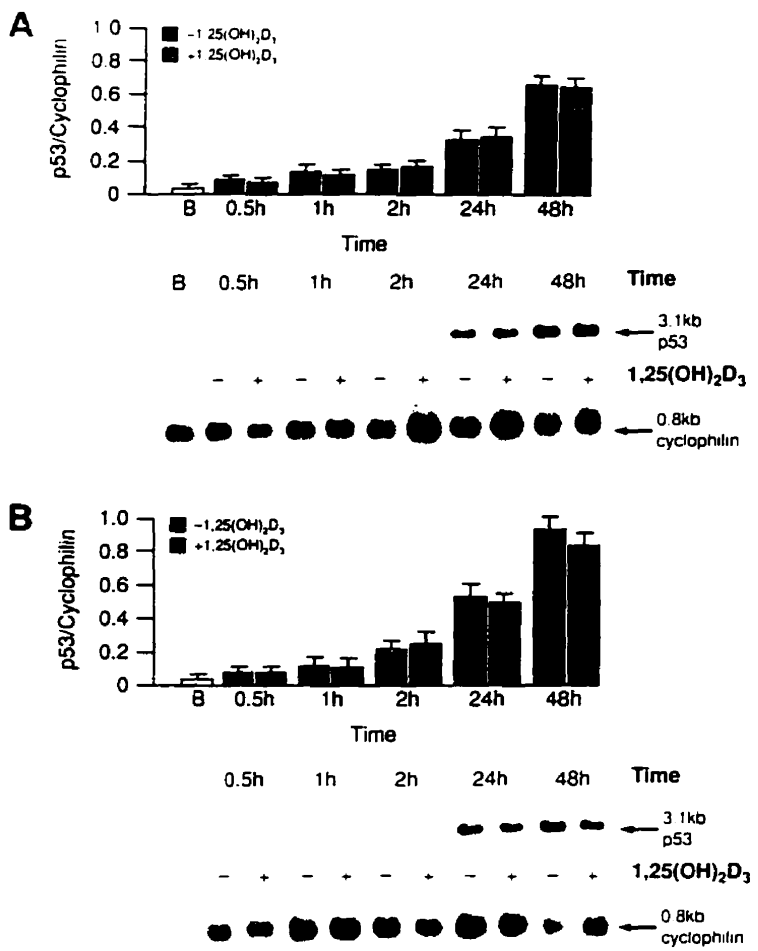

A
p53/Cyclophilin
1.0
0.8
0.6
0.4
0.2
0
-1.25(OH)2D3
+1.25(OH)2D3
B
0.5h
1h
2h
24h
48h
Time
3.1kb
p53
1,25(OH)2D3
0.8kb
cyclophilin
B
-1,25(OH)2D3
+1,25(OH)2D3
C
0.15mM Ca++
2.0 mM Ca++
a

Figure 2.6

$1,25(OH)_2D_3$and Ca^{++} effects on involucrin and p53 gene transcription. Nuclear run-on assays were performed as described in materials and methods. ^{32}P labeled run-on transcripts were prepared from nuclei isolated from cells cultured for 2 h (panel A) and 24h (panel B) in low (0.15mM) or high (Hi), (2.0mM) Ca^{++} concentrations in the presence or absence of $10^{-8}M$ $1,25(OH)_2D_3$. Autoradiographs of a representative experiment are shown in the upper panel [p53, involucrin, GAPDH and plasmid pBR322 with no insert to asses non-specific binding]. The relative transcription rate for involucrin and p53 over GAPDH are shown in the lower panel. Results are representative of three different experiments.

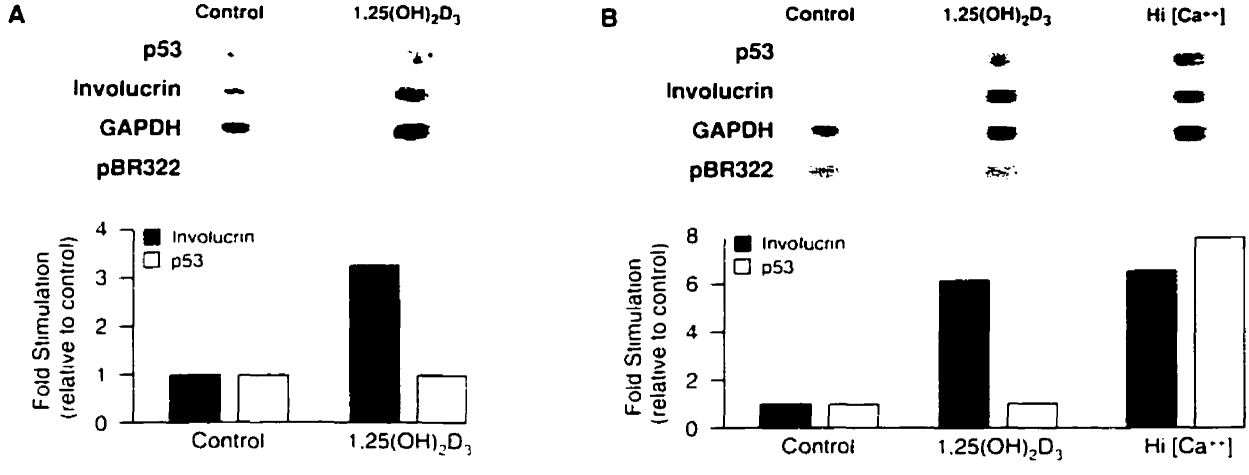

A
Control
1.25(OH)2D3
p53
Involucrin
GAPDH
pBR322
Fold Stimulation (relative to control)
Involucrin
p53
Control
1.25(OH)2D3
B
Control
1.25(OH)2D3
Hi [Ca++]
p53
Involucrin
GAPDH
pBR322
Fold Stimulation (relative to control)
Involucrin
p53
Control
1.25(OH)2D3
Hi [Ca++]

Table 2.1: Effect of calcium and $1,25(OH)_2D_3$ on EGF-stimulated keratinocyte proliferation[a].

	G_0G_1 (%)	S (%)
Untreated Cells	77.5±0.8[c]	9.2±0.6[c]
EGF + 0.15mM Ca^{++}	69.5±2.0[b]	13.8±0.8[b]
EGF + 0.15mM Ca^{++} +$1,25(OH)_2D_3$	74.1±1.2[b,c]	11.6±0.6[b,c]
EGF + 2.0mM Ca^{++}	73.3±1.1[b,c]	11.1±0.7[b,c]
EGF + 2.0mM Ca^{++} +$1,25(OH)_2D_3$	77.0±2.0[c]	9.3±0.6[c]

[a] results are expressed in percentage (%) of cells in the different phases of the cell cycle and represent the mean±SEM of three different experiments done in duplicate.

[b] significant difference from untreated cells $p<0.05$

[c] significant difference from incubations with EGF and 0.15mM Ca^{++}

RESULTS

1) Effect of Ca^{++} and 1,25 $(OH)_2D_3$ on cell proliferation and [^{3}H] Thymidine incorporation.

Normal human keratinocytes were incubated in the presence and in the absence of 10^{-8}M $1,25(OH)_2D_3$ in 0.15, 0.5 or 2.0mM Ca^{++}. In the absence of $1,25(OH)_2D_3$ cells responded to 2.0mM Ca^{++} by decreasing their growth rate (Fig. 2.1 C); no difference was noted between 0.15 and 0.5 mM Ca^{++} (Fig. 2.1 A,B). Cells treated with $1,25(OH)_2D_3$ showed a markedly reduced growth rate compared to untreated cells at all three calcium concentrations tested. Similarly [^{3}H] thymidine incorporation was inhibited by 10^{-8}M $1,25(OH)_2D_3$ at each of the three Ca^{++} levels, whereas ambient Ca^{++} had to be raised to 2.0mM to see inhibition in the absence of $1,25(OH)_2D_3$ (Fig. 2.1 D,E,F). Similar inhibitory effects of $1,25(OH)_2D_3$ on growth rate and [^{3}H] thymidine incorporation of rapidly proliferating keratinocytes were noted at low (0.15mM) and intermediate (0.5mM) Ca^{++} concentrations. However, at a high Ca^{++} concentration (2.0mM), $1,25(OH)_2D_3$ totally abolished cell proliferation.

2) Effect of $1,25(OH)_2D_3$ and calcium on keratinocyte differentiation.

a) Immunocytochemistry.

Immunocytochemical studies using the 56.5kD/65-67kD Keratin pair as markers of keratinization showed that $1,25(OH)_2D_3$ induced staining at low Ca^{++} concentration (0.15mM) (Fig. 2.2B); both the intensity of staining and the number of stained cells were maximally increased at high (2.0mM) Ca^{++} with $1,25(OH)_2D_3$ (fig 2.2C).

b) Keratinocyte differentiation assessed by involucrin mRNA expression.

Involucrin was seen as a faint message in non differentiated keratinocytes, incubated in KBM, as a single-2.1kB transcript. Addition of $1,25(OH)_2D_3$ in the presence of low calcium (0.15mM) caused a significant increase in involucrin mRNA, maximal at 48 h (Fig. 2.3A). Simultaneous addition of 2.0mM calcium and $1,25(OH)_2D_3$ produced a marked increase of involucrin mRNA at 24h which remained stable thereafter (Fig. 2.3B). However, the maximal level of expression achieved at 48h was not significantly different between 2.0mM calcium and $1,25(OH)_2D_3$ added separately or with the combination of $1,25(OH)_2D_3$ and 2.0mM calcium. Finally, there was a marked increase in involucrin mRNA with addition of 2.0 mM calcium to the culture medium; it was maximal at 48h (Fig. 2.3C).

3) Effect of $1,25(OH)_2D_3$ on the cell cycle

Using flow cytometry, we next examined the effect of $1,25(OH)_2D_3$ on the progression of normal human keratinocytes through the cell cycle. In the quiescent state the majority of cells were in G_0/G_1 phase. The addition of EGF for 24h resulted in a shift into S phase. The addition of 10^{-8} M $1,25(OH)_2D_3$ to culture medium containing 0.15 mM Ca^{++} and EGF inhibited the shift. Addition of 2.0mM Ca^{++} along with EGF to the culture medium also inhibited the shift into the S phase. Finally, the combination of 2.0mM Ca^{++}, 10^{-8}M $1,25(OH)_2D_3$ and EGF had a more pronounced effect than 2.0mM calcium or $1,25(OH)_2D_3$ alone. (Table 2.1).

4) Effect of EGF, Calcium and of $1,25(OH)_2D_3$ on c-*fos* and p53 mRNA

After 24 hours incubation in KBM, the medium was removed and replaced with KBM containing EGF (10ng/ml) and Ca^{++} (0.15 or 2.0mM) with or without 10^{-8}M of

$1,25(OH)_2D_3$. EGF addition in the presence of 0.15mM Ca^{++} produced a rapid and transient increase of c-*fos* mRNA (Fig. 4A). Addition of $1,25(OH)_2D_3$ markedly enhanced c-*fos* mRNA levels above the one seen with EGF alone as early as 30 min and up to 24h both in low (0.15mM) (Fig. 2.4A) or high (2.0mM) medium Ca^{++} concentrations (Fig. 2.4B). Addition of 2.0mM calcium did not significantly alter the level nor the time course of c-*fos* mRNA expression (Fig. 2.4C). We next examined the effect of EGF, calcium and $1,25(OH)_2D_3$ on p53 mRNA expression. Addition of EGF in the presence of 0.15mM Ca^{++} produced an early (60 min) and progressive increase of p53 mRNA, maximal at 48h (Fig. 2.5A). However, $1,25(OH)_2D_3$ did not alter p53 mRNA levels significantly at any of the time points analyzed whether the cells were incubated with 0.15mM (Fig. 2.5A) or 2.0mM medium calcium concentrations (Fig. 2.5B). In contrast to the absence of an effect seen on c-*fos* mRNA after addition of 2.0mM calcium, p53 mRNA was significantly increased at 24 hours and was maintained at 48 h after calcium addition (Fig. 2.5C).

5) Effect of Ca^{++} and $1,25(OH)_2D_3$ on involucrin and p53 gene transcription

To determine whether the effects of these various factors were acting at the transcriptional level, we performed nuclear transcription run-on assays. After exposing the cells to 0.15 and 2.0mM Ca^{++} with and without 10^{-8}M $1,25(OH)_2D_3$ for 2h and 24h, nuclear extracts were labeled and hybridized to specific target DNA sequences, as shown in Fig. 2.6. Hybridization to pBR322 was absent and transcription of GAPDH, used as internal control was unaffected by Ca^{++} or $1,25(OH)_2D_3$. By contrast $1,25(OH)_2D_3$ increased involucrin gene transcription approximately 3.5 fold at 2h (Fig 2.6A) and 6 fold at 24h (Fig 2.6B), but did not affect p53 gene transcription. Ca^{++} increased both involucrin

and p53 gene transcription approximately 6 fold and 8 fold respectively, at 24h (Fig 2.6B).

DISCUSSION

In this study we examined the effect of $1,25(OH)_2D_3$ and calcium on parameters of cell growth and differentiation and on c-*fos* and p53 expression in normal human keratinocytes. Previous studies showed both the existence of a specific receptor for $1,25(OH)_2D_3$ (Pillai et al., 1988) and local production of the hormone by keratinocytes (Bikle et al., 1986). This secosteroid could therefore regulate growth and differentiation (Hosomi et al., 1983; Smith et al., 1986) of keratinocytes in an autocrine manner.

We observed that $1,25(OH)_2D_3$ is a potent growth inhibitor at low (0.15 mM) and intermediate Ca^{++} concentration (0.5 mM). However, the growth inhibition observed was similar for both Ca^{++} concentrations. In contrast, the combination of 2.0mM Ca^{++} and 10^{-8}M $1,25(OH)_2D_3$ completely abolished cell division. Therefore, it appeared that $1,25(OH)_2D_3$ initiated specific cellular events under conditions where normal human keratinocytes would normally proliferate and not differentiate. This calcium independent effect was supported by the synergistic effect of calcium and $1,25(OH)_2D_3$ seen at higher Ca^{++} concentrations. This effect is consistent with previous findings reported in normal human keratinocytes showing dose dependent inhibition of cell growth by $1,25(OH)_2D_3$ with a minimal effective dosage of around 10^{-8}M (Smith et al., 1988), a concentration used throughout the present study. The human keratinocytes used in our study displayed cell specific arrest in the G_0/G_1 phase of the cell cycle in response to $1,25(OH)_2D_3$. The arrest in the resting phase of the cycle was early and independent of the Ca^{++} concentration, demonstrating the specificity of the effect of $1,25(OH)_2D_3$ on growth inhibition by blocking entry into the S phase of the cell cycle.

We then assessed the effect of $1,25(OH)_2D_3$ and calcium on keratinocyte

differentiation. Normal human keratinocytes in culture proliferate in low Ca^{++} concentration and differentiate when ambient Ca^{++} is raised to or above 1.0mM (Hennings et al., 1980). Epidermal differentiation has previously been extensively studied using several key markers, including keratins (Fuchs and Green, 1980), involucrin (Rice and Green, 1977; Rice and Green, 1979) and fillagrin (Harding and Scott, 1983). Involucrin is a 68 kd precursor protein of the keratinocyte cornified envelope (Rice and Green, 1977; Rice and Green, 1979) and correlates positively with the process of differentiation. Involucrin mRNA expression was recently shown to be an excellent marker of keratinocyte differentiation under the influence of calcium or phorbol esters (Younous and Gilchrest, 1992). In the present study, we analyzed involucrin expression over time in the presence of various concentrations of Ca^{++} and 10^{-8}M $1,25(OH)_2D_3$. Calcium or $1,25(OH)_2D_3$ strongly stimulated involucrin expression to similar levels, corroborating our immunocytochemical data which demonstrated positive staining of differentiated cells with markers of keratinization. These observations are consistent with the increased involucrin protein levels observed previously in differentiated cells (Banks-Schlegel and Green, 1980). Mitogenic stimuli and $1,25(OH)_2D_3$, or its metabolites, are also known to upregulate the abundance of vitamin D receptors (Krishnan and Feldman, 1991; Costa et al., 1985). This may contribute to the sustained overexpression of involucrin mRNA, in the presence of $1,25(OH)_2D_3$ and EGF, in our model. Furthermore nuclear run-on analysis demonstrated that the effect of Ca^{++} and $1,25(OH)_2D_3$ occur at the level of gene transcription. Nevertheless, it should be noted that the observed effect of $1,25(OH)_2D_3$ in the nuclear run-on assays is much more pronounced than the effect observed in the Northern blot analysis. Since steady state mRNA levels seen on Northern blots reflect a balance between the rates of transcription and degradation, these results may suggest a

relatively more rapid rate of involucrin mRNA degradation. Further studies on Involucrin mRNA stability should help clarify this issue. The transcriptional regulation of involucrin by $1,25(OH)_2D_3$ suggests the presence of a Vitamin D response element(s) (VDRE(s)) in the promoter region of this gene. Such cis-acting elements have been previously identified in the osteocalcin (Kerner et al., 1989) and osteopontin genes (Noda et al., 1990) but have not yet been defined in the 5' flanking region of the involucrin gene.

We also showed that the EGF effects on keratinocyte cell growth were preceded by a rapid and transient increase in c-*fos* mRNA levels. This is consistent with the effects of various growth stimulants on the proto-oncogenes, c-myc and c-*fos*, in these and other cell types (Tramontano et al., 1986; Heldin and Westmark, 1988), suggesting that these proto-oncogenes may play an important role in the growth and differentiation of a variety of cells (Müller et al., 1982; Alt et al., 1986). Earlier studies from this laboratory showed that $1,25(OH)_2D_3$ inhibited c-myc expression in primary cultures of parathyroid cells (Kremer et al., 1989), which preceded the growth inhibition observed with $1,25(OH)_2D_3$. More recently, $1,25(OH)_2D_3$ was shown to inhibit c-myc proto-oncogene expression in normal human keratinocytes (Matsumoto et al., 1990). Furthermore, inhibition of c-myc expression by addition of antisense oligonucleotides to the culture medium of HeLa cells produced a growth arrest in G_0/G_1 (Heikila et al., 1987), implying a causal relationship between c-myc expression and cellular growth. However, the relationship between c-*fos* and cellular differentiation was less clear. *fos* is known to modulate gene transcription by association with another proto-oncogene product, *jun*, to form a heterodimer or AP_1 protein complex (Sassone-Corsi et al., 1988). Earlier studies showed that the c-*fos* gene was stimulated by factors that strongly influence cellular differentiation in PC12 cells, such as nerve growth factor (Milbrandt, 1986). It was also shown that $1,25(OH)_2D_3$ produced a

sustained elevation of c-*fos* mRNA in HL 60 leukemic cells (Brelvi et al., 1986), associated with their monocytic differentiation. However, a direct effect of *fos* on cellular differentiation was not yet demonstrated. In the present study, addition of $1,25(OH)_2D_3$ to growth factor stimulated keratinocytes increased c-*fos* expression; this effect was sustained over a long period. A sharp rise in *fos* expression was seen as early as 60 min after addition of growth factor but the combination of $1,25(OH)_2D_3$ and growth factors were clearly additive on c-*fos* mRNA levels. In contrast, addition of Ca^{++} did not significantly change *fos* mRNA levels whether $1,25(OH)_2D_3$ was present or not. The absence of effect of calcium on c-*fos* mRNA was consistent with previous observations in mouse (Dotto et al., 1986) and human keratinocytes (Younus and Gilchrest, 1992) and suggested that $1,25(OH)_2D_3$ could exert a specific effect on the expression of a proto-oncogene frequently associated with cellular differentiation. It is tempting to conclude that these results are mechanistically related. But further studies, such as those using antisense oligonucleotides (Heikkila et al., 1987; Pietenpol et al., 1990) should be used to assess the specificity of c-*fos* induced promotion of cell differentiation by $1,25(OH)_2D_3$ in normal human keratinocytes.

Finally we examined the influence of EGF, calcium and $1,25(OH)_2D_3$ on expression of p53 in keratinocytes. Recent evidence suggest that wild type p53 functions as a tumor suppressor gene (Baker et al., 1990; Finlay et al., 1989; Eliyahu et al., 1989; Lane and Benchimol, 1990) and that its inactivation by mutation or deletion is associated with neoplastic growth. However, p53 may also play an important role in growth of normal cells, as suggested by its regulated expression in a number of normal cells (Rotter et al., 1980; DeLeo et al., 1979; Oren et al., 1981). Previous studies showed that wild type p53 is necessary for the mitogenic response of lymphocytes (Milner 1984) and 3T3 cells

(Mercer et al., 1982; Reich et al., 1984). p53 subcellular localization varies throughout the cell cycle, accumulating in the nucleus following the initial step of DNA synthesis, around the beginning of the S phase; re-accumulating in the cytoplasm during the resting phase. This suggests that the protein is spatially regulated during the cell cycle (Shaulsky et al., 1990). In the present study we observed that p53 mRNA is rapidly induced by EGF, which is a necessary mitogen for the growth of keratinocytes in serum free conditions (Tsao et al., 1982). This close cell-cycle association also supports a role for p53 in normal keratinocyte cell growth. Since p53 acts normally as a growth suppressor, its induction by mitogenic stimuli, that we and others have observed, may represent a critical step in the control of cell growth to counteract the effect of mitogenic stimuli. However, the precise mechanism by which EGF modulates p53 gene transcription remains elusive and requires further studies. We also observed that p53 mRNA levels were sustained long after EGF stimulation; in sharp contrast c-*fos* mRNA stimulation by mitogens returns to basal levels quickly after its early peak. Recent data indicate that the p53 gene product may mediate the repression of c-*fos* mRNA, possibly acting as a transcriptional regulator of the c-*fos* promoter (Ginsberg et al., 1991). The temporal pattern of c-*fos* and p53 mRNA observed in our study would be consistent with such a mechanism. Finally, we observed that raising medium Ca^{++} from 0.15 to 2.0 mM significantly enhanced both steady-state p53 mRNA levels and the rate of p53 gene transcription, indicating that p53 is at least in part under the transcriptional control of Ca^{++}. These results are highly suggestive of a role for p53 in calcium mediated growth inhibition and induction of differentiation in keratinocytes but will require more direct evidence, such as the inhibition of intra-cellular p53 content by micro-injection of p53 monoclonal antibodies (Mercer et al., 1982) or introduction of plasmids coding for antisense p53 (Shohat et al., 1987).

Our data nevertheless clearly suggest that $1,25(OH)_2D_3$ and calcium, which are essential in the control of growth and differentiation of normal human keratinocytes, act through a network of specific genes which are thought to be important in the control of the cell cycle. These co-ordinated responses, under the influence of $1,25(OH)_2D_3$ and calcium, seem to simultaneously control entry into the S phase of the cell cycle and at the same time trigger signals to initiate the differentiation process. Further studies aimed at blocking and/or activating *fos* and/or p53 genes should help clarify whether $1,25(OH)_2D_3$ and calcium affect growth and differentiation by a direct modulation of the expression of these cell-cycle associated-genes.

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Since the effects of 1,25-dihydroxyvitamin D_3 on normal human keratinocytes have been well characterized by us as well as other groups, we next looked at these effects on keratinocytes as they progress from the normal to the malignant phenotype.

CHAPTER 3

RELATIVE RESISTANCE TO 1,25-DIHYDROXYVITAMIN D_3 IN A KERATINOCYTE MODEL OF TUMOR PROGRESSION

ABSTRACT

We have examined the effect of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) on mitogen stimulated growth and on c-*myc* proto-oncogene expression in a keratinocyte model of tumor progression. A dose-dependent inhibition of cell growth by $1,25(OH)_2D_3$ was demonstrated in both established (HPK1A) and malignant (HPK1A-*ras*) cells. However this inhibition was observed with the addition of $1,25(OH)_2D_3$ at a higher concentration in HPK1A-*ras* cells than in HPK1A cells. Cell cycle analysis revealed a blockage of the normal progression of the cell cycle from G0 to S phase in the presence of $1,25(OH)_2D_3$. A higher concentration of $1,25(OH)_2D_3$ was required in HPK1A-*ras* cells to overcome the mitogen stimulated progression into S phase, when compared to HPK1A cells. Analysis of c-*myc* messenger RNA revealed a strong inhibition of its expression at early time points with higher concentrations of $1,25(OH)_2D_3$ being required to obtain an inhibition in HPK1A-*ras* cells similar to that obtained in HPK1A cells. $1,25(OH)_2D_3$ receptor characterization by sucrose gradient analysis and equilibrium binding demonstrated the presence of a single 3.7S protein with similar receptor numbers and affinity in both cell lines. These observations therefore demonstrate that an alteration of the growth inhibitory response to $1,25(OH)_2D_3$ occurs when keratinocytes acquire the malignant phenotype and suggest that the alteration lies beyond the interaction of the ligand and its steroid receptor. In additon, resistance to $1,25(OH)_2D_3$ was also observed in the expression of the cell-cycle associated oncogene c-*myc*. These studies may therefore have important implications *in vivo* in the development and growth of epithelial cell cancers.

INTRODUCTION

Carcinogenesis is a multistep process (Nowell, 1970) and epidemiological studies have suggested that five or six independent steps are required for acquisition of the malignant phenotype (Peto, 1977). One of the mechanisms which has been implicated in neoplastic development is the co-operative action of two or more oncogenes (Land et al., 1983) which are thought to act in a positive way to control cell growth in response to known mitogenic factors (Alt et al., 1986). However, equally important to the tumor development process may be negative regulators of cell growth which oppose the action of known growth factors. 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) has been identified recently as an important factor controlling the growth of HL-60 human promyelocytic leukemic cells and their differentiation into the monocytic cell type (Rigby et al., 1984). This steroid has also been shown to have antiproliferative capabilities in normal human keratinocytes (Hosomi et al., 1983; Smith et al., 1986). Specific receptors for $1,25(OH)_2D_3$ have been demonstrated in both normal and malignant murine keratinocytes (Clemens et al., 1981) which are thought to mediate the biological effects of the hormone. In addition to its inhibitory action on growth, previous studies have demonstrated an altered expression of specific oncogenes, such as c-*myc*, in response to $1,25(OH)_2D_3$ (Kremer et al., 1989; Katakami et al., 1988; Sebag et al., 1994). In normal human keratinocytes alterations in growth elicited by $1,25(OH)_2D_3$ were shown to be accompanied by a rapid inhibition of c-*myc* (Matsumoto et al., 1990) expression. In the present study, we have examined the effects of $1,25(OH)_2D_3$ on growth and c-*myc* expression in a human keratinocyte model of tumor cell progression (Dürst et al., 1987; Dürst et al., 1989). In this model, primary human foreskin keratinocytes were established

as an immortalized cell line following transfection with HPV type 16 and subsequently tranformed by an activated *ras* oncogene. Using this model system, we have studied the effects of factors previously shown to modulate the growth of normal human keratinocytes (Kremer et al., 1991), such as epidermal growth factor (EGF), fetal bovine serum (FBS) and $1,25(OH)_2D_3$.

MATERIALS AND METHODS

1) Culture of human keratinocyte cell lines

The HPK-1A cell line was established from normal human keratinocytes by stable transfection with human papillomavirus type I6 (HPV16) (Dürst et al., 1987). Despite acquiring an indefinite lifespan in culture these cells are non-tumorigenic when injected into nude mice (Table 1). These immortalized cells were subsequently transformed into the malignant *HPK1A-ras* cell line following transfection with a plasmid carrying an activated *H-ras* oncogene (Dürst et al., 1989). In addition to forming colonies in soft agar the malignant HPK1A-*ras* cells produce invasive squamous cell carcinoma when transplanted into nude mice (Table 1). Both immortalized (HPK1A) and malignant (HPKIA-*ras*) cell lines were seeded and grown in Dulbecco's modified Eagle medium (DMEM) (GIBCO Labs; Grand Is., NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and passaged once or twice weekly.

2) XTT-Microculture Tetrazolium assay for cell growth.

This assay, performed as described previously (Scudiero et al., 1988), assesses cellular growth on the basis of the intensity of a colorimetric reaction resulting from the reduction of a tetrazolium reagent (XTT, Polysciences, Warrington, PA) to a soluble formazan salt by growing cells, absorbance being directly proportional to cell density. Linearity of the reaction over a wide range of cell numbers was assessed in preliminary experiments. Briefly, cells were seeded at $2x10^3$ cells/lOO CLI into 9-well microtiter plates in DMEM containing 10% FBS. After 24h in basal conditions, medium was removed and replaced with fresh DMEM supplemented with 10ng/ml EGF and varying concentrations o f

$1,25(OH)_2D_3$. After 24h 5 ml of XTT was made at 1 mg/ml in pre-warmed basal medium and mixed with 25 µL of phenyl-methyl- sulfate (PMS) (1.53 mg/ml in PBS, Sigma Chem. Co., St. Louis, MO). 50 µl of this mixture was added to each well and incubations continued for 4 h. at 37°C. Absorbance at 450 nm was measured using a Biorad microplate reader (Biorad Canada Ltd., Mississauga, ON). Background absorbance was subtracted at each point using absorbance at 655 nm.

3) [^{3}H]thymidine incorporation into DNA

Cells were seeded at a density of $2x10^4$ cells/well for HPK1A and lx104 cells/well for HPK1A-*ras* in 24 well cluster plates and grown to 20% confluence. Following 24h in basal conditions fresh medium containing 10 ng/ml EGF without or with varying concentrations of $1,25(OH)_2D_3$ was added to cultured cells and incubations continued for 24-72h. [^{3}H]Thymidine (1µCi/ml; New England Nuclear, Boston, MA) was added to cultured cells during the last two hours of incubation. After aspiration of the medium cells were washed twice with cold Hank's Balanced Salt Solution and then incubated in 5% cold trichloroacetic acid for 15 min. After aspiration of the trichloroacetic acid, the cell pellets were dissolved in 1 ml 0.6N NaOH and an aliquot removed for liquid scintillation counting. Triplicate wells/plate were trypsinized and aliquots counted to correct the [^{3}H} thymidine cpm for cell number. Results were then expressed as % of EGF stimulated activity.

4) Flow cytometry

Cells were seeded at a density of 10^5 and $5x10^4$ cells/well for HPK1A and HPK1A-*ras* respectively in 6 well cluster plates and grown to 20% confluence. Following a 24h period

in basal conditions fresh DMEM supplemented with 10 ng/ml EGF and varying concentrations of $1,25(OH)_2D_3$ was added to the cultures for 24h. Following trypsinization, cells were centrifuged at low speed (600g), rinsed once with PBS and stained according to the technique described by Vindeløv (Vindeløv, 1977). The pellet was resuspended in 1 ml of the following solution added dropwise while vortexing: 3.5 mM Tris, 7.5 µM propidium iodide (Calbiochem) 0.1% NP40 (Sigma Chem. Co., St. Louis, MO) 700 u/L RNAse (Boehringer Mannheim Canada) and 10 mM NaCl. After standing at least 10 minutess on ice, the nuclei were analyzed in a FACScan (Becton Dickinson Inc., Oxnard CA). Calculation of percentage distribution in different phases of the cell cycle was performed with CellFit software (Becton Dickinson Inc.) using a SOBR (sum of broadened rectangles) fit.

5) RNA analysis

Cells were plated in the same manner as for flow cytometry studies, trypsinized at timed intervals, centrifuged at low speed (6OOg), rinsed with phosphate buffered saline (PBS) and lysed with a mixture of 4 M guanidine thiocyanate, 25 mM trisodium citrate, 1 mM EDTA, and 0.1 M ß-mercaptoethanol (GTC mixture). GTC extracts were stored at 700C for subsequent RNA analysis by dot blot hybridization or Northern blot hybridization.

For Northern Blot analysis, GTC extracts were purified by cesium chloride gradient ultracentrifugation (Chirgwin et al., 1979) and 10µg of total RNA electrophoresed on a 1.1% agarose-formaldehyde gel. Ethidium bromide was added to each sample before electrophoresis to permit detection by UV transillumination and to ensure equivalent quantities of RNA were loaded into all lanes. RNA was transferred by blotting to a nylon membrane (Nytran). For dot blot hybridization, samples were processed as described

earlier (Kremer et al., 1989). The filters were air-dried, baked at 80°C for 2 h, and then hybridized with a ClaI-EcoRI restriction fragment encoding exon III of the human c-*myc* gene (Dalla-Favera et al., 1982). This probe was labelled with [^{32}P] dCTP (ICN Biomedicals of Canada Ltd., Mississauga, ON) using a random primer kit (Amersham, Canada). After incubation at 42'C for 24 h, filters were washed twice at room temperature for 30 min each in 2xSSC, 0.1% SDS (1 x SSC is 0.15 M sodium chloride, 0.015 M trisodium citrate) and then twice for 30min each in 0.5xSSC, 0.1% SDS at 55°C. Autoradiography of filters was carried oul at -70°C using Kodak XAR (Eastman Kodak Co., Rochester, NY) films and two intensifying screens. The intensity of the dot blots was analyzed by laser densitometry (Ultroscan XL, LKB instruments Inc., Gaithersburg, MD). Filters were also probed with an 800bp BamH1 restriction fragment of rat cyclophilin as a control for c-*myc* mRNA changes.

6) 1,25 dihydroxyvitamin D, receptor characterization.

Binding studies were performed according to a previously published method (Malloy et al., 1989). Confluent cells were scraped and suspended in 1 ml of a buffer containing 300 mM/L KCL, 10 mM/L Tris, 1 mM/L EDTA, 5 mM/L dithiothreitol and 10 mM/L sodium molybdate pH 7.4 (KTEDM). Cells were sonicated in the buffer for 5 x 30 sec intervals on ice at maximum setting using a Brinkman sonicator and centrifuged at 80,000 g for 60 minutes at 4°C. 180µl of cytosol adjusted to contain 500 µg of protein containing vitamin D receptors (VDR) was incubated with 10,000 cpm [^{3}H] $1,25(OH)_2D_3$ (New England Nuclear, Boston, MA) in 20µl of ethanol and increasing concentrations of unlabeled $1,25(OH)_2D_3$ for 16 hours at 4°C. Non-specific binding was assessed in the presence of 200 fold excess of non-radioactive $1,25(OH)_2D_3$. Bound and unbound

hormones were separated using 100 µl of dextran-coated charcoal (0.15% dextran and 1.5% charcoal in KTEDM). After 15 minutes of incubation followed by centrifugation, 100 µl of the supernatant was counted by liquid scintillation spectroscopy.

Sucrose density gradient analysis was performed using a linear 4-20% gradient of sucrose solutions in KTEDM buffer. 180 µl of cytosol was incubated 16h at 4°C with 20µl of ethanol containing 10,000 cpm [^{3}H] 1,25$(OH)_2D_3$ with or without excess 1,25$(OH)_2D_3$ (60 nM). Following removal of unbound hormone with dextrancharcoal supernatants were layered on top of gradients and centrifuged at 257,000 g for 18 h at 4°C in a Beckman ultracentrifuge (Palo Alto, CA). The radioactivity in 0.1 ml fractions was determined by liquid scintillation spectroscopy.

8) Statistical analysis

Statistical significance was determined by one way analysis of variance (ANOVA) or by Student's t-test and results were expressed as mean±SEM of replicate determinations. A probability value of <0.01 was considered to be significant.

Figure 3.1

Effect of $1,25(OH)_2D_3$ On EGF stimulated cell growth in HPK1A and HPK1A-*ras* cells.

Following 24 h. in basal conditions (no growth factors), fresh DMEM containing EGF (10 ng/ml) without or with increasing concentrations of $1,25(OH)_2D_3$ was added to cultures of HPK1A ( ) and HPK1A-*ras* ( ) cells at time 0. Panel A represents formazan production assessed at 24h using the XTT assay described in **Materials and Methods**. Panel B represents [^{3}H]thymidine incorporation corrected for cell numbers and expressed as a percent of EGF stimulated activity (100%). Panel C represents cell numbers assessed at 96h and expressed as percent of EGF stimulated cell numbers (100%). Each value represents the mean±SEM of 4-6 determinations and is representative of 3 different experiments. Asterisks indicate a significant difference from control values (EGF stimulated activity) and open circles indicate a significant difference between HPKIA and HPK1A-*ras* cells at the $1,25(OH)_2D_3$ concentration indicated.

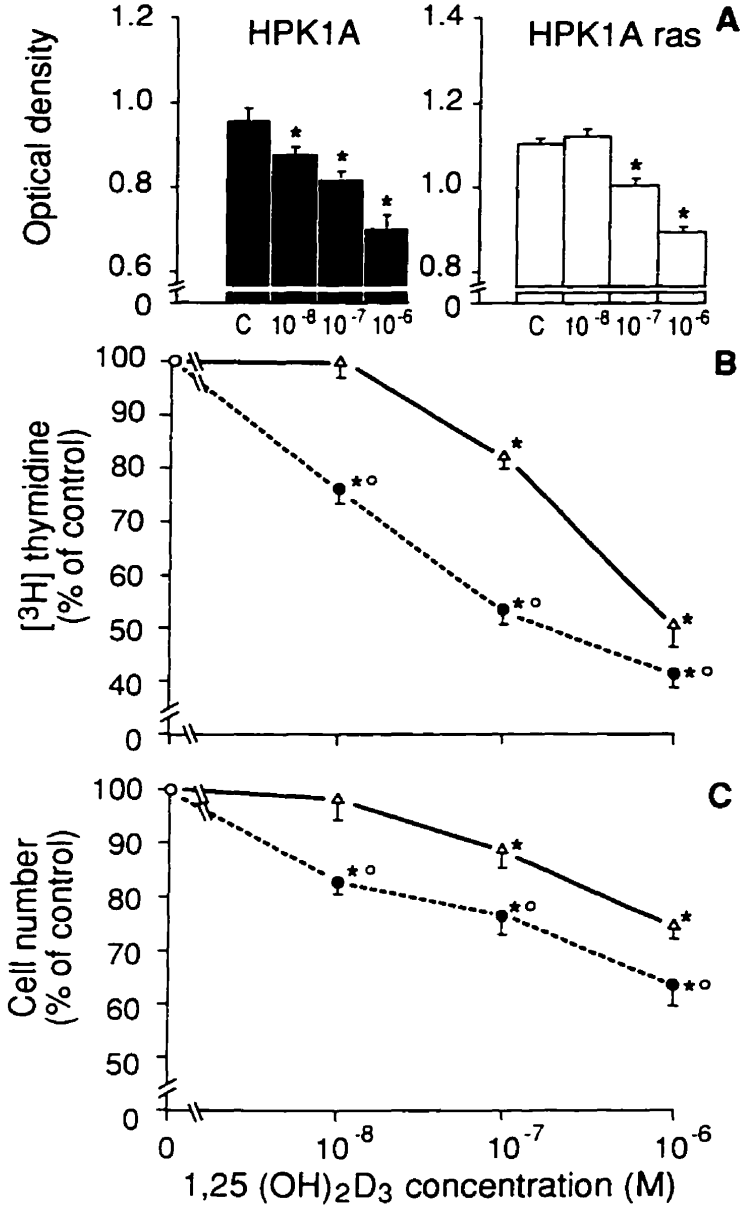
A
HPK1A
HPK1A ras
Optical density
1.2
1.0
0.8
0.6
0
1.4
1.2
1.0
0.8
0
C
10-8
10-7
10-6
B
[3H] thymidine (% of control)
100
90
80
70
60
50
40
0
C
Cell number (% of control)
100
90
80
70
60
50
0
0
10-8
10-7
10-6
1,25 (OH)2D3 concentration (M)

Figure 3.2

Cell cycle analysis of EGF stimulated HPK1A and *HPKIA-ras* cells in the absence and presence of $1,25(OH)_2D_3$. Following 24 h. in basal conditions (no growth factors) ( ), fresh DMEM supplemented with EGF (10 ng/ml) ( ) or EGF plus $1,25(OH)_2D_3$ at 10^{-8} M( )or 10^{-6} M ( ) was added to cultures of HPK1A (panel A) and *HPK1A-ras* (panel B) cells at time 0. 24h later cells were trypsinized and analyzed by flow cytometry as described in **Materials and Methods.** Results are expressed as percentage of cells distibuted in G0/G1 phase (left panel) and S phase (right panel) of the cell cycle. Each bar represents the mean±SEM of 4 determinations and is representative of duplicate experiments.

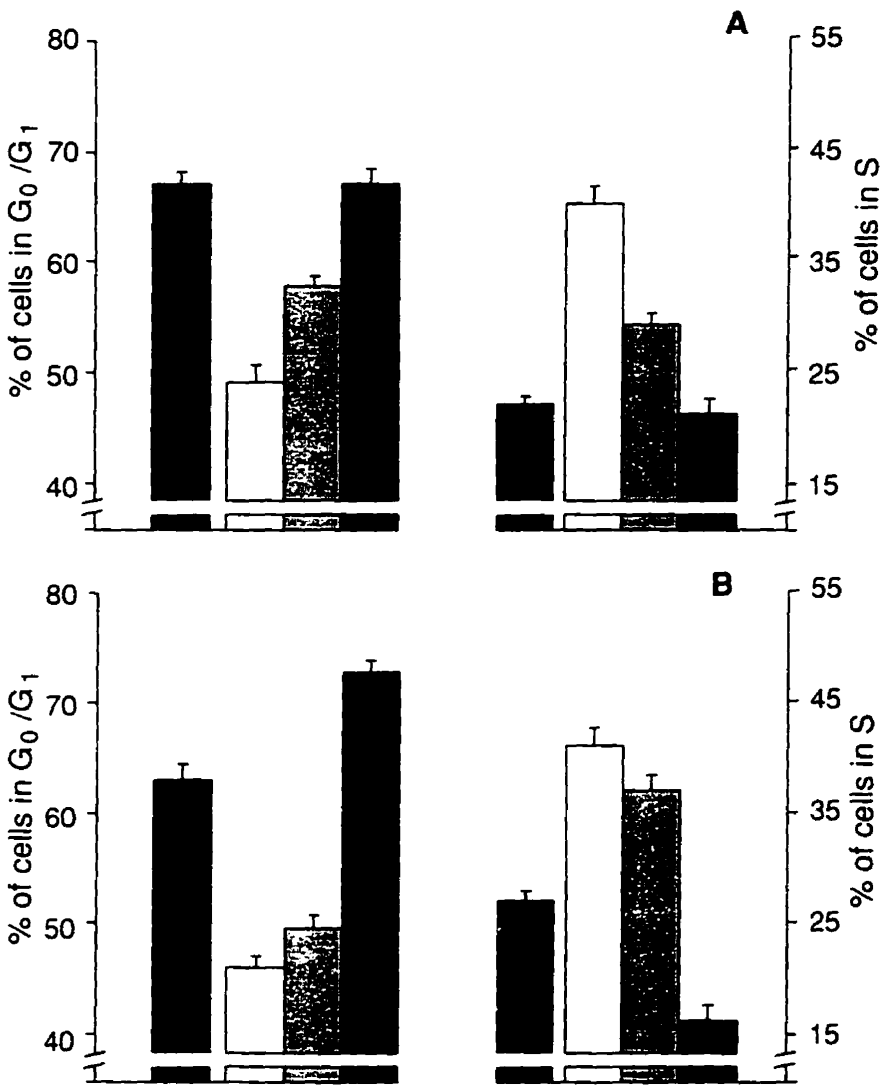
A
B
80
70
60
50
40
55
45
35
25
15
% of cells in G_0/G_1
% of cells in S

Figure 3.3

Dot blot analysis of c-*myc* mRNA in HPK1A and HPK1A-*ras* keratinocytes.

Following 24h in basal conditions (B), fresh DMEM supplemented with 10% FBS was added to cultures of HPK1A ( ) and HPK1A-*ras* ( ) cells at time 0. Total cellular RNA was extracted from equal numbers of cells removed at timed intervals as described in **Materials and Methods**. Nytran filters were probed with a ClaI-EcoRI restriction fragment encoding exon III of the c-*myc* gene. *Data is representative of 3 different experiments.*

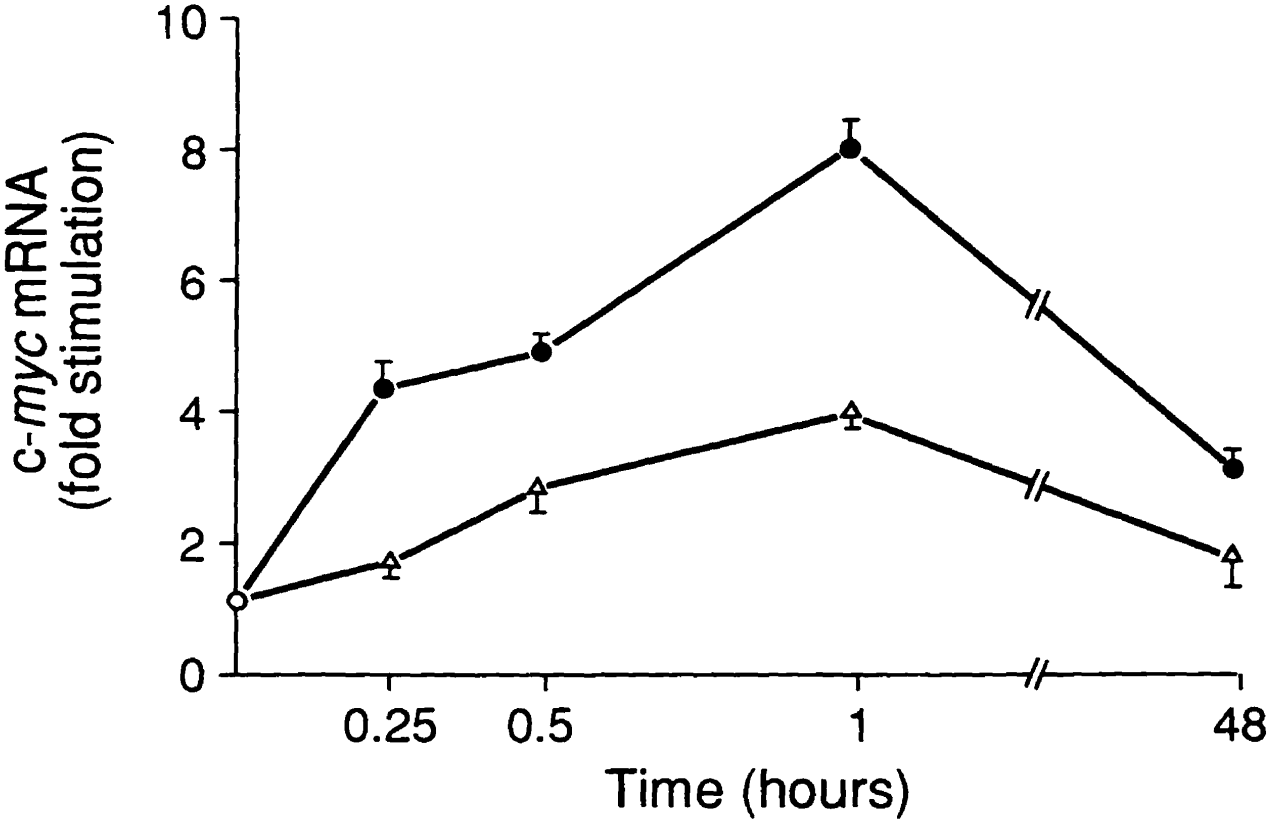
c-myc mRNA
(fold stimulation)
10
8
6
4
2
0
0.25
0.5
1
48
Time (hours)

Figure 3.4

Dot blot analysis of c-*myc* mRNA in HPK1A and HPK1A-*ras* cells treated with varying concentrations of $1,25(OH)_2D_3$. Following 24 h. in basal conditions (c), fresh DMEM containing 10% FBS without (0) or with increasing concentrations of $1,25(OH)_2D_3$ was added to cultures of HPK1A ( ) and HPK1A-*ras* ( ) keratinocytes. GTC extracts of equal numbers of cells collected at 6h were subjected to dot blot analysis as described in Materials and Methods. Filters were hybridized with a Cla1EcoR1 restriction fragment encoding exon III of the c-*myc* gene. Each bar represents the mean± SEM of triplicate determinations and data is representative of 3 different experiments. Asteriks indicate a significant difference from incubations performed in the absence of $1,25(OH)_2D_3$ (0) and open circles indicate a significant difference from basal (C).

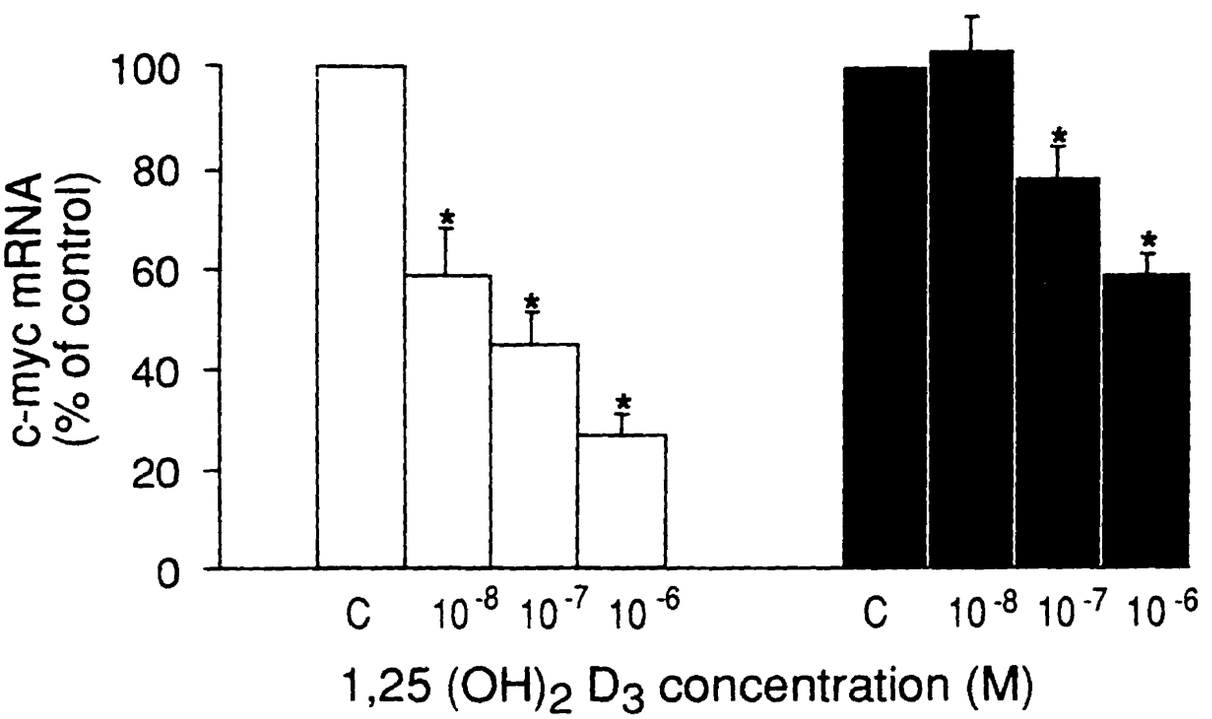
c-myc mRNA
(% of control)
100
80
60
40
20
0
C
10-8
10-7
10-6
C
10-8
10-7
10-6
1,25 (OH)2 D3 concentration (M)

Figure 3.5

Northern analysis of c-*myc* mRNA in HPK1A and HPKIA-*ras* keratinocytes.

Following 24h in basal conditions (0), fresh DMEM supplemented with10%FBS with (+) or without (-) 10^{-7}M $1,25(OH)_2D_3$ was added to cultures of HPK1A (panel A) and HPK1A-*ras* (panel B) cells at time 0. Total cellular RNA was extracted from cells removed at timed intervals as described in Materials and Methods. 10μg/lane of RNA was electrophoresed on a 1,1% Agarose-Formaldehyde gel, blotted onto a Nytran filter and probed as in figure 3.4. Ethidium bromide stained gels demonstrated equivalent quantities of RNA loaded into all lanes.

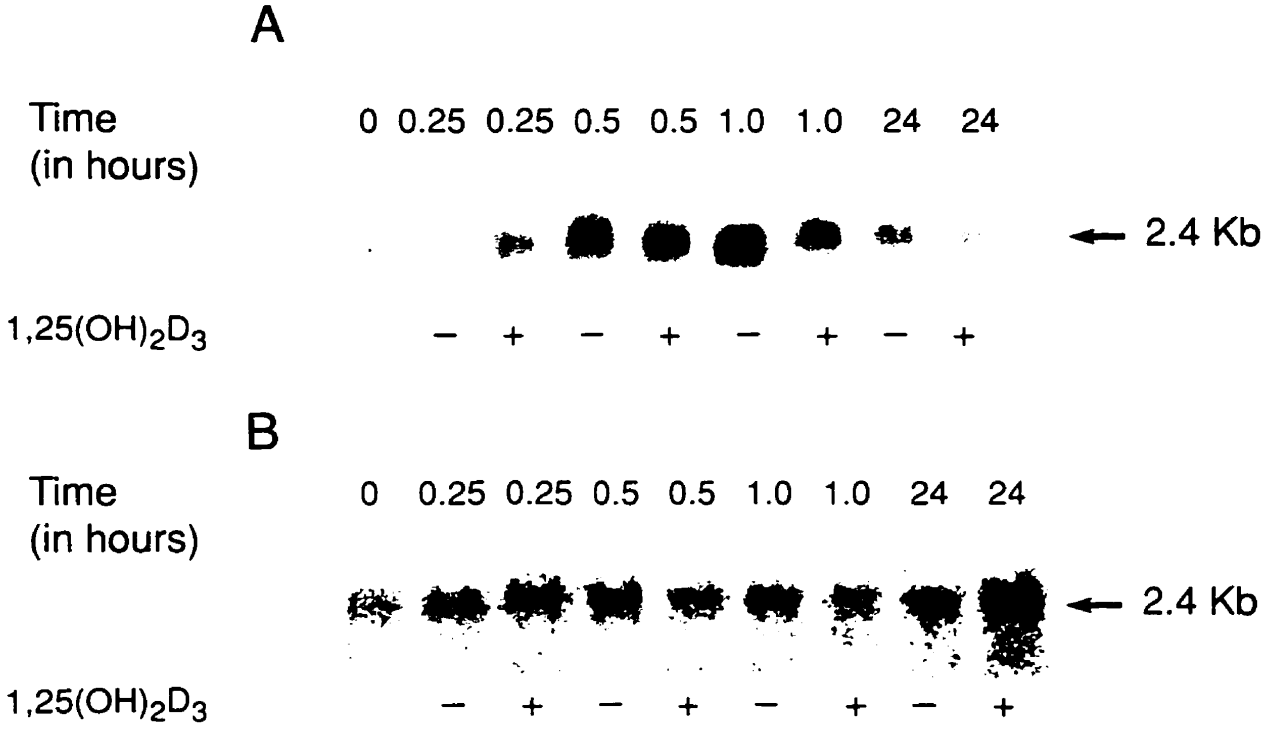
A
Time
(in hours)
0 0.25 0.25 0.5 0.5 1.0 1.0 24 24
2.4 Kb
1,25(OH)2D3
– + – + – + – +
B
Time
(in hours)
0 0.25 0.25 0.5 0.5 1.0 1.0 24 24
2.4 Kb
1,25(OH)2D3
– + – + – + – +

Figure 3.6

Sucrose density gradient analysis of [^{3}H]1,25(OH)$_2$D$_3$ binding to cytosolic receptors in HPK1A and HPK1A-*ras* cells. Binding studies were performed using 500 μg of cytosolic extract from HPK1A (panel A) and HPK1A-*ras* (panel B) incubated with 10,000 cpm [^{3}H]1,25(OH)$_2$D$_3$ in the absence ( ) or presence ( ) of 60nM unlabeled 1,25(OH)$_2$D$_3$ as described in Materials and Methods. Unbound hormone was removed with dextran-coated charcoal before centrifugation through linear 4-20% sucrose gradients. Sedimentation coefficients (3.7S) were estimated using a ^{14}C ovalbumin standard.

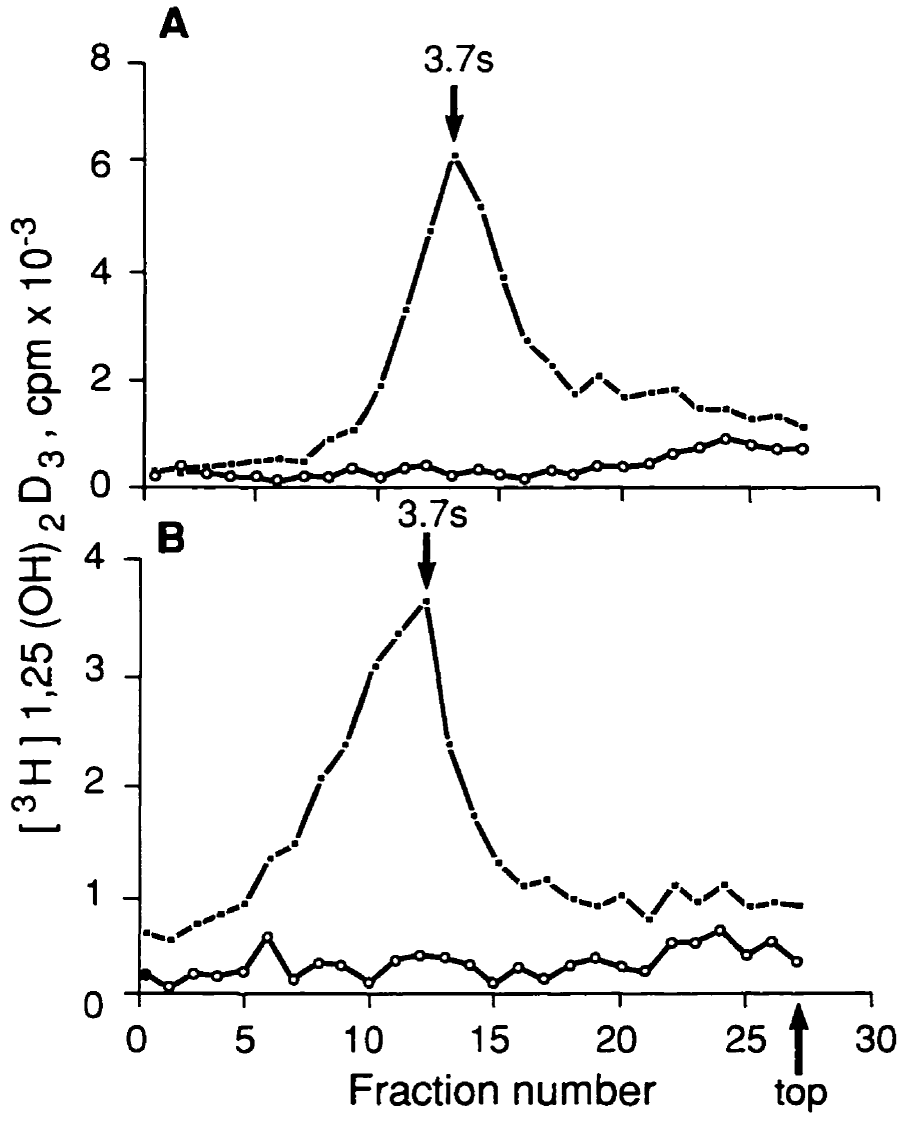
A
3.7s
8
6
4
2
0
B
3.7s
4
3
2
1
0
0
5
10
15
20
25
30
Fraction number
top
[3H] 1,25 (OH)2 D3, cpm x 10-3

Figure 3.7

Scatchard analysis of binding of [^{3}H]1,25(OH)$_2$D$_3$ to cytosolic receptors in HPK1A and HPK1A-*ras* cells. Binding studies were performed using 500μg of cytosolic extract from HPK1A (panel A) and HPK1A-*ras* (panel B) in the absence or presence of increasing concentrations of unlabeled 1,25(OH)$_2$D$_3$. Non-specific binding was assessed in the presence of 60nM unlabeled 1,25(OH)$_2$D$_3$.

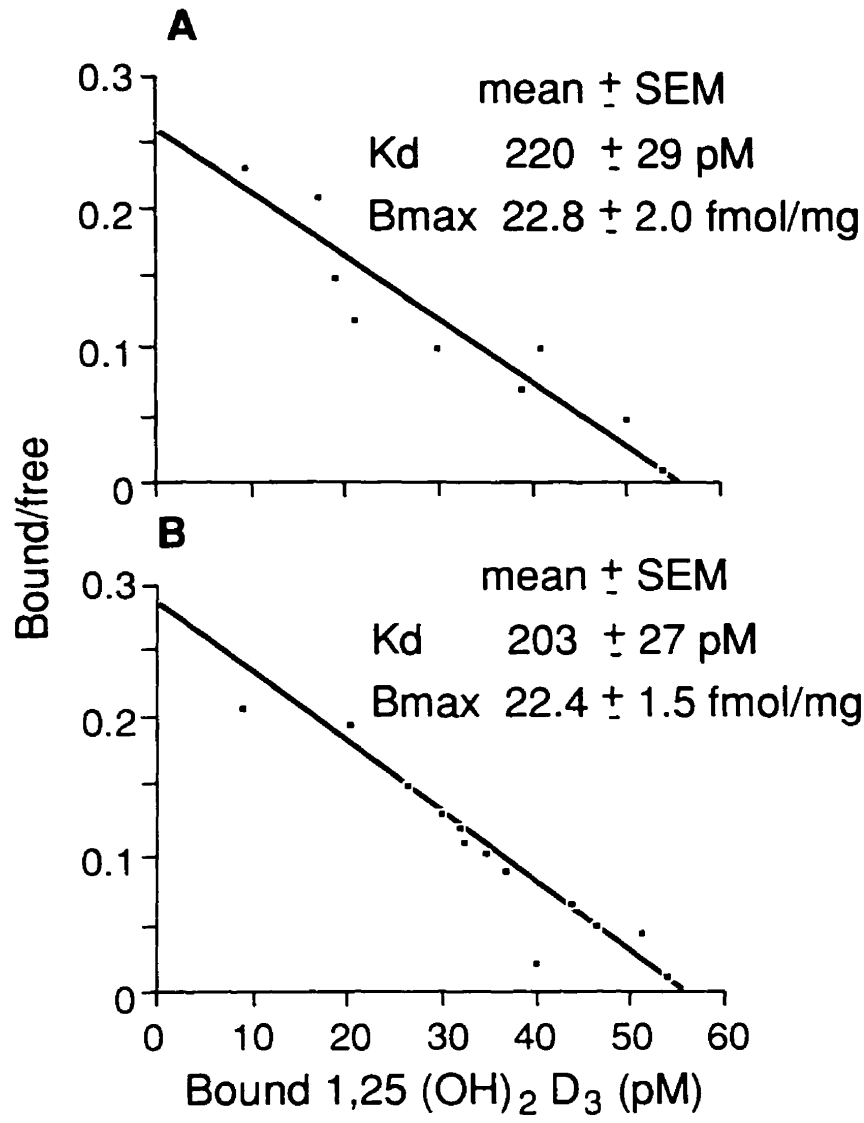
A
mean ± SEM
Kd 220 ± 29 pM
Bmax 22.8 ± 2.0 fmol/mg
B
mean ± SEM
Kd 203 ± 27 pM
Bmax 22.4 ± 1.5 fmol/mg
Bound/free
0.3
0.2
0.1
0
0 10 20 30 40 50 60
Bound 1,25 (OH)2 D3 (pM)

Table 3.1

Human Keratinocyte Model of Tumor Progression

	Normal		Immortalized		Transformed
		HPV-16		*pSV2ras*	
	NHK	→	HPK1A	→	HPK1A-*ras*
1. Stratification	+++		–		–
2. Differentiation	+++		++		+
3. Passages in Culture	+		+++		+++
4. Tumoreginicity	–		–		+++

Table 3.2

Time course of $1,25(OH)_2D_3$ effect on EGF stimulated [3H]thymidine uptake.

Following 24h in basal conditions (no growth factors), fresh medium containing EGF (10ng/ml) and 10^{-7}M $1,25(OH)_2D_3$ was added to cultures of HPK1A and HPK1A-*ras* cells. [3H]thymidine uptake was assessed at timed intervals and results expressed as a percentage of EGF stimulated activity (C). Each value represents the mean±SEM of 6 determinations and is representative of 3 separate exeriments.

	[3H]thymidine (% of control)			
Time (hours)	C	24	48	72
HPK1A	100	72±4[a]	55±2[a]	47±2[a]
HPK1A-ras	100	70±1[a]	64±4[a,b]	88±1[a,b]

a. Significant difference from control (C)

b. Significant difference from HPK1A cells at the times indicated.

RESULTS

Effects of $1,25(OH)_2D_3$ on cell growth

Addition of increasing concentrations of $1,25(OH)_2D_3$ to the culture medium produced a dose-dependent inhibition of cell growth in cultures of both established (HPK1A) and malignant (HPK1A-*ras*) keratinocytes (Fig 1). Formazan production (Fig 3.1A), [^{3}H]thymidine incorporation (Fig 3.1B) and cell numbers (Fig 3.1C) were all significantly reduced by addition of 10^{-8}M 1 $1,25(OH)_2D_3$ to cultures of HPK1A cells whereas 10^{-7}M $1,25(OH)_2D_3$ was required to inhibit these parameters of cell growth in cultures of HPKIA-*ras* cells. In addition, the degree of inhibition at any one dose of $1,25(OH)_2D_3$ was greater in HPK1A than in HPK1A-*ras* cultures. The time course of inhibition of [^{3}H]thymidine incorporation was also different in the two cell lines (Table 3.2). The established (HPK1A) cells demonstrated a progressive and sustained response to 10^{-7}M $1,25(OH)_2D_3$ over 72h compared with the transient effect noted in the malignant (HPK1A-*ras*) cells.

Effect of $1,25(OH)_2D_3$ on the cell cycle,

Using flow cytometry we next examined the effect of $1,25(OH)_2D_3$ on the progression of HPKIA and HPK1A-*ras* cells through the cell cycle (Fig 3.2). In the quiescent state the majority of cells were in the Go/G1 phase in both HPKIA (Fig 3.2A) and HPKIA-*ras* (Fig 3.2B) cultures. Addition of 10 ng/ml EGF to the culture medium for 24h resulted in a shift into S phase in both cell lines. Addition of 10^{-8}M $1,25(OH)_2D_3$ along with the EGF inhibited the shift into S phase in HPK1A cells but had little effect in HPK1A-*ras* cells. However, addition of 10^{-6}M $1,25(OH)_2D_3$ with EGF resulted in an

accumulation of cells in Go/G1 and a reduction of cells in S phase in both HPKIA and HPKIA-*ras* cultures.

Dot-Blot analysis.

Total cellular RNA extracted from equal numbers of HPK1A and HPKIA-ras cells at timed intervals, following stimulation with 10% FBS, revealed a rapid and transient induction of c-*myc* mRNA in both cell lines. When expressed as fold stimulation above basal, the induction was greater in HPKIA than in HPKIA-ras cultures (Fig 3.3). Dose-dependent decreases in this FBS stimulated activity in response to $1,25(OH)_2D_3$ were noted in both cell lines, although the inhibitory response was greater in the HPK1A than in HPK1A-*ras* cells (Fig 3.4).

Northern analysis.

Northern analysis of total RNA removed at timed intervals from HPKIA and HPK1A-*ras* cells cultured in the absence or presence of 10^{-7}M $1,25(OH)_2D_3$ revealed one major transcript of 2.4kb in both cell lines (Fig 3.5). Addition of 10% FBS resulted in a stimulation of c-*myc* mRNA expression which was greater in HPK1A (Panel A) than in HPK1A-*ras* (Panel B). Although strong early inhibition of this activity was noted in the established cells (HPK1A) treated with $1,25(OH)_2D_3$, minimal inhibition uias noted in the malignant cells (HPK1A-*ras*) with an apparent stimulation in the presence of $1,25(OH)_2D_3$ at 24 hours.

$1,25(OH)_2D_3$ receptor analysis.

To determine if alterations in the ligand binding domain of the $1,25(OH)_2D_3$

receptor could be responsible for the diminished efficacy of $1,25(OH)_2D_3$ in HPKIA-*ras* cells, we then compared the characteristics of binding of $[^3H]1,25(OH)_2D_3$ to its cytosolic receptor in both HPKIA and HPK1A-*ras* cells. Sucrose gradient analysis of $[^3H]1,25(OH)_2D_3$ binding in cytosols of HPK1A (Fig 5.6A) and HPKIA-ras (Fig 5.6B) revealed a single peak of 3.7S, corresponding to the known size of the $1,25(OH)_2D_3$ receptor. Scatchard analysis of the equilibrium binding studies were consistent with a single class of binding sites (Fig 5.7). The number of receptor molecules and the dissociation constants (Kd) calculated from the slopes of the Scatchard plots were not significantly different in HPKIA (Fig 5.7A) and HPKIA-ras (Fig 5.7B) cells.

DISCUSSION

We have examined the effect of $1,25(OH)_2D_3$ on parameters of mitogen stimulated cell growth and c-*myc* proto-oncogene expression in a keratinocyte model of tumor progression. Previous studies have identified $1,25(OH)_2D_3$ as a regulator of cell growth and differentiation in normal human keratinocytes (8,12,13). Keratinocytes in culture have been shown to convert 25 hydroxyvitamin D_3 to its active metabolite, $1,25(OH)_2D_3$ (Bikle et al., 1986) and cytosolic receptors for $1,25(OH)_2D_3$ have been identified in keratinocytes Clemens et al., 1981; Simpson et al., 1980; Feldman et al., 1980). This steroid, therefore, has the potential to inhibit growth and stimulate differentiation in epidermal cells in an autocrine manner.

In previous studies we demonstrated a sustained inhibition of mitogen stimulated growth in normal human keratinocytes by 10^{-8} $1,25(OH)_2D_3$ (Sebag et al., 1994). This inhibition was shown to be due to a blockade in the transition from G1–S, with an accumulation of cells in G0/G1 phase. In the present study, 10^{-8} M $1,25(OH)_2D_3$ was capable of inducing a similar sustained inhibition of mitogen-stimulated growth in the established HPK1A cell line, which was a result of blockage into S phase. However, the neoplastic HPK1A-*ras* keratinocytes demonstrated a transient growth inhibitory response which required 10-100 fold higher concentrations of $1,25(OH)_2D_3$ to achieve the same degree of inhibition as that seen in the established cells with 10^{-8} $1,25(OH)_2D_3$. Analysis of the cell cycle of the malirnant HPK1A-*ras* cells revealed a similar pattern of resistance, where 10-8 M $1,25(OH)_2D_3$ was incapable of preventing the mitogen stimulated passage of cells into S phase. A similar resistance to the anti-mitogenic effects of TGFß, a known negative regulator of keratinocyte grrowth (Pietenpol et al., 1990), has been reported previously in malignant keratinocytes (Woodworth and DiPaolo) although the mechanism

remains undefined.

Having demonstrated resistance to $1,25(OH)_2D_3$ as a negative regulator of the cell cycle in the malignant keratinocytes, we then assessed and compared the levels of c-*myc* mRNA in response to mitogens and $1,25(OH)_2D_3$ in the HPKIA and HPK1A-*ras* cell lines. Recent studies have focussed on a potential role for c-*myc* in the regulation of the cell cycle. This proto-oncogene encodes a highly conserved nuclear protein which is expressed in a tissue specific manner in both fetus and adult (Shilo and Weinberg, 1981). The *myc* gene product is thought to mediate a signal associated with cell division and appears to be required for normal cell growth (Müller et al., 1982). The rapid induction of c-*myc* following stimulation by agents such as growth factors and serum (Alt et al., 1986; Tramontano et al., 1986) suggests a potential role for the protein in the passage of cells from the resting phase into the actively dividing phase of the cell cycle.

In the present study, a characteristic time course of c-*myc* mRNA expression in response to stimulation by mitogens was observed in both cell lines with an early peak and a slow decline over 24-48 hrs. However, maximum levels of induction were greater in HPK1A than in HPK1A-*ras* cells. This apparent independence from exogenous growth promoters could be a function of overproduction of endogenous TGFα (Coffey et al., 1987), constitutively active EGF receptors (Weinberg, 1985) or altered positive responsive elements located in the promoter region of the c-*myc* gene (Chung et al., 1986). In previous studies, we and others have shown that expression of c-*myc* mRNA was negatively regulated by $1,25(OH)_2D_3$ in several cell types (Kremer et al., 1989; Katakami et al., 1988; Dürst et al., 1987). In addition, $1,25(OH)_2D_3$ has been shown to regulate c-*myc* gene expression at the transcriptional level (Simpson et al., 1987). In the present study, the immortalized HPK1A cells showed a strong inhibition of c-*myc* mRNA

expression similar to that described previously in normal keratinocytes in response to 10^{-8} M $1,25(OH)_2D_3$ (R. Kremer, unpublished results). The neoplastic HPK1A-*ras* cells, on the other hand, responded to a 10 fold greater dose with minimal inhibition of serum-stimulated c-*myc* mRNA levels at early time points and with an apparent rebound at 24 hrs.

We have previously noted the same type of altered response at the mRNA level of another $1,25(OH)_2D_3$-responsive gene, the PTH-related peptide (PTHRP) gene, in the HPK1A-*ras* keratinocytes (Henderson et al., 1991). This resistance could occur at the level of receptor-ligand interaction as a function of altered intra-cellular metabolism of the steroid, altered receptor function or a constitutional absence or decreased expression of the $1,25(OH)_2D_3$ receptor in the malignant cells. Purified receptors from HPKIA and HPK1A-*ras* cells demonstrated similar $1,25(OH)_2D_3$ binding characteristics including receptor number, receptor affinity and migration pattern of the receptor/ligand complex on a sucrose density gradient which were similar to those found in normal human keratinocytes (Pillai et al., 1988). Analysis of medium conditioned by HPK1A and HPKIA-*ras* cells for 48 hrs. in the presence of 10^{-7} $1,25(OH)_2D_3$ showed no significant difference in the levels of the steroid (data not shown). Taken together, these observations suggest that the resistance to $1,25(OH)_2D_3$ demonstrated in the malienant keratinocytes lies somewhere beyond the interaction of the ligand with its cytosolic receptor, perhaps at the point of interaction between receptor and target gene.

The preceeding studies therefore demonstrate that a resistance to an important negative regulator of keratinocyte cell growth is acquired in the passage from established to malignant phenotype. These findings may have important implications *In vivo* in the development and unrestrained growth of squamous carcinomas.

ACKNOWLEDGEMENTS

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We have identified a resistance to the growth inhibitory influences of 1,25-dihydroxyvitamin D_3 in *ras* transformed keratinocytes. Our next objective was to identify and characterize the nature of this resistance.

CHAPTER 4

DISRUPTION OF VDR/RXR HETERODIMER FORMATION FOLLOWING RAS TRANSFORMATION OF HUMAN KERATINOCYTES

ABSTRACT

A partial resistance to the growth inhibitory influence of 1,25 dihydroxyvitamin D_3 is apparent when immortalized keratinocytes are transformed by the ras oncogene. The vitamin D receptor (VDR) was isolated, analyzed and found to be identical in normal, immortalized and ras-transformed keratinocytes. Subsequently, nuclear extracts from immortalized and *ras* transformed keratinocytes were analyzed in gel mobility shift assays using labeled osteocalcin or osteopontin vitamin D response elements (VDREs). A specific VDR-DNA complex was identified in both type of extracts using an anti-VDR antibody. The addition of an anti-retinoic acid X receptor (RXR) antibody confirmed the presence of the RXR heterodimer partner in the same protein-DNA complex in normal and immortalized keratinocyte cell extracts, but not in ras-transformed keratinocytes. Similar results were observed using a labeled thyroid hormone responsive element (TRE). Furthermore, transfection of ras-transformed keratinocytes with wild type RXRα rescued VDR/RXR and TR/RXR complexes as demonstrated by a supershift in the presence of anti-RXR antibody. Both cell lines were found to express RXRα message in equal amounts. The cDNA encoding RXRα was cloned from *ras*-transformed keratinocytes, sequenced and found to be identical to the published sequence of RXRα. Western blot analysis of RXRα protein from *ras*-transformed keratinocytes indicate post-translational modifications quite distinct from control cells. These results suggest a novel way in which malignant cells can become resistant to the growth inhibitory influences of 1,25 dihydroxyvitamin D_3.

INTRODUCTION

1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active metabolite of vitamin D, is a potent inhibitor of keratinocyte cell growth and stimulates its differentiation (Hosomi et al., 1983; Smith et al., 1986). It exerts these effects by binding to its receptor, found principally in the nuclei of its target cells, then modulating the transcription of specific target genes involved in cell growth and differentiation, such as the proto-oncogenes c-*myc* and c-*fos* and involucrin, a marker of keratinocyte differentiation (Haussler, 1986; O'Malley, 1984; Sebag et al., 1994). The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily with which it shares structural homology in the DNA binding domain, the hormone binding domain, the dimerization domain and the transactivation domains (Evans, 1988). Recent studies indicate that in order to effect gene transcription, VDR first requires protein-protein interaction with another but distinct member of this receptor family, the retinoid X receptor (RXR) (Zhang et al., 1992). Once dimerized, this VDR/RXR complex recognizes and binds to specific bipartide DNA sequences found on the promoter regions of vitamin D target genes also known as vitamin D response elements (VDRE) (Ozono et al., 1990). Bound to these elements, the VDR/RXR complex can have either a stimulatory or inhibitory effect on gene transcription.

In previous studies we have characterized the effects of $1,25(OH)_2D_3$ on human keratinocytes as they progress from the normal to the malignant phenotype (Sebag et al., 1992). In our model of tumor progression, normal human keratinocytes were established as an immortal cell line by transfection with human papilloma virus type 16 and subsequently transformed with an activated *ras* oncogene. We determined that the malignant keratinocytes were resistant to not only the growth inhibitory effects of $1,25(OH)_2D_3$, but also to its transcriptional influences.

In the present work we have studied the mechanism responsible for vitamin D resistance in *ras* transformed keratinocytes. We report the first incidence of vitamin D resistance caused by an abnormally processed RXR protein.

MATERIALS AND METHODS

Cell culture and transfections.

The HPK1A cell line was established from normal human keratinocytes by stable transfection with human papilloma virus type 16. These cells have an indefinite life span in culture but retain differentiation properties characteristic of normal keratinocytes and are nontumorigenic when injected into nude mice. These immortalized cells were then transformed into the malignant HPK1A*ras* cell line after transfection with a plasmid carrying an activated H-*ras* oncogene. In addition to forming colonies in soft agar the malignant HPK1A*ras* cells produce invasive tumors when transplanted into nude mice (Dürst et al., 1987). All cell lines were grown in Dulbeco's modified Eagle's medium DMEM (GibcoBRL) supplemented with 5% FBS (GibcoBRL) and passaged once or twice weekly. Cells grown in these conditions were then seeded at a density of $2.5x10^4$ cells/cm^2 in either 6-well plates, for CAT assays, or in 150mm culture dishes for transfections and nuclear extract preparation. Transfections were performed by incubating plasmid DNA (5-20μg) with Lipofectamine (GibcoBRL) (10μg) for 20h in serum free DMEM, then replacing the medium with fresh DMEM containing 10%FBS.

CAT assays.

HPK1A and HPK1A*ras* cells were plated at a density of $1x10^4$ cell/ml into 6-well plates and transfected with 5μg of a MOP3 plasmid, containing 3 repeats of the mouse osteopontin vitamin D response element (CAAGGTTCACGAGGTTCAC) in front of a CAT reporter gene and 2μg of a ß-galactosidase (ß-gal) plasmid (Ferrara et al., 1994). Cells were then treated with increasing concentrations of $1,25(OH)_2D_3$ (10^{-8}-10^{-6}) for 24

hours. Following this incubation period the cells were scraped, washed in PBS, resuspended in PBS containing 1.0mM Phenylmethylsulfonylfluoride (PMSF) and finally lysed by 3 freeze thawing cycles. The cell debris was spun down, and aliquots of cell extracts were used for CAT assay. Assays were performed using a CAT ELISA kit (5 Prime-3 Prime, Boulder, CO). Statistical significance was determined by ANOVA. A probability value of <0.01 was considered to be significant.

Nuclear extracts.

African Green Monkey kidney cells, COS-7, and HPK1A*ras* cells at 30-50% confluence in 100mm culture plates were transiently transfected with either 10μg of psG5 plasmid DNAs expressing the human retinoic acid X receptor (hRXRα) and the human vitamin D receptor (hVDR) (kind gifts of Drs. R. Evans and M. Haussler respectively) or the hRXRα receptor alone using the Lipofectamine technique, as described above. After 20h the medium was changed and cells were further incubated for 48h, washed with chilled PBS and collected. These cell pellets as well as cell pellets collected from untransfected HPK1A and HPK1A*ras* cells were disrupted in a dounce tissue homogenizer in buffer A (10mM HEPES, 1mM EDTA, 26mM KCl, 0.15mM spermine, 0.5mM spermidine, 2M sucrose, 10% glycerol, 1mM DTT, 0.1mM phenylmethylsulfonylfluoride, 15μg/ml aprotinin, 1μg/ml leupeptin). Nuclear pellets were obtained by centrifugation at 85,000xg for 30min at 2°C, resuspended in buffer B (20mM HEPES, 1.5mM $MgCl_2$, 1mM DTT 0.42M NaCl, 25% Glycerol) and then dounce homogenized. Following a 20min centrifugation at 25,000xg nuclear extracts were dialysed for 10h to 12h against two changes of buffer C (20mM HEPES, 1mM $MgCl_2$, 20mM KCL, 17% Glycerol 2mM DTT). Protein content was determined and samples

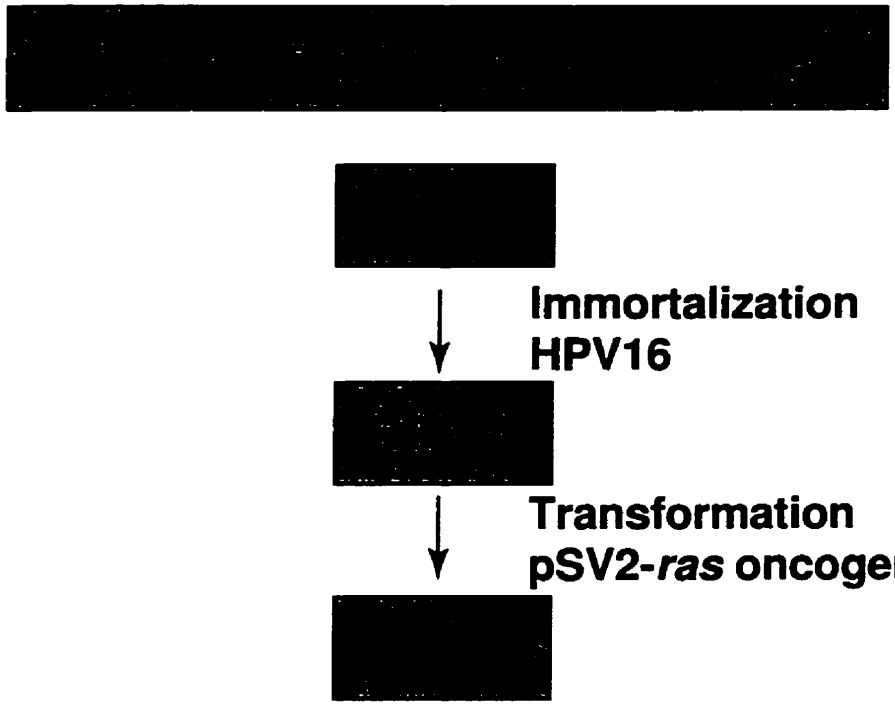
were then aliquoted and stored at -70°C.

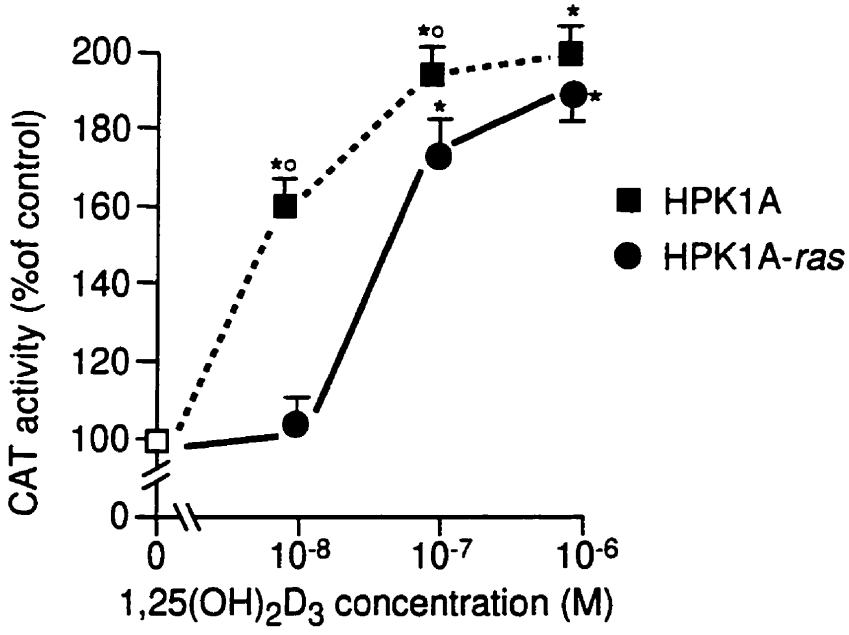
Gel mobility shift analysis.

2μg of nuclear extracts were incubated for 30 min at room temperature with 200nM $1,25(OH)_2D_3$ or 200nM thyroxine, and 3.7μg of polydIdC in a binding buffer (10mM Tris, 100mM KCL, 5mM $MgCl_2$, 5% glycerol, 1mM EDTA, and 1mM DTT). 5 fmol of the appropriate ^{32}P labeled DNA response element was then added and incubated 20 minutes at room temperature. When required, the anti-RXR antibody (4X1D12- a monoclonal antibody recognizing a common region in the E-domain of all three types of RXR) (a kind gift of Dr. P. Chambon) antibody or the anti-VDR antibody (Affinity Bioreagents) were added to the incubationions. In some experiments 1-5ng of baculovirus expressed hVDR (Panvera, Madison, WI) was added to the above reactions. The samples were then electrophoresed on 5% non-denaturing polyacrylamide gels in 0.5XTBE at 6V/cm. Following electrophoresis the gels were dried and exposed to Kodak XAR-5 film without intensifying screens.

RNA analysis.

Total cellular RNA was prepared using the Trizol reagent (GibcoBRL, Burlington ON) and chloroform extractions. For Northern Blot analysis, 5-10μg of total RNA was electrophoresed on a 1.1% agarose-formaldehyde gel. RNA was transferred onto a nylon membrane (Nytran), air dried, baked at 80°C for 1h, then hybridized with RXRα probe labelled with ^{32}P dCTP (ICN) by the random primer method (Amersham, Ontario, Canada). After 24h of incubation at 42°C, filters were washed successively in 1XSSC, 1%SDS for 15min at room temperature, 0.1XSSC, 0.1%SDS twice for 30 min at 55°C.


Immortalization
HPV16
Transformation
pSV2-*ras* oncogene


CAT activity (%of control)
200
180
160
140
120
100
0
0
10-8
10-7
10-6
1,25(OH)2D3 concentration (M)
HPK1A
HPK1A-*ras*

Autoradiography of filters was carried out at -70°C using Kodak XAR films and two intensifying screens.

For RT-PCR, first strand cDNA synthesis, 1μg of RNA in DEPC treated water was denatured at 80°C for 10min, quick cooled on ice and reverse-transcribed from random hexamer primers (PdN6, Pharmacia), 50pmol, using 50U of M-MLV reverse transcriptase (GibcoBRL), in 1X PCR Buffer (Perkin Elmer, ON, Canada), containing 500μmol/L dNTPs (Perkin Elmer) and 20U RNasin (Pharmacia). The reaction was carried out in a volume of 20μl at 25°C for 10min, then 41°C for 30min, followed by heating to 95°C for 5 min. For amplification of first-strand cDNA derived from RXRα and RXRß mRNA, each RT reaction was expanded to a volume of 100μl to contain 1X PCR buffer, 200mmol/L dNTPs, and 50pmol each of the upstream and downstream PCR primers for RXRα and ß. The reactions were heated to 72°C, then supplemented with 2.5U of Taq DNA polymerase (GibcoBRL). The reactions were subjected to 30 cycles of denaturation (94°C, 30sec), annealing (55°C, 30sec) and extension (72°C, 1min)-except for denaturation at 94°C for 1 minute, annealing for 1.5 minutes and extension for 2 minutes during the first cycle and extension for 15 minutes during the last cycle. PCR products were resolved on a 1.5% agarose gel run in 1XTBE buffer and visualized with ethidium bromide.

Cloning and Sequencing.

PCR products were directly cloned in the vector pCRII, according to the manufacturer's specifications (Invitrogen, San Diego, CA). Plasmid clones of interest were identified by restriction endonuclease size analysis and/or PCR analysis, and the inserts were sequenced from primers complementary to the flanking T7 and SP6 RNA

polymerase promoter sites, using the T7 DNA sequencing kit (Pharmacia). The reaction mixtures, radiolabeled with ^{35}S-dATP (ICN) were electrophoresed on 5% Long Ranger sequencing gel (AC Biochem, Malvern, PA) for 2 hours at 60W. Gels were dried and exposed to Kodak XAR film at room temperature.

Western Blot Analysis.

60μg of nuclear proteins were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon membranes (Millipore, Bedford, MA) for 2h at 200 mAmp in 25mM Tris (Ph8.3)-0.192M glycine-20%methanol. Blots were incubated overnight at 4°C in a blocking solution containing PBS, 3.5%BSA and 0.2% NP-40, followed by 1 hour incubation with an anti-RXRα polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA) diluted to the manufacturers specifications in blocking solution. The blots were washed three times for 15 min each time in PBS 0.1% NP-40 and then incubated with secondary horseradish peroxidase conjugated antibody (goat anti-rabbit immunoglobulin G, (BioRAD) diluted to 1:3000 in blocking solution. After the blots were washed as described above, they were incubated for 2 minutes in 20ml of methanol containing 60mg 4'chloronapthol and 1.5% of freshly diluted H_2O_2 then photographed using Polaroid Type57 film (Polaroid, Canada).

Synthetic Oligonucleotides Used

Gel shift experiments:

mouse osteopontin vitamin D response element (mOPVDRE)

5'-TGACAAGGTTCACGAGGTTCACGG-3'

thyroid responsive element ß (TRE-ß)

NHEK	ggcaatggcg gccagcactt ccctgcctga ccctggagac tttgaccgga acgtgccccg gatctgtggg gtgtgtggag accgagccac
HPK1A	ggcaatggcg gccagcactt ccctgcctga ccctggagac tttgaccgga acgtgccccg gatctgtggg gtgtgtggag accgagccac
HPK1A*ras*	ggcaatggcg gccagcactt ccctgcctga ccctggagac tttgaccgga acgtgccccg gatctgtggg gtgtgtggag accgagccac
NHEK	tggctttcac ttcaatgcta tgacctgtga aggctgcaaa ggcttcttca ggcgaagcat gaagcggaag gcactattca cctgcccctt
HPK1A	tggctttcac ttcaatgcta tgacctgtga aggctgcaaa ggcttcttca ggcgaagcat gaagcggaag gcactattca cctgcccctt
HPK1A*ras*	tggctttcac ttcaatgcta tgacctgtga aggctgcaaa ggcttcttca ggcgaagcat gaagcggaag gcactattca cctgcccctt
NHEK	caacggggac tgccgcatca ccaaggacaa ccgacgccac tgccaggcct gccggctcaa acgctgtgtg gacatcggca tgatgaagga
HPK1A	caacggggac tgccgcatca ccaaggacaa ccgacgccac tgccaggcct gccggctcaa acgctgtgtg gacatcggca tgatgaagga
HPK1A*ras*	caacggggac tgccgcatca ccaaggacaa ccgacgccac tgccaggcct gccggctcaa acgctgtgtg gacatcggca tgatgaagga
NHEK	gttcattctg acagatgagg aagtgcagag gaagcgggag atgatcctga agcggaagga ggaggaggcc ttgaaggaca gtctgcggcc
HPK1A	gttcattctg acagatgagg aagtgcagag gaagcgggag atgatcctga agcggaagga ggaggaggcc ttgaaggaca gtctgcggcc
HPK1A*ras*	gttcattctg acagatgagg aagtgcagag gaagcgggag atgatcctga agcggaagga ggaggaggcc ttgaaggaca gtctgcggcc

5'- GATCCTGGAGGTGACAGGAGGACAGCGATC)

RT-PCR:

VDR:

upstream: 5'-ATGGCGGCCAGCACTTCCCTGCCTGAC-3'

downstream: 5'-CTCCTCCTTCCGCTTCAGGATCATCTC-5'

RXRα

upstream: 5'-TGGCAAGGACCGGAACGAGAAT-3'

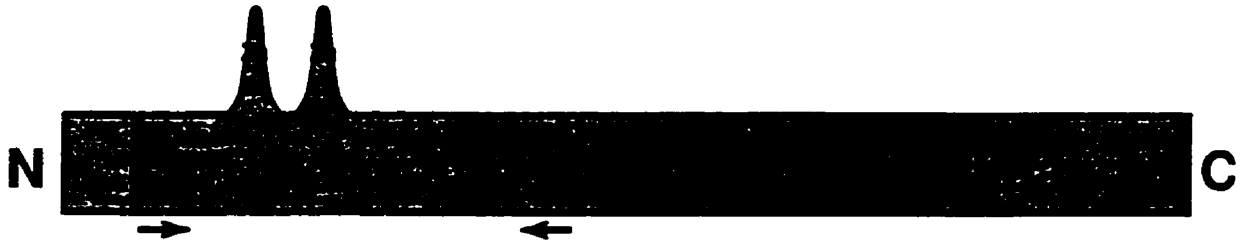
downstream: 3'-TCCATAAGGAAGGTGTCAATGGG-3'

RXRß

upstream: 5'-TGCGGGGACAGAAGCTCAGGCAAA-3'

downstream: 5'-GTAGGTCTCCAGTGATGCATACAC-3'

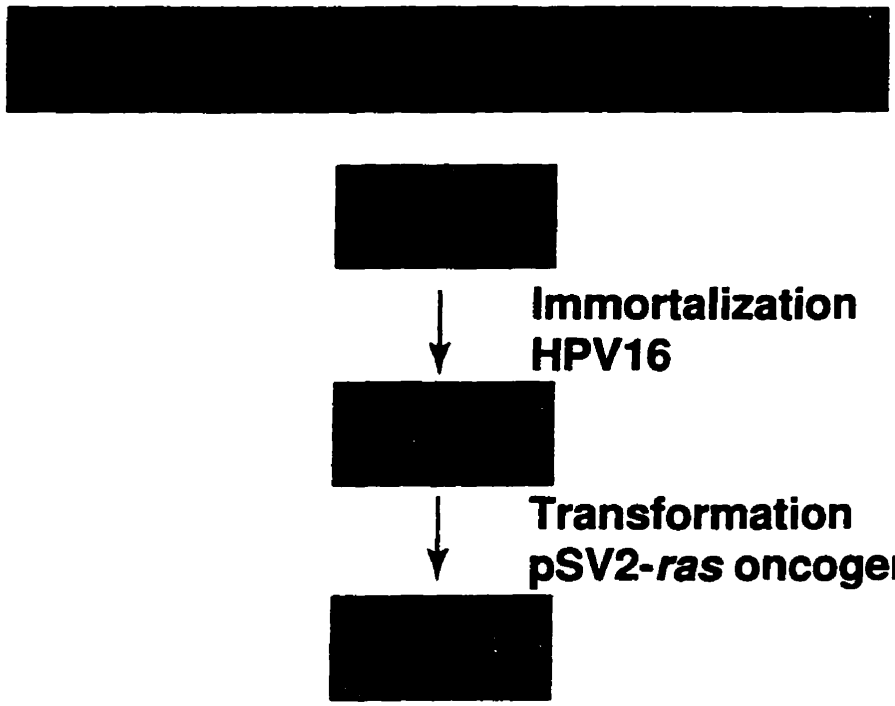
Figure 4.1. Resistance to $1,25(OH)_2D_3$ action in *ras*-transformed keratinocytes. Cells were transfected with reporter plasmid as described in materials and methods. Cells were then treated with or without increasing concentrations of $1,25(OH)_2D_3$. CAT activity was assayed and normalized for transfection efficiency by the corresponding ß-galactosidase activity. Results are expressed as mean±SEM. *indicate significant differences from untreated cells, °indicate significant difference from corresponding HPK1A*ras* cells.



NHEK	ggcaatggcg gccagcactt ccctgcctga ccctggagac tttgaccgga acgtgccccg gatctgtggg gtgtgtggag accgagccac
HPK1A	ggcaatggcg gccagcactt ccctgcctga ccctggagac tttgaccgga acgtgccccg gatctgtggg gtgtgtggag accgagccac
HPK1A*ras*	ggcaatggcg gccagcactt ccctgcctga ccctggagac tttgaccgga acgtgccccg gatctgtggg gtgtgtggag accgagccac
NHEK	tggctttcac ttcaatgcta tgacctgtga aggctgcaaa ggcttcttca ggcgaagcat gaagcggaag gcactattca cctgcccctt
HPK1A	tggctttcac ttcaatgcta tgacctgtga aggctgcaaa ggcttcttca ggcgaagcat gaagcggaag gcactattca cctgcccctt
HPK1A*ras*	tggctttcac ttcaatgcta tgacctgtga aggctgcaaa ggcttcttca ggcgaagcat gaagcggaag gcactattca cctgcccctt
NHEK	caacggggac tgccgcatca ccaaggacaa ccgacgccac tgccaggcct gccggctcaa acgctgtgtg gacatcggca tgatgaagga
HPK1A	caacggggac tgccgcatca ccaaggacaa ccgacgccac tgccaggcct gccggctcaa acgctgtgtg gacatcggca tgatgaagga
HPK1A*ras*	caacggggac tgccgcatca ccaaggacaa ccgacgccac tgccaggcct gccggctcaa acgctgtgtg gacatcggca tgatgaagga
NHEK	gttcattctg acagatgagg aagtgcagag gaagcgggag atgatcctga agcggaagga ggaggaggcc ttgaaggaca gtctgcggcc
HPK1A	gttcattctg acagatgagg aagtgcagag gaagcgggag atgatcctga agcggaagga ggaggaggcc ttgaaggaca gtctgcggcc
HPK1A*ras*	gttcattctg acagatgagg aagtgcagag gaagcgggag atgatcctga agcggaagga ggaggaggcc ttgaaggaca gtctgcggcc

Figure 4.2. Cloning and Sequencing of hVDR.

Schematic representation of the isolation, cloning and sequencing of the cDNA encoding the DNA binding domain of the VDR from normal human keratinocytes (NHEK), HPK1A and HPK1A*ras* cells. Arrows indicate the relative position of the primers used to isolate and amplify the target sequence. Sequences obtained are aligned and displayed to indicate that no mutation was found in the amplified region/target.


Immortalization
HPV16
Transformation
pSV2-*ras* oncogene

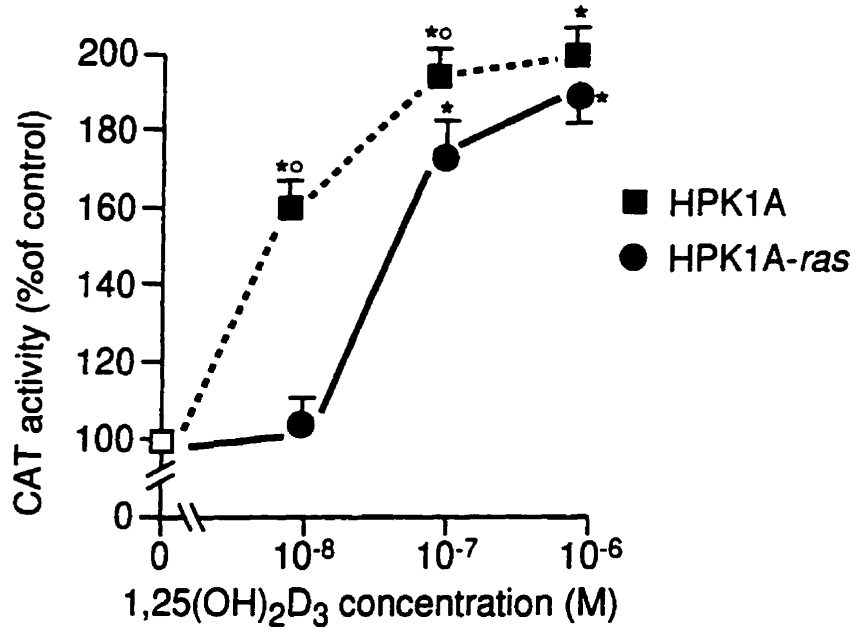

CAT activity (%of control)
200
180
160
140
120
100
0
0
10^{-8}
10^{-7}
10^{-6}
1,25(OH)$_2$D$_3$ concentration (M)
HPK1A
HPK1A-*ras*

Figure 4.3. Gel shift analysis of VDR/RXR binding.

(A) An OP vitamin D response element (OPVDRE) was ^{32}P labeled and incubated with either control extracts (COS1 cells transfected with hVDR and hRXRα) (lanes 2, 3, 4), HPK1A nuclear extracts (lanes 5 ,6, 7) or HPK1A*ras* nuclear extracts (lanes 8, 9, 10) each in the presence or absence of either anti-VDR antibodies or anti-RXR antibodies. Diamonds indicate the presence of putative VDR/RXR complexes. Circles indicate the presence of supershifted complexes containing RXR.

(B) The identity of a VDR complex in HPK1A*ras*-nuclear extracts complexes is confirmed by the addition of increasing concentrations of Baculovirus expressed hVDR to reactions. As noted, in the presence of labelled OPVDRE, nuclear extracts from HPK1A cells (lanes 6, 7) and nuclear extracts from HPK1A*ras* cells (lanes 1, 2, 3, 4) both form VDR complexes with dose dependent increases when incubated with varying concentration of hVDR.

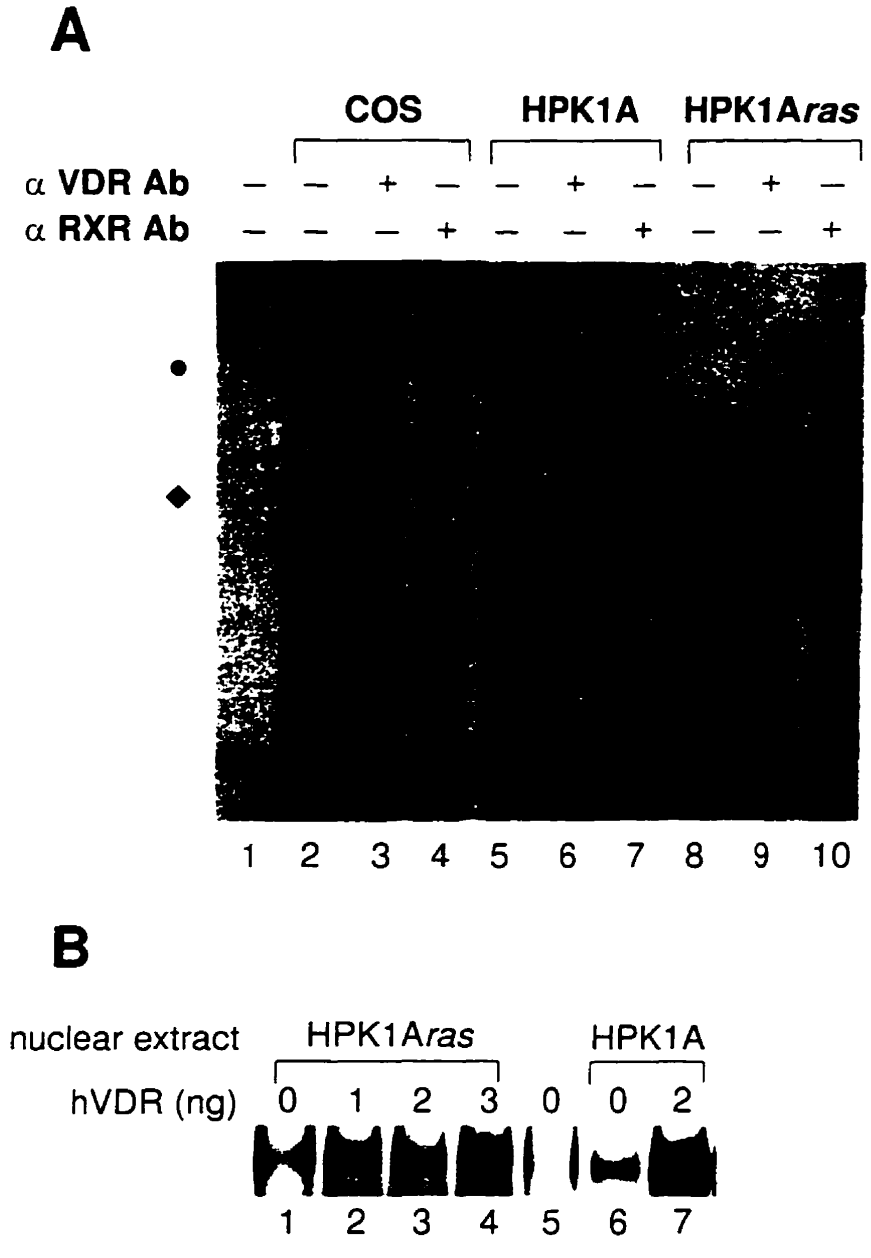
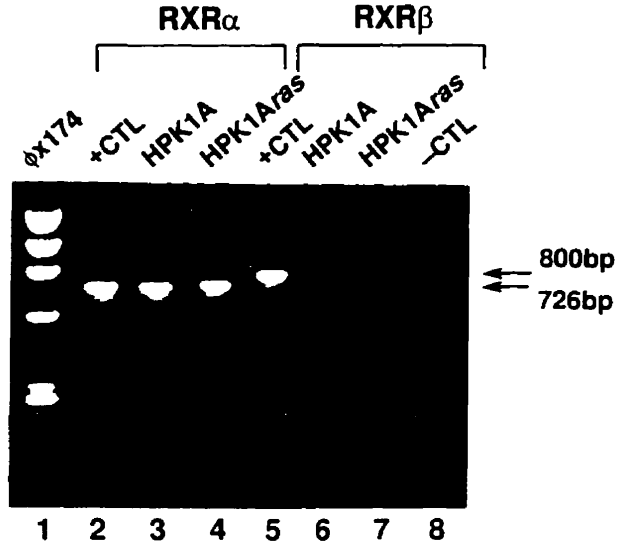

A
COS
HPK1A
HPK1Aras
α VDR Ab
α RXR Ab
1 2 3 4 5 6 7 8 9 10
B
nuclear extract
HPK1Aras
HPK1A
hVDR (ng)
0 1 2 3 0 0 2
1 2 3 4 5 6 7

Figure 4.4 Analysis of RXRα and RXRß mRNA.

(A) RNA from HPK1A and HPK1A*ras* cells was prepared and used in RT PCR reactions using primers designed to specifically amplify either RXRα (lanes 1-4) or RXRß (lanes 5-7). Positive controls used are DNA plasmids containing either hRXRα (lane 1) or hRXRß (lane 5). Negative controls were carried out in absence of either DNA or RNA. PCR products are resolved on a 1.5% agarose ethidium bromide stained gel along with ϕx174 HaeI DNA size markers.

(B) Northern blot analysis of RXRα using RNA from HPK1A and HPK1A*ras* cells. Filters are hybridized with a ^{32}P labelled hRXRα probe.

A


RXRα
RXRβ
φx174
+CTL
HPK1A
HPK1Aras
+CTL
HPK1A
HPK1Aras
-CTL
800bp
726bp
1 2 3 4 5 6 7 8

B

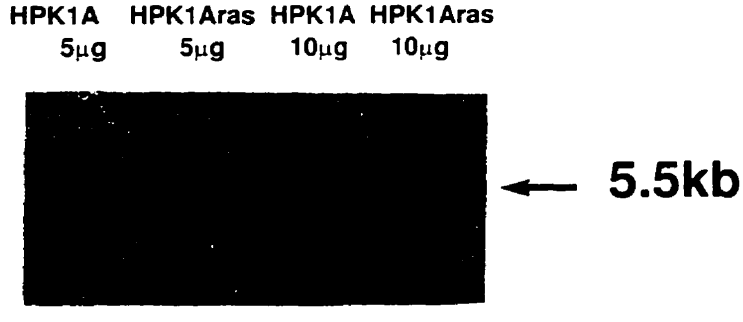

HPK1A 5μg
HPK1Aras 5μg
HPK1A 10μg
HPK1Aras 10μg
5.5kb

Figure 4.5 Rescue of VDR/RXR complexes in HPK1A*ras* nuclear extracts.

(A) HPK1A*ras* nuclear extracts were supplemented with COS-1 cell extracts overexpressing hRXRα (COS-RXR) (lanes 5, 6) before incubation with a labelled OPVDRE oligonucleotide. Control reactions consist of COS-RXR extracts incubated with or without anti-RXR Ab (lanes 7, 8).

(B) Extracts from HPK1A*ras* (lanes 1, 2) and HPK1A*ras* cells transfected with an hRXRα plasmid (HPK1A*ras*-RXR, lane 3) with or without anti-RXR-Ab.

Diamonds indicate the presence of VDR/RXR complexes and circles indicate the presence of supershifted complexes containing RXR.

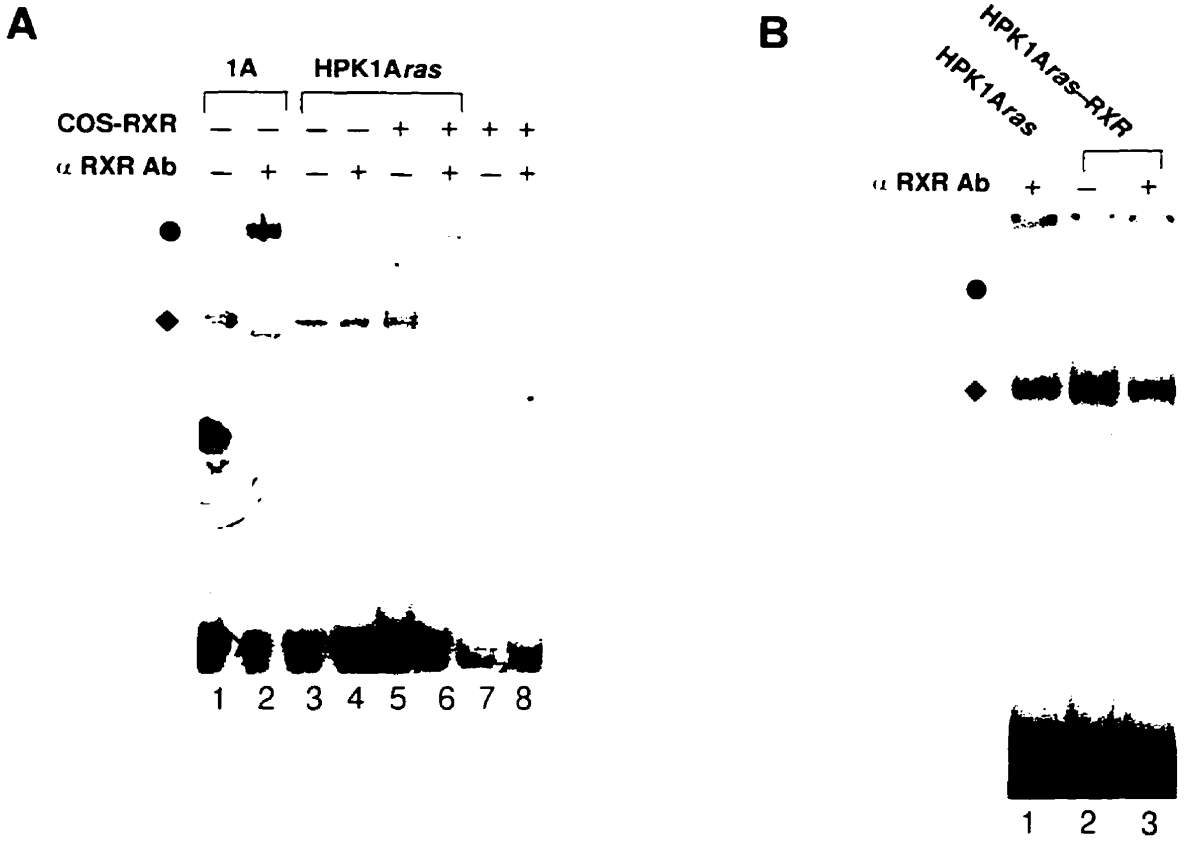

A
1A
HPK1Aras
COS-RXR
α RXR Ab
1 2 3 4 5 6 7 8
B
HPK1Aras
HPK1Aras–RXR
α RXR Ab
1 2 3

Figure 4.6. Gel shift analysis of RXR binding to a TRE.

A thyroid resonse element TREß was labelled and incubated with nuclear extracts prepared from either HPK1A (lanes 2, 3), HPK1A*ras* (lanes 4, 5) or HPK1A*ras* cells overexpressing hRXRα (HPK1A*ras*-RXR, lanes 6, 7) in the presence or absence of anti-RXR-Ab or a control, pre-immune, IgG (lane 7).

Diamonds indicate the presence of TR/RXR complexes. Circles indicate the presence of supershifted complexes containing RXR.

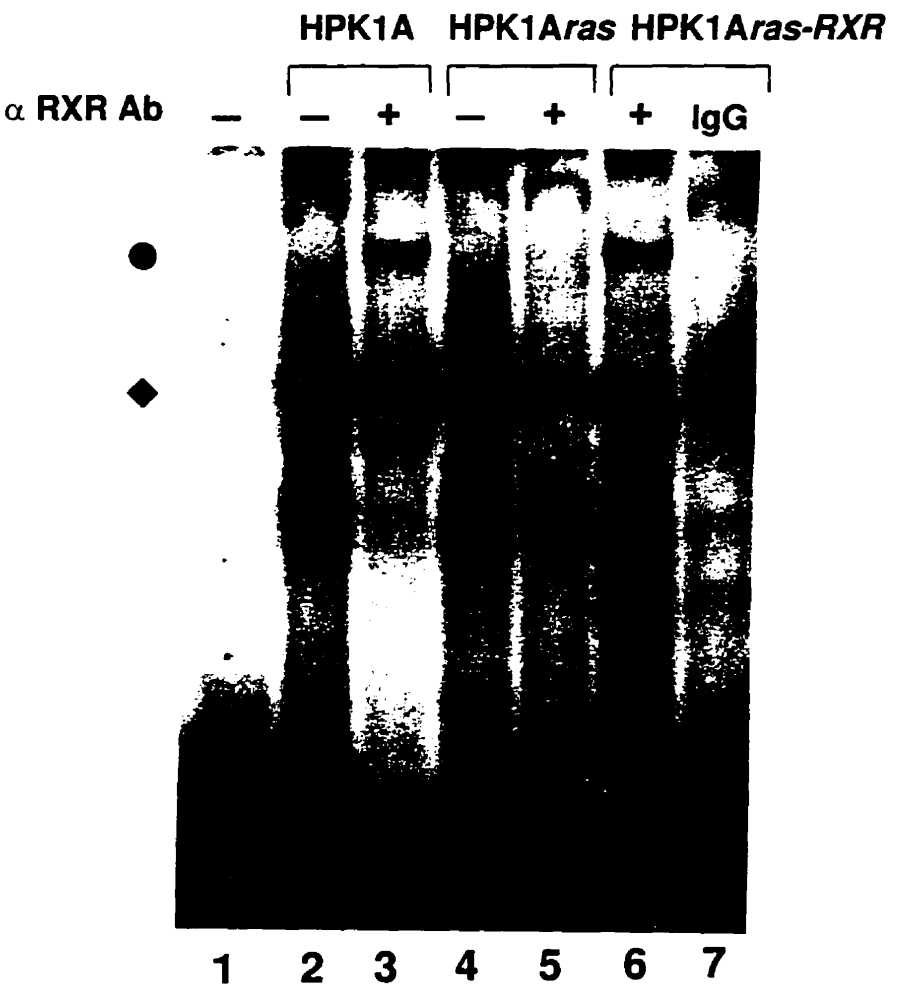
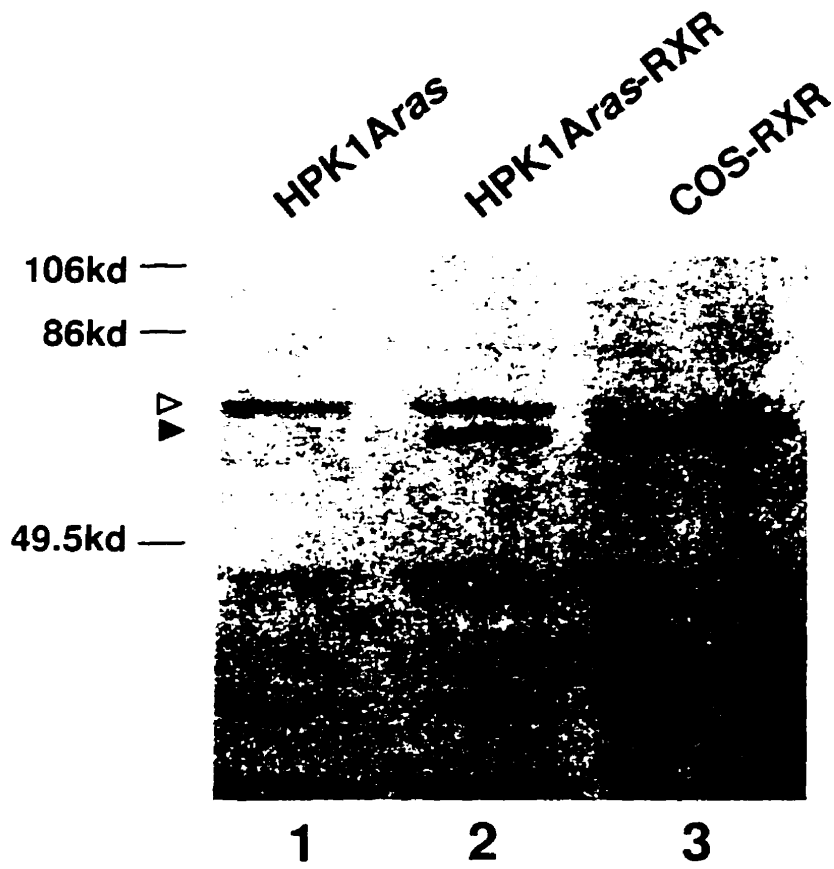

HPK1A
HPK1Aras
HPK1Aras-RXR
α RXR Ab
–
–
+
–
+
+
IgG
1
2
3
4
5
6
7

Figure 4.7 Western Blot analysis of RXRa protein in HPK1A*ras* cells.

Nuclear extracts prepared from HPK1A*ras* cells (lane 1), HPK1A*ras* cells overexpressing hRXRα (HPK1A*ras*-RXR,lane 2) and COS-1 cells overexpressing hRXRα (COS-RXR, lane 3) were electrophoresed on an SDS-Page gel and processed as described in materials and methods. Triangles indicate the position of specific RXRα bands. Endogenous RXRα protein expressed in HPK1A*ras* migrated at a slightly higher position (open triangle) as exogenously expressed wild type RXRα (closed triangle).


HPK1Aras
HPK1Aras-RXR
COS-RXR
106kd
86kd
49.5kd
1
2
3

RESULTS

Effects of $1,25(OH)_2D_3$ on gene transcription in HPK1A and HPK1Aras cells.

To determine if the resistance of HPK1A*ras* cells to the growth inhibitory influence of $1,25(OH)_2D_3$ occurs at the transcriptional level, we transfected a reporter plasmid (MOP3) containing three repeats of the mouse osteopontin vitamin D response element cloned in front of a thymidine kinase driven bacterial chloramphenicol acetyl transferase gene (Fig. 4.1). A 1.6 fold stimulation of CAT expression was observed in the presence of 10^{-8} M of $1,25(OH)_2D_3$ in HPK1A cells. In HPK1A*ras* cells, 10^{-7}M concentration of $1,25(OH)_2D_3$ was required to reach a similar increase in CAT expression.

Cloning and sequencing of the cDNA encoding VDR from HPK1A and HPK1A*ras* cells.

We previously reported that both the number of vitamin D receptors and their affinities for ligand were not significantly different in HPK1A and HPK1A*ras* cells (Sebag et al., 1992). We concluded that the hormone binding domain was functionally intact. Therefore, we first analyzed the DNA binding domain of VDR by cloning the VDR cDNA from HPK1A and HPK1A*ras* cells using RT-PCR. After sequencing the cloned DNAs we determined that sequences from both cell lines were identical to the known sequence of human VDR (Fig. 4.2).

Gel mobility shift analysis of VDR/RXR binding.

The ability of nuclear extracts derived from HPK1A and HPK1A*ras* cells to form specific VDR/RXR protein complexes interacting with known VDREs was next examined. We first determined that nuclear extracts from both HPK1A and HPK1A*ras* formed complexes with a mOPVDRE ^{32}P labeled probe (Fig 4.3A). These complexes co-migrate with those formed by control nuclear extracts (ie.: COS-7 cells co-transfected

with plasmids encoding hVDR and hRXRα). The addition of an anti-VDR antibody which targets the DNA binding domain of VDR inhibited the formation of retarded complexes in all nuclear extracts. The extracts were also incubated with an anti-RXR, which recognizes the D and E domain RXR and normally retards (supershift) any complexes containing RXR. A supershift was evident with control extracts (COS) as well as in extracts prepared from immortalized HPK1A cells. These results constrasted sharply with the absence of a supershift with HPK1A*ras* nuclear extracts in the presence of RXR antibody. Similar results were also observed using an osteocalcin VDRE as labeled probe in the same conditions (data not shown).

To verify the presence of VDR in the complexes formed by HPK1A*ras* nuclear extracts purified hVDR expressed in baculovirus was added in increasing concentrations to HPK1A*ras* nuclear extracts and to HPK1A extracts (Fig. 4.3B). A dose dependent increase in complex formation was observed, therefore confirming the VDR's presence in nuclear extracts prepared from HPK1A*ras* cells.

We next investigated the possibility that the level of RXR mRNA expression in HPK1A*ras* cells was low or absent. Subsequently RT-PCR confirmed the presence of RXRα message in both HPK1A and HPK1A*ras* cells and the absence of RXRß message in both these cell lines (Fig 4.4A). Northern Blot analysis revealed an equal amount of RXRα mRNA in both these cell lines (Fig 4.4B).

We then examined the possibility that an inhibitory factor present only in HPK1A*ras* cells could prevent the formation of VDR/RXR complexes. To test this hypothesis we supplemented HPK1A*ras* nuclear extracts with COS cell extracts enriched with hRXRα (Fig. 4.5A). The complexes formed by this supplementation were recognized by anti-RXR antibody. Similar results were observed when HPK1A*ras* cells

were transiently transfected with an expression plasmid enabling over-expression of hRXRα (Fig. 4.5B).

Finally we examined the interaction of RXR with another dimerization partner. To this effect we examined its role in enabling thyroid hormone (TR) to bind to its DNA recognition sequence (TRE) (Fig. 4.6A). Results with a TRE ^{32}P labeled probe were similar to those observed with a VDRE. HPK1A and HPK1A*ras* cells formed complexes that co-migrated in gel retardation assays. However, the complexes formed by HPK1A*ras* cells were not recognized by the anti-RXR antibody in as much as no supershift was observed with this antibody. Overexpressing hRXRα in HPK1A*ras* cells restored the ability of anti-RXR antibody to supershift complexes as priviously observed with VDREs(Fig 4.6B).

Cloning and sequencing of the cDNA encoding RXRα from HPK1A and HPK1A*ras* cells.

The C-terminal domain of the RXRα cDNA derived from HPK1A and HPK1A*ras* cells was amplified and cloned into a sequencing vector. This area, from amino acid 706 to amino acid 1426, comprises the D and E domains of the receptor, domains which are responsible for ligand binding, but which also play a critical role in transactivation and dimerization. No genetic alterations were found in the RXRα cDNA of HPK1A*ras* cells. The sequencing data is not shown.

Western Blot analysis of RXRα

Western blot analysis was performed using nuclear extracts prepared from HPK1A*ras*, HPK1A*ras* cells transfected with hRXRα and COS cells transfected with hRXRα (fig 4.7). An antibody specific for the human RXRα revealed a single protein complex in the control, COS extracts. A single protein complex was also observed in the HPK1A*ras*

extracts, however it migrated to a higher position (higher molecular weight) than the protein complex present in control COS cells. HPK1A*ras* cells transfected with hRXRα revealed two protein complexes migrating at both the higher and lower molecular weight positions.

DISCUSSION

Keratinocytes, in culture, have been shown to hydroxylate $25(OH)D_3$ to its active metabolite $1,25(OH)_2D_3$ (Bikle et al., 1986) and its receptor (the vitamin D receptor) has been identified in skin and in keratinocyte cultures (Pilai et al., 1988). It has therefore been proposed that $1,25(OH)_2D_3$ acts in an autocrine fashion to suppress growth and stimulate the differentiation of keratinocytes, making it a crucial player in keratinocyte cell biology.

In this study we first demonstrated that the vitamin D resistance observed in *ras* transformed keratinocytes occurs at the level of gene transcription. Conditioned medium from HPK1A and HPK1A*ras* cells was collected and assayed for $1,25(OH)_2D_3$. The levels of $1,25(OH)_2D_3$ were found to be the same in both cell lines, indicating that abnormal vitamin D metabolism could not be the cause of the observed resistance. Our CAT reporter plasmid experiments also support the hypothesis that resistance in HPK1A*ras* cells was due to a disruption in the VDR signalling pathway. Vitamin D receptor isolated from HPK1A*ras* cells appeared functionally intact, and present in amounts equal to those found in HPK1A cells. Consequently, we focused our attention on the DNA binding domain of the VDR.

To date several mutations have been found in this region in the clinical condition described as type II vitamin D resistant rickets (Sone et al., 1980; Malloy et al., 1994). The consequence of these mutations is a non-fuctional VDR, unable to modulate gene transcription. Our present study indicates that no such mutations are found in the VDR DNA binding domain cloned from HPK1A*ras* cells. Furthermore, several clones were sequenced and we ruled out the possibility of mutation in only one allele. Consequently we focused our attention on the interaction between VDR, RXR and target DNA response elements.

Using gel mobility shift assays, we demonstrated that nuclear extracts prepared from HPK1A and HPK1A*ras* cells and control COS extracts bind to a VDRE in a similar fashion. Addition of an anti-VDR antibody which targets the DNA binding domain of the receptor confirmed the presence of VDR in the complex seen in gel retardation assays using nuclear extracts derived from both established and transformed keratinocytes. Further confirmation of VDRs presence was obtained by the addition of exogenous baculovirus produced hVDR to nuclear extracts. From these studies, we can therefore conclude that in HPK1A*ras*, VDR is present in the protein complex that retards a VDRE probe.

Recently, the dimerization partner of VDR has been identified in gel retardation assays as the RXR (Zhang et al., 1992), and at least in vitro, RXR is required for VDR binding to a VDRE (Jin and Pike, 1996). Using an antibody that recognizes all three major types of RXR (α,β,γ) we determined that RXR was present in the VDR containing complexes from control and HPK1A nuclear extracts. However, the same antibody did not bind to the protein complex obtained from *ras* transformed keratinocytes. It is therefore likely that RXR is not present in VDRE binding complexes formed with HPK1A*ras* nuclear extracts.

Much of the work supporting the notion that VDR has a weak affinity for VDRE in absence of RXR was done using either bacterial or yeast expressed vitamin D receptors (Jin and Pike, 1986). However, this notion is still disputed and some groups have proposed that VDR can indeed form homodimers and effectively bind to VDREs in the absence of RXR (Freedman, et al., 1994; Carlberg et al., 1993; Mackey et al., 1996). Our present studies seem to indicate the lack of RXR in HPK1A*ras* complexes but not to rule out the possibility that another as of yet unidentified protein can participate in VDR binding

to a VDREs. Further investigations are required to clarify this issue.

To further clarify RXR interaction in HPK1A*ras* cells we next analyzed its interaction with another well characterized dimerization partner (ie.: the thyroid receptor, TR) (Zhang et al., 1992). Our hypothesis that RXR interaction with that receptor was abnormal in *ras* transformed keratinocytes was confirmed by the absence of a supershift in presence of anti-RXR antibody. As TR has been clearly shown to form transcriptionally active homodimers *in vitro* (Hollenberg et al., 1995), it is not clear what are the physiological implications of RXR absence. Further work needs to be done on HPK1A*ras* cells to identify any biological consequences contributed by the lack of RXR involvement in thyroid receptor heterodimerization.

Another potential mechanism of vitamin D resistance is the presence of interfering proteins preventing VDR/RXR interaction with VDREs. Such inhibitory accessory proteins have been previously identified for the thyroid hormone receptor (Chen and Evans, 1995; Hörlein et al., 1995) and vitamin D resistant New World Primate cells were recently reported to contain a protein or complex of proteins capable of inhibiting VDR-RXR heterodimer binding to a VDRE (Arbelle et al., 1996). Consequently, we tested this possibility in our system by adding COS extracts enriched for RXRα to HPK1A*ras* nuclear extracts and observed heterodimer formation and binding to a VDRE. We observed that the same VDR containing band was present but also that addition of this exogenous RXRα rescued the supershift of this complex following addition of the anti-RXR antibody. A similar rescue phenomenon was observed in gel shift experiments using nuclear extracts of HPK1A*ras* cells transfected with a plasmid overexpressing hRXRα. The presence of the expected VDR/RXR complex these experiments rules out the presence of an inhibitory protein in HPK1A*ras* extracts. Similar results obtained using

a TRE labelled probe therefore indicate that the addition of exogenous RXRα is sufficient to form both RXR-VDR and RXR-TR heterodimers in HPK1A*ras* cells.

RT-PCR analysis of RNA extracted from both HPK1A and HPK1A*ras* cells reveals that the predominant form of RXR expressed in these cell lines is RXRα. Although RXRß has been shown to increase and facilitate vitamin D receptor binding, it is RXRα that has the greatest effect on vitamin D-dependent transcription (Kephart et al., 1996). RXRα's importance in skin biology is underscored by previous reports which claim that it is the predominant form of RXR in skin and cultured normal human keratinocytes (Xiao et al., 1995; Elder et al., 1992). Northern blot analysis of RXRα message revealed that this receptor is expressed in equal amounts in both HPK1A and HPK1A*ras* cells, therefore eliminating low levels of RXRα expression as one potential mechanism of vitamin D resistance.

Another distinct possibility to explain the inability of RXRα obtained from HPK1A*ras* cells to heterodimerize could be explained by genetic alteration of the RXRα dimerization domain. We investigated this possibility by amplifying, cloning and sequencing the D and E regions of the RXRα. These regions contain functional domains responsible for ligand binding, transactivation, and dimerization (Evans 1988; Forman and Samuels, 1990). No mutations in these regions were found.

Finally we analyzed RXRα protein expression by Western blot analysis. Surprisingly, RXRα derived from HPK1A*ras* cells migrates at a slightly slower rate than that of control cells, indicating a slightly higher molecular weight of the receptor. Almost all nuclear receptors are known to be phosphoproteins and an altered migration on SDS-gel analysis suggests that RXRα expressed in HPK1A*ras* cells could be in a hyperphosphorylated state as compared to the RXRα from control cells. Such a

mechanism could be responsible for the inability of RXRα to form complexes with VDRE and TRE. It is interesting to note that both the higher and lower molecular weight bands were present in the HPK1A*ras* extracts following overexpression of hRXRα, indicating that saturation of the mechanism by which RXRα is abnormally phosphorylated in HPK1A*ras* cells probably occurs. Phosphorylation is known to play a crucial role in regulating the activity of other nuclear receptors and transcriptional factors by altering their ability to translocate to the nucleus, by modulating their DNA binding capabilities, by affecting their dimerization ability or by affecting their interaction with other transcriptional factors (Weigel et al., 1995; Zhang et al., 1995; Mason and Housley, 1993). Inhibition of receptor-DNA interaction by phosphorylation has previously been reported in a brain isotype of the thyroid receptor (TRα2), in which the phosphorylation of a carboxy terminal residue results in the inability of TRα2 to bind DNA (Katz et al., 1995). DNA binding abilities of the monomeric nuclear receptor NGFI-B and VDR have also been described to be inhibited by phosphorylation (Hirata et al., 1993; Hsieh, 1993).

The present study would be the first incidence of post-translational modification(s) resulting in inhibition of RXR function. The crucial role of this protein in modulating VDR function is likely to expand our knowledge as to how malignant cells can evade normal growth arresting signals.

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CHAPTER 5

GENERAL DISCUSSION

5.1 Calcium Signalling in Keratinocytes

Signal transduction is critical in the control of cell proliferation and differentiation. Not all cells will respond the same way to a given signal. Many signals, especially those that are extracellular in origin, require the use of second messengers to transduce their message to intracellular effectors. Calcium, which exists in mM quantities outside the cell and in μM amounts inside, can act as a signal in certain cell types. Second messengers and effector proteins are just some of the ways in which this molecular signal is thought to work.

Calcium has been shown to attenuate keratinocyte cell proliferation and stimulate their differentiation. We have reported that raising the extracellular calcium concentration of cultured normal human keratinocytes results in increased transcriptional rates for both involucrin, a marker of differentiation, and p53, an anti-mitogen (Sebag et al., 1994). Other groups have shown that by simply varying the extracellular calcium concentration (Cao), the levels of involucrin and transglutaminase mRNA increase in a dose dependent fashion (Su et al., 1994). These effects have been supported *in vivo* by the finding that there exists a gradient of increasing intracellular calcium concentration from the basal layers of the epidermis to the more differentiated outer layers (Menon et al., 1985). Calcium exerts its effects on keratinocyte processes by modulating gene transcription as well as by post-transcriptional and post-translational modification of mRNA and protein (Gibson et al., 1996).

Calcium stimulates the transcription of another marker of differentiation, keratin 1 (K1) (Yuspa et al., 1991). The mechanism of keratin induction by calcium was somewhat clarified by the work of Roop et al. (Rothnagel et al., 1993). A 249 bp element, located 7.9

kb downstream from the K1 promoter, was isolated and shown to have calcium responsive transactivation properties (Rothnagel et al., 1993). This 249bp region was found to be active only in keratinocytes and not fibroblasts. Presumably a cell specific factor exists which enables calcium-dependent transcription of certain genes. However, the existence of this keratinocyte specific enabler has yet to be proven.

Phorbol esters are capable of inducing terminal differentiation of cultured normal keratinocytes. The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) mediates many of its cellular effects on keratinocytes through binding to a membrane receptor which can activate the calcium-activated protein kinase C (PKC) (Wille et al., 1985). Inhibitors of PKC have been shown to abrogate calcium induced keratinocyte differentiation (Denning et al., 1995). The mechanism of calcium induced transcription of loricrin, filaggrin and transglutaminase are all PKC dependent (Dlugosz and Yuspa, 1993; Dlugosz and Yuspa, 1994). We have reported that involucrin mRNA synthesis is stimulated by calcium, however, further work is necessary to determine whether this event is PKC dependent (Sebag et al., 1994).

Elevations of the early-immediate gene c-*fos* have been associated with the onset of keratinocyte differentiation (Dotto et al., 1986; Fisher et al., 1991). Calcium regulation of gene expression is well characterized in neuronal cells. Furthermore, calcium influx into neuronal PC12 cells through voltage-sensitive calcium channels leads to a rapid induction of c-*fos* (Ghosh et al., 1994). The influx of calcium through these channels stimulates phosphorylation of the cAMP response element-binding protein (CREB) transcription factor at serine-133 leading to an activated transcription complex capable of stimulation.

CREB is usually associated with a G-protein initiated signal transduction cascade. G-proteins are associated with the receptors of certain hormones and bind guanine

nucleotides. Upon hormone binding, the G-protein dissociates from the receptor and its stimulatory subunit will activate adenylate cyclase which in turn increases intracellular cyclic AMP concentration. cyclic AMP binds the regulatory subunit of protein kinase-A (PKA) whose catalytic subunit phosphorylates many cellular proteins including CREB (Gonzalez and Montminy, 1989). Phosphorylated CREB is active and regulates the transcription of target genes by binding to specific promoter DNA sequences known as cyclic-AMP response element (CRE) (Mitchell and Tjian, 1989). Calcium can bypass this cascade and directly stimulate CREB.

Calcium stimulated PKA-independent phosphorylation of CREB has been reported a variety of cell types. A family of calcium dependent enzymes known as calcium/calmodulin-dependent protein kinases (CaMK) have been shown to directly phosphorylate and activate CREB (Enslen et al., 1995). Subcellular distribution analysis revealed that CaMK can even enter the nucleus to turn on CREB. In neuronal cell lines, CaMKIV is responsible for the induction of c-*fos* in response to calcium.

In cultured human keratinocytes, c-*fos* stimulation does not occur following an increase in extracellular calcium (Sebag et al., 1994). TPA has been shown to increase c-*fos* mRNA in cultured mouse keratinocytes (Dotto et al., 1986). Unlike in mouse keratinocytes, calcium stimulated PKC activity in human keratinocytes does not result in c-*fos* transcription. Furthermore, CaMKIV does not appear to be expressed in keratinocytes (Enslen et al., 1995). The absence of this calcium based effector system in keratinocytes could also explain their failure to induce c-*fos* following calcium stimulation.

In our study, we found that in keratinocytes, calcium was able to stimulate p53 production. Calcium not only increased steady state levels of p53 mRNA, but it also increased the transcriptional rate of p53. The mechanism by which p53 transcription is

stimulated remains unknown. The importance of p53 in the calcium initiated differentiation of keratinocytes is underscored by recent experiments using p53 deficient mice. Keratinocyte cultures from mice expressing a null mutation in the p53 gene demonstrated a decreased responsiveness to elevated calcium concentrations (Weinberg et al., 1994).

The discovery of the calcium receptor in keratinocytes opens up interesting possibilities concerning calcium signalling. This receptor is believed to be coupled to the phospholipase C (PLC) pathway (Bikle et al., 1996). Activation of PLC leads to the production of two second messengers, inositoltriphosphate (IP3) and diacylglycerol (DAG). IP3 is thought to bind to an intracellular receptor and mediate the release of calcium from intracellular stores. It is interesting to note that in the human leukemic cell line HL-60, $1,25(OH)_2D_3$ has been shown to rapidly increase both the transcriptional rate of the IP3 receptor gene and the steady state levels of its mRNA (Bradford et al., 1993). The pro-differentiating effects of $1,25(OH)_2D_3$ and calcium are said to be synergistic. This synergy could be due to simultaneous calcium-dependented increases in IP3 production and $1,25(OH)_2D_3$-dependent increases in the synthesis of the IP3 receptor. The importance of this pathway in the intracellular signalling of calcium in keratinocytes remains to be proven. Analysis of keratinocytes from mice transgenically induced to express a null mutation in the calcium receptor gene could be used to further understand this signalling pathway in keratinocytes.

5.2 Diversity of VDR/Nuclear Receptor Response

The vitamin D receptor (VDR), like other nuclear receptors and transcriptional

factors, can function as transcriptional inducers as well as a repressors of transcription. For example, $1,25(OH)_2D_3$ bound VDR can stimulate the transcription of osteocalcin, osteopontin, c-*fos* and involucrin while repressing the transcription of c-*myc*, parathyroid hormone (PTH) and parathyroid hormone related protein (PTHRP). The DNA response elements that mitigate these diverging effects are often very similar in sequence and arrangement. Three mechanisms exist which can account for the differential effects of nuclear receptors. First, the presence transcriptional co-activators or co-repressors, which can associate with the nuclear factor, can direct ultimate outcome. Second, response element sequence may decide the effect of a nuclear receptor. Finally, response element environment in the context of the promoter can influence whether a nuclear factor can activate or repress.

5.2.1 Co-activators and Co-repressors

Overwhelming evidence has proven the existence of adaptor proteins that can act either as coactivators or corepressors for nuclear receptors Meyer et al., 1989). A co-activator has been recently identified for the estrogen and progesterone receptors that can also function for all steroid receptors (Onate et al., 1995; Baniahmad et al., 1995). The SRC-1 (steroid receptor co-activator) protein was identified using a yeast two hybrid system using the progesterone receptor as bait. It was found to interact with the C-terminal activation domain of steroid receptors to increase ligand dependent transcriptional activation by up to 10-fold. The presence of this coactivator in cells could therefore greatly enhance the transactivating ability of steroid receptors over those cells that lack this protein. Since this initial cloning, two other potential co-activators have been discovered,

both using the same yeast two-hybrid system. The first is the ligand dependent androgen receptor associated protein (ARA) that preferentially co-activates the androgen receptor and not the other steroid receptors (Yeh and Chang, 1996). The second is the glucocorticoid receptor-interacting protein 1 (GRIP1) which can interact with the hormone binding domain of the glucorticoid receptor. GRIP1 can also interact with a component of the RNA polymerase II complex, thereby acting as a bridge between the glucocorticoid receptor and the transcription machinery (Hong et al., 1996). A co-activator for VDR has yet to be identified.

Unoccupied thyroid and retinoic acid receptors are capable of repressing or silencing the transcription of certain target genes (Damm and Evans, 1993). Two factors have been identified to date which fulfill the role of co-repressors for unliganded thyroid and retinoid receptors, the silencing mediator for retinoid and thyroid hormone receptors (SMRT) and the nuclear receptor co-repressor (N-CoR) (Chen and Evans, 1995; Hörlein et al., 1995). Neither of them bind DNA, but when fused to a GAL4 DNA binding domain, they act as frank repressors of transcription of a GAL4-dependent reporter (Chen and Evans, 1995). The ligand binding domain of the thyroid and retinoid receptors have been proposed as putative sites for co-repressor binding. This is the same domain that is also responsible for both transactivation and dimerization with RXR (Tong, et al., 1996). In TR as in RAR, hormone binding results in the release of the co-repressor from the ligand binding domain and the reversal of transcriptional silencing, allowing TR and RAR/RXR to activate gene transcription.

5.2.2 Response Elements Context

The possibility that the sequence of the hormone response element itself can direct the trancriptional fate of bound hormone receptors is the least supported of all transcriptional-modulation theories. In theory, the differences between a response element that can activate transcription versus one that can repress transcription can presumably be attributed to nucleotide variations. For example, N-CoR-dissociates from RAR/RXR complexes in the presence of retinoic acid leaving RAR/RXR heterodimers bound to DR5 retinoic acid response elements (RARE). If the repeat spacing of the response element is decreased to 1 (DR1), N-CoR remains associated with RAR/RXR thereby preventing transactivation. Keeping in mind that N-CoR does not bind DNA, this is evidence that the actual nucleotide sequence of a response element can direct co-factor association with a nuclear receptor thereby selecting activation or repression (Kurokawa et al., 1995).

The two half sites that comprise the VDRE for the chicken PTH and the rat PTHRP genes are remarkably similar to those found in the human osteocalcin VDRE (Ozono et al., 1990; Liu et al., 1996; Kremer et al., 1996). All three VDREs were shown to bind to the same VDR/RXR complexes. The osteocalcin VDRE stimulates transcription while the PTH and PTHrP VDREs repress transcription. Variations found in methylation interference assays demonstrate that different nucleotides serve as binding sites for the 'negative' cPTH VDRE as compared to the 'positive' osteocalcin VDRE (Liu et al., 1996). It is possible that these differences in protein-nucleotide contacts could determine the way in which a VDR/RXR heterodimer could affect gene transcription.

The presence of a response element within the context of a gene promoter plays an important role in determining the outcome of nuclear receptor binding. Repression of the glycoprotein α-subunit gene occurs through an interaction between glucorticoid and CREB (Akerblom et al., 1988; Diamond et al., 1990). The overlapping cAMP response element

and of a glucocorticoid response element, causes the GR to displace the stronger-transactivator CREB.

Another example of context specific inhibition is the mechanism of $1,25(OH)_2D_3$ induced inhibition of interleukin-2 gene transcription in T-lymphocytes (Alroy et al., 1995). A negative-VDRE was found within a 40 base pair region that also holds an important positive regulatory element. Normally the T-lymphocyte specific transcription factor, NFATp and an AP-1 complex (c-*fos*/c-*jun*) bind to the 40 base pair region known as the NF-AT-1. Binding by either or both of these factors/complexes, results in the transactivation of interleukin-2. Addition of $1,25(OH)_2D_3$ abolishes this transactivation by allowing VDR to bind to the NF-AT-1 site thereby preventing NFATp and AP-1 induced transactivation. A truncated VDR possessing only a DNA binding domain was not able to repress interleukin-2 transcription, but was able to prevent its stimulation. This suggests that in addition to antagonizing transcriptional factors, VDR mediated repression must involve some as of yet undertermined mechanism or factor.

5.3 VDR Resistances

Analysis of the mechanisms responsible for vitamin D resistance has given us much information concerning the vitamin D signalling pathway.

5.3.1 Type II Rickets

The first, and by far the most commonly reported vitamin D resistances, are those found in the clinical condition known as rickets. Patients with type II rickets are

insensitive to vitamin D despite the presence of elevated circulating concentrations of $1,25(OH)_2D_3$. These patients display a target-organ resistance to $1,25(OH)_2D_3$ which is inherited in an autosomal recessive manner (Liberman, et al., 1983; Liberman, et al., 1980). Up to now it has been shown that almost all cases of hereditable type II rickets are associated with a point mutation within coding region of the VDR gene. Ten mutations have been identified to date, two are in the $1,25(OH)_2D_3$ binding domain, two in the variable-hinge region and six more have been found in the DNA binding domain of the VDR (Sone et al., 1980; Malloy et al., 1994). These mutations either disable ligand or DNA binding or prevent receptor translocation to the nucleus. In one unique case of end-organ resistance to the effects of $1,25(OH)_2D_3$, no VDR mutations whatsoever were found (Hewison, et al., 1993). Skin fibroblasts were isolated from this patient, and it was found that there were lower but adequate cytosolic concentrations of VDR. However, this unaltered VDR was absent from the nucleus in cells of this patient. Since little is known about the translocation process of nuclear factors, understanding the defect in this case of rickets could help us clarify this process.

5.3.2 Tissue/Cell Resistances

Several cell lines have been characterized that show resistance to the gowth inhibitory influences of $1,25(OH)_2D_3$. Most of these cell lines are either derived from *in vivo* tumors or are part of artificially established tumor models. It is not surprising to see the spontaneous development of resistance to a growth inhibitory substance. Cancerous tissues or cells that have been reported to demonstrate vitamin D resistance include those deriving from the breast (Narvaez et al., 1996), skin (Sebag et al., 1992; Ratnam e al.,

1996; Park et al., 1994), colon (Schumaker et al., 1996) and hematopoetic system (Lasky et al., 1994; Iwata et al., 1996). With the exception of a point mutation found in the VDR of colon cancer cells, all other incidences of resistance do not or have not yet been shown to inculpate a genetic alteration of the VDR.

An inhibitor, or repressor, of VDR function has been postulated to account for the $1,25(OH)_2D_3$ resistance reported in New World primate cells (Arbelle et al., 1996). Nuclear extracts from these cells were shown to have impaired binding characteristics to a radiolabelled VDRE in electrophoretic mobility shift analysis (EMSA). Although a complex was present that could bind specifically to the VDRE, it did not contain VDR nor RXR. Normal VDR/RXR-VDRE formation by old world primate nuclear extracts was disrupted by supplementing these with extracts from the resistant cells. So far no protein(s) has been purified or isolated that could compete with VDR/RXR binding to a VDRE thereby confering the observed $1,25(OH)_2D_3$ resitance.

5.4 Nuclear Receptor Phosphorylation

We have proposed that a phosphorylation event involving the RXRα could be responsible for the resistant phenotype seen in ras transformed keratinocytes (Sebag et al., 1996). To date aberrant phosphorylation has never been reported to be the cause of *in vivo* steroid receptor dysfunction. Even though all nuclear receptors are known to be phosphoproteins, the physiological relevance of this post-translational modification is unclear and under recent active investigation.

The chicken progesterone receptor (PR) is phosphorylated at 9 different sites. Five of these sites are phosphorylated in basal conditions but enhanced in response to

progesterone, while the four others are only phosphorylated in the presence of hormone (Weigel et al., 1995; Beck et al., 1996). Two enzymes have been reported to phosphorylate the chicken progesterone receptor *in vivo*, protein kinase A (PKA), and a novel DNA-dependent protein kinase (Weigel et al., 1995). The chicken progesterone can be activated in absence of hormone by modulating these kinases through treatment of cells with EGF or dopamine, which activate PKA. Phosphorylation of the PR above basal levels, or hyper-phosphorylation, results in a net increase in transcription from a progesterone response element-driven promoter (Bagchi et al., 1992). Full phosphorylation of the PR was achieved only in the presence of a PR-response element, implicating the DNA-dependent protein kinase in PR activation. Progesterone antagonists can differ in the way in which they influence PR phosphorylation. While one progesterone antagonist was shown to induce the same level of phosphorylation as progesterone itself, another was found to only weakly stimulate phosphorylation (Beck et al., 1996). Finally, differential phosphorylation of different PR forms may be able to explain their diverging biological activities. PR exists in two forms, PR-B, which acts as a transcriptional activator and PR-A which is shorter than PR-B by 164 residues at its amino-terminus and can function as a transcriptional repressor. PR-B has an extra phosphorylation site at ser102 which may contribute to its inability to repress transcription (Zhang et al., 1995).

At least seven phosphorylation sites have been identified in the glucorticoid receptor (GR) (Mason and Housley, 1993). Unlike the PR, PKA does not seem to play a role in GR phosphorylation (Orti et al., 1993). Inhibition of protein phosphatases are the only mechanisms proposed to date that modulate glucorticoid phosphorylation (Moyer et al., 1993). The enzyme(s) responsible for GR phosphorylation remain undefined. Glucocorticoid phosphorylation at these sites does not appear to modulate the

transcriptional properties of the liganded receptor (Orti et al., 1993). It has been proposed that hormone-dependent phosphorylation of the GR modulates GR transport in and out of the nucleus (Orti et al., 1993; Kuiper and Brinkmann 1994).

A link between the GR phosphorylation state and the various events of the cell cycle has been proposed. Most cells are believed to be resistant to the effects of glucocorticoids when they are in the G2 phase of the cell cycle. GR basal phosphorylation is three times higher in the G2/M phase of the cell cycle as compared to the S phase. However, addition of hormone results in an increase in GR phosphorylation only in cells that are in the S phase. GR receptors are refractory to ligand-dependent phosphorylation when cells are in the G2 phase of the cell cycle. Differences in these phosphorylation levels are likely due to the actions phosphatases as well as kinases (Hsu and DeFranco, 1995; Hu et al., 1994).

Transformation of cells with the v-*mos* oncogene results in disruption of normal GR movement. Activation of v-*mos*, an oncogenic transcription factor that normally functions in the induction of meiosis and regulation of cell-cycle progression in oocytes (RhoLenormand and Leibovitch, 1995), causes hyperphosphorylation of GR which results in GR accumulation in the cytoplasm (Borror et al., 1995). The disruption of GR function by oncogenic activation is similar to our report of VDR disruption. However, cytoplasmic localization is not suspected to be the defect in ras transformed keratinocytes as RXR protein is readily detectable in HPK1A*ras* nuclear extracts (Sebag et al., 1996).

Phosphorylation plays an important role in VDR function. VDR is phosphorylated on at least two residues and by two different enzyme systems. The most important phosphorylation site is the one on serine 51 (Hsieh et al., 1993). Protein kinase C is responsible for the phosphorylation of VDR at this site. The Ser 51 is located

between the two zinc fingers within the DNA binding domain of hVDR. Mutation of ser51 to a negatively charged Asp, results in a hVDR that is unable to bind to VDRE and unable to be phosphorylated by purifed PKC. PKC phosphorylation at Ser51 disables DNA binding. Mutation of Ser51 to alanine results in a hVDR that can normally bind DNA and transactivate, but cannot be negatively regulated by PKC. PKC, when activated, therefore acts as a negative regulator of VDR function (Hsieh et al., 1993).

The other important phosphorylation site of VDR is at position 208. This serine is phosphorylated by the catalytic subunit of casein kinase II (Jurutka et al., 1993). Phosphorylation at this site does not alter hormone binding, nuclear translocation nor VDRE binding. Co-expression experiments with plasmids containing hVDR, casein kinase II and a VDRE-driven reporter plasmid, demonstrate that the presence of casein kinase II, while not essential, greatly enhance the transactivating abilities of VDR. As such, the mutation of Ser208 to a glycine or alanine does not result in complete disruption of VDR, but does abrogate casein kinase II inducibility and enhancement (Jurutaka et al., 1996).

Perhaps one of the best studied receptor with respect to phosphorylation is the estrogen receptor (ER). ER is the only nuclear receptor known to date that is phosphorylated on a tyrosine residue. A 67 kDa kinase was identified in calf uterine extracts that could be responsible for ER Tyr537 phosphorylation (Castoria et al., 1993). This enzyme appears to bind to and be activated by calmodulin in a calcium dependent manner. The enzyme was also shown to be inactive without the presence of the estrogen receptor itself. *In vitro*, ER can also be phosphorylated at Tyr537 by the frequently oncogenic src family of tyrosine kinases (Arnold et al., 1995). Breast cancer cells overexpressing p60c-src would therefore gain a definate growth advantage by

constitutively activating the growth promoting estrogen receptor.

The ER is also phosphorylated on a number of serine residues. Serines 122, 156, 158 and 298 have been identified as possible phosphorylation sites (Lahooti et al., 1995). As well, casein kinase II was shown to phosphorylate ER at serine 167 in an estrogen dependent manner (Arnold et al., 1994). The functional significance of these modifications to the ER remain nebulous.

It is now believed that phosphorylation at ser118 plays an extremely important role in ER biology. Ser118, within the A/B binding domain of of ER, is phosphorylated by mitogen activated protein kinase (MAPK) (Kato et al., 1995). This phosphorylation can be stimulated by addition of mitogens such as EGF. MAPKK, the activator of MAPK, was also shown to stimulate the phosphorylation ER at Ser118. Phosphorylation at this site had no effect on response element recognition and binding, it did however have an impact on estrogen mediated transcriptional activation. This is not surprising as Ser118 lies within the A/B domain which contains a transactivation region. In addition, ER transactivation function was demonstrated to be stimulated by *ras*, the upstream 'controller' of MAPK.

It has been known that estrogen is mitogenic for breast cancer cells. *Ras* and its related proteins, are frequently mutated or upregulated in breast cancers (see section 1.4). By constitutively activating the ER through MAPK mediated phosphorylation, the *ras* oncogene can confer a growth advantage to *ras* positive breast cancer cells.

5.4.1 RXR as Target for MAPK

Can the cross talk described between *ras* and nuclear receptors be applied to our

keratinocyte model of tumor progression? *Ras* transformed keratinocytes are resistant to the growth inhibitory effects of vitamin D. This resistance appears to be mediated by a disruption of the normal VDR/RXR-VDRE complex formation. Furthermore, RXR may be hyperphosphorylated in HPK1A*ras* cells, as demonstrated by retarded mobility through an SDS-gel. Scanning of the RXRα amino acid sequence reveals two MAPK consensus phosphorylation sites (ProXXSer/ThrPro). Both are in the same region as the MAPK target in ER, one at Ser32, the other at Thr82. As not much is known about the phosphorylation pattern of RXR and there exist no reports to date of phosphorylation in this region. Ras transformation of keratinocytes leading to a constitutively active *ras*/MAPK pathway could lead to a permanently hyperphosphorylated RXR. Future experiments could include the transfection of a constitutively active MAPKK into the normally responsive HPK1A cells to see if a resistant phenotype can be duplicated. Another experiment needed to clarify this issue would involve the transfection of RXRα mutated at Ser32 and/or Thr82 into HPK1A*ras* cells. The inability of *ras* to stimulate the phosphorylation of the mutant RXRα could restore HPK1A*ras* sensitivity to $1,25(OH)_2D_3$.

If *ras* were to play a role in this disruption, it would have to inhibit VDR/RXR function, rather than to stimulate it as it does with ER. Had the putative RXR MAPK-consensus phosphorylation sites been situated within the DNA binding domain of RXR, it would be easy to imagine a disruption of complex formation in the presence of a VDRE. Although MAPK mediated inhibition of RXR/RXR-RXRE transactivation has yet to be proven, phosphorylation at these two amino-terminal sites could still prevent DNA binding or VDR dimerization.

A brain specfic variant of the thyroid hormone receptor, TRα2 has been shown to

be phosphorylated by casein klinase II on serine residues at position 474 and 475 (Katz et al., 1995). A unique C-terminus prevents TRα2 from binding thyroid hormone. Its only known function is to antagonize the binding of ligand bound TR. As TRα2 is unliganded, when it is bound to a TRE it represses transcription. Phosphorylation of Ser474 and Ser475 results in an inhibition of this activity. Specifically, phosphorylation at the carboxy terminal end of TRα2 results in the disruption of DNA binding, mediated by the amino terminal portion. This scenario sets a precedent for the mechanism we are proposing to explain the *ras* induced phosphorylation of RXRα and its subsequent inability to form complexes with VDR on a VDRE.

Inhibition of the RXR/VDR signalling pathway confers a growth advantage to cells that have been transformed by *ras*. As $1,25(OH)_2D_3$ is a powerful inhibitor of cell growth, it would therefore be advantageous for a cancer cell to turn off this inhibitory pathway.

In conclusion, these results suggest that VDR/RXR heterodimer disruption occurs in *ras*-transformed keratinocyte and may at least in part explain the mechanism(s) by which *ras* transformed keratinocytes escape growth inhibitory influences.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. $1,25(OH)_2D_3$ and calcium act in concert to modulate the expression of c-*fos* and p53, two cell cycle related genes, in normal human keratinocytes.

2. Unlike in other cell types, varying the extracellular calcium concentration has no effect on c-*fos* mRNA expression in normal human keratinocytes.

3. Calcium stimulates the transcription of the p53 gene in normal human keratinocytes.

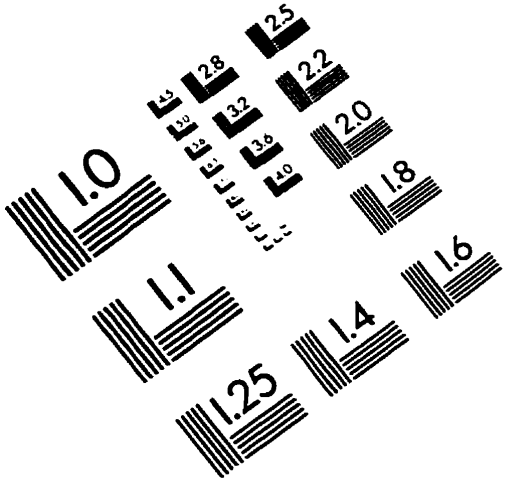
4. *ras* transformed human keratinocytes are resistant to the growth inhibitory influence of $1,25(OH)_2D_3$.

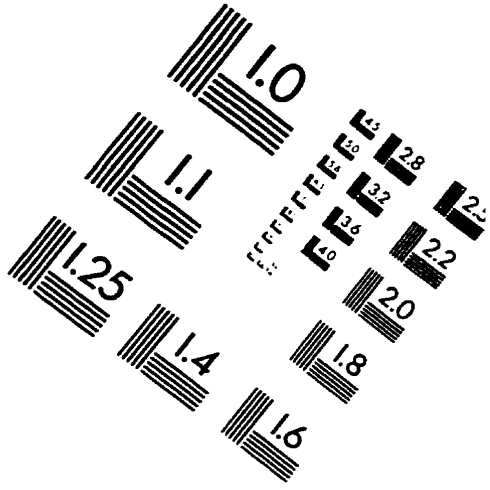
5. The resistance of *ras* transformed human keratinocytes to the effects of $1,25(OH)_2D_3$ occurs at the level of gene transcription.

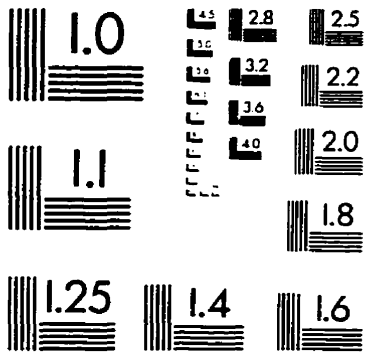
6. A disruption of normal VDR/RXR heterodimer formation in the presence of a VDRE occurs following *ras* transformation of HPV immortalized keratinocytes.

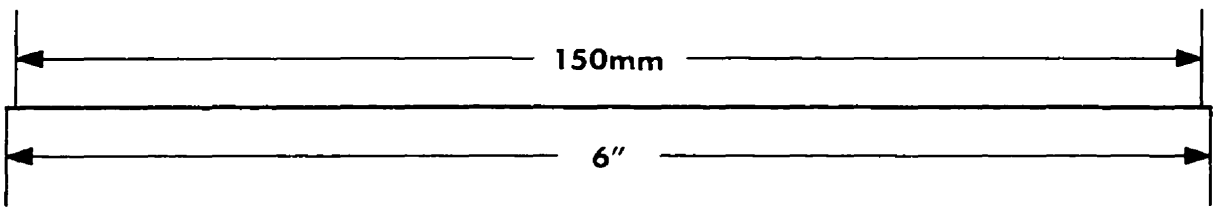
7. *ras* transformation of HPV immortalized keratinocytes induces aberrent post translational modification(s) of the RXRα protein.

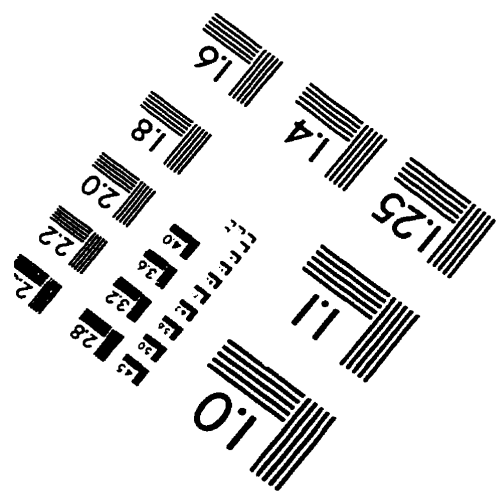
IMAGE EVALUATION
TEST TARGET (QA-3)

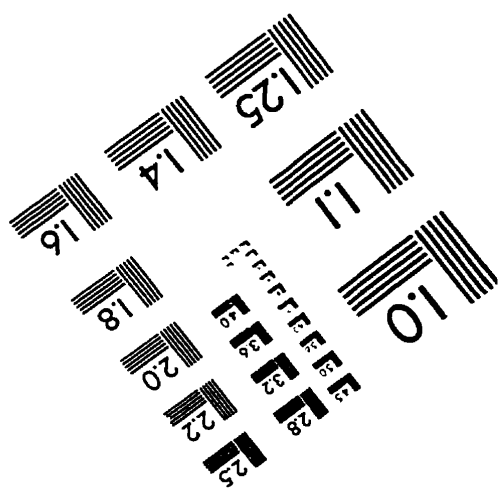












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