CCND1 a Cell Cycle Regulator and Proto-oncogene.

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Abstract.

The *CCND1* gene codes for a recently discovered member of the cyclin D family of proteins and is thought to function during the G1 phase of the cell cycle. Amplification and transcript over-expression of *CCND1* have in the past two years been reported in several types of human tumours, providing the initial evidence for the contribution of its product in the development of cancer. This investigation was aimed at clarifying the involvement of the *CCND1* gene in transformation.

The *CCND1* gene dosage, transcript and protein levels were characterised in a panel of newly derived SCC cell lines, revealing the same frequency of amplification as reported in tumour biopsies. The frequency differed from the previous analyses of established cell lines and may reflect the methods used to derive the cell lines. In all cases amplification resulted in upregulated levels of the *CCND1* mRNA and protein ($p34^{cycD1}$), although amplification was not necessary for over-expression of *CCND1*. These findings further emphasise the status of this gene as the target of 11q13 amplification. Interestingly, the cell lines that exhibited *CCND1* amplifications had also increased expression of the tumour suppressor protein pRb, a potential regulator or substrate of $p34^{cycD1}$.

SCC derived cell lines, original tumour biopsies and xenografts revealed an exclusively nuclear localisation of $p34^{cycD1}$, as previously determined for fibroblast cell lines. Serum stimulation of G0 arrested fibroblasts induced an increase of $p34^{cycD1}$ levels four hours from exposure, which reached a maximum between 8-12 hours and declined thereafter. The same oscillation pattern was observed in EGF stimulated keratinocytes where cell cycle delay was achievable through serum deprivation.

To analyse the stage during transformation at which a cell might find it beneficial to over-express $p34^{cycD1}$, two types of transfection analyses were carried out. An established, poorly tumourigenic SCC cell line (SCC12) that originated from a skin SCC, was lipofected with sense or antisense *Cyl-1* cDNA expression vectors and the derived clones examined for their ability to form tumours in immunodeficient mice. No alterations in growth patterns or tumourigenicity of the transfected cells were observed. Alternatively, human foreskin primary keratinocytes transfected with *Cyl-1* cDNA expression vectors alone or in combination with *HPV-16 E6* or *E7* expression constructs exhibited the ability to overcome the M1 restrictive stage and therefore achieve an extended life span. It remains speculative if *in vivo*, over-expression of *CCND1* may contribute to immortalisation of epithelial cells. Examination of rodent fibroblast cell lines and their *fos* over-expressing clones revealed the lack of correlation between $p34^{cycD1}$ levels and *fos* induced transformation.

In conclusion, these studies may suggest a significant contribution of *CCND1* over-expression in the initial stages of epithelial transformation. Increased levels of $p34^{cycD1}$ could permit escape of epithelial cells from growth control and therefore differentiation, resulting in their indefinite lifespan which allows the occurrence and fixation of other genetic alterations. However, these results do not contradict the potential involvement of *CCND1* in later stages of tumour development.

To my family and David

for all the happy times we have had together.

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DECLARATION.

The work contained in this thesis was carried out personally, unless otherwise stated.

Table of Contents.

List of Figures.

List of Tables.

Abbreviations.

A_x	$Absorbance_{wavelenght}$
amp	ampicillin resistance marker
ATP	adenosine 5'triphosphate
bp	base pair
BrdU	5'-bromo-2'deoxyuridine
BSA	bovine serum albumin
C	centigrade
cAMP	3',5' cyclic adenosime monophosphate
cm	centimeter
CSF	colony stimulating factor
cpm	counts per minute
CTP	cytosine 5'triphosphate
d	deoxy
dH_2O	distilled water
Da	Dalton
dd	dideoxy
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleid acid
ECL	enhanced chemiluminescent
EDTA	ethylenediaminetetra-acetic acid, disodium salt
EGF	epidermal growth factor
FBS	foetal bovine serum
FGF	fibroblast growth factor
fig.	figure
G418	geneticin sulphate
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTP	guanine 5'triphosphate
HBS	HEPES buffered saline
HCL	hydrochloric acid
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
hr	hour
k	kilo
KGM	keratinocyte growth media
l	litre
LTR	long terminal repeat
μ	micro
m	milli
M	molar
mA	milliamps
min	minute
mol	mole
MOPS	sodium morpholinopropane sulphonic acid
mRNA	messenger RNA

Mr	molecular weight
n	nano
neo	neomycin resistance marker
nm	nanometre
o/n	overnight
oligo	oligonucleotide
PAGE	polyacrilamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PMSF	phenylmethylsulphonylfluoride
rpm	revolutions per minute
RNA	ribonucleic acid
RNase	ribonucease
SDS	sodium dodecyl sulphate
sec	second
SFM	serum free media
p	pico
TEMED	tetramethylenediamine
TPA	12-O-tetradecanoylphorbol-13-acetate
tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
TTP	thymidine 5'triphosphate
UV	ultraviolet
V	volts
W	watts
v/v	volume for volume
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Amino acid one and three letter codes

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine

W	Trp	Tryptophan
Y	Tyr	Tyrosine

INTRODUCTION

CHAPTER 1 : Introduction.

1.1 The plasticity of chromosomes in cancer.

The human genome consists of approximately $7.1x10^9$ base pairs, the entire length of which is packed into 23 pairs of chromosomes. In these structures lies most of the information a cell needs to live and proliferate and it is here that accumulative changes may bring about disordered cellular growth resulting in transformation and cancer.

The abnormalities most commonly seen in chromosomes have been classified into structural (translocations, insertions, inversions, deletions and amplifications) and numerical (changes in chromosome number). In the past decade great progress has been made to link consistent chromosomal aberrations with certain types of cancer, leading to the discovery and isolation of many new genes.

1.1.1 Changes in chromosome number.

Polyploidy is not commonly seen in cancer and is a term used to characterise an increase in the number of haploid sets of chromosomes. A more common phenomenon in transformation is aneuploidy, the loss of a single chromosome and the gain of one or more (Yang-Feng 1991). These changes cause alterations in huge blocks of DNA making it hard to identify specific genes involved in the phenotype of the cancer cell. In several instances a link between aneuploidy and poor prognosis has been suggested although this is still under debate (von Rosen 1989, Suchy *et al* 1992, Johan *et al* 1992).

Restriction fragment length polymorphism (RFLP) analysis has revealed the deletion of chromosome 11 in a significant number of neuroblastomas (Strivatsan *et al* 1993). The loss of this chromosome is also seen in rare cases of parathyroid adenomatosis (Arnold and Kim 1989). Trisomy, hyperploidy to near tetraploidy and even rare cases of polyploidy were observed in a study of 59 ovarian carcinomas (Pejovic *et al* 1992). The most frequent chromosome gain was +20. Specific loss of chromosomes 8, X, 13, 14, 17 and 22 were also reported (Pejovic *et al* 1992).

What causes aneuploidy ? In a study of colorectal neoplasms point mutations in the *Kirsten-ras* proto-oncogene (*K-ras*) were proposed to contribute to the development of aneuploidy (Suchy *et al* 1992). Loss of functional tumour suppressor protein p53 is a frequent event in most cancers and may indirectly lead to gross mutations and rearrangements of the genome (Weinberg 1991, Lane 1992). p53 is a sequence specific DNA binding protein proposed to act as the "guardian of the genome", protecting cells from DNA damage (Lane *et al* 1993). Several reports have implicated p53 in the G1 check point (Lane 1992) and it may also be involved in apoptosis (Yonish-Rouach *et al* 1993). The removal of this "guardian" is predicted to lead to genome instability and accumulation of many genetic alterations, of which aneuploidy may be one.

A majority of cell lines that have been derived from tumour tissues show aneuploidy, although alterations caused by adaptation to tissue culture conditions must be taken into account (Edington *et al* submitted). For example aneuploidy has been observed during later stages of rodent and human fibroblast or keratinocyte immortalisation (Hawley-Nelson *et al* 1989, Rice *et al* 1993, Edington *et al* submitted). One of the potential causes of several types of

chromosomal abnormalities such as rearrangements, deletions and aneuploidy in these cells is thought to be telomere shortening, due to the absence of functional telomerase, a ribonucleoprotein containing the ability to compensate for sequence loss during replication (Harley 1991, Counter *et al* 1992).

1.1.2 Deletions.

In 1971 Knudson (Knudson 1971) proposed a mechanism by which tumours arose from specific DNA deletions. Today the theory is widely accepted and chromosomal losses in human tumours are regarded as evidence that the affected regions contain tumour suppressor genes. The malignancies are thought to arise from two distinct genetic events, one inherited from a parent or taking place during gametogenesis and the other occurring somatically. Conversely, both events may be somatic.

The del (13)(q14;q14) is associated with retinoblastomas, a rare type of childhood tumour and results in the loss of the tumour suppressor gene, *Rb* (Knudson 1971, Friend *et al* 1986, Varmus 1989, Sikora *et al* 1990, Solomon *et al* 1991). The fact that the protein pRb is a target for inactivation by viral oncogenes such as the *SV40 T* antigen, *human papilloma virus (HPV-)16 E7* and *adenovirus 5 E1a* suggests its importance as a negative regulator of cellular proliferation (Weinberg 1991).

The *WT1* gene is a potential transcription factor and tumour suppressor gene (Hastie 1993). Deletions of chromosome 11p13 result in the loss of one *WT1* allele predisposing the individuals to a disease known as Wilms' tumour that results from the absence of functional *WT1* products (Sikora *et al* 1990; Solomon *et al* 1991).

Work on colorectal carcinomas has revealed the existence of several potential negative regulators of cell growth. The loss of 17q proved to remove a now well known tumour suppressor gene, *p53* (Baker *et al* 1989, Finlay *et al* 1989) and occurs in more than 70% of cases. The absence of functional p53 releases the cell from an important G1 check point and will allow for the accumulation of DNA mutations (Lane 1992, Prives 1993). Deletion of 18q takes place at a similar frequency (~70%) resulting in the loss of the *DCC* gene, the product of which is related to *NCAM*, a neural cell adhesion molecule (Bishop 1991, Srivatsan *et al* 1993, Porfiri *et al* 1993). A dominantly inherited disorder, familial adenomatous polyposis (FAP) is closely linked to the loss of chromosome 5q in ~20% of studied cases (Solomon *et al* 1987, Miki *et al* 1992, Sen-Gupta 1993). Heterozygous individuals develop multiple benign polyps that frequently progress to malignant adenocarcinomas (Solomon *et al* 1987, Sikora 1990) following a mutational event that inactivates the other *FAP* allele.

1.1.3 Chromosomal amplification.

In some organisms at specific points during development a requirement for copious amounts of certain gene products must be reached. Transcription from single genes would not be sufficiently rapid to reach the requirements and in these instances DNA amplification takes place. The best known examples of developmentally regulated amplification are the accumulation of rDNA in *Xenopus laevis* germ cells and of the chorion genes in *Drosophila* egg chambers. In these instances gene dosage may increase a 1000-fold or up to 60-fold, respectively (Stark and Wahl 1984). In mammals no examples of developmentally regulated amplification have been found, however it has been well documented as an abnormal process in cancer (Stark 1986, Stark *et al*

1989). Specific regions of the human genome have proved to be common targets for amplification in certain types of cancer and this is linked to poor prognosis for the patient (Tsuda *et al* 1989a, Kitagawa *et al* 1991, Schuuring *et al* 1992b).

The gene dosage of *N-myc* is increased in a high proportion (~50%) of neuroblastomas, some retinoblastomas and Wilms' tumours (Schwab and Amler 1990, Solomon *et al* 1991). The extent of amplification may vary from 3-300 fold

in different tumours and strongly correlates with more advanced stages of neuroblastomas. The high frequency of these events suggests that it is advantageous to have increased levels of *N-myc*. *N-myc* is located on chromosome 2p23-p24, however copies of the gene may be found at several other chromosomal homologous staining regions (HSRs) in tumours where amplification has taken place (Schwab *et al* 1984). The other two members of the myc-box family, *c-myc* and *L-myc,* have increased gene dosage in several types of lung carcinoma. *c-myc* is also amplified in breast carcinomas and Burkitt's lymphoma (Stark 1986).

The epidermal growth factor receptor (EGFR) is an important component of the signal transduction pathway, the gene of which (*c-erbB*) is located on chromosome 7q22 and is amplified in ~10-20% of squamous cell carcinomas (SCC), 1% of breast cancers, 80-90% of late stage glioblastomas and occasional meningiomas (Libermann *et al* 1985, Wong *et al* 1987, Haley *et al* 1987, Ro *et al* 1987, Malden *et al* 1988, Harris and Nicholson 1988, Ishitoya *et al* 1989). A related gene *c-erbB2*, also known as *HER-2/neu*, commonly has increased gene dosage in human mammary carcinomas and derived cell lines (Schwab and Amler 1990, Stanton *et al*, submitted).

The size of the amplified DNA fragment is generally much greater than one gene and can involve up to 10,000kb, suggesting that many sequences are co-amplified with a key gene, the increased expression of which is selected for (Stark *et al* 1989). A good example is the commonly amplified 11q13 region reported in ~50% of head and neck squamous cell carcinomas (HNSCC), ~15% mammary tumours and oesophageal carcinomas (Zhou *et al* 1988, Berenson *et al* 1989, Tsuda et al 1989b, Somers *et al* 1990, Kitagawa *et al* 1991). The identity of the key gene in this amplicon still remains debatable. This region will be described in greater detail in section 1.2.

The location of amplified DNA in mammalian cells can be:

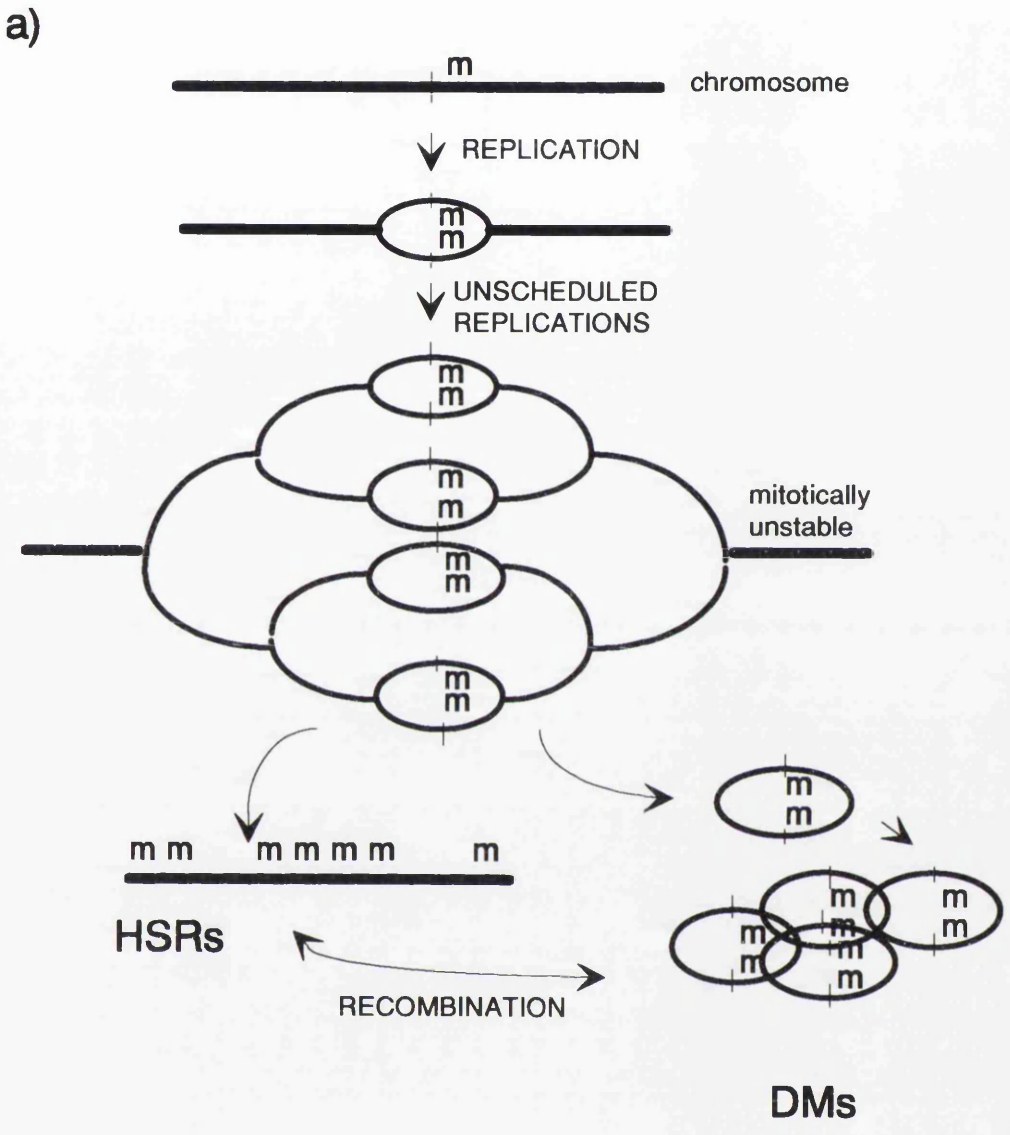
(i) chromosomal - a stable form and more common in cancer. It is often found integrated into chromosomes as HSRs or extended chromosomal regions (ECR) (Stark *et al* 1989), or

(ii) extrachromosomal - DNA found free of chromosomes usually in double minutes (DMs), a transient type of amplification frequently seen in clinical resistance to drug treatment and tissue culture conditions (Stark *et al* 1989). In cancer, amplified DNA is often found near to or at the site of the original gene from which it was derived, although this is not always the case (Roelofs *et al* 1993).

The exact mechanisms of amplification are thought to be specific for each event. Likely models include unequal sister chromatid exchange during mitosis, unscheduled replication and chromosomal rearrangements see Figure 1. Commonly, other gross abnormalities of the genome such as chromosome deletions, rearrangements, translocations and even loss have been coupled with amplification.

Figure 1. Two mechanisms of DNA amplification.

Schematic diagram representing **a)** DNA amplification due to unscheduled replication and subsequent recombination; **b)** DNA amplification due to chromatid misalignment during mitosis (Stark and Wahl 1984).


a)
m
chromosome
REPLICATION
m
m
UNSCHEDULED
REPLICATIONS
mitotically
unstable
m m m m m
HSRs
RECOMBINATION
DMs
b)
chromatids
MISALIGNMENT
RECOMBINATION

1.1.4 Translocations, insertions and inversions.

Chromosomal rearrangements affect genes in the proximity of the translocation breakpoint resulting in their deregulation, or the formation of oncogenic fusion genes. Although this type of chromosomal abnormality is most common in cancer the mechanism by which the events take place are not known, probably due to their diversity.

The consequences of chromosomal rearrangements have been separated into two categories:

(i) Rearrangements that result in the formation of a new chimeric product which contains coding sequences and transforming potential from both partners.

A well known example is the Philadelphia chromosome in chronic myelogenous leukaemia (CML), present in over 90% of patients with this disease (Bishop 1989, Solomon *et al* 1991, Secker-Walker and Craig 1993). It results from a reciprocal translocation between 9p34 and 22q11 causing the formation of the *bcr-abl* fusion mRNA and protein. The breakpoint cluster region (*bcr*) encodes a guanosine triphosphatase (GTP-ase) binding protein (GAP) for $p21^{rac}$. *c-abl* is a specific protein tyrosine kinase, the activity of which is unmasked by the fusion to *bcr*, this is attributed to the replacement of the normal N-terminal domain of *c-abl* by the domain derived from *bcr* (Bishop 1989).

(ii) Rearrangements that place a cellular proto-oncogene under the control of a strong promoter causing over-expression of the gene.

The immunoglobulin heavy (IgH) chain locus on 14q32 is frequently involved in rearrangements with several regions: 8q24, 11q13 and 18q21. The t(8;14)(q24;q32) translocation is a characteristic feature of Burkitt's lymphoma (BL), a malignancy of immature B cells evident in ~80% of patients suffering

from the disease (Bishop 1989, Hiyama *et al* 1991). The consequence of the rearrangement is upregulation of the *c-myc* transcripts and protein (Petroni *et al* 1992). Other less common events in Burkitt's lymphoma, involving 8q24 and 2p11 or 22q11 have a similar effect on *c-myc* expression and may encompass the *PVT* gene located 260kb downstream of *c-myc* (Shtivelman and Bishop 1990) .

The t(11;14)(q13;q32) and t(14;18)(q32;q21) rearrangements are proposed to link the IgH chain promoter and genes located in these two regions. The former will be described in greater detail in section 1.2.1.b.

1.2 *CCND1*, the gene coding for human cyclin D1.

1.2.1 The 11q13 region.

The q13 band on chromosome 11 is a dynamic area commonly amplified or rearranged in human tumours (Lammie and Peters 1991, Gaudray *et al* 1992). 11q13 is suggested to be ~15 million base pairs (mb) long and may be divided into five sub-regions by the use of cytogenetic analysis of extended pro-metaphase spreads (Lammie and Peters 1991, Brookes *et al* 1993). Most of the genes expected to reside in this area remain unknown, although rapid progress is under way leading to their elucidation.

1.2.1.a *FGF-3* and *FGF-4.*

The mouse mammary tumour virus (*MMTV*) is capable of inducing mammary carcinomas in susceptible mice after a long latency period. This was proposed to be by activating host genes in the vicinity of the viral DNA integration site and was used for the isolation of new proto-oncogenes (Nusse and Varmus 1982, Peters *et al* 1983, Dickson *et al* 1984, Dickson and Peters

1987). Following this strategy, *MMTV* integration into murine chromosome 7 led to the identification of the activated *int-2* gene (Peters *et al* 1983, Dickson *et al* 1984). The human homologue of *int-2* was mapped to chromosome 11q13 (Casey *et al* 1986).

In 1986 Sakamoto caused transformation of a mouse fibroblast cell line, NIH3T3, by transfection with specific DNA sequences obtained from patients with stomach cancer (Sakamoto *et al* 1986). The transforming sequences were isolated, cloned and the new gene initially named *hst*, was later termed *hstf-1* for heparin binding secretory transforming factor (Yoshida *et al* 1988). *hstf-1* mapped to chromosome 11q13.3 and the vicinity of *int-2* (Yoshida *et al* 1988). These were the first candidates for the key genes of 11q13. Following the new nomenclature, the *int-2* and *hstf-1* genes will be referred to in subsequent sections as *FGF-3* and *FGF-4*, respectively.

1.2.1.b Rearrangements involving 11q13.

One of the first observed rearrangements of 11q13 were made in small or large cell diffuse lymphoma and B-cell chronic lymphocytic leukaemia patients and involved chromosome 14q32 (Erikson *et al* 1984). The events were postulated to activate a proto-oncogene near to or at the breakpoint region on 11q13, by placing it under the control of the strong IgH chain locus (Erikson *et al* 1984). The breakpoint region was named *bcl-1* for B-cell leukaemia/lymphoma. It was initially cloned from a case of chronic lymphocytic leukaemia (CLL) and successfully used as a probe to detect translocations of the same DNA sequences in other types of B-cell malignancies (Tsujimoto *et al* 1984). In 1988 and 1989 it became clear that the chromosomal break can occur at 1kb, 2kb, 36kb or even 63kb away from the major translocation

cluster, examples of which were observed in different types of B-cell malignancies (Rabbits *et al* 1988, Koduru *et al* 1989, Meeker *et al* 1989, Lammie and Peters 1991).

Since its discovery, the t(11;14)(q13;q32) rearrangement has been reported in several B-neoplasms, including non-Hodgkin's lymphoma (Vanderberghe *et al* 1992), centrocytic lymphoma (Williams *et al* 1991, Rosenberg *et al* 1991a) and myeloma (Seto *et al* 1992). In rare cases of benign parathyroid adenomatosis 11q13 is rearranged with the parathyroid hormone gene on 11p15 (Arnold *et al* 1989, Rosenberg *et al* 1991b). A t(8;11)(p21;q13) translocation was reported in one case of B-cell non-Hodgkin's lymphoma (Vanderberghe *et al* 1992).

1.2.1.c Amplification of 11q13.

Initially, reports of chromosome 11q13 amplification were made in primary breast carcinoma (Varley *et al* 1988, Zhou *et al* 1988), HNSCC (Zhou *et al* 1988) and patients with lymph node metastasis of stomach cancer (Yoshida *et al* 1988). This stimulated the interest in *FGF-3* and *FGF-4*, revealing increased gene dosage in oesophageal carcinomas (Tsuda *et al* 1989b, Kitagawa *et al* 1991), transitional cell carcinomas of the bladder (Theillet *et al* 1989, Proctor *et al* 1991), lung carcinomas (Berenson *et al* 1990), melanomas (Theillet *et al* 1989) and many new cases of breast carcinoma and HNSCC (Linderau *et al* 1988, Liscia *et al* 1989, Berenson *et al* 1989, Theillet *et al* 1989, Berenson *et al* 1990, Theillet *et al* 1990, Saint-Ruf *et al* 1991). Even though *FGF-3* and *FGF-4* were not normally expressed in adult murine or human tissue, their homology to fibroblast growth factors and apparent

transforming potential made them good candidates for the targeted gene/genes of chromosome 11q13 (Lammie and Peters 1991).

It is widely believed that the key transforming gene of an amplicon must fulfil the following criteria: (i) the amplification unit contains the essential gene(s) in all examined cases, (ii) the essential gene(s) is amplified to the greatest level when compared to other genes in the amplicon, (iii) the essential gene(s) is expressed to the highest extent when compared with the other members of the amplicon, (iv) the structure and function of the gene(s) is consistent with the implied role in oncogenesis and (v) no other marker should fulfil the same criteria (Schuuring *et al* 1992a).

Expression of *FGF-3* and *FGF-4* were shown to rarely accompany amplification of chromosome 11q13, eliminating both genes as potential key targets of this genomic region (Liscia *et al* 1989, Theillet *et al* 1989, Faust and Meeker 1992).

The mechanisms by which a cell may increase the dosage of a specific gene(s) was described briefly in sections 1.1.1 and 1.1.3. Analysis of the 11q13 region and *FGF-3* in a panel of human cell lines that have amplified these sequences, was carried out using the method of fluorescence *in situ* hybridisation (FISH) (Roelofs *et al* 1993). Low levels of amplified sequences were detected only at the original gene location, suggesting that formation of extrachromosomal structures was not the mechanism by which increased gene dosage had taken place in the majority of examined tumours. This was based on the proposal that DMs and similar extrachromosomal particles often integrate at non-native genome locations (Roelofs *et al* 1993). Amplification of 11q13 was suggested to take place by an *in situ* process and was coupled with

specific translocations, duplications and inversions detected in the examined cell lines (Roelofs *et al* 1993).

1.2.2 The identification of D11S287.

In a survey of 297 mammary tumours, amplification of *bcl-1* alone was seen in 6 patients (Theillet *et al* 1990). Two other studies showed amplification of *FGF-3* but not of *FGF-4* in breast and oesophageal carcinomas, a surprising observation considering the small distance separating the genes (Ali *et al* 1989, Tsuda *et al* 1989b). These data, coupled with the frequent involvement of *bcl-1* in B-cell chromosomal rearrangements suggested the presence of a gene located between the *FGF* tandem and *bcl-1* that may be the key gene of the amplicon (Theillet *et al* 1990).

The first indications of the identity of this putative gene came from rare cases of parathyroid adenomatosis (Arnold *et al* 1989). Examination of the high molecular weight DNA isolated from a parathyroid adenoma obtained from a 68 year old patient, revealed an abnormal *BamH1* restriction fragment that hybridised to a parathyroid hormone (PTH) specific probe. The abnormality was clonal and resulted in a 50% loss of the normal PTH gene in all the tumour cells. Digestion of the DNA with combinations of several restriction enzymes suggested that a break had separated non-coding exon-1 from the downstream sequences of the gene.

Further characterisation of the involved DNA was carried out with the use of probes specific to the sequences immediately 5' (A) or 3' (B and C) of the PTH gene breakpoint. The non-PTH gene sequences were mapped to chromosome 11q13.3-11q13.5 by *in situ* hybridisation studies and Southern blot analysis of human/mouse hybrids. In accordance with the Human Gene

Mapping Library system the new locus was termed D11S287. All three probes detected single copy sequences in normal human DNA, with A and C also hybridising to mouse sequences at conditions of high stringency. The sequences specific to this locus were completely unrelated to *bcl-1*, *FGF-3* and *FGF-4*. This suggested that the D11S287 locus was a well conserved, new gene, the expression of which had been influenced by the translocated, highly active 5' PTH gene region.

Probe B was used to confirm the proposal. It detected D11S287 transcripts (D11S287E) in parathyroid adenomas with or without the t(11;11)(p15;q13) rearrangement and normal placenta (Rosenberg *et al* 1991b). However, in adenomas carrying the t(11;11)(p15;q13) rearrangement, dramatic over-expression was evident, suggesting upregulation of D11S287E by the displaced PTH regulatory elements. The full length transcript of D11S287E (4242bp) was isolated from a human placental cDNA library and a new name proposed for the gene, PRAD-1 for parathyroid adenomatosis (Motokura *et al* 1991). The longest reading frame starting from the first ATG codon was estimated to encode a protein of 295 amino acids and 33,729D in size. Screening the Genbank peptide database with the identified sequence, revealed extensive homology only with members of cyclin families (Motokura *et al* 1991).

Cyclins were initially discovered in marine invertebrates and proved to be essential regulatory compartments of the cell cycle (Rosenthal *et al* 1980, Evans *et al* 1983) It is easy to imagine how increased expression of cell cycle promoting proteins may contribute to deregulated cellular proliferation. However, until the discovery of PRAD1 (later referred to as cyclin D1 or *CCND1*) very little evidence existed for the involvement of cyclins in cancer.

1.3 Cyclins.

1.3.1 The Maturation promoting factor.

Unfertilised, fully grown frog oocytes are arrested at the G2/M boundary of meiosis I. *In vivo*, this arrest is broken by the action of progesterone, secreted by the surrounding follicle cells, causing oocyte passage through meiosis I and arrest at metaphase of meiosis II. The second arrest point is overcome by insemination (Smith and Ecker 1971, Minshull 1993). In 1971 it was postulated that exposure to progesterone activated an intracellular factor termed maturation promoting factor (MPF), causing nuclear envelope breakdown, chromosome condensation and passage of fully grown oocytes through meiosis I (Masui and Market 1971). Injection of mature progesterone-induced *Rana pipiens* oocyte extracts into immature oocytes of the same species, caused the latter to mature without the presence of progesterone, consistent with the above proposal (Masui and Market 1971, Smith and Ecker 1971). In 1970 a phenomenon resembling initiation of mitosis, termed premature chromosome condensation (PCC), was observed in non-mitotic human *HeLa* cells, upon fusion with mitotic cells of the same line (Johnson and Rao 1970). PCC was also achieved in unstimulated mosquito cells, *Xenopus laevis* kidney cells, embryonic chick fibroblasts and erythrocytes, Chinese hamster ovary cells, bovine spermatozoa and horse lymphocytes by fusion to mitotic *HeLa* cells (Johnson and Rao 1970). The discovery that almost all meiotic and mitotic cells contained interchangeable MPF activity led to the idea that the factor was conserved in all eukaryotes (Reynhout and Smith 1974, Sunkara *et al* 1979, Kishimoto *et al* 1982, Kishimoto *et al* 1984, Sorensen *et al* 1985).

Eukaryotes depend on *de novo* protein synthesis for cell cycle progression, a requirement which is bypassed by injection of MPF (Wasserman and Masui 1975, Minshull 1993). *In vivo*, MPF activity is specific only to late G2 and M-phase cells, a characteristic convincingly shown by induction of *Xenopus laevis* oocyte maturation with extracts from *HeLa* cells synchronised to late G2 or M phases, whereas G1 and S phase *HeLa* cell extracts had no effect on the oocytes (Sunkara *et al* 1979). Partial purification of MPF revealed it to be a protein complex of approximately 100kD, which was subsequently shown to consist of a 34kD protein homologous to the *Schizosaccharomyces pombe* cdc2 (this will be further described in section 1.3.3.a) and a cyclin (Dunphy *et al* 1988, Gautier *et al* 1988, Draetta *et al* 1989).

1.3.2 The discovery of cyclins.

Mitotic cyclins were initially recognised in marine invertebrates due to their oscillation patterns at specific stages of the cell cycle and ability to induce mitosis. Tim Hunt and his students were the first to identify cyclins A and B, in embryos of two sea urchin species *Arbacia punctulata* and *Lytechinus pictus* and surf clam, *Spisula solidissima* oocytes (Evans *et al* 1983). The initial experiments on ^{35}S-methionine labelled *Arbacia punctulata* eggs, revealed that upon fertilisation or treatment with parthenogenic reagents NH_4Cl or A23187, the levels of one protein significantly increased. In cells that had been fertilised, the protein, termed cyclin A, was destroyed more or less completely every time the embryos divided, as opposed to weak base and Ca^{++} ionophore treated eggs that were unable to pass through mitosis. Similar analysis of *Lytechinus pictus* and *Spisula solidissima* revealed the presence of two cyclin proteins A and B that oscillated in different phases (Evans *et al* 1983). The two cyclins,

known as proteins A and B, had been previously identified in *Spisula solidissima*. Following fertilisation the surf clam embryos were exposed to ^{35}S-methionine or ^{3}H-leucine and the labelled proteins analysed on one dimensional acrylamide gels (Rosenthal *et al* 1980). This revealed a substantial increase of proteins A and B, while proteins termed X, Y and Z decreased in amount, however in 1980 the role of proteins A and B in cell cycle regulation had not been realised (Rosenthal *et al* 1980).

MPF activity coincided with entrance of cells into mitosis (Gerhart and Kirshner 1984, Sunkara *et al* 1979) a characteristic described in section 1.3.1 of this chapter. The requirement for *de novo* protein synthesis in each cell division, even in amphibian oocytes despite the great abundance of mitotic proteins present in these cells, suggested that a component of the MPF may be a protein synthesised prior to each round of cell division. Cyclins proved to be the best candidates for the MPF regulatory component, however until 1989 there was no evidence to confirm these speculations. The first *in vitro* cell cycle, carried out using cytoplasmic preparations of *Rana pipiens* oocytes and demembranated *Xenopus laevis* sperm nuclei, was the key to the analysis of this phenomenon. A crude cytoplasmic extract obtained by centrifugation of activated oocytes, termed "the heavy ooplastic fraction", caused morphological changes of the sperm nuclei that resembled those seen during pronuclear formation in intact cells (Lohka and Masui 1984). The results of the experiments confirmed previous reports of MPF activity, while the reconstruction of the cell cycle by using cell-free extracts from amphibian eggs allowed a whole new approach to the analysis of protein interactions that lead to cell division. Thus, in 1989, Murray and Kirshner were able to demonstrate that cyclin B was a component of the MPF (Murray and Kirshner 1989).

Treatment of activated *Xenopus laevis* oocytes with pancreatic RNase caused destruction of the endogenous mRNA and prevented the cells from entering mitosis. However, the addition of *in vitro* transcribed sea urchin cyclin B mRNA completely restored the morphological and biochemical events characteristic of mitosis, a phenomenon which was not achieved by the use of tobacco mosaic virus RNA.

1.3.3. Cyclin B and mitosis, the mechanism of action.

Since 1983 and the discovery of cyclins, our knowledge of the cell cycle has increased dramatically. Today, seven cyclin families have been isolated, which have the potential to interact with over 11 identified catalytic subunits at different points of the cell cycle. Due to the structural similarities of the involved proteins, the catalytic and regulatory subunit interaction and kinase activation are thought to follow a similar pathway. Currently, most is known about the associations of the two cyclin B proteins (B1 and B2) with $p34^{cdc2}$, the mechanisms of which are described in the following sections and are used as an example of the interactions that may take place between the other known cell cycle regulators.

1.3.3.a The association of $p34^{cdc2}$ and cyclin.

The 34kD protein cdc2 was initially identified in *Schizosaccharomyces pombe* as a critical regulator of the M phase (Nurse and Thuriaux 1980), six years later it was shown to function as a protein kinase (Simians and Nurse 1986). The human homologue of *cdc2* was cloned with the use of yeast genetics. Rescue of the non-viable *S. pombe cdc2-33 leu1-32* mutants was attempted by the introduction of a *LEU2* selectable marker and a full-length

human fibroblast cDNA library made in an *SV40* expression vector (Okayama and Berg 1983, Lee and Nurse 1987). Cell cycle restoration was achieved by the presence of a cDNA that upon examination showed extensive homology to the *S.pombe cdc2* and related *S. cerevisiae CDC28* genes.

Work carried out by two research groups independently showed the presence of the *Xenopus laevis* cdc2 homologue in the MPF. In one study, the specific association of cdc2 and a 13 kD protein suc1, previously described in yeast, was used to isolate cdc2 from *Xenopus* cell extracts (Brizuela *et al* 1987; Dunphy *et al* 1988). When crude MPF was chromatographed on a suc1 agarose column, the activity of MPF was quantitatively depleted, suggesting that cdc2 was a component of the maturation factor (Dunphy *et al* 1988). These results were confirmed by detection of cdc2 in purified *Xenopus* oocyte MPF, with the use of antibodies to *S.pombe* cdc2 in immunoblot and immunoprecipitation analyses (Gautier *et al* 1988). The proof that cyclins A or B complex with cdc2 to form the MPF, yet again came from experiments on amphibian cell free lysates (Draetta *et al* 1989). Isolation of cyclins A and B was achieved by incubation of M phase surf clam embryo extracts with suc1-Sepharose beads already known to precipitate cdc2. The identity of the two cyclins was proved by treatment with N-chlorosuccinimide, a reagent that specifically cleaves at tryptophan residues creating an array of short peptides specific to individual proteins. Both cdc2-cyclin A and cdc2-cyclin B complexes exhibited H1 kinase activity (Draetta *et al* 1989).

1.3.3.b The current model of mitosis.

Cyclin B is now recognised as an essential positive regulator of the cell cycle that functions by associating with cdc2, thus enabling the onset of mitosis

(Westendorf *et al* 1989). Unlike cyclin B, the cdc2 protein does not oscillate with the cell cycle, however its activity is tightly regulated by specific phosphorylations that are dependent on the association with cyclin B (Solomon *et al* 1990, Krek and Nigg 1991). In middle to late G2, newly synthesised cyclin B binds to inactive, monomeric cdc2 triggering phosphorylation of cdc2 on residues Thr-14 and Tyr-15, maintaining the inactive state of this kinase (Solomon *et al* 1992). The reaction is thought to be catalysed by two homologues of *S.pombe* kinases wee1 and mik1, the activity of which is also regulated by phosphorylation (Lundgren *et al* 1991, Parker and Piwnica-Worms 1992, Parker *et al* 1993, Wu and Russell 1993, Tang *et al* 1993). The Tyr-15 residue is located within a consensus sequence (GXGXXG) of the cdc2 protein which represents a putative nucleotide binding site, suggesting that phosphorylation of Tyr-15 inhibits the kinase activity of cdc2 by preventing the binding of ATP (Gould and Nurse 1989). However, a recently derived three dimensional model of cdc2 based on the crystal structure of cAPK (the catalytic subunit of cyclic AMP-dependent protein kinase), suggested that phosphorylation of Tyr15 displaces a highly conserved cdc2 domain necessary for cyclin binding away from the cdc2 substrate recognition site, preventing interaction between the two domains and peptide recognition (Marcote *et al* 1993). These results confirmed preceding observations determined by FSBA (the nuclear analogue 5'-*p*-fluorosulphonyl-benzoyladenosine) labelling experiments that Tyr15 phosphorylation does not prevent ATP binding (Atherson-Fessler *et al* 1993).The cdc2/cyclin B complex will remain in this inactive state until the end of G2 when DNA synthesis, repair and other essential processes have been completed (Dasso and Newport 1990, Enoch and Nurse 1990). Activation of cdc2 involves removal of the

phosphates on Thr-14 and Tyr-15, a reaction catalysed by a family of specific phosphatases termed cdc25 (Gautier *et al* 1991, Izumi *et al* 1992, Lee *et al* 1992). In humans three related cdc25 proteins have been identified and termed cdc25A, cdc25B and cdc25C of which all three are able to rescue yeast cdc25 deficient mutants (Sadhu *et al* 1990, Galaktionov and Beach 1991). The catalytic activity of the cdc25 proteins is considerably weaker than other known PTPases which is possibly due to the absence of a potential activating domain shared by other known PTPases. However, cyclin B has been shown to contain the PTPase specific activating domain which is thought to contribute to cdc25 activation "in trans" by intermolecular interactions (Galaktionov and Beach 1991, Zheng and Ruderman 1993). Interestingly, recent reports have suggested that the phosphatase activity of cdc25C is also enhanced by Ser/Thr phosphorylation catalysed by the functional cyclin B/cdc2 complex, thus generating a positive feedback loop (Hoffmann *et al* 1993). Activation of the cyclin B/cdc2 complex at the G2/M transition will not take place in the absence of a positive phosphorylation of cdc2 at residue Thr-161. There is evidence that the CAK (cdc2 activating kinase) protein also known as the *Xenopus* $p40^{MO15}$ cdc2-related kinase, catalyses this reaction (Solomon *et al* 1992, Solomon *et al* 1993, Poon *et al* 1993). The activated cyclin B/cdc2 complex proceeds to function as a Ser/Thr kinase phosphorylating specific nuclear substrates and allowing mitosis to take place. Two major components of the nuclear envelope, the lamin intermediate filament proteins A and C are specifically phosphorylated by the cyclin B/cdc2 kinase (Ward and Kirshner 1990, Heald and McKeon 1990). Phosphorylation of these proteins causes their disassembly and solubilisation, resulting in the nuclear envelope breakdown. The cyclin B/cdc2 complex is also thought to be responsible for other mitotic

events such as spindle assembly and chromosome condensation (Janssen *et al* 1991, Verde *et al* 1992, Leiss *et al* 1992). Potential substrates of this kinase include tumour suppressor protein p53, $pp60^{c\text{-}src}$ and numatrin - a nuclear matrix phosphoprotein (Bischoff *et al* 1990, Feuerstein 1991, Shenoy *et al* 1992). Dephosphorylation of Thr-161 and inactivation of the cyclin B/cdc2 complex is necessary for the cell to enter anaphase (Lorca *et al* 1992, Holloway *et al* 1993). This is followed by the dissociation of cdc2 and cyclin B of which the former remains in the cell in the inactive monomeric form, while the latter is rapidly degraded by the ubiquitin pathway (Draetta and Beach 1988, Krek and Nigg 1991, Glotzer *et al* 1991, Hunt 1991).

1.3.4 The cell cycle.

"The cell cycle is the period during which events required for successful cell reproduction are completed" (Nurse 1990). The two major phases S and M characterised by chromosome replication and segregation respectively, are separated by the G1 and G2 gap phases. Proteins active during G1 and G2 tightly regulate the two major phases. In terms of duration G1 is the most variable in length. It may last several hours or longer, when it is frequently referred to as G0, or be completely absent as in rapidly dividing cells during development (Wintersberger 1991).

The importance of cyclin B for the onset and passage through mitosis was described in detail in the preceding sections. In the recent years increasing attention has also been focused on the G1 phase of the cell cycle where several cyclin families are proposed to function. However, the protein interactions that take place in this phase of the cell cycle have proved to be far more complicated than in mitosis, a major cause being the many growth

stimulators and repressors that act in the G1 phase by binding to specific cell surface receptors and activating a cascade of events that regulate proliferation. In view of the multitude of interactions that take place in this phase, it is clear that it may also be the most common source of mutations that could lead to transformation and cancer, a topic that will be discussed in later sections.

1.3.4.a Mammalian G1 cyclins.

The G1 cyclins were initially discovered in budding yeast *Saccharomyces cerevisiae* (Hadwiger *et al* 1989, Richardson *et al* 1989). Today at least four families of mammalian cyclins (C, D, E and G) are proposed to regulate G1.

The *Saccharomyces cerevisiae CLN* family of cyclins consists of members *CLN1*, *CLN2* and *CLN3*, initially proposed to be partially redundant, although contradicting evidence now exists (Richardson *et al* 1989, Cross and Tinkelenberg 1991, Tyers *et al* 1993). *S. cerevisiae* haploid cells have three possible fates: when nutrients are limiting they may enter the resting phase G0; if exposed to mating factors the cells differentiate into gametes, capable of conjugating with neighbouring cells of an opposite mating type; the last option available is to continue cycling and divide when sufficient nutrients are present (Nasmyth 1990). During the G1 phase of the cell cycle there is a decision point termed START where the haploid cell must decide whether to proceed with the cell cycle or not. The decision is determined by cell size and the presence of nutrients or secreted mating factors (Lew *et al* 1991, Xiong *et al* 1991). Just one functional *CLN* protein is sufficient to allow passage through START, upon which the cell is committed to division (Richardson *et al* 1989). This potential redundancy created an excellent system for the isolation of novel mammalian

genes and led to the discovery of several G1 cyclins (Xiong *et al* 1991, Lew *et al* 1991).

In one study a yeast strain was constructed in which *CLN3* was altered to allow conditional expression from a galactose-inducible, glucose-repressible *GAL1* promoter (Xiong *et al* 1991). *CLN2* and *CLN3* were rendered inactive by insertional mutagenesis. When grown on 1% galactose, the yeast strain had a doubling time indistinguishable from the *CLN* parental wild type strain, although differed in size. Removal of galactose or addition of glucose caused accumulation of cells in the G1 phase and cell division ceased. Introduction of a human glioblastoma cDNA library into the yeast was attempted with the aim to prevent the G1 block using a human functional equivalent. The expression vector that carried the cDNA library contained a *LEU2* selectable marker which enabled detection of spontaneous revertants. From the ~$3x10^6$ transformants screened, only two revertants contained the *LEU2* marker. Restriction enzyme digestion and partial DNA sequencing were able to show that in both cases the same human gene was involved. A full length cDNA was subsequently isolated from a HeLa cell line cDNA library (1325bp) and was calculated to correspond to a 33,670D protein. The new protein (cyclin D1) was identical to the previously described PRAD-1 (Motokura *et al* 1991).

An independent, but very similar study also led to the isolation and cloning of cyclin D1 (Lew *et al* 1991). Two strains of *S.cerevisiae* (DL1 and YCS1) were constructed where all three *CLN* genes were disrupted by insertional mutagenesis. Survival of DL1 and YCS1 was enabled in the presence of galactose due to an integrated chimeric gene containing the coding region of *CLN2* expressed from the regulatable *GAL1* promoter. Under growth repressible conditions the DL1 strain was rescued by introduction of two

human cDNA libraries derived from either HepG2 or HeLa cells. A human glioblastoma cDNA library was introduced into the YCS1 strain. Transformed *S.cerevisiae* were selected for either in the absence of uracil, as HepG2 and HeLa libraries contained the *URA3* gene, or due to the presence of the *LEU2* marker in the glioblastoma cDNA library. Eighteen colonies from the HepG2 transformants, 23 from the HeLa and 3 colonies containing glioblastoma cDNA were dependent on library plasmids for growth. The majority of cDNAs were derived from cyclins B1, B2 and A, however three novel genes were identified and named cyclin C (HepG2 library), cyclin D (glioblastoma library) and cyclin E (HeLa library) and were predicted to code for proteins of 303, 295 and 396 amino acids, respectively. Cyclin D was identical to the previously described PRAD-1 gene (Motokura *et al* 1991, Xiong *et al* 1991).

The simultaneous discovery of the D-type cyclins in mouse macrophages provided further evidence for their importance as G1 phase regulators. The dependency of bone-marrow macrophages on colony stimulating factor-1 (CSF-1) for growth, was used for the isolation of the PRAD-1/cyclin D1 mouse homologue (Matsushime *et al* 1991). CSF-1, also known as the macrophage growth factor, belongs to a family of glycoprotein regulators that control proliferation and differentiation of granulocytes, macrophages and certain haematopoietic cells by binding to a specific receptor at the cell surface. In doing so the receptor intrinsic tyrosine kinase is triggered and will subsequently phosphorylate specific protein substrates activating the signal transduction pathway (Metcalf 1990). The CSF-1 receptor (CSF-1R) is coded for by the *c-fms* proto-oncogene and is specific to macrophages (Sherr *et al* 1985).

In the absence of CSF-1, bone-marrow macrophages arrest in the G1 phase of the cell cycle. This invariably results in cell death if the growth factor is withdrawn for longer periods (Tushinski and Stanley 1985). However, cell cycle synchronisation of mouse macrophages can be achieved by transient starvation followed by stimulation with CSF-1. Cells treated in such a way will move synchronously through G1 and into the S phase (Matsushime *et al* 1991). This allows the identification of specific genes that respond to CSF-1, and was used in the following experiments (Matsushime *et al* 1991) :

The mouse macrophage cell line BAC1.2F5A was not viable following growth in the absence of CSF-1 for over 36 hours. When deprived of growth factor for 18 hours and then stimulated with CSF-1 for 3 hours, the cell line responded by synchronised entry into the S phase 10-12 hours following exposure to CSF-1. A cDNA library was constructed from the messenger RNA isolated from stimulated cells which was in turn screened with a subtracted cDNA probe enriched for sequences expressed in induced cells. Due to its homology to known cyclins, the newly identified gene was named *Cyl-1* for cyclin-like 1 gene and represented the mouse homologue of the human cyclin D1 gene. During the screening, phage plaques that weakly hybridised to probes derived from the most highly conserved regions of *Cyl-1* were also identified. To further investigate this phenomenon, cDNA libraries from mouse thymus cells and a fibroblast cell line (NIH3T3) were screened with a probe derived from nucleotide sequences of one such phage and the previously used *Cyl-1* cDNA probe. This led to the isolation of two related but distinct genes *Cyl-2* and *Cyl-3* that were 63% and 49% identical to *Cyl-1*, respectively and 61% homologous to one another in regions of the proteins termed the "cyclin box" known to be evolutionarily conserved in all cyclins.

The existence of three mouse *Cyl* genes and the extensive homology between human (proposed new nomenclature *CCND1*) and mouse (*Cyl-1*) cyclin D1 equivalents suggested the presence of two additional human D-type cyclins. Cyclins D2 and D3 (new nomenclature *CCND2* and *CCND3*) were according to predictions independently isolated in several laboratories, also leading to the identification of two pseudogenes proposed to originate from them (Xiong *et al* 1992a, Inaba *et al* 1992, Motokura *et al* 1992a, Kiyokawa *et al* 1992). Interestingly comparison of *CCND1*, *CCND2* and *CCND3* with *Cyl-1*, *Cyl-2* and *Cyl-3* revealed a greater degree of identity of the human and mouse counterparts than between the D-family members of one species, implying a high degree of evolutionary conservation and distinct functions of the three genes (Xiong *et al* 1992a, Inaba *et al* 1992).

Complementation analysis of *S. cerevisiae* triple *CLN* mutants was also used in an independent isolation of human cyclin E (Koff *et al* 1991). A yeast strain was derived that contained *CLN3* expressed from the *GAL1* regulatable promoter, all three endogenous *CLN* genes were mutated. Rescue at restrictive growth conditions (growth on glucose) was achieved by the introduction of a human glioblastoma cell line (U118) cDNA library. The isolated 1.7kb cDNA predicted to encode a 395 amino acid protein was designated cyclin E. Only human cyclin B1 was able to fully rescue the *CLN* deficient yeast strain, cyclin A proved to be lethal and cyclin E was much less efficient. This was attributed to the ability of the three cyclins to associate with CDC28, the *S. cerevisiae* homologue of the *S.pombe* cdc2 kinase. CDC28 is required in *S.cerevisiae* for the function of START and the entry into mitosis, cyclin E was postulated to only be effective in G1 therefore allowing DNA replication but not passage through the G2/M transition phase. The hypothesis

was confirmed with the use of a *cdc28-13* mutant strain that was nonfunctional at restrictive temperatures of 38°C, but allowed growth at 30°C. The strain was used as a host to screen the glioblastoma cell line cDNA library for human genes that associated with cyclin E to perform START. Five clones that depended on exogenous DNA sequences for survival were tested, four contained human homologues of *cdc2* and one contained a new, related gene termed *cdk2*. *cdk2* had previously been isolated in *Xenopus laevis* and will be further described in section 1.3.4.e of this chapter. In conjunction with *cdc2* or *cdk2*, cyclin E was able to perform all the functions of START providing evidence for its function in the G1 phase of the cell cycle (Koff *et al* 1991).

The existence of at least one more potential G1 cyclin has recently been reported. Cyclin G was isolated in an effort to identify new rat homologues of the *c-src* kinase family (Tamura *et al* 1993). A rat fibroblast cell line (NRK) cDNA library was screened with a mixture of probes containing the domains of rat *c-src*, *c-fyn* or *c-yes* cDNAs. The predominant cDNA isolated was 3.17kb in size and showed homologies to known cyclins especially *Cig1*, a B-type cyclin found in *Schizosaccharomyces pombe* and thought to function at the G1/S boundary (Tamura *et al* 1993). The discovery of cyclin F, was reported by Simon Elledge at "The Cell Cycle Meeting" in Cold Spring Harbor, 1991. The protein consists of 786 amino acids making it the largest known cyclin. It is not yet clear at what stage of the cell cycle cyclin F might function, however within the cyclin box region it displayed greatest homology to cyclin A (39%) suggesting a functional relatedness of these two proteins.

1.3.4.b The oscillation patterns of G1 cyclins.

The first indications for the oscillation behaviour of the cyclin D proteins came from the analysis of mouse macrophage cell lines (Matsushime *et al* 1991). Advantage was made of the ability of these cells to arrest in G1 when deprived of CSF-1. The time of entry into the S phase was determined by flow cytometric analysis of propidium iodide stained cells. Synchronous DNA replication was observed 10 hours after stimulation with a maximum percentage of cells in S phase at 13-16 hours. *Cyl-1* mRNA species of 3.8 and 4.5kb were detected with the use of *Cyl-1* cDNA probes in northern blot analysis 1 hour after release from the G1 arrest. The maximum levels were reached 4-6 hours post stimulation and the mRNA species remained elevated at 23 hours which was the duration of the experiment. A 6kb *Cyl-2* mRNA specie appeared several hours after *Cyl-1* and peaked at the G1/S transition phase. The oscillation pattern of *Cyl-3* was not determined. A polyvalent antibody raised against bacterially produced *Cyl-1* was used to analyse the cell cycle pattern of protein expression. CSF-1 starved cells had low amounts of the *Cyl-1* protein which rose 2 hours after stimulation and the highest levels were seen at 10-12.5 hours. The protein levels declined during the S and G2 phases reaching the lowest point in mitosis (~22.5 hours post stimulation).

These observations were subsequently confirmed in several independent studies of human fibroblasts synchronised to G0 by serum starvation (Surmacz *et al* 1992, Won *et al* 1992, Baldin *et al* 1993). In quiescence cyclin D1 mRNA and protein levels were found to be low. Upon stimulation with serum, the BB form of platelet derived growth factor (PDGF-BB), epidermal growth factor (EGF), fibroblast growth factor (FGF) or TPA, expression of cyclin D1 and cyclin D3 transcripts was upregulated. In all

instances except after TPA treatment the cells entered S phase, indicating that cyclin D1 or D3 expression alone was not sufficient for cell cycle progression (Won *et al* 1992). Exposure of quiescent cells to insulin-like growth factor 1 (IGF-1), insulin, the AA form of PDGF or transforming growth factor beta (TGF-β) had no effect on the mRNA levels of these two cyclins (Surmacz *et al* 1992, Won *et al* 1992). However, IBMX an inhibitor of cyclic nucleotide phosphodiesterases and forskolin an activator of adenylate cyclase, caused a decrease of cyclin D1 and D3 mRNA levels and [3H]thymidine incorporation, suggesting that the activated cAMP pathway may have negatively regulated cyclin D expression (Won *et al* 1992). In serum or growth factor treated fibroblasts where a rise in expression was seen, levels of cyclin D1 or D3 mRNA increased gradually reaching a plateaux at ~12 hours from stimulation and declining over the next 26 hours. At 20 hours from stimulation, most of the cells had entered the S phase, which was determined by the degree of [3H]thymidine incorporation.

The cell cycle regulated expression of cyclin D1 protein was also confirmed in quiescent human fibroblasts (Baldin *et al* 1993). The duration of the G1 phase in the examined cells was determined by 5-bromodeoxyuridine (BrdU) incorporation and flow cytometry. Exposure to serum induced proliferation and by 24 hours post stimulation ~80% of the cells had entered the S phase. Cyclin D1 levels were analysed by immunoblotting and immunoprecipitation techniques, revealing low levels in quiescence that rapidly increased at 8-10 hours, reaching a plateaux 20 hours after exposure to serum. At ~24 hours a decrease was observed that lasted until the 32 hour time point, when accumulation of the cyclin D1 protein began again, however further analysis was hindered by the loss of cell synchrony.

Immunofluorescence analysis was able to reveal an exclusively nuclear localisation of the protein during the G1 phase. Quiescent cells or those where DNA synthesis was taking place had undetectable cyclin D1 protein levels, as determined by this technique.

It is becoming increasingly evident that cyclin D1 may function in cells during the central part of the G1 phase. However, an exception to the rule arose from the analysis of human HeLa cells synchronised to G1/S by sequential thymidine-amphidicolin treatment. After release, cyclin D1 mRNA levels peaked in the G2/M transition phase as well as in G1, the synchrony of the cells was monitored by determining [3H]thymidine incorporation and expression of histone H4 transcripts (Motokura *et al* 1991). The altered oscillation pattern of cyclin D1 transcripts was attributed to accumulated abnormalities over the long period HeLa cells have resided in culture. Currently these are the only reported cells where cyclin D1 levels do not follow the previously described oscillation pattern.

In a separate study, G1/S arrested HeLa cells were used to determine the periodicity of cyclin C and cyclin E expression (Lew *et al* 1991). The mRNA levels of cyclin C did not vary more that 2-3 fold during the cell cycle, a modest peak was evident in G1/S arrested cells followed by a slow decline during the S, G2 and M phases. Cyclin E transcripts also predominated in arrested cells, although in this case the levels oscillated more dramatically and a sharp decrease was observed as they entered the S phase. In both cases the detected transcripts were 2-2.5 kb in size. The oscillation patterns of the cyclin G protein were determined in NRK rat fibroblasts synchronised to G1 by serum starvation and contact inhibition. Upon release, transcription from the cyclin G

gene was induced 3 hours after stimulation, however, the levels did not change thereafter (Tamura *et al* 1993).

1.3.4.c Structural comparison of cyclins.

A major feature of all cyclins is a highly conserved region termed the "cyclin box", initially identified by amino acid sequence comparison of the budding yeast cyclins with their surf clam and frog counterparts (Minshull *et al* 1989, Draetta 1990). The "cyclin box" is defined by ~100-200 amino acids of which only five well spaced residues Arg-Asp-Leu-Lys-Phe (single letter code - RDLKF) show complete conservation from yeast to man, with the exception of human cyclin C in which Arg and Asp residues are altered (Draetta 1990, Kobayashi *et al* 1992, Tamura *et al* 1993). The position of the cyclin box within the proteins varies between G1 and G2/M cyclins where the location may be N-terminal (rat cyclin G is the most extreme example) or central, respectively. In general, reported identities between different cyclins are largely confined to comparisons of amino acid sequences within the "cyclin box". However, a number of homologous islands have been identified outside this region, of which the most significant are the PEST, destruction and P boxes (Rogers *et al* 1986, Nugent *et al* 1991, Hunt 1991, Glotzer *et al* 1991, Zheng and Ruderman 1993).

The destruction box is specific to the N-terminal domain of A and B cyclins, it is identifiable by a partially conserved Arg-X-X-Leu-X-X-Ile-X-Asn sequence (RXXLXXIXN) followed by a Lys-rich stretch (Hunt 1991, Nugent *et al* 1991). This motif was shown to be recognised by the highly conserved ubiquitin protein, resulting in rapid cyclin degradation (Glotzer *et al* 1991, Hershko *et al* 1991). Mutational analysis of both cyclins A and B revealed the

importance of this region for the specific destruction of the two proteins, allowing anaphase initiation and the completion of mitosis in *Spisula solidissiama* and *Xenopus laevis* egg extracts (Luca *et al* 1991, Kobayashi *et al* 1992, Holloway *et al* 1993) and HeLa cells (Gallant and Nigg 1992).

The PEST hypothesis was based on the sequence analysis of forty five distinct proteins with different intracellular half-lives (Rogers *et al* 1986). Proteins with half-lives less than two hours, such as c-myc, p53, c-fos adenovirus 5 E1A and ornithine decarboxilase (ODC), contained one or more regions rich in Pro, Glu, Ser and Thr (termed PEST regions) which were absent from the majority of more stable proteins. The presence of these sequences correlated much better with rapid degradation than with compartmentalisation, function or common ancestry. To date PEST sequences have been identified at the C-termini of all the mammalian G1 cyclins, *S.pombe* Cig1 and *S.cerevisiae* CLN1, CLN2 and CLN3, an exception to the rule is rat cyclin D1, while cyclin F has not been examined (Hadwiger *et al* 1989, Lew *et al* 1991, Tamura *et al* 1993).

A small conserved region of *Xenopus* cyclin B termed the P-box, was recently proposed to contribute part of a phosphatase-activating domain to cdc25 (Galaktionov and Beach 1991, Zheng and Ruderman 1993). Three highly conserved residues Arg202, Glu221 and Asp231 within this 33 amino acid stretch, were shown to be essential for the functioning of the domain. Mutation of the P-box had no effect on cdc2 binding and subsequent inhibitory Thr14 and Tyr15 phosphorylations, however the mutated cyclin B/cdc2 complex arrested in its inactive state and cdc25 remained in the low activity form preventing progression through mitosis (Zheng and Ruderman 1993). Two of the functional P box residues, Arg202 and Asp231 were found in

equivalent positions in all cyclins except cyclin C and HCS26 (an *S.cerevisiae* mitotic cyclin) while cyclins F and G have not been examined. HCS26, cyclin C and the CLNs also lack the third, Glu221 residue which is conserved in the A, B, D and E-type families (Zheng and Ruderman 1993). This suggests that the P box may represent the second functional domain common to most cyclin proteins.

Several mutation studies have been carried out in the effort to determine the specific regions of cyclins required for binding to their catalytic subunits (Luca *et al* 1991, Kobayashi *et al* 1992). Removal of the first 161 N-terminal amino acids from *Xenopus* or bovine cyclin A had no effect on the ability to associate with cdc2 or cdk2 in frog cell-free extracts, although deletions beyond the highly conserved Tyr169 abolished binding (Luca *et al* 1991, Kobayashi *et al* 1992). Alterations of any amino acids within the "cyclin box" or small C-terminal deletions also prevented formation of the protein complex. Kinase functional assays revealed the loss of the ability to phosphorylate histone H1 in all the mutants that were unable to associate with subunits cdc2 or cdk2 (Kobayashi *et al* 1992). Similar results were observed in the analysis of cyclin B (Luca *et al* 1991, Zhang *et al* 1993).

1.3.4.d The phosphorylation of cyclins.

The discovery that cyclins are also kinase substrates was initially shown for cyclin B (Lohka *et al* 1988). Tryptic peptide mapping of [^{32}P]orthophophosphate-labelled cyclin B of human or *Xenopus* origin, revealed specific Ser residues as sites of phosphorylation (Pines and Hunter 1989, Izumi and Maller 1991). In extracts derived from fertilised sea urchin oocytes, histone H1 kinase activity correlated well with the appearance of

phosphorylated forms of cyclin B (Meijer *et al* 1989). Suggestions that cdc2 may have catalysed this reaction were confirmed by the *in vitro* ability of purified MPF to phosphorylate cyclin B, although significant phosphorylation was also demonstrated in resting oocytes prior to maturation and the activation of cdc2 (Gautier *et al* 1990, Gautier and Maller 1991). Cyclin B may also be phosphorylated by c-mos and microtubule associated protein (MAP) kinase at sites indistinguishable from those targeted by MPF (Roy *et al* 1990, Izumi and Maller 1991). The *c-mos* proto-oncogene codes for a Ser/Thr kinase, the function of which is essential for germinal vesicle breakdown during the maturation of frog eggs, it is normally expressed only in germ cells and undetectable in most sommatic cells (Sagata *et al* 1988, Sagata *et al* 1989, Freeman *et al* 1989). MAP kinase is a proposed major component of the ras signalling pathway and a member of a larger family of related proteins. Meiotic activation of frog and clam oocytes leads to a one-time activation of MAP kinase that will function as a Ser/Thr kinase for many hours until the metaphase II arrested egg is fertilised (Ruderman 1993). This kinase was able to phosphorylate *Xenopus* cyclin B1 *in vitro*, however, it was largely inactive towards cyclin B2 (Izumi and Maller 1991).

Recent reports have questioned the importance of cyclin B phosphorylation in mitosis. Mutations of amino acids Ser90 in cyclin B1 and Ser94 or Ser96 in cyclin B2 did not alter the activation or efficiency of the cdc2 histone H1 kinase, nuclear envelope breakdown and chromosome condensation. The rate of cyclin degradation was also unaltered and the only observed difference was a decreased translation efficiency of the mutant forms in *Xenopus* oocyte extracts (Izumi and Maller 1991).

Several independent studies have reported Tyr phosphorylation of cyclins A, B and D (Tamura *et al* 1990, Matsushime *et al* 1991, Hall *et al* 1993). Cyclin A phosphorylation was observed as a response to mitogen treatment of a human vulvar SCC cell line A431, and the same was catalysed *in vitro* and *in vivo* by purified c-src, a Tyr-specific protein kinase (Hall *et al* 1991). The homologue of c-fms found in the McDonough strain of feline sarcoma virus (*SM-FeSV*) also induced the *in vitro* phosphorylation of cyclins A and B (Tamura *et al* 1990). Immunoprecipitation of cyclin D1 from [^{32}P]orthophosphate labelled mouse macrophages revealed two phosphate containing forms of the protein, the labelling index of which was highest during the G1 phase (Matsushime *et al* 1991). These observations were confirmed by the detection of cyclin D1 with anti-phosphotyrosine antibodies in western blot analysis of extracts derived from foetal and adult rat alveolar epithelial cells, a human lung carcinoma cell line and Ewing's sarcoma cells (Hall *et al* 1993). Purified recombinant c-src was able to phosphorylate bacterially produced cyclins A and D *in vitro* and the same was true for the cyclin A/cdc2 kinase although it exhibited an apparent preference for cyclin A (Hall *et al* 1993).

1.3.4.e The current model of the mammalian cell cycle.

The turning point in the study of the cell cycle was the discovery that cyclins function as regulators of specific Ser/Thr kinases, the activation of which depends on direct interactions between the two types of proteins. Cyclin B activation of cdc2 was the first to be identified and currently the best understood (see section 1.3.3.b). In yeast a single 34kD protein (CDC28 in *S. cerevisiae* and cdc2 in *S. pombe*) interacts with both G1 and mitotic cyclins to regulate the G1/S and G2/M transitions, respectively (Reed 1991, Solomon

1993, Nasmyth 1993). The discovery of the cdk2 kinase in *Xenopus laevis* and mammalian cells suggested that cell cycle regulation in higher eukaryotes was far more complex than in yeast. Human cdk2 was able to rescue budding yeast that lacked functional CDC28, a characteristic that led to its isolation (see section 1.3.4.a) (Koff *et al* 1991, Elledge and Spottswood 1991, Ninomiya-Tsuji *et al* 1991). In *Xenopus laevis*, cdk2 (initially known as Eg1) was identified as a maternal protein by the differential screening of an unfertilised oocyte cDNA library (Paris *et al* 1991). In the most extensive study, degenerate oligonucleotides synthesised from highly conserved regions of cdc2 were used in the search for new genes by screening human HeLa and Nalm-6 (a pre-B leukaemia) cell lines, leading to the isolation of eight genes of which five were novel (Meyerson *et al* 1992, Tsai *et al* 1991). Following a proposed convention, the cdc2-like kinases shown to associate with cyclins were given the synonym cdk for cyclin dependent kinase. The remaining proteins were named after their amino acid sequences within the highly conserved region of the cdc2 PSTAIRE motif (Lee and Nurse 1987). Comparison of the predicted amino acid sequences allowed grouping of the proteins into subfamilies, represented in Table 1 (Lee and Nurse 1987, Meyerson *et al* 1992, Hanks 1987, Bunnell *et al* 1990, Solomon 1993, Lew *et al* 1992).

The activity but not the levels of cdk2 were shown to oscillate with the cell cycle in a distinct pattern to that of cdc2 (Draetta and Beach 1988). In synchronised HeLa cells cdk2 activity increased to a peak at the time of DNA synthesis, four hours after release from the G1/S arrest, fell slightly and increased again two hours prior to the peak of mitotic cdc2 activity (Rosenblatt *et al* 1992). This was shown to be due to the formation of active complexes with cyclin E in late G1 and the G1/S transition, followed by cyclin A

Table 1. Cyclin dependent kinase families.

Currently known cyclin dependent kinases can be subgrouped into five families based on their sequence homologies (Meyerson *et al* 1992, Solomon 1993).

Table 1.

kinase subfamilies	Thr14 & Tyr15 aa. residues	Thr161 aa. residue
cdc2 cdk2 cdk3 cdk5	conserved	all kinases have a Ser or Thr amino acid residue as an equivalent of Thr161
PCTAIRE-1 PCTAIRE-2 PCTAIRE-3	conserved	
cdk4 cdk6	Thr14 replaced by Ala	
KKIALRE	Thr14 replaced by Ser	
p58-GTA		

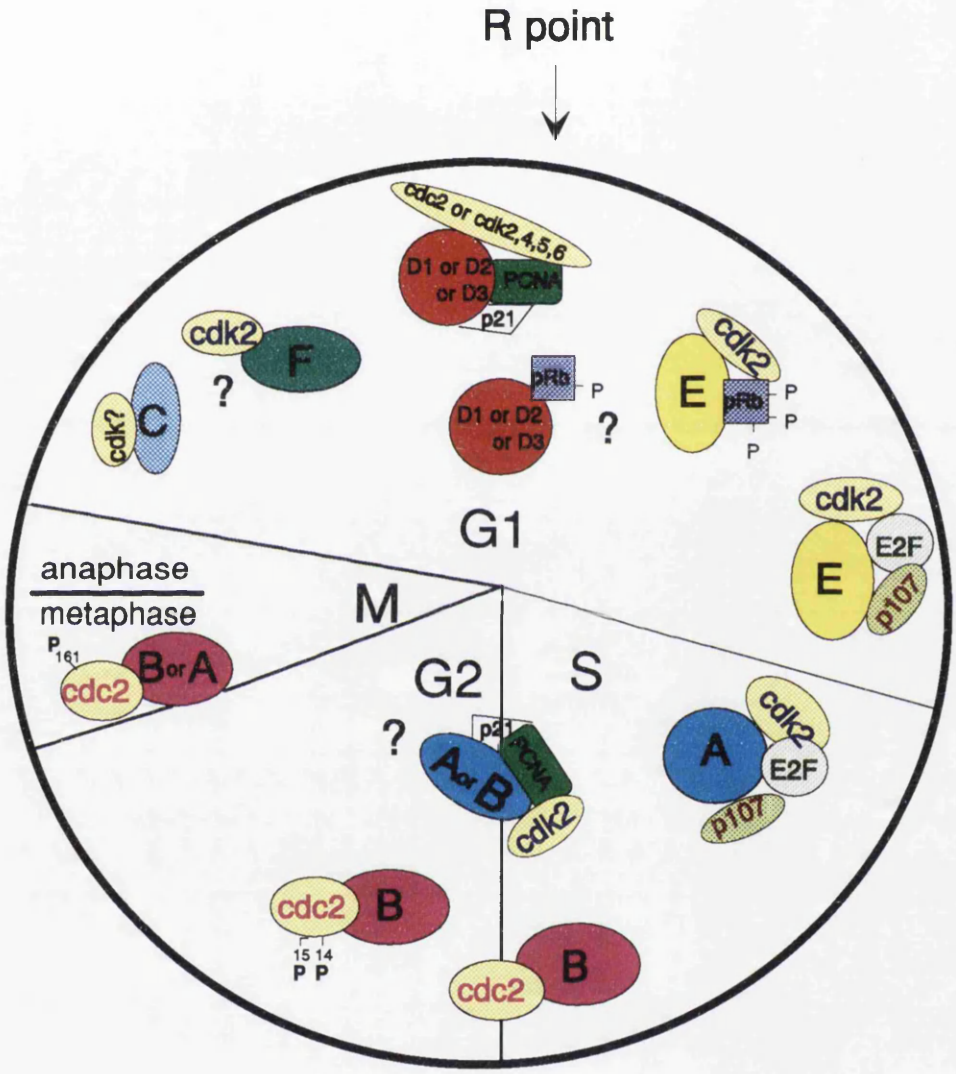
in S and G2 phases (Dulic *et al* 1992, Koff *et al* 1992, Rosenblatt *et al* 1992, Tsai *et al* 1993, Pagano *et al* 1993). A high degree of diversity in the choice of subunits is characteristic of the D-type cyclins, which have been shown to complex with cdc2, cdk2, cdk4, cdk5 or cdk6, although contradicting evidence exists for most of these associations with exception of cdk4 (Motokura *et al* 1991, Elledge and Spottswood 1991, Xiong *et al* 1992b, Matsushime *et al* 1992, Kato *et al* 1993, Sherr 1993, Hall *et al* 1993). Substrate specificity of the D-family of cyclins may vary in dependence of the catalytic subunit present in the complex, a proposal recently supported by the predominance of cyclin D1 associated with cdk5 or cdk6 in breast tumour cells as opposed to the majority of cyclin D1 bound to cdk2 or cdk4 in normal cells (Peters *et al*1993). The identities of the kinases regulated by cyclin C, cyclin F or cyclin G are unknown. Figure 2 represents the currently accepted model of the mammalian cell cycle, depicting the coordinate oscillation patterns of the known protein kinase complexes.

1.3.4.f What are the substrates of G1 and G1/S cyclins?

Currently the substrates of only the mitotic cyclin B/cdc2 kinases are known (see section 1.3.3.b), while the function of other cyclin families are under constant debate, the most puzzling of which are the D-type cyclins. Association of D cyclins with the proliferating cell nuclear antigen (PCNA), a well characterised DNA replication and repair factor, was recently described in a human diploid fibroblast cell line (WI38) (Xiong et al 1992b). Antibodies raised against cyclins D1 or D3 selectively co-precipitated the two cyclins with PCNA, cdk2, cdk4, cdk5 and an unrelated 21kD protein that did not correspond to human max or ras. *In vitro* kinase assays did not reveal phosphorylation of

Figure 2. The mammalian cell cycle.

Schematic representation of most of the known cyclins, specific catalytic subunits and other cellular proteins they associate with at different points of the cell cycle (Pines 1993).


R point
cdc2 or cdk2,4,5,6
D1 or D2 or D3
PCNA
p21
cdk2
F
?
cdk?
C
pRb
P
D1 or D2 or D3
?
cdk2
E
pRb
P
P
P
G1
cdk2
E2F
E
p107
anaphase
metaphase
M
P161
cdc2
B or A
G2
S
?
p21
PCNA
A or B
cdk2
cdk2
A
E2F
p107
cdc2
B
15 14
P P
cdc2
B

PCNA or p21 by various combinations of cyclin D/cdk subunits, suggesting that these two proteins were not primary cyclin D substrates and could function as members of larger complexes. Subsequent immunoprecipitation analyses have suggested that PCNA and p21 are universal components of cyclin A, B or D dependent kinases in all normal human diploid fibroblasts. However, upon transformation with the *SV40 T* antigen, the p21 protein was replaced by two unrelated proteins : p19 associated with cyclins A or B1 and p16 bound to cdk4 (Xiong and Beach 1993).

The retinoblastoma protein pRb is a potential substrate of cyclins A, E and D. pRb was identified as a nuclear target of three distinct viral oncogenes and a common site of inactivating mutations in several types of cancer (Whyte *et al* 1988, DeCaprio *et al* 1988, Dyson *et al* 1989, Weinberg 1991). Human papilloma virus-16 (*HPV-16*) oncoprotein E7, adenovirus-5 (*Ad-5*) oncoprotein E1a and simian virus-40 (*SV40*) T antigen share two conserved regions (CR-I and CR-II) both of which are essential for transformation (Ruley 1983, Phelps *et al* 1988, DeCaprio *et al* 1988, Jones *et al* 1990, Raychaudhuri *et al* 1991). CR-II contains a highly conserved core motif Leu-X-Cys-X-Glu (LXCXE) preceded by an acidic amino acid. Mutations of any amino acid residues in this motif will reduce or abolish binding to pRb (Jones *et al* 1990, Heck *et al* 1992). The products of three viral oncogenes, *E7*, *E1a* and *T* antigen, recognise specific sequences of the pRb protein termed the "pocket" (Livingston *et al* 1993). The region was mapped with the use of *in vitro* translation systems and mutational analysis and divided into two subdomains A (amino acids 373-579) and B (amino acids 640-771). The subdomains are separated by a spacer sequence the length but not the amino acid content of which is important for protein binding (Huang *et al* 1990, Ewen *et al* 1993a). Substitution or deletion

of any residues located in subdomains A or B abolished oncoprotein-pRb complex formation *in vitro*, these were also the sites of pRb mutations detected in human tumours (Huang *et al* 1990).

The function of pRb in the suppression of transformation was shown in several studies by the introduction of wild type sequences into established tumour cell lines where the endogenous pRb was mutated. In all cases this resulted in a significant decrease of transformation and tumourigenicity (Huang *et al* 1988, Banerjee *et al* 1992, Hinds *et al* 1992). During the cell cycle pRb switches between a Ser/Thr hyper-phosphorylated and a relatively under-phosphorylated state (DeCaprio *et al* 1989, Weinberg 1991). The hypo-phosphorylated form, present in the G1 phase was shown to be the exclusive target of viral oncogenes (Ludlow *et al* 1989, Ludlow *et al* 1990). Entry into the S phase was characterised by accumulation of hyper-phosphorylated pRb which remained in this state throughout G2 and was lost during the M phase, when the dephosphorylated protein reappeared (DeCaprio *et al* 1989, Ludlow *et al* 1990, DeCaprio *et al* 1992). The observed oscillation patterns of pRb phosphorylation suggested that it may regulate the cell cycle and represent a substrate for cell cycle dependent kinases. Confirmation of the former proposal came from the observed effects of injected wild type pRb on synchronised human osteosarcoma cell lines Saos-2 (containing truncated pRb) and SR-40 (Goodrich *et al* 1991). Cell cycle progression and 5-bromodeoxyuridine (BrdU) incorporation were blocked for up to four days in Saos2 and SR-40 cells, if the injections took place early in the G1 phase. However, pRb injected six to ten hours prior to the G1/S transition or during the S phase had little or no effect on cellular proliferation, suggesting that pRb functioned as a growth suppressor in G0 and G1. A significant discovery supporting the second proposal was the

identification of an intact LXCXE motif (LLCCE) preceded by the Glu residue at the N-termini of cyclins D1, D2 and D3 (Dowdy *et al* 1993). Currently these are the only discovered cellular proteins that contain the conserved pRb binding motif characteristic of viral transforming genes. However, conflicting data has been generated in several laboratories, thus it is still unclear whether the D-cyclins bind to and/or cause pRb phosphorylation *in vivo*.

Several studies addressing this question made use of the distinct morphological changes and growth inhibition induced in Saos2 cells by the wild type form of pRb (Hinds *et al* 1992, Ewen *et al* 1993a, Dowdy *et al* 1993). Co-transfection of cyclin A or E *cytomegalovirus* (*CMV*) expression vectors with wild type pRb resulted in a 90%-100% reduction in the pRb mediated flat morphology as was the effect of the adenovirus-5 E1a oncoprotein. Cyclin D1 or D3 expression vectors had a reduced rescuing ability (~60%-80% and 52%, respectively) which was increased to 90% for cyclin D1 upon mutation of the LLCCE pRb binding domain, suggesting that *in vivo* pRb is a negative regulator of cyclin D1 (Hinds *et al* 1992, Dowdy *et al* 1993). The B-type cyclins were unable to revert the flat morphology of inhibited Saos2 cells (Hinds *et al* 1992).

Hypo-phosphorylated pRb exhibits tight nuclear association in comparison to the hyper-phosphorylated form of the protein. This can be detected by treating cells with weak non-ionic detergents which will cause the release of only the highly phosphorylated pRb protein. In Saos2 cells transfected with wild type pRb virtually all of the tumour suppressor protein was tightly tethered to the nucleus and remained so upon the introduction of cyclins B1, B2 or D1, however, expression of cyclins A or E reduced the degree of nuclear association by half (Hinds *et al* 1992). Immunoprecipitation analysis of [^{35}S]methionine or [^{32}P]orthophosphate labelled Saos2 cell extracts indicated

that E1a, cyclin A, cyclin D2, cyclin E and to a lesser extent cyclin D3, induced the phosphorylation of pRb, while cyclins B1 and B2 did not. Some controversy existed with regards to the ability of cyclin D1 to phosphorylate pRb in Saos2 cells, although even in the positive observations the levels of phosphorylation were small (Hinds *et al* 1992, Ewen *et al* 1993a, Dowdy *et al* 1993). The G1 block normally induced by pRb was overcome in cells containing E1a, cyclin A or cyclin E and to a lesser extent by cyclin D2. Expression of cyclins D1 or D3 was observed to have little or no effect on the arrested Saos2 cells, although mutation of cyclin D1 at the LLCCE domain increased the number of cells in S, G2 and M phases, suggesting the absence of pRb-mediated control (Hinds *et al* 1992, Ewen *et al* 1993a, Dowdy *et al* 1993). In summary, the results obtained from analysis of Saos2 cells suggested that cyclins A and E were the most effective in phosphorylating pRb and preventing its growth suppressive function, the B-type cyclins had no effect and the D family, especially cyclin D1, functioned through a different pathway.

In a separate set of experiments, cyclin D3 produced in insect cells from baculovirus expression vectors was able to bind cdk4 *in vitro* and function as an active pRb and p107 (a pRb related protein) kinase. Cyclin D1 also readily complexed with cdk4 while the affinity of cyclin D2 for the kinase subunit was reduced in comparison, both complexes lacked the ability to phosphorylate pRb or p107 (Matsushime *et al* 1992). However, a subsequent report from the same laboratory contradicted these results. Lysates of insect Sf9 cells coinfected with baculovirus vectors encoding all three D-type cyclins and cdk4, exhibited a protein kinase activity that was able to phosphorylate a glutathionine S-transferase (GST)-pRb fusion protein *in vitro* (Kato *et al* 1993). Mutational analysis of pRb revealed the Ser807 residue as a major

phosphorylation site. Cyclins A or B1 could activate cdc2 pRb kinase activity while cyclin E was a potent activator of cdk2, all three cyclins were unable to cause pRb phosphorylation in the presence of cdk4 (Kato *et al* 1993, Ewen *et al* 1993a).

To address the possibility of direct interactions between pRb and cyclin D1, bacterially produced recombinant cyclin D1 was used to make an affinity matrix and a number of binding proteins were identified in extracts from Ewing's sarcoma (EW-1) cells of which pRb was one. The pRb suppressor protein was also able to bind cyclin A affinity columns of a similar type (Hall *et al* 1993). Confirmation for the *in vivo* pRb-cyclin D1 association came from immunoprecipitation experiments and site directed mutagenesis of the cyclin D1 LLCCE motif which were carried out in human diploid fibroblasts and Saos2 cells (Dowdy *et al* 1993). A separate set of experiments revealed the ability of cyclins D2 and D3 to associate with pRb *in vitro* with much greater affinity than cyclin D1 (Ewen *et al* 1993a, Kato *et al* 1993).

A major target of pRb during the G1 phase of the cell cycle is the E2F transcription factor, originally encountered through its ability to transcribe the adenovirus-5 *E2* gene by recognising two 5'-TTTCGCGC-3' motifs in the promoter region (Kovesdi *et al* 1986, Kovesdi *et al* 1987, Bagchi *et al* 1990, Raychaudhuri *et al* 1991, Chellappan *et al* 1991). E2F binding sites have been mapped in the promoters of several cellular genes which include *c-myc*, *N-myc*, *c-myb* (Lipp *et al* 1987, Thalmeier *et al* 1989, Mudryj *et al* 1990), dihydrofolate reductase (*DHFR*) (Blake and Azaizkhan 1989, Mudryj *et al* 1990), thymidine kinase (Kim and Lee 1991) DNA polymerase α (Pearson *et al* 1991), EGFR (Mudryj *et al* 1990) cyclin A (Brechot 1993) and *CCND1* (Motokura and Arnold 1993). Recent reports have suggested a dual role for

E2F, the activity of which may alternate between a positive and negative transcriptional factor during the progression of the cell cycle depending on its association with other cellular proteins (Weintraub *et al* 1992). Free E2F, of which three different forms have recently been identified, is believed to function as a positive transcription factor (Mudryj *et al* 1991, Nevins 1992, Chittenden *et al* 1993). However, expression from the *E1a* oncogene was shown to be negatively regulated by E2F in the presence of hypo-phosphorylated pRb, suggesting that this complex actively functions as a repressor of transcription (Chellappan *et al* 1991, Weintraub *et al* 1993). Inhibitory activity of E2F-pRb was confirmed on a simple promoter consisting of a TATA box and an ATF transcription factor site or the more complex SV40 early gene promoter, both of which contained 5'-TTTCGCGC-3' binding sequences (Weintraub *et al* 1992). Co-transfection of Saos-2 cells with pRb and cyclin D1 or D3 expression vectors considerably reduced the abundance of detectable pRb-E2F in comparison to cells that were transfected with pRb alone, while full inhibition was achieved in the presence of cyclin D2 (Ewen *et al* 1993a). Therefore, inhibition of E2F and pRb association was achieved in the presence of cyclins that potentially phosphorylate the tumour suppressor protein. In the same type of analysis carried out in Sf9 cells, only cyclin D1 prevented association between the transcription factor and suppressor protein, while cyclins D2 and D3 effectively abolished E2F-pRb complex formation only in the presence of cdk4 expression vectors (Kato *et al* 1993). One proposed mechanism for the cyclin action is the conversion of pRb to the hyper-phosphorylated state, however since there is wide controversy on the ability of different D-family members to phosphorylate and/or bind to pRb, the mechanisms may involve structural alterations of E2F.

pRb is not the only cellular protein that interacts with E2F. Extracts from synchronised NIH3T3 murine fibroblasts revealed the presence of two E2F protein complexes one of which contained cyclin A and was present exclusively during the S phase, while the other was G1 specific (Mudryj *et al* 1991). The E2F-cyclin A complex was later shown to contain cdk2 and the pRb related protein p107 (Cao *et al* 1992, Devoto *et al* 1992, Shirodkar *et al* 1992, Pagano *et al* 1992c), while in G1 E2F was found in association with cyclin E, cdk2 and p107 or with p107 alone (Lees *et al* 1992, Nevins 1992). Recently, p107 was shown to suppress gene expression in the G1 and S phases acting by cell-type and cell-cycle dependent mechanisms distinct from those of pRb (Schwarz *et al* 1993, Zhu *et al* 1993, Zamanian and La Thangue 1993). Transfection of p107 into Saos2 cells caused them to arrest in G1, although the distinct flat morphology induced by wild type pRb was not evident in this case. Interestingly co-transfections with cyclin A or cyclin E expression vectors did not overcome the growth inhibitions as was seen in the case of pRb, confirming the above proposal (Zhu *et al* 1993). p107/cyclin A/cdk2/E2F complexes isolated from S-phase synchronised NIH3T3 fibroblasts were shown to posses both DNA binding and histone H1 kinase activities, suggesting the involvement of cyclins in a negative control of proliferation (Devoto *et al* 1992). Cyclin A/cdk2 and cyclin E/cdk2 kinases were also able to phosphorylate p107 *in vitro* at most of the Ser/Thr sites phosphorylated in human cells *in vivo* (Lees *et al* 1992, Peeper *et al* 1993). In summary, association of E2F with cyclin/cdk kinases and the potential E2F dependent transcription from the cyclin A and cyclin D1 genes, has suggested a new, important component of the cell cycle machinery. E2F is proposed to direct cyclin dependent kinases to specific promoter regions where phosphorylation of other DNA-binding proteins might take place.

This activity may in turn be negatively regulated by the association of E2F or cyclins with suppressor proteins such as pRb or p107.

1.3.5 The cell cycle machinery is linked to the signal transduction pathway.

Signal transduction from the cell surface to targets inside the nucleus plays an important role in many cellular and developmental processes. There is no doubt that a link must exist between the rapid, cytoplasmic response and the nuclear cell cycle machinery, although, due to the complexity of mammalian systems analysis of these pathways has proved difficult. However, the mating pheromone signal transduction pathway in yeast is an easily manipulated, genetically tractable model system that has recently revealed the missing link.

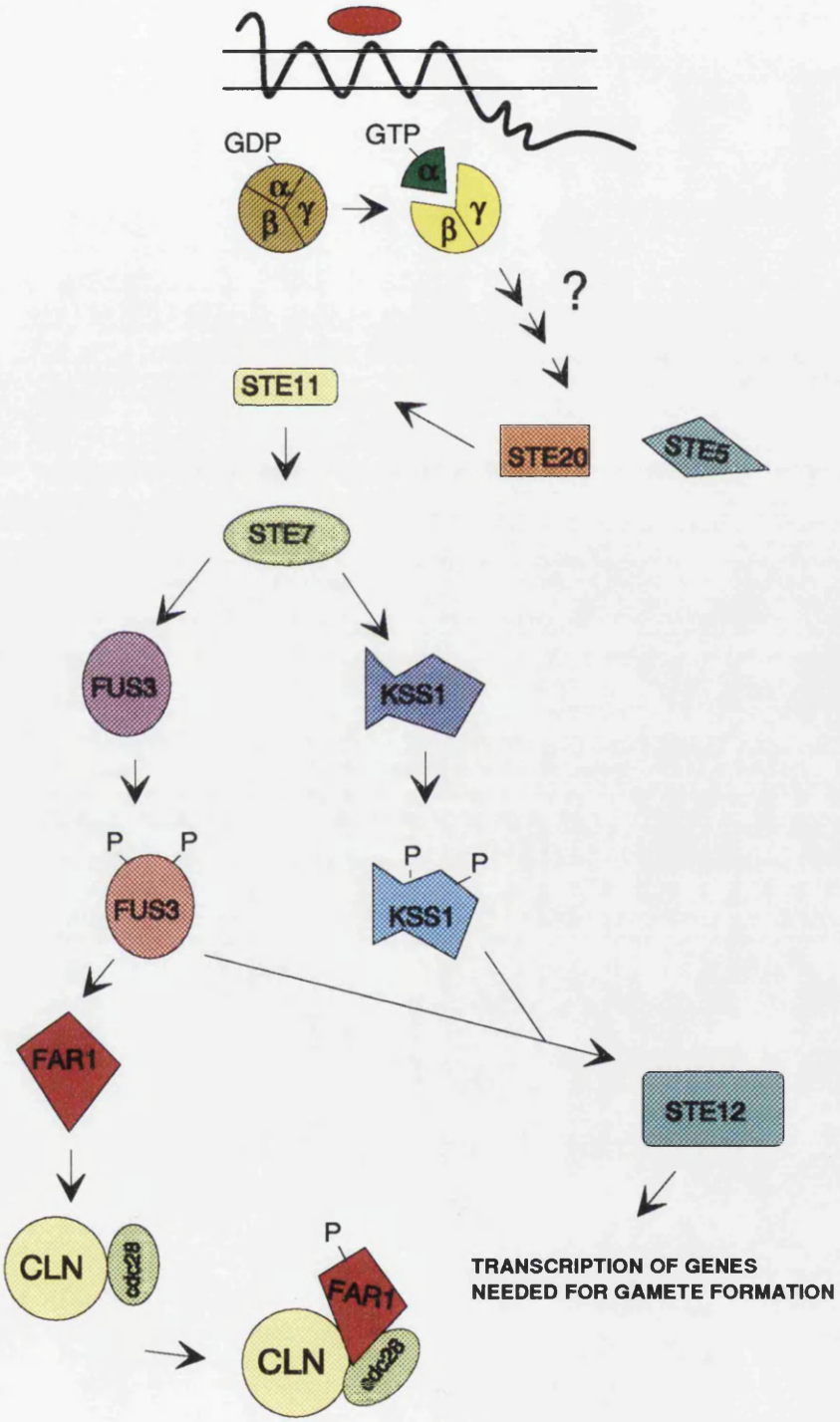
1.3.5.a FAR-1.

Haploid *S.cerevisiae* cells have three possible fates, they may enter a G0 arrested state, divide, or in the presence of secreted pheromones arrest in G1 and differentiate into gametes that are capable of conjugating with cells of opposite mating type. Cells exposed to pheromones were shown to arrest at the same point as yeast mutants defective in the function of all three CLN cyclins or CDC28, suggesting that the mating factors operated by inhibiting the function of G1 cyclin/CDC28 kinases (Reed 1980, Richardson *et al* 1989, Cross 1990). Pheromone response in yeast starts with the binding of extracellular mating factors to specific cell surface receptors (Nasmyth 1990, Marsh *et al* 1991). Two types of conjugating *S.cerevisiae* exist, those of **a** mating type that secrete the **a**-factor and have α-factor receptors, or those of α

mating type that produce the α-factor and **a**-factor receptors. Therefore **a** mating type *S.cerevisiae* will respond to the presence of α-factor and the opposite is true for the α mating type cells (Marsh *et al* 1991). Both **a**- and α-factors are short polypeptides that act by binding complex cell surface receptors linked to an αβγ heterotrimeric G protein (guanine nucleotide binding protein). Ligand/receptor association triggers release of the $G_{\beta\gamma}$ subunit which will propagate the signal by activating specific cytoplasmic Ser/Thr kinases (Cairns *et al* 1992). Two components of this pathway FUS3 and KSS1 are related to mammalian MAP kinases, however only FUS3 is able to mediate the pheromone induced G1 arrest (Elion *et al* 1991). Initially FUS3 was thought to transcriptionally repress CLN1 and CLN2 while its effect on CLN3 was unknown and hypothesised to be post-transcriptional
(Elion *et al* 1991). Recently a more general role for FUS3 was proposed, *in vitro* kinase assays revealed the ability of FUS3 to phosphorylate FAR1, a protein that is rapidly phosphorylated in haploid *S.cerevisiae* upon exposure to mating factors (Chang and Herskowitz 1992). Immunoprecipitation analysis revealed specific association of activated FAR1 with the CLN1/CDC28, CLN2/CDC28 and CLN3/CDC28 complexes and the importance of these interactions for cell cycle arrest was shown by mutational analysis (Peter *et al* 1993, Tyres and Futcher 1993). The three CLN/CDC28 complexes were also able to function as FAR1 kinases, the consequences of which remain unknown (Peter *et al* 1993, Tyres and Futcher 1993). The current model of pheromone induced *Saccharomyces*
cerevisiae cell cycle arrest based on the recent discoveries is depicted in Figure 3, this pathway may be analogous to that of negative growth factors that

Figure 3. Pheromone induced signal transduction in *S.cerevisiae.*

Schematic representation reflecting the proteins shown to be involved in the mating factor induced cytoplasmic signal transduction pathway in *S. cerevisiae*, leading to cell cycle arrest. $\alpha\beta\gamma$ represents the heterotrimeric G protein, while STE5, STE20, STE11 and STE7 are some of the cytoplasmic proteins that lead to the activation of FUS3 and KSS1 (two human MAP kinase homologues). FUS3 contains the ability to activate FAR1 by phosphorylation, which will subsequently associate with CLN/CDC28 complexes resulting in cell cycle arrest (Marsh *et al* 1991, Peter *et al* 1993).



CELL CYCLE ARREST IN G1

control differentiative responses in higher eukaryotes.

1.3.5.b In mammalian cells TGFβ acts to uncouple cyclin E/cdk2 complexes.

Transforming growth factor-β (TGFβ) is a paracrine polypeptide that regulates the production of extracellular matrix, cell proliferation and differentiation (Massague 1990, Moses *et al* 1990). TGFβ is prototypic of a larger family of proteins : three isoforms are known in man (TGFβs 1, 2 and 3) and five closely related proteins have been found in vertebrates (TGFβs 1, 2, 3, 4 and 5). The family also includes many structurally highly related proteins such as inhibins, activins, embryonic morphogens and Mullerian inhibiting substance (Sporn and Roberts 1992, Massague 1990). TGFβ functions as a homo- or hetero-dimer composed of the same or different TGFβ gene products that can bind to three receptors present on the surface of most cells, of which only types I and II are involved in signal transduction (Cheifetz *et al* 1987, Massague 1990, Massague 1992, Sporn and Roberts 1992).

Type II receptor codes for a transmembrane Ser/Thr kinase protein recently shown to require association with the type I receptor for the binding of TGFβ. The two receptors are proposed to initiate diverse cellular responses, with type I accounting for the effects on the extracellular matrix and type II affecting nuclear proteins (Wrana *et al* 1992, Chen *et al* 1993). The proposed dual functioning of TGFβ has been the subject of great interest. Currently the protein is viewed as a mesenchymal cell growth stimulatory factor and a growth inhibitor for epithelial cells (Moses *et al* 1985). Exposure of fibroblast cells to low concentrations of TGFβ induces synthesis of the AA and BB forms of PDGF, confirming the stimulatory role of this factor, however, inhibition of the

same cell type can be achieved by increasing the concentrations of TGFβ and is thought to be due to the loss of PDGF receptor α subunit expression (Moses *et al* 1990). Equivalent observations have been made in several other instances and suggest that the mitogenic effects of TGFβ seen in cells of mesenchymal origin may be secondary to other (inhibitory) processes (Massague 1990).

TGFβ prevents proliferation of almost all non-neoplastic epithelial cells and is able to overcome the EGF-induced mitogenic effects (Moses *et al* 1990, Howe *et al* 1991, Abraham *et al* 1992). Recent studies have revealed rapid reduction of *c-myc* transcript levels and predominance of hypo-phosphorylated forms of pRb in cultured epithelial cells as a consequence of the exposure to TGFβ, although in the case of pRb conflicting evidence now exists (Coffey *et al* 1988, Moses *et al* 1990, Pietenpol *et al* 1990a, Pietenpol *et al* 1990b, Laiho *et al* 1990, Yan *et al* 1992).

The effects of TGFβ mediated growth inhibition have been studied most extensively in a mink lung epithelial cell line (Mv1Lu). Exposure of Mv1Lu cells to TGFβ for 24 hours was shown to cause reversible inhibition of DNA synthesis and proliferation, with ~90% of the cells accumulating in G0/G1 phases, an observation that correlated well with the absence of pRb phosphorylation and most importantly with the loss of functional cyclin E/cdk2 kinase (Laiho *et al* 1990, Koff *et al* 1993). Cells treated with TGFβ did not exhibit altered expression levels of cyclin E or cdk2, however, a phosphorylated form of cdk2 (proposed to be the Thr160 phosphorylated species) that normally predominates in G1 disappeared if TGFβ treatment was carried out at the start of, or up to six hours after the onset of G1. Once formed, the cyclin E/cdk2 complexes were resistant to subsequent effects of

TGFβ, suggesting that this factor acts to prevent recognition and stable association of the two subunits.

It would not be unreasonable to assume that TGFβ may act on other cyclin-dependent kinases that form during G1 or even in later phases of the cell cycle, such a proposal has already gained support from the observed inability of exogenous cyclin A to activate cdc2 or cdk2 from extracts of TGFβ treated Mv1Lu cells (Koff *et al* 1993). Loss of cdc2 kinase activity had been previously shown to result from a reduction in mRNA expression levels as well as the absence of post-translational modifications of the cdc2 protein, resulting in the absence of detectable histone H1 kinase activity (Howe *et al* 1991, Abraham *et al* 1992). TGFβ mediated reduction of cdk4 expression in Mv1Lu cells, subsequently leading to the loss of cdk2 kinase and G1 arrest has also been reported (Ewen *et al* 1993b).

The cellular growth advantage of some carcinomas, brain tumours, melanomas and some haematologic tumours is thought to derive at least partially from resistance to the inhibitory effects of TGFβ (Massague 1990, Game *et al* 1992). Transfection of viral oncogenes *E1a*, *T* antigen or *E7* expression vectors (TGFβ prevents transcription of the *HPV-16* early genes) and cellular proto-oncogenes such as *ras* or mutant *p53* into epithelial cells that respond to TGFβ by arresting in G1, may result in the acquired resistance to this factor (Pietenpol *et al* 1990b, Woodworth *et al* 1990, Game *et al* 1992, Missero *et al* 1991a, Missero *et al* 1991b, Abraham *et al* 1992, Reiss *et al* 1993). Confirming observations were made following transfection of TGFβ antisense expression vectors into a poorly tumourigenic human colorectal carcinoma cell line. Reduction of endogenous TGFβ expression in the derived clones, resulted in their increased transformation and tumourigenicity potential,

determined by growth in semisolid medium and the formation of xenografts in athymic mice (Wu and Russell 1992). Resistance to TGFβ has been associated with the loss of cell surface receptors and/or a reduction of its autocrine production (Massague 1990).

1.4 Cyclins and cancer.

Cyclins and their dependent kinases are central to the regulation of the eukaryotic cell cycle, therefore they represent good targets for deregulation that may lead to neoplasia. Currently there is substantial evidence only for the involvement of cyclin D1 in transformation. This protein was identified in several independent studies as a cyclin and a potential proto-oncogene, the evidence being the different names it was initially known by : D11S287 (Arnold *et al* 1989, Rosenberg *et al* 1991b), *PRAD-1* (Motokura *et al* 1991, Schuuring *et al* 1992a), *Cyl-1* (Matsushime *et al* 1991), cyclin D1 (Lew *et al* 1991, Xiong *et al* 1991) and *bcl-1* (Withers *et al* 1991). Loss of regulated expression of cyclins A and E have also been reported, although only in a small number of examined cases (Keyomarsi and Pardee 1993, Buckley *et al* 1993, Leach *et al* 1993).

1.4.1 *CCND1* is most likely the "*bcl-1* linked oncogene".

The first indications that cyclin D1 may be a proto-oncogene came from its identification in chromosomal rearrangements specific to parathyroid adenomatosis (Bale *et al* 1990, Motokura *et al* 1991, Rosenberg *et al* 1991b). Further evidence supporting this proposal was obtained by mapping the exact chromosomal location of the gene to the proximity of the *bcl-1* breakpoint marker on 11q13, a common site of rearrangements with chromosome 14q32

(Erikson *et al* 1984, Rosenberg *et al* 1991b, Withers *et al* 1991, Seto *et al* 1992).

The majority (60-90%) of CpG nucleotide doublets found in eukaryotic genomes are methylated (Bird 1986). However, approximately 1% of vertebrate DNA, known as the HTF-fraction, is highly rich in unmethylated CpG sequences (Cooper *et al* 1983, Bird 1986, Lindsay and Bird 1987). The HTF-islands are found surrounding transcription start sites of the majority of known genes, although not exclusively. In a few examples, such as the glucose-6-phosphate dehydrogenase gene (*G6PD*) the islands are located elsewhere, in this case the 3' end of the gene (Bird 1986). Since HTF islands are most commonly found 5' of genomic coding sequences, one way of locating new genes would be to identify new islands. This hypothesis was used in the isolation of the gene targeted by the t(11;14)(q13;q32) translocation in B-cell lymphocytic malignancy and therefore in proximity of the breakpoint marker *bcl-1*. The first telomeric HTF-island was located 110kb downstream from the breakpoint marker by the technique of chromosome walking (Withers *et al* 1991). Subsequent Southern and northern blot analysis of the cloned region revealed it to be the new cyclin *CCND1* gene, the expression of which had become deregulated by the t(11;14)(q13;q32) translocation. The absence of any other CpG islands between the *bcl-1* major translocation cluster and *CCND1*, strongly suggested that the cyclin D1 gene was the "*bcl-1* proto-oncogene" (Bale *et al* 1990, Lammie *et al* 1991, Withers *et al* 1991, Brookes *et al* 1992).

Since its characterisation, the t(11;14)(q13;q32) translocation has been identified in a number of B-cell neoplasms of which most frequently (up to 70%) in centrocytic lymphoma (Williams *et al* 1991; Williams *et al* 1993). In all cases

the coding regions of the cyclin D1 gene were left intact, although in some instances loss of the 3' non-translated regions was observed (Rosenberg *et al* 1991a, Withers *et al* 1991, Seto *et al* 1992). Furthermore, both t(11;14)(q13;q32) and t(11;11)(p15;q13) rearrangements were uniformly associated with over-expression of *CCND1* transcripts, postulated to be due to the juxtaposed strong promoter regions of the IgH chain locus or the parathyroid hormone promoter regions, respectively (Rosenberg *et al* 1991a, Withers *et al* 1991, Motokura *et al* 1991, Seto *et al* 1992). The levels of over-expression varied in the different cases examined and could reach up to 37-fold more than in a range of control, non-neoplastic cell lines (Rosenberg *et al* 1991a, Rosenberg *et al* 1991b, Withers *et al* 1991, Seto *et al* 1992).

D-type cyclins show a degree of tissue type specificity with cyclin D1 and cyclin D3 transcripts predominating in epithelial and mesenchymal cells or in T- and B-lymphocytes, respectively, while cyclin D2 mRNA species have been reported in epithelial and lymphoid cell lines (Palmero *et al* 1993, Matsushime *et al* 1991, Inaba *et al* 1992). The proposed non-overlapping functions of D-type cyclins during the development of different cell lineages has suggested an even greater significance of *CCND1* over-expression seen in transformed lymphoid cells (Palmero *et al* 1993).

1.4.2 Amplification of cyclin genes.

1.4.2a The incidence of cyclin amplification in human carcinoma and the effects on patient prognosis.

Currently the gene dosages and expression levels of cyclins A, B1, B2, D1, D2, D3 and E have been examined in a variety of human tumours, as previously mentioned, only the cyclin D1 gene is amplified and subsequently

over-expressed in a significant number of cases (Jiang *et al* 1992, Schuuring *et al* 1992a, Buckley *et al* 1993). Independent clinical studies on breast, oesophageal and SCCs have implicated the amplification of chromosome 11q13 as an important prognostic factor. In breast carcinomas increased gene dosage of the 11q13 markers was associated with lymph node metastasis, a short relapse free period following curative surgery and poor patient prognosis, while in most cases no correlations were found with patient age, tumour size, histological grade, or menstrual status (Linderau *et al* 1988, Tsuda *et al* 1989a, Adnane *et al* 1989, Fantl *et al* 1990, Schuuring *et al* 1992b). Breast tumours with increased copy numbers of chromosome 11q13 were in several studies also shown to have highly elevated oestrogen receptor (ER) levels (Adnane *et al* 1989, Fantl *et al* 1990, Borg *et al* 1991). Amplifications of chromosome 11q13 in oesophageal and HNSCC are more common than in breast carcinomas, in these tumours increased gene dosage was correlated with poor differentiation, a higher incidence of distant organ metastasis and low patient survival rates (Tsuda *et al* 1989a, Berenson *et al* 1989, Berenson *et al* 1990, Kitagawa *et al* 1991). The use of mouse skin carcinogenesis models allowed an experimental analysis of the role of cyclin D1 in tumour development. Following TPA treatment, modest amplifications of the murine *CCND1* homologue (*Cyl-1*) and highly elevated transcript levels were observed in the majority of late papillomas (38 to 39 weeks of promotion with TPA), suggesting that *Cyl-1* played an important role in tumour progression and the acquisition of autonomous growth (Bianchi *et al* 1993).

The human cyclin D2 gene (*CCND2*) has been mapped to the short arm of chromosome 12 at 12p13 and cyclin D3 (*CCND3*) to 6p21 (Motokura *et al* 1992a, Inaba *et al* 1992, Xiong *et al* 1992a, Motokura *et al* 1992b). To date,

there is little evidence for the direct involvement of these genes in cancer, however, isochromosomes of 12p and 6p were observed in ~90% of germ cell tumours (GCTs) and 40-60% retinoblastomas, respectively (Samaniego *et al* 1990). Increased copy numbers of the two regions were also observed in several cases of CLL, malignant lymphoma, hyperploid acute lymphocytic leukaemia and one colorectal carcinoma cell line, suggesting the potential involvement of *CCND2* and *CCND3* in transformation (Han *et al* 1984, Leach *et al* 1993).

Increased gene dosage and/or over-expression of one or more of the cyclin A, B1 or E genes were observed in to independent studies of breast cancer cell lines and one case of colorectal carcinoma (Keyomarsi and Pardee 1993, Buckley *et al* 1993, Leach *et al* 1993). The prognostic significance of these abnormalities have not yet been analysed.

1.4.2.b *CCND1* and *EMS1* are commonly co-amplified.

The incidence and levels of 11q13 amplification in breast, lung, oesophageal and SCCs were described in section 1.2.1.c. Increased gene dosage and most significantly up-regulated expression of the cyclin D1 gene were observed in all the tumours harbouring 11q13 amplifications, suggesting that *CCND1* may represent the targeted ("key") gene of the amplicon (Lammie *et al* 1991, Faust and Meeker 1992). However, in a study of human breast carcinomas and HNSCCs, a new gene termed *EMS1* (chromosome eleven, band q13, mammary tumour and squamous cell carcinoma-associated gene) was identified and shown to co-amplify and co-express with *CCND1* in most tumours with an amplification of the 11q13 region, despite the large distance between the two genes (Schuuring *et al* 1992a).

EMS1 was cloned in an effort to isolate the putative oncogene of chromosome 11q13. A cDNA library derived from a SCC cell line (UMSCC2) harbouring 20 copies of the 11q13 region was differentially screened using [^{32}P]dCTP - labelled cDNAs obtained from UMSCC2 or a low 11q13 copy number cell line (UMSCC1), the two identified genes corresponded to *CCND1* and *EMS1* (Schuuring *et al* 1992a). The predicted EMS1 protein sequences showed greatest identity (85%) with the chicken p80/85 proteins, previously described as substrates of the oncogenic *src* tyrosine kinase (Wu *et al* 1991, Schuuring *et al* 1993). The fact that *EMS1* may represent the human homologue of chicken p80/85 was further supported by the detection of a p80/85 protein doublet in denaturing acrylamide gels with the use of affinity purified antibodies raised against EMS1 (Schuuring *et al* 1993).

In a study of 127 breast carcinomas, 41 HNSCCs and derived cell lines amplification of chromosome 11q13 markers (*FGF3*, *FGF4* and *bcl-1*) was detected in 40 tumours and nine cell lines, all of which had increased the *CCND1* gene dosage (Schuuring *et al* 1992a). The absence of co-amplified *EMS1* was evident in only three cases, although in four tumours the *EMS1* gene was amplified to a greater extent than *CCND1*. Northern blot analysis revealed over-expression of the two genes in all cell lines with corresponding amplifications, however increased copies of 11q13 were not a prerequisite for transcriptional upregulation, as observed in two cell lines and one tumour, indicating that mechanisms other than increased gene dosage may activate these putative proto-oncogenes (Schuuring *et al* 1992a).

In normal chicken cells p80/85 proteins are phosphorylated on Ser and Thr residues and exhibit a predominantly cytoplasmic location. However, in cells expressing activated forms of the src protein, substantial Tyr

phosphorylation is easily detectable and p80/85 co-localises with actin in rosette-like structures representative of membrane contact sites with the substratum (Wu *et al* 1991). This distribution of p80/85 is characteristic of cells transformed with tyrosine kinase oncogenes and the rosette structures termed podosomes also contain a variety of other cytoskeletal proteins such as α-actinin, vinculin, talin and fibrin (Burridge *et al* 1988). Immunocytochemical analysis of human SCC cell lines with or without amplifications of chromosome 11q13 revealed the same pattern of EMS1 protein subcellular localisations as seen in transformed and non-transformed chicken cells, respectively (Schuuring *et al* 1993).

Recently several lines of experimental evidence have suggested that over-expression of only *EMS1* or *CCND1* genes is not sufficient to induce transformation (Schuuring *et al* 1993, Dr Gordon Peters, ICRF, London, England, unpublished data; Vladimir Grigorijev, Beatson Institute, personal communication). However, the presence of numerous potential phosphorylation sites mapped to the EMS1 protein and the common co-amplification of *EMS1* and *CCND1* genes observed in a significant number of carcinomas, has indicated that cyclin D1/cdk complexes might be one of the EMS1 protein kinases (Schuuring *et al* 1993). Therefore, the over-representation of both proteins may have significant effects on cell cycle regulation, the organisation of the cytoskeleton and focal adhesion.

The *EMS1* gene was mapped to a position 800-1000kb telomeric from *CCND1* with the use of pulsed field gel electrophoresis, chromosome walking and chromosome jumping techniques (Schuuring *et al* 1992a, Brookes *et al* 1993). Based on the presence of non-methylated CpG islands, the ~1000kb region was postulated to contain at least five genes of which two are the

fibroblast growth factors *FGF3* and *FGF4* (described in section 1.2.1.a), while the other candidates remain unknown and also represent potential targets of the 11q13 amplicon (Brookes *et al* 1992, Brookes *et al* 1993). In summary, the pathogenic significance of *CCND1* involvement in tumours with 11q13 amplifications is less certain than in parathyroid adenomas and centrocytic lymphomas with detectable clonal rearrangements.

1.4.3 Viral insertion causes elevated cyclin expression.

Epidemiological studies have revealed a significant correlation between chronic infection by the hepatitis B virus (*HBV*) and development of liver cancer (Brechot 1993). The observed frequent integration of *HBV* into the genome of primary liver cancer cells led to the proposal that this may result in the activation of a cellular proto-oncogene. In a comparative study, the region of *HBV* integration was cloned from a very early, well-differentiated primary hepatocellular carcinoma and its germline counterpart (Wang *et al* 1990). Analysis of the sequences revealed that the virus had disrupted the cyclin A gene by integrating into its second intron (Wang *et al* 1990, Wang *et al* 1992). *In vitro* translation of several hybrid cyclin A-*HBV* cDNAs isolated from the tumour, produced a 50kD protein. The N-terminal 152 amino acids of cyclin A that normally contain the destruction box, had been replaced with 150 amino acids corresponding to the viral PreS2 and S proteins, while the cyclin C-terminus of cyclin A (including the cyclin box) had remained intact. A frog oocyte *in vitro* degradation assay of the chimeric protein, confirmed the suspicions that the loss of the amino-terminal of the cyclin A protein had rendered it nondegradable by ubiqitination (Wang *et al* 1992). Analysis of the tumour tissue revealed a large quantity of the *HBV*-cyclin A mRNAs, however,

for several proposed reasons no hybrid protein was detected in the examined samples (Wang *et al* 1992, Brechot 1993). The resulting implications for the effects of a undegradable cyclin A species that may exhibit altered subcellular localisation (the chimeric protein contains two hydrophobic helices necessary for membrane anchorage of the viral proteins) in developing liver cancer is considerable, although as yet no direct evidence has been found.

The murine *Cyl-1* gene was mapped to mouse chromosome 7 in a region highly homologous to human chromosome 11q13 and in the proximity of a Friend murine leukaemia virus (*MuLV*) integration site (*Fis-1*) (Lammie *et al* 1992). Normal lymphoid cells predominantly lack *Cyl-1* transcripts, however, expression of the gene was induced in all the examined tumours that contained *MuLV* sequences integrated into *Fis-1* of chromosome 7. *Friend MuLV*-induced erythroleukaemias and myelogenous leukaemias that were negative for insertion into the *Fis-1* locus were also negative for *Cyl-1* expression, suggesting that the presence of *Cyl-1* mRNA was tightly linked to *MuLV* induced transcription (Lammie *et al* 1992).

Another common integration site of *MuLV* in T-cell leukaemias was mapped to murine chromosome 12 and the proximity of the *Vin-1* gene, which was subsequently identified by sequence analysis as the murine homologue of *CCND2* (*Cyl-2*). Transcriptional activation of the *Cyl-2* gene, as a consequence of viral insertion, was proposed to contribute to the growth disregulation of the malignant T cells (Hanna *et al* 1993). Therefore, as in the case of cyclin A, the over-expression of at least two members of the D-family of cyclins resulting from *MuLV* insertion proposes a mechanism by which this virus may act to transform cells, however many more studies will be necessary to elucidate the function of the cyclin proteins in these processes.

1.4.4 The consequences of cyclin over-expression on the control of cellular proliferation.

Cell cycle events of most organisms are ordered into pathways in which the intitiation of later events depends on the completion of early ones. To illustrate this, in eukaryotes entry into mitosis is dependent on the completion of DNA synthesis, which in turn is regulated by a number of DNA repair proteins that operate during G1 (Hartwell and Weinert 1989). The majority of decisions regarding proliferation of mammalian cells are made during the G1 phase and depend on mitogenic stimulation (Pardee 1989). Analogous to START in budding yeast, a mammalian cell can switch between proliferation and arrest at a specific stage during the G1 phase, termed the restriction- (R-) point (Pardee 1974, Rossow *et al* 1979, Cross *et al* 1989). Once the R-point is passed the cell becomes committed to proliferation despite any extracellular signals, therefore it is temporarily growth factor independent. At least two stages in the cell cycle, the G1-S and G2-M transitions, are responsible for the detection and repair of DNA damage (Hartwell 1992, Murray 1992). Cells are able to delay cell cycle progression at these two checkpoints to allow repair before entering the S phase or mitosis, where DNA damage would cause the accumulation of mutations and the loss or gain of genomic material, respectively. Currently, the evidence for the involvement of the tumour suppressor protein p53 in the G1-S checkpoint is rapidly accumulating, there are also some indications that p53 is a substrate of cyclin dependent kinases (Bishoff *et al* 1990, Lane 1992, Hartwell 1992, Wang and Eckhart 1992).

G1 cyclins represent one of the best candidates for the regulators of progression through G1 and the timing of DNA synthesis, thus the major consequences of deregulated cyclin expression are suggested to be the

shortening of specific cell cycle phases causing the abolishment of check point controls (Dou *et al* 1993). This proposal has been analysed in two independent studies in which constitutive over-expression of cyclins D1, D2 or E were achieved in several fibroblast cell lines (Ohtsubo an Roberts 1993, Quelle *et al* 1993). Increased cyclin E expression was achieved in a rat fibroblast cell line and primary human foreskin fibroblasts by infection with a Moloney murine sarcoma virus expression vector, also containing the *neomycin phosphotransferase* gene as a selectable marker (Ohtsubo and Roberts 1993). At least two forms of the endogenous cyclin E protein were detectable in the examined fibroblasts, of which the smaller 50kD species predominated during G1 and exhibited increased expression in the transfected cells. In exponentially growing cells the over-expression of the cyclin E protein resulted in a three- to five-fold increase of cyclin E associated histone H1 kinase activity, which was relatively independent of the cell cycle. The duration of G1 was on average three hours shorter in infected cells when compared to the controls, although the overall length of the cell cycle was unchanged due to secondary delays in S and G2. Upon forty eight hours of serum starvation, most of the control Rat-1 and primary fibroblasts were in G1 (92% and 84%, respectively) the median length of which had increased from eleven to twenty three hours. However, the same experiment revealed a smaller number of cyclin E infected cells in G1 (77% of Rat-1 and 64% of primary fibroblasts) which lasted only forteen hours, suggesting a reduction in serum requirement of these cells. Over-expression of cyclin E also resulted in up to a 30% decrease of fibroblast cell size, an analogous phenomenon to budding yeast that have increased levels of CLN cyclins (Hadwiger *et al* 1989).

The effects of cyclin D1 and D2 over-expression in two rodent fibroblast cell lines (NIH3T3 and Rat-2) were similar to those previously observed for cyclin E, although transfections were hampered by apparent cyclin D toxicity (Quelle *et al* 1993). A shortening of the G1 and G0 to S phase intervals by up to four hours was evident only in cells that had increased levels of cyclin D1, while the smaller effects of cyclin D2 were potentially less significant. Microscopic and Coulter counter analyses of cyclin D over-expressers revealed a reduction in cell size. NIH3T3 and Rat-2 cyclin D transfectants also exhibited a decreased serum requirement which was determined by the timing of DNA synthesis following serum stimulation of starved cells. Opposite to the previously described consequences of cyclin E over-representation, cyclin D transfected cells did not compensate for the shortening of G1 by extending subsequent phases and therefore exhibited a reduced generation time.

In 1974 Arthur Pardee proposed the G1 restriction point model which allowed distinction between "normal" cells that were able to arrest in G1 as a response to low serum, high cell density or partial inhibition of protein synthesis and tumourigenic cells that had lost this ability (Pardee 1974, Rossow *et al* 1979). Subsequently the control of growth at the R point was suggested to depend on the accumulation of a specific "initiator" protein the expression of which would be deregulated in transformed cells (Rossow *et al* 1979, Campisi *et al* 1982). Currently it is becoming apparent that not one but a whole set of G1 specific proteins satisfy the criteria for the R factor (Dou *et al* 1993). The G1-S cyclins (A, C, D and E) accumulate throughout the G1 phase of the cell cycle, triggering the activation of a range of Ser/Thr kinases (Xiong and Beach 1991, Pines 1993). Several reports have revealed associations of these proteins with tumour suppressor, DNA repair and transcription factors such as

pRb, p53, PCNA or E2F (Xiong *et al* 1992b, Hinds *et al* 1992, Lees *et al* 1992, Zhang *et al* 1993, Hall *et al* 1993). Constitutive over-expression of cyclins D and E induced by transfection experiments, causes premature DNA replication in cells and a reduced serum requirement (Quelle *et al* 1993, Ohtsubo and Roberts 1993). Taken together with the observed over-expression of cyclins in a variety of mammalian tumours, these data suggest that cyclins may form a new class of proto-oncogenes the function of which is important for the proliferation of "normal" and transformed cells.

1.5 Summary and aims.

Cyclins were initially given this name due to the periodic changes in expression during the cell cycle (Rosenthal *et al* 1980, Evans *et al* 1983). However, the classification is currently determined to a greater degree by the presence of specific evolutionarily conserved domains within these proteins, termed "the cyclin box". Furthermore, many newly discovered families, such as cyclins C and G, do not exhibit periodic oscillations in expression levels and depend on growth stimulation signals rather than the cell cycle itself (Tamura *et al* 1993)

Currently, cyclin D1 is the only cell cycle regulator revealing extensive involvement in human cancer. The gene (*CCND1*) is amplified and over-expressed in HNSCCs, breast carcinomas, oesophageal carcinomas and transitional carcinomas of the lung (Zhou *et al* 1988, Berenson *et al* 1989, Tsuda *et al* 1989b, Berenson *et al* 1990, Somers *et al* 1990, Lammie and Peters 1991, Kitagawa *et al* 1991). Rearrangements involving the immunoglobulin heavy chain and the parathyroid hormone loci with chromosome 11q13, result in *CCND1* over-expression in B-cell leukaemias

and lymphomas, therefore cell types that normally lack any expression of this gene.

Despite extensive studies, there has been a lack of published data investigating the expression levels and subcellular localisation of the *CCND1* protein product in original tumours or derived cell lines. Furthermore, the HNSCC cell lines examined revealed a higher frequency of *CCND1* amplification than that reported in tumour tissue samples, suggesting growth selection during cell line establishment (Lammie *et al* 1991, Schuuring *et al* 1992a, Edington *et al* submitted). In addition, the relevant studies failed to include normal epithelial cells during the gene dosage and mRNA expression analyses, potentially reducing the accuracy of the determined results (Lammie *et al* 1991, Schuuring *et al* 1992a).

This study was aimed at the characterisation of *CCND1* gene dosage, transcript and protein expression levels in a panel of new SCC cell lines which had been derived under conditions that minimised selection of fitter variants. The obtained values were compared to those determined for human foreskin primary keratinocytes. The intent was to, by using the technique of lipofection, analyse the effects of increased *CCND1* protein expression on the growth and transformation of human primary keratinocytes or established, non-tumourigenic epithelial cell lines.

MATERIALS AND METHODS

CHAPTER 2 : Materials and Methods.

2.1 Materials.

2.1.1 Chemicals.

The chemicals were of "AnalaR" grade, the majority of which were obtained from BDH Chemicals Ltd., Poole, Dorset, England or Sigma Chemical Co. Ltd., Poole, Dorset, England, except those obtained from the suppliers listed below :

Supplier - Amersham International PLC, Amersham, Bucks, England.

$\alpha[^{32}P]$ dCTP (3000Ci/mmol) Hybond-N+

$[^{35}S]$ dATP αS (600Ci/mmol) $[^{125}I]$ EGF (50μCi/ml)

Supplier - B.R.L. (UK), Gibco Ltd, Paisley, Scotland.

All restriction enzymes and buffer concentrates with the exception of where otherwise stated.

Select agar

Supplier - Biogenesis Ltd., Bournemouth, England.

RNAzol B

Supplier - Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.

Caesium chloride

DNA molecular weight markers IV and VI

proteinase K

primer $p(dT)_{15}$

RNase DNase free, from bovine pancreas

Supplier - J.Burrough (FAD) Ltd., Witham Essex, England.

Ethanol

Supplier - Central Services, Beatson Institute.

CT buffer (6g NaCl, 2.96g trisodium citrate, 1.76g tricine, 5mg phenol red, 700ml water, pH7.8)

Sterile distilled water

L-broth

Sterile PBS

PE buffer (0.04g KCl 0.04g, KH_2PO_4, 1.6g NaCl, 0.276g $Na_2HPO_4x2H_2O$, 0.07g EDTA, 200ml water)

Supplier - Difco Labs., Detroit, Michigan, USA.

Bacto-tryptone

Bacto-yeast extract

Supplier - Gateway PLC, Glasgow, Scotland.

Marvel, dried non-fat milk powder

Supplier - GIBCO Europe, Life Technologies Ltd., Paisley, Scotland.

10x DMEM

keratinocyte-SFM

foetal calf serum

penicillin

200mM glutamine

streptomycin

hydrocortisone

2.5% trypsin

7.5%sodium bicarbonate

geneticin sulphate

protein molecular weight markers low and high range

Supplier - ICN Biomedicals Ltd.,Irvine, Ayrshire, Scotland.

mycoplasma removal reagent

Supplier - Oxoid Ltd., Basingstoke, England.

PBS tablets

Supplier - Pharmacia Ltd., Milton Keynes, England.

ultrapure dNTP set

restriction enzymes *Sal1* and *Pst1* including appropriate buffer concentrates.

NICK^{TM} columns, sephadex G50

Supplier - Rathburn Chemicals Ltd., Walkeburn, Scotland.

phenol (water saturated)

2.1.2 Kits.

Supplier - Amersham International PLC, Amersham, Bucks, England.

ECL Western Blotting analysis system

Supplier - Bio 101 Inc., Stratech Scientific, Luton, England.

Geneclean kit

Supplier - Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.

Transfection Reagent DOTAP

Supplier - Cruachem Ltd., Glasgow, Scotland.

Cruachem oligonucleotide purification cartridges

Supplier - Invitrogen corporation, Abingdon, Oxon, England.

TA Cloning™ kit

Supplier - Perkin Elmer Cetus Ltd., Beaconsfield, Bucks., England.

PCR Gene amplification kit RNA PCR kit

Suppler - Pharmacia Ltd., Milton Keynes, Bucks., England.

Oligo-labelling kit

Supplier - Promega Ltd., Southampton, England.

Magic Minipreps DNA purification system

Magic PCR* Preps DNA purification system

Supplier - Sigma Chemical Co. Ltd., Poole, England.

Bicinchroninic acid protein concentration kit

Supplier - Clonetics, TCS Biologicals Ltd., Buckingham, England.

KGM Bulletkit™

Supplier - United States Biochemical Corporation, Cleveland, USA.

Sequenase version 2.0 DNA sequencing kit

Supplier - Vector Laboratories, Bretton, Peterborough, England.

ABC kit, peroxidase mouse IgG

ABC kit, peroxidase rabbit IgG

2.1.3 Equipment.

In most cases the main pieces of equipment are referred to in the appropriate sections. The Beatson Institute stores provided the general plasticware, while the following companies supplied some of the most commonly used items:

Supplier - Becton Dickinson Labware, Plymouth, Devon, England.

tissue culture dishes (35, 60 and 90mm)

falcon tubes (15 and 50ml)

polystyrene round bottom tubes (12x75mm and 17x100mm)

Supplier - Bibby-Sterilin Ltd., Stoney, Staffs., England.

bacteriological dishes (90mm)

Supplier - Chance Propper Ltd., Warley, England.

gold star microslides (76x26mm)

microscope glass cover slips (22x50mm)

Supplier - Costar, Cambridge, Massachusetts, USA.

6, 24 and 96 well plates disposable cell scrapers

Supplier - Eastman Kodak Co., Rochester, New York, USA.

duplicating film X-ray film (XAR-5)

Supplier - Fuji Photo Co., Ltd., Japan.

X-ray film (RX)

Supplier - GIBCO Europe, Life Technologies Ltd., Paisley, Scotland.

Nunc cryotubes (1 and 1.5ml)

Nunc flasks (25, 80 and 175cm^3) Nunc glass chamber slides

Supplier - Griener Labortechnik Ltd., Dursley, England.

Eppendorf tubes

Supplier - Labsystems, Basingstoke, England.

pipette tips (200 and 500µl)

Supplier - Millipore UK Ltd., Middlesex, England.

immobilon P transfer membrane

Supplier - Sartorius Instruments Ltd., Surrey, England.

collodion bags

Supplier - Whatman International Ltd., Maidstone, England.

3mm chromatography paper

2.1.4 Plasmids.

pA6	A 190bp cDNA fragment complementary to 7S RNA cloned into the pAT153 vector. A gift from Dr David Prowse, Beatson Institute (Balmain *et al* 1982).
pAM91	A 1360bp *Pst1* cDNA fragment from an actin mRNA expressed in adult skeletal muscle cloned into the *Pst1* site of the plasmid pBR322. A gift from Dr Rob Nibbs, Beatson Institute (Minty *et al* 1982).
pHR28-1	A 7kb fragment containing sequences homologous to 28S, the 3' end of 18S and 5.8S rRNA cloned into the *EcoR1* site of the plasmid pAT153. A gift from Frances Fee, Beatson Institute.
pHSG274	A gift from Dr Kim Hawker, Beatson Institute (Brady *et al* 1984).

pJ5Ω-D1 A 1290bp fragment containing the *Cyl-1* cDNA was cloned into the *EcoR1* site of the pJ5Ω vector. A gift from Dr Gordon Peters, ICRF, London, England.

pJ4Ω16.E6 A 1.77kb fragment containing the *HPV-16 E6* open reading frame was inserted into *EcoR1/BamH1* restriction enzyme digested vector pJ4Ω. At the C-terminal of the *E6* fragment, amino acid Leu was replaced by His-Gly. A gift from Dr Lionel Crawford, ICRF, London, England (Storey *et al* 1988).

pJ4Ω16.E7 A 1.9kb fragment containing the *HPV-16 E7* open reading frame and 1.6kb of 3' sequences beyond the *E7* stop codon was cloned into the *BamH1* site of plasmid pJ4Ω. A gift from Dr Lionel Crawford, ICRF, London, England (Storey *et al* 1988).

pMoE6 A 0.63kb fragment of *HPV-16* DNA containing the intact *E6* open reading frame linked to the Molony murine leukaemia virus long terminal repeat, inserted into the pUC19 vector. A gift from Dr Karen Vousden, Ludwig Institute for Cancer Research, London, England (Vousden and Jat 1989, Hawley-Nelson *et al* 1989).

pMoE7 A 0.671kb of *HPV-16* DNA containing the intact *E7* open reading frame linked to the Molony murine leukaemia virus long terminal repeat, inserted into the pUC19 vector. A gift from Dr Karen Vousden, Ludwig Institute for Cancer Research, London, England (Vousden and Jat 1989, Hawley-Nelson *et al* 1989).

pSP64-ets1 Human *c-ets1* cDNA cloned into the *EcoR1* site of the pSP64 vector. A gift from Dr Rob Nibbs, Beatson Institute.

pJ7Ω-D1 A 1290bp *Cyl-1* cDNA fragment inserted in the correct orientation into the *EcoR1* site of the pJ7Ω vector. A gift from Dr Gordon Peters, ICRF, London, England (Smith *et al* unpublished data).

pJ7Ω-antiD1 A 1290bp *Cyl-1* cDNA fragment inserted in the incorrect orientation into the *EcoR1* site of the pJ7Ω vector. A gift from Dr Gordon Peters, ICRF, London, England (Smith *et al* unpublished data).

pHR28-1 A 7kb fragment containing homologous sequences to 28S, the 3' end of 18S and 5.8S. A gift from Frances Fee, Beatson Institute.

2.1.5 DNA probes.

The probes for cyclin D1, *ets-1*, *GAPDH*, 7S, 18S and 28S ribosomal RNA, human skeletal actin and *HPV-16 E7* are listed below. The origin of most probes is schematically depicted in Figure 4.

cyclin D1	1.3kb *EcoR1* fragment of mouse *Cyl-1* cDNA.
ets1	4kb *Pst1* fragment of human *ets1* cDNA.
GAPDH	0.7kb PCR fragment of rat *GAPDH*.
7S	0.6kb *EcoR1/Sal1* fragment containing a 190bp stretch complementary to 7S RNA.
18S and 28S	A 7kb fragment containing homologous sequences to 28S, the 3' end of 18S and 5.8S.

actin	1.36kb *Pst1* fragment of adult human skeletal muscle specific actin cDNA.
HPV-16 E7	A 313bp region of the *E7* open reading frame isolated by *BamH1* restriction of the vector pURHPV-16. A gift from Angeliki Malliri, Beatson Institute.

2.1.6 Immunological reagents.

Rabbit polyclonal anti-cyclin D1 (287-3). Used at dilutions 1:500 and 1:3000.

A gift from Dr Gordon Peters, ICRF, London, England.

Rabbit polyclonal anti-cyclin D1. Used at a 1:500 dilution.

Pharmingen, AMS Biotechnology UK Ltd., Oxon, England.

Mouse monoclonal anti-cyclin D2. Used at a 1:500 dilution.

Pharmingen, AMS Biotechnology UK Ltd., Oxon, England.

Mouse monoclonal anti-cyclin D3. Used at a 1:500 dilution.

Pharmingen, AMS Biotechnology UK Ltd., Oxon, England.

Rabbit polyclonal anti-cdk4 (10165). Used at a 1:1000 dilution.

A gift from Dr Li Huei Tsai , Massachusetts General Hospital, Boston, USA.

Mouse monoclonal anti-pRb (XZ77). Used at a 1:10 dilution.

A gift from Dr Li Huei Tsai, Massachusetts General Hospital, Boston, USA.

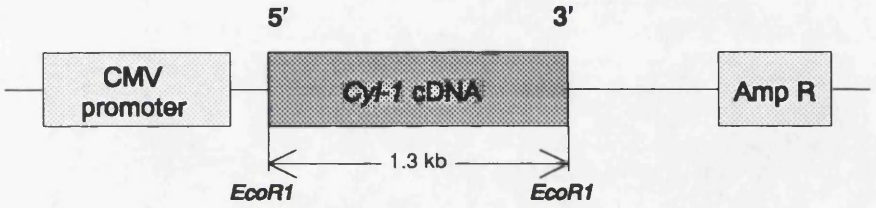
Mouse monoclonal anti-PCNA. Used at a 1:1000 dilution.

Pharmingen, AMS Biotechnology UK Ltd., Oxon, England.

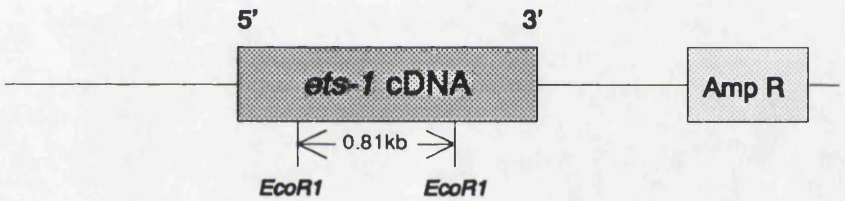
Figure 4. Schematic representation of DNA fragments used as probes in Southern and northern blot analyses.

Figure 4.

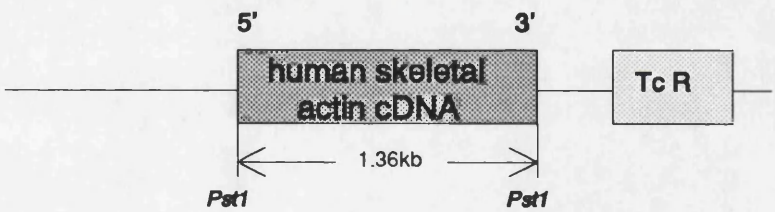
a) cyclin D1 : 1.3kb fragment of *Cyl-1* cDNA



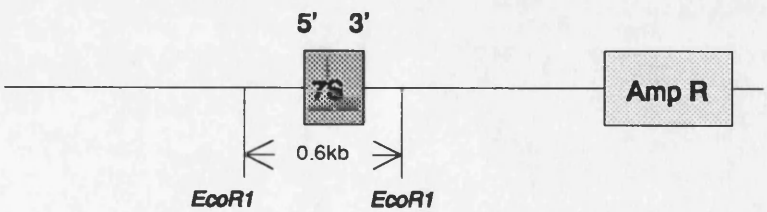
b) *ets-1* : 0.81kb fragment of *ets-1* cDNA



c) actin : 1.36kb fragment of actin



d) 7S : 0.6kb fragment containing 7S cDNA



Mouse monoclonal anti-*HPV16* E7. Used at a 1:10 dilution.
Triton diagnostics, Alameda, California, USA.
Mouse monoclonal anti-BrdU. Used at a 1:2000 dilution.
Sigma, Chemical Co. Ltd., Poole, Dorset, England

Donkey anti-mouse horseradish peroxidase-linked whole antibody (1:5000 dilution). Amersham International PLC, Amersham, Bucks, England.
Sheep anti-rabbit horseradish peroxidase-linked whole antibody (1:3000 dilution). Amersham International PLC, Amersham, Bucks, England.

Anti-mouse IgG biotinylated antibody
Vector Laboratories, Bretton, Peterborough, England.
Anti-rabbit IgG biotinylated antibody
Vector Laboratories, Bretton, Peterborough, England.

2.1.7 Bacterial strains.

E.coli DH5α cells. Obtained from B.R.L.(UK), Gibco Ltd, Paisley, Scotland.
E.coli INVαF' cells. Obtained from Invitrogen corporation, Oxon, England.

2.1.8 Cell lines.

BICR 3-22 Seven cell lines derived from human squamous cell carcinomas of the head and neck by Kirsten Edington at the Beatson Institute. A gift from Kirsten Edington.

	Maintained in DMEM containing hydrocortisone in the presence of terminally irradiated fibroblasts.
MS-2	A cervical squamous cell line derived by Margareta Nikolic at the Beatson Institute. Maintained in DMEM containing hydrocortisone in the presence of terminally irradiated fibroblasts.
A431	A vulvar squamous cell carcinoma cell line obtained from Dr Brad Ozanne, Beatson Institute. Maintained in DMEM containing hydrocortisone.
SCC12-F	A skin squamous cell carcinoma cell line obtained from Dr Ken Parkinson, Beatson Institute. Maintained in DMEM containing hydrocortisone.
TFK104	*HPV-16 E6/E7* immortalised human foreskin primary keratinocytes. A gift from Dr Karen Vousden, Ludwig Institute for Cancer Research, London, England. Maintained in GIBCO SFM.
HEK b	Human foreskin primary keratinocytes. A gift from Caroline Chapman, Beatson Institute. Maintained in Clonetics KGM.
HEK34	Human foreskin primary keratinocytes. A gift from Dr Ken Parkinson, Beatson Institute. Maintained in GIBCO SFM.
HEK124	Human foreskin primary keratinocytes. A gift from Dr Ken Parkinson, Beatson Institute. Maintained in GIBCO SFM.
Saos-2	A human osteosarcoma cell line. A gift from Dr Ken Parkinson, Beatson Institute. Maintained in DMEM.
x3T3	Terminally irradiated Swiss 3T3 fibroblasts used to provide optimal growth conditions for the BICR and MS-2 cell lines.

208F	An immortalised rat fibroblast cell line. A gift from Dr Bob Hennigan, Beatson Institute. Maintained in DMEM.
MMV	*c-fos* transfected 208F fibroblasts. A gift from Dr Bob Hennigan, Beatson Institute. Maintained in DMEM.
A1C	*FBJ* infected 208F fibroblasts expressing *v-fos*. A gift from Dr Bob Hennigan, Beatson Institute. Maintained in DMEM.
FBR	*FBR* infected 208F fibroblasts expressing *v-fos*. A gift from Dr Bob Hennigan, Beatson Institute. Maintained in DMEM.
Rat1	An immortal rat fibroblast cell line. A gift from Dr Bob Hennigan, Beatson Institute. Maintained in DMEM.
1302	*FBJ* infected Rat1 fibroblasts. A gift from Dr Bob Hennigan, Beatson Institute. Maintained in DMEM.
Swiss 3T3	An immortal murine fibroblast cell line. A gift from Dr Ken Parkinson, Beatson Institute. Maintained in DMEM.
Balb 3T3	An immortal murine fibroblast cell line. A gift from Dr Brad Ozanne, Beatson Institute. Maintained in DMEM.
NIH3T3	An immortal murine fibroblast cell line. A gift from Dr Brad Ozanne, Beatson Institute. Maintained in DMEM.
T45	Swiss 3T3 murine fibroblasts exposed to high doses (60Gy) of cobalt irradiation. A gift from Dr Garry Sibbet, Beatson Institute. Maintained in DMEM.

2.1.9 Tissue culture media.

DMEM	Dulbecco's modified Eagles media supplemented with 0.5%, 2% or 10% FBS, 0.4μg/ml hydrocortisone, 25μg/ml streptomycin and 18.75μg/ml penicillin.

SFM Keratinocyte serum free media supplemented with 5ng/ml human epidermal growth factor, 2ml bovine pituitary extract, 25µg/ml streptomycin and 18.75µg/ml penicillin.

KGM Keratinocyte serum free media supplemented with 0.1ng/ml human epidermal growth factor, 0.5µg/ml hydrocortisone, 2ml bovine pituitary extract, 5µg/ml bovine insulin, 50µg/ml gentamicin sulphate, 50ng/ml amphotericin-B, 25µg/ml streptomycin and 18.75µg/ml penicillin.

2.1.10 Water.

Deionised water for solutions was obtained from a Millipore MilliRO 15 system. For nucleic acid, protein and tissue culture manipulations, the water was further purified on a Millipore MilliQ system.

2.2 Methods.

2.2.1 Growth and manipulation of eukaryotic cells.

2.2.1.a Establishment of cell lines from human SCCs.

A total of twenty five cervical SCC biopsies were obtained from patients of a wide age group (a gift from Dr Brendan Bolger, Royal Infirmary, Glasgow, Scotland.). Half of the samples were snap frozen in liquid nitrogen for histological examination and future DNA extraction, while the remaining portions were bisected into 1mm^3 particles, under sterile conditions. The fragments were rinsed in foetal bovine serum (FBS) and, following serum drainage, maintained on plastic petri dishes in a non-humidified incubator at 37°C for 45 minutes, to allow tissue attachment. Subsequently 5ml of DMEM

containing 10% FBS were added to the dishes and the explants incubated for 5 days at 37°C in an atmosphere of 7% CO_2 until firm attachment to the matrix was evident. At the first media change, terminally irradiated Swiss 3T3 fibroblasts (x3T3) were added to the growing cells in order to minimise selection for fitter variants (Rheinwald and Beckett 1981; Edington *et al* submitted). The explants were also exposed to 10μg/ml mycoplasma removal reagent to prevent and where applicable cure cellular infection during the initial stages of culturing. Upon establishment the MS-2 cell line was tested for optimal proliferative conditions by varying the serum levels and attempting growth in the absence of x3T3 cells.

2.2.1.b Mammalian cell line culturing.

The BICR and MS-2 cell lines were maintained on 90mm plastic petri dishes in the presence of x3T3 cells at 37°C in an atmosphere of 7% CO_2. The FBS levels were varied in dependence of individual cell line growth requirements. At confluence the cells were passaged at a 1:2-1:5 ratio onto fresh x3T3 containing petri dishes, this took place every 3-5 days and was determined by the cell type. The same conditions were used for the culturing of cell lines A431, SCC12, P-1 and A3, except that they did not require the presence of x3T3 fibroblasts. The human foreskin primary keratinocytes (HEKs) and TFK104 cells were cultured in keratinocyte serum free media (GIBCO or Clonetics) on 90mm plastic petri dishes at 37°C in an atmosphere of 7% CO_2.

Passaging was carried out by aspiration of the growth media from the cell monolayers, followed by two successive exposures to 2.5ml of 0.25% trypsin solution in CT buffer (see section 2.1.1). The majority of trypsin was

immediately removed by aspiration and the petri dishes incubated at 37°C until the cells detached. In the case of SCCs, the detached cells were resuspended in the appropriate DMEM and replated at the required dilution. For the cells growing in serum free media, trypsin inactivation was carried out by resuspention in ice cold DMEM containing 2-10% FBS, followed by the pelleting of cells by centrifugation in a Beckman GP centrifuge at 1000rpm and subsequent plating in the appropriate media at the required dilution.

All of the cell lines were routinely examined for mycoplasma infection as part of the Beatson Institute mycoplasma test programme (Joe Winnie and Mary Freshney, Beatson Institute). Where appropriate, the cells were treated for one week with 0.5μg/ml mycoplasma removal reagent or until the complete removal of contaminants.

Keratinocyte cell cycle delay was achieved following trypsinisation, trypsin inactivation in ice cold DMEM containing 2-10% FBS and subsequent plating in DMEM containing 0.5% FBS for 24 hours. Due to increased cell death following plating in low serum, to obtain G0 arrest, sub-confluent cultures of fibroblast cell lines were washed 3-5 times in 10 ml of sterile ice cold PBS and subsequently cultured in DMEM containing 0.5% FBS for 72 hours.

For immunocytochemical analysis $1x10^4$-$3x10^4$ cells were plated on glass chamber slides in 200μl of the appropriate media, depending on their growth properties. Culturing took place in humidifying conditions at 37°C in an atmosphere of 7% CO_2 for 24-48 hours subsequent to which the cells were processed for immunocytochemistry as described in section 2.2.4.d. To obtain G1 arrest, fibroblast and keratinocyte cell lines were initially cultured in DMEM containing 0.5% FBS for 72 or 24 hours, respectively.

2.2.1.c Frozen cell stocks.

A large frozen stock was maintained for all the examined cell lines. Upon trypsinisation, the detached cells were resuspended in 1ml of appropriate growth media supplemented with 20% FBS and 10% DMSO at approximately $1x10^6$ cells/ml. These cells were rapidly aliquoted into 1 or 1.5ml Nunc cryotubes and frozen at -70°C, where they were kept for up to one month. Subsequently, the frozen cryotubes were placed for long term storage into liquid nitrogen.

2.2.1.d EGF binding assays.

Cells were plated at a density of $1x10^5$ cells/well in 24 well tissue culture plates 24 hours prior to the assay. Each well was washed 3 times with PBS and duplicates were incubated with increasing amounts of [^{125}I] EGF (typically 11 concentration points from 0.1μg/well to 50μg/well) in the presence or absence of a hundred fold excess of unlabelled competitor EGF in 0.5ml of PBS containing 1% BSA. Incubation was carried out at room temperature for 1 hour. Cells were subsequently washed 5 times in ice cold PBS containing 5% BSA, solubilised in 1ml of 1M NaOH and the amount of radioactivity determined in a Beckman 5500B gamma counter. The amount of specifically bound EGF was determined by subtracting the figures obtained by analysing the competitor samples (revealing non-specific binding) from the figures obtained for wells lacking non-radioactive EGF (total EGF binding). The results were processed using the LIGAND programme.

2.2.2 Transfection, selection and cloning of cell lines.

2.2.2.a Transfection of established SCC12 cells.

SCC12 cells were co-transfected with sense or antisense cyclin D1 expression constructs in combination with a neomycin resistance conferring vector (see section 2.1.4). Lipofection was used as a means of introducing exogenous DNA into these cells. Diluted transfection reagent DOTAP (70µl of DOTAP were added to 180µl of HBS buffer, containing 20mmol/l Hepes, 150mmol/l NaCl, pH7.4) was mixed with 5µg of appropriate DNA in 250µl HBS buffer incubated for 10 minutes at room temperature to allow association between the DNA and cationic lipid particles. The old tissue culture media was subsequently removed SCC12 cells growing in 90mm plastic petri dishes at a 60-80% confluency and the cells exposed to the transfection mixture diluted in 14ml of serum free DMEM. Incubation was carried out for 12 hours at 37°C in humidifying conditions and an atmosphere of 7% CO_2, following which the media was replaced by fresh DMEM containing 10%FBS. After a period of 48-72 hours, during which plasmid expression took place, SCC12 cells were passaged at a 1:10 split into growth media containing 250µg/ml G418. Antibiotic resistant colonies were observed following a period of 4-5 weeks, during which selection was maintained and media changes were carried out every 4-5 days. Resistant colonies were cloned as follows:

(i) A felt-tip mark was made on each petri dish outlining the position of selected clones.

(ii) The growth media was removed by aspiration.

(iii) Sterile metal or plastic ring clones were greased with sterile Vaseline and placed onto the dish isolating individual clones.

(iv) 100µl of trypsin solution were applied to each ring and maintained for approximately 1 minute at room temperature.

(v) The trypsinised cells were placed into separate wells of a 24 well plate in 1ml of DMEM containing 10% FBS. This media was replaced with fresh DMEM one day from cloning and subsequently at 4-5 day intervals.

(vi) Proliferating clones were sequentially expanded into larger plates, maintaining selection throughout culturing.

2.2.2.b Transfection of human foreskin primary keratinocytes.

HEK cells were co-transfected with varying combinations of cyclin D1, *HPV-16 E6* or *E7* expression constructs in the presence or absence of G418 resistance conferring expression vectors (as depicted in Tables 8, 9 and 10). Lipofection was used as a means for introducing exogenous DNA into the cells and the same methods applied as described in section 2.2.2.a with a few alterations listed bellow:

(i) Lipofection was carried out in GIBCO SFM (HEK124 and HEK34) or Clonetics KGM (HEKb).

(ii) The cells were exposed to the transfection mixture only for a period of 8 hours.

(iii) Transfection of HEK124 cells was carried out in the absence of G418. Instead the cells were selected for resistance to senescence and differentiation.

(iv) HEK34 and HEKb cells were selected by a 48 hour exposure to 10µg/ml G418 followed by culturing in keratinocyte growth media.

(v) Colony formation was evident approximately 10 days from lipofection.

(vi) Due to previously observed Vaseline toxicity, HEKb transfectants of the same type were pooled and maintained on 90mm petri dishes.

2.2.3 Nucleic acid procedures.

2.2.3.a Transformation of competent cells.

Competent *E.coli* DH5α or INVαF' cells were thawed in dry ice and to each 20μl aliquot, 100ng of appropriate plasmid DNA were added. Following a 20 minute incubation on ice, the cells were heat shocked for 2 minutes at 42°C, placed on ice for 2 minutes and finally incubated in SOC medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) for 1 hour at 37°C with constant shaking at 225rpm allowing expression of antibiotic resistance markers. Transformed cells were briefly pelleted by spinning for 5 minutes in a microfuge and resuspended in 100μl of L-broth. 1:10,1:100 dilutions and the remaining mixture of resuspended cells were spread onto separate agar plates (1-2% agar in L-broth) containing 100μg/ml of ampicillin. Incubation was carried out at 37°C overnight. The following day representative colonies were picked using a heat sterilised bacterial loop and bacteria inoculated into 12ml of L-broth containing 100μg/ml ampicillin and incubated with constant shaking (225rpm) overnight at 37°C. Subsequent DNA extraction was carried out according to procedures described in section 2.2.3.b-1 to identify colonies harbouring the required expression vectors. These were expanded into 500ml cultures for the preparation of large scale plasmid preparations (as described in section 2.2.3.b-2).

Storage of transformed bacteria was achieved by mixing 500µl of overnight cultures with 500µl of glycerol in 1ml Nunc cryotubes which were frozen and maintained at -70°C.

Upon transformation with recombinant plasmids containing exogenous DNA interrupting the *lacZ* gene, INVαF' cells were cultured on agarose plates overlaid with 25µl of fresh X-Gal (40mg/ml stock solution) 1 hour prior to use. IPTG was not required since the INVαF' cells did not express the *lacI* repressor. Following overnight incubation bacteria containing introduced successfully ligated plasmids were distinguished by their inability to break down the chromogenic X-Gal compound, therefore giving rise to white colonies. INVαF' cells harbouring re-ligated, non-disrupted plasmids formed distinct blue colonies under the same conditions.

2.2.3.b Preparation of nucleic acids.

2.2.3.b-1 Small scale preparations of plasmid DNA.

Modifications of the alkali lysis methods proposed by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981) were used to extract plasmid DNA from small bacterial cultures. Single bacterial colonies expanded overnight in 12ml of L-broth containing 100µg/ml ampicillin were separated into 1.5ml aliquots and spun in Eppendorf tubes at maximum speed for 5 minutes. The pellet was resuspended in 200µl of solution I (50mM glucose, 25mM TrisHCl pH8.0, 10mM EDTA pH8.0, 2mg/ml of lysozyme in water) and kept at room temperature for 5 minutes. Cell lysis was continued by the addition of 400µl of solution II (0.2M NaOH, 1%SDS) and the Eppendorf tubes stored on ice for 10 minutes. Lysis was terminated by the addition of 300µl of ice cold solution III (3M CH_3COONa pH4.8), and the tubes stored on ice for 3-5 minutes

subsequent to which they were centrifuged at maximum speed for 10-15 minutes. To induce DNA precipitation 800µl of supernatant were mixed with 480µl of ice cold isopropanol and the samples frozen at -20°C for a minimum of 30 minutes. The precipitate was pelleted by centrifugation at top speed in a bench top microfuge at 4°C for 20 minutes. Following supernatant aspiration, the pellet was washed in ice cold 70% ethanol and respun at the same conditions for 15 minutes. The DNA was dried under vacuum and subsequently resuspended in 50µl of MilliQ water at 37°C for 30 minutes. In some cases RNase treatment was employed and involved incubation with 50µg/ml RNase for 30 minutes at 37°C. 1µl aliquots were subjected to appropriate restriction enzyme digestion (see section 2.2.3.c-1) and electrophoretic analysis (see section 2.2.3.d-1), while the nucleic acid concentration was determined by spectrophotometric analysis of 1:100 dilutions (see section 2.2.3.e). For short term storage the DNA was kept at 4°C.

2.2.3.b-2 Large scale plasmid preparations.

The alkali lysis method proposed by Sambrook *et al* 1989 was used to obtain plasmid DNA from large bacterial cultures. Overnight 500-1000ml cultures were collected by centrifugation (6000rpm) in polypropylene bottles for 5 minutes at 4°C using a Beckman J2-21 centrifuge. The pellets were washed in 100ml of bugwash solution (150mM NaCl, 10mM TrisHCl and 1mM EDTA pH8.0) and respun at the same conditions. Following supernatant removal, the pellets were vigorously resuspended in 20ml of solution I (50mM glucose, 25mM TrisHCl pH8.0 and 10mM EDTA pH8.0) and kept on ice for 5 minutes subsequent to which 80ml of solution II (0.2M NaOH and 1% SDS) were added. The samples were mixed by gentle inversion at room temperature for 5-

10 minutes. Lysis was terminated by the addition of 30ml of solution III (5M CH_3COOK - 60ml, glacial acetic acid - 11.5ml and water - 28.5ml), the samples kept on ice for 10 minutes followed by centrifugation at 4000rpm, 4°C for 15 minutes. A 0.6 volume of ice cold isopropanol was mixed with the supernatant which had been filtered through gauze and the DNA precipitated at room temperature for 10-20 minutes. The DNA was pelleted by centrifugation at 5000rpm, room temperature for 15 minutes in a Beckman J2-21 centrifuge and subsequently washed in 70% ice cold ethanol. The vacuum dried pellet was resuspended in 12ml of TE buffer (1mM EDTA, 10mM TrisHCl pH8.0) and subjected to $CsCl_2$ gradient nucleic acid separation as follows:

(i) 12ml of resuspended DNA were mixed with 12.9g $CsCl_2$ and 0.8ml of ethidium bromide (stock solution 10mg/ml).

(ii) The refractive index was adjusted to 1.39 with $CsCl_2$ or TE buffer and the solutions transferred to appropriate centrifuge tubes.

(iii) Centrifugation was carried out at 40,000rpm for 48 hours at 20°C in a Beckman L-60 ultracentrifuge (T-50 rotor).

(iv) Removal of closed, circular plasmid DNA was carried out with the use of an ultraviolet lamp and blunt ended needles.

(v) The ethidium bromide was discarded by vigorously mixing equal volumes of sample and $CsCl_2$ saturated isopropanol, the top red layer removed and the process repeated 3-4 times.

(vi) Plasmid DNA was precipitated in glass Corex tubes by adding 3M CH_3COONa - 1/10th volume, 100% ethanol - 2.5 volumes and placing overnight at -20°C.

(v) Precipitates were pelleted by centrifigation at 8000rpm for 30 minutes in a Beckman J2-21 centrifuge, washed in 70% ethanol, vacuum dried and

resuspended in 0.5ml TE buffer (pH8.0) or MilliQ water. They were stored at 4°C.

2.2.3.b-3 Genomic DNA extraction from mammalian cell lines

Preparation of genomic DNA was carried out following the procedure suggested by Laird *et al* 1991. Cells grown to confluence on 90mm dishes were submerged in 3ml of lysis buffer (100mM TrisHCl pH8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl and 100µg proteinase K/ml), removed with the use of disposable cell scrapers and the samples placed into 5ml plastic sterilin tubes where lysis continued at room temperature for up to 2 hours. Upon the addition of 3ml cold isopropanol the DNA rapidly precipitated and was removed from the solution with the use of a 200µl Gilson pipette. The DNA was further resuspended in 0.5ml-3ml of TE buffer (10mM TrisHCl, 0.1mM EDTA pH7.5) and the concentration determined by spectrophotometry of 1:100 dilutes (see section 2.2.3.e). The genomic DNA was stored at 4°C.

2.2.3.b-4 Total RNA extraction from mammalian cell lines.

Extraction of total RNA from mammalian cell lines was carried out following the manufacturer's protocol for RNasol B (Biogenesis Ltd.) which was based on the guanidium thiocyanate-phenol-chlorophorm method established by Chomczynski and Sacchi 1987. Sub-confluent cell cultures growing on 90mm dishes were washed in ice cold PBS which was completely removed by aspiration and subsequently lysed in 1ml of RNasol B. A disposable cell scraper was implemented to homogenise the cells and the sample added to Eppendorf tubes containing 80µl of chloroform. Following a 5 minute incubation on ice the phases were separated by top speed centrifugation in a microfuge

for 15 minutes at 4°C. The upper colourless layer was removed and the RNA precipitated by mixing with 0.4ml of ice cold isopropanol that had previously been purified by filtration. The lower blue phase was discarded. RNA precipitation was carried out overnight at -20°C and pelleted by centrifugation in a microfuge for 20 minutes at 4°C. The supernatant was carefully removed by aspiration and the pellet washed once in ice cold 70% ethanol. A subsequent 15 minute spin at 4°C reduced the chances of pellet loss upon ethanol removal. The RNA was dried under vacuum and resuspended in 40µl of DEPC treated, autoclaved MilliQ water. Spectrophotometric analysis of 1:250 dilutes determined the sample concentrations (see section 2.2.3.e). They were stored at -20°C.

2.2.3.b-5 Oligonucleotide preparation.

Designed oligonucleotides were synthesised on an Applied Biosystems mode 381A DNA synthesiser. The 5' methoxy-trytil group remained in place after incorporation of the final nucleotide, in order to insure efficient purification of the oligonucleotides from any aborted synthetic products. Elution from the synthesis column was achieved by overnight incubation of the resins in approximately 2ml of concentrated ammonia solution (29%) at 55°C in airtight glass tubes, at the same time removing the side chain protective groups. Purification of trytil protected oligonucleotides was carried out the following day with the use of trytil affinity columns (Cruachem). The columns were precleaned with 2ml of concentrated acetonitrile gently drawn through under vacuum, followed by 2ml of 2M triethylamine acetate pH7.0. Prepared columns were exposed to the oligonucleotide samples which were slowly drawn through twice to insure maximum entrapment. Unbound oligonucleotides were removed

by flushing the columns with 3ml of dilute ammonium hydroxide in water (1:10), 2ml of MilliQ water, 2ml of 2% trifluoroacetic acid and 2ml of MilliQ water. Purified oligonucleotides were eluted with 1ml of 20% acetonitrile. The DNA was collected by lyophilisation in a centrifugal evaporator system (Hectovac) and resuspended in 50µl of MilliQ water. Oligonucleotide concentrations were determined by spectrophotometric analysis (see section 2.2.3.e), and the samples stored in aliquots at -20°C.

2.2.3.c Enzymatic manipulations of nucleic acids.

2.2.3.c-1 Restriction endonuclease digestion of DNA.

Plasmid DNA (usually 1-5µg) was digested using 1-10 units of appropriate restriction endonucleases in the presence of buffers supplied by individual manufacturers, the total volume of the reaction not exceeding 10µl. Due to the inhibitory effects of high glycerol concentrations, the volume of enzyme used never exceeded 1/10th of the total reaction. Incubation was carried out for a minimum duration of 30 minutes at 37°C and the reactions terminated by the addition of 3µl of DNA loading buffer (0.25% bromophenol blue, 30% glycerol in water).

Genomic DNA (25-50µg) was digested following the same principle and the volumes were increased in proportion. Following overnight incubation the DNA was analysed by gel electrophoresis procedures (see section 2.2.3.d) to determine the extent of digestion, where necessary a further aliquot of appropriate restriction enzyme was added and the incubation continued overnight. The volume of enzyme used never exceeded 1/10th of the total volume.

2.2.3.c-2 PCR amplification.

Two types of polymerase chain reaction (PCR) amplifications were carried out and differed in the starting material which was either genomic DNA or total RNA extracted from the appropriate mammalian cell lines. In both cases the conditions in which the reactions took place were determined following manufacturer's suggestions (Perkin Elmer Cetus) (Saiki *et al* 1988; White *et al* 1989).

Genomic DNA isolated from SCC12, P-1 and transfected HEK cells was subjected to PCR amplification with the use of 24bp oligonucleotides (b2 and y4) that spanned *CCND1* exon boundaries 2-3 and 4-5, respectively and were therefore specific for exogenously introduced cyclin D1 cDNA (Table 2). A master mix containing 200mM concentrations of each nucleotide (dATP, dCTP, dTTP and dGTP) in 1x reaction buffer (10x stock contains 100mM TrisHCl pH8.3, 500mM KCl, 15mM $MgCl_2$ and 0.01% gelatine) was aliquoted into separate capped 0.5ml polypropylene microcentrifuge tubes containing correct amounts of double distilled water to make the final reaction volume a total of 100µl. Into each reaction mixture 0.5µg of appropriate genomic DNA was added and 100pg of oligonucleotides b2 and y4, with the exception of control samples. The samples were heated to 95°C for 6 minutes to inactivate any proteases that may be present in the crude DNA extracts, subsequent to which 0.5µl of AmpliTaq DNA polymerase were added and the mixtures overlayed with 100µl of mineral oil to prevent evaporation. Reactions were carried out at high stringency for 40 cycles in a Perkin Elmer Cetus DNA Thermal Cycler as follows :

1minute melt at 95°C

1 minute anneal at 60°C

Table 2. Oligonucleotides used in PCR amplification reactions.

Oligonucleotides 3 and 4 were homologous to a *CCND1* transcript 3' untranslated region, while oligonucleotides b2 and y4 were specific for *CCND1* cDNA and were used to distinguish exogenous from endogenous *CCND1* DNA in transfected cell cultures.

Table 2.

5' 1730 -ATT CCG TAG GTA GAT GTG TAA CC- 1753 3' (3)

5' 1959 -AGG GCC GTT GGG TAG AAA ACC A- 1939 3' (4)

5' 547 -GAG GAG CTG CTG CAA ATG GAG CTG- 573 3' (b2)

5' 880 -TTC ACA CTG GGC CTG ACG GAG GCC- 856 3' (y4)

1 minute extend at 72°C

The resulting samples were examined by gel electrophoresis (as described in section 2.2.3.d-1) and Southern blot analysis (as described in section 2.2.3.g). Remaining mixtures were stored at -20°C.

Total RNA isolated from A431 cells was subjected to reverse transcription and subsequently PCR amplification following the methods suggested by the

RNA PCR kit (Perkin Elmer Cetus). Reverse transcription samples containing 5mM $MgCl_2$, 1x PCR buffer II, 1mM dGTP, 1mM dCTP, 1mM dATP, 1mM dTTP, 1mM RNase inhibitor, 1units/µl of reverse transcriptase, 2.5µM oligo $d(T)_{16}$,1µg of total RNA and sterile distilled water to a final volume of 20µl, were placed into capped 0.5ml polypropylene microcentrifuge tubes and overlayed with 100µl of mineral oil to prevent sample evaporation or refluxing. The tubes were exposed to 42°C for 15 minutes, 99°C for 5 minutes and 5°C for 5 minutes for one cycle, to achieve reverse transcription from the poly-A RNA species. To each reverse transcription reaction tube 78µl of a prepared PCR master mix (2mM $MgCl_2$, 1x PCR buffer II, 2.5 units/100µl of AmpliTaq DNA polymerase and water to a final volume of 78µl) were added followed by appropriate "upstream" and "downstream" primers (Table 2) each at a final concentration of 0.15mM making the total reaction volume 100µl by the addition of sterile water where necessary. The tubes were briefly spun in a microcentrifuge and subjected to PCR amplification in a Perkin Elmer Cetus DNA Thermal Cycler as follows:

2 minutes at 95°C for 1 cycle

1 minute at 95°C and 1 minute at 60°C for 35 cycles

7 minutes at 60°C for 1 cycle

storage at 4°C until sample retrieval

The samples were subsequently examined by gel electrophoresis (see section 2.2.3.d-1), purified (see section 2.2.3.d-2) and cloned (see section 2.2.3.c-3).

2.2.3.c-3 Ligation of DNA fragments.

PCR products obtained by initial reverse transcription from total RNA extracts were cloned taking advantage of the non-template dependent activity of AmpliTaq DNA polymerase adding single deoxyadenosines to the 3'-end of all duplex molecules provided by PCR. The A-overhangs were used to achieve direct ligation of PCR products into the specifically designed linearised vector pCR™ II (Invitrogen), that contains single 3' T-overhangs in the central part of a multiple cloning site. Ligation reactions contained 1µl 10x ligation buffer, 50ng of linear vector pCR™ II, Xµl of diluted PCR product, 1µl of T4 DNA ligase and water to make the final volume 11µl. For maximum efficiency, the molar ratio of vector : PCR insert was kept from 1:1 to 1:3. Reactions were incubated overnight at 4°C 1ml of which were used immediately to transform bacteria (as described in section 2.2.3.a). The unused ligation mix was stored at -20°C.

2.2.3.d Agarose gel electrophoresis and DNA purification.

2.2.3.d-1 Agarose gel electrophoresis.

Non-denaturing 0.8-1.5% agarose gels were used for the visualisation of DNA fragments of >200bp. Agarose was dissolved by heating in 50ml (mini-gel) or 200ml (maxi-gel) of 1xTAE buffer (10x stock contains 48.4g of Tris, 11.42ml of glacial acetic acid and 40ml of 0.25M EDTA pH8.0 in 1l of water) for

further DNA purification or 1xTBE buffer (10x stock contains 108g of Tris, 55g of boric acid and 9.5g of EDTA in 1l of water) for fragment resolution. The hot gel mix was cooled to approximately 50°C upon which ethidium bromide was added to a final concentration of 0.4µg/ml. TAE gels were cast at 4°C while TBE gels at room temperature. Electrophoresis was carried out in the same buffer used for the gel formation, therefore either 1xTAE or 1xTBE. Prior to loading into the wells, the DNA samples were mixed with 3µl of DNA loading buffer (0.25% bromophenol blue and 30% glycerol in water) and subsequently electrophoresed in the presence of appropriate size markers at 1-5V/cm. A 312nm transilluminator was used for the resolution of the fragments and records were taken by Polaroid camera photography on Polaroid Type 57 high speed film. Further purification of DNA from TAE gels is described in the following section.

2.2.3.d-2 Recovery of DNA fragments from agarose gels.

Two distinct methods were employed for the purification of DNA fragments >200bp from low melting point TAE agarose gels : "Genecleaning" (Bio 101 Inc. Statech) which was found to result in smaller yields and "Magic Minipreps DNA purification system" or "Magic PCR* Preps DNA purification system" (Promega), both equally applicable and effective in DNA purification.

"Genecleaning" involved weighing the gel piece containing the DNA fragment of interest and addition of $2^1/_2$ volumes of NaI. The mixture was incubated at 60°C up to 10 minutes to induce gel melting, following which 5µl of manufacturer's GLASSMILK solution were added and incubation continued at 4°C for 5 minutes. The GLASSMILK bound DNA was pelleted by centrifugation in a microfuge and washed in 450µl of ice cold NEW wash, 4

times. Subsequent to the final spin and supernatant removal, the DNA was eluted from the resin by resuspending the pellet in 20µl of MilliQ water, the sample was heated to 55ºC for 5 minutes and centrifuged.

DNA purification using the "Magic systems" involved the addition of 1ml of DNA purification resin to molten agarose and vortexing the mixture for 1 minute. The resin containing bound DNA was slowly drawn through a mini-column by applying vacuum and the column subsequently washed with 2ml of 70% isopropanol. Residual wash solution was removed by centrifugation in a microfuge, followed by placing at room temperature for 5-15 minutes allowing isopropanol evaporation. Bound DNA was eluted with 50µl of MilliQ water at room temperature for 1 minute and the elute collected by centrifugation at top speed for 20 seconds. The purified DNA fragments were analysed by gel electrophoresis (see section 2.2.3.d-1) and stored at 4ºC.

2.2.3.e Nucleic acid quantitation.

A Beckman DU-64 spectrophotometer was used to determine the concentrations of individual nucleic acid samples. Typically 1:100 dilutions of DNA extracts or PCR products and 1:250 dilutions of RNA extracts were subjected to analysis. Each reading was initially calibrated with a blank containing the solution the nucleic acids were dissolved in, commonly MilliQ water or TE buffer. Optical density readings at A_{260} of 1 were taken to correspond to 50µg/ml for plasmid and genomic DNA, 40µg/ml for RNA and 30µg/ml for oligonucleotides. The A_{260}/A_{280} ratio was calculated to determine the degree of protein contamination of the samples and values of 1.8-2.0 were taken to indicate a sufficiently pure extract.

2.2.3.f The construction and purification of radioactive DNA probes.

$\alpha[^{32}P]$dCTP labelled DNA fragments applied in Southern and northern blot analyses were derived by the technique of random priming. In most cases a commercially available kit (Pharmacia) was used. The principle of the method was based on the synthesis of new DNA strands in the presence of radioactively labelled bases (in all cases $\alpha[^{32}P]$dCTP was used) following denaturation of double stranded DNA fragments by boiling. For the construction of one probe 50-100ng of appropriate DNA were linearised in a volume of 34µl and briefly cooled on ice prior to the addition of 10µl of reaction buffer (containing required salt concentrations, random primer sequences, dATP, dGTP and dTTP), 5µl od $\alpha[^{32}P]$dCTP (3000Ci/mmol-Amersham) and 1µl of Klenow fragment. The reaction mix was incubated at 37°C for 30 minutes - 1 hour.

Alternatively, in one case where the use of specific primers was required the same reactions were carried out with separately made solutions following the protocol suggested by Sambrook et al 1989. The reaction buffer was made by combining 100µl of solution A (composed from 1 ml of a 1.25M TrisHCl pH8.0 and 0.125M $MgCl_2$ solution, 18µl of neat β-mercaptoethanol, 5µl of 100mM dATP pH8.0, 5µl of 100mM dTTP pH8.0 and 5µl of 100mM dGTP pH8.0), 250µl of solution B (2M HEPES buffer titrated to pH6.6 with 4M NaOH) and 150µl of solution C (0.6µg of primer 3 and 0.6µg of primer 4 diluted in TE buffer pH8.0) and used in the same way as described above.

Removal of unincorporated nucleotides was carried out by purification in Sephadex-G50 columns obtained from Pharmacia (Nick columns) or made with the use of Eppendorf tubes. The Nick columns were allowed to drip under the force of gravity, they were precleared with approximately 5ml of TE buffer

(pH8.0) following which the samples were eluted with 400µl aliquots of TE buffer. The first radioactive aliquot obtained from each column contained the purified probe. Alternatively, Eppendorf tubes pierced at the bottom were packed with Sephadex-G50 equilibrated in TE buffer (pH8.0), excess liquid was removed by centrifugation at 2500rpm for 2 minutes. Samples containing non-purified probes were subsequently spun at 2500rpm for 5 minutes, and the unincorporated nucleotides retained in the beads. The degree of $\alpha[^{32}P]$dCTP incorporation was determined by the analysis of 1µl aliquots in a Beckman LS5000CE counter.

2.2.3.g Southern blot analysis.

20µg of restriction endonuclease digested genomic DNA (see section 2.2.3.c-1) were electrophoresed overnight through a 0.8% agarose TBE gel at a constant 30V (see section 2.2.3.d-1). The gel was subsequently visualised under ultraviolet light and a photographic record taken using Polaroid Type 57 high speed film. Transfer of the DNA onto Hybond N+ membrane was achieved under vacuum (50-60mbar). A piece of membrane the size of the gel was immersed in MilliRO water, placed onto a vacuum blotter (Pharmacia) and overlayed with the agarose gel. The area not occupied with the gel had been covered with a plastic sheet which contained an opening the size of the gel, 1% molten agarose was used to seal the gel to the sheet. Depurination of the DNA was achieved by the suction of 0.2M HCl through the gel until the visible bromophenol blue representing the dye front changed to a yellow colour, this typically took 30-45 minutes. Subsequent DNA denaturation was carried out by the suction of a 0.5M NaOH, 1.5M NaCl solution (35 minutes) followed by neutralisation with 1M TrisHCl pH7.5, 1.5M NaCl (35 minutes). After

neutralisation the DNA was transferred to the Hybond N+ membrane with 20x SSC blotting buffer (3M NaCl, 0.3M CH_3COONa, pH 7.0) for $1^1/_2$ hours under constant vacuum. The membrane was briefly dried at room temperature and the DNA UV-crosslinked using a UV Stratalinker 1800 (Stratagene). Hybridisation to specific probes was carried out as described in section 2.2.3.i.

2.2.3.h Northern blot analysis.

Prior to electrophoresis all RNA samples were reconcentrated to 4µg/µl by lyophilisation in a centrifugal evaporator system (Hectovac) and resuspended in appropriate amounts of DEPC treated MilliQ water. 5µl of each sample (20µg) were mixed with 3 volumes (15µl) of RNA loading buffer (350µl formaldehyde, 1µl formamide, 150µl DNA loading buffer, 10µl ethidium bromide - stock solution 10µg/µl and 200µl 5x MOPS buffer containing 0.1M MOPS, 40mM CH_3COONa and 5mM EDTA, pH7.0) and heated at 65°C for 15 minutes. The prepared samples were subsequently loaded onto a 150ml 1% agarose gel containing 30ml of 5x MOPS buffer and 26.3ml of formaldehyde. Electrophoresis took place overnight at 30V in 1x MOPS buffer which was constantly recirculated. Gel resolution was enabled with the use of a 312nm transilluminator and a photographic record taken with a Polaroid camera on Polaroid Type 57 high speed film. Transfer of the RNA to a Hybond N+ membrane was achieved under vacuum in 20x SCC blotting buffer (3M NaCl, 0.3M CH_3COONa, pH 7.0) for $1^1/_2$ hours under constant vacuum. The membrane was briefly dried at room temperature, the DNA UV-crosslinked using a UV Stratalinker 1800 (Stratagene) and the positions of 18 and 28S ribosomal RNA marked with a permanent felt tip marker. Hybridisation to specific probes was carried out as described in section 2.2.3.i.

2.2.3.i Hybridisation of labelled DNA probes to membrane bound nucleic acids.

The detection of specific DNA or RNA species that had been transferred onto Hybond N+ membranes was achieved by hybridisation with appropriate radiolabelled probes. The membranes were prehybridised at 42°C for 2 hours in sealed plastic bags containing approximately 0.1ml/cm3 of SCC hybridisation buffer (45% deionised formamide, 5% dextran sulphate, 5x SCC, 0.3% SDS, 0.1mg/ml boiled salmon sperm DNA, 5x Denhardt's solution {50x stock contains 5g ficoll, 5g polyvinyl pyrolidone, 5g bovine serum albumin in 500ml} and 50mM Na-phosphate buffer pH6.6 {1M stock contains 35.2ml of 1M Na_2HPO_4 and 64.8ml of 1M NaH_2PO_4}), upon which probes denatured by boiling for 10 minutes were added to the mixture. Hybridisation was carried out overnight at 42°C in a shaking water bath. Following the disposal of the radioactive solution, the membranes were washed at high stringency (two 10 minute washes at room temperature in copious amounts of 2x SCC, 0.1% SDS followed by two 30 minute washes at 65°C in copious amounts of 0.2x SCC, 0.1% SDS) or low stringency (two 10 minute washes at room temperature in copious amounts of 2x SCC, 0.1% SDS followed by one 35 minute wash at room temperature in copious amounts of 0.2x SCC, 0.1% SDS). The membranes were then dried on 3mm Whatman paper to remove excess fluid, wrapped in Saran wrap, placed in a cassette with a suitable X-ray film and subjected to autoradiography at -70°C.

2.2.3.j Stripping membranes of radioactive label.

In cases when rehybridisation of a Southern or northern blot with a different probe was required, the previously bound probes were removed as listed below:

(i) Southern blots were stripped by shaking in 0.1% SDS that had been heated to 100°C when applied to the membranes and subsequently cooled during a period of 30 minutes to 1 hour. The process was repeated twice where required.

(ii) Northern blots were stripped at 65°C for 1 hour while shaking in a 5mM TrisHCl pH 8.0, 2mM EDTA and 0.1x Denhardt's solution. The process was repeated once where required.

2.2.3.k Sequencing of double stranded DNA.

Sequencing of DNA was carried out to determine the authenticity of PCR amplification products according to the chain termination reaction of Sanger *et al* 1977 adapted into a marketed kit (Sequenase version 2.0 - U.S.B.). Plasmid DNA (4µg) purified from contaminating RNA by RNase treatment (see section 2.2.3.b-1) was denatured in 0.2mM NaOH, 2mM EDTA at 37°C for 30 minutes. The mixture was neutralised by the addition of 0.1 volume of 3M CH_3COONa pH4.5-5.5 and the DNA precipitated using 2-4 volumes of 100% ethanol at -70°C for 15 minutes. The precipitated DNA was pelleted in a microfuge at 4°C for 15 minutes, washed in 70% ethanol, respun, briefly dried and resuspended in 7µl of MilliQ water. To this solution 1µl of M13 (-40) oligonucleotide sequencing primers and 2µl of 5x sequenase buffer were added, incubation was initially carried out at 65°C (2 minutes), followed by a slow cool to <35°C for approximately 30 minutes allowing annealing to take

place. The tubes were stored on ice. Sequencing was carried out following the manufacturer's instructions and with the use of provided reagents. The labelling reaction consisted of 10μl of ice cold annealed DNA mixture, 1μl of 0.1M DTT, 2μl of diluted labelling mix (4μl of dGTP, 16μl of double distilled water), 0.5μl [^{35}S] dATP (600Ci/mmol), 2μl of sequenase (1:8 dilution in TE buffer). Incubation took place for 2-5 minutes at room temperature. Subsequently 3.5μl of the labelling reaction were transferred into 4 termination tubes containing ddA, ddT, ddC or ddG mixes and incubated at 37°C for 5 minutes. The addition of 4μl of Stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF) and a 2 minute boil, prepared the samples for electrophoresis. Denaturing 6% polyacrylamide gels (containing 42% urea) were used to separate the labelled, terminated fragments and electrophoresis was carried out at 1500V/30-40mA for 2-4 hours. The gels were fixed in a solution of 12% methanol and 10% acetic acid for 1 hour at room temperature and dried under vacuum at 80°C for approximately 2 hours. Autoradiography was carried out as described in section 2.2.3.i.

2.2.4 Protein procedures.

2.2.4.a Protein extraction from mammalian cell lines.

Sub-confluent cell cultures growing on 90mm plastic dishes were washed in ice cold PBS and lysed in appropriate solutions, depending on the identity of analysed proteins. For the future detection of cyclin D1, lysis was carried out with 1ml of a) 100mM NaCl, 50mM TrisHCl pH7.4, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 5mM $CaCl_2$, 25μg/ml aprotinin, 10pg/ml pepstatin, 0.5mM PMSF and 10μg/ml leupeptin or b) 50mM TrisHCl

pH7.4, 150mM NaCl, 20mM EDTA, 0.5% NP-40, 1mM PMSF, 25μg/ml leupeptin, 25μg/ml aprotinin, 10pg/ml pepstatin, 1mM benzamidine, 10μg/ml trypsin inhibitor and 1mM sodium ortho-vanadate. For the detection of cdk4, PCNA and *HPV-16* E7, cells were lysed in 1ml of 50mM TrisHCl pH7.4, 150mM NaCl, 20mM EDTA, 0.5% nonidet P-40 (NP-40), 1mM PMSF, 25μg/ml leupeptin, 25μg/ml aprotinin, 10pg/ml pepstatin, 1mM benzamidine, 10μg/ml trypsin inhibitor and 1mM sodium ortho-vanadate, while for the detection of pRb cells were lysed in 1ml of 50mM HEPES pH7.0, 250mM NaCl, 0.1% NP-40, 1mM PMSF, 25μg/ml aprotinin, 25μg/ml leupeptin, 5mM NaF and 1mM sodium ortho-vanadate. Lysis was carried out ice for 20-30 minutes, the extracts transferred to Eppendorf tubes with the use of disposable cell scrapers and the supernatants collected by centrifugation in a microphuge at 4°C for 5 minutes. The protein extracts were stored in aliquots at -20°C.

Protein concentration was determined by analysis of original sample and 1:10 dilution aliquots (10μl) with the use of a Dynatech MR7000 spectrophotometer. Calibration was carried out with a range of BSA standards (0ng/μl, 80ng/μl, 100ng/μl, 200ng/μl, 400ng/μl, 1μg/μl, 2μg/μl). 10μl of each standard were aliquoted into specific position on a 96 well plate, 200μl of reaction mix (1 volume of $CuSO_4$ and 50 volumes of bicinchroninic acid) were added to every well and the colour allowed to develop at 37°C for 30 minutes-1 hour. Spectrophotometric analysis was carried out at A590nm.

2.2.4.b Acrylamide gel electrophoresis.

The acrylamide concentration in the gels was varied depending on the identity of the analysed proteins, therefore 10% gels were used for the resolution of cdk4, cyclin D1, PCNA and *HPV-16* E7, while 7.5% gels were

used for the resolution of pRb. The components listed bellow apply for the formation of one 10% gel, the same ingredients were used for 7.5% gels that differed only in the amount of acrylamide and water used:

3M TrisHCl pH8.8	6.25ml
30% acrylamide/*bis*-acrylamide	16.75ml
MilliQ water	26.63ml
20% SDS	0.25ml
10% ammonium persulphate	0.25ml
Temed	0.1ml

The mixture was poured inbetween two glass plates, overlayed with water saturated butanol and allowed to polymerase at room temperature for approximately 30 minutes. Following the removal of butanol traces by washing with MilliRO water, the gel was overlayed with the stacking gel (4% acrylamide/bis-acrylamide mix in 50mM TrisHCl pH6.8) and appropriate combs inserted. The polymerised gel was placed into an electrophoresis tank and covered with 1x Tris-glycine electrophoresis buffer (5mM Tris, 50mM glycine pH8.3, 0.02% SDS in MilliRO water) taking care to remove air bubbles caught in the wells.

Aliquots containing 20-50μg of extracted protein were mixed with an equal volume of 2x SDS protein loading buffer (100mM TrisHCl pH6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 2% β-mercaptoethanol in MilliRO water) and the samples boiled for 5 minutes prior to loading onto the denaturing gel. B.R.L. high molecular weight markers were included on all gels. Electrophoresis was carried out for 6 hours at 30mA/gel or 24 hours at 10-30mA/gel. Overnignt protein transfer onto Immobilon P membranes was carried out in Towbin buffer (24mM Tris, 192mM glycine 20% methanol, 1.2ml

conc. HCl, pH8.3) using a Bio Rad Trans-Blot Cell according to the manufacturer's instructions. Western blot was carried out as described in the following section.

2.2.4.c Western blot analysis.

Following protein transfer, the Immobilon P membrane was pre-blocked in PBS containing 5% marvel, 0.1% Tween-20 at room temperature for 30 minutes. The same solution was used to dilute the appropriate primary antibody (see section 2.1.6) small volumes of which (typically 2.5-5ml) were sealed with the membrane into a plastic bag and incubated at 4°C for a minimum of 3 hours with constant vigorous shaking. The membrane was washed in PBS containing 5% marvel, 0.1% Tween-20 1x 10 minutes and 3x 5 minutes at room temperature with constant shaking, followed by incubation in the presence of the appropriate horseradish peroxidase labelled secondary antibody (see section 2.1.6) at room temperature for 45-60 minutes. The final wash was carried out in PBS containing 0.1% Tween-20 1x10 minutes and 3x 5 minutes following which the membrane was incubated with an equal volume of ECL detection reagents 1 and 2 for 1 minute at room temperature. Upon removal of excess fluid, the membrane was covered in Saran wrap and placed in a cassette including an X-ray film for 1 second-15 minutes.

2.2.4.d Immunological staining procedures.

2.2.4.d-1 Bromodeoxyuridine incorporation and immunocytochemical detection.

Growing cells were cultured on Nunc glass chamber slides in the presence of 10μM bromodeoxyuridine (BrdU) for a minimum of 1 hour. Prior to

fixation, cells were washed two times in ice cold PBS and once in a 1:1 mix of methanol : acetone. Fixation in a 1:1 mix of methanol : acetone was carried out for 10 minutes at room temperature upon which the slides were air dried for 10-15 minutes. The slides were either used immediately or stored at -20°C in airtight conditions.

Prior to the detection of BrdU, the cells were permeabilised in PBS containing 0.25% Tween-20 for 5 minutes at room temperature. The slides were washed three times in PBS for a total of 10 minutes and incubated at 60°C in 1M HCl for 5-10 minutes to denature the DNA and therefore allow antibody binding. PBS rinsed slides (3 washes lasting 10 minutes in total) were rapidly exposed to diluted anti-BrdU antibody (1:2000 dilution in PBS containing 0.5% BSA) and incubated at room temperature in humidifying conditions for a minimum of 3 hours. Unbound antibody was washed off by shaking the slides 3x 10 minutes in PBS containing 0.1% Tween. They were subsequently exposed to 1:100 diluted biotin bound anti-mouse antibody in PBS containing 0.5% BSA with constant gentle shaking at room temperature for 45 minutes. Washed slides were exposed to a avidin, biotinylated horseradish peroxidase amplification mix (solutions A and B from the Vectastain ABC kit) for 45 minutes, washed as previously described and the colour developed using a Ni-DAB solution (4ml of PBS and 1.67µl of 30% hydrogen peroxide were added to 1ml of a 0.07% $NiCl_2$, 0.025% diaminobenzidine-tetrahydrochloride solution) for 10 minutes. The cells were dehydrated by sequential immersion into 70% ethanol, 90% ethanol, 2x 100% ethanol and 100% xylene, overlayed with DPX and a glass coverslip.

2.2.4.d-2 Cyclin D1 immunocytochemical and immunohistochemical detection.

Expression of the cyclin D1 protein in cell lines and tissue biopsies was examined with the use of the rabbit Vectastain ABC kit (Vector Laboratories) following the manufacturer's methods. Cells grown on Nunc glass chamber slides or tissue sections were washed two times in ice cold PBS and subsequently fixed following the same procedures described in section 2.2.4.d-1. 200µl of blocking serum (1 drop of goat serum in 1ml of PBS containing 0.5% BSA) were added to the cells and incubation took place at room temperature in humidifying conditions for 30 minutes. The serum was drained off and the excess fluid gently removed using a tissue. The primary antibody was added at a 1:3000 dilution to half of the wells while the same dilution of pre-immune rabbit serum was added to the other half (both were made up in PBS containing 0.5% BSA) and the cells incubated at room temperature for a minimum of 3 hours. The slides were then washed 3x10 minutes in PBS containing 0.1% Tween-20 and 0.15-0.2M NaCl to eliminate undesirable ionic interactions. A biotinylated anti-rabbit secondary antibody provided by the kit was diluted (1 drop in 10ml of PBS containing 0.5% BSA) and added to the cells. Subsequent manipulations were the same as previously described in section 2.2.4.d-1 with the exception of the wash solution which was kept at a higher stringency.

RESULTS

CHAPTER 3 : The establishment of new cell lines derived from human carcinomas.

3.1 Introduction.

The incidence of *CCND1* gene amplification in human cancer, reviewed in sections 1.2.1.c and 1.2.2, has been most extensively analysed in breast carcinomas and in fewer cases of SCCs, oesophageal and colorectal carcinomas (Lammie *et al* 1991, Jiang *et al* 1992, Schuuring *et al* 1992a, Faust and Meeker 1992, Buckley *et al* 1993, Leach *et al* 1993). Currently, only two separate studies have concentrated in greater detail on the analysis of human HNSCC, revealing *CCND1* gene dosage and/or transcript levels determined by Southern and northern blot analysis, respectively (Table 3) (Lammie et al 1991, Schuuring *et al* 1992a). Unfortunately, seven out of the eight SCC cell lines examined overlapped in the two reports. In addition, little was known of the culture conditions used for their establishment and therefore if they were good representatives of the original tumours. Due to the above mentioned reasons and the lack of published *CCND1* data on cervical or vulvar tumours (only HeLa and A431 cell lines have been examined), an attempt was made in this study to derive and analyse cell lines from human cervical carcinomas using methods established for the growth of primary keratinocytes (Edington *et al* submitted, Rheinwald and Becket 1981). A panel of HNSCC independently derived by Kirsten Edington (Beatson Institute) using the same methods, were also examined (Edington *et al* submitted).

3.2 The MS-2 cervical carcinoma cell line.

An attempt was made to develop a panel of cell lines derived from primary cervical tumours isolated from patients of a wide age group. Unfortunately the success rate was very small, consistent with previous reports stating the infrequent outgrowth of cells from such types of tumours. A total of 25 biopsies were dissected and further subjected to methods favouring the growth of primary keratinocytes (described in section 2.2.1.a), in this way avoiding selection of fitter variants (Edington *et al* submitted). Under these conditions approximately 50% of the samples revealed outgrowth of cells during the initial period of two weeks. However, the subsequent deterioration and inviability of the majority of cells attributed to crisis, senescence or terminal differentiation, resulted in the loss of all but one sample. The newly derived cell line, MS-2 (named after the first initials of the two establishers), had exhibited a remarkably rapid outgrowth from the tumour tissue (3-4 days following dissection) which had originated from a 21-year old patient with severe neoplasia. The presence of terminally irradiated Swiss 3T3 fibroblasts (x3T3) was found to be necessary for the growth of MS-2 cells in media containing 10% foetal bovine serum (FBS), however, x3T3-free culturing was achievable in conditions of low serum (2% FBS). In 10% FBS a population of the MS-2 cells took up a more spindle-type, fibroblast cell-like morphology that was lessened or lost with the reduction of serum, when all the cells flattened out and assumed the typical "paving stone" morphology observed for keratinocyte cells (Edington *et al* submitted).

Subsequent examination using PCR techniques revealed the presence of *HPV-16* specific sequences integrated into the genome of MS-2 cells (work carried out by Dr Garry Sibbet, Beatson Institute). These results were

confirmed by the detection of the *E7* oncogene using Southern blot analysis of *EcoR1* restriction endonuclease digested genomic DNA (Figure 5), while transcripts of the *E6* and *E7* oncogenes were detected by northern blot analysis (the northern blots were carried out by Angeliki Malliri, Beatson Institute). Human primary keratinocytes and two HNSCC cell lines (BICR6 and BICR18) were used as controls for the absence of *HPV-16* specific sequences. A commercially available mouse monoclonal antibody raised against E7 revealed expression of this 21kD onco-protein in MS-2 cells and *HPV-16 E6, E7* immortalised human keratinocytes (TFK104, a kind gift from Dr Karen Vousden, Ludwig Institute for Cancer Research, London, England) the latter of which were analysed for control purposes (Figure 5). The MS-2 cell line also exhibited a five-fold over-expression of EGFRs at the cell surface, which was observed with the use of an EGF binding assay and Scatchard analysis, as described in section 2.2.1.d. Injection of 10^7 cells into the flanks of three immunodeficient nude mice revealed these cells to be tumourigenic in all three cases (Table 4).

3.3 The HNSCC cell lines, BICR.

The BICR (Beatson Institute for Cancer Research) cell lines were derived and analysed by Kirsten Edington and Dr Ken Parkinson, Beatson Institute. Tumour staging, caryotyping, the detection of keratinocyte specific markers, optimal growth conditions in tissue culture and growth in immunodeficient mice were determined using a variety of different techniques (Table 4 represents the most relevant results for this study) (Edington *et al* submitted).

Table 3. Previously examined SCC cell lines.

Eight SCC derived cell lines were examined in two previous studies for *CCND1* gene dosage and mRNA expression levels (Lammie *et al* 1991, Schuuring *et al* 1992a).

Table 3.

cell lines	*CCND1* amplification	*CCND1* mRNA over-expression	reference
UMSCC1	1-Lammie *et al.* 2-Schuuring *et al*	-	Lammie *et al.* (1991) Schuuring *et al.* (1992)
UMSCC2	11	++	
UMSCC11A	3	+	
UMSCC11B	2	+	
UMSCC14C	3	+	
UMSCC22B	6	+	
A431	2	+	
UMSCC14B	3	+	Schuuring *et al.* (1992)

Figure 5. Detection of the *HPV-16 E7* DNA sequences and protein product in the MS-2 cell line.

20μg of genomic DNA from primary keratinocytes and cell lines MS-2, BICR6 and BICR18 were electrophoresed through a 0.8% agarose gel, blotted and probed with a 313bp fragment of the *E7* ORF (as described in materials and methods). The same membrane was stripped from radioactivity and reprobed with a 1.3kb fragment of *Cyl-1* cDNA as a control for DNA loading (as described in materials and methods). Arrows point to the ~3.5kb *E7* sequences and the 4kb, 2.2kb and 2kb *CCND1* bands obtained upon *EcoR1* restriction enzyme digestion of the genomic DNA.

15μg of protein extracts from primary keratinocytes, TFK104 and MS-2 (25μg of extracts) cell lines were electrophoresed through a 10% denaturing polyacrylamide gel, blotted and exposed to an anti-*HPV-16* E7 monoclonal antibody at a 1:10 dilution (as described in materials and methods). The E7 21kD protein was detected in both TFK104 and MS-2 cells. In the figure the band is pointed to with an arrow.

Figure 5.

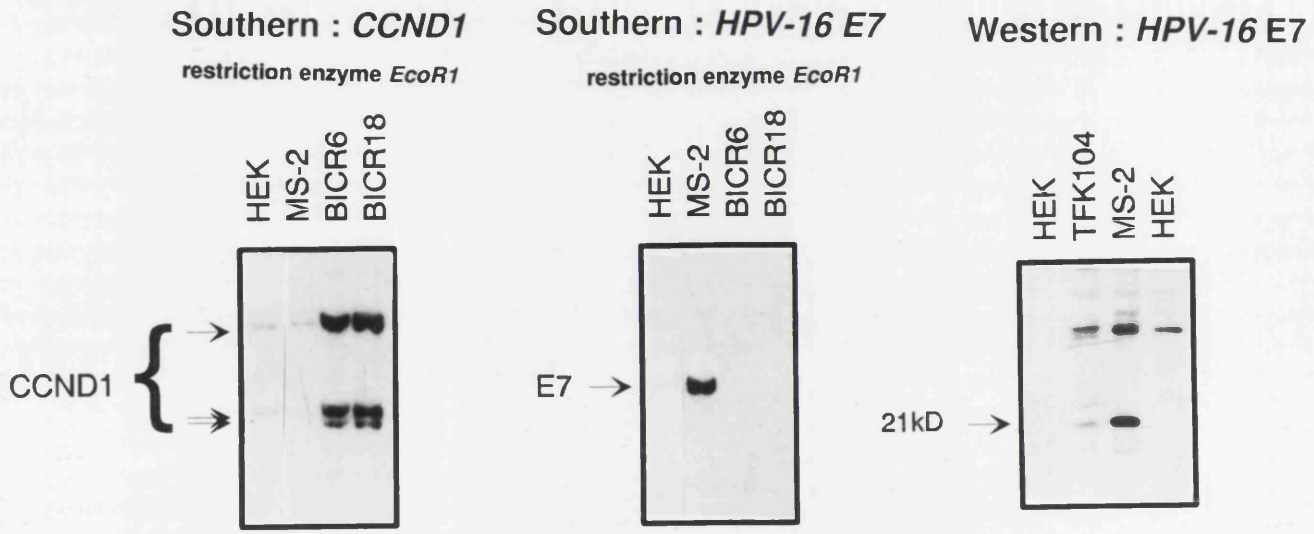

Southern : CCND1
restriction enzyme EcoR1
HEK
MS-2
BICR6
BICR18
CCND1
Southern : HPV-16 E7
restriction enzyme EcoR1
HEK
MS-2
BICR6
BICR18
E7
Western : HPV-16 E7
HEK
TFK104
MS-2
HEK
21kD

Table 4. The origin and characteristics of BICR and MS-2 cell lines.

TNM = tumour, node, metastasis staging (UICC 1987).

b = recurrent tumours.

(Edington *et al* submitted).

Table 4.

cell line	tumour site	tumour TNM staging	chromosome number	multiplication without x3T3	multiplication in low serum	tumour form. in nude mice
HEK	/	/	46	-	-	-
BICR3	alveulus	$T_2N_{2b}M_0$	49	-	+/-	-
BICR6	hypopharynx	$T_4N_1M_0$	66	+	+	+
BICR10[b]	buccal mucosa	$T_4N_0M_0$	78	+/-	+	+
BICR16[b]	tongue	$T_2N_0M_0$	63	-	+	+
BICR18	larynx	$T_4N_1M_0$	58	+/-	+	+
BICR19	epidermis	staging not applicable	44	-	+	+/-
BICR22	tongue	$T_4N_3M_0$	42	-	+	+
MS-2	cervix	nd.	nd.	+	+	+

3.4 Discussion.

To date only a few cervical cell lines have been established, of which the adenocarcinoma HeLa cell line is the most commonly described, however, despite the extensive analysis of these cells, very little is known of how they were originally derived. From the literature it is evident that most SCC cell lines have been obtained from recurrent or irradiated tumours and were cultured under conditions that favour the growth of fitter or more aggressive cells, therefore resulting in the growth of cell lines that are poor representatives of the original tumours (Easty *et al* 1981a, Easty *et al* 1981b, Rheinwald and Beckett 1981). For these reasons attempts were made to derive cell lines from primary, untreated cervical tumours using non-selective conditions optimal for the growth of primary keratinocytes (Rheinwald and Beckett 1981, Edington *et al* submitted). The low success rate of cell line establishment from cervical tumours (only 4% in this study) and the lack of good correlations between the number of HNSCC and derived cell lines with *CCND1* amplifications in published literature, triggered the analysis of *CCND1* gene dosage and expression levels in seven newly established HNSCC cell lines (Edington *et al* submitted).

CHAPTER 4 : Characterisation of *CCND1* gene dosage and expression levels.

4.1 Introduction.

In two previous reports, the frequency of *CCND1* amplification observed in HNSCC cell lines (83% or 100%), was considerably greater than that detected in unrelated HNSCC tumour samples (44% showed *CCND1* amplifications), a phenomenon that was postulated to reflect growth selection during the establishment of cell lines (Lammie *et al* 1991, Schuuring *et al* 1992a). The MS-2 and BICR cells examined in this study, were derived under conditions of low stringency (described in the previous chapter), therefore Southern blot analysis was a means of determining whether the frequency of *CCND1* amplification was equivalent for the new and previously established cell lines. Similarly, northern blot analysis was used to determine if over-expression of the cyclin D1 gene was restricted to those cells which had amplified *CCND1*. To date, the level of expression or intracellular localisation of the cyclin D1 protein (termed $p34^{cycD1}$), has not been reported for any human tumours, a surprising fact considering the potential post-transcriptional and post-translational modifications of this protein. The presence of $p34^{cycD1}$ in the BICR and MS-2 samples was demonstrated by western blot, immunocytochemical and immunohistochemical analyses.

4.2 *CCND1* amplification.

Southern blot analysis was performed on DNA extracts from MS-2 and BICR cell lines (as described in section 2.2.3.g) with the aim to look for the presence of *CCND1* amplifications and 11q13 rearrangements. Genomic DNA isolated from the new cell lines, A431, human primary epithelial keratinocytes

(HEK) and an *HPV-16 E6, E7* immortalised, non-transformed keratinocyte cell line (TFK104), was subjected to digestion with restriction endonuclease *BamH1*, electrophoresed, transferred onto a membrane and the resulting blot sequentially hybridised with probes for *CCND1* and *ets-1* (Figure 6). From this analysis *CCND1* amplification was evident in cell lines BICR6, BICR18, BICR22 and A431, while, no increased gene dosage was detected in the remaining cell lines, HEK or TFK104. There was also no evidence for rearrangements of 11q13. The *ets1* transcription factor gene is located on chromosome 11, band q23, the dosage of which was analysed with the aim to look for chromosomal duplication or aneuploidy. The observed constant gene dosage of *c-ets1* indicated that the *CCND1* amplification did not extend to 11q23. Densitometric analysis was used to quantitate the degree of *CCND1* amplification, the extent of which was based on the ratio of *CCND1* to *c-ets1* (Table 5). From the data it is evident that the frequency of *CCND1* amplifications in the examined BICR cell lines (48%) corresponds better to the figures previously reported for tumour samples rather than established cell lines.

4.3 *CCND1* mRNA expression.

Cyclin D1 mRNA species of 4.4-4.8kb and 1.7kb have previously been reported in cells of human origin (Xiong *et al* 1991, Motokura *et al* 1991, Withers *et al* 1991). The predominating larger transcripts were shown to contain the full-size message, while loss of the 3' non-coding sequences resulted in shorter species. Both small and large *CCND1* mRNAs were found to contain a full length protein coding region (Xiong *et al* 1991, Withers *et al* 1991, Seto *et al* 1992). In cells harbouring rearrangements of chromosome 11q13,

other *CCND1* transcripts were detected all of which showed loss of 3' sequences, while the 5' regions remained intact. A 2.5kb cyclin D1 mRNA was reported in a chronic lymphocytic leukaemia (CLL) cell line (MO2058), while 3.4kb and 2.6kb species were observed in a B-cell lymphoma cell line (SP-49). Both cell lines were characterised by the t(11;14)(q13;q32) translocation (Withers *et al* 1991, Seto *et al* 1992). Alternative splicing and differential polyadenilation were the two mechanisms suggested to cause the presence of *CCND1* mRNA species of diverse lengths (Xiong *et al* 1991, Motokura *et al* 1991, Withers *et al* 1991).

Northern blot analysis was used to examine the *CCND1* mRNA in the BICR, MS-2 and control cell lines (as described in section 2.2.3.h). A larger 4.5kb transcript was predominant in all of the tested cell lines, while the 1.7kb specie was evident only in cases of mRNA over-expression (Figure 7). Transcripts were barely detectable in the HEK and TFK104 cells, which served as an indication of how low keratinocyte *CCND1* mRNA baseline expression levels actually are. Interestingly, two additional mRNA species of 4.0 and 3.5kb were also observed in MS-2 and all of the BICR cell lines, while in HEK, TFK104 and A431 cells these bands were absent (Figure 7). Southern blot analysis had shown the lack of chromosome rearrangements in the new cell lines, suggesting that this had not been the cause of divergent transcripts. To minimise growth selection the BICR and MS-2 cell lines were cultured on lethally irradiated mouse feeder cells (x3T3). Murine macrophage cells were previously reported to contain *Cyl-1* mRNA species of 4.5 and 3.8kb in size (Matsushime *et al* 1991), however northern blot analysis of the Swiss 3T3 cells revealed high levels of 4.0 and 3.5kb *Cyl-1* transcripts, which corresponded to the additional species observed in the BICR and MS-2 cell lines. The extensive

levels of *Cyl-1* mRNA expression observed in the Swiss 3T3 fibroblasts would suggest that the presence of only a few contaminating cells was sufficient for transcript detection on the northern blots, therefore despite the effort made to remove most of the x3T3 cells from the cell line cultures, it was concluded that the most likely source of the 4.0 and 3.5kb cyclin D1 transcripts were the murine feeder cells. To determine the level of feeder contamination in the BICR and MS-2 RNA extracts, decreasing amounts of x3T3 total cellular RNA were subjected to northern blot analysis and probed with cyclin D1 cDNA or *GAPDH*, demonstrating that *Cyl-1* mRNA was detectable in 5μg of x3T3 RNA (Figure 8). Densitometric analysis of the signals allowed an estimation of feeder contamination in the human cell extracts and subsequent correction for *CCND1* expression levels.

In previous reports levels of *GAPDH* expression were used as a control for equal RNA loading in northern blot analysis (Lammie *et al* 1991, Schuuring *et al* 1992a). In this study differential expression of the *GAPDH* gene was evident in most of the examined cell lines, rendering the use of this probe as a control in northern blot analysis unsatisfactory. For these reasons ribosomal 28S and 18S RNA or actin probes were used to determine the loading of extracted cellular RNA, supported by the ethidium bromide (EtBr) staining of the gel (Figure 7). After correction for x3T3 contamination, the ratio of the human 4.5kb transcript to 18S RNA was used as an estimate of *CCND1* expression levels in the examined cell lines. The figures were determined as an average of several northern blots. Greatest *CCND1* over-expression was evident in cell lines BICR6, BICR18 and BICR22, which was lower in BICR3, BICR10, BICR16 and BICR19, while MS-2 consistently had "normal" *CCND1* mRNA levels, similar to those observed for HEK cells (Table 5). Although the

Figure 6. *CCND1* gene dosage in the examined SCC cell lines.

20μg of *BamH1* restriction endonuclease digested genomic DNA from primary keratinocytes, TFK104, BICR3, BICR6, BICR10, BICR16, BICR18, BICR19, BICR22, MS-2, A431 and SB (a pre B-cell leukaemia) cell lines were electrophoresed through a 0.8% agarose gel, blotted and probed with a 1.3kb fragment of *Cyl-1* cDNA (as described in materials and methods). Arrows point to the 12kb, 4kb, and 1.75kb bands specific to the human *CCND1* gene (the two bands marked 3T3 were of x3T3 origine). The membrane was stripped from radioactivity and reprobed with a 810bp fragment of human *ets-1* cDNA as a control of equal loading. Amplification was observed in cell lines BICR6, BICR18, BICR22 and A431.

Figure 6.

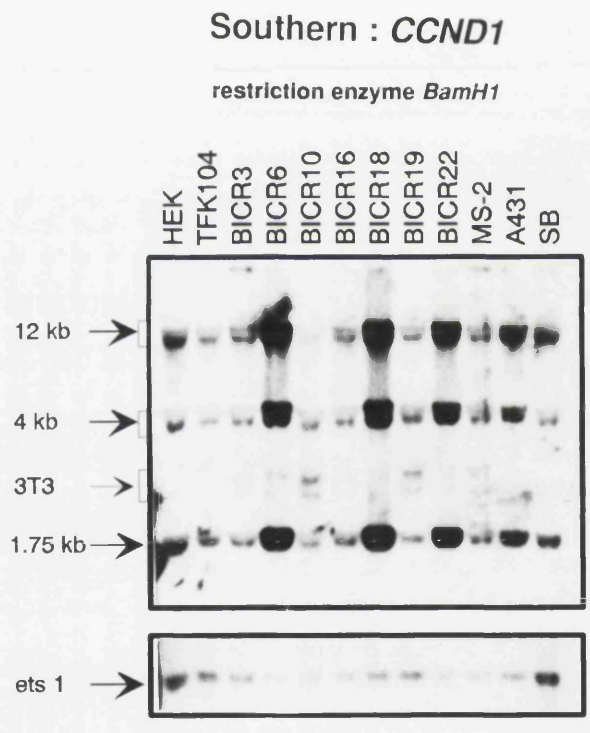

Southern : CCND1
restriction enzyme BamH1
HEK
TFK104
BICR3
BICR6
BICR10
BICR16
BICR18
BICR19
BICR22
MS-2
A431
SB
12 kb
4 kb
3T3
1.75 kb
ets 1

Figure 7. *CCND1* mRNA expression varies between the examined SCC cell lines.

20μg of total RNA from TFK104, BICR3, BICR6, BICR10, BICR16, BICR18, BICR19, BICR22, MS-2, A431, primary keratinocytes and 3T3 cell lines were electrophoresed through a 0.8% acrylamide gel containing formaldehyde and probed with a 1.3kb fragment of *Cyl-1* cDNA. Arrows point to the 4.5kb and 1.7kb human *CCND1* mRNA specific species and the 4.0kb murine *Cyl-1* specific transcript. The membrane was stripped from radioactivity and reprobed with a 1.36kb fragment of human skeletal muscle actin and a 7kb fragment specific to 28S RNA as a control for RNA loading and transfer (as described in materials and methods). Ethidium bromide staining of the agarose gel was also used as control for RNA loading.

Figure 7.

Northern : *CCND1*

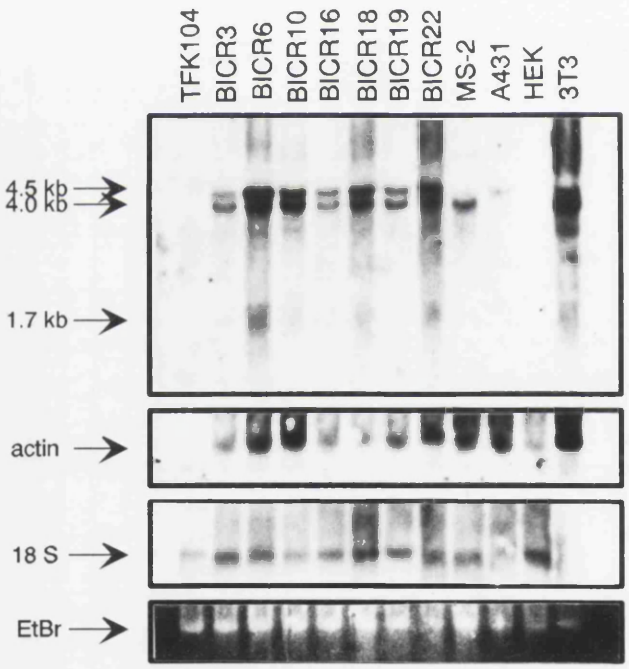
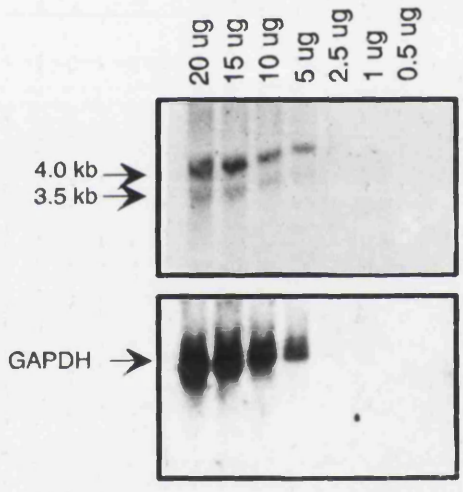


Figure 8. Concentration gradient analysis of *Cyl-1* expression in x3T3 cells.

Decreasing amounts of total RNA from x3T3 cells (20μg-0.5μg) were electrophoresed through a 0.8% agarose gel containing formaldehyde, blotted and probed with a 1.3kb fragment of *Cyl-1* cDNA (as described in materials and methods). Arrows point to the 4.0kb and 3.5kb murine specific *Cyl-1* mRNA species. The membrane was stripped from radioactivity and reprobed with a 0.7kb *GAPDH* probe as a control for RNA loading (as described in materials and methods).

Figure 8.

Northern : *Cyl-1*



presence of x3T3 RNA complicated quantitation, from the data it was evident that the cell lines that had amplified the *CCDN1* gene exhibited the greatest degree of *CCND1* mRNA expression, however increased gene dosage was not the only means for over-expression, as elevated transcripts were also detected in cells that did not display *CCND1* amplifications.

In an attempt to overcome the species cross-reactivity problem, a human specific probe was isolated. Previous reports have revealed a greater than 90% identity of the *CCND1* and *Cyl-1* cDNAs (Xiong *et al* 1991, Matsushime *et al* 1991). Comparison of human and mouse cyclin D1 mRNA sequences revealed extensive homology in the 5' protein coding regions and divergence at the 3' ends of full length transcripts (Matsushime *et al* 1991, Xiong *et al* 1991, Withers *et al* 1991, Motokura *et al* 1991). For these reasons five short fragments (200-380bp in length) were isolated by PCR techniques from the 3' non-coding regions of the human *CCND1* cDNA. The amplified regions were subsequently cloned, sequenced and tested for human specificity by northern blot analysis. One fragment (termed 3,4) was used for the determination of *CCND1* mRNA expression levels in the examined tumour and control cells (Figure 9). From the northern blot analysis of total RNA extracts, the most extensive over-expression of *CCND1* transcripts was evident in BICR6, while BICR18 and BICR22 also had elevated expression of cyclin D1 in comparison to HEK and TFK104 cells, which was in agreement with previous results. The ratio of the human 4.5kb transcript to 7S RNA was used as an estimate of *CCND1* expression levels in all the examined cell lines (Figure 10, Table 5).

4.4 Expression of $p34^{cycD1}$.

The predicted molecular masses of mouse and human cyclin D1 proteins based on cDNA sequences, were estimated to be 33.4k and 33.7k, respectively (Matsushime *et al* 1991, Xiong *et al* 1991, Motokura *et al* 1991). Both species were shown to migrate on denaturing SDS-polyacrylamide gels at approximately 34kD, relative to molecular weight standards. However, in several studies the cyclin D1 protein is referred to as p36 or p35 due to its slower migration than that of $p34^{cdc2}$ (Matsushime *et al* 1991, Xiong *et al* 1992b, Quelle *et al* 1993, Baldin *et al* 1993). The human and mouse counterparts show a high degree of identity, react specifically with the same antibodies and are considered functionally interchangeable (Matsushime *et al* 1991, Withers *et al* 1991, Dr Gordon Peters, ICRF, London, England, personal communication).

To date no reports have analysed the expression levels of *CCND1* protein (in this study referred to as $p34^{cycD1}$) in human tumours. Western blot analysis with a polyclonal antibody, 287-3 (a kind gift from Dr Gordon Peters, ICRF, London, England), raised against the C-peptide of the mouse *Cyl-1* protein ($p34^{cyl-1}$) was used to analyse $p34^{cycD1}$ expression in the various cell lines (Figure 11). Due to the species cross-reactivity of the antibody it was necessary to determine the degree of x3T3 contribution to the p34 band observed in the western blots. Fortunately, a fibroblast cell type specific protein of approximately 100kD was recognised by antibody 287-3 and was present at higher levels than $p34^{cyl-1}$ in murine fibroblast extracts. The ratio of ~100kD to $p34^{cyl-1}$ protein bands was determined by densitometric analysis of a western blot with varying amounts of feeder cell extracts (Figure 12). This ratio was used to correct for the contribution of x3T3 cells to the observed level of

Figure 9. DNA sequencing of a PCR amplified fragment from the 3' non-coding region of *CCND1* mRNA.

A 320bp fragment of the 3' non-coding region of *CCND1* mRNA was amplified using the technique of PCR, subcloned and sequenced as described in materials and methods. The diagrammatic representation highlights the amplified region within the full length *CCND1* transcript.

Figure 9.

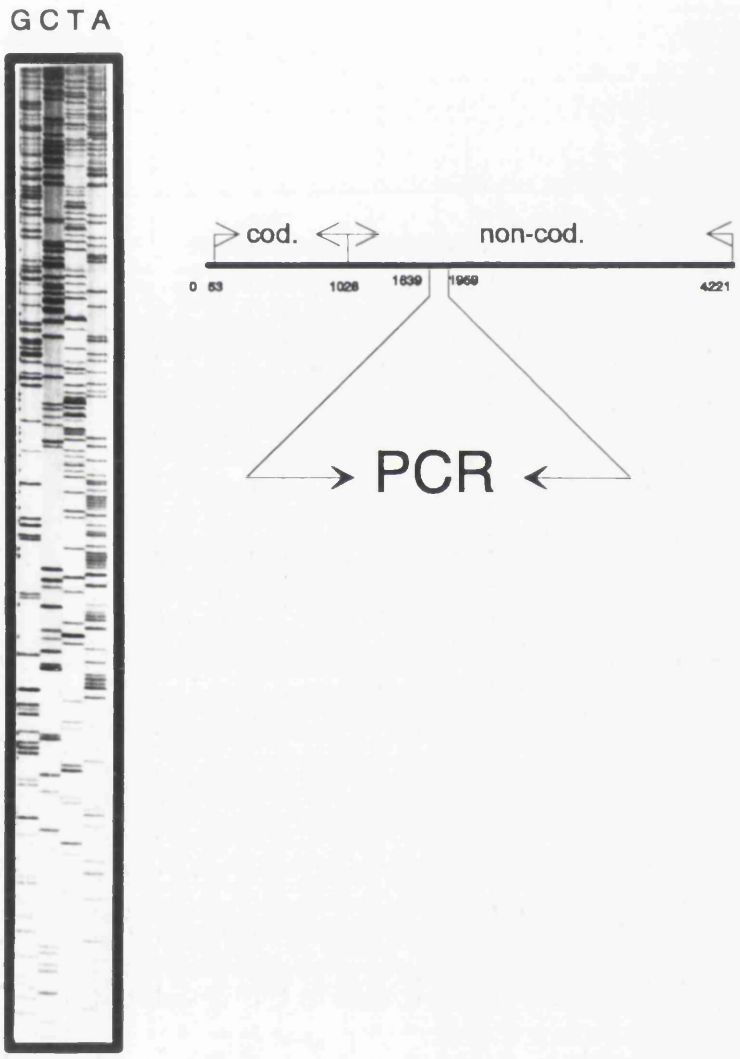


Figure 10. *CCND1* mRNA expression in the examined SCC cell lines.

20μg of total RNA from primary keratinocytes, TFK104, BICR3, BICR6, BICR10, BICR16, BICR18, BICR19, BICR22, MS-2, A431 and T45 cell lines were electrophoresed through 0.8% agarose gel containing formaldehyde, blotted and probed with a *CCND1* 4.5kb mRNA-specific 320bp fragment (as described in materials and methods). The membrane was stripped from radioactivity and reprobed with a 0.6kb fragment homologous to 7S rRNA as a control for equal RNA loading and transfer.

Figure 10.

Northern : *CCND1*

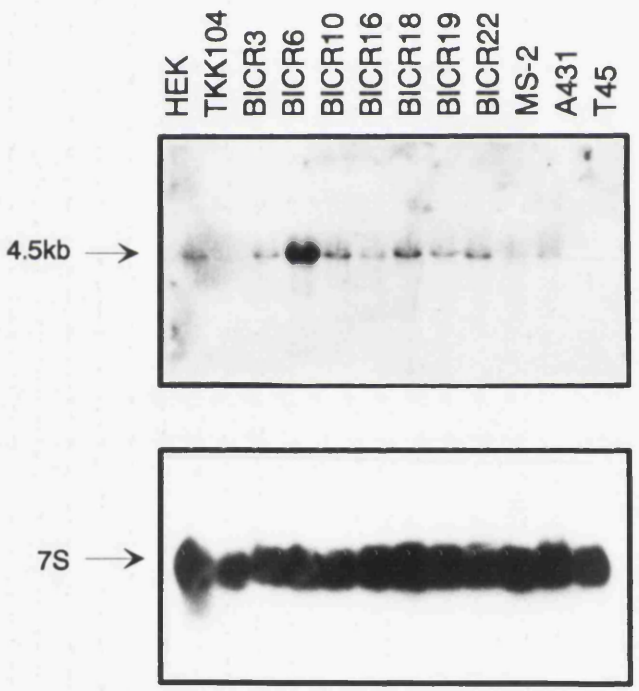


Figure 11. $p34^{cycD1}$ expression in the examined SCC cell lines.

Protein extracts were prepared from primary keratinocytes, TFK104, BICR3, BICR6, BICR10, BICR16, BICR18, BICR19, BICR22, MS-2 and A431 cell lines and TFK104. 35µg of extracts were electrophoresed through a 10% denaturing polyacrylamide gel and blotted, as described in materials and methods. A rabbit polyclonal antibody (287-3) was used at a 1:500 dilution to analyse for the expression levels of $p34^{cycD1}$. Arrows point to a murine specific ~100kD peptide and the cyclin D1 specific 34kD protein.

Figure 11.

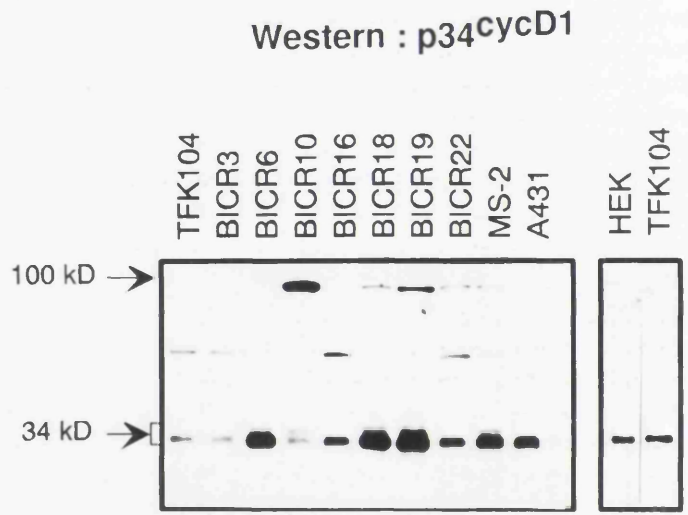

Western : p34cycD1
TFK104
BICR3
BICR6
BICR10
BICR16
BICR18
BICR19
BICR22
MS-2
A431
HEK
TFK104
100 kD
34 kD

Figure 12.The expression of p34^{cyl-1} and an unknown ~100kD peptide in x3T3 cells.

Varying amounts of protein extracted from x3T3 cells (35-1μg) and 35μg of protein isolated from the BICR6 cell line were electrophoresed through a 10% denaturing polyacrylamide gel and further subjected to western blot analysis as described in materials and methods. The intensities of the ~100kD and 34kD proteins, determined and compared by densitometric analysis, are diagramatically represented.

Figure 12.

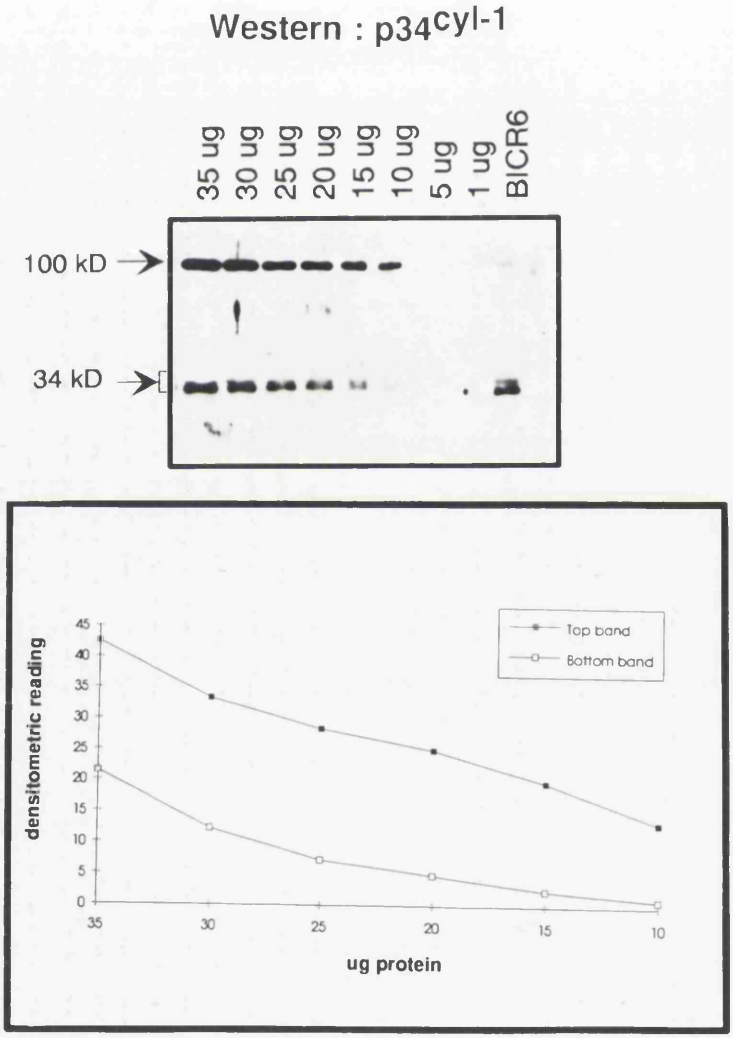

Western : p34cyl-1
35 ug
30 ug
25 ug
20 ug
15 ug
10 ug
5 ug
1 ug
BICR6
100 kD
34 kD
densitometric reading
Top band
Bottom band
ug protein
45
40
35
30
25
20
15
10
5
0
35
30
25
20
15
10

Table 5. *CCND1* gene dosage, mRNA and protein expression levels in the SCC cell lines.

Figures represent the levels of *CCND1* amplification, transcript and protein expression in the examined cell lines, when compared to human foreskin primary keratinocytes.

h.s. = human specific

Table 5.

Cell lines	*CCND1* fold amplification	*CCND1* mRNA	*CCND1* mRNA h.s.	p34 cycD1 fold overex.
HEK	1	+	1	1
TFK104	1	+	1	1
BICR3	1	++	3	1
BICR6	12	++++	15	10
BICR10	1	++	4	1
BICR16	1	++	2	4
BICR18	11	++++	4	9
BICR19	1	++	3	13
BICR22	9	+++	4	3
MS-2	1	+	1	4
A431	3	++	2	5
SB	1	nd.	nd.	nd.

$p34^{cycD1}$ in the western blots of tumour cell lines. Corrections were necessary only in cases where the murine specific ~100kD band was detectable. The greatest over-expression of $p34^{cycD1}$ was evident in BICR6, BICR18 and BICR19, increased expression was also present in BICR10, BICR16, BICR22, A431 and MS-2, while BICR3 appeared to have equal or lower levels of $p34^{cycD1}$ than that observed in HEK and TFK104 cells (Table 5).

The western blot analysis was repeated using a different cell lysis buffer and $p34^{cycD1}$ specific antibodies as a means to test for the exactness of the obtained data. The alternative lysis method used was recommended by Xiong *et al* 1992b and revealed similar results to those previously observed. Unsuccessful attempts were made to use a monoclonal antibody raised against the human $p34^{cycD1}$ peptide (kindly provided by Dr Ed Harlow, Massachusetts General Hospital, Boston, USA), while a commercially available polyclonal antibody did yeald results although also exhibited a high degree of protein cross-reactivity.

In summary, parallel with the data obtained for *CCND1* mRNA levels, tumour cell lines with the highest *CCND1* gene dosage also revealed increased amounts of the protein product, however amplification was not a prerequisite for over-expression as exemplified by BICR19 cells.

4.5 Nuclear localisation of $p34^{cycD1}$.

Previous reports have revealed the subcellular localisation of cyclins A, B1 and B2 in human primary fibroblasts, HeLa cells and chick embryo fibroblasts (Pines and Hunter 1991, Gallant and Nigg 1992). Immunofluorescence analysis with the use of appropriate antibodies had revealed a predominantly nuclear localisation of cyclin A from S phase

onwards, while the B cyclins accumulated in the cytoplasm of interphase cells, only entering the nucleus at the beginning of mitosis. Assynchronously growing tumour and normal cell lines were examined in this study with the aim to determine the subcellular distribution of $p34^{cycD1}$. Immunocytochemical detection of $p34^{cycD1}$ in methanol/acetone fixed cells with the antibody 287-3, revealed predominantly nuclear localisation of the protein (Figure 13). The variation in intensity of staining observed within individual cell lines was attributed to their passage through the cell cycle, with the most intensely stained cells residing in late G1 (supporting data is described in chapters 5 and 6). Recent reports have confirmed these observations in normal diploid human fibroblasts by the techniques of indirect immunofluorescence and the use of polyclonal anti-cyclin D1 antibodies (Baldin *et al* 1993).

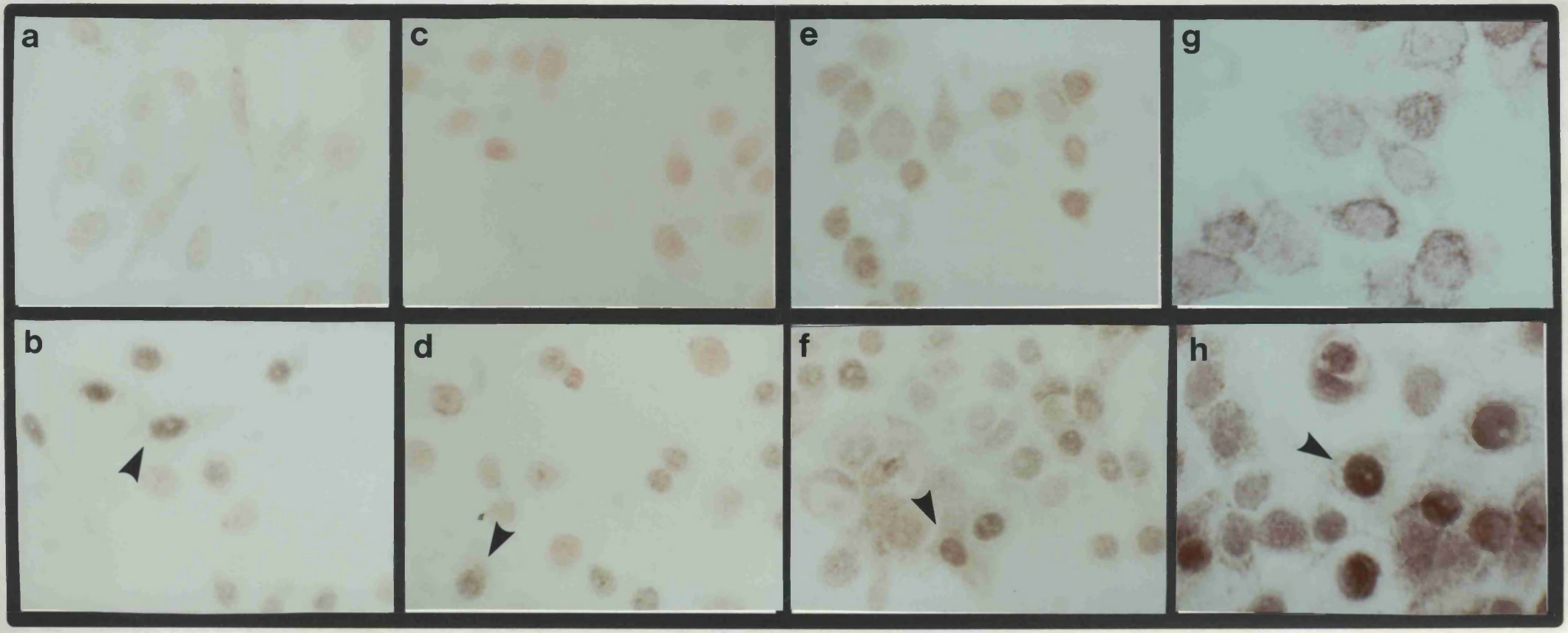
The expression and subcellular localisation of $p34^{cycD1}$ was also examined in original tumour biopsies (BICR3, BICR6 and BICR18), biopsies of xenograft tumours (BICR10, BICR16, BICR19, BICR22 and MS-2) and normal human skin (Figure 14). Immunohistochemical analysis revealed similar levels and patterns of staining as were observed in the established cell lines. No obvious staining was seen in normal skin and MS-2 xenografts, presumably due to the undetectable basal levels of expression. The results obtained from western blots had suggested a higher level of $p34^{cycD1}$ in BICR19 than what was observed in immunological analysis of both cell line and tumour xenografts. This phenomenon was attributed to reduced permeability and/or epitope masking of $p34^{cycD1}$ in BICR19. To test this hypothesis, immunocytochemical analysis was repeated on cells fixed in paraformaldehyde and with the use of an alternative antibody. Unfortunately in both cases the

Figure 13. $p34^{cycD1}$ localises primarily in the nucleus.

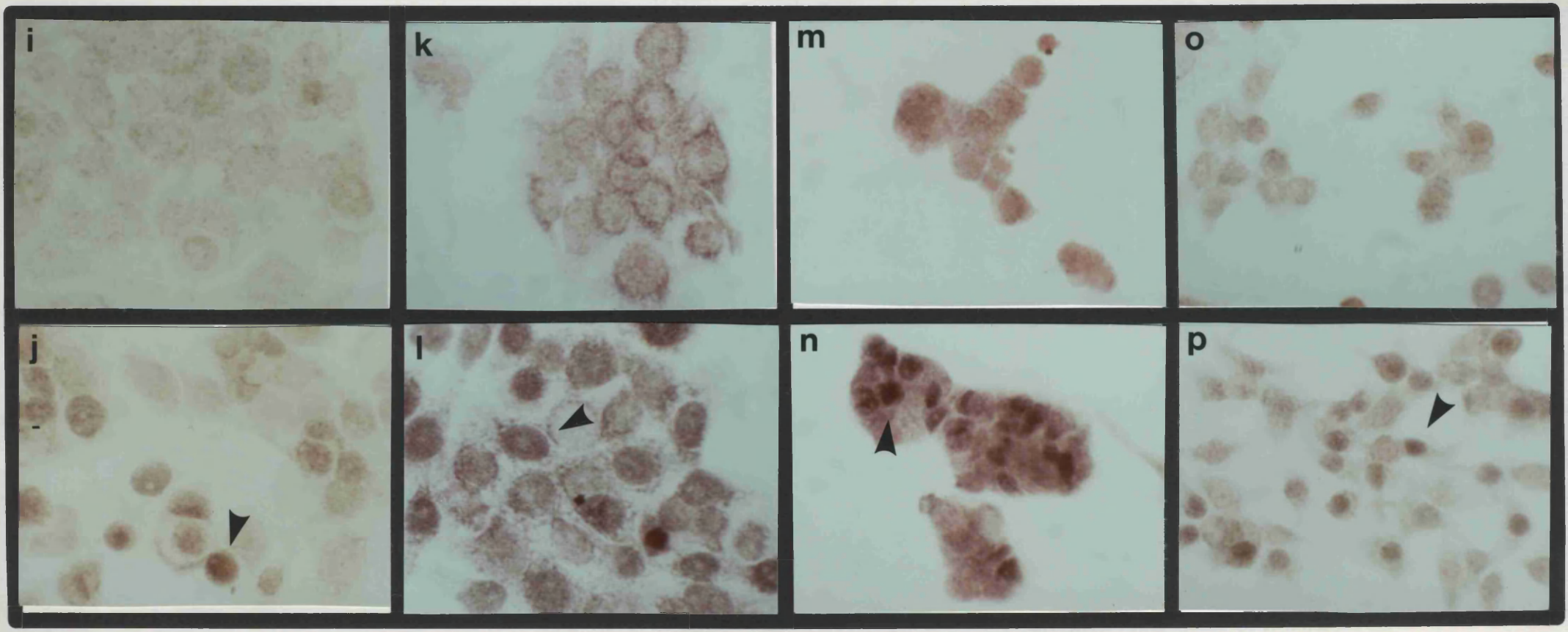
Assynchronously growing cells were fixed in a methanol:acetone solution and subjected to immunocytochemical analysis for $p34^{cycD1}$ with the use of an anti-cyclin D1 rabbit polyclonal antibody, 287-3 (as described in materials and methods). The analysed cell lines are depicted by letters : HEK (**a**,**b**); TFK104 (**c**,**d**), BICR3 (**e**,**f**), BICR6 (**g**,**h**), BICR10 (**i**,**j**), BICR16 (**k**,**l**), BICR18 (**m**,**n**), BICR19 (**o**,**p**), BICR22 (**q**,**r**), MS-2 (**s**,**t**) and A431 (**u**,**v**). In **a**, **c**, **e**, **g**, **i**, **k**, **m**, **o**, **q**, **s** and **u** pre-immune rabbit serum was at a 1:3000 dilution, as a control for nonspecific binding. In **b**, **d**, **f**, **h**, **j**, **l**, **n**, **p**, **r**, **t** and **v** antibody 287-3 was used at a 1:3000 dilution to detect $p34^{cycD1}$. Arrows point out strongly positive cells for $p34^{cycD1}$.

Figure 13.

Immunocytochemistry $p34^{cycD1}$



Immunocytochemistry p34^{cycD1}



Immunocytochemistry $p34^{cycD1}$

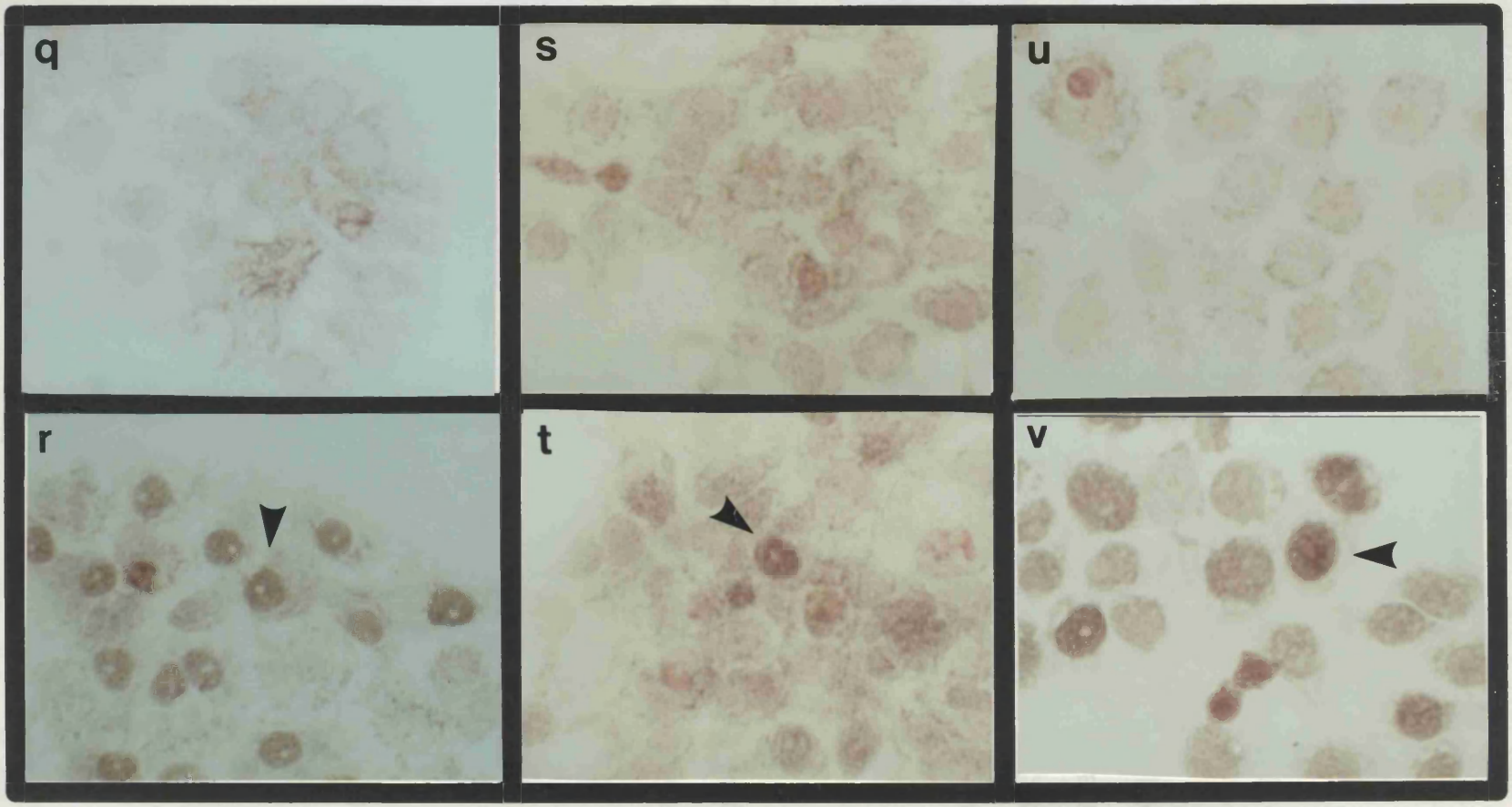


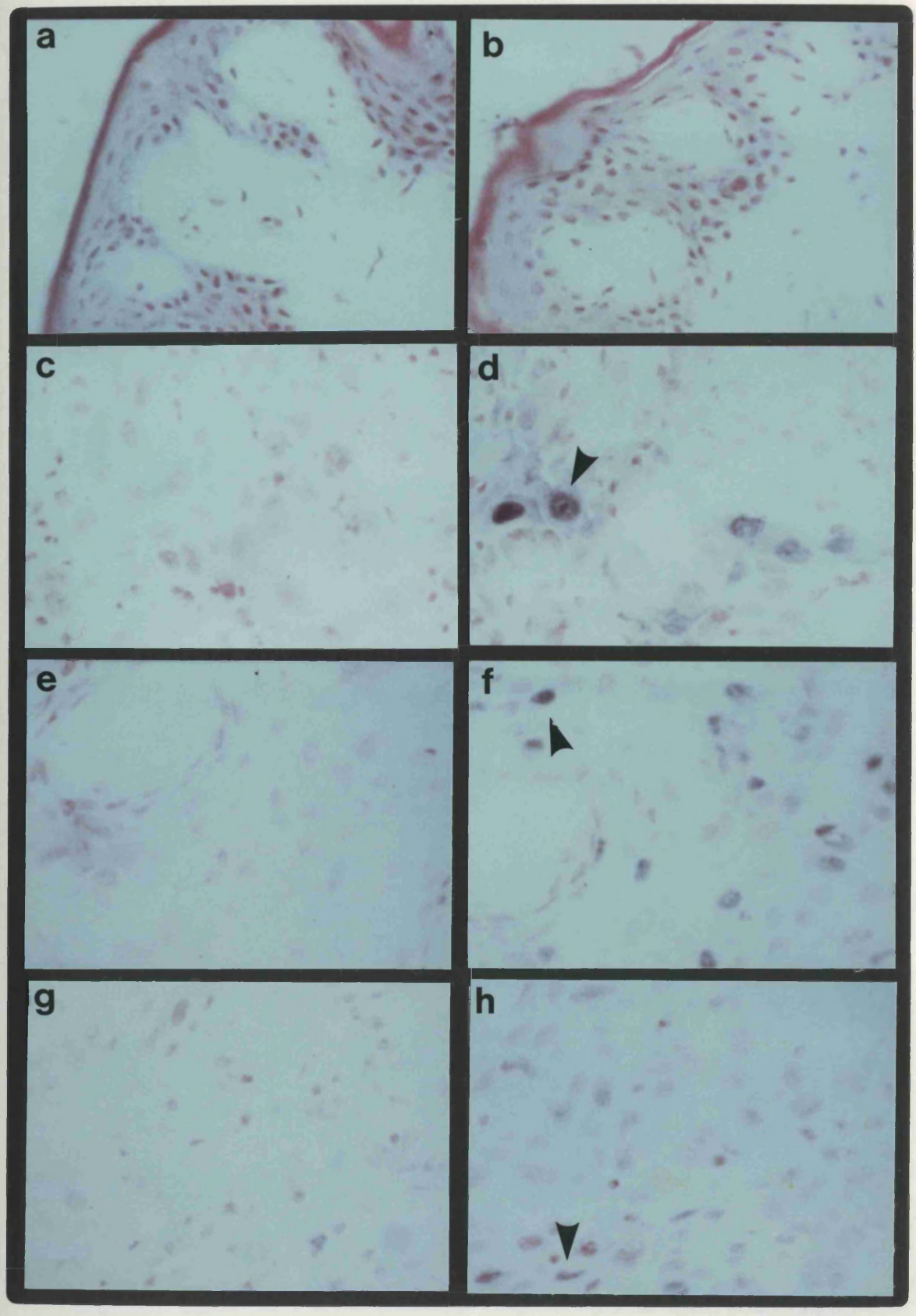
Figure 14. Nuclear localisation of $p34^{cycD1}$ in tumour xenografts and original biopsies.

Immunohistochemical analysis using the 287-3 antibody was carried out on tumour sections fixed in a methanol:acetone solution as described in materials and methods. **a)** original biopsies of normal skin (**a**,**b**), BICR3 (**c**,**d**), BICR6 (**e**,**f**) and BICR18 (**g**,**h**) were incubated with pre-immune rabbit serum (**a**, **c**, **e** and **g**) to determine the extent of non-specific staining; levels of $p34^{cycD1}$ were undetectable in normal skin nevertheless clearly nuclear in the remaining sections (**b**, **d**, **f** and **h**). **b)** tumour xenografts BICR10 (**i**,**j**), BICR16 (**k**,**l**), BICR19 (**m**,**n**), BICR22 (**o**,**p**) and MS-2 (**q**,**r**) were incubated with pre-immune serum (**i**, **k**, **m**, **o** and **q**) or anti-cyclin D1 antibody (**j**, **l**, **n**, **p** and **r**). Arrows point out strongly positive cells for $p34^{cycD1}$.

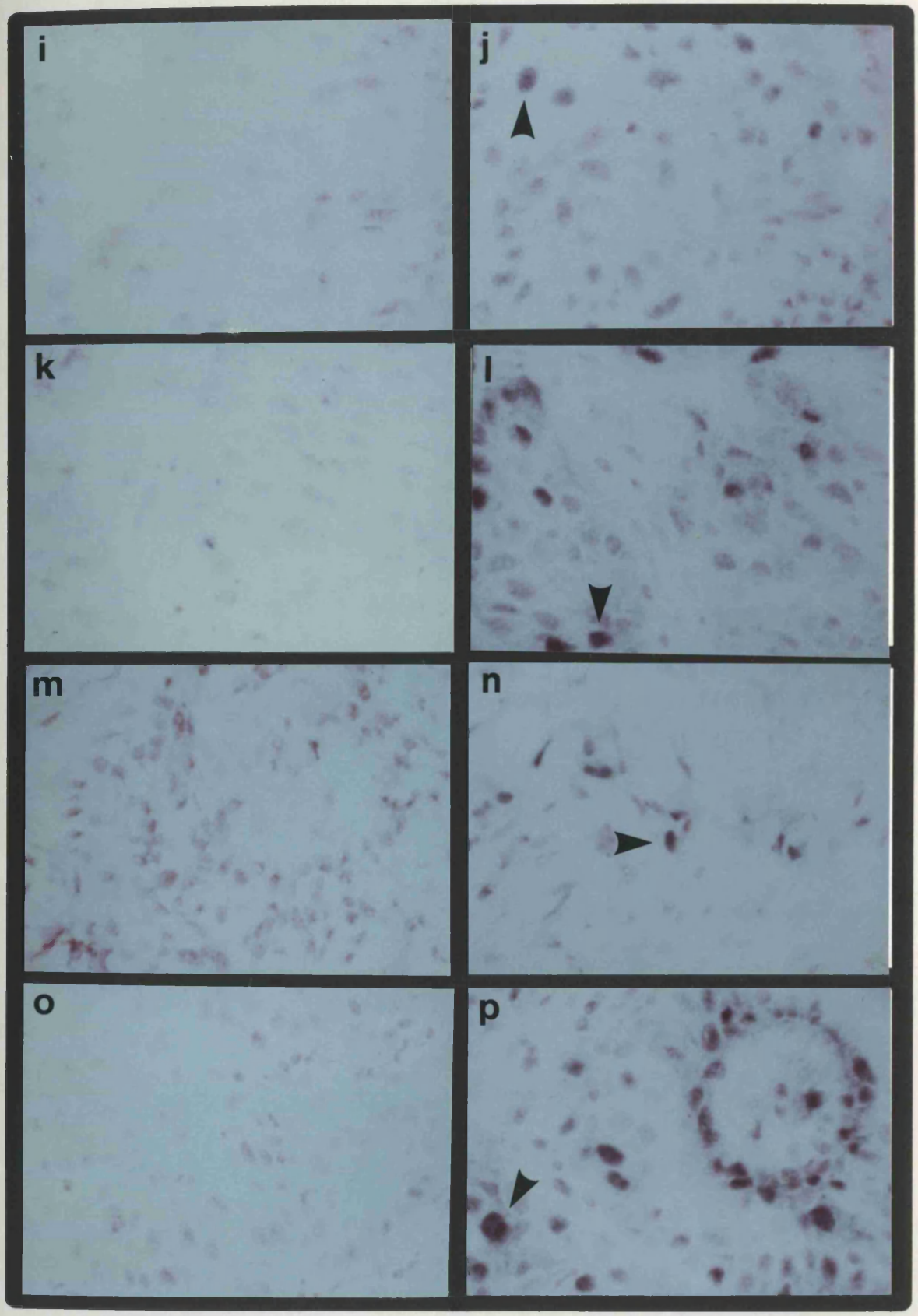
Figure 14.

Immunohistochemistry : $p34^{cycD1}$

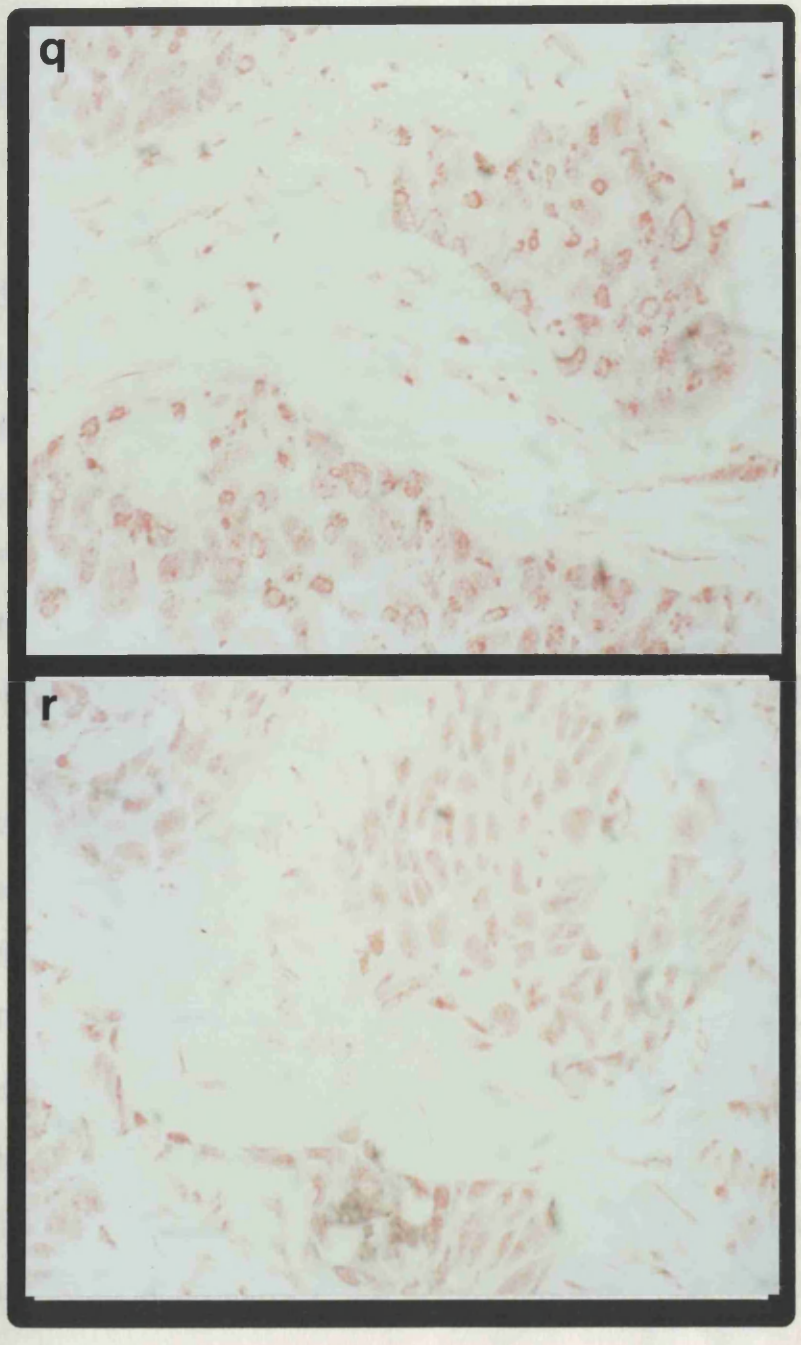
a)



b) Immunohistochemistry : $p34^{cycD1}$



Immunohistochemistry $p34^{cycD1}$



antibodies were unable to detect $p34^{cycD1}$ even in highly expressing cell lines such as BICR6, potentially due to epitope distruction.

4.6 cdk4 and pRb protein expression levels in the tumour cell lines.

The recently detected interactions between $p34^{cycD1}$ and cdk4 and the potential action of this complex or of $p34^{cycD1}$ alone on pRb initiated the examination of cdk4 and pRb levels in the BICR and MS-2 cell lines (Xiong *et al* 1992b, Matsushime *et al* 1992, Kato *et al* 1993, Dowdy *et al* 1993). Detection of the two proteins was achieved by western blot analysis of extracts from assynchronously growing cells and the use of a mouse monoclonal or rabbit polyclonal antibody raised against pRb or cdk4 peptides, respectively (both antibodies were a generous gift from Dr Li Huei Tsai, Massachusetts General Hospital, Boston, USA). cdk4 protein levels did not significantly vary between the tumour cell lines, confirming the suggestions of specific post-translational regulations of cdk4 activity by cyclin D1 or other cyclins (Figure 15). However, with the exception of MS-2, levels of apparently wild type pRb exhibited an increase in most of the tumour cell lines, of which BICR6, BICR18 and BICR22 were most dramatic (Figure 16). Upregulated pRb expression is a phenomenon that has not yet been reported in literature, in this study the levels correlated well with *CCND1* amplification and expression status. The absence of detectable pRb in the *HPV-16* positive cell line (MS-2) was not thought to be due to the viral oncoprotein E7, but to independently occurring mutations in the *Rb* gene.

Figure 15. cdk4 expression in the SCC cell lines.

40μg of protein extracted from TFK104, BICR3, BICR6, BICR10, BICR16, BICR18, BICR19, BICR22, MS-2 and A431 cell lines were subjected to western blot analysis, as described in materials and methods. A 1:1000 dilution of an anti-cdk4 rabbit polyclonal antibody was used for the detection of cdk4 expression levels. Arrows specify the cdk4 protein and an unknown ~43kD peptide.

Figure 15.

Western : cdk4

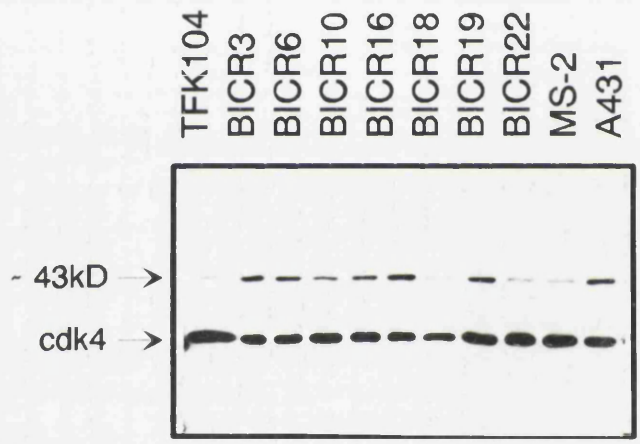
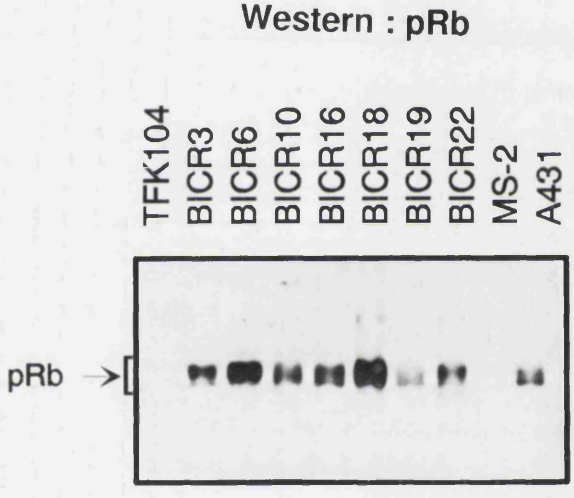


Figure 16. pRb expression is upregulated in cells containing *CCND1* amplification.

45µg of protein extracts from TFK104, BICR3, BICR6, BICR10, BICR16, BICR18, BICR19, BICR22, MS-2 and A431 were subjected to western blot analysis as described in materials and methods. An anti-pRb monoclonal antibody (XZ77) was used at a 1:10 dilution to detect the expression levels and different pRb phosphorylation forms present in the examined cell lines.

In this experiment the levels of pRb in the SCC cell lines were compared to those seen in TFK104 cells which were used as a normal control. Subsequent studies carried out by Angeliki Malliri (Beatson Institute), showed equivalent expression levels of pRb in human foreskin primary keratinocytes and TFK104 cells, suggesting that the over-expression of pRb observed in some of the BICR cell lines was real.

Figure 16.


Western : pRb
TFK104
BICR3
BICR6
BICR10
BICR16
BICR18
BICR19
BICR22
MS-2
A431
pRb

4.7 Discussion.

The previously reported levels of *CCND1* gene dosage in HNSCC cell lines have not been obtained from comparisons with primary keratinocytes but with other cell lines or unrelated tissues (Lammie *et al* 1991, Schuuring *et al* 1992a). In the most extreme case cell line UMSCC1, judged to be normal in one report (Lammie *et al* 1991) was subsequently shown to exhibit a low level of *CCND1* amplification and over-expression (Schuuring *et al* 1992a). For these reasons especial care was taken in this study to include human primary keratinocytes and an *HPV-16 E6, E7* immortalised, non-transformed keratinocyte cell line (TFK104), in this way determining the *CCND1* gene dosage in normal and "near-to-normal" squamous epithelial cells. In several previous reports A431, a vulvar carcinoma cell line, was demonstrated to contain a two-fold amplification of *CCND1* and was therefore included in this study for comparative purposes (Lammie *et al* 1991, Withers *et al* 1991, Schuuring *et al* 1992a, Faust and Meeker 1992, Jiang *et al* 1992). Upon examination of the BICR and MS-2 cell lines, only three (BICR6, BICR18 and BICR22) revealed increased *CCND1* gene dosage, corresponding well to the frequency of *CCND1* amplifications previously observed in tumour samples rather than established cell lines (Lammie *et al* 1991, Schuuring *et al* 1992a). The degree of amplification in the cell lines ranged from tree- to twelve-fold relative to primary keratinocytes and the gene dosage of *c-ets1*, representing the highest range of amplification when compared to previous studies (Lammie *et al* 1991, Schuuring *et al* 1992b, Faust and Meeker 1992, Jiang *et al* 1992).

It is becoming evident that *CCND1* fulfils the prerequisites for the key gene of the chromosome 11q13 amplicon by revealing transcript over-expression in cells that have increased gene dosage (Lammie *et al* 1991,

Schuuring *et al* 1992b, Faust and Meeker 1992). In one report a parallel was drawn between the levels of *CCND1* gene amplification and mRNA upregulation observed in cell lines derived from breast and HNSCCs (Schuuring *et al* 1992a). In this study over-expression of *CCND1* was observed in all three cell lines that had amplified the gene, confirming the potential "key" status of *CCND1* within chromosome 11q13. However, increased transcript levels were not only caused by amplification, demonstrated by cell lines BICR3, BICR10, BICR16 and BICR19, coupled with previous reports (Schuuring *et al* 1992a). Again the levels of cyclin D1 mRNA expression were compared with human foreskin primary keratinocytes, a measure not used in previous studies, while more appropriate probes than differentially expressed *GAPDH* were used as a determinant of RNA loading.

To date there have been no reports on the *CCND1* protein levels in human tumours. As with the mRNA, this study revealed a good correlation between the degree of amplification and expression of $p34^{cycD1}$, although examples such as BICR19 indicate that upregulation of protein levels can take place in the absence of increased gene dosage. Furthermore, $p34^{cycD1}$ predominantly localised in the nuclei of examined cells with no apparent differences in the frequency of positively staining tumour derived and "normal" cells. The pattern of subcellular $p34^{cycD1}$ distribution observed in the cell lines was mimicked in the tumour biopsy specimens whether they originated from the primary samples or xenografts. With the exception of MS-2, most tumour biopsies exhibited elevated levels of $p34^{cycD1}$ in comparison to normal skin, possibly reflecting the low rate of proliferation in the basal layers of skin. The observed predominant nuclear localisation of $p34^{cycD1}$, regardless of the

degree of transformation, is consistent with its role as a cell cycle regulator and effector or target of other nuclear regulatory proteins.

CHAPTER 5 : Cyclin D1 oscillations during the fibroblast cell cycle.

5.1 Introduction.

The pattern of cyclin D1 mRNA appearance following release of arrested cells was initially described in CSF-starved mouse macrophages and subsequently shown in human fibroblast cell lines (Matsushime *et al* 1991, Surmacz *et al* 1992, Won *et al* 1992, Baldin *et al* 1993). In all of the examined cases cyclin D1 mRNA levels peaked in late G1, suggesting a role for the protein during this stage of the cell cycle (see chapter 6. for further details). To date, very little work has been carried out on the protein product of the cyclin D1 gene and its oscillation patterns in different cell types (Matsushime *et al* 1991, Baldin *et al* 1993). Only one recent report has addressed the question of $p34^{cycD1}$ levels and subcellular localisation following stimulation of human diploid fibroblasts, the results of which confirmed the observations made in this study (Baldin *et al* 1993).

The advantage of using fibroblast cell lines in cell cycle analysis is their ability to respond to serum starvation by arresting in G0. In all the examined cases subsequent serum stimulation resulted in *Cyl-1* mRNA and protein upregulation at the expected stages of G1. The study was extended to examine *c-fos* and *v-fos* over-expressing cell lines with varying degrees of transformation, aiming to determine whether *Cyl-1* expression is dependent on fos levels and/or the activity of the AP-1 transcriptional factor (Hawker *et al* 1993).

5.2 *Cyl-1* mRNA levels in quiescent and growing rodent fibroblasts.

To obtain G0 synchrony, the analysed rodent fibroblast cell lines were cultured in conditions of reduced serum (0.5% FBS) for a period of 72 hours, a method used for achieving cell cycle arrest in several previous reports (Won *et al* 1992, Surmacz *et al* 1992, Baldin *et al* 1993). Extracts of total RNA obtained from assynchronously growing and G0 arrested cells were examined by northern blot analysis for *Cyl-1* transcript expression, as described in section 2.2.3.h. Figure 17 represents the comparison of four murine 3T3 cell lines (Balb3T3, Swiss3T3, NIH3T3 and T45) of which T45 are Swiss3T3 cells that had received and survived high doses of irradiation, thought to approximate 60Gy. In all four cases a reduction in the levels of both murine specific *Cyl-1* transcripts (4.0 and 3.5kb) were observed in quiescent cells. Arrested Balb3T3 fibroblasts exhibited a greatest decline (9-fold) of *Cyl-1* mRNA expression, determined by densitometric analysis, while this decrease was less evident in T45 cells.

The same type of analysis was repeated for six rat fibroblast cell lines, the characteristics of which are described in Table 6. Again, comparison of growing and arrested cells revealed a clear correlation of *Cyl-1* mRNA levels and the state of cell growth (Figure 18). Detection of 28S or 7S ribosomal RNA levels were used as determinants of RNA loading and transfer for rat and murine cell line extracts, respectively. Densitometric analysis allowed calculation of the ratio of rodent 4.0kb *Cyl-1* mRNA to 28S or 7S RNA in the appropriate cell lines and subsequently the expression levels of *Cyl-1* transcripts, presented in Table 6.

5.3 $p34^{cyl-1}$ expression in arrested and growing fibroblasts.

Western blot analysis was used to determine the levels of *Cyl-1* protein in quiescent and assynchronously growing murine and rat fibroblast cell lines described in the previous section. Total protein cell extracts were electrophoresed through denaturing 10% SDS-polyacrylamide gels, transferred to a membrane and subsequently exposed to antibody 287-3 for the detection of $p34^{cyl-1}$ (see section 2.2.4.c). Upon examination, the murine fibroblast cell lines revealed significantly reduced $p34^{cyl-1}$ expression in arrested cells, a similar pattern to the one observed for the cyclin D1 transcripts. Balb3T3 cells responded to serum starvation with the greatest decrease of $p34^{cyl-1}$ levels (6-fold), determined by densitometric analysis. T45 cells had upregulated expression of this cyclin in comparison to parental Swiss3T3 fibroblasts, suggesting an irradiation induced effect (Figure 19). Subsequent examination of 208F, MMV, A1C, FBR, Rat-1 and 1302 cell lines did not reveal such dramatic down-regulation of $p34^{cyl-1}$ during G0. Perplexingly, expression of this cyclin decreased (MMV, FBR), remained unaltered (208F, A1C, 1302), or even fractionally increased three fold (Rat-1) in arrested cells, potentially reflecting their ability to quiesce (Figure 20).

MMV and FBR fibroblasts contain the highest levels of *fos* expression, although only the later were transformed, in both cases down-regulation of $p34^{cyl-1}$ was evident in G0, however, the cell line A1C an intermediate between MMV and FBR cells in the degree of transformation, expressed elevated levels of $p34^{cyl-1}$ and lacked the ability to down-regulate this cyclin. Therefore, attempts to correlate *fos* expression levels or induced transformation with the loss of $p34^{cyl-1}$ down-regulation were unsuccessful. Table 6 represents comparison of $p34^{cyl-1}$ expression levels in the examined

Figure 17. *Cyl-1* mRNA expression is downregulated in quiescent murine fibroblasts.

20μg of total RNA were obtained from assynchronously growing or G0 arrested murine fibroblast cell lines Balb3T3, Swiss3T3, NIH3T3 and T45 and subjected to northern blot analysis for *Cyl-1* mRNA expression using a [^{32}P]dCTP-labelled 1.3kb fragment of the *Cyl-1* cDNA (as described in materials and methods). Rehybridisation of the membrane with a 7S homologous probe was used as a control for equal RNA loading and transfer.

Q = G0 arrested cells.

Figure 17.

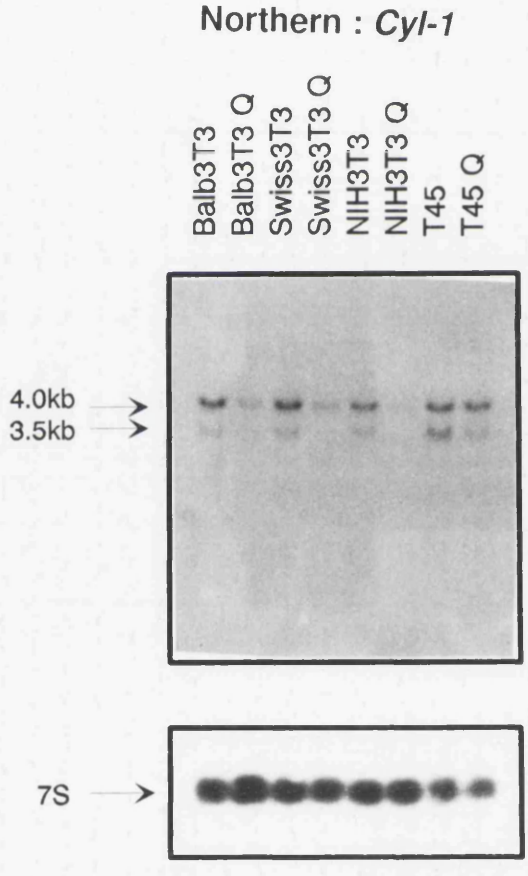

Northern : *Cyl-1*
Balb3T3
Balb3T3 Q
Swiss3T3
Swiss3T3 Q
NIH3T3
NIH3T3 Q
T45
T45 Q
4.0kb
3.5kb
7S

Figure 18. *Cyl-1* mRNA is downregulated in quiescent rat fibroblast.

20μg of total RNA were extracted from G0 arrested and assynchronously growing rat fibroblast cell lines 208F, MMV, A1C, FBR, Rat1 and 1302, blotted and probed with a 1.3kb *Cyl-1* cDNA fragment. The blot was stripped from radioactivity and reprobed with a 28S rRNA homologous fragment as a control for equal loading.

Q = G0 arrested cells.

Figure 18.

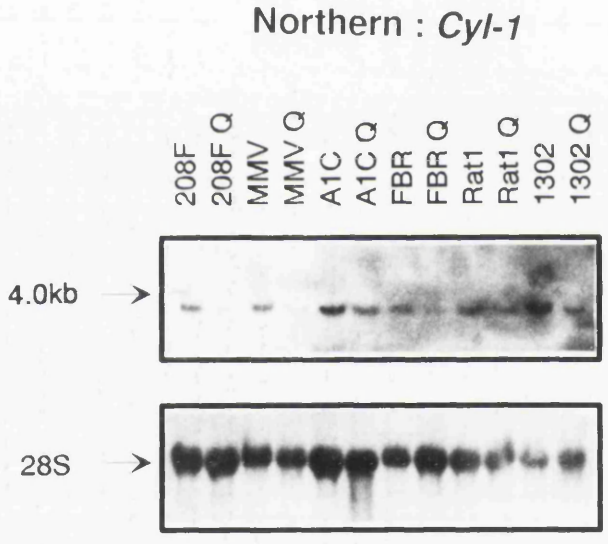

Northern : *Cyl-1*
208F
208F Q
MMV
MMV Q
A1C
A1C Q
FBR
FBR Q
Rat1
Rat1 Q
1302
1302 Q
4.0kb
28S

Figure 19. $p34^{cyl-1}$ expression is reduced in quiescent murine fibroblasts.

35μg of protein were extracted from assynchronously growing and G0 arrested Swiss 3T3, Balb3T3, NIH3T3 and T45 cell lines and subjected to western blot analysis, as described in materials and methods. Arrows point to the $p34^{cyl-1}$ protein doublet and the fibroblast specific ~100kD band.

Q = G0 arrested cells.

Figure 19.

Western : p34^{cyl-1}

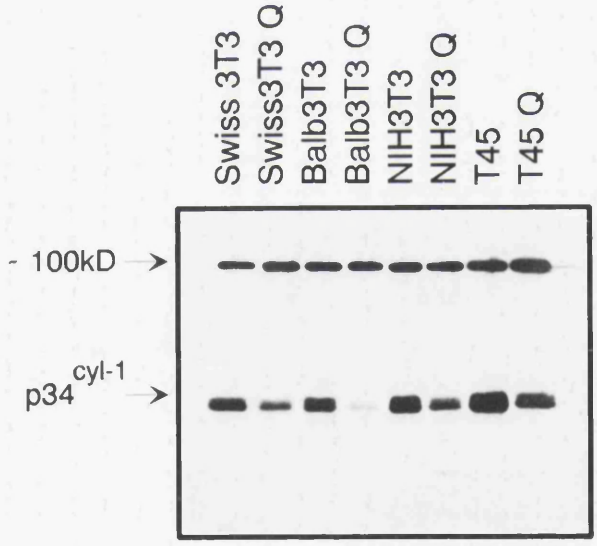


Figure 20. $p34^{cyl-1}$ expression in quiescent and growing rat fibroblast cell lines.

$p34^{cyl-1}$ expression in assynchronously growing and G0 arrested 208F, MMV, A1C, FBR, Rat1 and 1302 rat fibroblast cell lines were compared to murine NIH3T3 fibroblasts. 35µg of protein extracts were subjected to western blot analysis using the 287-3 anti-cyclin D1 polyclonal antibody. $p34^{cyl-1}$ and the ~100kD peptide are labelled with arrows.

Q = G0 arrested cells.

The degree of G0 arrest was based on the results obtained from separate experiments (see Figures 23 to 27) therefore full cell cycle synchrony may not have been obtained.

Figure 20.

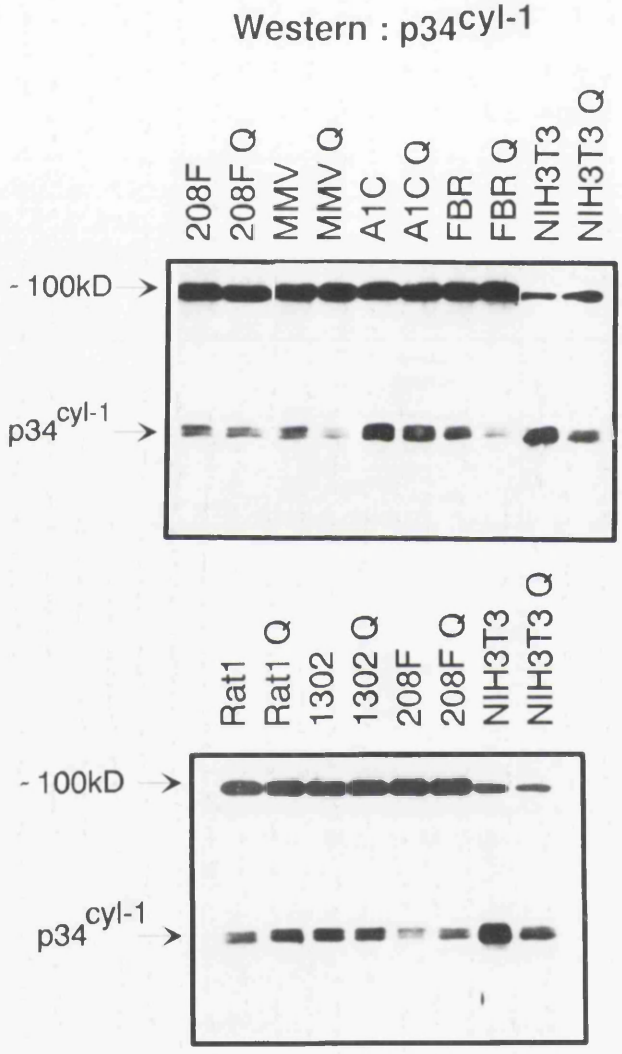

Western : p34cyl-1
208F
208F Q
MMV
MMV Q
A1C
A1C Q
FBR
FBR Q
NIH3T3
NIH3T3 Q
- 100kD
p34cyl-1
Rat1
Rat1 Q
1302
1302 Q
208F
208F Q
NIH3T3
NIH3T3 Q
- 100kD
p34cyl-1

Table 6. Comparison of *Cyl-1* mRNA and protein expression levels in G0 arrested and assynchronously growing rodent fibroblast cell lines.

Gr = assynchronously growing cells

Q = G0 arrested cells.

Table 6.

cell lines	origin	*Cyl-1* mRNA Gr/Q expression	cyl-1 p34 Gr/Q expression
208F	rat fibroblast cell line	3	1
MMV	*c-fos* over-expressing 208F cells	1	3
A1C	FBJ infected 208F cells	2	1.5
FBR	FBR infected 208F cells	2	4
Rat1	rat fibroblast cell line	1	0.4
1302	FBJ infected Rat1 cells	3	1
NIH3T3	murine fibroblast cell line	4	2
Balb3T3	murine fibroblast cell line	9	6
Swiss3T3	murine fibroblast cell line	4	4
T45	irradiated Swiss3T3 cells	1	1.5

Figure 21. cdk4 levels do not oscillate with the cell cycle.

35μg of protein extracts from assynchronously growing and G0 arrested Swiss 3T3, Balb3T3, NIH3T3 and T45 murine fibroblasts were subjected to western blot analysis as described in materials and methods. cdk4 was detected with the use of a 1:1000 dilution of an anti-cdk4 rabbit polyclonal antibody.

Q = G0 arrested cells.

Figure 21.

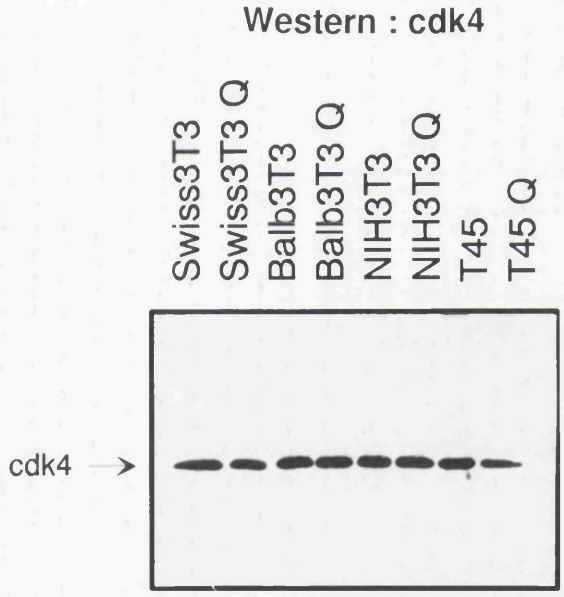
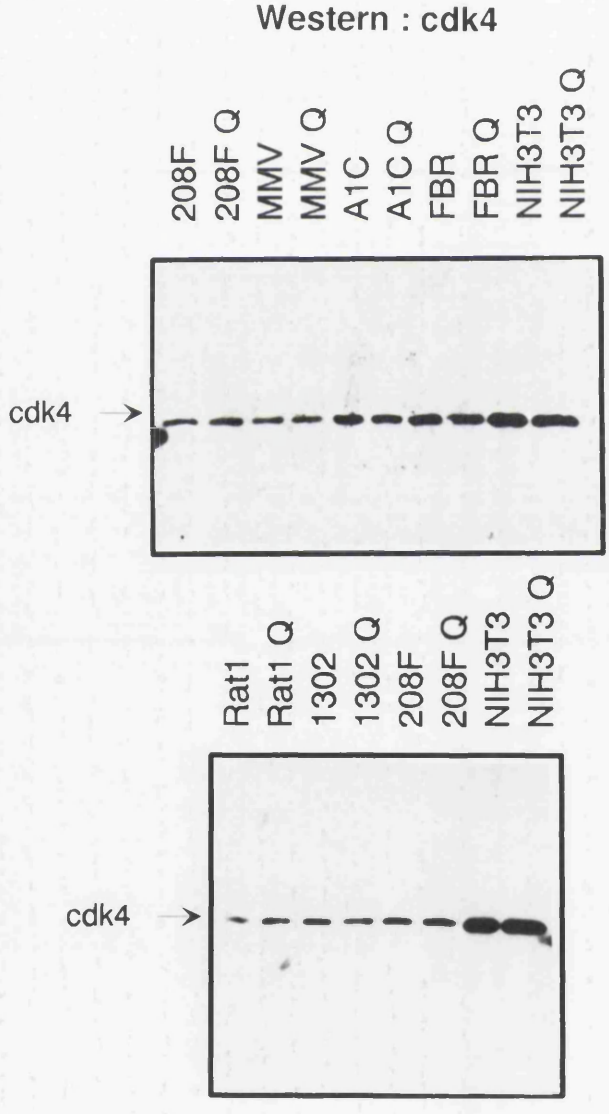

Western : cdk4
Swiss3T3
Swiss3T3 Q
Balb3T3
Balb3T3 Q
NIH3T3
NIH3T3 Q
T45
T45 Q
cdk4

Figure 22. cdk4 expression levels in quiescent and growing rat fibroblast cell lines.

cdk4 expression levels in G0 arrested and assynchronously growing 208F, MMV, A1C, FBR, Rat1 and 1302 cell lines were compared to the expression levels observed in NIH3T3 murine fibroblasts. 35µg of protein extracts were electrophoresed through a 10% denaturing polyacrylamide gel, blotted and subjected to cdk4 western blot analysis as described in materials and methods. The position of the cdk4 protein is shown.

Q = G0 arrested cells.

Figure 22.


Western : cdk4
208F
208F Q
MMV
MMV Q
A1C
A1C Q
FBR
FBR Q
NIH3T3
NIH3T3 Q
cdk4
Rat1
Rat1 Q
1302
1302 Q
208F
208F Q
NIH3T3
NIH3T3 Q
cdk4

assynchronously growing and G0 arrested fibroblast populations, determined by densitometric analysis.

Subsequent western blot analysis of the same cell extracts for cdk4 expression, revealed no differences between growing and arrested cells (Figure 21 and Figure 22). Interestingly, in comparison to parental 208F fibroblasts, cdk4 expression was upregulated two fold in *v-fos* transformed cell lines A1C and FBR, however the same was not true for 1302 and Rat-1 cells (Figure 22). The eight to ten fold higher levels of detectable cdk4 in the murine cell lines was proposed to result from differences in the basal expression levels, or more probably due to increased antibody affinity for mouse sequences.

NIH3T3 cell extracts were included in all the western blots, allowing densitometric analysis and the comparison of $p34^{cyl-1}$ and cdk4 expression levels in the examined cell lines.

5.4 $p34^{cyl-1}$ levels oscillate with the fibroblast cell cycle.

The subcellular distribution and oscillation patterns of the cyclin D1 protein were examined in both murine and rat fibroblast cell lines with the use of western blot and immunocytochemical analyses (as described in sections 2.2.4.c and 2.2.4.d-2). Representative cell lines (208F, FBR, NIH3T3, Rat1 and T45) were synchronised to G0 by serum starvation (as described in section 2.2.1.b) and were subsequently released following exposure to 10% FBS. The degree of cell synchrony was assessed by 5-bromodeoxyuridine (BrdU) incorporation over a 24 hour period, revealing on average less than 5% of growing cells in the arrested populations. Initiation of DNA synthesis took place at approximately 12 hours post-stimulation, with the exception of NIH3T3

fibroblasts that exhibited a 2-4 hour delayed entry into the S phase. At 24 hours from exposure to serum more than 80% of the cells were synthesising DNA (Figures 23, 24, 25 and 26).

Antibody 287-3 was used to detect $p34^{cyl-1}$ in methanol/acetone fixed cells (Figures 23, 24, 25, 26 and 27) or by western blot analysis (Figure 28) of cell extracts derived at set time intervals from stimulation. $p34^{cyl-1}$ was evident in the nuclei of Balb3T3, T45, 208F and FBR cell lines 4 hours post-stimulation, peaking during the 8-12 hour interval and subsequently declining at 24 hours (Figures 24, 25, 26 and 27). The previously determined cell cycle delay of NIH3T3 fibroblasts was also evident in $p34^{cyl-1}$ expression, in this case maximum levels were observed at 12-16 hours following release from G0 (Figure 23). In T45 cells the analysis was extended to 56 hours from exposure to serum, during which period cells were postulated to have re-entered subsequent cell cycles and loss of synchrony was observed (Figure 27). In all the examined time points the dramatic decline of detectable $p34^{cyl-1}$ observed in G0 was not repeated, suggesting that cycling cells retain basal levels of the *CCND1* protein throughout proliferation.

5.5 Discussion.

Prior reports have suggested minimum oscillations of *Cyl-1* mRNA levels throughout the mouse macrophage or mammalian fibroblast cell cycles, in one case drawing a parallel with the behaviour of *CLN3* transcripts that remain relatively constant throughout the *S.cerevisiae* cycle (Wittenberg *et al* 1990, Matsushime *et al* 1991, Won *et al* 1992, Surmacz *et al* 1992, Lew *et al* 1992). To date all reports have detected a reduction of cyclin D1 transcript levels in G0 arrested cells, in agreement with observations made for rodent fibroblasts

Figure 23. Immunocytochemical analysis of NIH3T3 fibroblasts.

G0 arrested NIH3T3 murine fibroblasts were stimulated with serum and at the same time half of the samples were exposed to 10μM BrdU. The cells were subsequently fixed in a methanol:acetone solution at set time points from stimulation (as described in materials and methods). Immunocytochemical analysis with an anti-BrdU mouse monoclonal antibody or an anti-cyclin D1 antibody 287-3 was used to determine the timing of S phase and the expression levels and subcellular localisation of $p34^{cyl-1}$, respectively. Minus signs represent negative controls in which the primary antibody was not included and in the case of $p34^{cyl-1}$, pre-immune rabbit serum was used at the same (1:3000) dilution as antibody 287-3; **-Br** represents cells that did not contain incorporated BrdU and were exposed to the anti-BrdU antibody. Numbers specify hours from serum stimulation.

Figure 23.

Immunocytochemistry : $p34^{cyl-1}$ and BrdU

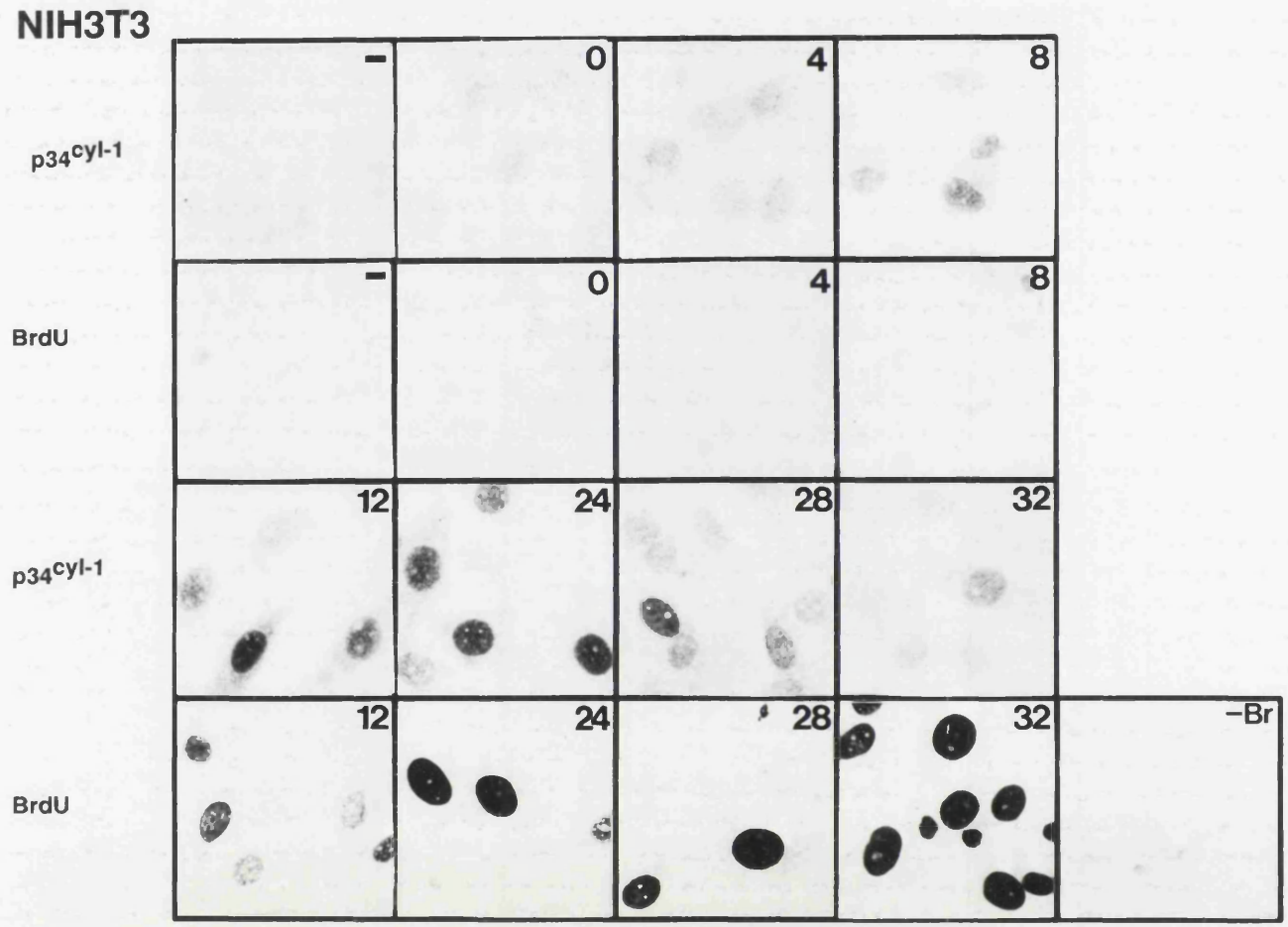


Figure 24. Immunocytochemical analysis of 208F fibroblasts.

G0 arrested cells were stimulated with serum and at the same time half of the samples exposed to 10μM BrdU. Cells fixed at set time intervals from stimulation were subjected to immunocytochemical analysis for BrdU incorporation and $p34^{cyl-1}$ cell cycle dependent oscillation pattern and subcellular localisation, as described in materials and methods. Minus signs represent negative controls in which the primary antibody was not included and in the case of $p34^{cyl-1}$, pre-immune rabbit serum was used at the same (1:3000) dilution as antibody 287-3; **-Br** represents cells that did not contain incorporated BrdU and were exposed to the anti-BrdU antibody. Numbers specify hours from serum stimulation.

Figure 24.

Immunocytochemistry : p34^{cyl-1} and BrdU

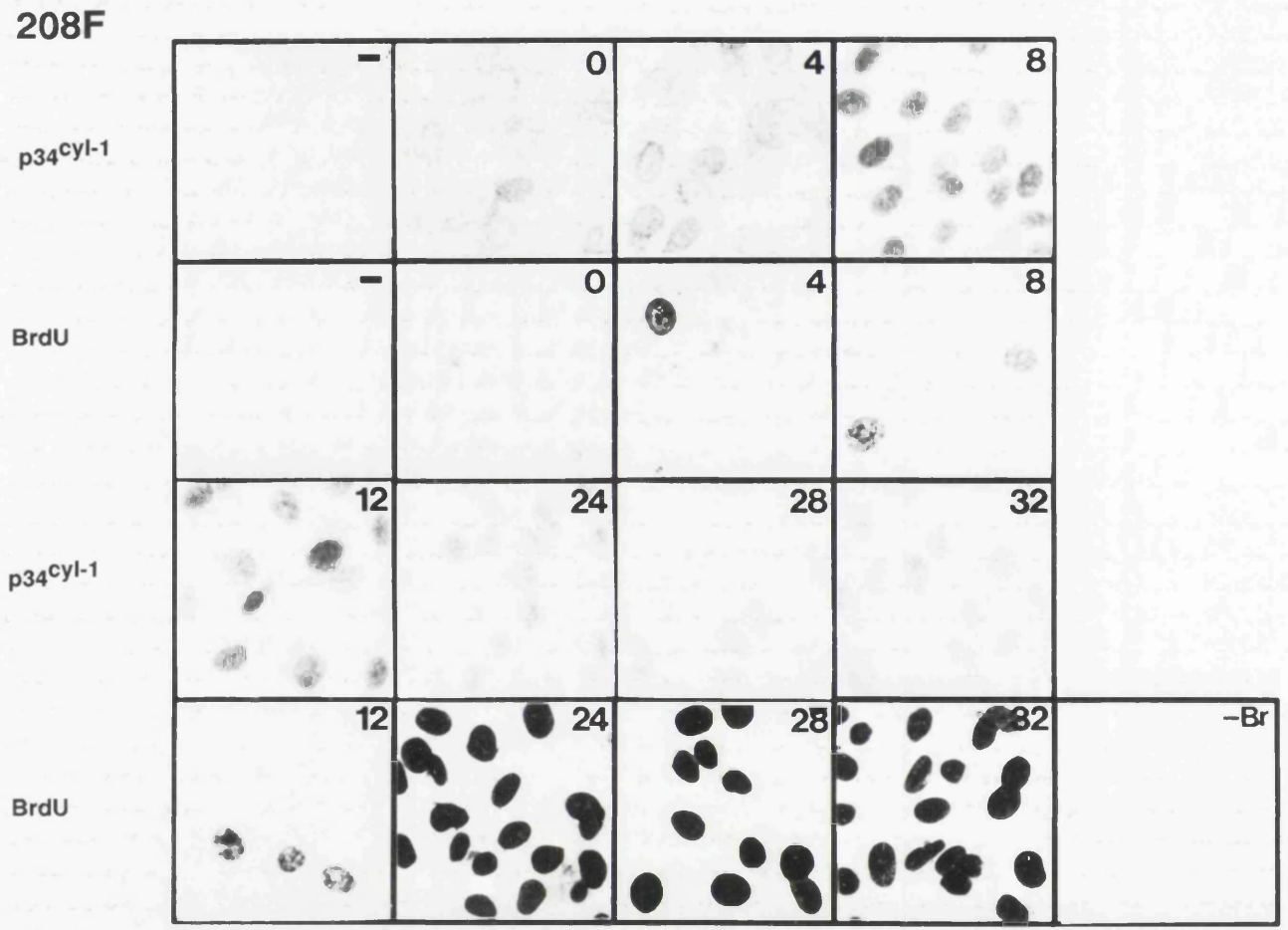


Figure 25. Immunocytochemical analysis of FBR fibroblasts.

G0 arrested FBR rat fibroblasts were subjected to the experimental analysis described in Figures 23 and 24 and in materials and methods. Minus signs represent negative controls in which the primary antibody was not included and in the case of $p34^{cyl-1}$, pre-immune rabbit serum was used at the same (1:3000) dilution as antibody 287-3; **-Br** represents cells that did not contain incorporated BrdU and were exposed to the anti-BrdU antibody. Numbers specify hours from serum stimulation.

Figure 25.

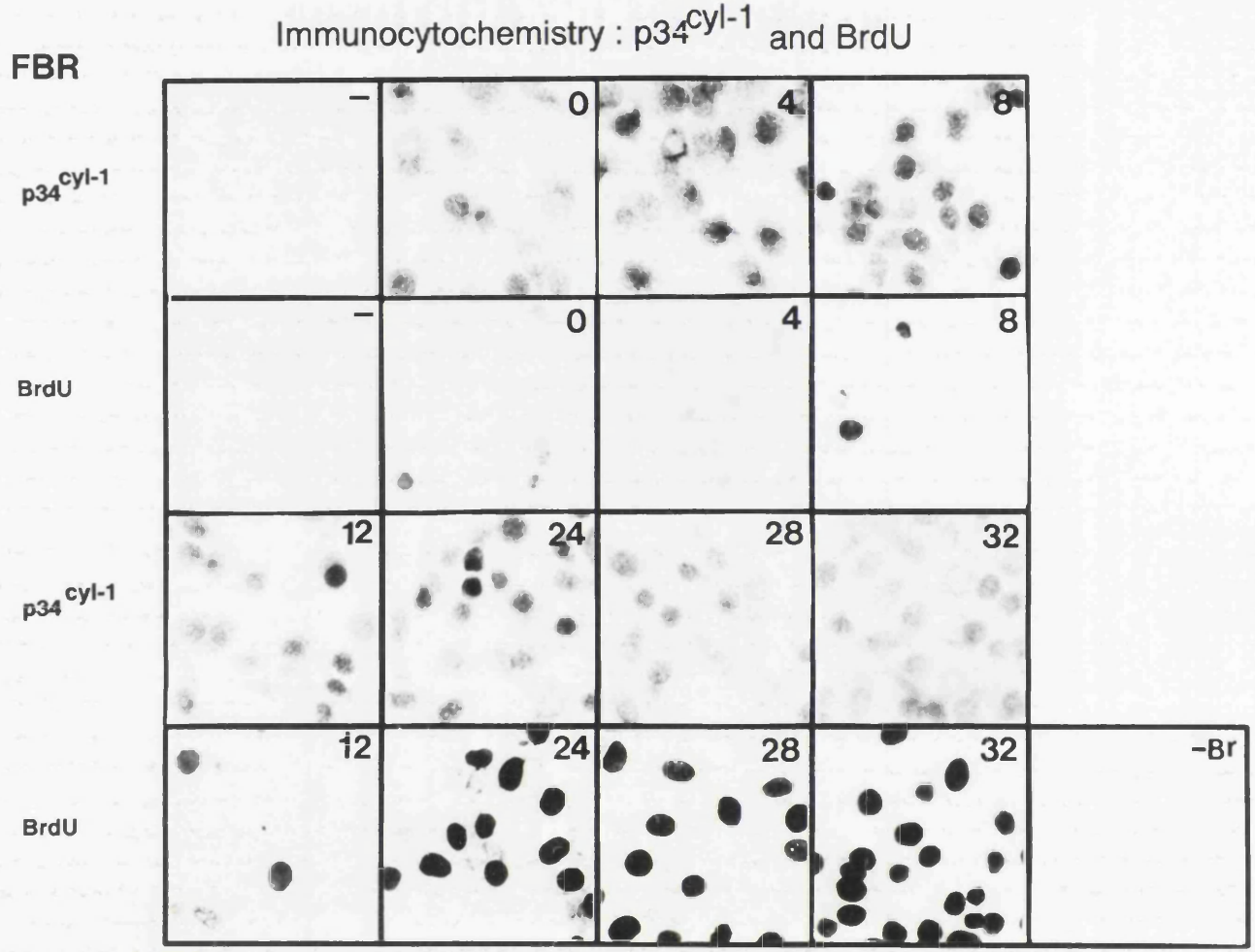

Immunocytochemistry : p34cyl-1 and BrdU
FBR
p34cyl-1
BrdU
p34cyl-1
BrdU
–
0
4
8
–
0
4
8
12
24
28
32
12
24
28
32
-Br

Figure 26. Immunocytochemical analysis of Rat1 fibroblasts.

G0 arrested Rat1 fibroblasts were subjected to the experimental analysis described in Figures 23 and 24 and in materials and methods. Minus signs represent negative controls in which the primary antibody was not included and in the case of $p34^{cyl-1}$, pre-immune rabbit serum was used at the same (1:3000) dilution as antibody 287-3; **-Br** represents cells that did not contain incorporated BrdU and were exposed to the anti-BrdU antibody. Numbers specify hours from serum stimulation.

Figure 26.

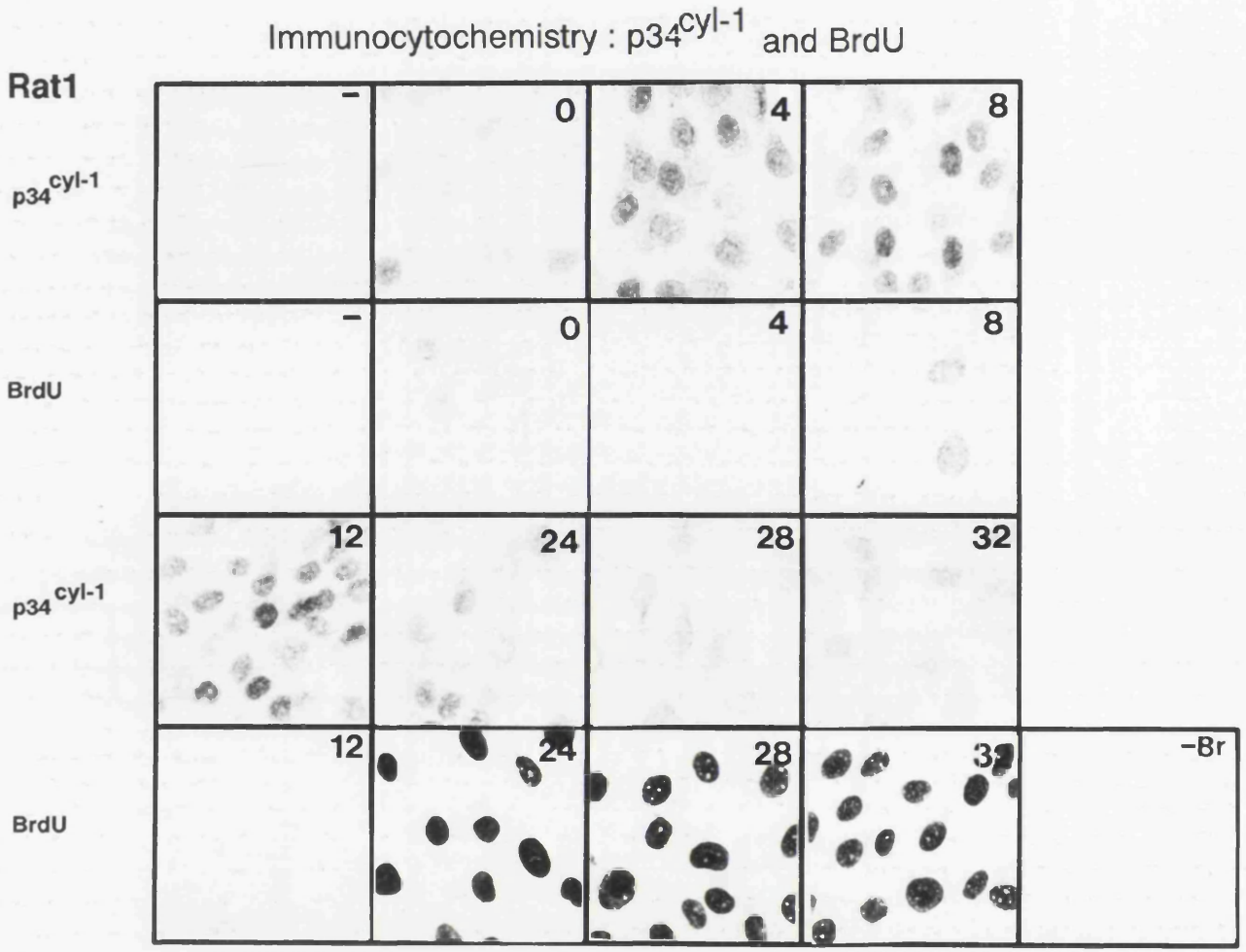

Immunocytochemistry : p34cyl-1 and BrdU
Rat1
p34cyl-1
BrdU
p34cyl-1
BrdU
–
0
4
8
–
0
4
8
12
24
28
32
12
24
28
32
–Br

Figure 27. Immunocytochemical analysis of T45 fibroblasts.

G0 arrested T45 murine fibroblasts were stimulated with serum and fixed in methanol:acetone at set time intervals from stimulation, depicted by numbers of hours (as described in materials and methods). Immunocytochemical analysis was carried out with a 1:3000 dilution of antibody 287-3. Minus sign represents the control sample for nonspecific binding, these cells were exposed to preimmune rabbit serum at a 1:3000 dilution.

Figure 27.

T45 Immunocytochemistry : $p34^{cyl-1}$

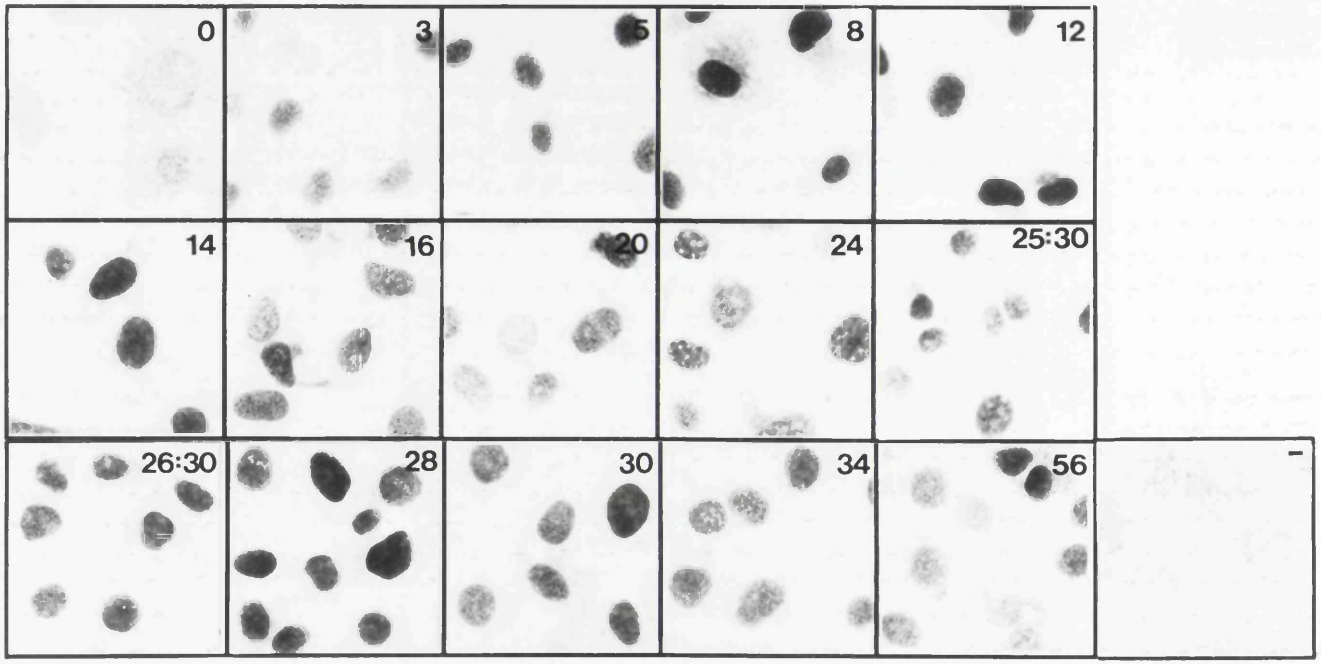
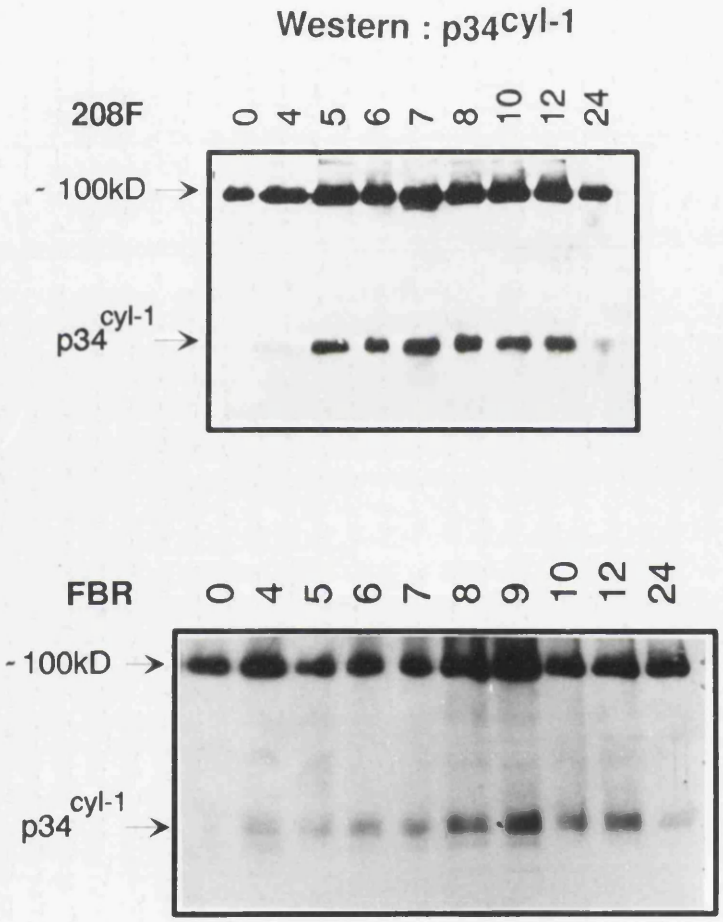


Figure 28. Cell cycle dependent oscillation pattern of $p34^{cyl-1}$ in rat fibroblasts.

Western blot analysis for $p34^{cyl-1}$ expression was carried out on protein extracts derived from G0 arrested and stimulated 208F (top) or FBR (bottom) rat fibroblast cell lines (as described in materials and methods). Numbers represent hours from serum stimulation when the extracts were obtained. Arrows specify the $p34^{cyl-1}$ protein and the ~100kD fibroblast-specific polypeptide of unknown origin.

Figure 28.


Western : p34cyl-1
208F
0 4 5 6 7 8 10 12 24
- 100kD
p34cyl-1
FBR
0 4 5 6 7 8 9 10 12 24
- 100kD
p34cyl-1

in this study. Unexpectedly, the same was not evident for $p34^{cyl-1}$ expression in a portion of analysed rat fibroblasts in culture where similar levels of the cyclin protein were observed in growing and arrested cells. No correlations were found between cyclin D1 levels and the degree of *fos* expression or induced transformation in the fibroblast cell lines, suggesting that the lack of $p34^{cyl-1}$ down-regulation in G0 was due to the frequency of arrest in the examined populations. Subsequent analysis of cells at specific time points from serum stimulation confirmed previous observations in CSF-stimulated mouse macrophages, where metabolic labelling with [^{32}P]orthophosphate or [^{35}S]methionine revealed that the level and overall phosphorylation of $p34^{cyl-1}$ (referred to as $p36^{cyl}$ in this report) increased before the G1/S transition phase and declined thereafter (Matsushime *et al* 1991).

The cellular substrates of the cyclin D1 protein remain unclear. To date most of the predicted targets such as pRb, p107, PCNA and p53 function in the nucleus (Morris and Mathews 1989, Bravo and Macdonald-Bravo 1985, Goodrich *et al* 1991, Hamel *et al* 1992, Cobrinik *et al* 1992, Lane 1992). The exclusive nuclear localisation of $p34^{cyl-1}$ at all stages of the cell cycle in fibroblast cell lines examined in this study and one previous report coupled with the observed peak in expression during late G1, supports the predicted functioning of cyclin D1 during the G1 to S transit (Baldin *et al* 1993). Further evidence for this proposal was obtained from interference with cyclin D1 expression by microinjection of antisense plasmids or antibodies into serum stimulated fibroblasts emerging from quiescence (Baldin *et al* 1993). The reported cell cycle arrest following injections prior to or eight hours from serum stimulation and the lack of similar responses in cells injected sixteen hours post-stimulation, suggested that a critical cyclin D1-induced event(s) had taken

place early to mid G1 and was essential for DNA synthesis, thus providing sound support for a specific G1/S role of cyclin D1 (Baldin *et al* 1993).

CHAPTER 6 : $p34^{cycD1}$ oscillations during the keratinocyte cell cycle.

6.1 Introduction.

To date there have been no reports of $p34^{cycD1}$ expression levels and oscillation patterns in normal or transformed keratinocyte cells. In humans amplification and subsequent over-expression of the *CCND1* gene is linked predominantly to this cell type in head and neck, breast, lung, colon and oesophageal carcinomas (Lammie *et al* 1991, Faust and Meeker 1992, Jiang *et al* 1992, Schuuring *et al* 1992a, Buckley *et al* 1993, Leach *et al* 1993). Due to their inability to efficiently arrest in response to serum starvation, cell cycle analysis of SCC-derived cell lines is potentially problematic.

Under certain circumstances, a SCC cell line analysed in this study (SCC12-F) was able to slow down proliferation in response to low serum conditions for a sufficient time period to determine the oscillation pattern of $p34^{cycD1}$. Further examination of cervical or HNSCC-derived cell lines, revealed different levels of serum requirement that could not be correlated with their tumourigenicity potential or TNM (tumour, node, metastasis staging) of the original biopsies.

6.2 The SCC12-F AC3 cells.

The SCC12-F cell line was derived from a facial SCC of a 60-year old male kidney transplant recipient who had been treated with immunosupressive agents for a period of seven years (Rheinwald and Green 1975). Upon establishment, the cell line was tested for growth properties, revealing a reduced dependence on fibroblast support and the ability to form colonies in

semi-solid methylcellulose medium with an efficiency of approximately 0.02%. Injection of $3x10^6$ cells into immunodeficient mice resulted in the formation of well differentiated cysts that did not progress during the life of the mouse (Rheinwald and Beckett 1981). Subsequent subcloning of cells was carried out to select for variants with the most similarities to primary keratinocytes leading to the derivation of cell line SCC12-F AC3, some properties of which were examined in this study (Dr Ken Parkinson, Beatson Institute, personal communication).

SCC12-F AC3 cells (subsequently referred to as SCC12) were cultured in the absence of x3T3 fibroblasts in 10% FBS containing growth media. However, upon trypsinisation and plating in low serum (0.5% FBS) an approximately 100 hour delay in growth was observed (Figure 29) that was subsequently overcome, suggesting a long-term low growth factor requirement of these cells for proliferation.

The temporary delay in cell cycle progression was used in this study as a means to obtain partial cell cycle synchrony. SCC12 cells that had been exposed to low serum for 24 hours were stimulated with 10ng/ml EGF and pulsed for 1 hour with BrdU prior to fixation in methanol/acetone, as described in section 2.2.4.d. Increased BrdU incorporation was evident at 16 hours from growth factor stimulation and was maximal by 24 hours, suggesting that the majority of cells had entered the S phase (Figure 30). Therefore, the temporary arrest caused by serum starvation was proposed to have taken place in SCC12 cells during G1. Detection of $p34^{cycD1}$ expression by western blot (Figure 31) or immunocytochemical (Figure 30) analyses in starved and stimulated SCC12 cells revealed a similar pattern to the one previously described in rodent fibroblasts (section 5.4). Starved cells exhibited a reduction in detectable

$p34^{cycD1}$, the expression of which increased four hours from growth factor stimulation, peaking at eight hours and subsequently declining. Equivalent to prior observations, minimal levels of $p34^{cycD1}$ were observed only during arrest, while in proliferating cells a basal level of the protein appeared to be retained regardless of their cell cycle positions.

6.3 Effects of serum starvation on some tumour cell lines.

Cell lines BICR6, BICR16, BICR18, BICR22, MS-2 and A431 were subjected to the same type of analysis as previously described for SCC12 cells (section 6.2). Protein extracts were obtained from assynchronously growing populations and cells that had been exposed to reduced serum conditions for 24 or 48 hours. Examination of PCNA expression in BICR16 and MS-2 cells by western blot analysis, revealed a fractional reduction in the levels of this protein following 24 hours of starvation in comparison to the cells treated with low serum for 48 hours, suggesting at least a partial although very short cell cycle delay. The increase in PCNA protein levels at 48 hours in the absence of any stimulation, revealed the low requirement of these cell lines for growth factors. BICR6, BICR18, BICR22 and A431 cells exhibited unaltered PCNA expression upon serum deprivation, with A431 cells revealing increased levels of this protein in comparison to assynchronously growing populations (Figure 32). Examination of $p34^{cycD1}$ levels in the same extracts revealed largely unaltered expression of this cyclin upon serum removal, with slight decreases evident in BICR16 and MS-2 cells (Figure 33). The overall conclusions obtained from these studies implied that serum deprivation was not a good means to induce cell cycle synchrony in most SCC cell lines.

Figure 29. The growth properties of SCC12 cells in low serum.

$3x10^2$ cells were plated onto 24 well dishes in low serum and the rate of growth monitored at set time intervals by the use of a Coulter counter. The rate of growth is represented diagramatically.

Figure 29.

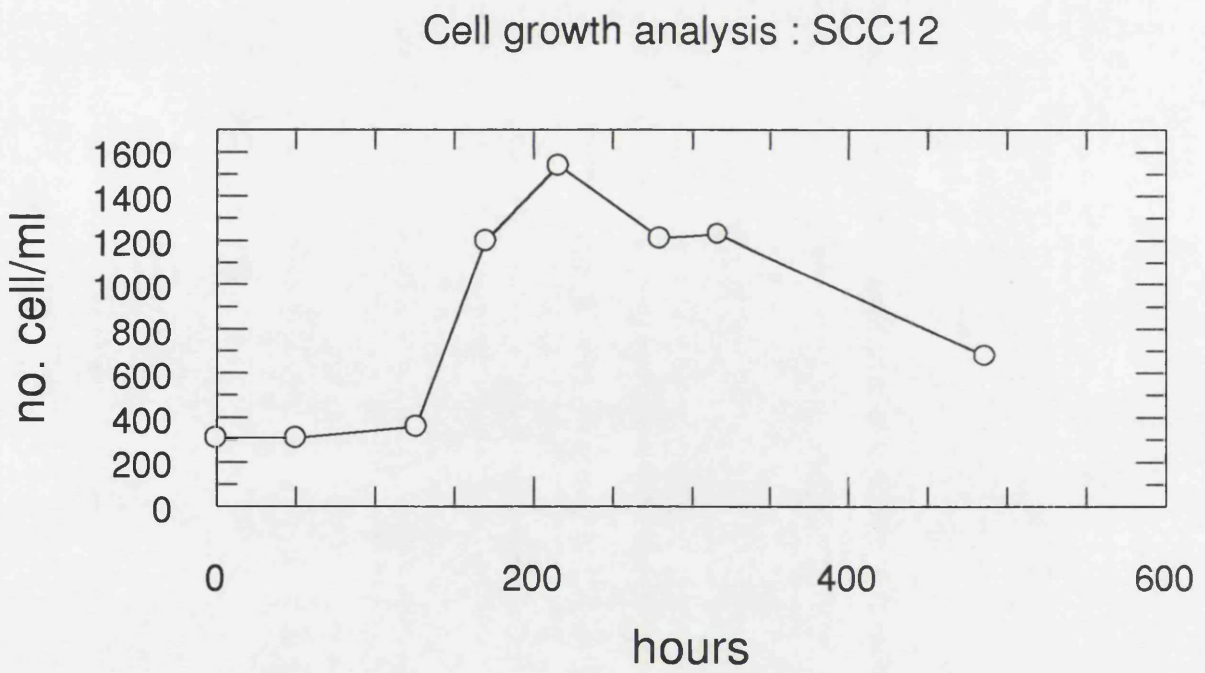

Cell growth analysis : SCC12
no. cell/ml
1600
1400
1200
1000
800
600
400
200
0
0
200
400
600
hours

Figure 30. Immunocytochemical analysis of the SCC12 cell line.

SCC12 cells were exposed to 10ng/ml of EGF 24 hours after plating in low serum. Half of the samples were exposed to 10μM BrdU 1 hour prior to fixation in methanol:acetone. Fixation was carried out at different time intervals from stimulation and the cells were subjected to immunocytochemical analysis for BrdU incorporation. The other half were immunocytochemically analysed for $p34^{cycD1}$ subcellular localisation and cell cycle dependent oscillation pattern. Minus signs represent negative controls in which the primary antibody was not included and in the case of $p34^{cycD1}$, pre-immune rabbit serum was used at the same (1:3000) dilution as antibody 287-3; **-Br** represents cells that did not contain incorporated BrdU and were exposed to the anti-BrdU antibody. Numbers specify hours from serum stimulation and **as.** were assynchronously growing cells.

Figure 30.

Immunocytochemistry : p34^{cycD1} and BrdU

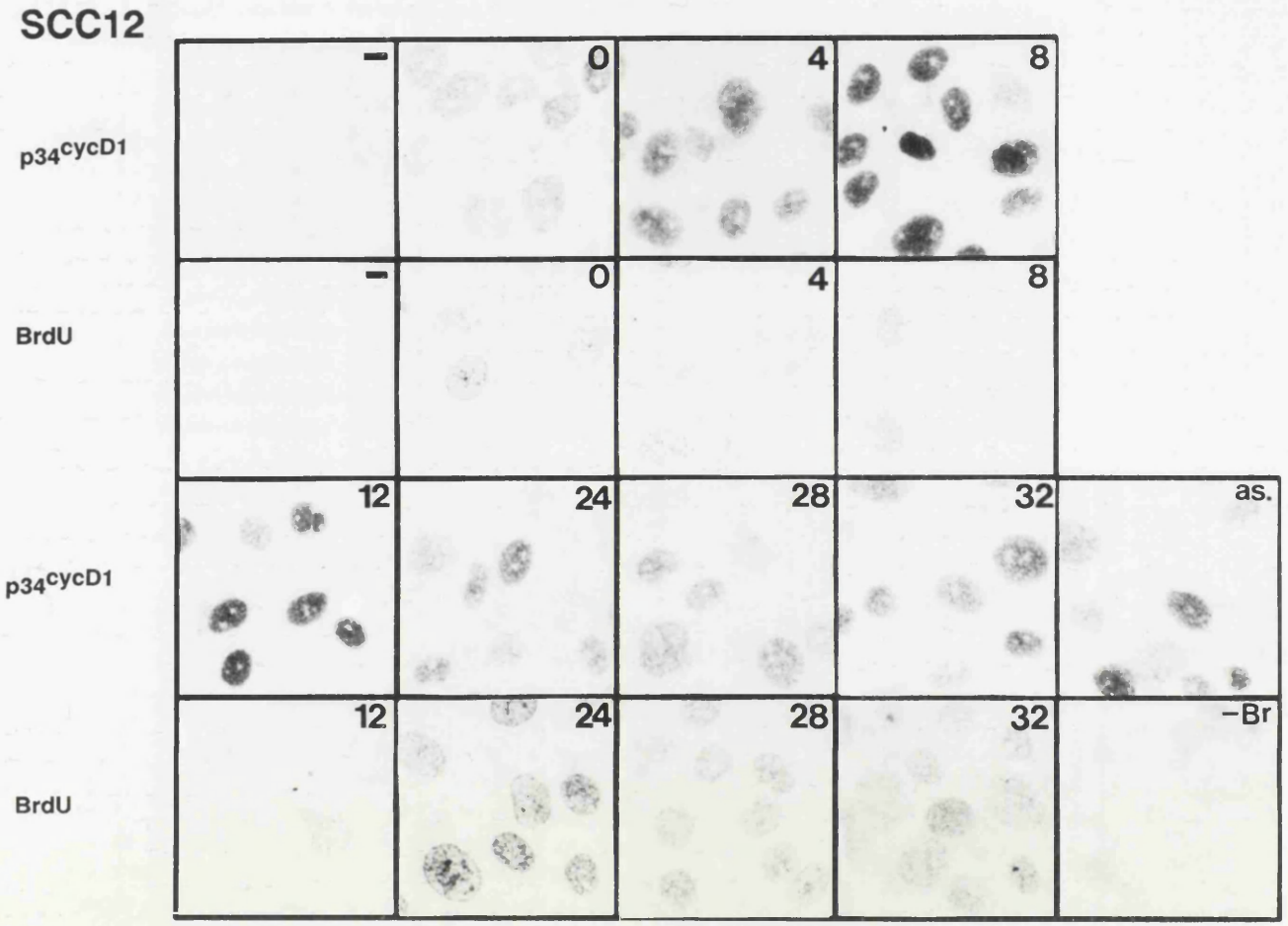


Figure 31. Cell cycle dependent oscillation pattern of $p34^{cycD1}$.

SCC12 cells were exposed to 10ng/ml of EGF 24 hours from plating in low serum and protein extracts were obtained at set time intervals from stimulation (hours from stimulation are depicted by numbers). 40μg of each protein extract were electrophoresed through a 10% denaturing polyacrylamide gel, blotted and subjected to western blot analysis as described in materials an methods. The position of $p34^{cycD1}$ is shown.

Figure 31.

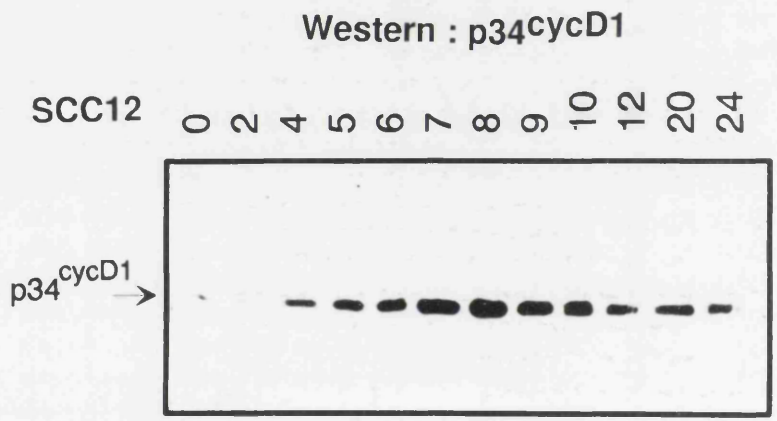


Figure 32. Serum requirement of SCC cell lines.

Cell lines BICR6, BICR16, BICR18, BICR22, MS-2 and A431 were plated in low (0.5%) serum and protein extracts obtained 24 and 48 hours from plating. 35μg of extracts were electrophoresed through a 10% denaturing polyacrylamide gel in parallel with protein samples obtained from assynchronously growing cells, blotted and subjected to western blot analysis (as described in materials and methods). PCNA was detected with the use of 1:1000 dilution of an anti-PCNA mouse monoclonal antibody.

Figure 32.

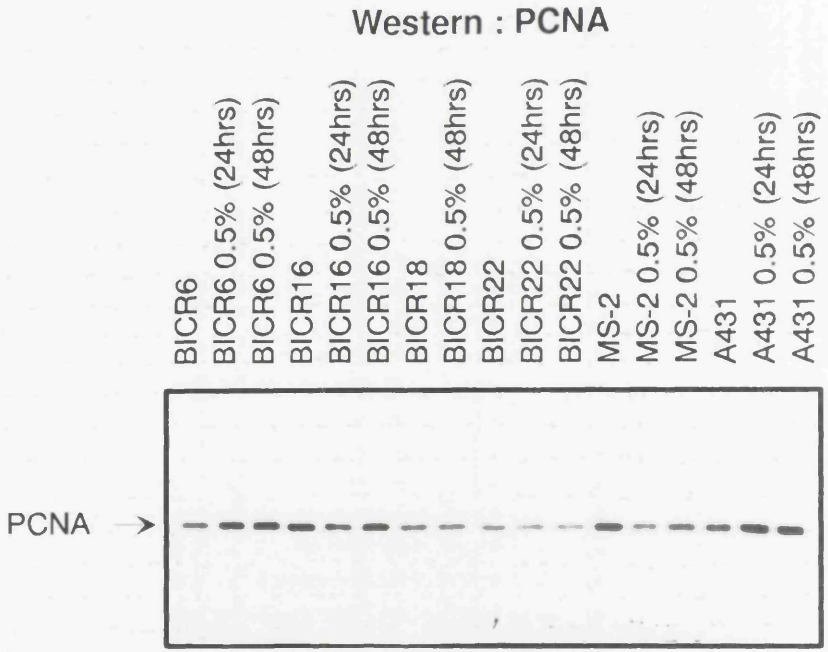
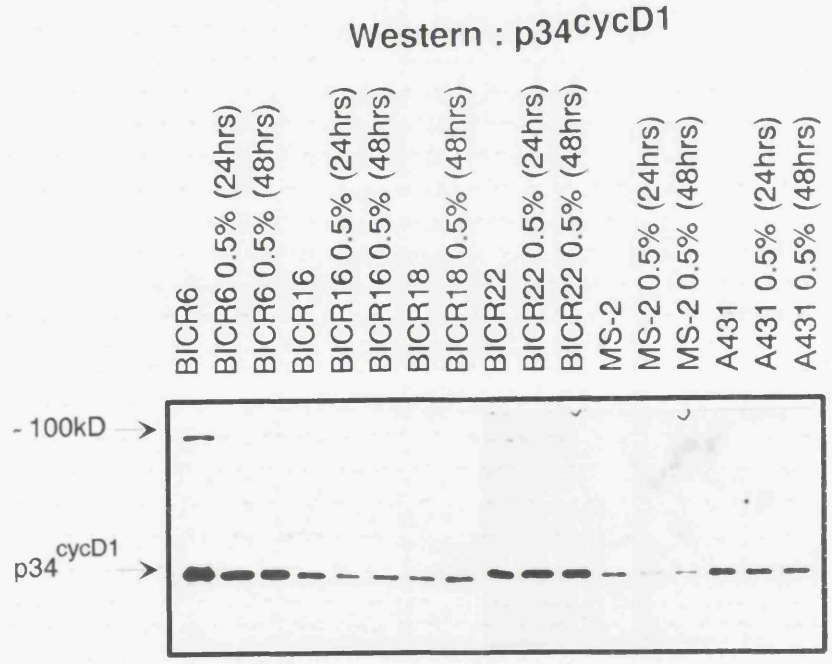

Western : PCNA
BICR6
BICR6 0.5% (24hrs)
BICR6 0.5% (48hrs)
BICR16
BICR16 0.5% (24hrs)
BICR16 0.5% (48hrs)
BICR18
BICR18 0.5% (48hrs)
BICR22
BICR22 0.5% (24hrs)
BICR22 0.5% (48hrs)
MS-2
MS-2 0.5% (24hrs)
MS-2 0.5% (48hrs)
A431
A431 0.5% (24hrs)
A431 0.5% (48hrs)
PCNA

Figure 33. $p34^{cycD1}$ expression in serum deprived SCC cell lines.

The same protein extracts described in Figure 32 were subjected to western blot analysis with the use of an anti-cyclin D1 rabbit polyclonal antibody 287-3. Arrows point to the $p34^{cycD1}$ protein band and the contaminating fibroblast-specific ~100kD polypeptide.

Figure 33.


Western : p34cycD1
BICR6
BICR6 0.5% (24hrs)
BICR6 0.5% (48hrs)
BICR16
BICR16 0.5% (24hrs)
BICR16 0.5% (48hrs)
BICR18
BICR18 0.5% (48hrs)
BICR22
BICR22 0.5% (24hrs)
BICR22 0.5% (48hrs)
MS-2
MS-2 0.5% (24hrs)
MS-2 0.5% (48hrs)
A431
A431 0.5% (24hrs)
A431 0.5% (48hrs)
- 100kD
p34cycD1

6.4 Discussion.

The oscillation pattern of the cyclin D1 protein, following growth factor stimulation of some serum deprived human keratinocyte cell lines, is consistent with previous observations made for fibroblast cultures. This would imply that upon serum starvation these cells delay further cell cycle progression and temporarily reside in the G1 phase, a proposal reinforced by the appearance of BrdU incorporation approximately 12 hours from stimulation. However, the proliferative delay revealed for SCC12 cells was absent from the majority of HNSCC derived cell lines. BICR18 and BICR22 originated from metastatic tumours and in tissue culture exhibited optimal growth in 2%FBS containing media. Consequently the cells were not highly growth factor dependent and exposure to 0.5%FBS had no apparent effect on their rate of proliferation. The BICR6 HNSCC cell line was derived from a T4 stage tumour and exhibited preference for 10% FBS for optimal growth. However, the cells were able to multiply in low serum, had a low requirement for the presence of x3T3 cells, were the only cell line that formed large colonies in soft agar and were highly tumourigenic in nude mice (Edington *et al* submitted). Subsequently detected increased expression of EGFR, pRb and *CCND1* and mutations of the p53 gene, coupled with the previously listed observations, clearly points out the high proportion of accumulated abnormalities of BICR6 cells and several potential causes for growth factor independence (Stanton *et al* submitted, Dr Ken Parkinson, Beatson Institute, personal communication).

Cell cycle delay by serum starvation was achieved in only two cell lines BICR16 and MS-2, with evidence of recovery within 48 hours from treatment. For these reasons serum deprivation was not considered suitable for cell cycle arrest of the BICR and MS-2 cell lines and subsequent analysis of $p34^{cycD1}$

oscillation patterns were not performed. In future, other methods, such as drug treatment would have to be employed to achieve G1 synchrony.

Currently, predictions of $p34^{cycD1}$ oscillation patterns in keratinocytes can therefore be based on the data obtained from the analysis of the SCC12 cell line.

CHAPTER 7 : The effects of *CCND1* over-expression.

7.1 Introduction.

To date, over-expression of cyclin D1 in recipient cells following stable transfection experiments has been reported in only one study. Clones derived from the two transfected murine fibroblast cell lines, NIH3T3 and Rat-2, exhibited reduced generation times and morphological alterations in response to increased *Cyl-1* transcript and protein levels (Quelle *et al* 1993). Clinical studies have revealed amplification and subsequent over-expression of *CCND1* in a significant proportion of SCCs, rendering the analysis of artificially increased cyclin D1 gene dosage essential in these cell types.

The previously described SCC12 cell line was observed to express low (equivalent to normal) levels of cyclin D1 (section 6.2). Upon injection into immunodeficient mice the cells exhibited a poor tumourigenicity potential (Rheinwald and Beckett 1981). These two characteristics made SCC12 cells a good target for transfection analysis and over-expression of *CCND1* was achieved with the use of lipofection techniques. However, despite a resulting three-fold excess of $p34^{cycD1}$, the derived cell line exhibited no alterations in the oscillation pattern of the cyclin D1 protein nor in its tumourigenicity potential, suggesting a role for cyclin D1 in earlier stages of cellular transformation. Highly increased constitutive expression of *CCND1* was thought to have toxic effects on proliferating cells, an observation confirmed in later studies of fibroblast cell lines (Quelle *et al* 1993).

7.2 Expression of exogenous cyclin D1 in SCC12 cells.

The SCC12 cell line was co-transfected (as described in section 2.2.2.a) with one of two *Cyl-1* cDNA expression vectors, in which expression of the coding region of *Cyl-1* in the 5'-3' or 3'-5' orientation was under the control of an internal *cytomegalovirus* (*CMV*) promoter (a kind gift from Dr Clive Dickson, ICRF, London, England; Smith *et al* unpublished data) and the neomycin expression construct pHSG274 (Brady *et al* 1984).

The first attempt to obtain geneticin sulphate resistant ($G418^R$) colonies was unsuccessful due to cell death, correlating well with the proposed function of $p34^{cycD1}$ as a cell cycle regulator and with later reports of cyclin D1 toxicity when constitutively expressed from the same type of vector (Quelle *et al* 1993). However, subsequent attempts to transfect the cells did yield a low success rate. Ninety six colonies were ring cloned from cells transfected with *Cyl-1* cDNA antisense and sense expression vectors of which 4% and 12% grew out into established cell lines, respectively.

Genomic DNA was prepared from the cell lines (as described in section 2.2.3.b-4), digested with restriction endonuclease *EcoR1* that was able to release a 1.3kb *Cyl-1* cDNA fragment from the *CMV* expression vector and subjected to Southern blot analysis. The presence of a 1.3 kb band specific to the cyclin D1 expression vector was detected only in one cell line, termed P1 (Figure 34). The low frequency of stable cyclin D1 cDNA transfections (only one positive cell line out of 48 clones) was consistent with the previous observations reported for NIH3T3 and Rat-2 fibroblasts (Quelle *et al* 1993). In the published report, introduction of several vectors in which cyclin D1 expression was governed by strong promoters such as the *Moloney murine leukaemia virus* (*MuLV*) long terminal repeat (LTR), *myeloproliferative sarcoma*

virus LTR and an internal *CMV* promoter, caused cell death. However, transfections using weak promoters (such as the avian *Rous sarcoma virus* promoter) from which cyclin D1 expression was regulated did not appear to harbour toxic effects on the cells, suggesting that high constitutive over-expression of this protein is fatal for cell viability (Quelle *et al* 1993).

Northern blot analysis was used for the detection of cyclin D1 transcripts in the examined cell lines (as described in section 2.2.3.h). Increased hybridisation of the labelled *Cyl-1* cDNA probe (previously used in Southern blot analysis) to RNA species of 1.1-1.7 kb was observed for the P-1 and E3 cell lines, both of which had been transfected with cyclin D1 sequences in the correct orientation. Interestingly a ~2.0kb transcript was also evident exclusively in the P-1 and E3 cell lines the origin of which is unknown (Figure 34). Reduced levels of the 4.5kb *CCND1* mRNA species were observed in one clone (D2) into which exogenous *Cyl-1* cDNA in the antisense orientation had been introduced (Figure 34). The same membrane was subsequently hybridised with a *GAPDH* specific probe to determine RNA loading.

Western blot analysis of extracts obtained from assynchronously growing cells was carried out with the use of the previously described cyclin D1 protein specific polyclonal antibody, 287-3. This revealed a three fold over-expression of the protein in cell line P-1, while the levels were equal to those observed for SCC12 cells in all the remaining clones (Figure 34). The reduction in *CCND1* transcript levels previously observed for cell line D2 were not followed by similar behaviour of the protein, suggesting poor or absent interference of the 3'-5' *Cyl-1* sequences with the endogenous expression of *CCND1*.

7.3 Analysis of two SCC12-derived clones P-1 and A3.

Microscopic analysis of confluent and subconfluent SCC12 cells and derived clones revealed morphological differences. Previous reports had suggested a cyclin D1 mediated reduction in cell size in fibroblast cultures that were over-expressing exogenous *Cyl-1* (Quelle *et al* 1993). Paradoxically, a significant increase in cell volume was observed for the P-1 clone that exhibited a three fold over-expression of exogenous cyclin D1 (Figure 35). Examination of the parental SCC12 cell line revealed considerable variation between individual cell sizes, suggesting clonal selection as a cause of the diversity observed in the newly derived cell lines.

In an attempt to compare growth factor requirements of the individual cell lines, the same type of assay as described in section 6.2 was used. SCC12, P-1 and A3 cells were trypsinised and cultured in conditions of reduced serum (DMEM containing 0.5% FBS). Cells were harvested at regular time intervals and subjected to Coulter counter analysis to determine their rate of proliferation. The examination revealed no differences in the growth patterns of all the cell lines from those previously described for SCC12 (see section 6.2). A temporary cell cycle delay was observed during the initial 100 hours from starvation which was subsequently overcome, regardless of the differences in cyclin D1 expression (Figure 36).

The cell cycle position of serum starved SCC12 cells, determined by the degree of BrdU incorporation coupled with the oscillation pattern of the $p34^{cycD1}$ protein, were described in section 6.2, Figure 30. Exposure of P-1 and A3 cells, that had been plated in 0.5% FBS, to 10ng/ml of human EGF and subsequent BrdU pulsing one hour prior to fixation, revealed the same pattern and timing of DNA synthesis as described for SCC12 cells (Figures 37 and 38).

Previous reports on *Cyl-1* over-expressing fibroblast cultures had suggested cyclin D1 mediated reductions in cell generation time caused by a 2-4 hour shortening of G1 (Quelle *et al* 1993). The data obtained in this study did not reveal similar observations for the P-1 cell line.

The temporary G1 delay induced by serum starvation was used to compare $p34^{cycD1}$ oscillation patterns in the parental and derived subclones. Results obtained from western blot and immunocytochemical analyses of the SCC12 cells were described in section 6.2. Although the basal levels of $p34^{cycD1}$ were greater in the P-1 cell line, the cell cycle dependent expression pattern, determined by western blot analysis, had remained unaltered (Figure 39). The same $p34^{cycD1}$ oscillation patterns were observed in A3 cells (Figure 39). Immunocytochemical analysis of serum starved cells that had been stimulated with EGF and subsequently fixed at set time intervals (as described in section 2.2.4.d-2), revealed exclusively nuclear localisation of the cyclin D1 protein and the same pattern of cell cycle dependent appearance as determined by western blot analysis (Figures 37, 38 and 39). The conclusions that can be drawn from these experiments are that increased levels of $p34^{cycD1}$ do not alter the time of appearance or subcellular localisation of the cyclin D1 protein, however, some indications were found for a reduction in growth factor independence in SCC12 cells (A3) transfected with 3'-5' Cyl-1 cDNA expression vectors (Figure 36).

7.4 The effects of cyclin D1 over-expression on tumourigenicity.

The incidence of *CCND1* over-expressing HNSCC and breast carcinomas is high, suggesting that increased levels of cyclin D1 may confer a growth advantage to a developing tumour. The tumourigenicity potential of the

Figure 34. Over-expression of exogenous cyclin D1 in SCC12 cells.

G418 resistant clones obtained from transfection experiments in which SCC12 cells were lipofected with 5'-3' or 3'-5' *Cyl-1* cDNA expression vectors were analysed for the presence and expression of exogenous cyclin D1. SCC12 = parental SCC cell line; P-1 and E3 = 5'-3' *Cyl-1* cDNA transfected clones; A3 and D2 = 3'-5' *Cyl-1* cDNA transfected clones.

20μg of genomic DNA were digested with *EcoR1* restriction endonuclease and subjected to Southern blot analysis (as described in materials and methods). Arrows point to the endogenous (4.0kb, 2.3kb and 2.0kb) and exogenous (1.3kb) *CCND1* bands.

20μg of total RNA extracts were subjected to northern blot analysis, as described in materials and methods. The blot was rehybridised with *GAPDH* as a control for equal loading. Arrows point to the endogenous 4.5kb and 1.7kb transcripts, exogenous 1.1-1.7kb transcripts and an ~2.0kb mRNA specie of unknown origin.

40μg of protein extracts were subjected to western blot analysis, as described in materials and methods. The position of $p34^{cycD1}$ is shown.

Figure 34.

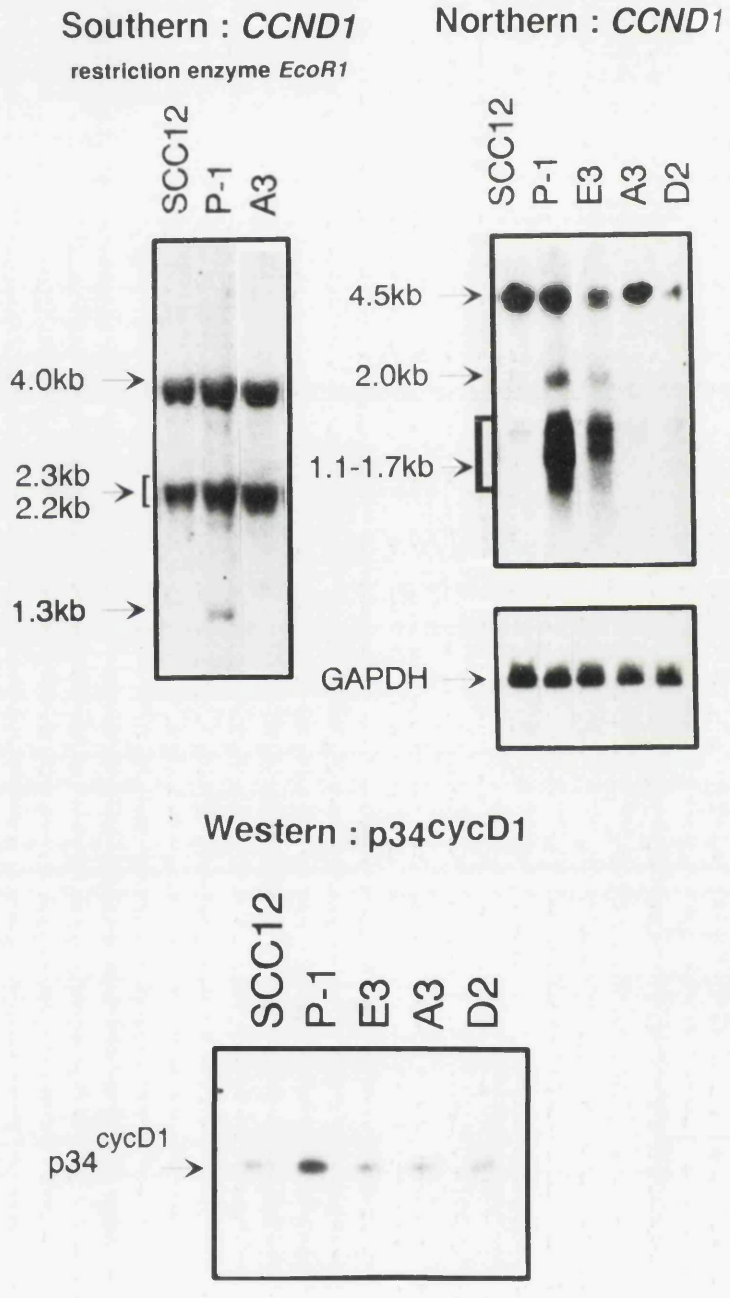

Southern : CCND1
restriction enzyme EcoR1
SCC12
P-1
A3
4.0kb
2.3kb
2.2kb
1.3kb
Northern : CCND1
SCC12
P-1
E3
A3
D2
4.5kb
2.0kb
1.1-1.7kb
GAPDH
Western : p34cycD1
SCC12
P-1
E3
A3
D2
p34 cycD1

Figure 35. Morphological comparison of SCC12 cells and derived clones.

Monolayer morphology of parental SCC12 cells and derived clones (P-1 = 5'-3' *Cyl-1* cDNA transfectants and A3 = 3'-5' *Cyl-1* cDNA transfectants).

Figure 35.

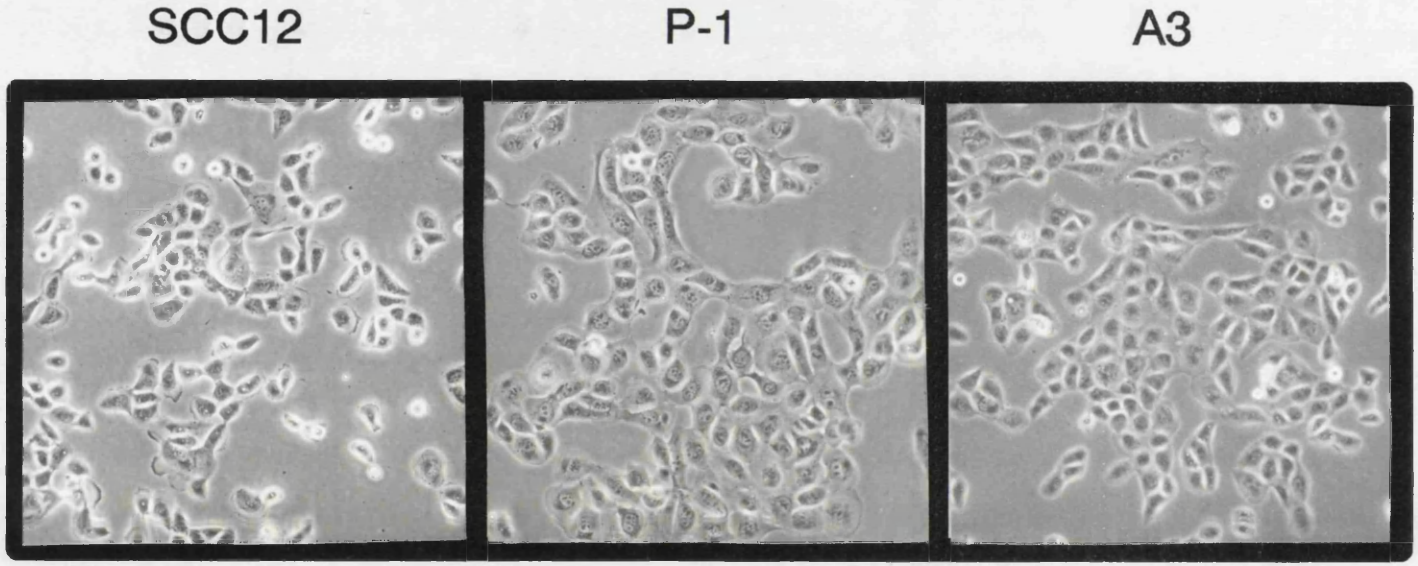


Figure 36. The growth properties of SCC12 cells and derived clones in low serum.

SCC12, P-1 and A3 cells were plated into 24 well dishes ($3x10^2$ per well) in conditions of low serum and subsequently monitored for rates of proliferation. The diagrammatic representation reveals the growth properties and serum requirement of these cell lines.

Figure 36.

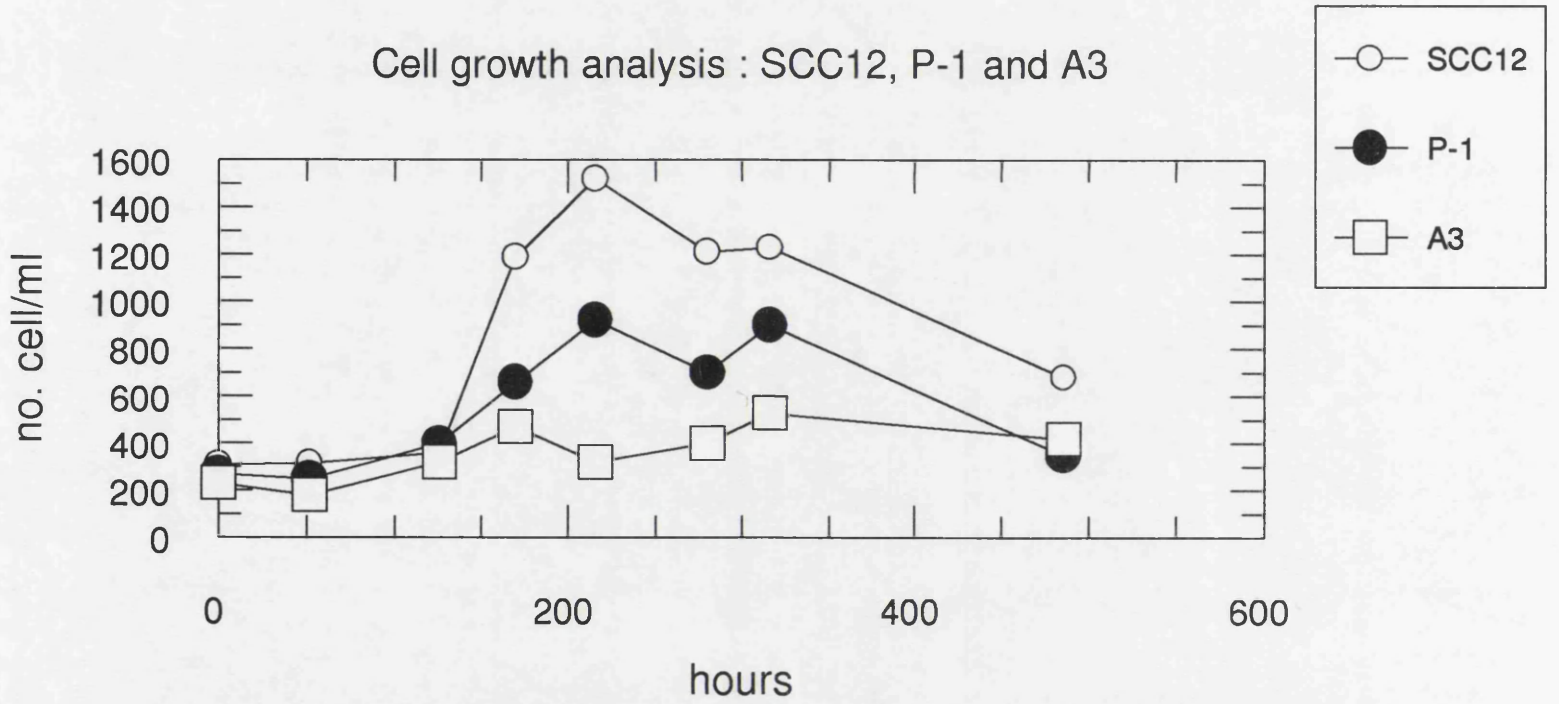

Cell growth analysis : SCC12, P-1 and A3
SCC12
P-1
A3
no. cell/ml
1600
1400
1200
1000
800
600
400
200
0
0
200
400
600
hours

Figure 37. Immunocytochemical analysis of the P-1 cell line.

P-1 cells were exposed to 10ng/ml of EGF 24 hours after plating in low serum. One hour prior to fixation, half of the samples were exposed to 10μM BrdU. Immunocytochemical analysis was used to detect BrdU incorporation and $p34^{cycD1}$ cell cycle dependent oscillation pattern and subcellular localisation as described in materials and methods. Minus signs represent negative controls in which the primary antibody was not included and in the case of $p34^{cycD1}$, pre-immune rabbit serum was used at the same (1:3000) dilution as antibody 287-3; **-Br** represents cells that did not contain incorporated BrdU and were exposed to the anti-BrdU antibody. Numbers specify hours from serum stimulation and **as.** were assynchronously growing cells.

.

Figure 37.

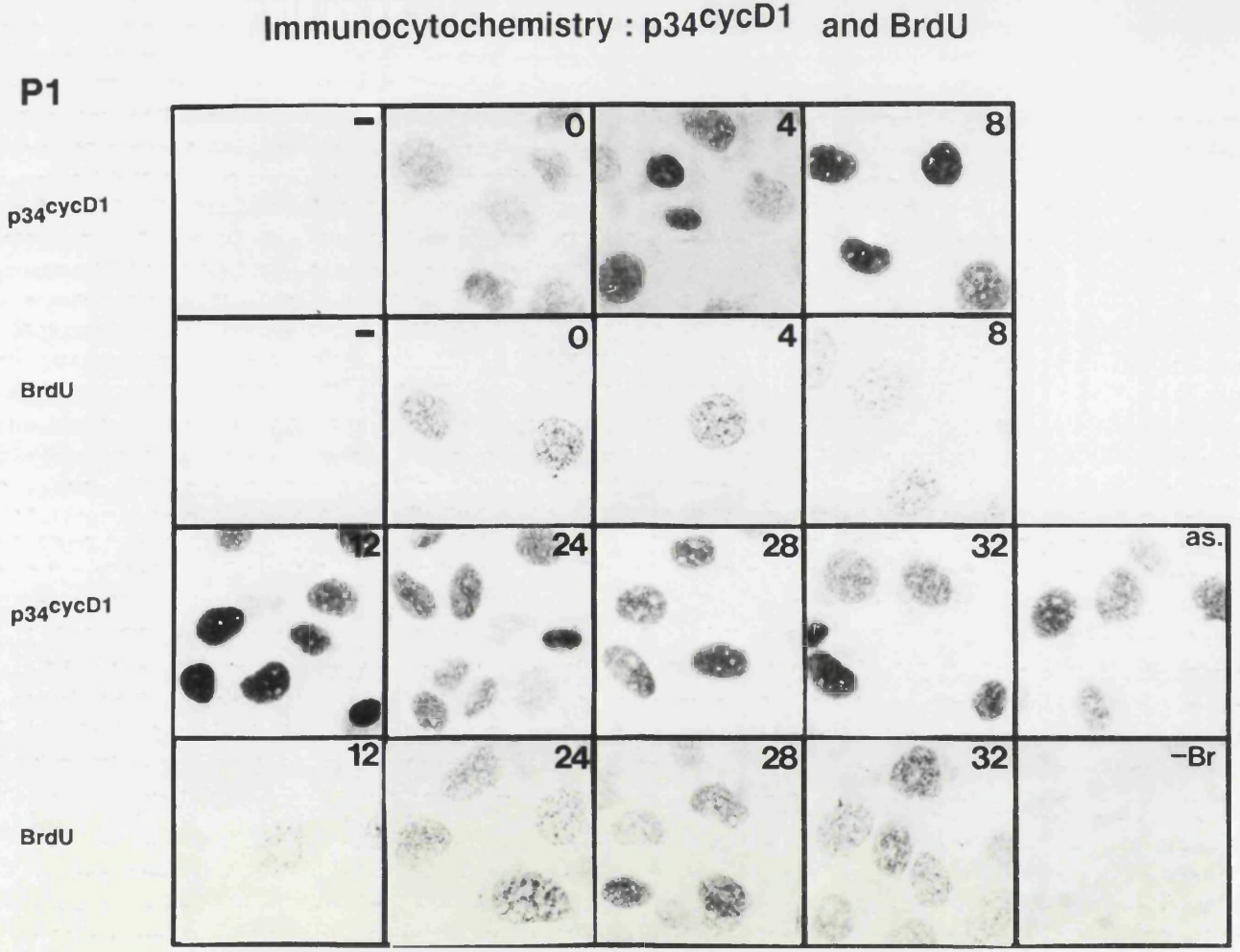

Immunocytochemistry : p34cycD1 and BrdU
P1
p34cycD1
BrdU
p34cycD1
BrdU
-
0
4
8
-
0
4
8
12
24
28
32
as.
12
24
28
32
−Br

Figure 38. Immunocytochemical analysis of the A3 cell line.

A3 cells plated in low serum were subjected to the experimental analysis described in Figures 30 and 37 and in materials and methods. Minus signs represent negative controls in which the primary antibody was not included and in the case of $p34^{cycD1}$, pre-immune rabbit serum was used at the same (1:3000) dilution as antibody 287-3; **-Br** represents cells that did not contain incorporated BrdU and were exposed to the anti-BrdU antibody. Numbers specify hours from serum stimulation and **as.** were assynchronously growing cells.

.

Figure 38.

Immunocytochemistry : p34^{cycD1} and BrdU

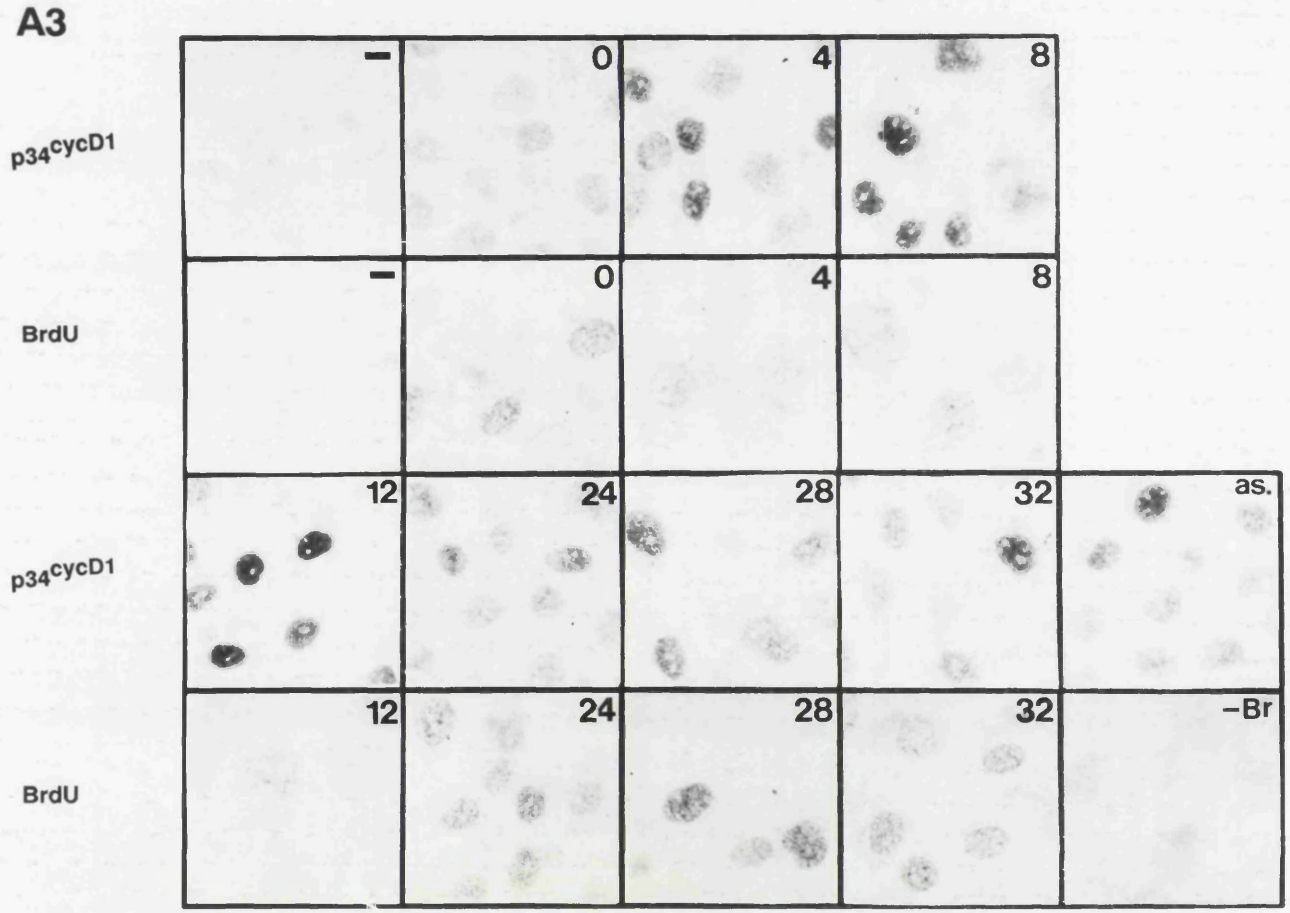
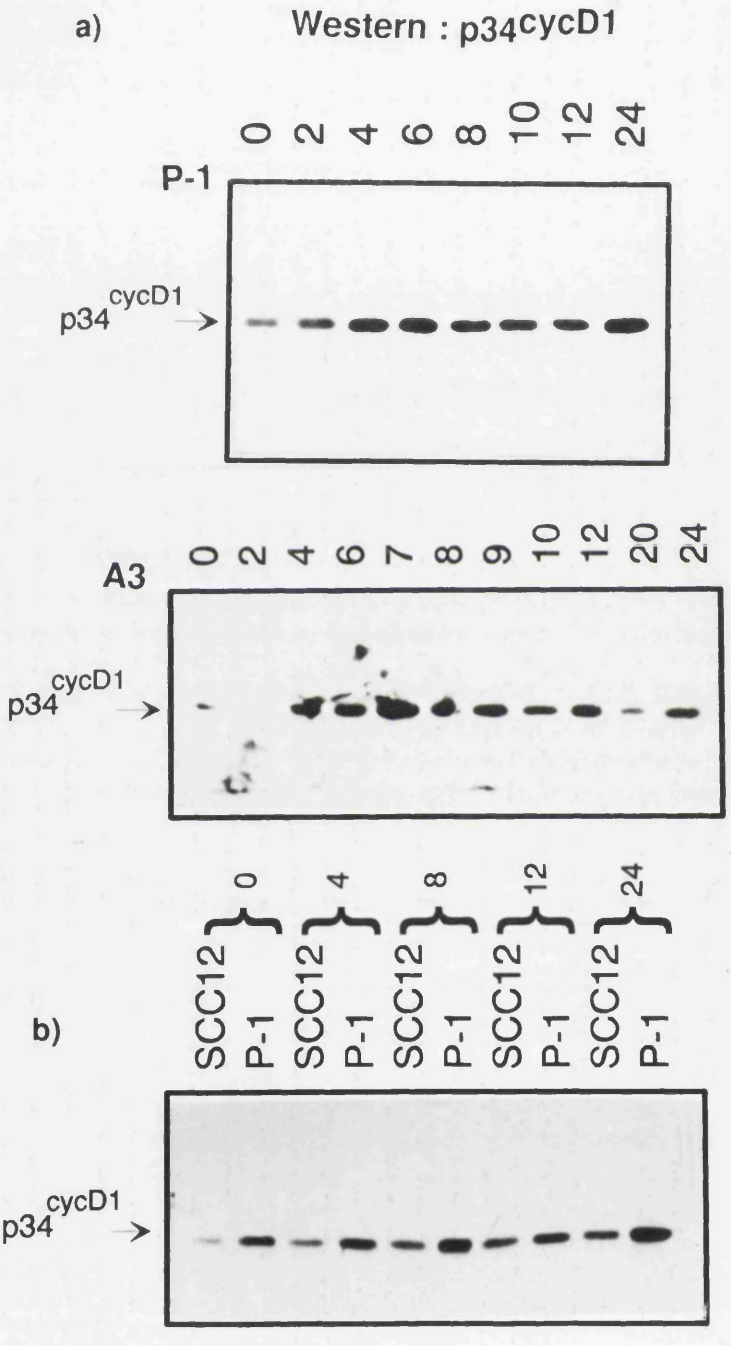


Figure 39. Cell cycle dependent $p34^{cycD1}$ expression levels in P-1 and A3 cell lines.

a) P-1 and A3 cells were exposed to 10ng/ml of EGF 24 hours after plating in low serum and protein extracts were obtained at set time intervals from stimulation (hours from stimulation are depicted by numbers). 40µg of each protein extract were electrophoresed through a 10% denaturing polyacrylamide gel, blotted and subjected to western blot analysis as described in materials an methods. Arrows specify the $p34^{cycD1}$ protein.

b) Protein extracts from P-1 and SCC12 cell lines were compared for levels of $p34^{cycD1}$ expression at 0, 4, 8, 12 and 24 hours from EGF stimulation.

Figure 39.


a)
Western : p34cycD1
0 2 4 6 8 10 12 24
P-1
p34 cycD1
0 2 4 6 7 8 9 10 12 20 24
A3
p34 cycD1
b)
0 4 8 12 24
SCC12 P-1 SCC12 P-1 SCC12 P-1 SCC12 P-1 SCC12 P-1
p34 cycD1

SCC12 cell line had previously been examined and was shown to form well differentiated cysts in immunodeficient mice that failed to progress throughout the rodent life span (Rheinwald and Beckett 1981). It was therefore necessary to examine if a three fold over-expression of cyclin D1 in these cells had increased their ability to proliferate in nude mice. Table 7 represents the number of cells injected into the flanks of immunodeficient mice and the subsequent frequency of outgrowth. From the obtained data no alterations were evident in the tumourigenicity potential of the P-1 or A3 cell lines when compared to SCC12 cells, suggesting that increased expression of *CCND1* may not be advantageous at this stage of transformation. Conversely, a three fold over-expression of the protein may have not been sufficient to alter the tumourigenicity of these cells.

7.5 Discussion.

Currently only two reports have concentrated on determining the function and necessity of the cyclin D1 protein in proliferating mammalian cells (Quelle *et al* 1993, Baldin *et al* 1993). $p34^{cycD1}$ was shown to be essential for the transit from G1 to S phases in human and rodent fibroblasts while constitutive over-expression of cyclin D1 was suggested to result in premature DNA synthesis and subsequent generation shortening of the same cell types. To date no data has been published on $p34^{cycD1}$ in keratinocyte cell types.

The previously determined characteristics of SCC12 cells and the three fold over-expression of $p34^{cycD1}$ achieved in this study, rendered this cell line and the derived P-1 clone excellent comparative material (Rheinwald and Beckett 1981). Interestingly, the subsequent observations largely contradicted previous reports made for fibroblast cells, potentially reflecting the differences

Table 7. Tumourigenicity potential of SCC12 cells and derived clones.

$1x10^7$ cells from each cell line were injected into immunodefficient mice and the degree of tumour formation recorded within two months.

Table 7.

cell line	no. mice	no. injections	no. tumours
SCC12	7	11	1
P1	7	11	1
A3	7	11	0

in cell type or the experimental methods used (Quelle *et al* 1993). Over-expression of $p34^{cycD1}$ may have altered the growth properties of P-1 cells, however the reduced growth factor requirement characteristic of most SCC derived cell lines could have hampered the detection of any marginal changes. The induced cell size observed for the P-1 clone opposed previous suggestions of cyclin D1 mediated reduction of rodent fibroblast cell volumes (Quelle *et al* 1993). However, clonal selection of the SCC12 cells which are characterised by differences between individual members within a population, were thought to be responsible for the morphological alterations of the P-1 and A3 cell lines, although clearly the P-1 cells did not exhibit reduced volumes. The use of further methods such as flow cytometry and detailed serum requirement analysis, would be necessary to address the cell size and growth factor requirement inconsistencies between fibroblast and keratinocyte cell lines.

To date several unsuccessful attempts have been made to obtain *CCND1* transgenic mice with detectable phenotypes (Vladimir Grigorijev, Beatson Institute, unpublished data; Dr Clive Dickson and Dr Gordon Peters, ICRF, London, England, personal communication). In this study the injection of P-1 cells into the flanks of immunodeficient mice did not alter the poor tumourigenicity potential of the SCC12 cells. The conclusions drawn from both types of experiments would suggest that cyclin D1 over-expression alone is insufficient to cause transformation or increase the ability of immortal cells to form tumours. A more likely explanation for the frequently observed over-expression of $p34^{cycD1}$ in HNSCC and breast carcinomas is a growth advantage (such as shortened G1 or reduced serum requirement) conferred to these cells at early stages of transformation, contributing to immortalisation.

CHAPTER 8 : The effects of *CCND1* over-expression on immortalisation.

8.1 Introduction.

Normal diploid vertebrate cells have a limited capacity to proliferate, a phenomenon termed replicative senescence characterised by a decreasing mitogen responsiveness and eventual cellular arrest in G1 (Hayflick and Morehead 1961, Wright and Shay 1992). However, *in vitro* transformed cells or cell lines derived from tumour tissues are able to proliferate indefinitely. Species comparative studies have revealed a greater tendency for rodent fibroblast and keratinocyte cell types to overcome senescence than their human counterparts (Wright and Shay 1992). The only agents consistently shown to immortalise human epithelial cells are DNA tumour viruses such as papillomavirus *HPV-16* or *HPV-18*, adenovirus and *SV40* (Ruley 1983, Durst *et al* 1987, Schlegel *et al* 1988, Woodworth *et al* 1989, Pecoraro *et al* 1989, Kaur and McDougall 1989, Wright *et al* 1989, Shay and Wright 1989, Wright and Shay 1992).

A model has been proposed for the programmed mechanism of cellular senescence that consists of two distinct stages (Wright *et al* 1989, Shay and Wright 1989, Wright and Shay 1992; Shay *et al* 1993). Mortality stage 1 (M1) is a process commonly viewed as *in vitro* senescence and involves loss of mitogen responsiveness, the production of a DNA synthesis inhibitor and arrest in G1. Introduction of oncogenes *HPV-16 E6/E7*, *adenovirus-5 E1a/E1b* or *SV40 T* antigen into primary human keratinocytes was shown to frequently bypass or overcome M1 (Ruley 1983, Hawley-Nelson *et al* 1989, Munger *et al* 1989, Wright *et al* 1989, Halbert *et al* 1991, Shay *et al* 1993). The second

mortality stage (M2) encompasses a mechanism(s) that prevents proliferation. Phenomena such as telomere shortening and alterations in DNA methylation are believed to be involved in cell death during M2 (Catania and Fairweather 1991, Harley 1991, Counter *et al* 1992, Wright and Shay 1992, Holliday and Grigg 1993). Inactivation of M2 is postulated to be a mutational event, independent of M1, that may lead to subsequent activation of telomerase (a ribonucleoprotein believed to be responsible for maintaining constant telomere lengths during DNA replication), or an equivalent phenomenon that will free the cells from proliferative control (Wright *et al* 1989, Wright and Shay 1992, Shay *et al* 1993).

p53 and pRb proteins represent direct or indirect host targets for inactivation by the above mentioned viral onco-proteins, implicating the tumour suppressors as functional contributors to M1 and M2 (Whyte *et al* 1989, Scheffner *et al* 1990, Band *et al* 1991, Hubbert *et al* 1992, Vousden *et al* 1993). Both pRb and p53 operate as cell cycle regulators during the G1 phase and are considered potential substrates of the cyclin D1 directed kinase(s) (Goodrich *et al* 1991, Cobrinik *et al* 1992, Lane 1992, Wang and Eckhart 1992). Constitutive over-expression of $p34^{cycD1}$ may therefore cause the previously observed shortening of G1 by inactivating the two growth suppressory proteins (Quelle *et al* 1993). In the long term this may cause the cells to overcome M1 and potentially lead to immortalisation.

The immortalisation properties of $p34^{cycD1}$ were investigated in this study by transfection of *Cyl-1* expression vectors alone or in combination with *HPV-16 E6* and/or *E7* into primary human foreskin keratinocytes. The aim was to determine the effects of $p34^{cycD1}$ over-expression on the lifespan of normal keratinocytes and whether it may function in co-operation with viral E6 and/or

E7 onco-proteins. The observed bypass of M1 was the first evidence for a role of cyclin D1 in early stages of cellular transformation.

8.2 Transfection analyses of HEK cells.

Two types of transfections were carried out on HEK cells, derived from three individuals. Cells were selected for longevity or for clone formation in the presence of G418, the results from which are described below.

8.2.1 HEK cells with extended life spans over-express $p34^{cycD1}$.

HEK124 cells, derived from human foreskin (kindly provided by Dr Ken Parkinson, Beatson Institute) were co-transfected at passage five with various combinations of *Cyl-1* cDNA, *E6* and *E7* expression vectors as described in Table 8. In view of previous reports on the toxicity of cyclin D1 (Quelle *et al* 1993), two alternative expression systems were used : the *CMV* vector described in section 2.1.4 and the same 1.3kb *Cyl-1* cDNA fragment inserted downstream of the inducible *MMTV* LTR in the plasmid pJ5Ω (a kind gift from Dr Clive Dickson and Dr Gordon Peters, ICRF, London, England). The *HPV-16 E6* and *E7* expression constructs pJ4Ω16.E6 and pJ4Ω16.E7, respectively, contained open reading frames of the two viral oncogenes linked to the *Moloney murine leukaemia virus* LTR and were a kind gift from Dr Lionel Crawford (Storey *et al* 1988).

Upon lipofection, the cells were cultured in GIBCO keratinocyte serum free media in the absence of selection (as described in section 2.2.2.b). Samples were frozen at -70°C followed by immersion into liquid nitrogen (as described in section 2.2.1.c) for subsequent comparative purposes with later passages. Initially, rapid outgrowth was observed for HEK124 cells transfected

with *E6/E7*, *E6* alone and the two cyclin D1 expression systems, however the growth advantage of cells into which pJ5Ω-D1 or pJ4Ω16.E6 were introduced was lost after one month of growth. Non-transfected control HEK124 cells entered crisis at passage twelve and subsequently rapidly died. Keratinocytes lipofected with pJ4Ω16.E6, pJ5Ω-D1 and pJ4Ω16.E6/pJ7Ω-D1 stopped proliferating in M1 at passages sixteen, seventeen and nine, respectively, following the same fate as HEK124 cells. However, extended lifespan was observed for the remaining cultures of which cells lipofected with pJ4Ω16.E7/pJ7Ω-D1, pJ7Ω-D1 and pJ4Ω16.E7/pJ4Ω16.E6 survived the longest, although no immortalisation was evident (Table 8).

Genomic DNA and total RNA were extracted from proliferating keratinocytes at early passages following transfection, with the aim to carry out Southern and northern blot analyses for the presence of exogenous cyclin D1 DNA and transcripts, respectively. Unfortunately, the two methods proved to be inappropriate for the analysis of these cells, probably due to the mixed populations and the low titre of transfected cells in culture. For these reasons the more sensitive technique of PCR was employed with the use of short primer sequences specific to cyclin D1 cDNA. The primers were designed to span exon/intron junctions of the *CCND1* gene, joining exons 2 and 3 (primer b2), or 4 and 5 (primer y4), in this way specifically binding to exogenous cDNA at high stringency (Figure 40) (Motokura and Arnold 1993). Optimal conditions (as described is section 2.2.3.c-2) for the cyclin D1 cDNA amplification were established with the use of SCC12 and P-1 cell extracts. Figure 41 represents the results obtained from PCR reactions with the use of primers b2 and y4 on genomic DNA isolated from these two cell lines. The amplified DNA sequences specific to P-1 extracts were of the expected 332bp size and hybridised to

1.3kb [α ^{32}P]dCTP labelled *Cyl-1* cDNA probes at conditions of high stringency (Figure 42). The same methods were used for the examination of transfected keratinocyte cultures. Southern blot analysis of 1:10 dilutions of the PCR amplification products revealed the presence of exogenous cyclin D1 sequences in early passages of the *Cyl-1* lipofected cell populations (Figure 43).

In confirmation of the previously observed decline of *CCND1* mRNA expression in senescent human diploid fibroblasts (Won *et al* 1992), western blot analysis of parental and transfected HEK cells revealed reduced p34^{cycD1} levels in extracts obtained from later passages (Figure 44). Interestingly, up-regulated expression of p34^{cycD1} was not only observed in cells lipofected with *Cyl-1* cDNA expression vectors, but also in keratinocytes into which *HPV-16* sequences had been introduced. Coupled with the recently reported associations between *HPV-16* E7 and cdk2 and the functioning of E7 during the G1 to S transition phase, the results of this study suggest direct or indirect action of viral transforming sequences on the function and regulation of the cyclin D1 protein (Banks *et al* 1990, Tommasino *et al* 1993). Conversely, a transient induction of p34^{cycD1} levels may be due to a lipofection artifact.

Unfortunately, expression of the viral onco-proteins by western blot analysis was not successful, probably due to low protein levels and heterogeneous cell populations. For these reasons subsequent co-transfection experiments were carried out in the presence of G418^{R} markers allowing selection of resistant cells.

Table 8. Transfection experiments in HEK124 cells.

The expression vectors used to tranfect HEK124 keratinocytes and the lifespan of individual populations prior to reaching crisis are represented.

Table 8.

Expression vectors	passage no. reached
none	12
pJΩ16.E6	16
pJΩ16.E7	21
pJΩ16.E6 pJΩ16.E7	19
pJ7Ω-D1	26
pJ5Ω-D1	16
pJ4Ω16.E6 pJ7Ω-D1	9
pJ4Ω16.E7 pJ7Ω-D1	27

Figure 40. The origin of *CCND1* cDNA-specific primers.

24bp primer sequences were designed to span the boundaries between exons 2 and 3 (primer b2) or exons 4 and 5 (primer y4) resulting in an 332bp amplified region from *CCND1* cDNA. The diagram represents the location of the primers and the amplified sequence in the *CCND1* cDNA. Arrow heads mark the exon boundaries.

Figure 40.

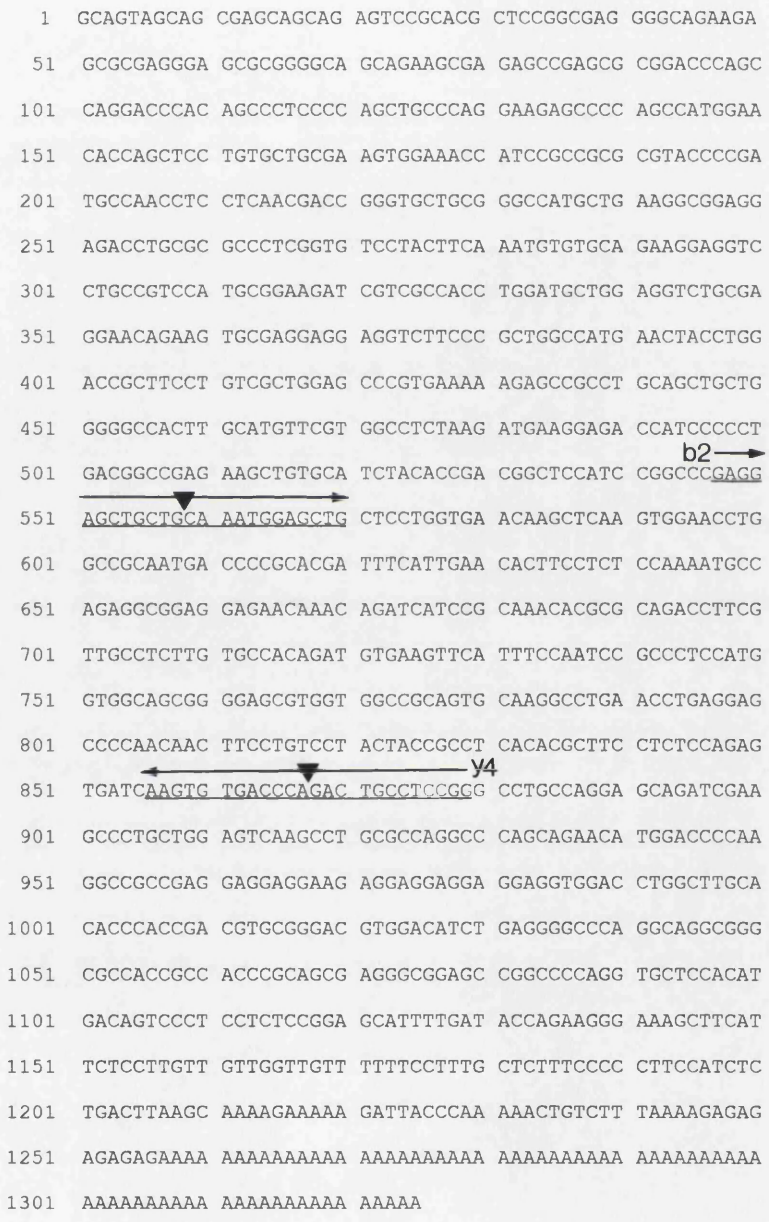

1 GCAGTAGCAG CGAGCAGCAG AGTCCGCACG CTCCGGCGAG GGGCAGAAGA
51 GCGCGAGGGA GCGCGGGGCA GCAGAAGCGA GAGCCGAGCG CGGACCCAGC
101 CAGGACCCAC AGCCCTCCCC AGCTGCCCAG GAAGAGCCCC AGCCATGGAA
151 CACCAGCTCC TGTGCTGCGA AGTGGAAACC ATCCGCCGCG CGTACCCCGA
201 TGCCAACCTC CTCAACGACC GGGTGCTGCG GGCCATGCTG AAGGCGGAGG
251 AGACCTGCGC GCCCTCGGTG TCCTACTTCA AATGTGTGCA GAAGGAGGTC
301 CTGCCGTCCA TGCGGAAGAT CGTCGCCACC TGGATGCTGG AGGTCTGCGA
351 GGAACAGAAG TGCGAGGAGG AGGTCTTCCC GCTGGCCATG AACTACCTGG
401 ACCGCTTCCT GTCGCTGGAG CCCGTGAAAA AGAGCCGCCT GCAGCTGCTG
451 GGGGCCACTT GCATGTTCGT GGCCTCTAAG ATGAAGGAGA CCATCCCCCT
b2
501 GACGGCCGAG AAGCTGTGCA TCTACACCGA CGGCTCCATC CGGCCCGAGG
551 AGCTGCTGCA AATGGAGCTG CTCCTGGTGA ACAAGCTCAA GTGGAACCTG
601 GCCGCAATGA CCCCGCACGA TTTCATTGAA CACTTCCTCT CCAAAATGCC
651 AGAGGCGGAG GAGAACAAAC AGATCATCCG CAAACACGCG CAGACCTTCG
701 TTGCCTCTTG TGCCACAGAT GTGAAGTTCA TTTCCAATCC GCCCTCCATG
751 GTGGCAGCGG GGAGCGTGGT GGCCGCAGTG CAAGGCCTGA ACCTGAGGAG
801 CCCCAACAAC TTCCTGTCCT ACTACCGCCT CACACGCTTC CTCTCCAGAG
y4
851 TGATCAAGTG TGACCCAGAC TGCCTCCGCG CCTGCCAGGA GCAGATCGAA
901 GCCCTGCTGG AGTCAAGCCT GCGCCAGGCC CAGCAGAACA TGGACCCCAA
951 GGCCGCCGAG GAGGAGGAAG AGGAGGAGGA GGAGGTGGAC CTGGCTTGCA
1001 CACCCACCGA CGTGCGGGAC GTGGACATCT GAGGGGCCCA GGCAGGCGGG
1051 CGCCACCGCC ACCCGCAGCG AGGGCGGAGC CGGCCCCAGG TGCTCCACAT
1101 GACAGTCCCT CCTCTCCGGA GCATTTTGAT ACCAGAAGGG AAAGCTTCAT
1151 TCTCCTTGTT GTTGGTTGTT TTTTCCTTTG CTCTTTCCCC CTTCCATCTC
1201 TGACTTAAGC AAAAGAAAAA GATTACCCAA AAACTGTCTT TAAAAGAGAG
1251 AGAGAGAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
1301 AAAAAAAAAA AAAAAAAAAA AAAAA

Figure 41. Specific amplification of *CCND1* cDNA.

1μg of genomic DNA isolated from SCC12 and P-1 cell lines was subjected to PCR amplification with the use of primers b2 and y4 (as described in materials and methods). A control sample was included in the analysis that lacked the presence of primer sequences (blank). DNA molecular weight markers (BRL DNA-VI) were used to determine the size of the amplified DNA product specific to the P-1 cell line.

Figure 41.

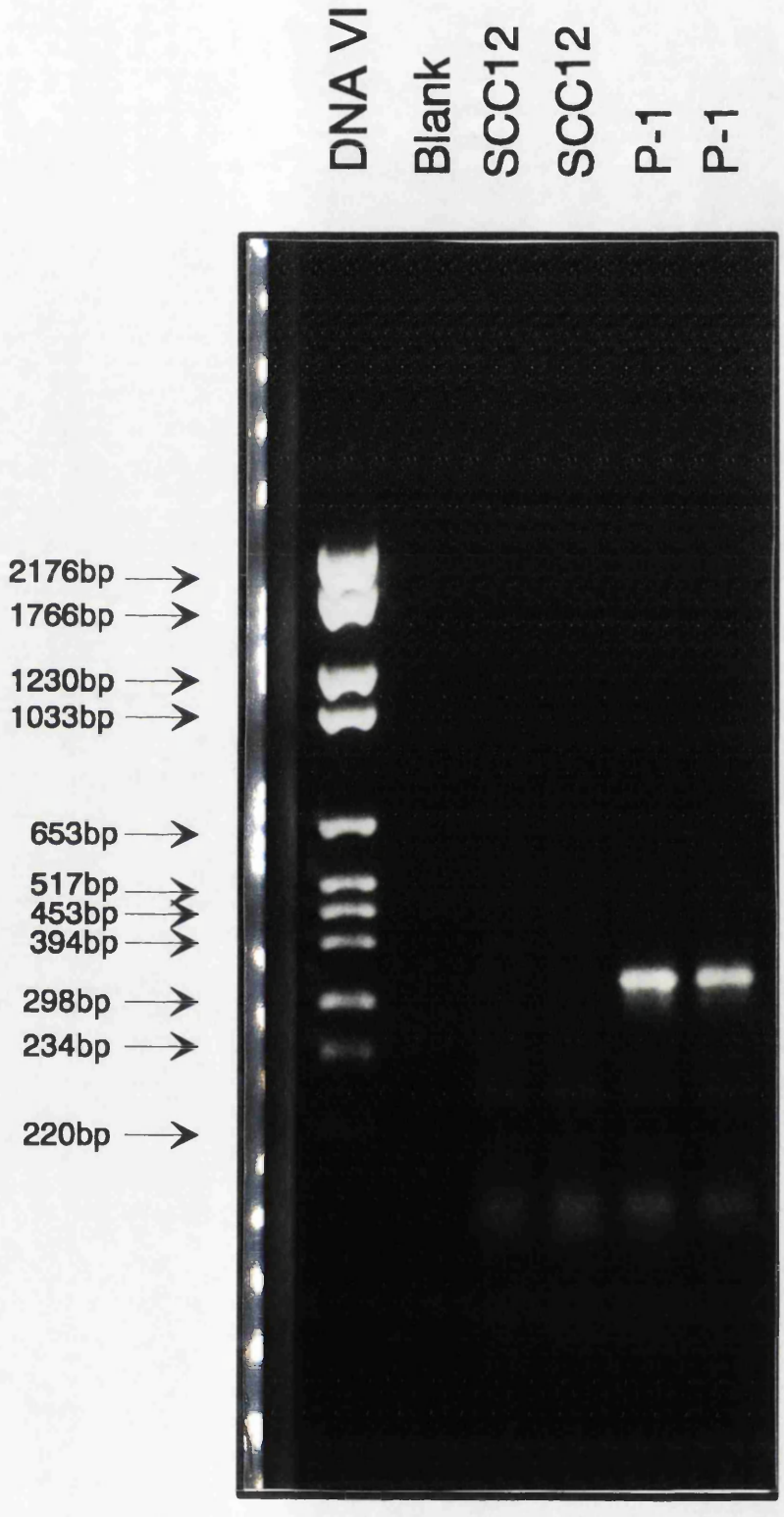

DNA VI
Blank
SCC12
SCC12
P-1
P-1
2176bp
1766bp
1230bp
1033bp
653bp
517bp
453bp
394bp
298bp
234bp
220bp

Figure 42. Southern blot analysis of the PCR amplification products.

0.5μg of genomic DNA obtained from SCC12, P-1, TFK104 and Saos2 cell lines was subjected to PCR amplification with the use of *CCND1* cDNA specific primers b2 and y4. 1μl aliquots of 1:500 diluted products were analysed by Southern blot analysis and probed with a 1.3kb *Cyl-1* cDNA fragment. 1μl of 1:100 diluted PCR product from P-1 cells was included as a strong positive control. The arrow points to the 332bp band specific for P-1 cells.

Figure 42.

Figure 43. Southern blot analysis of the PCR amplification products from HEK124 transfectants.

0.5μg of genomic DNA isolated from *Cyl-1* cDNA transfected HEK cells were subjected to PCR amplification with the use of primers b2 and y4. Two blank control samples were also included : one lacking the primers (blank) and one lacking the genomic DNA (blank-o). The products were compared to those obtained for SCC12 and P-1 cell lines as negative and positive controls respectively, by Southern blot analysis. 1μl aliquots of 1:10 diluted PCR products were probed with a 1.3kb *Cyl-1* cDNA fragment, revealing a *CCND1* cDNA specific 332bp band. A 1:500 diluted PCR amplification product from P-1 cells was also included for comparative purposes. Letters a, b and c mark individual extracts of the analysed HEK transfectants.

Figure 43.

Southern : *CCND1*

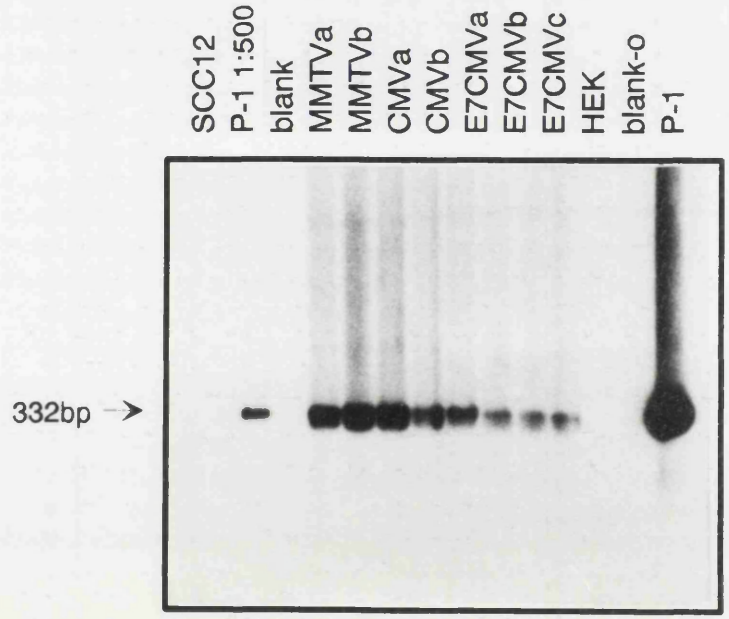
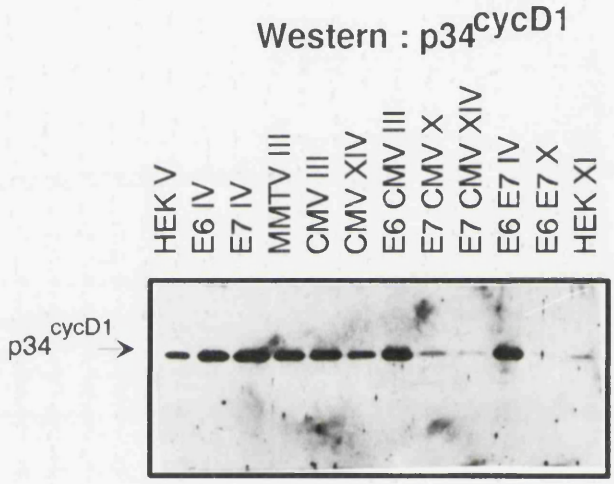


Figure 44. $p34^{cycD1}$ expression in the HEK124 transfectants.

50μg of protein extracts were subjected to western blot analysis with the use of an anti-cyclin D1 rabbit polyclonal antibody 287-3, as described in materials and methods. The position of the $p34^{cycD1}$ protein band is shown. Roman figures depict the passage number of the cell populations from which the extracts were made.

Figure 44.


Western : p34cycD1
HEK V
E6 IV
E7 IV
MMTV III
CMV III
CMV XIV
E6 CMV III
E7 CMV X
E7 CMV XIV
E6 E7 IV
E6 E7 X
HEK XI
p34cycD1

8.2.2 Clone formation frequencies of transfected HEK cells.

HEK34 cells, derived from a different individual than HEK124, were co-transfected with the same expression vectors as described in section 8.2.1 (Table 9) and the G418^R conferring vector pHSG274 (as described in section 2.2.2.b). Clone formation was evident 29 days from transfection and the greatest number of resistant cells were present in pJ4Ω16.E7/pJ7Ω-D1, pJ7Ω-D1, pJ4Ω16.E7/ pJ5Ω-D1 and pJ4Ω16.E6 lipofected cells (see Table 9). Contrary to expectations, a low number of resistant cells was evident in keratinocytes co-transfected with pJ4Ω16.E7/pJ4Ω16.E6 expression vectors suggesting plasmid toxicity or incompatibility, although such observations had not been previously reported (Storey *et al* 1988). However, the *HPV-16 E6* open reading frame cloned into the pJ4Ω plasmid did harbour an abnormality. The C-terminal Leu residue was replaced in the expression system by His-Gly that may have caused loss or altered function of the E6 onco-protein and unexpected results in this study (Storey *et al* 1988). None of the clones survived to form immortal cell lines.

Subsequently, a third lipofection was attempted on a newly derived HEK cell population, HEKb (a kind gift from Caroline Chapman, Beatson Institute), with the use of two different *HPV-16 E6* and *E7* expression vectors pMoE6 and pMoE7, respectively (a kind gift from Dr Karen Vousden, Ludwig Institute for Cancer Research, London, England) (Hawley-Nelson *et al* 1989; Edmonds and Vousden 1989, Vousden and Jat 1989). HEKb cells, cultured in Clonetics serum free media, were co-transfected at passage four with the expression vector combinations depicted in Table 10, including *Cyl-1* cDNA in the incorrect 3'-5' orientation linked to the *CMV* early promoter (pJ7Ω-D1$^{3'\text{-}5'}$). Due to a degree of lipofection reagent toxicity, geneticin selection was carried out one

month from co-transfections during which period the control cells senesced and died. pMoE6 transfected cells initially revealed an increased rate of proliferation that appeared to be temporary, the cells exhibited entry into crisis approximately one month from lipofection (Table 10), in agreement with the previously reported inability of E6 to overcome M2 (Vousden *et al* 1988, Hawley-Nelson *et al* 1989). Significant growth advantage was also temporarily evident for keratinocytes transfected with pJ7Ω-D1 expression vectors, predominantly exhibiting islands of tightly packed cells that outlived the control samples (Figure 45). Transfection with *Cyl-1* expression vectors alone proved to be insufficient to cause immortalisation. Perplexingly, after a period of delay, cells lipofected with pJ7Ω-D1$^{3'\text{-}5'}$ expression vectors alone or in combination with pMoE6 expression constructs exhibited
successful growth in tight clusters and a reduction in cell size (Figure 45). The same phenomenon was absent from pMoE7/pJ7Ω-D1$^{3'\text{-}5'}$ co-transfected cells, suggesting lack of co-operation between the two expression vectors in overcoming growth abortion of primary keratinocytes. The most successful HEKb transfectants are listed in Table 10

Due to the preliminary state of this data no further analysis has been carried out on the transfected keratinocytes. Further examination of proliferative rates, degrees of immortality and exogenous DNA expression levels will be necessary to determine with greater certainty the function and potential co-operation of cyclin D1 and viral onco-proteins in the initial stages of transformation.

Table 9. Transfection experiments in HEK34 cells.

The expression vectors used to transfect HEK34 cells and colony formation efficiencies are represented.

Table 9.

Expression vectors	no. clones formed
pHSG274	4
pJ4Ω16.E6 pHSG274	29
pJ4Ω16.E7 pHSG274	10
pJ4Ω16.E6 pJ4Ω16.E7 pHSG274	7
pJ7Ω-D1 pHSG274	9
pJ5Ω-D1 pHSG274	9
pJ4Ω16.E6 pJ7Ω-D1 pHSG274	12
pJ4Ω16.E6 pJ5Ω-D1 pHSG274	26
pJ4Ω16.E7 pJ7Ω-D1 pHSG274	22
pJ4Ω16.E7 pJ5Ω-D1 pHSG274	23

Table 10. Transfection experiments in HEKb cells.

The expression vectors used to transfect HEKb cells and the lifespan of individual populations are depicted. Blank spaces indicate cells that are still proliferating.

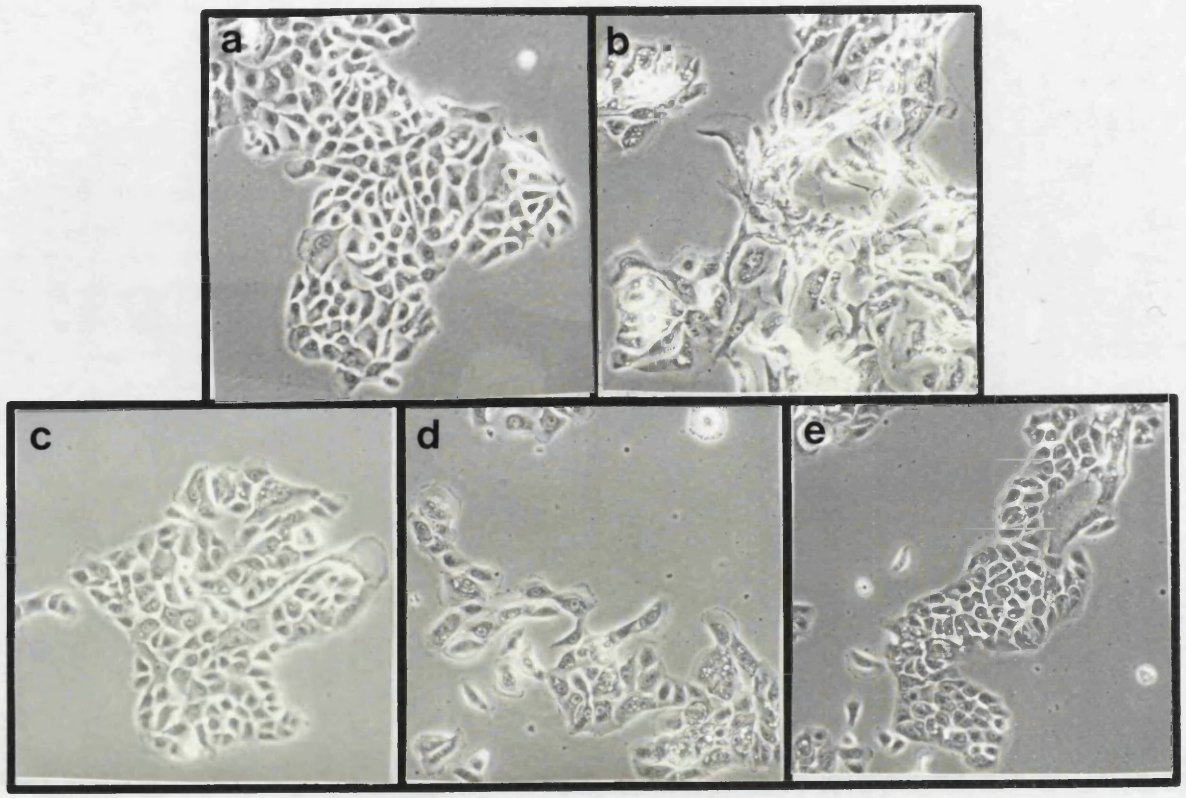
Table 10.

Expression vectors	no. days survived
pHSG274	41
pMoE6 pHSG274	50
pMoE7 pHSG274	60
pMoE6 pMoE7 pHSG274	
pJ7Ω-D1 pHSG274	52
pJ5Ω-D1 pHSG274	55
pMoE6 pJ7Ω-D1 pHSG274	
pMoE6 pJ5Ω-D1 pHSG274	
pMoE6 $^{3'\text{-}5'}$ pJ7Ω-D1 pHSG274	
pMoE7 pJ7Ω-D1 pHSG274	47
pMoE7 pJ5Ω-D1 pHSG274	
pMoE7 pJ7Ω-D1 $^{3'\text{-}5'}$ pHSG274	38

Figure 45. Morphological comparison of *Cyl-1* cDNA and *HPV-16 E6* and/or *E7* transfected HEKb cells.

Monolayer cultures of *Cyl-1* cDNA and *HPV-16 E6* and/or *E7* transfected HEKb cells were compared. **a** - cells transfected with the pJ7Ω-D1 vector containing *Cyl-1* cDNA sequences in the 5'-3' orientation; **b** -differentiating cells characteristic of all populations; **c** - cells co-transfected with vectors pJ5Ω-D1/pMoE7, representing the morphology characteristic of primary HEK cells and pMoE6/pMoE7or pMoE6/pJ5Ω-D1 co-transfectants; **d** - cells transfected with the pJ5Ω-D1 expression vector; **e** - cells transfected with the pJ7Ω-D1$^{3'-5'}$ vector containing *Cyl-1* cDNA sequences in the 3'-5' orientation, characteristic of cells co-transfected with pMoE6/pJ7Ω-D1(3'-5') vectors.

Figure 45.



8.3 Discussion.

The ability of viral oncogenes to immortalise human keratinocytes has on several occasions been linked to their ability to bind and inactivate host suppressor proteins p53 and pRb (Band *et al* 1991, Hubbert *et al* 1992, Pagano *et al* 1992a, Tedesco *et al* 1993, Vousden *et al* 1993), although some contradictions do exist (Jewers *et al* 1992). In the case of *HPV-16*, co-operation of onco-proteins E6 and E7 is thought to result in the highest frequency of immortalisation when compared to the effects of E6 or E7 alone (Hawley-Nelson *et al* 1989, Munger *et al* 1989). The E6 onco-protein was shown to *in vitro* and *in vivo* cause rapid degradation of the host p53 protein via the ubiquitin-dependent protease system, resulting in a significant decrease in the half life (from 4 hours to 15-30 minutes) of the tumour suppressor protein (Scheffner *et al* 1990, Crook *et al* 1991, Band *et al* 1991, Hubbert *et al* 1992, Lechner *et al* 1992). Onco-protein E7 contains a sequence motif (LXCXE) that allows tight binding to the host pRb protein and subsequently resulting in the release of free, transcriptionally active E2F (Pagano *et al* 1992a).

A variety of mammalian proto-oncogenes contain the ability to immortalise murine primary cells, however only one case of *ras*-mediated immortalisation of human epithelial cells has been reported (Sager 1984, Yoakum *et al* 1985). The recently derived nucleotide sequences of the *CCND1* gene have revealed the presence of a pRb-binding LXCXE motif, making it the first mammalian gene harbouring this region and implicating cyclin D1 as a potentially immortalising protein (Dowdy *et al* 1993). The proposed functioning of $p34^{cycD1}$ during the G1 to S phase transition, frequent over-expression in certain types of cancer and potential association with pRb provides indirect evidence for the contribution of deregulated cyclin D1 to immortalisation.

Results obtained from this study have contributed the first indications for the effects of constitutively over-expressed $p34^{cycD1}$ on the life span of human primary keratinocytes. Preliminary results may suggest cyclin D1-induced bypass or blockage of M1.

In view of the proposed pRb-$p34^{cycD1}$ binding and the known effects of E6 on p53, a co-operation of the two onco-proteins was looked for in keratinocyte immortalisation. The *E6* expression constructs in two of the transfection assays were not satisfactory, rendering interpretation of the obtained results uncertain. However, the results of the most recent transfections of HEKb cells, using a different *E6* expression vector (Vousden and Jat 1989, Edmonds and Vousden 1989, Hawley-Nelson *et al* 1989) may suggest a joint effect of E6 and cyclin D1 on cell longevity.

Co-operation between the major *HPV-16* immortalising oncogene, *E7* and cyclin D1 in overcoming M1 and M2 was also hypothesised. In preliminary support for this theory the cells transfected with *Cyl-1* cDNA and *E7* expression vectors revealed a degree of longevity in comparison with the controls. However, the results must be treated with caution and further analysis is essential before any conclusions can be made. A likely disadvantage of the experiments in this study were the low numbers of cells lipofected with each expression vector combination, in this way decreasing the chances of observing positive results. Subsequent co-transfection repeats will therefore be carried out to a larger scale.

The observations obtained following transfection of antisense *Cyl-1* cDNA expression vectors may contradict the concept of cyclin D1 as a proto-oncogene, raising a new aspect to the study. The functional roles of cyclin

D1-directed kinases most likely lie within the catalytic subunits which are only regulated by cyclin D1 association. Due to the observed wide diversity in subunit association, the cyclin D1/cdk kinases may differ greatly in substrate specificity and function. The reduction in cyclin D1 levels at certain points during the cell cycle may allow normally less probable associations between the free cdk subunits and other cyclins and/or new substrates which may in turn contribute to immortalisation, however these findings are currently highly speculative and extremely uncertain, it would be necessary to examine established cell lines to show the efficiency and effects of the transfections with antisence *Cyl-1* expression vectors.

DISCUSSION

CHAPTER 9 : Discussion.

9.1 *CCND1* dosage and expression in the newly derived SCC cell lines.

The significance of *CCND1* over-expression in transformation currently remains unclear, most conclusions have been speculative and often contradictory. To date only two reports have analysed the gene dosage and transcript levels of *CCND1* in human HNSCCs, although frequent chromosome 11q13 amplifications in these types of tumours were observed (Zhou *et al* 1988, Berenson *et al* 1989, Somers *et al* 1990, Lammie *et al* 1991, Schuuring *et al* 1992a). A major aim of the thesis was to examine and understand the consequences of up-regulated $p34^{cycD1}$ in SCCs. A number of omissions made in the two preceding reports influenced the approaches and enquiries used in this study:

(i) Repeated data was generated with regards to the examined HNSCC cell lines of which all were the same, with the exception of UMSCC14B. Furthermore, cell line UMSCC1, initially reported to have normal *CCND1* gene dosage and therefore used as a control (Lammie *et al* 1991), subsequently revealed a two fold amplification of this gene (Schuuring *et al* 1992a). In this study the amplification levels in SCC cell lines were compared to the *CCND1* gene dosage in human foreskin primary keratinocytes,.

(ii) The recorded incidence of *CCND1* amplifications in tumours (30-50%) did not correlate well with observations made in SCC cell lines (~100%) (Schuuring *et al* 1992a). This phenomenon is thought to be due to the conditions in which the cell lines were established. Most of the existing SCC cell lines have been derived from recurrent or irradiated tumours and have been cultured under conditions that favour growth of fitter variants, generating cell lines which may

be non-representative of the original tumour tissues. The advantage of the BICR and MS-2 cell lines is that they were derived and cultured under conditions favouring the growth of normal keratinocytes (Edington *et al* submitted). The frequency of *CCND1* amplifications was ~40%, consistent with the previously reported values in SCC tumours.

(iii) Inappropriate controls were used for RNA loading and transfer during northern blot analysis. Normal cells such as primary keratinocytes were not included in the *CCND1* expression studies. Work carried out in this study revealed differential expression of *GAPDH* in the examined SCC cell lines, an observation supported by the analysis of fibroblast cell types (Dr Bob Hennigan, Beatson Institute, personal communication). For these reasons hybridisation with *GAPDH*-specific probes was carried out only in the analysis of clones derived from the same parental cell line. In the case of the SCC cell lines, hybridisation with ribosomal RNA-specific probes was used for control purposes.

(iv) There has been no published data concerning expression levels, oscillation patterns and subcellular localisation of the *CCND1* protein product in SCCs or any transformed tissues and cell lines. Therefore, part of this study was directed at the analysis of $p34^{cycD1}$ in the newly derived SCC cell lines and tumour or xenograft biopsies. Previous analysis of murine macrophages revealed a peak in $p34^{cycD1}$ expression evident during the second part of G1 (Matsushime *et al* 1991), while only one study has examined the subcellular localisation of this protein in human diploid fibroblasts (Baldin *et al* 1993). The SCC cell lines analysed in this study appeared not to have altered the exclusive nuclear localisation and G1 specific appearance of $p34^{cycD1}$, although problems were encountered attempting to synchronise the cells by

serum starvation (see section 6.3). For these reasons, the cell cycle dependent oscillation patterns of $p34^{cycD1}$ in the tumour cell lines would in future have to be examined in cells arrested by other means, such as the use of blocking reagents, TGFβ or anti-EGF_R antibodies.

In this study the observed *CCND1* gene dosage ranged from tree- to twelve-fold higher than the levels recorded in primary human foreskin keratinocytes, representing the highest range of amplification when compared with previous reports (Lammie *et al* 1991, Schuuring *et al* 1992a). *CCND1* transcript and protein over-expression were evident in all cases exhibiting increased gene dosage, supporting the previously proposed key status of *CCND1* in the 11q13 amplicon (Lammie *et al* 1991, Schuuring *et al* 1992a). However, amplified *CCND1* was not a prerequisite for over-expression, as observed for cell line BICR19 and in previous reports, indicating alternative, although rarer mechanisms for cyclin D1 upregulation in SCCs (Shuuring *et al* 1992a).

Comparisons between the derived *CCND1* sequences from several independent studies has revealed single base changes within the coding region of this gene when compared to normal sequences obtained from human placenta (Motokura *et al* 1991, Withers *et al* 1991, Motokura and Arnold 1993). This observation would suggest other potentially modulating events that could affect the function, kinase subunit and subsequently substrate specificity of $p34^{cycD1}$. Recently *in vitro* constructed dominant-negative cyclin B mutants were able to compete with wild type forms of the protein and successfully abolish the functioning of the cyclin B-directed kinase (Zheng and Ruderman 1993). *In vivo* occurring mutations of *CCND1* may have the reverse effect and result in a dominant positive mutation which could lead to transformation.

However, this hypothesis was recently partially disproved by the sequence comparison of two *CCND1* transcripts derived from a benign parathyroid adenoma and a malignant centrocytic lymphoma with the previously determined normal *CCND1* sequences; no mutations were revealed (Motokura *et al* 1991, Rosenberg *et al* 1993). In this study Southern blot analysis of the restriction enzyme digested SCC cell line genomic DNA did not disclose any alterations in the specific *CCND1* banding pattern (Figure 6). Subsequent sequence analysis could be employed to look for the presence of potential point mutations within the SCC *CCND1* genes, which may account for the high levels of $p34^{cycD1}$ in the BICR19 cell line, potentially altering the half life of this protein.

A more frequently observed *CCND1* abnormality is the shortening of transcript 3' non-coding regions by alternative splicing or additional polyadenylation sequences, suggested to cause an altered stability of the cyclin D1 mRNA (Withers *et al* 1991, Seto *et al* 1992, Motokura and Arnold 1993). In this study northern blot analysis revealed full length *CCND1* mRNA species in all the examined SCC cell lines regardless of the expression levels, therefore indicating the absence of abnormal *CCND1* transcripts (Figures 7 and 10). One of the ways to examine for the potential contributions of mutated *CCND1* to the development of transformation is the *in vitro* construction of mutants and subsequent transfection into primary cells or non-transformed immortal keratinocytes such as the SCC12 cell line (see section 6.2). Subsequent analysis of proliferation rates, longevity and tumourigenicity of the surviving cells would contribute to our understanding of the cyclin D1 role in cell cycle regulation and transformation. Conversely, it is not impossible to postulate a cooperative nature of pRb and $p34^{cycD1}$ interactions in cellular

growth regulation during G1 which may account for the over-expression of pRb in tumours that have amplified *CCND1*. A contradiction to this hypothesis the apparent absence of pRb over-expression in BICR19 cells which lack *CCND1* amplification but do not exhibit over-expression of $p34^{cycD1}$.

Interestingly the three HNSCC cell lines exhibiting increased *CCND1* gene dosage and expression levels, also harboured pRb over-expression, a phenomenon that has not been previously reported. In view of the recently proposed targeting of $p34^{cycD1}$ by pRb, it could be hypothesised that tumours BICR6, BICR18 and BICR22 had upregulated production of the pRb protein in an attempt to over-come the increased $p34^{cycD1}$ expression during earlier stages of transformation (Dowdy *et al* 1993). Conversely, it is not impossible to postulate a cooperative nature of pRb and $p34^{cycD1}$ interactions in cellular growth regulation during G1 which may account for the over-expression of pRb in tumours that have amplified *CCND1*. A contradiction to this hypothesis is the apparent absence of pRb over-expression in BICR19 cells which lack *CCND1* amplification, but do exhibit over-expression of $p34^{cycD1}$, therefore the former hypothesis would appear more likely. Clearly, high levels of apparently wild type pRb in cell lines BICR6, BICR18 and BICR22 were not sufficient to prevent tumour development, probably due to the accumulation of other abnormalities as a consequence of p53 mutations which were evident in all of the BICR cultures (Edington *et al* submitted). The cervical SCC cell line MS-2 exhibited no detectable pRb in the western blot analysis (Figure 16), potentially due to the presence of integrated *HPV-16* early regions into the host genome. However, E7 onco-protein expression is commonly low in infected cells and thought not to be sufficient to sequester all of the endogenously expressed

pRb, suggesting mutations may cause the loss of detectable pRb (Vousden and Jat 1989).

$p34^{cycD1}$ is currently the only discovered mammalian protein that contains the viral onco-protein specific amino acid motif (LXCXE) thought to be essential for the binding of pRb (Dowdy *et al* 1993). This would imply that at least one aspect of cyclin D1 functioning may be mimicked by transforming viral onco-proteins, potentially to a more successful level. Following this argument, the presence of E7 in the MS-2 cell line may have rendered over-expression of endogenous *CCND1* unnecessary. The same explanation could account for the low frequency of *CCND1* amplifications observed in cervical cancer, taking into account the high incidence of *HPV-16*, *-18* and *-33* infections in these carcinomas (DiPaolo *et al* 1993). Furthermore, upon *SV40* transformation, normal human diploid fibroblasts exhibited a two- to three-fold down-regulation of $p34^{cycD1}$ and the same was observed in *HPV-16* infected HeLa cells and *adenovirus* transformed primary rat kidney cells (Xiong and Beach 1993). None of the viral oncogene containing cells exhibited $p34^{cycD1}$ association with the cdk catalytic subunits, suggesting complete loss of the cyclin D1-directed kinases (Xiong and Beach 1993). However, the observed increase in cyclin D1 protein expression in this study following transfection of primary keratinocytes with *E6* and/or *E7* expression vectors, lies in contradiction of the above described proposal. Nevertheless, the presence of E6 and E7 onco-proteins was not shown in transformants potentially due to low expression levels, suggesting that $p34^{cycD1}$ upregulation might represent a transient effect of the lipofection experiments. A more likely explanation is the requirement of other viral genome sequences for specific $p34^{cycD1}$ downregulation. This inconsistency can be explained by the expression of viral

oncogenes from vector promoters in the transfected cells. The apparently "normal" expression levels of $p34^{cycD1}$ in the TFK104 cells is consistent with this proposal. Further examination will be necessary to address these phenomena such as transfection of cells with expression vectors containing different parts of the *HPV-16, SV40* or *adenovirus-5* genomes.

It is not unlikely that cell cycle regulators such as cyclin D1 may exhibit similar types of self-regulation as observed for cyclin B during mitosis. The entry into and progression of a cell through the M phase depends on a negative feedback loop in which cyclin B binds to and activates cdc2. This complex will in turn initiate the cyclin destruction system allowing anaphase completion and the onset of G1. It is widely thought that the negative feedback loop is regulated by specific and currently unknown domains of the cyclin B protein (Prof. Joan Ruderman, Harvard Medical School, Boston, USA, personal communication). The previously observed down-regulation of $p34^{cycD1}$ in virally transformed cells may reflect the activation of the cyclin D1 negative feedback loop induced by the replacement of $p34^{cycD1}$ with highly active viral onco-proteins in specific kinase complexes. Therefore, due to sequence and functional similarities between $p34^{cycD1}$ and viral transforming onco-proteins, $p34^{cycD1}$ may represent the only exception to the general viral-induced cyclin upregulation as part of the mechanism of increased cellular proliferation (Xiong and Beach 1993). However, the proposal directly contradicts the observed $p34^{cycD1}$ increase in this study of *HPV16 E6* and/or *E7* transfected human foreskin primary keratinocytes and is therefore considered unlikely. Analysis of normal human primary keratinocytes and their virally immortalised and /or transformed counterparts, would contribute to the verification of this hypothesis. In previous reports $p34^{cycD1}$ was isolated from a diploid human

lung fibroblast cell line (WI-38) complexed with one of the catalytic subunits (cdk2, cdk4 or cdk5), PCNA and an unknown p21 protein (Xiong and Beach 1993). However, *SV40*, *HPV16* or *adenovirus* transformed WI-38 cells exhibited a loss of these complexes, revealing association of cdk4 and an unknown p16 protein. The same was true for p53 deficient cells derived from Li-Fraumeni patients carrying no known tumour viruses, suggesting an altered cyclin D1 role during transformation.

$p34^{cycD1}$ is currently the most promiscuous cyclin with regards to catalytic subunit specificity and associates with five related but distinct proteins, cdc2, cdk2, cdk4, cdk5 and cdk6 (Motokura *et al* 1991, Xiong *et al* 1992b, Xiong and Beach 1993, Dr Gordon Peters, ICRF, London, England, personal communication). Transformation specific expression of two cyclin D1 regulated catalytic subunits cdk5 and cdk6 has recently been proposed (Peters *et al* 1993). Increased expression of the cdk5 protein (normally expressed only in neurones) as a response to *v-fos* transformation of a rat fibroblast cell line confirmed the previous observations (Lew *et al* 1992, Dr Bob Hennigan, Beatson Institute, unpublished data). To date the general opinion has been that the inactive kinase subunits reside in the nucleus of a cell until the synthesis of a specific cyclin which will in turn regulate the timing and activity of the kinase (Minshull 1993, Pines 1993, Solomon 1993). However, differential expression of certain kinase subunits that may exhibit an increased specificity for cyclin binding or be present at higher concentrations, could successfully compete against normally associated subunits (such as cdk2 or cdk4) leading to altered substrate specificity of the functionally active kinase and subsequent transformation. Over-expression of cyclin D1 observed in several types of tumours may serve to enhance this effect. In this study altered cdk4 expression

was not observed in the BICR and MS-2 cell lines when compared to primary keratinocytes (see section 4.6). However, the examination of cdk5 and cdk6 levels and degree of cell cycle dependent association with $p34^{cycD1}$ in the newly derived SCC cell lines would contribute to the understanding of the involvement of these proteins in transformation. Transfection experiments of mammalian primary fibroblast or keratinocyte cell types with cdk5 or cdk6 expression systems alone or in combination with cyclin D1 cDNA expression vectors, would reveal any immortalising or transforming properties of these kinase complexes. Subsequent immunoprecipitation analysis of the derived clones may determine specific substrates of the different cyclin D1-directed kinases.

9.2 The potential consequences of elevated $p34^{cycD1}$.

9.2.1 Over-expression of exogenous cyclin D1.

Co-transfection of *Cyl-1* cDNA and neomycin sulphate expression vectors into a poorly tumourigenic SCC cell line (SCC12) exhibiting normal levels of $p34^{cycD1}$ and the absence of increased *CCND1* gene dosage (described in section 7.2), was used to address the significance of *CCND1* over-expression in transformation.

Consistent with previous reports, constitutive over-expression of cyclin D1 was observed to have toxic effects on proliferating cells (Quelle *et al* 1993). Only one clone (P-1) was derived that exhibited a three-fold increase of $p34^{cycD1}$ levels at all stages of the cell cycle when compared to parental SCC12 cells, reflecting a low (2%) success rate (see section 7.2). Introduction of *Cyl-1* cDNA in the incorrect orientation also proved to be toxic and the two derived cell lines, D2 and A3, did not contain any detectable plasmid DNA by

Southern blot analysis, suggesting stable transfection only of neomycin sulphate expression vectors. The extensive cell death observed for both sense and antisense types of transfections, implicated tight regulation of endogenous *CCND1* expression levels and the requirement for $p34^{cycD1}$ in normal proliferating cells. The observed tolerance of extensively over-expressed $p34^{cycD1}$ in some of the examined BICR tumours (described in section 4.4) is therefore somewhat perplexing. However, these cells also harbour many additional abnormalities that may have abolished the toxicity of increased cyclin D1 levels (such as the increased levels of pRb), reflecting the progressive stages of transformation. In previous reports five to ten-fold induction of $p34^{cyl-1}$ was achieved in normal human diploid fibroblasts, following the optimisation of transfection conditions, implying that under certain circumstances higher expression of this cyclin can be achieved avoiding any toxic effects (Quelle *et al* 1993). Transfection into keratinocyte cell types is further complicated by their sensitivity to metal ions or calcium which induce differentiation, therefore methods such as previously employed calcium phosphate precipitation techniques cannot be used with these cells (Quelle *et al* 1993). Lipofection as a means of introducing foreign DNA is tolerated by keratinocytes, however this technique generally gives a low copy number of inserted expression vectors per cell and is therefore less efficient. Recently a new, more effective lipofection product has appeared on the market (GIBCO) and could be used to repeat the above described transfection experiments, in addition, electroporation or retroviral infection are two other applicable techniques. Inducible cyclin D1 expression systems could also be used in transfection analysis, potentially achieving higher expression levels in

keratinocyte cells coupled with a more precise means of analysing the levels and effects of cyclin D1 toxicity.

Contrary to previous reports, no reduction in cell size, generation time or serum requirement were evident as a consequence of upregulated cyclin D1 expression, although additional experiments should be carried out to determine the serum responsiveness of these cells and the timing of DNA synthesis (Quelle *et al* 1993). Paradoxically, the cyclin D1 over-expressing cells (P-1) had increased in volume (Figure 35). The obvious explanation for this discrepancy is clonal selection of the parental SCC12 cells, the populations of which are characterised by size differences between individual members. However, taking into account the large volumes of the P-1 cells it is hard to imagine that they may have originated from an even larger parental cell. The low levels of over-expression may have been insufficient to cause any alterations, although none of the HNSCC derived BICR cell lines containing extensive $p34^{cycD1}$ over-expression exhibited exceptionally small cells, casting some doubt on cyclin D1-induced morphological changes in keratinocyte cell types.

9.2.2 Cyclin D1 expression during the cell cycle.

The previously described cell cycle dependent oscillation pattern of the cyclin D1 protein in mouse macrophages and normal human diploid fibroblasts was confirmed in this study (Matsushime *et al* 1991, Baldin *et al* 1993). Serum starved, quiescent murine fibroblasts were nearly completely devoid of $p34^{cyl-1}$ expression, as described in section 5.3. Upon serum stimulation, $p34^{cyl-1}$ levels increased, peaking at eight to twelve hours post-stimulation and subsequently declining. The same observations had previously been made in

human diploid fibroblasts and mouse macrophage cell lines, although studies on mouse macrophages had revealed constantly elevated cyclin D1 transcript levels, the down-regulation of which only took place following removal of CSF-1 from the growth media (Matsushime *et al* 1991, Baldin *et al* 1993).

The difficulties in achieving keratinocyte cell cycle synchrony by serum starvation in this study were described in sections 6.2 and 6.3. Fortunately, the skin SCC cell line SCC12 exhibited a temporary although vastly reduced rate of proliferation following trypsinisation and culturing in conditions of low serum (as described in section 6.2). Consequently, the levels of $p34^{cycD1}$ declined, while subsequent induction of this protein was achieved by the exposure of the cells to saturating amounts of human EGF and exhibited a similar cell cycle dependent pattern to that observed in fibroblasts (see section 5.4). Over-expression of cyclin D1 in the P-1 cell line did not alter the oscillation pattern of this protein, as described in section 7.3. Specific induction of cyclin D1 transcripts following the exposure to this mitogen had previously been observed in normal human diploid fibroblast cultures synchronised to G0 by serum deprivation (Won *et al* 1992).

9.2.3 The effects of cyclin D1 on tumourigenicity.

Despite the substantial supporting evidence for the proto-oncogenic properties of *CCND1* and its activation through gene rearrangement or amplification, the ability of *CCND1* to transform cells in culture or induce tumours *in vivo* has not yet been established. An insufficient number of HNSCC cell lines were examined in this study to determine any correlations between cyclin D1 over-expression and tumour staging (UICC 1987). However, all three BICR cell lines exhibiting increased *CCND1* gene dosage, transcript and

protein levels (BICR6, BICR18 and BICR22) were derived from either T4 or metastatic SCCs (Table 4). The only examined early stage (T2) tumour-derived cell line (BICR3) lacked detectable *CCND1* amplification or increased expression of the mRNA and protein products, implying that over-representation of $p34^{cycD1}$ may be advantageous at later stages of transformation (Edington *et al* submitted). However, an insufficient number of cells were examined to allow general conclusions to be made. In view of the cyclin D1 induced lifespan of primary keratinocytes observed in this study, it would appear more likely that cyclin D1 amplification and over-expression confers a growth advantage at the initial stages of immortalisation and that this advantage may extend to later stages of transformation, maintaining the selection for *CCND1* over-expressing cells.

Injection of P-1 and SCC12 cells into immunodeficient mice was carried out in this study, with the aim of testing for potentially induced tumour formation by cyclin D1 over-expression. No alterations were observed in the ability of P-1 cells to form tumours in comparison to parental SCC12 cells (described in section 7.4), suggesting that cyclin D1 over-expression alone is insufficient to induce tumourigenesis, consistent with the above proposal. An alternative explanation is based on the low increase of cyclin D1 expression in the P-1 cells when compared to the levels observed in the HNSCC derived cell lines (Table 7). BICR6, BICR18 and BICR22 were the most highly transformed cell lines and formed tumours in nude mice with relatively short latency periods (Edington *et al* submitted). All three cell lines exhibited *CCND1* amplifications and higher transcript and protein expression levels than were achieved in P-1 cells. Contrary to this hypothesis, BICR10-derived cells also exhibited a high tumourigenicity potential but contained equivalent $p34^{cycD1}$ expression levels

to those observed in P-1 cells (see section 4.4). However, the BICR10 cell line was derived from a recurrent T2 stage tumour and contained a heterogeneous mixture of two cell types in the one BICR10 population, therefore rendering the analysis of one cell line impossible (Edington *et al* submitted). The two BICR10 cell types were recently sub-cloned and subsequent analysis will reveal the individual properties of the two derived cell lines (Edington *et al* submitted). In summary, from the currently available data, it is impossible to accurately determine at what stage of cellular transformation increased expression of cyclin D1 is most likely to confer growth advantage.

9.3 The first links between cyclin D1 and the signal transduction pathway.

Extracellular growth factors and repressors act during the G1 phase by binding to cell surface receptors and triggering a cascade of cytoplasmic reactions which will eventually regulate the nuclear cell cycle machinery. Recently the pheromone induced *S.cerevisiae* arrest was shown to involve a set of yeast MAP-kinase and MAP-kinase-kinase homologues which specifically affected the functioning of the CLN cyclins and CDC28 complexes (Peter *et al* 1993). In several studies a parallel has been drawn between the α and **a** factor induced pathways in *S.cerevisiae* and TGFβ action in mammalian keratinocytes (Marsh *et al* 1991, Peter *et al* 1993). The recently described TGFβ-induced effects on cyclin E regulated kinase have confirmed in part these suggestions (Koff *et al* 1993). Exposure of mink lung epithelial cells (Mv1Lu) to TGFβ was not shown to affect cyclin E or cdk2 protein levels, however the loss of cyclin E-directed histone H1 kinase activity suggested loss of functional cyclin E/cdk2 complexes, supported by the absence of detectable

active cdk2 forms (Koff *et al* 1993). TGFβ induced G1 arrest was therefore hypothesised to results from the abolishment of cyclin/catalytic subunit associations and potentially could apply to all cyclins. However, preliminary results have revealed opposite effects of TGFβ on cyclin D1 in normal and non-transformed epithelial cells (Angeliki Malliri, Beatson Institute, unpublished data). TGFβ induced increase of $p34^{cycD1}$ levels determined by western blot analysis, was observed in human foreskin primary keratinocytes following a twenty four hour exposure to this growth inhibitory factor. The same phenomenon was not observed following the TGFβ treatment of the most highly transformed SCC cell lines, therefore appearing to depend on the degree of transformation (Angeliki Malliri, Margareta Nikolic, Beatson Institute, unpublished data). Furthermore, recent reports on Mv1Lu cells revealed specific TGFβ inhibition of cdk4 expression potentially resulting in loss of cdk2 kinase activity and subsequent cellular arrest in G1 (Ewen *et al* 1993b). No alterations of cyclin D1 or D2 protein levels were observed. Perplexingly, the decrease in cdk4 levels observed in contact inhibited Mv1Lu cells was not in agreement with the constant expression levels seen in quiescent and growing fibroblast cell lines examined in this study, a phenomenon that could reflect species and/or cell type differences (Ewen *et al* 1993b). These observations would imply a diversity in TGFβ induced cellular responses, potentially reflecting a dual role of cyclin D1 in cell cycle regulation or the alteration of specific TGFβ induced pathways as a result of transformation. Alternatively, keratinocyte exposure to TGFβ causes arrest in middle to late G1, exactly the period during which $p34^{cycD1}$ levels are at their highest. The increase in $p34^{cycD1}$ observed in normal cells may simply reflect the stage of the cell cycle they have arrested at. Transformed SCC cultures have often acquired

TGFβ resistance, a characteristic that may account for the lack of detectable increase of $p34^{cycD1}$ in these cells. However, preliminary examinations have only revealed TGFβ resistance of one SCC cell line analysed in this study (BICR19) (Angeliki Malliri, Beatson Institute, unpublished data),suggesting that the former hypothesis is more likely. Further analysis is required to resolve this controversy.

In mammalian systems, mitogens such as EGF, FGF, BB form of PDGF but not IGF-1 were able to activate G0 arrested fibroblasts and induce cyclin D1 expression (Won *et al* 1992, Surmacz *et al* 1992). This study revealed a similar response upon EGF stimulation of SCC cell lines (SCC12 and clones P-1 and A3) that exhibited a temporary decline in their rate of proliferation, following serum starvation (see section 6.2). The three-fold elevation in basal $p34^{cycD1}$ levels had no effect on the response of P-1 cells to this mitogen. Unfortunately, little is known of the signal transduction pathways that are activated following mitogenic stimulation of epithelial cells. Currently the MAP-kinase pathway represents one recognised system linking extracellular, cytoplasmic and nuclear events in mammalian cells, however the mechanism by which cyclins are induced is unclear.

9.4 The contribution of $p34^{cycD1}$ to cellular immortalisation.

Immortalisation of mammalian cells is thought to represent the first step in the development of cancer. Cells that have escaped normal proliferative limitations are predisposed to the accumulation of other abnormalities which may subsequently lead to transformation. To date, only viral onco-proteins are known to harbour the ability to modulate human primary keratinocyte regulatory systems, avoiding programmed host cell death and inducing

proliferation, presumably with the aim of promoting expansion of the viral population. In contrast, the over-expression of individual mammalian genes has not been shown to effectively cause a similar response in human primary keratinocytes.

In comparison with rodent cells, human keratinocytes exhibit a strong resistance against longevity, with currently only a few reported cases describing spontaneous resistance to M1 and M2 growth restriction points (Baden et al 1987, Boukamp *et al* 1988, Rice *et al* 1993). The most recent and significant finding revealed increased expression of cyclins A and B and cdk2 in a human line of spontaneously immortalising keratinocytes (SIK cells) (Rice *et al* 1993). These cells had been derived from a sample of normal human foreskin and cultured in the presence of x3T3 fibroblasts, under standard growth conditions. A six- and four-fold elevation in cyclin A or B expression, respectively was noted between passages ten and sixteen remaining so apparently indefinitely. Coupled with the gradual increase in cdk2 levels (passage twenty four exhibited a seven-fold elevation of this protein), these observations suggested a direct involvement of cyclin directed kinases in SIK cell immortality. During the early stages of cell line development, the chromosome complement was indistinguishable from normal keratinocytes, however, by passage seventy eight several abnormalities were evident involving chromosomes 6p, 7 and 8q. This finding suggests the ability of cyclin over-expressing cells to overcome the growth inhibitory effects of M1. Escape from the second, more restrictive regulation point (M2) is allowed by the occurrence of other abnormalities involving the cellular chromosome content. Nevertheless, late passage SIK cells had retained unaltered growth properties such as response to the inhibitory effects of TGFβ and TPA, lack of growth in

suspension and strong requirement for EGF, suggesting that the cells had only achieved longevity and were not malignantly transformed (Rice *et al* 1993).

The discovery of G1 cyclins introduced a new set of likely targets in immortalisation, of which cyclin D1 is a strong contender. Despite this, the ability of cyclin D1 to immortalise and/or transform cells in culture has not yet been established. Co-transfection experiments on normal human foreskin keratinocytes were therefore employed in this study with the aim to determine the extent with which cyclin D1 may overcome the M1 and M2 proliferative blocks in cells programmed for a definite life span. The results represented in chapter 8. reveal $p34^{cyl-1}$-induced longevity in human foreskin primary keratinocytes (see section 8.2.1). Unfortunately in this study an insufficient number of human primary keratinocytes were transfected with individual expression vector combinations, reducing the probability of obtaining immortalisation. The low frequency of *HPV-16 E6/E7* immortalisation and/or transformation reported in previous studies further supports this argument (Vousden *et al* 1988). Furthermore, cells transfected with *E6* and *E7*, *Cyl-1*, *E7* or *E7* and *Cyl-1* expression systems were able to efficiently bypass M1. Future analysis will require transfection of a larger population of cells. In addition, co-transfection of cyclin D1 expression vectors with *cdc2*, *cdk2*, *cdk4*, *cdk5* or *cdk6* expression vectors into primary keratinocytes may increase or decrease the immortalising properties of *CCND1*.

The transforming competence of viral onco-proteins was shown in previous reports to be strongly linked with their ability to associate with host tumour suppressors p53 and pRb (Band *et al* 1991, Hubbert *et al* 1992, Pagano *et al* 1992a; Tedesco *et al* 1993, Vousden *et al* 1993). Recently, a series of contradictory reports have analysed the ability of $p34^{cycD1}$ to

associate with pRb (Ewen *et al* 1993a, Dowdy *et al* 1993, Hall *et al* 1993). Two alternative hypotheses (pursued in separate laboratories) differed with respect to which protein represented the substrate and which was the effector. One proposed mechanism suggested the targeting of active hypophosphorylated pRb during G1 by cyclin D1, the over-expression of which would occupy all of the available pRb pockets preventing association with other cellular proteins. *In vitro* studies have revealed cyclin D1 induced pRb phosphorylation providing an additional means for the tumour suppressor inactivation, however, these reactions do not appear to take place *in vivo* (Matsushime *et al* 1992, Hinds *et al* 1992, Kato *et al* 1993). Loss of pRb was proposed to abolish control of some positive proliferative regulators (such as free E2F) and promote cell cycle progression (Nevins 1992, Weintraub *et al* 1992). Alternatively, cyclin D1 itself may represent a centrally important promoter of cell cycle progression and therefore an object for pRb sequestration. Consequently over-expression of cyclin D1 could saturate the binding ability of pRb, allowing free $p34^{cycD1}$ to promote proliferation and the lack of functional pRb ultimately resulting in a similar fate as proposed by the former hypothesis. In view of the observed pRb over-expression in the BICR cell lines harbouring *CCND1* amplifications (described in chapter 4.6), currently the latter explanation would appear more likely. This is further supported by the recent findings revealing an increased ability of mutated cyclin D1 unable to associate with pRb and overcome the inhibitory effects of this tumour suppressor when co-transfected into Saos2 cells (Hinds *et al* 1992, Dowdy *et al* 1993). In this example cyclin D1 may have rendered itself inaccessible to the sequestering actions of pRb.

Based on the perplexing results obtained in this study following transfection of antisense *Cyl-1* cDNA expression vectors into primary

keratinocytes, a new hypothesis can be made regarding the function of cyclin D1 in mammalian epithelial cells. The fact that HEK cells into which 3'-5' *Cyl-1* cDNA expression vectors were introduced exhibited a growth advantage forty one days from lipofection, suggests that a reduction of endogenous $p34^{cycD1}$ levels may also confer a growth advantage in certain circumstances. The diversity of catalytic subunits with which $p34^{cycD1}$ is known to associate would be consistent with $p34^{cycD1}$ having a number of different functions. As substrate specificity and hence stimulatory or inhibitory effects on cellular proliferation may be determined exclusively by the kinase subunit, in a proliferating cell important decisions involving growth or senescence could be determined by the availability and affinity of alternative cdk proteins to $p34^{cycD1}$. The previously suggested inhibitory role of p107/cyclin A/cdk2/E2F complexes on cellular proliferation further supports a diverse role for cyclins in cell cycle regulation (Devoto *et al* 1992).

The same type of argument could be used to explain the frequent occurrence of *CCND1* amplification and over-expression in human SCCs. Chromosome 11q13 is a very dynamic region commonly involved in amplification and rearrangements linked to distinct tumour types. Several proto-oncogenes are known to map to this area which is large enough to encompass many as yet undiscovered genes. Evolutionarily it would be of great advantage for a cell to co-amplify genes (the products of which may be growth suppressory) with transforming oncogenes, in this way preventing or slowing down transformation at the early stages. Following this argument *CCND1* amplification and subsequent over-expression could be viewed as a programmed cellular response to transformation in an attempt to prevent uncontrolled growth. The role of cyclin D1 as an exclusively negative regulator

of cellular proliferation is however hard to imagine, taking into account the currently available evidence for its proto-oncogenic properties and the absence of observed "normal" or "near-to-normal" cells harbouring increased *CCND1* gene dosage and expression. A more likely picture that is emerging is the combined functioning of cyclin D1 as a positive and negative cell cycle regulator, which may be determined by the cell type, the availability of catalytic partners, regulatory proteins (such as PCNA and potentially p21) and substrates. The potential association of pRb and $p34^{cycD1}$ need not occur to inactivate one or both of these proteins but may be of collaborative nature and therefore account for the increase of pRb expression in cells that have amplified *CCND1*. To date cyclin D1 directed inactivation and subsequent phosphorylation of pRb has only been revealed *in vitro* studies reflecting a potential although not necessarily real function of the kinase. Nevertheless, the majority of published reports provide data that directly contradict any growth suppressory functions of cyclin D1, such as the proximity of the *CCND1* gene to the *bcl-1* region, the effects caused by constitutive over-expression of exogenous cyclin D1 in human diploid fibroblasts and the inability for cells to proliferate following a reduction in endogenous $p34^{cycD1}$ levels. However, these observations may simply reflect the complex role of cyclin D1 and directed kinases in regulating the fate of a single cell in dependence of environmental factors, cell type, availability of catalytic subunits and state of cellular growth.

To date there is no direct evidence for the effects cyclin D1 may have on p53. This tumour suppressor protein is known to function during later stages of G1 and at the G1/S boundary as a major part of the G1 checkpoint control (Lane 1992, Hartwell 1992). However, despite the overlapping time of action

coupled with p53 phosphorylation on Ser residues, currently the evidence linking cyclin D1 and p53 is circumstantial (Lane 1992).

The complex regulatory role of cyclin D1 in cellular proliferation and its coupling with alternative kinase subunits is rapidly becoming clear. In the event of devising a uniform cure for cancer, cyclin proteins and their associated kinases represent an ideal target for inactivation which would bypass the complex cytoplasmic signal transduction pathways and effectively prevent proliferation of selected cells.

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