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Abbreviations

A	adenine
bp	base pairs
BPV	Bovine Papillomavirus
C	cytosine
cm	centimetres
ATP	adenosine triphosphate
CTP	cytosine triphosphate
GTP	guanosine triphosphate
dNTP	deoxynucleoside triphosphate (with reference to the three above)
TTP	thymidine triphosphate
UTP	uridine triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
G	guanine
g	grammes
HBS	HEPES-buffered saline
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HPV	Human Papillomavirus
hr	hours
kb	kilobase pairs
kDa	kiloDaltons
µg	microgrammes
µl	microlitres
µM	micromolar
mg	milligrammes

ml	millilitres
mm	millimetres
mM	millimolar
min	minutes
M	molar
M_r	molecular weight
MOPS	3-(N-morpholino)propane sulphonic acid
neo^r	neomycin resistant
ng	nanograms
OAc	acetate
ORF	open reading frame
pers.comm.	personal communication
pg	picograms
RNA	ribonucleic acid
rpm	revolutions per minute
sec	seconds
SDS	sodium dodecyl sulphate
S/N	supernatant
T	thymine
TPA	12-O-tetradecanoyl phorbol-13-acetate
Tris	tris(hydroxymethyl)aminomethane
TAE	Tris Acetate/EDTA
tRNA	transfer RNA
V	Volts
% (v/v)	volume in ml per 100ml water
%(w/v)	weight in g per 100ml water

ABSTRACT

An *in vitro* assay system utilising non-established bovine fibroblasts derived from foetal palate was developed for the detection of BPV4-encoded transforming functions. This involved cotransfection of a dominant coselectable marker gene **neo** and recombinant BPV4 DNA linearised within the E1 ORF. The presence of BPV4 DNA resulted in the formation of colonies of diameter greater than 5mm (macrocolonies) which displayed a contact-inhibited phenotype.

Cloning of regions of BPV4 into two vectors, pSV2neo and pZIPneoSV(X1), indicated that macrocolony formation could be induced by two regions of the genome. The first, a 2.0kb XhoII fragment (nts 6487-1275) encoded two complete ORFs, namely E7 and E8. A construct lacking 233bp (nts 906-1139), resulting in interruption of the E7 ORF, was inactive in this assay, indicating a requirement for the E7 ORF for induction of the phenotype. The second region, encoded by a 3.9kb XhoII fragment (nts 2597-6487), contained the E2,E3,E4,E5 and L2 ORFs. In this case, colonies contained cells showing a more elongated phenotype.

Cotransfections of linear BPV4 with an activated **ras** gene did not lead to a loss of contact-inhibition. In contrast, overexpression of the 2.0kb fragment, in the presence of **ras** did lead to the formation of non-contact-inhibited neo^r macrocolonies. An intact E7 ORF was again required for this activity.In the case of the 3.9kb construct, a single non-contact-inhibited colony was observed in constructs utilising the BPV4 promoters upon cotransfection with **ras**.

SECTION 1

INTRODUCTION

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1.1 Cancer research: an historical perspective

The collection of diseases known as cancer arise due to the uncontrolled proliferation of an individual cell type within a multicellular organism. Such a tumour often spreads subsequently to secondary sites within the body, leading rapidly to the death of the host organism. With the advances in clinical medicine and the subsequent eradication of many diseases of childhood and early adulthood over the past century, the number of clinical cases of human cancer has risen considerably. As a result, much research has been carried out concerning the biology of tumour induction and its subsequent treatment. This introduction will not consider clinical treatment, since it plays no part in the work to be described in this thesis.

The development of cancer is presumed to occur due to the cumulative effect of several hereditable, somatic mutations (defined here as any change in the composition of the genetic material) within one individual cell, a theory first put forward by Boveri in 1907 and one which is still believed today. The two main questions researchers have been interested in over the subsequent eighty years are: firstly, what are these genetic changes at the molecular level? and secondly, what agents cause these changes to occur?

Research has proceeded using three broad strategies: firstly, a study of epidemiology (i.e. the statistical analysis of the variables which affect the frequency of tumour formation such as geography, diet, age, social habits, working practices etc) has predicted that the the number of somatic mutations required for

full human tumour development to be six or seven. It has also allowed the identification of certain aetiological agents, notably cigarette smoking in the induction of lung cancer and asbestos in the development of mesothelioma. Secondly, researchers have developed both *in vivo* animal model systems and *in vitro* tissue culture systems to mimic some of the processes occurring in the development of human tumours. Finally, the direct molecular analysis of human tumours has been coming to the forefront increasingly in order to test the relevance of results obtained using animal models to naturally occurring neoplasias.

In regard to the first question, three broad aetiological parameters have been shown to play a role in the development of tumours. The first is the group of agents known as viruses, originally defined as a tumour- inducing activity able to pass through filters which rendered solutions bacteriologically sterile (Rous (1911), Shope (1935)). Such viruses were initially demonstrated to be active in the development of acute and latent leukaemias in chickens and mice (later shown to be RNA tumour viruses or **retroviruses**), the development of benign epithelial tumours of the rabbit skin (a DNA tumour virus) and the development in female mice of mammary tumours after multiple pregnancies (another retrovirus). Their role in human disease, however, has taken longer to prove, but the role of Hepatitis B virus in the development of hepatocellular carcinoma, that of Epstein-Barr Virus in the development of Burkitt's lymphoma and nasopharyngeal carcinoma, those of certain Human Papillomaviruses (HPVs) in the development of certain epithelial carcinomas and that of HTLV-I in the development of human

leukaemia are now well established.

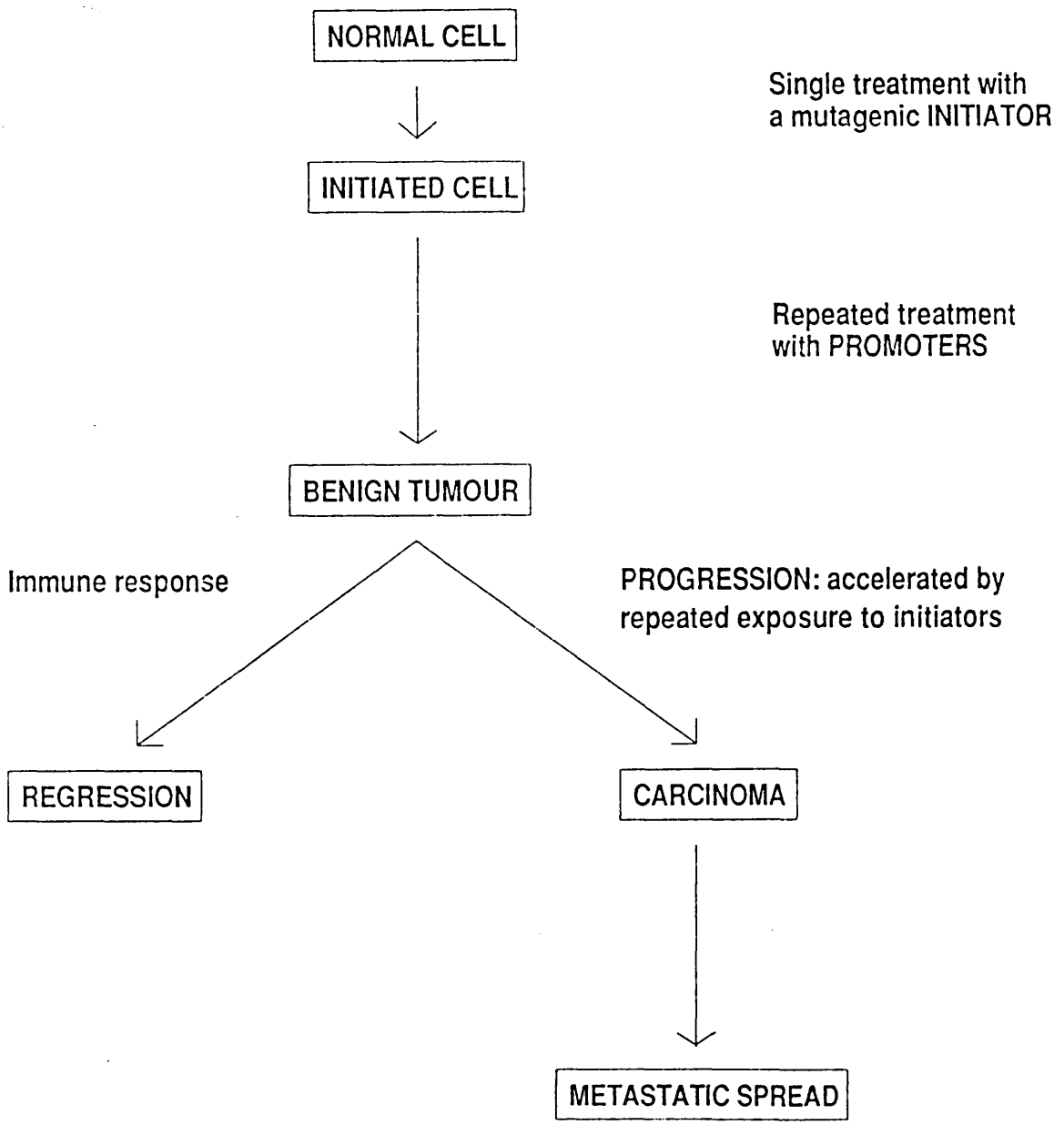
The second group are those known as the environmental agents. These include chemical compounds and short wavelength radiation. These were originally identified using epidemiological studies, but more recently, animal models have been used to test their activity in the development of a wide range of tumour types. Thus ultraviolet light (often as a result of several doses of 7 days' sun in the Mediterranean) has been shown to be a factor in the development of malignant melanoma in humans, whereas nickel was shown to be a causative agent of nasal sinus cancer found among miners early this century.

The third factor is more a passive one: that of genetic susceptibility. It is well known that predisposition to certain cancers is hereditable: the best examples are those of childhood retinoblastoma and Wilms' tumour.

With this knowledge, experimenters were then able to develop animal model systems with well-defined stages in the progression to malignancy. Perhaps the best known is that of the mouse skin: using chemical carcinogens, four distinct states could be identified, as shown in Figure 1.1: **normal** tissue, **benign** tumours, **primary carcinomas** and **metastatic deposits**. The discovery that a large percentage of benign tumours regressed naturally, and that such processes were mediated by the immune system, brought a fourth factor into disease development: that of the host immune status.

All these findings could be deduced from experiments on whole animals. In order to determine the mechanism of action of

Figure 1.1: Schematic representation of multistage carcinogenesis in vivo



tumour-inducing agents, a knowledge of the molecular nature of the mutations they induce was required. From the discovery that DNA was the genetic material in 1944 (Avery *et al* .), over thirty years of intense research into molecular biology elapsed before the identification of the first human **oncogene** (defined here as genes whose activation contributes to the tumorigenic phenotype). This proved to be the human analogue (human **c-Ha-ras**) of the gene found to be responsible for the induction of sarcomas in mice by the retrovirus Harvey Murine Sarcoma Virus (Ha-MSV). In this case, the mechanism of activation proved to be a point mutation resulting in a single amino acid change in the protein concerned (Santos *et al* . (1982)).

As a result of this discovery, the human analogues of the other retroviral oncogenes have also been studied and over the last ten years, more than twenty such oncogenes have been characterised and an amalgamation of the studies of oncogene function and normal cell growth *in vitro* has occurred, with the realisation that these oncogenes are genes involved in the response of cells to external growth-regulatory signals. It has become apparent that cellular division is preceded by the transduction of external mitogenic signals from the cell surface to the nucleus, leading to subsequent DNA synthesis and cell division. Several proteins are involved in this process and many appear to be encoded by cellular proto-oncogenes, i.e. those genes which may become oncogenes upon activation.

With the identification of such genes and their modes of activation, a myriad of diagnostic markers for clinical disease status and prognosis have been developed. These genes, however,

appear to be only a subset of those involved in the tumorigenic process, for it has become apparent, initially through the study of hereditary tumours, that loss of gene activity also plays a role, sometimes critical, in the development of tumours. Many tumours exhibit characteristic, non-random losses of chromosomes, but the best characterised example of gene loss to date is that of retinoblastoma (reviewed by Weinberg (1988)), where inheritance of only a single functional allele encoding the 105kDa protein referred to as Rb predisposes such people to the development of full retinoblastoma within the first 5 years of age, the development of which correlates with loss of this second Rb allele. Very recently it has become clear that a study of the interactions between the products of oncogenes and the so-called **tumour suppressor** genes will also occur, since it has been shown that the products of oncogenes of HPVs associated with human genital carcinomas may associate with the Rb gene product *in vitro* , although inactivation of the protein has not yet been proven. The identification of tumour suppressor genes and studies on their function will be one of the major efforts in the field of tumour biology over the next decade.

From this body of data, it has become clear that activation of individual oncogenes appears to be associated with the development of only a subset of tumour types. Furthermore, certain tumour types, such as retinoblastoma, develop only at certain stages in life (in this example, only in childhood). This indicates a role for **epigenetic** factors in tumour development. This has led to a study of the expression of proto-oncogenes during the process of normal cell differentiation (the maturation

of a self-renewing stem cell to form mature cells, either capable or incapable of further division) and the means by which oncogene activation may subvert such a process.

It has also become clear that other epigenetic factors concern the rate at which activating mutations occur. It has been found that metabolism of chemicals within cells, either the inactivation of carcinogens or the activation of pro-carcinogens, or the import and export of such molecules in and out of cells, occurs at differing rates within individuals and may play a role in differing susceptibilities towards tumour formation. Another factor to consider is the rate of repair of such activating mutations: the DNA replication machinery has been shown to have a finite accuracy and that such mistakes as it does make are picked up by DNA repair systems. Loss of such activities gives rise to certain heritable diseases such as Xeroderma pigmentosum, which arises due to the a loss of the ability to repair the thymidine dimers induced by ultraviolet light and which leads to development of skin tumours at an early age.

This project has been concerned with the role of a bovine member of the papillomavirus family in the development of cancer of the Upper Alimentary Canal in cattle. To place this in context, I will now give a brief historical review of papillomavirus research in general.

1.2 Historical context of papillomavirus research

Papillomaviruses are circular double stranded DNA viruses which cause benign epithelial, and occasionally fibroblastic, lesions in a species- and site-specific manner, more commonly known as warts. In certain cases, including some human lesions, these may progress to carcinoma. They are thus of interest both scientifically and clinically to cancer researchers.

The papillomaviruses are now enjoying a second phase of intensive research concerning their life cycle and the association of some members of the family with epithelial malignancies. During the 1930s, -40s and early -50s, much research was carried out on Cottontail Rabbit Papillomavirus (CRPV) and its role in the development of skin malignancies in both the cottontail rabbit of the American Midwest and in domestic rabbits (for review, see Kreider and Bartlett (1981)). In those early days of tumour biology, this system offered several attractive features, when considering the small number of workers active in the field:

(a): The skin was visible and thus a distinct series of characteristic phenotypes prior to invasive carcinoma could be discerned, unlike the leukaemias, where only the end point was detectable.

(b): The isolation of CRPV from naturally occurring papillomas, the scarification and inoculation of rabbits with virus and the topical application of chemical carcinogens such as tar were all

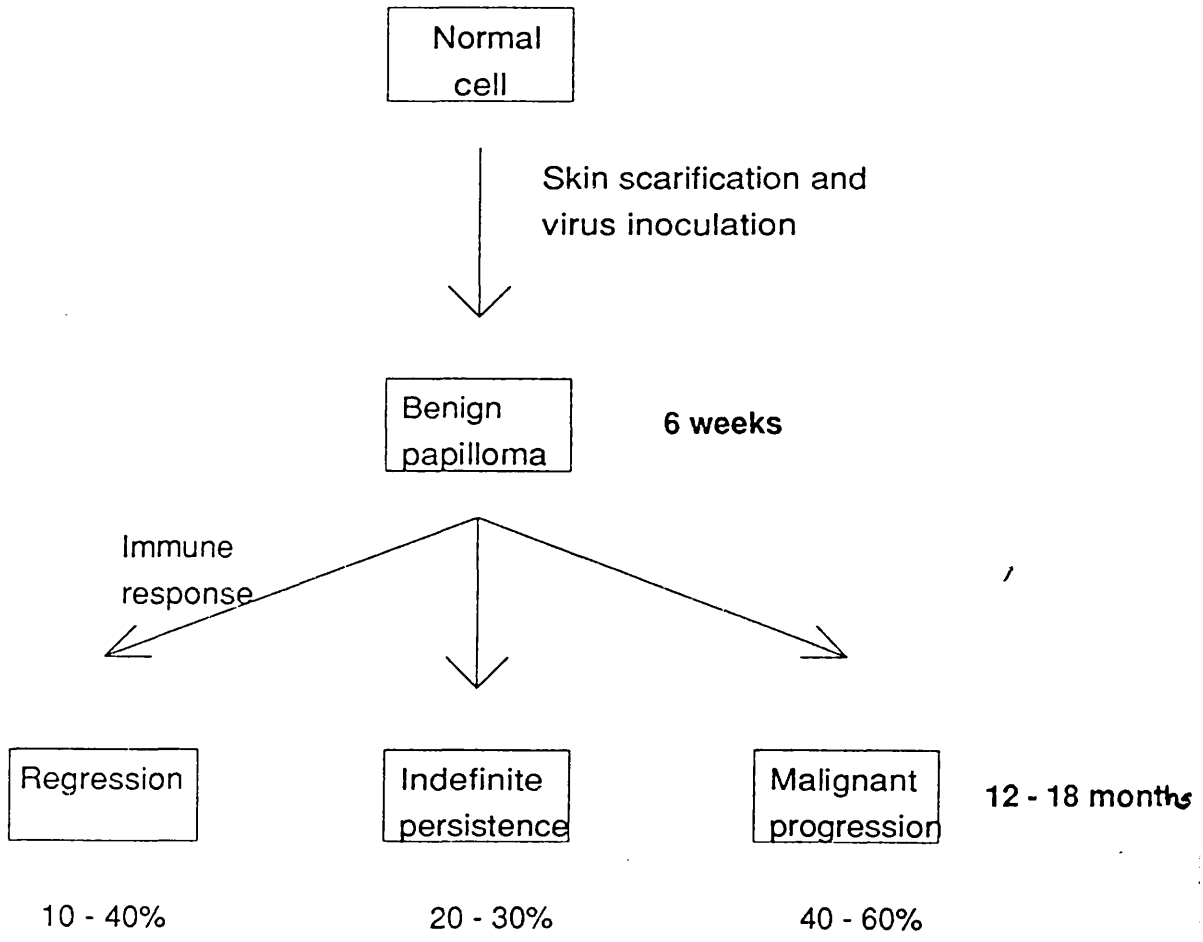
simple processes technically.

(c): Virus isolates were of high titre and were stable for long periods; thus continuous hunting for natural isolates in the wild was unnecessary.

With this system, it was possible to demonstrate that scarification of skin and inoculation of CRPV led to the development of benign papillomas in both cottontails and domestic rabbits in the skin, but not in other sites of epithelial tissue (Kidd and Parsons (1936)). No virus, or very poor titres could be recovered from papillomas of domestic rabbits (Shope (1935)). This was a problem, since it was also rapidly found that cottontails did not thrive in captivity and were thus unsuitable for the experimental procedures which took up to 18 months. Routine production of virus stocks in the laboratory was therefore not trivial.

By keeping the rabbits in captivity for periods up to two years, development of carcinomas could be monitored and a characteristic spectrum of papilloma fates could be discerned statistically, as shown in Figure 1.2. In the case of the domestic rabbits, 10 - 40% regressed, 20 - 30% persisted indefinitely while 40 - 60% progressed to carcinoma within 12 - 18 months. This spectrum suggested that secondary events distinct from virus infection were necessary, firstly to allow indefinite persistence and secondly in the development of carcinoma. In 1936, Rous and Kidd showed that topical application of tar to benign papillomas accelerated the onset of carcinoma

Figure 1.2: Spectrum of tumour formation in CRPV-associated carcinogenesis in domestic rabbits



development, thus demonstrating for the first time a synergistic action betwen a virus and chemical carcinogens in the development of malignancy. Furthermore, they demonstrated that scarification and virus inoculation of rabbits previously treated with tar led to the direct development of carcinoma, thus showing that, at least in this system, the order of genetic changes required for carcinoma development was not crucial(Rous and Beard (1935), Rous and Kidd (1936)). Analysis of carcinomas also showed that virus production could not take place under such conditions (Kidd and Rous (1940)). (Already, therefore, the principle of strict conditions of tissue- and host specificity as well as the state of differentiation permissive for virus production was recognized, although not expressed in such modern jargon).

Due to the long latency period prior to carcinoma formation, researchers were interested in developing methods for maintaining a record of the carcinomas through time.[f] Two important systems resulted; the transplantable carcinomas VX2 and VX7, which have now been propagated serially through animals for over 30 years, and cell lines derived from these carcinomas, as well as from CRPV-induced papillomas, which have been similarly propagated in tissue culture. With the advent of more modern techniques, these lines have been invaluable in the analysis of the molecular biology of CRPV-associated carcinogenesis and are a telling reminder that the full fruit of given experimental systems may not be apparent for 1 or even 2 generations.

Towards the end of the 1950s most of the fundamental

principles of tumour development at an organism level had been worked out. With the discovery of DNA as the genetic material (Avery *et al.* (1944)) and subsequent determination of its structure and means of replication (Watson and Crick (1953), Meselson and Stahl (1958)), biology was moving towards the molecular era. With the rapid advances in the understanding of the principles regarding gene expression, the focus of research turned to questions concerning expression of individual viral genes and their role in tumour formation. For systematic analysis of viral systems, this required a method of generating viral mutants and recovery of such mutants as infectious viral particles. It was at this point that papillomavirus research dwindled and was not to raise its head again in a large-scale manner for nearly twenty years: despite many varied attempts, no method could be found, *in vitro* or *in vivo* , to generate infectious virus from mutant DNA. Since such methods had been developed for other DNA tumour viruses such as SV40, polyomavirus and adenovirus, the once-mighty CRPV dwindled in importance throughout the 1960s and early 1970s.

Two important events heralded the return of papillomavirus research from the late 1970s to the present day: a medical reason and a scientific technical advance. The discovery by Harald zur Hausen's group of an aetiological association of HPVs 16 and 18 with cervical cancer (Dürst *et al* . (1983), Boshart *et al* . (1984)), one of the largest killers of women worldwide and which presents currently at ≈500,000 per year, focussed the need for more detailed knowledge concerning these viruses, whilst the methods developed initially by Alex van der Eb's laboratory for the

introduction of cloned DNA into tissue culture cells (Graham and van der Eb (1973)) allowed the analysis, in controlled *in vitro* experiments, of the phenotypic properties of mutant viral DNAs produced using recombinant DNA technology.

At this stage, however, the only well-defined culture systems were those of established mouse fibroblasts and primary rodent embryo fibroblasts and attempts to induce focus formation with the HPVs or CRPV were inefficient or unsuccesful. It turned out, however, that another animal papillomavirus, namely Bovine Papillomavirus Type 1 (BPV1), did transform such cells efficiently (Dvoretzky *et al* . (1980)), and as a result, this virus has served as the prototype for the molecular analysis of PVs *in vitro* . It has long been realised, however, that this virus was not perhaps typical of the genital HPVs, since it showed a more relaxed spectrum of host- and tissue specificity *in vivo* , producing both fibroblastic and epithelial proliferations in infected cattle. Moreover, in its natural host it failed to produce malignant epithelial lesions. Nevertheless, much useful information has been derived from this virus and adapted as more information concerning the HPVs has emerged. With the standardisation of keratinocyte culture methods, the development of collagen raft systems which partially mimic stratified epithelium (Kopan *et al* .(1987)) and transformation assays sensitive to HPV gene functions (Matlashewski *et al* (1987b), Schlegel *et al* . (1988)), research on HPVs has burgeoned within the 3 years of my Ph.D. Nevertheless, the impossibility of performing controlled experiments on humans is a serious gap in desired knowledge, and thus animal model systems will continue

to provide insights into papillomavirus functions. In this regard, it is interesting to mention two other animal models which, I believe, will continue to be studied in the future. The first, as yet still embryonic, are the two mouse papillomaviruses (those of the European harvest mouse *Micromys minutus* (Sundberg *et al* .(1987)) and the hairless mouse *Mus musculus* (Tilbrook *et al* .(1989))), which will hopefully provide a well-defined genetic backgound, the battery of gene probes unavailable in the CRPV system and the ease of manipulation of the genetic background in defined ways using transgenic technology. The second, BPV4 (Jarrett *et al* . (1978a)), provides a large animal model which, unlike BPV1, does show strict tissue specificity and is associated with malignant lesions of epithelial origin in the wild. It is with the latter system that this project was concerned.

In the next section, I will set out briefly the areas of papillomavirus research about which knowledge is desired: I will then follow this up with reviews of the progress made in pursuit of such goals and will finally provide a background to the project I have been working on during the past three years.

1.3 Fields of research within the PV field

Since this project is not concerned with clinical aspects of papillomavirus research, I will concentrate solely on aspects concerning the understanding of the role of such viruses in tumour formation and not on any means of using such understanding in the development of treatments for sufferers or potential development of vaccines to prevent tumour formation.

The first major field of research concerns the detection of papillomavirus infection, if necessary by the identification of novel strains of virus, and the analysis of the means by which such viruses are transmitted from individual to individual.

The second major area of desired knowledge concerns the process of virus production in infected cells. Specifically this involves the analysis of viral gene expression at the levels of RNA and protein, viral DNA replication and virion assembly and the effect that such processes have on the differentiation programmes of the host cells in which they occur. Also of interest is the basis for the strict host- and tissue-specificity of individual viruses concerning virus production, more specifically involving cell-surface virus receptors and tissue-specific factors influencing the viral life cycle.

The third major area of interest concerns the host response to productive viral infection, with regard to the role of the immune system in the rejection of benign papillomas.

The fourth major area concerns the secondary changes occurring within infected cells which result in the progression to carcinoma. Of interest here are changes in patterns of viral and cellular gene expression, and the effects these have both on the cellular differentiation process and the response of the immune system to such cells.

Clearly, in order to answer such questions, strategies must be designed concerning the approaches to be taken in order to allow the posing of specific scientific questions. These have often depended on the technology available at a given time and may also include the development of new technical tools. The review which

now follows of progress made towards the understanding of the previously described areas of papillomavirus research tries to bear in mind the strategies which have been employed and the reasons for such decisions.

1.4 Papillomavirus detection and transmission

Papillomaviruses were initially detected in animal systems by:

(a): Functional assays of tumour induction after filtration of extracts derived from homogenised benign papillomas or after purification of virus on caesium chloride gradients.

(b): Visualisation of virus particles in electron micrographs of the upper layers of such papillomas.

Analysis of such virions indicated that they were icosahedral units of diameter ≈55nm. They contained a circular, double-stranded DNA molecule of ≈8kb within a coat of 72 identical capsomeres.

These methods, however, failed to detect viruses with low levels of virion production in the tissue of interest and DNA from presumed viruses (which have not, as yet, been isolated in virion form) such as HPVs 16 and 18 were isolated by detection using Southern blot hybridisation under non-stringent conditions. This technique has a detection limit of ≈0.1 copies of DNA per cell, and thus performing such analysis on DNA derived from biopsies was unable to detect any infection where viral DNA was present only

in a small percentage of epithelial stem- or basal cells. Detection of such infections has now become possible with the development of the Polymerase Chain Reaction (PCR) and results on clinically normal individuals of a variety of ages indicates that HPV infection, as for another human DNA tumour virus, Epstein-Barr Virus (EBV), appears to be a very common event, which leads on to clinical conditions only under certain circumstances in a small percentage of cases.

The clinical manifestations, particularly development of benign papillomas and progression to carcinomas, appear to occur in a species-, tissue- and site-specific manner for each individual virus. The viruses may be split into two groups, the true epitheliotropic viruses, including all the major HPVs, which are associated with clinical lesions only in epithelial tissues, and the fibropapillomaviruses, notably BPVs 1, 2 and 5, the Deer papillomavirus (DPV) and the European Elk Papillomavirus (EEPV), which, in addition to productive epithelial papillomas, also induce non-productive lesions in the underlying fibroblastic tissue. The former may be further subdivided into viruses associated with skin- and mucosal epithelium, as shown in Table 1.1 for the HPVs. It is noteworthy that the latency periods for progression are long, indicating rare secondary genetic alterations being required for such a process.

The mode of transmission of the viruses has not been absolutely defined in many cases. Nevertheless, the geographical correlation of CRPV-induced papillomas with the presence of mosquitos indicates a possible agent, and such insects have

Table 1.1: Lesions associated with HPV infection in vivo (from Broker and Botchan (1986))

Type	Disease	Oncongenic potential
1	deep plantar and palmar myrmecia	benign
2	common warts (verrucae vulgares), some associated with anogenital condylomata	benign
3, 10, 28	juvenile flat warts (verrucae planae); associated with some types of epidermodysplasia verruciformis and genital infections and some common warts	rarely malignant
4	plantar warts and common warts	benign
5, 8	pityriasis-versicolor macules; epidermodysplasia verruciformis in patients with congenital cell-mediated immune deficiency and those undergoing immunosuppression for transplantation	30% progress to malignancy
6, 11	ano-genital condylomata acuminata; atypical (flat) c.a. (particularly HPV-11); dysplasias and intraepithelial neoplasias, grades I and II; penile warts; juvenile and adult-onset laryngeal papillomas (particularly HPV-11)	usually benign
7	common warts of meat and animal handlers	benign
9, 12, 14 15, 17 19–25, 36, 40	epidermodysplasia verruciformis basilioma (type 20)	HPV-12, -17, and -20 lesions, at least, can progress to carcinomas
13, 32	oral focal epithelial hyperplasia (Heck's disease)	possible progression to carcinoma
16, 18, 31 33, 35, 39	high-grade dysplasias, intraepithelial neoplasias and carcinomas (CIS) of genital mucosa; Bowenoid papulosis, Bowen's disease; laryngeal, esophageal, and probably some bronchial carcinomas	high corelation with genital and oral carcinomas
26	cutaneous wart, patient with immune deficiencies	unknown
27	cutaneous wart, renal transplant recipient	unknown
29	cutaneous, intermediate warts	unknown
30, 40	larynx	carcinoma
34	nongenital Bowen's disease	carcinoma-in-situ
37	keratoacanthoma	benign
38	in a melanoma	malignant
41	multiple condylomata and cutaneous flat warts	benign
42	genital warts	benign

proven capable of transmitting the virus in laboratory experiments. The BPVs are presumed to be spread within herds by abrasion on commonly touched agents such as barbed wire. The association of HPVs 16 and 18 with genital cancers of both males and females suggests that these viruses are primarily spread with sexual intercourse, an hypothesis consistent with the facts firstly that nuns never contract cervical cancer (Rigoni-Stern (1842)) and secondly that spouses of known patients show an increased risk for contracting the disease. The fact, however, that many young, prepubertal children have been shown to be latently infected with HPVs implies that controlling virus spread may prove to be impossible, unless means of inhibiting virus production can be developed. As yet, no-one has unequivocally defined the site(s) of productive infection for the carcinogenic HPVs; such an approach, therefore, requires several more years of scientific study.

1.5 Early work concerning the productive viral life cycle *in vivo*

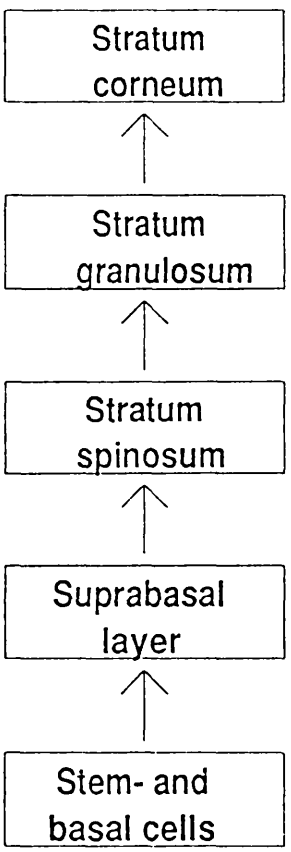
When considering the study of the productive life cycle, researchers were interested in analysing the mechanism of viral DNA replication and subsequent virion assembly. Most work has been done on the animal systems of BPV1 and CRPV: this is due to the highly productive infections associated with these viruses and their ease of induction when using experimental animals. Of the human viruses, only HPV1 has been analysed in any detail; the others have as yet not been addressed due to small size of

samples and the methods of treatment not often involving surgery.

When considering the physiological effects of PV infection, it is necessary to describe briefly the events which occur during the process of epithelial differentiation. The epithelium is a stratified layer of cells which may conveniently be divided into distinct layers on the basis of their differing growth properties and expression of certain genes. A diagramatic form is shown in Figure 1.3: the basal layer, separated from the underlying dermal layer by the basement membrane, is presumed to contain two types of cell: quiescent stem cells, which are stimulated into cycle only in response to tissue wounding, and basal cells, which divide continuously in order to replace the dead cells lost on the epithelial surface. One of the two daughter cells is presumed to be committed to differentiate, which involves the sequential passage, over a period of ≈ fourteen days, through the epithelial layer and concomitant expression of the epithelial marker genes. The suprabasal layer contains cells already committed to differentiate and which in general will not divide further. During the passage through the stratum spinosum , stratum granulosum, and in the case of cutaneous epithelium, to the quiescent stratum corneum, expression of distinctive,increasingly high molecular weight keratins occurs (Franke *et al* . (1986)), the precise nature of which appears to be distinct for each type of epithelium.

Analysis of histological sections of papillomas indicated that these lesions showed many varied morphological and histological features (Croissant *et al* .(1985)), which will not be reviewed in detail here: a few general points will however be made.

Figure 1.3: Schematic representation of epithelial differentiation



Early on, it was discovered from EM pictures of papilloma sections that virus production only occurs in fully differentiated epithelial cells i.e. those unable to divide again. This makes sense in terms of virus survival, since it can no longer be maintained within these host cells. Using *in situ* hybridisation techniques, it was also shown that viral DNA synthesis occurred predominantly in the stratum granulosum (Orth *et al* . (1971)), in contrast to cellular DNA synthesis, which occurs predominantly in the basal layer. Southern blotting of DNA isolated from papillomas indicated an overall copy number of ≈100 - 1000 per cell and that all this DNA appeared to be episomal (Amtmann *et al* . (1980)).

These results are derived by end-point measurements: of more interest was the role of cellular and viral gene expression in the induction of such phenomena. The relative lack of sensitivity of such techniques, however, made analysis of viral transcription by *in situ* hybridisation very difficult or impossible and thus research in this area has been limited to the analysis of RNA isolated from tumours as a whole, using Northern blotting and, more recently, primer extension and S1 nuclease analysis (Stenlund *et al* .(1985)) as well as the generation- and analysis by DNA sequencing of cDNA libraries (Baker and Howley (1987)).

Using such techniques, however, it was shown that transcription was limited to one strand of the viral DNA (Amtmann and Sauer (1982)) and that extensive splicing patterns existed. Furthermore, the identification of the region of the genome encoding the virion proteins was found by comparision of transcripts from productive papillomas, non-productive fibropapillomas and *in vitro* transformed cells, which are unable

to express virion proteins (Engel *et al* .(1983), Baker and Howley (1987)). The analysis of such transcripts, however, would obviously be aided by a knowledge of the nucleotide sequence of the viruses, which would also allow several predictions to be made concerning viral genome products. Results of such analyses are presented in the next section.

1.6 Genome organisation and transcription patterns of papillomaviruses

The complete nucleotide sequence of at least ten papillomaviruses is now known and these have revealed a common genome organisation (for review, see Giri and Danos (1986)). Furthermore, sequence conservation between certain viruses of specific areas of the genome has been correlated with common properties of such viruses *in vivo* .

The genome organisation of BPV1 is shown in Figure 1.4. This shows that potential open reading frames (ORFs) exist in all three frames. Comparison of the BPV1 sequence with the initial transcriptional analyses showed that the genome could be split into three parts:

the Early region,

which is transcribed in both productive and non-productive infections, as well as in *in vitro* transformed cells;

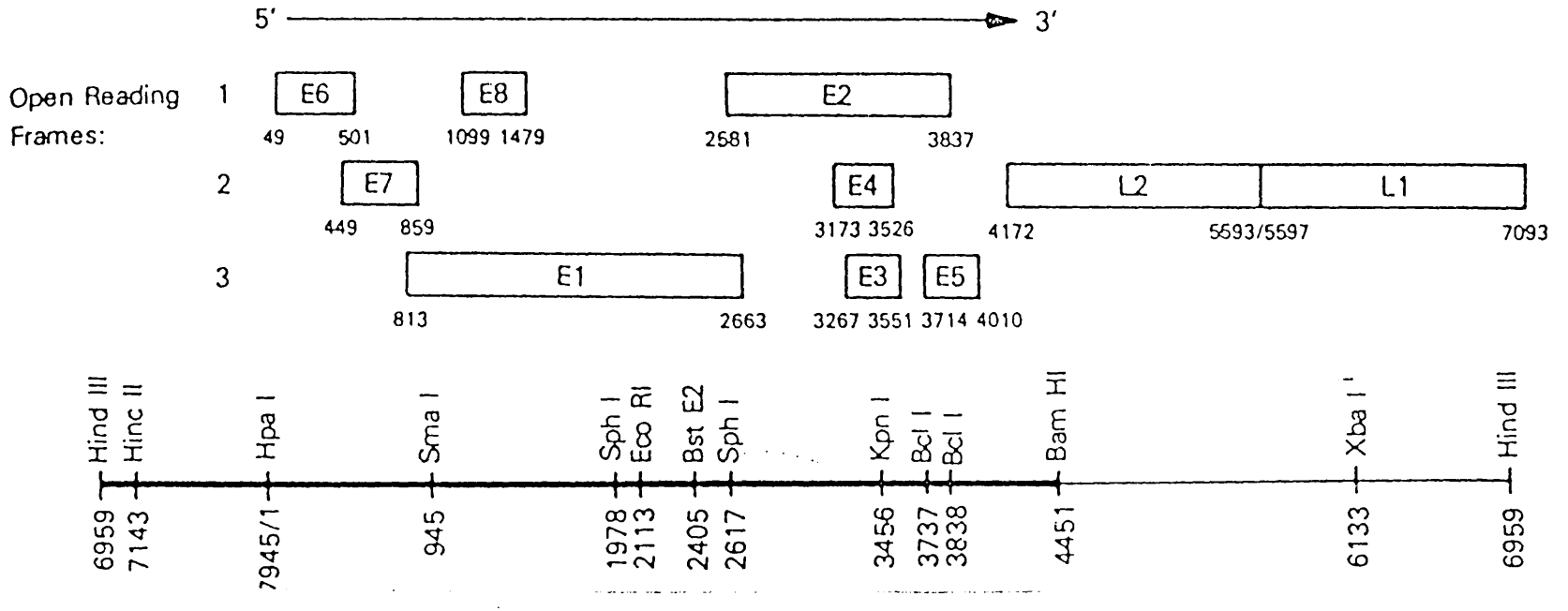


Figure 1.4: Genome organisation of Bovine Papillomavirus Type 1 (from Yang et al. (1985))

the Late region,

which is only transcribed in productive papillomas;

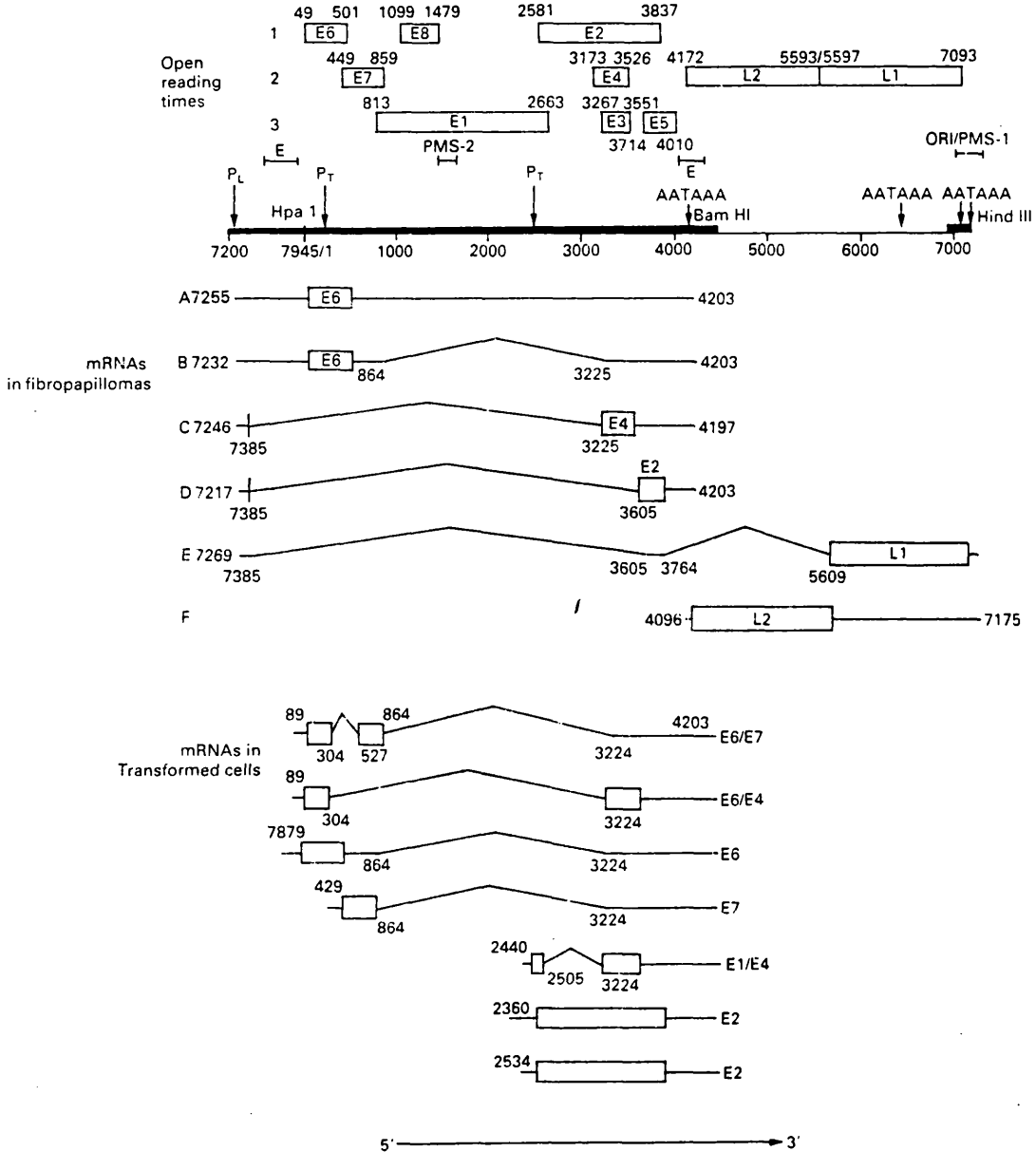
and a region which initially was not found to hybridise to any transcripts at all and was thus initially termed:

the Non-Coding Region (NCR),

but which more recently has been shown to encode very small leader sequences in certain transcripts (Choe *et al* .(1989)), but which, more importantly has been shown to contain transcriptional control sequences and is thus now more often referred to as the Long Control Region (LCR) or Upstream Regulatory Region (URR). The fact that this region was shown to act as an enhancer element **only in virally infected cells** indicated the presence of virally encoded transcription factors, which has subsequently been proven to be true (Spalholz *et al* .(1985)).

The transcriptional patterns, as presently understood, found within BPV1-induced papillomas and ID13 cells (C127 cells transformed by BPV1) are shown in Figure 1.5. These indicate immediately that assigning functions to 'Open Reading Frames' is not a trivial fact, since firstly many of these overlap and secondly many transcripts cover more than one such ORF. Ideally, therefore, analysis of gene function should come via the analysis of the activity of cDNAs in *in vitro* or *in vivo* assays. The great difficulty, however, in obtaining full-length viral cDNAs has made

Figure 1.5: Transcriptional patterns of Bovine Papillomavirus Type 1 in fibropapillomas and infected C127 cells (from Campo (1989))



such an undertaking difficult except in a few cases and thus the easier analysis of genetically engineered viral mutants or subgenomic fragments has been followed, with the proviso that careful evaluation of such results is important. The field of PV transcription has been reviewed in great detail in a recent thesis (A.C. Stamps (1987)) and as this area does not cover a central theme of my work, will not be reviewed further here.

Obviously, the ultimate executors of viral functions are the virally encoded proteins. The difficulty in identifying many such proteins either *in vivo* or *in vitro* was a block to research for nearly twenty years, since simple analysis of virally infected cells using traditional methods of two dimensional electrophoresis did not yield informative results. With the development of antibodies to bacterially produced fusion proteins or against synthetically produced peptides designed from viral DNA sequences, however, proteins have now be identified both in *in vitro* infected cells and in sections of naturally occurring tumour samples, but the low abundance of such proteins in *in vitro* infected cells has required detection by means of metabolic labelling, whereas the use of the immunoperoxidase staining method has allowed detection of proteins in tissue sections. The results of such methods will be described in later sections.

At this point, it should be emphasised that the analysis of individual viral gene functions *in vivo* is at present severely hampered by the inability to introduce mutant viruses into animals, either as packaged virions or as DNA molecules. The greatest challenge has thus been the development of artificial methods of generating infectious virus. In this regard, the work

of Kreider*et al* (1985) in the development of methods of generating HPV11 virions by the infection of human cervical keratinocytes with virus *in vitro* and subsequent injection of such cells into the renal capsule of athymic mice, leading to productive papilloma formation, is of interest. This system has recently been extended to bovine viruses (Gaukroger *et al* .(1989)), but its importance would be incomparably increased were methods devised to introduce DNA molecules into the tissue prior to injection, firstly since the HPVs associated with cervical cancer have never as yet been isolated as virions, and secondly to allow the possible generation of virions containing mutated PV DNA. To date, no succesful reports exist, but the use of retroviral vectors may in the future allow this to occur. Another approach, at least for BPV1, may come using the transgenic mouse model system, recently reported by Lacy *et al* . (1986).

1.7 Progression of PV-associated benign lesions to carcinoma

The progression of papillomas to carcinomas occurs naturally in a subset of PV-associated lesions. In all cases, such progression is associated with a loss of virion formation, but researchers have been more interested in other phenomena relating to this process.

In the study of the events involved in the progression of benign papillomas to carcinoma, three general approaches have been taken. Firstly, primary tumour samples have been analysed with

respect to viral DNA status and gene expression, alterations in cellular gene expression and non-random gross chromosomal alterations, an approach which has been complemented with similar work on cell lines derived from such tumours. Secondly, specific alterations in viral gene expression have been induced in such cell lines and these have been correlated with phenotypic changes in cellular physiology. More recently, attempts have been made to develop transgenic mouse models to study each stage of the disease with an easily generated supply of tumours of all types. Each virus appears to have characteristics unique to its own system, but the following broad guidelines appear to hold true:

(a): Different patterns of viral gene expression are found in more malignant lesions.

(b): Endogenous cellular proto-oncogenes often become activated.

(c): Non-random chromosome losses appear to occur

(d): Host immunosuppression increases the risk factor for progression.

In the field of molecular changes which occur during the progression to carcinoma, the best studied examples are cervical lesions associated with HPVs 16 and 18. This is due to the wide range of cell lines derived from cervical carcinomas, the large epidemiological studies performed on human populations and the

large available battery of oncogene probes, lack of which have hampered progress of researchers in the bovine systems, who have had to isolate such homologous probes themselves. The recent developent of a transgenic mouse model for BPV1-associated tumorigenesis (Lacy *et al* .(1986)) may have found a way round this limitation.

In independent studies on HPV16- and -18-associated lesions, progression has been correlated with viral DNA integration (Choo *et al* .(1987), Schneider-Maunoury *et al* . (1987)), resulting in the non-random inactivation of the E1 and E2 ORFs, although episomes are also maintained in many tumours. Progression is also correlated with an increase in E6 and E7 gene expression, possibly due to the loss of viral controlling genes but also possibly due to the loss of cellularly-encoded trans-repressors of viral gene expresssion. To my mind, this increase in expression of E6 and E7 is the key event, and the mechanism by which it is achieved may be variable. Of interest in this regard is a recent report indicating that deletions of sequences within the LCR have been found in episomal HPV16 molecules isolated from tumour material (Tidy *et al* .(1989)). It should be stressed, however, that the role of integration should be assessed primarily in tumour biopsies, since researchers have suggested that the passage of cells in tissue culture actively selects <u>against</u> episomal maintenance.

Good evidence for the role of the HPV18 E7 gene in the <u>maintenance</u> of the transformed phenotype comes from work performed on the cervical carcinoma cell line, C4-1 (von Knebel-Döberitz *et al* .(1988)). Introduction into this cell line of

an antisense RNA construct to E7 leads to decreased proliferative capacity of the cells. No analysis, however, of the cellular response to differentiation stimuli was reported. The control of HPV18 gene expression by cellular factors has also been analysed *in vitro* . Using non-tumorigenic hybrids of HeLa x normal human fibroblasts, and tumorigenic segregants isolated from such hybrids, it was possible to show that 5-azacytidine, a potent DNA demethylation agent, could induce specific inhibition of HPV18 gene expression in the nontumorigenic, but not the tumorigenic hybrids (Rösl *et al* .(1988)). Loss of HPV18 gene expression resulted in a decreased proliferative capacity. This indicated that HPV18 gene expression may be subject to negative regulation by cellular factors, but that this repressive activity has been lost in tumour cells. This view has been strengthened by the fact that such tumorigenic segregants show a specific, non-random loss of chromosomes 11 and 14 (Stanbridge *et al* .(1981)) and that reintroduction of chromosome 11 into such cells induces a non-tumorigenic phenotype (Saxon *et al* .(1986)). The role of chromosome 11 loss in the progression of HPV-associated lesions may in fact be more general: similar results have recently been shown to hold true for the cell line SiHa (Koi *et al* .(1989)), which contains a single copy of integrated HPV16 and it is thus reasonable to assume that loss of chromosome 11 may contribute, at least in tissue culture systems, to the tumorigenic phenotype. A third line of evidence, as yet still in its early stages, involves the comparison of HPV transforming activity in two lines of human embryonic fibroblasts (Smits *et al* .(1988)): one, del-11, contains a deletion in the short arm of chromosome 11, whereas

the other contains the normal diploid chromosome content. Using constructs containing E2 mutations, it was shown that after coselection for neomycin resistance, del-11 cells containing HPV16 were able to exhibit anchorage independent growth, whereas normal diploid fibroblasts containing HPV16 were not. This was correlated with high levels of HPV16 transcription in the del-11 derived lines, whereas in the normals, little or no transcription was detectable. This strengthens the view that chromosome 11 contains a repressor of HPV transcription *in vitro* and that loss of such a gene may be a factor in the progression of tumours *in vivo* .

Loss of other chromosomes may also be involved in the late stages of progression. In a second study on the hybrids used by Stanbridge, Klinger (1982) noted loss of chromosomes 2, 13 and 17, but not 14, in addition to chromosome 11.

To my mind, however, studies with regard to chromosome loss(es) in human tumour progression are best performed now on freshly isolated tumour material. In this regard, using Restriction Fragment Length Polymorphism techniques, workers in Japan studying fresh tumour samples have demonstrated a consistent loss of heterozygosity on the short arm of chromosome 3, indicating gene losses in this region (Yokata *et al* .(1989)). The fact that this was not identified at all in the hybrid cell studies indicates the limitation of the approach using only one cell line.

A further indicator for the loss of chromosomes being involved in the progression to malignancy comes from results using a BPV1-containing line of transgenic mouse. Development of fibromatosis was associated with the excision and subsequent

episomal amplification of the BPV1 DNA, whereas non-random partial loss, or translocation, of chromosome 14, as well as an internal chromosome 8 duplication, were shown to be found in the fibrosarcomas which subsequently developed (Lindgren *et al* .(1989)).These pieces of data indicate that loss of several tumour suppressor genes may be associated with progression *in vivo* . As yet their identity remains elusive. The isolation of the tumour suppressor gene on chromosome 11 may, however, be expected in the near future and those on the other chromosomes may well be isolated using other tumour systems. Acquisition of these probes will undoubtedly lead to a new set of markers with which to assess clinical progression, just as those of the oncogenes have been used in the past five years.

The third sphere of research in the progression of HPV-associated lesions has been concerning the activation of cellular proto-oncogenes. With the rapid expansion in oncogene probe availability, further screening will undoubtedly take place in the future, but to date, large-scale studies with positive results have been performed using only two probes: those of **c-Ha-ras** and **c-myc**. Two independent studies have shown an almost complete correlation of overexpression of the **c-myc** oncogene with advanced stages of cervical carcinoma, this resulting often, but not exclusively, from gene amplification or rearrangement (Ocadiz *et al* .(1987), Riou *et al* .(1985)). In the case of the **ras** gene, three diagnostic aberrations have been detected which increase in frequency with progression of the disease: point mutational activation, loss of normal alleles and gene amplification (Riou *et al* .(1988)), although other

researchers indicate that they have found no such changes in HPV-associated human tumours (T. Crook, unpublished results). It is noteworthy that in all cases where the mode of activation was point mutation, an overexpressed **myc** gene was also detected, indicating a cooperating effect between these two genes.

The final area concerning tumour progression concerns the role of immune surveillance. In humans, data has come primarily from studies on patients who have been immunosuppressed either for other medical reasons, notably allograft recipients, or due to a genetic disorder resulting in a lesion in the cell-mediated immune system. In the former case, patients show a much increased incidence of condyloma formation, development of HPV-associated anogenital carcinoma as well as skin carcinomas. The latter case, which causes the clinical syndrome Epidermodysplasia Verruciformis, results in the general development of skin warts associated with HPVs 3, 5, 8-10, 12,14, 15, 17 and 19-29. The papillomas associated with HPVs 5, 8 and 17 are known to progress to carcinomas in 30% of sufferers in areas exposed to sunlight (Orth *et al* .(1980)).

In animals, the best data comes from work concerning BPV4. All work concerning this virus, which formed the subject matter for this thesis, will be described at the end of this introduction.

1.8 Functions of Early region ORFs of BPV1: an *in vitro* analysis

In order to analyse the functions of individual viral gene products one requires assay systems to observe phenotypes and a

system capable of generating viral mutants. In the case of BPV1, such criteria were satisfied by the end of the 1970s. The two assays most used in the early days were both retrospective, end-point *in vitro* assays: the induction of morphologically transformed foci on the background of a confluent monolayer of normal cells (Dvoretzky *et al* . (1980)), and the analysis of viral DNA copy number and status with regard to episomal maintenance or integration into host chromosomes. Mutants were generated using recombinant DNA technology, resulting in the alteration of molecularly cloned viral DNA and, more recently, cDNAs.

Most early experiments used as recipient cells either NIH3T3 or C127 mouse fibroblasts: both are established cell lines and each may be transformed efficiently after transfection with recombinant BPV1 DNA, resulting in the formation of morphologically transformed foci, which upon expansion into cell lines proved to be tumorigenic in nude mice. Except in a few special cases mentioned later, however, the vast majority of recent work has been performed on the C127 cells, due to their lack of spontaneous transformation and the easier subsequent interpretation of data.

1.8.1 The transforming genes of BPV1

The results of a large body of work indicate that the main transforming protein of BPV1 in the C127 background, as detected by focus-forming assays, is that encoded by the E5 ORF. In the context of the full viral genome, however, mutations in other ORFs also lead to a loss of focus-forming activity. The most

important example in this context is that of the E2 ORF: mutants in the 5' end of this ORF invariably abolish transformation (Sarver*et al* .(1984)). The role of the E2 gene product has subsequently been shown to be that of a transactivator (Spalholz *et al* . (1985)), which binds to sequences in the Long Control Region (Androphy *et al* . (1987)) as a dimer (McBride *et al* .(1989)), inducing increased expression from several BPV1 promoters and expression of the E5 gene product in particular(Prakash *et al* . (1988)).

More recently, two further E2 proteins have been discovered which actually inhibit transformation. The first E2-TR, is a 3' truncation of the full length product, expressed from the nt3080 promoter (Lambert *et al* . (1987) originally isolated as a cDNA which inhibited 5-10-fold BPV1-mediated transformation of C127 cells when cotransfected with wild-type DNA. The protein binds to DNA, but contains no transactivation domain. Two mechanisms of action have been proposed: firstly, E2TR might compete with the full length product for E2-responsive element binding sites; secondly, E2TR might inactivate the full-length product by the formation of inactive heterodimers (McBride *et al* .(1989)).

The second repressor is an E8-E2 fusion protein originally isolated in a search for cDNAs encoding a replication function! (Choe *et al* .(1989)) It shows similar properties to E2TR in the assays described, but the actual activities of these two molecules have yet to be determined. They are both, however, expressed in C127 cells transformed by BPV1 (Hubbert *et al* . (1988)) and thus E2TR is not an artefact of the cDNA cloning

procedure, which often produces truncated cDNAs.

Four main bodies of evidence indicate unequivocally that the E5 gene product, and not the overlapping E2 gene product is indeed the transforming protein. Firstly, insertion of translation termination linkers (TTLs) into each individual ORF of a cDNA, C59, which contains ORFS E2, E3, E4 and E5 and which is expressed from the SV40 early promoter, indicates that only mutations in the E5 region abolish transformation (Yang *et al*. (1985)). Secondly, the reconstruction of such mutants into hybrid SV40-BPV1 viruses and subsequent infection of C127 cells has shown that acute overgrowth of the monolayer within 48hrs is only abolished by mutations in the E5 ORF, but not in the E2 or E4 ORFs (Settleman and DiMaio (1988)). Thirdly, E5 mutations within the context of the full viral genome abolish transforming activity in focus asays (DiMaio *et al* .(1986)). Finally, and perhaps most convincingly, the analysis of tranforming retroviruses isolated after transfection of the pZIPneoSV(X1) vector containing the 69T transforming region of BPV1 (Law *et al*. (1981)) into psi2 cells indicates that the only ORF contained within the mRNA transcribed from such viruses is that of the E5 ORF (Bergman *et al* .(1988)). The second assay described here has subsequently been used for an analysis of the E5 gene product by Site-Directed Mutagenesis (SDM).

A role for the E5 protein of BPV1 in *in vivo* transformation has recently been hinted at using a transgenic mouse model for BPV1-mediated tumour formation. It could be shown that detectable levels of E5 protein could only be discerned in the fibrosarcomas (i.e. the malignant tumours) and not in the

premalignant fibromas, implying an association of E5 overexpression with the later stages of disease (Lindgren *et al*. (1989)).

The mechanism of action of the E5 protein was initially a mystery, since its predicted size was only 44 amino acids. The development of antipeptide antibodies specific for the E5 protein have localised the protein mainly to membrane fractions (Schlegel *et al* .(1986)). This work was done on cells overexpressing the protein, however, so the actual location within an infected cell has yet to be demonstrated. The protein has been demonstrated to exist as a 15kDa homodimer (Schlegel and Wade-Glass (1987)) and SDM of the two cysteine residues at the C-terminal of the protein indicates that these residues mediate the dimerisation and that such dimerisation is essential for transforming activity (Horwitz *et al* . (1988)). Recent work has indicated that the protein acts by modulating the activity of growth factor receptors (Martin *et al* .(1989)). Introduction of high levels of human EGF-R using a retroviral vector into NIH3T3 cells along with an E5 expression vector allowed efficient focus formation or growth in agar in the absence of EGF. In the absence of the E5 gene, such phenotypes required exposure of the cells to exogenous EGF. Further analysis indicated that the receptor is maintained in an activated, phosphorylated form at the cell surface, presumably via an inhibition of the endocytotic down-regulatory mechanism. Analagous experiments involving the CSF-1 receptor indicate that this process may be a general one, and not specifically limited to the EGF receptor.

It was found fairly early on that transformation of C127 cells

by epitheliotropic HPVs was inefficient or non-detectable. Analysing the E5 regions of HPVs indicated that such viruses appeared not to have any recognisable E5 ORFs. Even before many HPVs were sequenced, however, a second BPV1-encoded transforming function had been defined in C127 focus assays, and this did show homology to recognisable ORFs predicted within the HPV genome sequences. This function was encoded by the E6 ORF and was originally uncovered only by overexpression of gene products of the 5' early region of BPV1 using a retroviral LTR construct (Schiller *et al* . (1984)). Interestingly, it was found that this activity was only discernible in C127 cells and not in NIH3T3 cells, unlike the E5 ORF, which was active in both cell lines.

A more recent analysis of E6 mutations within the context of the full viral genome (Neary and DiMaio (1989)) revealed that, in the presence of the E5 gene product, no effect on focus-forming activity was evident. Indeed, in the absence of E5 no foci could be induced. After coselection with a neomycin resistance gene, which allows the growth of colonies in the absence of surrounding normal cells, E5 mutants, but not E5 + E6 double mutants yielded ≈50% of colonies with a morphologically transformed phenotype. This indicated, that in the context of the full viral genome, the E6 ORF contains a weak transforming activity which is masked by the presence of the E5 product in the assays used. A more striking result was obtained, however, when the foci obtained using E6 mutants were expanded and tested for tumorigenicity in nude mice: none were ever tumorigenic. A third assay for transformation, that of growth in agar has also been

used to analyse such mutants. This showed that an intact E6 ORF is required for efficient anchorage-independent growth and also indicated an interesting phenomenon: interruption of the splice acceptor site required for the generation of the E6/E7 hybrid message actually increases the efficiency of agar growth. The role of the E6/E7 message will be discussed further later in this section.

How does the E6 protein act? The development of anti-E6 antibodies has allowed the localisation of the protein to the nuclear and membrane fractions (Androphy *et al* .(1985)), and analysis by SDM of E6 gene function using the original C127 transformation assay indicated that tight binding of the protein to the nuclear matrix correlates with its transforming activity (Androphy (unpublished)). The protein contains multiple cys-x-x-cys motifs, which have been shown in the transcription factor TFIIIA to be responsible for the binding of zinc (Miller *et al* . (1985)), and recently the E6 (and the E7) protein has been shown to bind zinc in a radioassay (Barbosa *et al* . (1989)). Finally, SDM of any of the cys residues within the protein abolishes its transforming function (Vousden *et al* .(1989a)). It seems likely, therefore, that the protein acts by altering the activity of cellular transcription factors, although it may also act within the context of full viral genome transformation to modulate viral gene expression. As yet, no specific reports concerning this have been published.

1.8.2 BPV1-encoded replication and plasmid maintenance functions

The second classical observation concerning papillomavirus replication was that the viral DNA was maintained as an episome and that specific DNA amplification took place during the differentiation process in papillomas. From this, one can predict that specific plasmid maintenance functions must exist, either encoded by the virus or supplied by cellular factors, and that a virus-specific DNA replication activator must also be active in differentiating keratinocytes.

In order to test this, assays must be devised. From the observation that infection of C127 cells with BPV1 virions resulted in foci containing 50 - 200 copies of episomally maintained DNA, it could be hypothesised that in this system, two functions were required: an initial replication function (R), which allowed amplification of the BPV1 DNA without being subject to the controls imposed on cellular DNA, and secondly a modulator function (M) which suppressed such replication activity and returned the BPV1 DNA to strict cell-cycle dependent replication. Whether these functions were also involved in plasmid maintenance was an open question.

Initial analysis of BPV1 sequences indicated that the plasmid maintenance function was mediated by two sequences, PMS-1 and PMS-2, which were only active in virus-infected cells (Lusky and Botchan (1984)). These two sequences mapped to the LCR and the E1 ORF respectively. Mutations in the E1 ORF all caused integration of BPV1 DNA in transfection assays, without often

affecting the transforming ability of the viral DNA (Lusky and Botchan (1985)). Mutations in the E7 ORF, however, caused a decrease in DNA copy number, without affecting the episomal state (Lusky and Botchan (1985)).

At this stage, more sensitive and rapid assays were required to probe deeper into the mechanisms concerning these phenomena. A transient DNA replication assay, taking advantage of the inhibition of the restriction enzyme DpnI by methylation, allowed analysis of the early stages of BPV1 DNA replication after transfection of C127 cells (Lusky and Botchan (1986a)). This identified a replication function encoded by the 3' end of the E1 ORF required for transient replication (Lusky and Botchan (1986b)) Furthermore, the inhibition of transient replication of wild-type DNA supertransfected into a line carrying a low copy-number mutant indicated the presence of a virally-encoded repressor of replication (Berg *et al* . (1986a)). Mutants able to replicate under such conditions were found to contain aberrations in the 5' end of the E1 ORF (Lusky and Botchan (1986b)). Such mutants were actually lethal in wild-type cells when selected under coselection for neomycin resistance and no foci were ever observed. This is consistent with loss of the modulator function resulting in runaway replication of BPV1 DNA and subsequent cell death. The ability of R^- and M^- mutants to complement one another in both replication and transformation assays indicates that the two functions are indeed distinct.

This assay system has certain similarities to the presumed vegetative cycle *in vivo* . The stable state resembles a latent infection, whereas the initial amplification is analagous to the

differentiation- induced amplification of viral DNA. The critical difference is, of course, that in the latent state *in vivo* , only a single, or at most very low copy number of viral DNA is maintained, whereas in the *in vitro* model system, the stable state maintains 50-200 copies.

One attempt to study the parameters affecting the switch from a stable state to an amplifying one, using this crude model system, has been published so far (Berg *et al* (1986b)). Using an $E7^-$ mutant which displays a low copy number phenotype, the authors studied the effect of transfecting a cDNA expressing an E6/E7 protein into cells either along with the $E7^-$ mutant or after such a mutant had been established in the cells. They found that transfection into stably established cells only caused a partial reamplification, whereas cotransfection of the two plasmids induced a wild-type phenotype. These results indicate, but do not prove, that expression of the E6/E7 gene product is required before the expression of E1M to allow induction of full amplification of the BPV1 DNA. This data is also consistent with the expression of E6/E7 augmenting the activity of the E1R protein, while the E1M protein may repress the activity of both.

A second, more interesting study (Burnett *et al* .(1989)) concerned the comparison of properties of virally-transformed C127 cells under conditions of exponential growth or maintenance of such cells in a quiescent state. After maintenance of BPV1-transformed C127 cells for up to 1 month in serum-free medium, readdition of serum led to two phenotypically observable events: firstly an overall 8-16 fold amplification of viral DNA copy number within 48hrs, and secondly the formation of

non-dividing 'giant' cells, which displayed both enlarged nuclear and cytoplasmic components. *In situ* hybridisation analysis indicated that most of the amplification occurred in these non-dividing cells. Such phenotypes were not observable either in control C127 cultures or in cells transformed by replication-defective mutants of BPV1. Interestingly, a cell line harbouring a mutant BPV1, generated spontaneously in tissue culture, which contained a 277bp deletion in the early poly(A) site and the 3' enhancer, displayed these phenotypes at confluence in the absence of serum starvation/restimulation.

What does this mean? The data is consistent with a model whereby a specific replication function is specifically expressed only at confluence. In the case of wild-type viral DNA, however, it might be the case that any M protein expressed prior to confluence might override such a signal by prior binding to viral DNA. Upon serum stimulation, inactivation of M might occur, allowing runaway replication to occur, leading to 'giant' cell formation. In the case of the viral mutant, one might postulate that in the absence of the 377bp sequence, replication function activity cannot be inhibited even in the presence of serum, thus leading to giant cell formation when simply maintained at confluence. This system shows potential for much more detailed studies concerning BPV DNA replication mechanisms becoming possible.

1.9 **HPV-mediated transformation *in vitro***

The identification and analysis of transforming functions of

HPVs *in vitro* has lagged behind that of BPV1: firstly simply due to the more recent isolation, cloning and sequencing of many of the HPVs and secondly due to their lack of activity in the C127 and NIH3T3 focus assays used originally. Recently, methods have been devised to detect HPV transforming functions within the background of established mouse fibroblasts, but other systems utilising primary cultures of epithelial, and occasionally fibroblastic origin have now been developed and these have actually yielded more information than those involving NIH3T3, C127 or the established rat line 3Y1. Before this work is described, however, it should be stressed that all of this work has been published since the start of my project in late 1986.

The primary aim of tissue culture assays for HPVs was to discriminate between viruses such as HPVs 6 and 11, which are primarily associated with benign cervical lesions *in vivo* , and those such as HPVs 16, 18, 31, 33 and 35 which are associated more often with malignancies. Three such assays have been used and their results will now be discussed.

The first assay simply involved transfecting primary keratinocytes derived from the susceptible genital tissues of both male and female with HPV DNA and subsequent analysis of growth properties *in vitro* (Dürst *et al* . (1987), Woodworth *et al* . (1988), Pecoraro *et al* . (1989)). Simple passaging of such cells induces senescence within 3 months in mock- or HPV6-transfected cells, but not in those transfected with HPVs16 or18. It was found that the phenotype induced by HPVs 16 and 18 required interruption of the E1 ORF, which correlates well with the interruption of the E1 ORF in the integrated forms of

HPVs found in the later stages of disease development *in vivo* . It is also noteworthy that coselection experiments involving selection for neomycin resistance yield essentially similar results (Kaur and McDougall (1988), Pirisi *et al* . (1988), Woodworth *et al* .(1989)) . Karyotypic analysis of such immortalised cell lines indicates aneuploidy, but no reports of consistent chromosomal loss or duplication are available. The implication, therefore, is that expression of HPV16 or 18 genes leads to a destabilisation of DNA replication fidelity, which may in some instances lead to development of the immortalised phenotype.

Analysis of any of these cell lines in nude mouse tumorigenicity assays led to negative results and thus researchers tried to devise assays in which HPV functions, perhaps in cooperation with other factors, could induce such a phenotype. Since it had been shown that **c-Ha-ras** and **c-myc** gene activation was often found in cervical carcinomas (Riou *et al* , (1987)), cotransfection assays were devised to test cooperation between HPVs and such oncogenes *in vitro* . The best characterised is that of primary Baby Rat Kidney (BRK) epithelial cells (Matlashewski *et al* . (1987a)). Using this system, workers in the laboratories of Crawford, Howley and Pater have shown that morphologically transformed foci may be obtained after cotransfection of oncogenic, but not non-oncogenic, HPVs and the activated **c-Ha-ras** oncogene, either on a background of a confluent monolayer or more efficiently by the use of selection for neomycin resistance, provided that efficient expression of the HPV early genes is achieved. This has been induced either by

cloning the HPV DNA downstream of retroviral LTRs (Storey *et al*. (1988)), by treating cells with dexamethasone (Pater *et al* .(1988)) or progesterone or via cotransfection of an HPV16 E2 expression vector (Lees *et al* . (1990)). Expansion of such foci into cell lines and injection of these cells into nude mice induces tumours efficiently.

This system has subsequently been used to identify the HPV gene function responsible for the phenotype and this has been mapped to the E7 ORF (Phelps *et al* . (1988), Storey *et al* . (1988)), a result initially surprising since the BPV1 E6, but not the E7, gene is transforming in C127 assays. The E7 ORF and the E6 ORF are thought to be derived from a common ancestral gene (Cole and Danos (1988)) and thus the fact that the oncogenic HPV E7 genes are transforming in this assay may not in fact be surprising in retrospect. After the identification of E7 activity in this system, SDM has been used to show that the cys-x-x-cys domain at the C-terminus of the protein is essential for activity (Storey *et al* .(1990)), thus pointing to a fundamentally similar mechanism of transformation of HPV E7 gene products and the E6 gene product of BPV1 and allowing the obvious prediction that the HPV16 E7 protein binds zinc.

It has further been shown, using E7 constructs employing the inducible Mouse Mammary Tumour Virus (MMTV) promoter, that continued expression of E7 is required for the maintenance of the transformed phenotype in such assays (Crook *et al* . (1989)). Indeed, such cells senesce if the E7 remains turned off for more than 3 days. This correlates well with the data of von Knebel-Döberitz *et al* . (1988), who showed using antisense RNA

constructs that abolition of HPV18 E7 expression caused decreased growth of the C4-1 cervical carcinoma cell line.

A further interesting result was obtained by the cloning of the E7 genes of HPV6 and -11 into expression vectors(Storey *et al* .(in press)). Cotransfection with pSV2neo and activated **c-Ha-ras** led to the formation of neomycin-resistant foci, but at a frequency 50-100-fold less than that observed for the oncogenic HPVs. Only a subset of such foci could be expanded, and therefore these lines may have arisen from rare cellular changes which may be induced by oncogenic HPV E7 gene products but not by those of HPVs 6 and 11. The indication is therefore that the E7 gene products of benign HPVs display a **subset** of the activities associated with the analogous proteins of the oncogenic HPVs, thus leading to less frequent association with tumours *in vivo* .

Using a slight modification of this system, two further questions have been addressed: namely, are any other oncogenes capable of cooperating with HPVs and secondly, what is the response of the immune system to the presence of such cells? Deriving the primary cells from baby BALB/C mice (BMK cells) instead of from Fischer rats allows the injection of cells into syngeneic, immunocompetent animals as well as into nude animals. Using this modified system it was shown that **v-fos**, but not activated **c-myc** was capable of cooperation with HPVs in the induction of foci and subsequent tumours in nude mice (Crook *et al* . (1988)). When injected into immunocompetent animals, however, a difference was observed: whilst the HPV + **ras** foci still induced tumours, none could be induced after transformation with HPV + **fos.** This appears to indicate that escape from

immune surveillance is a function of cellular changes and not a result of altered viral expression, but this is by no means certain.

What, therefore, is the role of **c-myc** activation in the tumorigenic process? Clearly, *in vivo* , the overexpression of the gene is correlated with late stages of cervical carcinoma: it was therefore considered likely that the lack of activity of **c-myc** in the assays described must be due to the design of the experiments. This has been shown to be the case recently. Cotransfection of an HPV16 plasmid pW12 with pSV-cmyc (a construct lacking exon 1, thus leading to overexpression of the protein) into BMK cells lead to efficient induction of foci after selection for neomycin resistance, which upon expansion proved to be tumorigenic in mice (T. Crook, unpublished results). It was found that treatment with dexamethasone decreased the efficiency of focus formation. This indicates that overexpression of HPV early genes in the presence of **c-myc** overexpression may well be lethal to cells *in vitro* . A second piece of evidence for **c-myc** overexpression in the progression of tumours comes from the observation that transfection of cells containing HPV16 and **v-fos**, whose tumorigenicity is dependent on continued treatment with dexamethasone, with pSV-cmyc leads to a dexamethasone-independent tumorigenic phenotype and also decreases the number of cells required to be injected into syngeneic animals in order to induce tumours.

This system has thus reproduced many of the observations found in primary tumour samples. It remains to be seen whether it can be used to examine the role of the other HPV genes in the transformation process.

Having identified the E7 protein as a transforming gene of oncogenic HPVs, workers have tried to define a function for this protein. The way forward was shown by the bringing together of three observations: firstly that the HPV16 E7 gene was shown to possess activities similar to the adenovirus E1a region, in that it could both activate transcription from the Adenovirus E2 promoter and cooperate with the activated **c-Ha-ras** gene in the transformation of primary cells (Phelps *et al* . (1988)); secondly, a highly conserved domain within the E7 ORFs of oncogenic HPVs was identical to domains in adenovirus E1a shown to be required for immortalising activity; and thirdly, it was shown that the protein product of E1a interacts with the 105kDa product of the retinoblastoma susceptibility gene (Rb), a protein shown to be associated with DNA on affinity columns (Lee *et al* . (1987)). Inactivation of both alleles of this gene has been shown to be the crucial determinant in the development of childhood retinoblastoma (for review, see Weinberg (1988)) and thus this gene has been assigned to the long-predicted group of tumour suppressor-, growth suppressor- or antioncogenes. It has now been shown that E7s from oncogenic HPVs are capable of binding Rb *in vitro* (Dyson *et al* . (1988)), which would presumably inactivate the protein in intact cells but as yet, the status of Rb activity in HPV-associated cancers has not been determined. However, SDM of the conserved region of HPV E7 analagous to the region of E1a shown to be necessary for Rb binding has shown that mutation of these sites causes loss of activity in either the Ad E2 promoter transactivation assay or the BRK **ras** cooperation assay (Phelps *et al* . (unpublished)), thus strongly implicating a

functional role for Rb binding in the transformation process.

Whether Rb inactivation actually occurs during the immortalisation process of human keratinocytes is unknown as yet, but evidence from another assay indicates that E7 alone is unable to induce the immortalised phenotype in this more homologous system. This assay, devised by Schlegel (Schlegel *et al* . (1988)), has three facets, involving sequential selection for stimulation of cellular proliferation, resistance to differentiation inducers and finally immortalisation after transfection of primary keratinocytes with linear HPV DNA. The discrimination between oncogenic and non-oncogenic HPVs occurs at the second stage and it is noteworthy that the percentage of colonies resistant to differentiation is very low, even when using the oncogenic HPVs (although for the non-oncogenic viruses there is absolutely no continued growth), indicating a rare second event being selected for at this stage. It was found that transfection of a region encoding solely the E6 and E7 ORFs was sufficient to produce immortalised clones (Münger *et al* . (1989)), whereas interruption of either of these ORFs, or introduction of clones containing only one of the two ORFs allowed formation of colonies resistant to differentiation inducers but not immortalisation. This indicates that E6 has functions similar to E7 in this assay, yet it is completely inactive in the BRK cooperation assay. It may be that both E6 and E7 interact with distinct tumour suppressor genes and that inactivation of both genes is required for the immortalisation of human keratinocytes. This double requirement would be consistent with the fact that spontaneous immortalisation of human keratinocytes has never

been observed, but at present this is mere speculation and not scientific fact.

Such assays have been informative in many respects, but they do not address the question of how HPV gene expression affects differentiation in the context of a stratified epithelium. From histological sections of biopsy material, it has been possible to define a number of stages between benign and frankly malignant tumours on the basis of the extent of the epithelium showing non-differentiated basal-like cells in the upper layers. These have been termed Cervical Intraepithelial Neoplasia grades I to III (CIN I - III). In the past two years, a novel method of keratinocyte cultivation has been developed using collagen rafts (Kopan *et al*. (1987)), which allows a mimicking of epidermal differentiation, and thus cell lines derived from each type of naturally occurring lesion may be analysed. Analysis of cells transfected with HPV16 showed traits such as parabasal cell crowding and enlarged nuclei in the upper layer characteristic of CIN I (McCance *et al* . (1988)). Undoubtedly, such an assay system will be used extensively in the future for the analysis of HPV gene function.

It was mentioned at the start of this section that assays for HPV gene function using established cell lines have now been developed. It has become clear that expression of HPV genes from their own promoters in cells such as NIH3T3, C127 or rat 3Y1 is inefficient. Full transformation of such cells has been achieved using retroviral vector constructs containing HPV16 (Matlashewski *et al* . (1987a)). In general, information derived from study on such cell lines has been similar to that derived from the systems previously mentioned, albeit more difficult to

detect, and thus only novel findings will be discussed here. The most interesting finding is that the E2-E5 region of HPV16, when expressed from a retroviral LTR, contains a weak transforming activity in an NIH3T3 assay (Vousden *et al* . (1989b)). This assay involved prior selection with the neomycin resistance gene and subsequent maintenance of the monolayer at confluence, scoring subsequently for focus formation. Using this region of DNA, focus formation was 50% that of the E6/E7: in both cases, however the efficiency was pitifully low. Such foci were unable to grow in agar, unlike those induced by E6/E7. What this result means, if anything, is unclear - obviously focus formation requires a second, rare event to occur, but to my mind, better systems for study of such phenomena should be sought - searching for 0.4 foci/μg plasmid DNA in NIH3T3 cells is not the most productive form of research ever undertaken!

One final direction of work relevant to this thesis concerns the tissue-specificity of HPV action. The discussion prior to this has described transfection of primary epithelial cells, but not that of primary fibroblasts. Pirisi *et al* .(1987) showed that transfection of an HPV16-containing plasmid extended the lifespan of human foreskin fibroblasts, but that rare (unidentified) secondary events were required to prevent subsequent senescence. This work was extended by Watanabe *et al* .(1989) who showed, using human embryonal lung fibroblasts, that both the E6 and E7 ORFs were required for an extension of *in vitro* lifespan, but the question of subsequent senescence was not addressed.

Two other studies are worthy of mention. Both involve cotransfection of HPV16 and activated **c-Ha-ras** sequences, one

into primary Rat Embryo Fibroblasts (REFs) and the other into what turned out to be human fibroblasts derived from the cervix. In the former (Chester and McCance (1989)), full focus induction was achieved and such foci, upon expansion, were tumorigenic in nude mice. In the latter case (Matlashewski *et al* . (1988)), cells were selected solely on the basis of extended lifespan and such cells were <u>not</u> tumorigenic. Since in the case of primary syrian hamster fibroblasts, specific chromosome loss has been shown to occur during the selection procedure, it may be that such losses do not occur so readily in cells of human origin, or alternatively that a greater number of gene losses are required to render human cells tumorigenic. The clear indication from this work, however, is that the tissue-specificity of HPVs may partly be due to the differing activities of their transforming proteins in different tissues and not solely due to different levels of expression.

1.10 Proposed model for PV life cycle in epithelial cells

In this section, I will attempt to bring together a large body of data into a model for PV-induced papillomatosis consistent with that which is known at present. To a certain extent this is speculative, but so is research work itself. The following facts are worthy of mention:

1. Within the epithelium, there exists a pool of predominantly non-dividing stem cells, which are presumably stimulated to divide in response to localised wounding to allow repopulation of

a depleted area of epithelium.

2. Many PVs are known to reside latently within cells. Development of papillomas during transmission often results from concurrent trauma at the site of infection (e.g. BPVs and CRPV).

3. Many viruses across the entire range of living organisms have two modes of existence:

(a): a latent existence (referred to as **lysogeny** in bacteriophages)

(b): a productive cycle.

4. In several cases, different programmes of viral gene expression have been identified in the two different modes of viral existence:

(a): In bacteriophage lambda, predominance of **cI** expression leads to the lysogenic state, where no other phage gene products are expressed, whereas predominant expression of **cro** leads to subsequent expression of further transactivators, virus-specific DNA replication and phage production (Ptashne *et al* . (1980)).

(b): In the case of EBV, at least 7 viral gene products have been characterised as being latent infection antigens (EBNAs 1-6

and LMP), whereas far more extensive gene expression is discernible during productive infection (Sugden (1989)).

From experiments performed in tissue culture, two, and possibly three BPV1 gene products may be proposed to be latency proteins:

(a): E2TR - this represses BPV-mediated transformation of C127 cells (Lambert *et al* .(1987)), binds to DNA sites previously identified as E2-dependent enhancers and thus presumably represses transcription from all promoters activated by the full-length E2 gene product (P97 and P2443 and possibly others as well). The as yet uncharacterised E8-E2 fusion protein (Choe *et al* .(1989)) may also have a role in such a scheme. In the case of HPV 16, the full-length E2 gene product may well be a repressor itself, since an E2 binding site resides between the CCAAT- and TATA boxes implicated in transcription from the early promoter.

(b): E1M - this protein inhibits BPV-specific DNA replication, but appears to permit cell-cycle associated replication. Furthermore, E1M mutants have increased transcription rates from the P97 promoter (Lambert and Howley (1988)), a promoter which transcribes genes associated with the BPV DNA amplification during the productive cycle. It is presumed that E1M binding to PMS-1 and/or PMS-2 maintains the viral DNA in an episomal form, although no evidence for this has been published to date. Expression of such a protein in infected stem cells might lead to stable maintenance of a single- or a few episomal copies of PV DNA.

The activity of many transcription factors has been shown to be modulated by phosphorylation. E2TR has been shown to be a nuclear protein (Hubbert *et al* .(1988)), and the E1M product has also been shown to be phosphorylated (Thorner *et al* .(1988)). It may therefore be the case that these proteins are inactivated by phosphorylation, and thus that agents inducing such events may act as promoters in the multistage carcinogenesis model. [It is of interest that TPA, a known tumour promoter, stimulates transcription of latent BPV1 genomes in tissue culture (Amtmann and Sauer (1982)), and that such stimulation leads to a return of the transformed phenotype. Furthermore, TPA has been shown to induce phosphorylation of the previously identified transcription factor AP1(Curran and Franza (1988)), leading to subsequent alterations in the pattern of cellular gene expression].

The proposed model for the virus life cycle is as follows:

In the absence of any cooperating factors, papillomavirus infection does not lead to any noticeable phenotype. The viral DNA is maintained stably, however, in stem cells and/or basal cells, but no virus-specific DNA synthesis takes place. Such a situation is maintained stably by the expression of a subset of viral proteins, as yet not completely identified. The critical event for papilloma (here I imply benign lesions and not necessariy productive infections) formation is stimulation of gene expression from the early promoter, leading to expression of E6, E6/E7 and, as a result of the latter, E1R, resulting in cellular hyperproliferation and PV-specific DNA replication. Expression of

some critical differentiation marker may lead to expression of the viral capsid proteins, leading to virion assembly in the quiescent stratum granulosum/corneum. How this might be achieved is left open: thus in the case of BPV1, activation of E2 gene expression is the most obvious course, and one presumes that the wounding response, induced by the abrasive mode of infection, leads to such stimulation. In the case of HPVs, however, activation might result from the infection of such cells by a second virus: it is noteworthy that activation of the HPV18 URR *in vitro* has been induced by transactivators of Herpes Simplex Virus Type 2 (HSV-2), a virus which has also been associated with cervical lesions *in vivo* (Gius and Laimins (1989)).

In the final section of this introduction, I will describe firstly the knowledge concerning the role of Bovine Papillomavirus Type 4 (BPV4) in the development of epithelial tumours of the Upper Alimentary Canal (UAC) in cattle, the state of knowledge as I started my Ph.D. project in late 1986 and finally the initial aims of that project.

1.11 BPV4 and Cancer of the upper alimentary canal in cattle

In the Western Highlands of Scotland there is a focus of cancers of the UAC in cattle, which represents a significant economic problem. Extensive research indicated the cooperation of two factors working over a period of at least 7 years (Jarrett *et al*.

(1978a)):

(a): Bovine Papillomavirus Type 4 (BPV4)

(b): the prolonged ingestion of bracken fern (which is known to contain mutagens and immunosuppressants).

The virus has been characterised (Campo *et al* .(1980)) and shown to be a member of the B subgroup of BPVs: these are strictly epitheliotropic viruses of molecular weight ≈7.2kb and which show no cross-hybridisation to members of the A subgroup, namely the bovine fibropapillomaviruses whose prototype is BPV1 (Jarrett (1985)).

Reproduction of the field results in animal experiments (see Campo and Jarrett (1987)) indicated firstly that infection of scarified epithelium with BPV4 led to formation of benign papillomas within a period of months, which regressed naturally due to a host immune response. Secondly, it was found that concomitant treatment of infected animals with the immunosuppressant azathioprine led to far more widespread and persistent papillomatosis; the development of malignant UAC tumours, however, could not be monitored since cattle died from bladder cancers within two years. Thirdly, feeding of cattle infected with BPV4 on bracken fern led also to far more widespread and persistent papillomatosis and to date, two cases fed with the bracken have led to the development of carcinoma.

Analysis of nucleic acids from papillomas and carcinomas from both field cases and experimental animals gave the extremely

surprising result that although BPV4 DNA was present as high copy number episomes in papillomas, no viral DNA could be detected in 99% of the transforming papillomas or carcinomas which were examined (Campo *et al* . (1985)). Two plausible explanations can be suggested to explain a result in direct contrast to those found in all HPV-associated tumours as well as those generated by CRPV: firstly, the carcinomas are of different clonal origin to the papillomas and therefore BPV4 has no role in the malignant transformation process in the UAC of cattle. Alternatively, the presence of BPV4 is not required for the progression from papilloma to carcinoma, but is required for an early initiating event in the carcinogenesis programme.

As yet, no formal examination of the clonality of tumours has been performed: no papilloma-specific markers have been identified whose status could be investigated in subsequently forming carcinomas (point mutational activation of **ras** sequences is an obvious candidate: sequence data of bovine **ras** genes is required to examine this. Such work has been carried out within the group (McCaffery *et al* . (1989)). In a detailed abattoir survey, however, Jarrett *et al* . (1978b)) found identical spectra of presentation sites for papillomas and carcinomas, indicating strongly a common origin of benign and malignant lesions.

This model system is interesting in several regards: firstly, it is a large animal model, which may therefore be expected to mimic human tumorigenesis more closely than rodent systems; secondly, unlike BPV1 but similar to the oncogenic HPVs, the virus is strictly epitheliotropic and induces malignant, epithelial lesions in its natural host; and thirdly, the system involves the

interaction of a virus, chemical mutagens and immunosuppressive agents, three of the classical components associated with cancer to date.

In attempts to elucidate the mechanism of BPV4-associated transformation, research has proceeded via two pathways: firstly via analysis of the transcriptional pattern of BPV4-induced papillomas *in vivo* and secondly via *in vitro* transformation of tissue culture cells, since the huge time periods required for animal experiments was prohibitive in terms of cost and practicality. The results of the former approach have been described extensively in a recent thesis (A.C. Stamps (1987)) and will not be described further here. The results of the latter approach will now be summarised.

1.11.1 *In vitro* transformation by BPV4

Since virions of BPV4 were readily available, it was natural to try to induce observable phenotypes via infection of cells *in vitro*. As yet, no primary bovine keratinocytes derived from the UAC have been succesfully cultured, and no phenotype could be induced by BPV4 using fibroblastic cells of the same origin, unlike the cases of BPVs 1 and 2, which readily induced focus formation, yielding cells which were tumorigenic in athymic mice (Moar *et al* . (1981)).

The next obvious cell types to try were the murine fibroblast lines NIH3T3 and C127. By this stage, the viral DNA had been molecularly cloned and thus subsequent experiments involved transfection of such DNA. Using NIH3T3 cells, BPV4 DNA was able

to induce foci at a frequency similar to that of BPV1 (Campo and Spandidos (1983)) and such cells were fully transformed using the criteria of anchorage-independence of growth and tumorigenicity in athymic mice. In light of recent results involving HPV-mediated transformation of NIH3T3 cells, it is noteworthy that the cloning of BPV4, also a strictly epitheliotropic virus, involved the interruption of the E1 ORF: the low focus-forming activity of HPVs in NIH3T3 assays might therefore be increased by interruption of this ORF.

The factors affecting BPV4-mediated transformation of C127 cells have proven to be much more complex and the results derived from it much more interesting. Firstly, full focal transformation required the separation of the viral DNA from plasmid sequences. Recircularisation (i.e. reconstruction of a functional E1 ORF) decreased transformation efficiency ten-fold. Secondly, transformation induced by complete linear viral DNA required either concomitant treatment of cells with the tumour promoter TPA or a period of exponential growth in the presence of a critical concentration of growth factors (Smith and Campo (1988)). Both of these procedures will cause expression of endogenous cellular genes, which is presumably required in addition to BPV4 in order to elicit the fully transformed phenotype. This is directly analagous to the *in vivo* finding that papilloma formation required both viral infection and concomitant induction of wound healing responses.

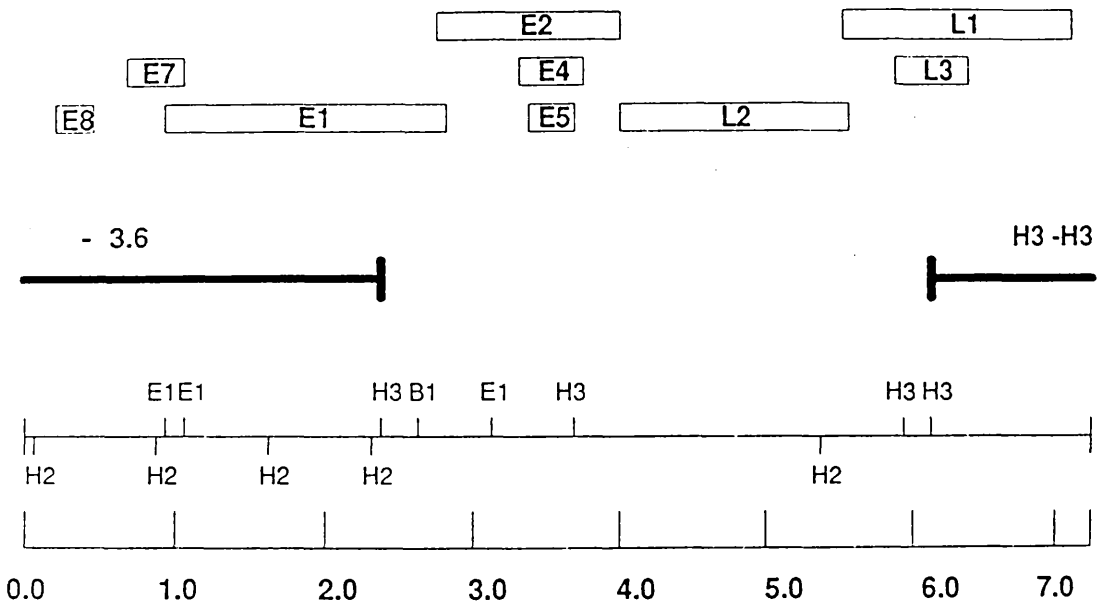
Thirdly, it was found that upon passaging the cells, viral DNA was lost, without affecting the transformed phenotype. Indeed, in some lines, BPV4 DNA could never be detected even at early

passage. No correlation between the presence of viral DNA and tumorigenicity could be found. The clear indication was that, as *in vivo* , BPV4 was only required for some initial 'hit', after which its presence was dispensable.

Recent work has led to the isolation of a 10kb murine sequence whose amplification correlates exactly with the tumorigenic phenotype of cell lines (Smith and Campo (1989)). This was isolated originally as a viral DNA-cellular sequence junction point from a cell line, C4Ta2a, which contained several hundred copies of integrated, rearranged BPV4 DNA. This murine sequence is active in the C127 focus assay and has also been found to be activated in chemically induced carcinomas of the laboratory mouse strain SenCar (K.T. Smith (unpublished results)). It is obviously of interest to determine whether a bovine analogue is activated in naturally occuring tumours of the bovine UAC and attempts to isolate the bovine equivalent are currently underway.

This system has also been used to determine the regions of the BPV4 genome required for transformation. The first approach used involved simple digestion of BPV4 DNA with various restriction enzymes - the appropriate sites and their relation to the genome organisation predicted from the viral nucleotide sequence (Patel *et al* . (1987)) are shown in Figure 1.6. This indicated that maintenance of the E7 ORF was essential for transformation: digestion with HindIII which leaves a 3.6kb sequence spanning this region intact, has no effect, whereas additional digestion with EcoRI, which cuts twice within this ORF, completely abolishes transformation. Cloning of this 3.6kb sequence has shown that this region alone is capable of inducing

Figure 1.6: Restriction sites within the BPV4 genome



Abbreviations: E1 EcoRI
B1 BamHI
H3 HindIII
H2 HincII

full transformation of C127 cells and amplification of the 10kb murine sequence, at a frequency similar to, or greater than that of wild-type DNA. This region of DNA contains three ORFs: the predicted amino acid sequence of the first, E8, has recently been proposed to share structural homology to the E5 ORF of BPV1, and thus this gene might also play a part in BPV4-mediated transformation. The second, E7, contains the motif cys-x-x-cys and is thus presumed to be a zinc-finger protein. The third is the 5' half of the E1 ORF: the function of this region of PV genomes is presumed to be concerned with viral DNA replication: however, since in C127 cells, maintenance of episomal BPV4 DNA molecules does not occur, any transforming function due to this region would be due to aberrant functions as yet not ascribed to any other E1 ORF of any PV.

While this system has yielded some very interesting results, it is in one way at odds with the *in vivo* situation: BPV4 *in vivo* acts at the earliest stages of the tumorigenic process, whereas, in these assays, the recipient cell lines are already established and thus might be considered to be analagous to premalignant tumour cells. A better assay system might therefore involve cells which have not yet been established as immortalised cell lines. This will be returned to a little later.

1.11.2 Cellular changes in the progression to carcinoma

The second major area of study in the field of molecular biology of BPV4-associated tumorigenesis has come from an examination of the status of cellular proto-oncogenes in naturally occurring

tumours. NIH3T3 transfection assays using DNA isolated from such tumours has, along with Southern blotting, shown that activated **ras** genes exist in at least two cases: in one case, amplification had also occurred (Campo *et al* . (1990)).

The second oncogene studied was that of the Epidermal Growth Factor Receptor (EGF-R). Using a [^{125}I-] EGF binding assay, it was shown that a cell line, 88529C, explanted from a naturally occurring carcinoma of the UAC, showed levels of receptor similar to that of the A431 cell line, which shows a 10-50-fold amplification of the EGF-R gene (Smith *et al* . (1987)). Furthermore, primary bovine fibroblasts transformed by the fibropapillomavirus BPV2 showed a 3-fold increase in EGF binding, which in the light of the results described earlier concerning the BPV1 E5 gene, may well be ascribed to the expression of the analagous gene of this virus.

When I joined the group in Glasgow in late 1986, almost nothing was known in detail about the transforming activities of strictly epitheliotropic viruses, of which BPV4 was a member. The aims of the project, the results of which are described in the remainder of this thesis, brought together some of the observations described previously in this section: to attempt to establish a culture system for the analysis of the transformation of non-established bovine cells by BPV4, to investigate the role of specific viral functions in such a process, and to investigate the effect of such changes on the expression of the EGF-R gene. The last part of this has proven to be relatively unproductive, and will thus be reported only briefly in this thesis.

SECTION 2:

MATERIALS AND METHODS

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2.1 Materials

2.1.1: Chemicals and solution preparation:

Analytical grade chemicals were used: most were purchased from BCL, BDH Ltd., Gibco-BRL Ltd., Pharmacia or The Sigma Chemical Co.. Distilled phenol was purchased from Rathburn Chemical Co. and formamide was purchased from Fluka AG. All chemicals were weighed in plastic boats on a Mettler PM300 balance to an accuracy of 0.01g.

2.1.1(i): Water:

All water had been purified prior to use by passage through the Milli-Q Reagent Grade Water System (Millipore).

2.1.1(ii): Preparation- and pH of solutions:

All chemicals were dissolved at room temperature by stirring generated by a magnetic flea or, if necessary, by heating to 65^{o}C. The pH of solutions were adjusted using appropriate acids or bases and measured using a Tris electrode Kent EIL 7045/46 (Russell).

2.1.1(iii): Sterilisation of solutions:

Heat-sensitive solutions were passed through a 0.22μm Acrodisc filter (Millipore) into sterile Universals (Millipore).

Other solutions were sterilised by autoclaving in a pressure cooker for 15 mins.

2.1.2: **Preparation- and storage of Organic reagents:**

Phenol, 'phenol/chloroform', 'chloroform' and formamide were prepared and stored as described in Maniatis *et al* (1982).

2.1.3 **Enzymes:**

Restriction enzymes were purchased from BCL, BRL and Pharmacia, most of which were supplied with appropriate buffers.

Other enzymes were purchased as follows:

Calf Intestinal Phosphatase (CIP) from BCL;
T4 DNA ligase from BRL;
lysozyme, proteinase K and ribonuclease A (RNase A) from Sigma;

2.1.4: **Kits for molecular biology:**

Random primer labelling kits were obtained from BCL.
GeneClean™ kits were obtained from Bio-101 Inc.

2.1.5: **Radioabelled compounds:**

These were purchased from Amersham International plc:

Compound	Catalogue No.	Specific Activity
[^{32}-P]-dCTP	PB10205	>111T Bqmmol^{-1}
D-Threo [dichloroacetyl-1-^{14}C]-chloramphenicol	CFA515	>1.85 GBqmmol^{-1}

2.1.6: **Other materials:**

Nucleic acid markers: HindIII-digested bacteriophage lambda
HaeIII-digested bacteriophage øX174

Biodyne nylon transfer membrane was obtained from Pall Process Filtration Ltd.

X-Omat XAR5 film was obtained from Kodak.

2.1.7. **Centrifuges:**

For large-scale, low speed preparations (>2ml), a Du Pont Instruments Sorvall RC-5B centrifuge was used. For pelleting bacteria and in situations where the use of disposable plastic tubes was called for, an MSE Mistral 4L low speed centrifuge was used. For small scale centrifugations (<1.6ml), an Eppendorf 5414 bench-top 'microfuge' was used. Preparative ultracentrifugation was performed using a Kontron Instruments Centrikon T-2070.

2.1.8. **Bacterial media and antibiotics:**

Luria Broth (L-Broth): 1% (w/v) bactotryptone (Difco)
0.5% (w/v) yeast extract (Difco)
170mM NaCl

L-Agar: 1.5% (w/v) agar in L-Broth.

Superbroth: Solution A: 1.33% (w/v) bactotryptone
2.66% (w/v) yeast extract
0.44% (v/v) glycerol

Solution B: 0.54M KH_2PO_4
0.27M K_2HPO_4

Each solution was autoclaved separately then 900ml A was mixed with 100ml B.

After autoclaving of such growth media and cooling to 50°C, ampicillin was added if desired to 50 µg/ml.

SOC:

2%(w/v)	bactotryptone
0.5%(w/v)	yeast extract
10mM	NaCl
2.5mM	KCl
10mM	$MgCl_2$
10mM	$MgSO_4$
20mM	glucose

Ampicillin: stock solution: 50mg/ml in water, stored at -20°C.

2.1.9 **Plasmid vectors and bacterial strains:**

Plasmids used in this study, along with their source, are described in Table 2.1. All novel plasmids generated during this study will be described in the results section. All plasmids were propagated in the E. coli strain DH-5A^{TM}, supplied as frozen stocks by BRL.

Table 2.1: Plasmids used in study (continued overleaf)

PLASMID	SOURCE	DESCRIPTION
pSV2neo	Southern and Berg (1982)	Neomycin resistance gene of Tn5 cloned under control of SV40 early promoter with 3' poly (A) site in a pBR322 background
pZIPneoSV(X)1	Cepko et al. (1984)	Mo-MuLV-derived retroviral vector containing neo gene and BamH1 cloning site.
pMS-2	M.S. Campo (unpublished)	Neomycin resistance gene cloned under the control of Herpes Simplex Virus thymidine kinase gene promoter with 3' poly(A) in pBR328
pBV4 B1	Campo and Spandidos (1983)	BPV4 cloned in BamH1 site of pAT153
pBV4 Sst1	M. O'Prey (unpublished)	BPV4 cloned in Sst1 site of pUC19
pBV4AB	M.S. Campo (unpublished)	2.0kb H3-E1 of BPV4 (nt 6110 - 906) ligated to 1.2kb E1-H3 of BPV4 (nt 1139 - 2356) cloned in pAT153
pIC20R.H3-H3 3.6	I. Doherty (unpublished)	3.6kb H3-H3 fragment of BPV4 (nt 6110 - 2356) cloned in pIC20R.

Table 2.1(continued)

PLASMID	SOURCE	DESCRIPTION
pCGBPV-MMTV-CAT-A2	Matthias et al. (1986)	Chloramphenicol acyltransferase gene under control of MMTV-LTR in a BPV1-based vector also containing a neomycin resistance gene.
pSVvmyc	Land et al (1983)	Retroviral gag-myc gene under MC29 LTR control cloned in pSVgpt
pT24	Santos et al (1982) and M. O'Prey (unpublished)	6.6kb genomic clone of the activated human c-Ha-ras gene derived from the T24 human bladder carcinoma cell line cloned in pUC13
pME2.0	Hung et al (1986)	2.5kb EcoRI fragment of the mouse EGF receptor gene cloned in pSP64

2.2 Methods in recombinant DNA technology

2.2.1 Basic methods:

2.2.1(i): Phenol/chloroform extraction:

Proteins may be removed from DNA-containing solutions by extraction with phenol/chloroform. An equal volume of phenol/chloroform (see 2.1.2(ii)) was added to the DNA solution and the tube was vortexed for 15 secs. After spinning for 5mins in the microfuge, the aqueous phase was transferred to a clean tube.

2.2.1(ii): Ethanol precipitation:

Concentration of dilute DNA samples and the changing of a sample buffer was achieved by ethanol precipitation. With concentrations greater than 1μg/ml, the procedure was almost quantitative.

1/10 volume (vol) of 3M NaOAc pH5.2 and 2 volumes of absolute ethanol (stored at -20^{o}C) were added. The tube was vortexed, placed on ice for 10mins and spun at 4^{o}C for up to 30mins in the microfuge. The supernatant(S/N) was carefully poured off. The remaining solute was removed by washing with 1ml 70% ethanol (stored at -20^{o}C) and centrifuging for 10mins. The S/N was poured off and the pellet was drained and dried in a freeze-drier.

2.2.1(iii): Quantitation of nucleic acids:

For concentrations of DNA greater than 5µg/ml, concentrations were determined by optical density readings of solutions at a wavelength of 260nm. An $O.D._{260nm}$ of 1.0 in a cuvette of pathlength 1cm corresponds to a DNA concentration of ≈50µg/ml.

For small amounts of DNA (such as that recovered from an agarose gel) a minigel was run and the intensity of UV-induced fluorescence was compared to that of standards of known concentration.

2.2.1(iv): Restriction enzyme digestion and Agarose gel electrophoresis:

The purification of a particular fragment of DNA found in a plasmid requires two processes: generating the desired fragment and separating it from the remaining unwanted DNA. These can be accomplished by restriction enzyme digestion and agarose gel electrophoresis.

Restriction enzymes were used according to manufacturer's specificities using the buffers provided. Typically, a digest was performed in 50µl using up to 50µg of DNA.

All agarose gel electrophoresis techniques were carried out according to Maniatis *et al* (1982). Agarose was melted in running buffer (TAE) in a microwave oven

[50 x TAE: 2M Tris.Cl, 50mM EDTA (pH8.0)
pHed to 7.5-7.8 using 57.1ml/litre glacial acetic acid]

and poured as a horizontal gel on a 20cm x 14.5cm plate using combs with dimensions 18mm x 8mm x 2mm.

One-sixth volume of 6 X Gel Loading Buffer (GLB)

6 X GLB:	15% (w/v)	Ficoll (w/v)
	0.25% (w/v)	Bromophenol blue
	0.25% (w/v)	Xylene cyanol

was added to the DNA samples to ensure that they remained at the foot of the wells. For preparative work, gels were run at 1V/cm overnight, while for rapid work, 4V/cm was more usual. In this case buffer was recirculated using a peristaltic pump. DNA was stained by soaking the gel in running buffer containing 0.5µg/ml ethidium bromide for 15 mins. It was visualised and photographed using a UV transilluminator.

2.2.2 Protocols for recombinant DNA cloning:

Generating novel recombinant DNA molecules involves cutting preexisting DNA, isolating the desired fragments, ligating these to each other, propagating the new recombinants in bacteria and subsequently identifying the desired clones. The following methods were used routinely in this process:

2.2.3 Bacterial growth and handling:

All bacterial work was carried out under Category 1 containment. Basic microbiological techniques were performed as

described by Maniatis *et al* (1982).

Bacteria from frozen stocks were streaked on agar plates and grown overnight at 37°C. Single colonies were expanded in liquid culture with vigorous shaking until confluent cultures were achieved. Frozen stocks were prepared by addition of glycerol to 20% (v/v), flash-freezing in liquid nitrogen and storage at -70°C.

2.2.4 **Large-scale plasmid preparation:**

The method used was based on the alkaline lysis procedure described by Birnboim and Doly(1979), but using a different growth medium.

The plasmid-harbouring bacteria were streaked out on a 1.5% agar plate supplemented with antibiotic and incubated O/N at 37°C. 5ml of Superbroth containing antibiotic was inoculated with a single colony and this was placed in a shaker at 225rpm for 8 hrs. The culture was then transferred into 500mls of the same medium and shaken for 36hrs.

The bacteria were harvested by centrifugation at 4000rpm for 10 minutes at 4°C. The S/Ns were discarded and the bacteria were resuspended in 2 X 30mls of 25mM Tris,10mM EDTA pH8.0, 50mM glucose to which lysozyme had been freshly added to 4mg/ml (Solution I). These were left at room temperature for 5 mins to allow disruption of the peptidoglycan layer of the outer cell membrane of the bacteria. To these were added 2 vols (i.e. 60mls) of ice-cold 0.2N NaOH, 1% SDS (Solution II). The buckets were inverted rapidly but gently to ensure a uniform pH of the whole solution while minimising the shearing of the chromosomal

DNA.

The addition of this solution has two effects: lysis of the cells occurs due to the SDS, and selective denaturation of the chromosomal DNA is achieved at the resulting pH of 12-12.5.

After incubating the buckets on ice for 10 mins, 0.5 vols (i.e. 45 mls) of ice-cold 3M KOAc pH4.8 (Solution III) was added. The buckets were shaken rapidly several times, and the chromosomal DNA, upon renaturation, was precipitated. After a further incubation on ice for 10 mins, the chromosomal DNA was pelleted by centrifugation at 10,000rpm for 10mins at 4°C. The S/Ns were filtered through a sterile tissue into 250ml buckets. [The resulting nucleic acid solution will be referred to as a CLEARED LYSATE]. 0.6 volumes (i.e. 72mls) of isopropanol was added to precipitate the nucleic acids. After mixing and standing at room temperature for 10 mins, the nucleic acids were pelleted by centrifugation at 10000rpm for 10mins at room temperature. After decanting the S/Ns, the pellets were washed with 50mls 70% ethanol (to remove adherent isopropanol and salt therefrom) and recovered by centrifugation at 10000rpm for 10 minutes at 4°C. After decanting of the S/Ns, these were air-dried for 5 minutes and resuspended in 8mls of TE.

Further purification was achieved using caesium chloride (CsCl) equilibrium density gradient centrifugation: 9.3g of CsCl was added and allowed to dissolve. To this solution, ethidium bromide was added to 600µg/ml. The refractive index of the solution was then measured using a refractometer and adjusted to a value of 1.4000. (This is above the value quoted in Maniatis *et al* (1982), but using solutions of refractive index 1.3960 as

suggested led to contamination of preparations with RNA).

The solution was then transferred to a 13.5ml Kontron tube, paraffin was added to fill the tube if necessary and the solution was centrifuged at 38,000rpm at 20°C for 48 hours. The RNA pelleted to the bottom of the tube and proteins floated to the top. In the intervening space, two red bands were normally visible: the lower band corresponds to the closed circular plasmid DNA. This was removed by puncturing the tube with a syringe needle and drawing the band out by the gentle suction of a 2ml syringe.

Since ethidium bromide in the presence of light induces single-strand breaks in DNA, the ethidium bromide was rapidly removed by 4 extractions with CsCl-saturated isopropanol. Caesium chloride was removed by dialysing the solution in Collodion bags (Sartorius) against 3 changes of TE. The DNA was then precipitated with ethanol and resuspended in 1-5ml of TE, according to yield. The solution was aliquoted and stored at -20°C.

By this method, routine yields of 5-20mg plasmid/litre of culture were obtained. These preparations were free of RNA and protein and were good substrates for restriction enzymes.

2.2.5 **Preparation of vector DNA for cloning:**

50µg of vector was digested for 4 hours at 37°C with 200U of appropriate restriction enzyme in a volume of 220µl. 50µl was removed to a new tube and returned to 37°C. (This served as a control for ligase activity later on). The remaining 170µl was incubated twice for 30 minutes at 37°C with 1U of Calf Intestinal

Phosphatase (CIP). Both phosphorylated and dephosphorylated vector were deproteinised by addition of Proteinase K to 100μg/ml and incubation for 15min at 37°C. The solution was extracted with phenol/chloroform, the DNA precipitated with ethanol, resuspended at ≈200ng/μl in TE pH 8.0 and stored frozen in small aliquots at -20°C.

2.2.6 **Preparation of insert DNAs for cloning:**

Recombinant plasmids were digested to completion with appropriate restriction enzymes, as checked by running a small aliquot on a minigel. The DNA was extracted with phenol/chloroform, then with chloroform and precipitated with ethanol. After resuspension in 1 X Gel Loading Buffer (GLB), the samples were loaded onto agarose gels of appropriate concentration and run overnight at 1V/cm. After ethidium bromide staining, the appropriate bands were located using long wavelength UV fluorescence and excised using a scalpel. The gel fragment was then chopped up into ≈1mm cubes ready for purification.

2.2.7 **Purification of DNA from agarose gels:**

DNA was purified using the GeneClean™ kit (Bio-101 Inc.) with a few modifications of the manufacturer's protocol. The principles of the method are as follows:

(i): melt the gel slice containing the DNA at 55°C using a

high concentration of chaotropic agents (in this case sodium iodide)

(ii): Bind DNA to a silica matrix under these high salt conditions.

(iii): Spin down the matrix and remove iodide by washing with an ice-cold salt/ethanol solution.

(iv): Elute the DNA at 55°C in a low salt buffer.

It was often found that after melting the gel slice, the DNA concentration was fairly dilute i.e. < 5µg/ml. As a result, it was discovered that efficient binding of DNA to the matrix required incubation periods of several hours or even overnight (more recent instructions with the kit acknowledge this). The matrix suspension was maintained by continuous rotation of tubes using a Burlimixer.

DNA recovery was assessed by running a small aliquot on a minigel using øX174 markers as indicators of DNA content. Typical yields were 20-50%.

2.2.8 Ligation of DNA with compatible ('sticky') ends

100fmol of dephosphorylated, linear vector DNA was mixed with a three-fold molar excess of insert DNA, ethanol precipitated and resuspended in 8µl of TE pH8.0. This was then heated to 65°C for

5 minutes to linearise any self-annealed sticky ends. The reaction mix was then incubated on ice for 60 mins to allow annealing of DNA. 1µl of 10 X ligase buffer:

10 X ligase buffer: 0.66M Tris.Cl pH 7.6

0.1M $MgCl_2$

10mM DTT

was then added, together with 0.5µl of 20mM ATP and 0.5U of T4 DNA ligase. The mixture was incubated overnight at 14°C. The reaction mix was then diluted 5-fold with TE pH 7.4 and 1µl was used to transform competent bacteria.

The following controls were routinely performed to pinpoint possible causes of failure:

TRANSFORMATION CONTROL:	100pg of supercoiled vector
LIGASE CONTROL:	100fmol of phosphorylated vector was self-ligated.
PHOSPHATASE CONTROL:	100fmol of dephosphorylated vector was self-ligated.
INSERT PURITY:	300fmol of insert was self-ligated (any colonies imply contamination with vector sequences)

2.2.9 Transformation of competent bacteria:

Competent E. coli strain DH5-A were purchased from BRL and stored at -70°C. After thawing on wet ice, 50µl aliquots were pipetted into prechilled Falcon 2059 polypropylene tubes. 1µl of the diluted ligation reaction was added, gently mixed with the cells and left to stand on ice for 30mins. The cells were then subjected to a heat shock for 45secs at 42°C, after which they were returned to ice for 2 minutes. 950µl of SOC medium was added and the cells were incubated for 60mins at 37°C with vigorous shaking (275rpm). Cells were then plated on agar containing antibiotic and transformants were selected by overnight growth at 37°C.

2.2.10 Screening of recombinant bacteria:

Colonies were picked and grown overnight as 5 ml cultures. (In general, it was found that screening 10-12 recombinants was sufficient to identify the desired clones). 1.6ml was then transferred to an Eppendorf tube and used to prepare mini-preparations of the recombinant DNA. The remainder was stored at 4°C.

The method used was that of Birnboim and Doly (1979). This produced plasmid DNA within 2hrs reliably free of chromosomal contamination which could be cut by a variety of restriction enzymes.

Cells were spun down by centrifugation for 2 minutes in a microfuge. The S/Ns were poured off, the pellets respun and final

traces of S/N were removed using a micropipette. Pellets were resuspended in 100µl of Solution I (see 2.2.2(ii)). The cleared lysate was then prepared as described in 2.2.2(ii). This was then extracted with phenol/chloroform and precipitated with ethanol at room temperature. The pellet was resuspended in 50µl TE pH8.0 containing 50µg/ml boiled RNase A.

Typical digests were: 17.5µl DNA
2µl 10 X restriction buffer
5U (0.5µl) restriction enzyme.

Incubation was at 37°C for 1hr.

4µl of 6 X GLB were added and the digest was loaded onto an agarose gel containing ethidium bromide. After visualisation and photography, appropriate recombinants were identified.

Using these protocols, 200-500 colonies were obtained/µl of diluted ligation mix, of which > 90% were recombinants.

2.3 Methods in tissue culture techniques

2.3.1 Materials

2.3.1(i): Plasticware

Nunclon flasks with growth areas of 25-, 80- and 175cm^2 were obtained from GIBCO, as were **1.8ml ampoules** for the long-term storage of cell lines.

Roller bottles of growth area 850cm^2 were obtained from Falcon Plastics.

24- and 96-well Costar Multiwell plates were obtained from Northumbria Biologicals Ltd (NBL).

2.3.1(ii): Stock solutions

All media- and nutrient stock solutions were obtained from GIBCO, except Foetal Calf Serum (FCS), which was obtained from NBL. These were stored at 4^{o}C, except for glutamine, FCS, antibiotics and trypsin, which were stored at -20^{o}C.

Phosphate Buffered Saline (PBS) pH7.3 was made at the Beatson Institute:

NaCl	137mM
KCl	44mM
KH_2PO_4	1.4mM
$Na_2HPO_4.7H_2O$	8.4mM

PBS/EDTA was identical to PBS, except that it contained 1mM EDTA.

Trypsin solution was made fresh from a 2.5% (w/v) frozen stock by dilution 1:10 in PBS/EDTA. It was stored at 4^{o}C for up to 2 weeks.

Geneticin (G418) was made up to 100mg/ml in water and stored at 4^{o}C until use.

Dexamethasone was made up to 100mg/ml in 100% ethanol and stored at 4°C until use.

TPA was made up to 100µg/ml in acetone and stored at -20°C until use.

2.3.1(iii): Cells

PAL cells were received after primary isolation from Dr J. Gaukroger (Dept. of Vet. Pathology, University of Glasgow Veterinary School)

C127 cells were obtained from Dr C. Bostock (M.R.C. Mammalian Genetics Unit, Edinburgh)

NIH3T3 cells were obtained from C. Marshall (Chester Beatty Institute, London)

2.3.1(iv): Other materials

Haemocytometers were obtained from Weber Scientific Int. Ltd.

Electroporation cuvettes were obtained from BioRad.

Thin Layer Chromatography (TLC) **plates** were obtained from Camlab.

2.3.2 Basic methods in tissue culture

2.3.2(i) Sterility

All work was carried out in vertical laminar flow hoods under

Category 2 containment. Aseptic techniques were performed as described by Freshney (1987).

2.3.2(ii): Growth medium preparation

Cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FCS. This was prepared as follows:

water	738.5ml
10 X DMEM	100ml
0.1M sodium pyruvate	10ml
7.5% (w/v) sodium bicarbonate	30ml
0.2M glutamine	10ml
penicillin (10000U/ml)	5ml
Foetal Calf Serum	100ml

The medium was pHed by the addition of 6.5ml 1N sodium hydroxide. It was routinely prewarmed to 37^{o}C before use.

2.3.2(iii): Initiation of cultures

Stocks were recovered from liquid nitrogen storage and thawed rapidly at 37^{o}C. The cell suspension was transferred to an 80cm^{2} flask and 15ml of medium was added. The flask was placed loose-capped in a 37^{o}C incubator with an atmosphere containing 5% CO_2 and the cells were left overnight to seed onto the plastic surface.

2.3.2(iv): Propagation and passaging of cultures

Cells were routinely fed twice a week with fresh medium warmed to 37°C, old medium having been removed by aspiration. For the purposes of this project, it was important that cells never remained at confluence (i.e. covering the entire surface) for any length of time. The cells were thus passaged once a week. Passaging involves two processes:

Removal of the cells from the plasticware by trypsinisation

Reseeding the cells at an appropriate density.

The medium was removed from the cells and they were then rinsed, first with PBS and then with the trypsin diluent (both prewarmed to 37°C). After removal of the trypsin, the flask was transferred to the 37°C room for 1 minute, then returned to the hood and growth medium was added to wash the cells off the growth surface. (The growth medium contains a trypsin inhibitor which prevents further damage to the cells). The cells were then transferred to a Universal, the flask was rinsed with PBS and the cells reseeded at an appropriate density.

C127 and NIH3T3 cells were replated at a 1:30 dilution
Pal cells were replated at a 1:10 dilution.

2.3.2(v): Long-term storage of cells

Cells may be stored frozen under liquid nitrogen for several years, maintaining their characteristics when recovered to active growth.

Cells were trypsinised and resuspended at $\approx 10^6$ cells/ml in growth medium. DMSO was added to 10% (v/v) (this acts as a cryoprotectant, preventing the formation of ice crystals within the cells), the cells were aliquoted into plastic ampoules, wrapped in cotton wool, placed in a polystyrene box and left at -70°C overnight. (This ensures a slow rate of cooling, which has been shown to enhance cell viability upon recovery), The next day, ampoules were transferred to racks which were stored under liquid nitrogen at -196°C.

2.3.3 Transfection of cells with recombinant DNA molecules

2.3.3(i) Calcium phosphate-mediated transfection

DNA may be taken up by eukaryotic cells when it is added to a subconfluent monolayer as a coprecipitate with calcium phosphate. The final pH of the solution overlying the cells is critical for the success of the procedure. It was thus crucial that both the pH of the solutions used in the preparation of the precipitate and that of the growth medium be correct. Furthermore, the process is inhibited by common contaminants of plasmid preparations such as tRNA and proteins. The following

precautions were thus routinely taken: firstly, plasmid DNA molecules were purified on two succesive CsCl gradients; secondly if DNAs were digested prior to transfection, the restriction enzymes were digested with proteinase K and the solutions were extracted with phenol/chloroform. This was followed by two ethanol precipitations; thirdly all solutions used for the formation of precipitates were warmed to room temperature before use; and fourthly, cells were not fed immediately prior to transfection to ensure that the pH of the growth medium was absolutely correct.

Stock solutions

2.5M $CaCl_2$:

Since the dihydrate form of $CaCl_2$ absorbs water very rapidly, weighing out the correct amount of salt may in fact yield a solution more dilute than 2.5M. Thus the refractive index was measured and adjusted to 1.401: this has been shown to give $CaCl_2$ suitable for transfection studies.

2 X Hepes-Buffered Saline (HBS) pH7.12:

HEPES	50mM
NaCl	280mM
Na_2HPO_4	1.5mM

The solution was adjusted to pH 7.12 using 0.1N NaOH, filter sterilised and used immediately.

Subconfluent cells were trypsinised, counted using a haemocytometer (as described in Freshney (1987)) and replated at 3 X 10^5 cells/T25 (C127 or NIH3T3) or 5 X 10^5 cells/T80 (Pal). They were incubated overnight before addition of the calcium phosphate-DNA coprecipitate.

The precipitates were formed in a total volume of 0.1 X volume of growth medium (i.e. 0.5ml for C127 and NIH3T3 and 1ml for Pal) at a DNA concentration of 20μg/ml. DNA was diluted in 0.1 X TE pH8 and $CaCl_2$ was added to 0.25M. This was added dropwise to an equal volume of 2 X HBS and left to stand for 30mins. This was then added dropwise onto the cells, which were then incubated for 16-20hrs at 37°C.

If left for a substantial time period, Ca^{2+} at such concentrations as used in transfections is toxic to cells. Thus after removal of the precipitate, the cells were washed twice in serum free medium. They were then refed and left for 24hrs before selection was started (see 2.3.3(v)).

2.3.3(ii): Transfection of cells by electroporation

At first, great problems were encountered with the calcium phosphate protocol. An alternative means of transfection, namely electroporation (EP), was therefore employed. The method used followed the protocol of Chu *et al* . (1987).

3 X 10^6 to 10^7 exponentially growing cells were resuspended in

1ml of EP buffer containing an appropriate amount of DNA (as described in the results section).

EP buffer:	HEPES	20mM
	NaCl	137mM
	KCl	5mM
	Na_2HPO_4	0.7mM
	dextrose	6mM

The solution was adjusted to pH 7.1 with 0.1N NaOH, filter sterilised and stored at 4^oC.

The cells were transferred to a sterile single-use cuvette and subjected to a single electrical pulse (using the Gene Pulser™ (BioRad)) at a capacitance of 960μF using voltages determined empirically (see results section). After standing for 10mins at room temperature, cells were plated out in T75s and grown for 48hrs at 37^oC. Cells were then either harvested (for transient expression) or placed under appropriate selection.

2.3.4: Chloramphenicol Acetyltransferase (CAT) assays

48hrs after transfection, cells were washed twice with ice-cold PBS and 1.5ml of 40mM Tris.HCl pH7.4, 1mM EDTA, 150mM NaCl was added. After incubation for 5mins at RT, cells were scraped off the surface using a rubber policeman. The cell suspension was transferred to an Eppendorf and the cells pelleted by centrifugation for 1min in the microfuge. The cells were

resuspended in 100µl 0.25M Tris.Cl pH8.0 and subjected to 3 cycles of freeze-thaw (5mins on dry ice followed by 5mins at 37°C).This causes lysis of the cells, allowing the nuclear and membranous fractions to be pelleted by centrifugation for 10mins in the microfuge. The supernatant (the CAT extract) was transferred to a fresh tube and stored at -20°C until use.

50µl of extract were added to 1µl of [^{14}C]-labelled chloramphenicol and the volume made up to 150µl with 0.25M Tris.HCl pH 8.0. The reaction was started by the addition of 20µl of 4mM acetyl CoA (made fresh in distilled water). The reaction was allowed to proceed for the desired time period at 37°C. This was stopped by addition of 1ml ethyl acetate, vigorous vortexing and separation of the phases by centrifugation. The upper organic layer was transferred to a fresh tube and freeze-dried using a SpeedVac. Samples were resuspended in 15µl ethyl acetate and loaded onto a TLC plate and the products were separated for 45mins using chloroform:methanol 95:5 as a solvent. The plates were air-dried and exposed overnight to X-Ray film.

2.3.5: Selection schemes

If selected for **focus formation**, cells were trypsinised, replated at 1:6 and grown until confluence was reached. The cells were fed for up to 21 days, when plates were scored for dense foci of non contact-inhibited cells.

If selected for **neomycin resistance**, cells were trypsinised, and replated at 1:6. (Pal cells) or 1:15 (C127- or NIH3T3 cells) in medium containing G418 at a concentration determined

empirically for Pal cells (as described in the results section), at 1.2mg/ml for C127 cells or 800µg/ml for NIH3T3 cells. Cells were selected for 21 days, with weekly changes of medium for C127- and NIH3T3 cells, or twice weekly for Pal cells, at which time colonies were counted and either picked or stained.

2.3.6: Picking of colonies

Colonies or foci were ringed with a felt-tip pen for identification and the top of the flask was cut away using a red-hot scalpel. Cells were washed with PBS and sterile stainless steel cloning rings of diameter 6mm were adhered around the desired area using high vacuum silicone grease. Cells were trypsinised and transferred to appropriately sized multiwell plates for expansion into cell lines.

2.3.7: Cell staining

Cells were washed with PBS and fixed for 10mins with ice-cold methanol. The methanol was removed and 0.1ml Giemsa stain was added per cm^2 of growth surface. After shaking gently for 5 mins at RT, the dye was diluted 5-fold with tap water and shaken for a further 5 mins. The dye was poured off and the flask washed with tap water to remove background dye. The flasks were dried overnight at 37^oC and then scored.

2.3 8: Cell tumorigenicity in athymic mice

Cells were harvested and resuspended at 2.5- to 5 X 10^7 cells/ml in PBS and stored on ice. 0.2ml of this suspension was inoculated subcutaneously into each of five athymic mice and animals were observed for up to 20 weeks for signs of tumour formation.

2.4. Methods in eukaryotic nucleic acid analysis

Analysis of genomic DNA by Southern blotting

The screening of cell lines for the presence of DNA sequences of interest, the detection of gene amplification and/or rearrangement may be performed by the hybridisation of single-stranded radiolabelled DNA sequences to single stranded genomic DNA. The most suitable methods currently available involve preparation of high molecular weight genomic DNA, digesting this DNA with restriction enzymes, separating this DNA on an agarose gel, denaturing the DNA and transfer to a nylon membrane, to which DNA may be covalently cross-linked by baking. This maintains the DNA in a stable form for repeated rounds of hybridisation with radiolabelled probes.

The following protocols were found to be reliable in the achievement of the above aims:

2.4.1: Preparation of high molecular weight cellular DNA

In order to generate pure cellular DNA of high molecular weight, steps must be taken to avoid shearing of the DNA during preparative manipulations. Two salient measures are significant in this regard:

(a): the cutting of the ends of disposable pipette tips to widen their entrance.

(b): the precautions taken for phenol extraction: the DNA solution was transferred to a 50ml Falcon tube and an equal volume of phenol/ chloroform was added. The flask was not vortexed, but rather was rotated on a Burlimixer for 20mins. The phases were separated by centrifugation at 3000rpm for 20mins at 20°C. The upper aqueous phase was transferred carefully to a fresh Falcon tube.

Cells grown in culture were washed once with PBS and were lysed and deproteinized in the plastic flask by addition of 5mls of 0.3M NaOAc pH7.5, 5mM EDTA, 0.5% SDS containing 100µg/ml Proteinase K. After incubation at 37°C for 1hr, the viscous solution was transferred to a 50ml Falcon tube, the flask was washed with a further 5ml of lysis buffer and the two solutions pooled. The solution was extracted once with phenol/chloroform and ≈80% of the aqueous phase was transferred to a fresh tube. By this means, no contaminating protein was carried through, and thus further extractions were unnecessary. The DNA was then

precipitated by addition of two volumes of ethanol. Gentle inversion of the tube for 5mins completed the precipitation. The DNA was pelleted by centrifugation at 3000rpm for 30mins at 4°C. The precipitate was washed once with 70% ethanol and left to air-dry for 5 minutes. The pellet was resuspended in 1ml TEpH8.0 containing 100mM NaCl by incubation for 15mins at 37°C folowed by gentle pipetting up and down. RNase A was then added to 100µg/ml and the solution was incubated at 37°C for 1hr. The solution was then diluted to 10ml total volume, phenol/chloroform extracted, ethanol precipitated and washed as before. The DNA was then resuspended to at ≈500µg/ml in TEpH8.0 and stored at 4°C.

Running of an aliquot of such DNA on a 0.3% agarose gel showed the DNA to have a molecular weight of 30 - 100kb.

2.4.2: Restriction digests, gel electrophoresis and Southern Transfer

20µg of genomic DNA was digested overnight with 100U restriction enzyme, extracted with phenol/chloroform and precipitated with ethanol. The DNA was resuspended in 60µl 1 X GLB, loaded onto a 250ml 0.8% agarose gel and electrophoresed overnight at 1V/cm. The gel was stained and photographed before preparation for Southern transfer.

The gel was placed for 10mins in 0.25N HCl, causing partial depurination of the DNA. After rinsing briefly in water, the gel was transferred to a 0.5N NaOH, 1.5M NaCl solution for 30mins. This causes two things: hydrolysis of the DNA at the sites of

depurination - resulting in fragmentation of high molecular weight DNA, easing its transfer from the gel - and denaturation of the DNA. After further rinsing with water, the gel was neutralised by soaking for 1hr in 0.5 M Tris.HCl pH 7.4, 3M NaCl.

A large tray was filled with a reservoir of 20 X SSC (3M NaCl, 0.3M NaCitrate pH7.0) and an inverted, flat-bottomed baking dish was placed thereon. A sheet of 3MM paper, presoaked in 20 X SSC, was placed over the dish, ensuring that its sides extended down into the reservoir. Any air bubbles underneath it were removed by rolling a Corex tube gently over the top of the dish. The gel was then placed on the filter paper. The area surrounding the gel was made water tight using X-Ray film. (This prevents any transfer of buffer not passing through the gel). On top of the gel was placed a sheet of Pall Biodyne membrane, precut to the dimensions of the gel and presoaked in 2 X SSC. Air bubbles were again carefully removed. Three identically sized pieces of 3MM paper, presoaked in 2 X SSC were then placed over the membrane and these were covered with a 10-15cm pile of paper towels. A large baking dish was placed over these and four filled 500ml bottles were placed on the top.

The effect of this set-up is to induce capillary flow of buffer from the reservoir, through the gel and into the paper towels. The DNA is concomitantly transferred to the biodyne membrane. The transfer was allowed to proceed overnight, to allow complete transfer of large-molecular weight DNA.

After transfer the membrane was carefully peeled from the gel, air-dried and baked at $80^{o}C$ for 2hrs to elicit cross-linking of the DNA to the membrane. It was then stored dry in a heat-sealed

plastic bag until hybridisation to suitable probes was performed.

2.4.3: Preparation and labelling of DNA hybridisation probes

DNA probes were labelled according to the method of Feinberg and Vogelstein (1983) using a kit supplied by BCL.

The probe-containing recombinant plasmid was digested with an appropriate restriction enzyme, separated on an agarose gel and the probe DNA was purified using the GeneClean kit (as described in 2.2.2(iv)). 50ng was then taken, the volume was made up to 9µl with water, and the DNA was denatured by boiling for 10mins followed by rapid cooling on ice. 2µl of 10 X buffer:

10 X buffer:	0.5M Tris.HCl pH 7.4
	50mM $MgCl_2$
	2M HEPES pH 6.6
	120mM ß-mercaptoethanol
	600µg/ml random hexanucleotides from calf thymus

were ~~was~~ added along with 3µl of a mixture containing 500µM dATP, dGTP, and TTP. 1.85MBq of [^{32}P-]dCTP was then added followed by 1U of the Klenow fragment of E. coli DNA polymerase I. The reaction mix was incubated for 4hrs at 37^{o}C. The unincorporated nucleotides were then removed using Elutip-d columns according to the supplier's instructions. DNA was then denatured as described above in preparation for hybridisation to the immobilised DNA.

2.4.4: Prehybridisation and hybridisation of probes to filters

The aim of any hybridisation protocol is the specific annealing of the radiolabelled probe to homologous (or semi-homologous) DNA with as little non-specific hybridisation as possible. The hybridisations were therefore carried out ≈5°C below the melting temperature of the homologous (or semihomologous) hybrids.

10ml of hybridisation solution (5 X Denhardt's (100X Denhardt's: 2%(w/v) Ficoll, 2%(w/v) Polyvinylpyrrolidone, 2% (w/v) BSA), 5 X SSC, 0.1% SDS, 500μg/ml denatured salmon sperm DNA) was added to the bag containing the filter, air bubbles were removed and the bag was heat-sealed. This was incubated at an appropriate temperature for 3hrs before addition of the radiolabelled probe. The bag was then hybridised overnight at the same temperature before washing the next day.

Stringent hybridisation: 65°C

Relaxed hybridisation: 42°C

2.4.5: Washing of filters and autoradiography

By washing the filters, it is desirable to remove non-specifically bound probe, whilst leaving the specific binding unaffected. The stringency of washing is thus determined by the stringency of hybridisation. Filters were first washed twice for 5mins at room temperature in 2 X SSC, 0.1% SDS to remove unbound probe. The washes were then carried out as follows:

Stringent washing: 1hr at 65^{o}C in 0.1 X SSC, 0.5% SDS with 3 changes of buffer

Relaxed washing: 1hr at 60^{o}C in 2X SSC, 0.1% SDS with 3 changes of buffer

After washing, the filters were air-dried and exposed to X-Ray film at -70^{o}C for up to 14 days.

SECTION 3

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3. BPV4-associated transformation of primary bovine fibroblasts

3.1 Introduction

Although much valuable information has been obtained from the studies of BPV4-mediated transformation of the established murine fibroblast cell lines NIH3T3 and C127, these lines suffer two distinct disadvantages when trying to extrapolate results to the development of malignant tumours *in vivo* : firstly, BPV4-mediated transformation of the cells *in vitro* leads to the development of the fully tumorigenic phenotype. In these systems, therefore, BPV4 is acting at a late stage of the carcinogenic process. *In vivo* , viral infection leads to the development of benign lesions (i.e. an **early** event): in the fully malignant tumours the virus is always absent; and secondly, the cell lines are fibroblastic and derived from a heterologous species: *in vivo* , the virus is species-specific and strictly epitheliotropic.

The ideal system for the study of BPV4-associated transformation *in vitro* , therefore, would be primary bovine keratinocytes derived from the UAC. At present, however, such cells are not available. It was therefore decided to use non-established bovine fibroblasts derived from the palate of a late term foetus (Pal cells) as a tool for further studies. The usefulness of such fibroblastic systems in the study of a strictly epitheliotropic virus has recently been demonstrated for HPV16 using secondary Rat Embryo Fibroblasts (REFs)(Chesters and McCance (1989)) and embryonic human lung fibroblasts (Watanabe *et al* . (1989).

Results obtained several years ago (Moar *et al* . (1981) and M.S. Campo (pers. comm.)) indicated that while the infection of Pal cells with virions of the fibropapillomaviruses BPV1 and -2 could induce complete tumorigenic conversion, no such activity could be obtained with BPV4 virions. Several possible explanations spring to mind:

(i): No viral infection was possible (For example, due to the lack of virion receptors on the cell surface).

(ii): Maintenance of viral DNA after infection was not possible in fibroblastic cells.

(iii): Expression of the required transforming genes could not be induced in the fibroblastic background.

(iv): The BPV4 genome does not encode transforming genes sufficient for the transformation of primary fibroblasts *in vitro* .

In this study it was decided to address the following questions: firstly, does BPV4 encode any genes **capable** of participating in the tumorigenic conversion of primary bovine fibroblasts and if so, what cooperating genes and/or cofactors are required to elicit this conversion?; and secondly, are these genes expressed efficiently in fibroblasts when placed in the context of the full viral genome and if not, does this have any significance for the epitheliotropic nature of the virus?

The following strategy was therefore decided upon, based on two broad themes: firstly, an analysis of the ability of the full

viral genome to elicit phenotypic alterations of Pal cells in cooperation with chemical cofactors and/or activated cellular protooncogenes; and secondly, the cloning of subgenomic fragments of BPV4 into expression vectors and subsequent analysis of their activity in the above assay.

3.2 Isolation and characterisation of Pal cells

The cells were obtained at passage 6 (p6) from Dr. J. Gaukroger (Dept. of Vet. Path., University of Glasgow Vet. School), were expanded until p9 and frozen down in bulk as aliquots of 5×10^5 cells in 0.5ml. Individual aliquots were passaged routinely until p12 for experimental purposes, after which they were discarded and fresh stocks recovered.

3.1.2(i): Growth characteristics and passaging

Since the cells were not established, it was important to determine a suitable dilution factor for their passaging. It was decided that weekly passaging was appropriate, and that since the cells were to be used in assays concerning altered growth properties after DNA transfection, it was important that no inadvertent selection for such properties should take place prior to transfection, which could occur upon prolonged maintenance of the cells as a confluent monolayer. it would thus be optimal for the cells to just return to confluence after seven days growth.

A confluent cell monolayer was trypsinised and plated at various dilutions in T25 flasks containing 5ml of growth medium. Cells were fed on days 2 and 4 after seeding and scored on day 7.

Confluence was judged by viewing the cells under the light microscope. From this simple experiment, it was determined that a **1:10 dilution factor** was appropriate for these cells.

The density of Pal cells at confluence was measured routinely at each passage: this appeared to vary slightly from aliquot to aliquot, possibly being affected by different batches of serum. This confluent density was normally between 3.0 and 4.5 x 10^4 cells/cm^2.

3.1.2(ii): Sensitivity toward geneticin (G418)

Since it was hoped to use the cells in cotransfection experiments involving the dominant selectable marker **neo** (which confers resistance to the antibiotic analogue geneticin), it was necessary to determine the concentration of drug which would kill the parental cells within a suitable time period (this was decided to be 7 days).

3 x 10^5 cells were plated in T75 flasks containing 0, 200-, 400-, 600-, 800-, 1000- or 1200μg/ml geneticin. These were fed on days 2 and 4 with the same concentration of drug and the plates were scored on day 7. The results are shown in Table 3.1. These show that at 400μg/ml G418, only ≈80% of the cells were killed, whereas at 600μg/ml 95% of cells died. The 5% surviving cells all appeared to be dying, appearing very elongated and vacuolar. **500μg/ml** was thus decided to be the working concentration of G418, since the minimum concentration required for the killing of normal cells would allow the maximum survival of cells which were expressing the **neo** gene.

Table 3.1: G418 kill curve for Pal cells

G418 conc./mg/ml	Cell viability
0	Cells confluent
0.2	Cells confluent
0.4	20% confluent
0.6	5% survival
0.8	ALL DEAD
1.0	ALL DEAD
1.2	ALL DEAD

3.3: Early transformation experiments of Pal cells by BPV4 and cooperating factors

As described in the introduction to this section, attempts to induce morphological transformation of Pal cells by BPV4 virion infection were repeatedly unsuccesful. To eliminate the possibility that infection was simply not possible due to a lack of virus receptor, it was decided to introduce linearised BPV4 DNA into cells by transfection. At first, great difficulty was encountered using the calcium phosphate coprecipitation method: the technique of electroporation was thus employed. Since, using this technique, transfection efficiency at a given capacitance varies with the applied voltage, it was necessary to perform a calibration curve. The most convenient method of doing this involves chloramphenicol acetyltransferase (CAT) assays using cytoplasmic protein extracts from cells transiently transfected with CAT expression vectors. (Although the ultimate goal was stable transfection, published data indicated that results of transient assays could be succesfully extrapolated to more long-term selection systems (Chu *et al* . (1987)).

3.1.3(i): Calibration of electroporation conditions

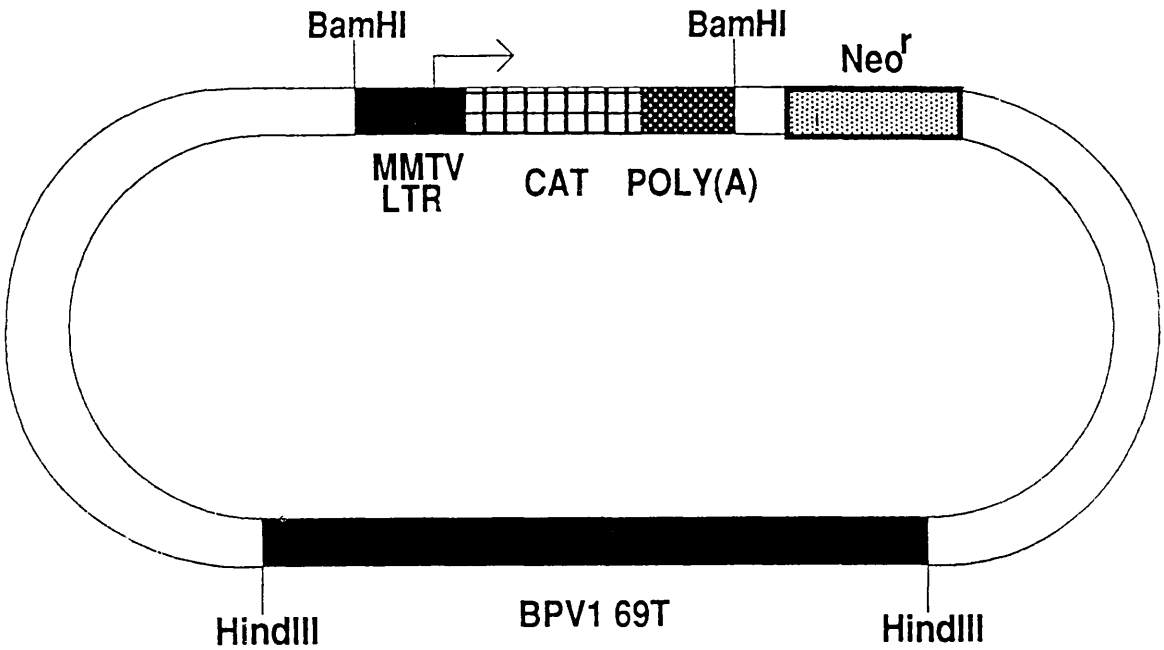
As a first experiment to check transfection- and CAT assay technique, NIH3T3 cells were used as recipients. The CAT expression vector used was pCGBPV-MMTV-CAT-A2 (Matthias *et al*. (1986)), whose structure is shown in Figure 3.1. The reporter gene in this construct may be induced by the synthetic glucocorticoid dexamethasone.

Duplicate aliquots of 3 x 10^6 exponentially growing cells were resuspended in 1 ml of transfection buffer containing 1, 10 or 20μg of plasmid DNA. Using the optimal conditions published by Chu *et al.* (1987), a voltage of 290V was used using the highest capacitance available (960μF). 24hrs after transfection, dexamethasone was added at a concentration of 2μM to one of each duplicate flask. (Such a concentration has been shown to induce maximal expression of the N-ras gene cloned under the control of the complete MMTV-LTR (McKay *et al* . (1986)). CAT extracts were harvested 48hrs post-transfection and the reaction was allowed to proceed for 1hr at 37°C. The results of the CAT assays are shown in Figure 3.2. These indicated that for NIH3T3 cells:

(a): The technique was effective at a DNA concentration of 10μg/ml, but not at 1μg/ml.

(b): In transient assays, the absolute requirement for ⟶ dexamethasone is overcome, presumably due to the titration of some negative trans-factor.

Figure 3.1: Structure of pCG-BPV-MMTV-CAT-A2



Legend: BPV1 69T refers to the 69% of the BPV1 genome capable of transforming NIH3T3- or C127 cells after transfection as a linearised recombinant DNA molecule (Law *et al* . (1981)).

MMTV LTR: Mouse Mammary Tumour Virus Long Terminal Repeat.

CAT: Chloramphenicol Acetyltransferase

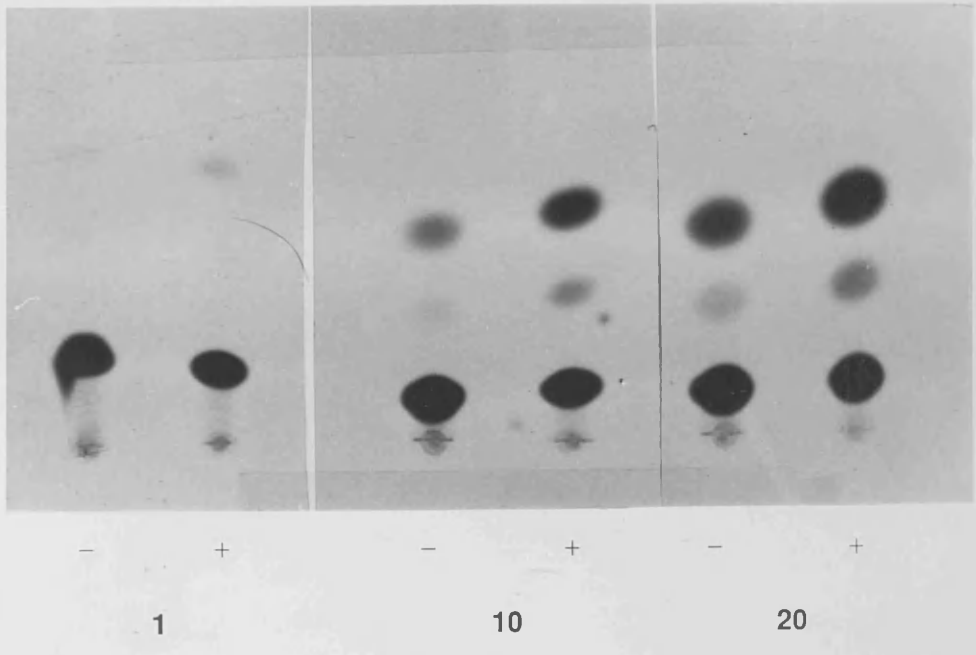
poly(A): polyadenylation signal

neo^r: neomycin resistance gene

Plasmid received from U. Böger-Brown and described in Matthias *et al.* (1986).

Figure 3.2: CAT assays of NIH3T3 cell extracts transfected with pCGBPV-MMTV-CAT-A2 by electroporation

Exponentially growing NIH3T3 cells were harvested and duplicate aliquots were resuspended in 1ml of electroporation buffer containing either 1, 10 or 20µg of pCGBPV-MMTV-CAT-A2 DNA. The cells were transferred to electroporation cuvettes and exposed to a voltage of 290V using a capacitance of 960µF. After a 10 minute incubation period at room temperature, the cells were plated in T75 flasks and grown overnight. 24hrs after electroporation, dexamethasone (2µM) was added to one of each of the duplicate flasks. 48hrs after electroporation, cells were harvested, cytoplamic protein extracts were prepared and 50% of the extract was used in the CAT assays.



Legend: the numbers refer to the number of microgrammes of plasmid DNA used in the electroporation.

- and + refer to the absence or presence of 2µM dexamethasone added 24hrs prior to harvesting the cells.

(c): Dexamethasone, nevetheless, did induce a 3-5-fold increase in CAT activity.

Since the technique appeared to be working succesfully, a voltage calibration for the Pal cells was then constructed. In the study of Chu *et al* . (1987), optimum voltages were always found to reside between 200- and 300V, 6 voltages equally spaced between 210V and 310V were chosen. The protocol was as for the 3T3 cells, except that 20μg of DNA was used in all cases. As the electroporation technique causes cell death, activities were **not** corrected for protein content: rather, 50% of the extract was used in the assay. This thus gives absolute levels of transfection. The results are shown in Figure 3.3. These indicated that:

(a): Below 250V, no significant CAT activity could be observed.

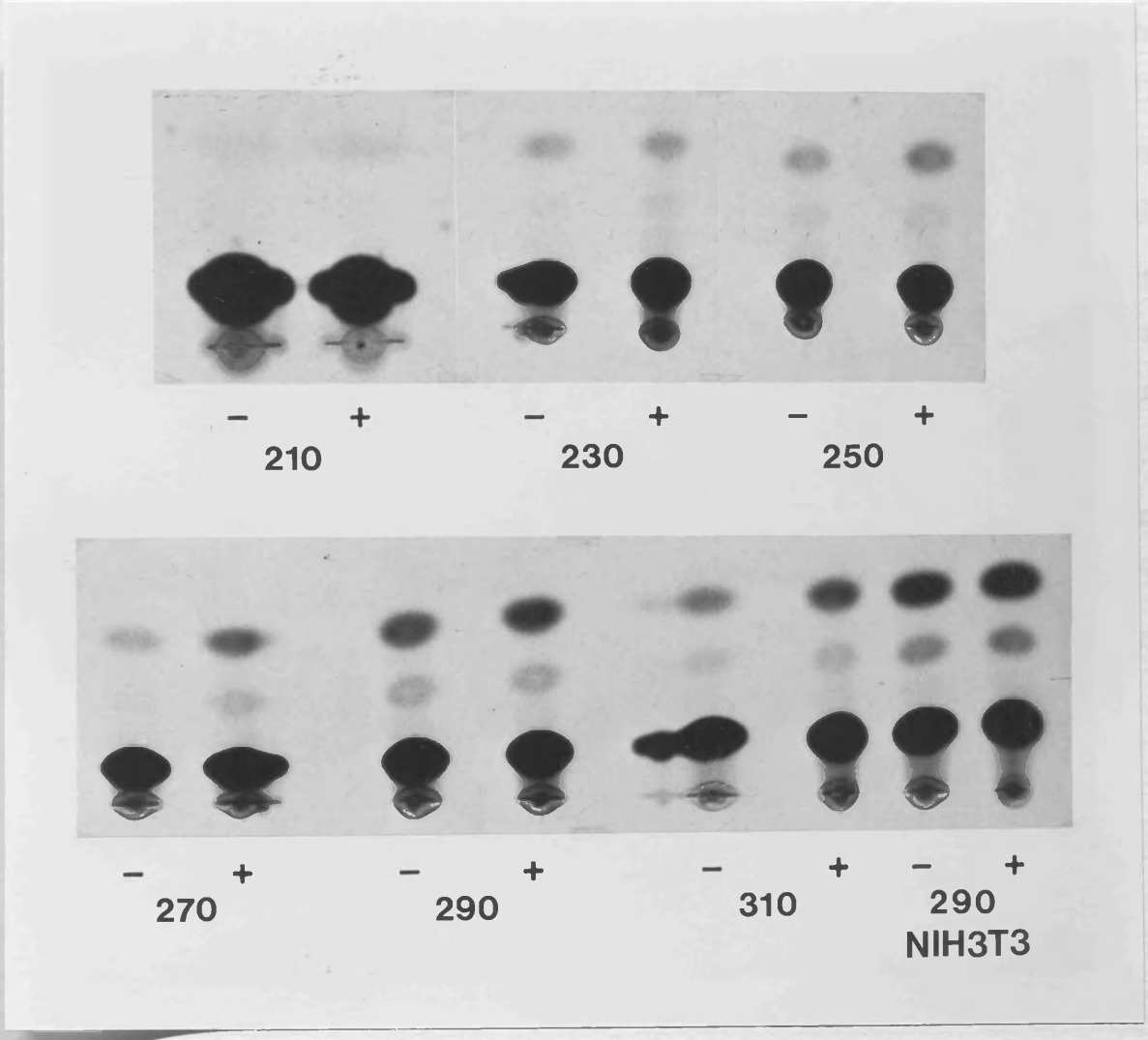
(b): The optimum voltage for transfection was 290V.

(c): CAT activity was ≈ 5-10 fold less than in NIH3T3 cells.

The final observation could be partially explained by comparing absolute levels of protein of the extracts: due to the slower growth rate of the Pal cells, considerably less cells were harvested when compared to the NIH3T3s.

Figure 3.3: Calibration of electroporation conditions for Pal cells

Exponentially growing Pal cells were harvested, resuspended at 3 x 10^6 cells/ml in electroporation buffer containing 20μg/ml pCGBPV-MMTV-CAT-A2 and 1ml aliquots were transferred into electroporation cuvettes. Duplicate cuvettes were exposed to 210, 230, 250, 270, 290 or 310V using a capacitance of 960μF. Subsequent treatment was identical to that described in Figure 3.2.



Legend: the numbers refer to the applied voltage across the electroporation cuvette.

- and + refer to the absence or presence of exogenously added dexamethasone (2μM) 24hrs after electroporation.

3.3(ii): Transformation of Pal cells by BPV4 in the absence and presence of TPA

The first attempts to generate morphologically transformed cells were transfection experiments using BPV4 DNA linearised at the BamHI site of pBV4 B1 (the BamHI site interrupts the BPV4 genome in the E1 ORF as shown in Figure 1.6), both in the presence and absence of 20ng/ml TPA. This tumour promoter has been shown to induce BPV4 gene expression in the murine fibrolast cell line C127 (Smith *et al.* (1987)) and, under certain conditions stimulates BPV4-mediated morphological transformation of such cells (Smith and Campo (1988)). Using the electroporation technique, with 20μg of linearised DNA and 5 x 10^6 cells, such experiments gave negative results (see Table 3.2); it was noted that the cells treated with TPA returned to confluence more slowly than the untreated controls, but appeared otherwise normal. This indicated that BPV4 DNA is unable to induce the full morphological transformation elicited by BPV1 and -2 virions at any measurable frequency, even in the presence of chemical cofactors.

Table 3.2: Electroporation of Pal cells by BPV4 in the presence of TPA

Exponentially growing Pal cells were harvested and resuspended at 10^7 cells/ml in EP buffer in the presence or absence of 20μg linearised BPV4 DNA. Cells were electroporated at 290V using a 960μF capacitance. After 10 minutes incubation at room temperature, cells were plated in duplicate T75 flasks and grown at 37°C for 48hrs. Cells were then split 1:6 and fed twice weekly for up to 8 weeks. Where appropriate, cells were treated with TPA at 20ng/ml for 48hrs prior to transfection with subsequent treatment twice a week throughout the period of selection.

DNA added	Foci
Calf thymus	0
BPV4 B1	0
BPV4 BI + TPA	0

Legend: BI: DNA digested with BamHI prior to transfection.

+ TPA: Treatment with 20ng/ml TPA 48hrs prior to transfection and subsequently twice a week during selection.

Foci expressed per 10^7 cells.

3.3(iii): Cooperation between BPV4 and activated proto-oncogenes in the transformation of Pal cells.

It was considered a possibility (since the BPV4 virus appears to lack an E6 ORF - see Figure 1.6) that the virus contained only one transforming gene and that this gene, when expressed in conjunction with an activated proto-oncogene, might cause full transformation of Pal cells. Such a finding has subsequently been shown to be the case for HPVs 16, 18, 31, 33 and 35 in rat kidney epithelial cells (Storey *et al* . (1988)) and for HPV16 in secondary rat embryo fibroblasts (Chesters and McCance (1989)). Following a similar strategy to these two groups led to the decision to cotransfect linearised BPV4 with either activated **c-Ha-ras-** (contained in the pT24 plasmid) or retroviral **myc** (contained in the plasmid pSVvmyc) genes. The structure of these plasmids are shown in Figures 3.4 and 3.5. Furthermore, since it was wished to exclude the possibility that interruption of the E1 ORF of BPV4 by cloning into the BamHI site caused an alteration in the transformation characteristics of the cloned viral DNA, a second BPV4 clone, pBV4 SstI (shown in Figure 3.6), which leaves the entire Early region of the virus intact, was used in identical transfection experiments.

10^7 cells were electroporated with 25µg of each linearised plasmid in all experiments. 48hrs after electroporation, the cells were split 1:6, grown to confluence and maintained thus for up to 10 weeks. The results are summarised in Table 3.3. After approximately 3 weeks, enlarged cells (as shown in Figure 3.7d, control cells shown in Figure 3.7a) began to appear in flasks containing **ras** alone, **ras** and **myc** or **ras** and BPV4, but not BPV4 alone, **myc** alone or BPV4 and **myc**. The results with both BPV4

clones were essentially identical. In the cases of BPV4 + **ras**, but not **ras** alone, these developed over the next 5 weeks into small 'foci' of enlarged cells (shown in Figure 3.7c). Similar 'foci' were observed in the **ras** + **myc** control (see Figure 3.7b). Explanting these 'foci' led to cell lines which at first appeared morphologically altered, but which rapidly reverted to a normal phenotype: they were contact-inhibited, did not display anchorage-independent growth and were non-tumorigenic in nude mice. Although these experiments did not yield conclusive results, they are interesting in two respects:

(a): any alteration in cell morphology was found only in flasks transfected with the activated **c-Ha-ras** oncogene.

(b): the process of transformation is very slow.

This could be due to inefficient expression of the BPV4 transforming gene, either due to the lack of activity of a positive trans-regulatory factor, or the presence of a trans-repressor. In the reports concerning HPV cooperation with **ras**, efficient transformation required expression of HPV gene functions either from a retroviral LTR (Matlashewski *et al* . (1987a)) or via steroid hormone induction (Pater *et al* . (1988)): it could therefore be the case that expression of the BPV4 transforming gene(s) is (are) naturally inefficient in bovine fibroblasts, when in the context of the full viral genome.

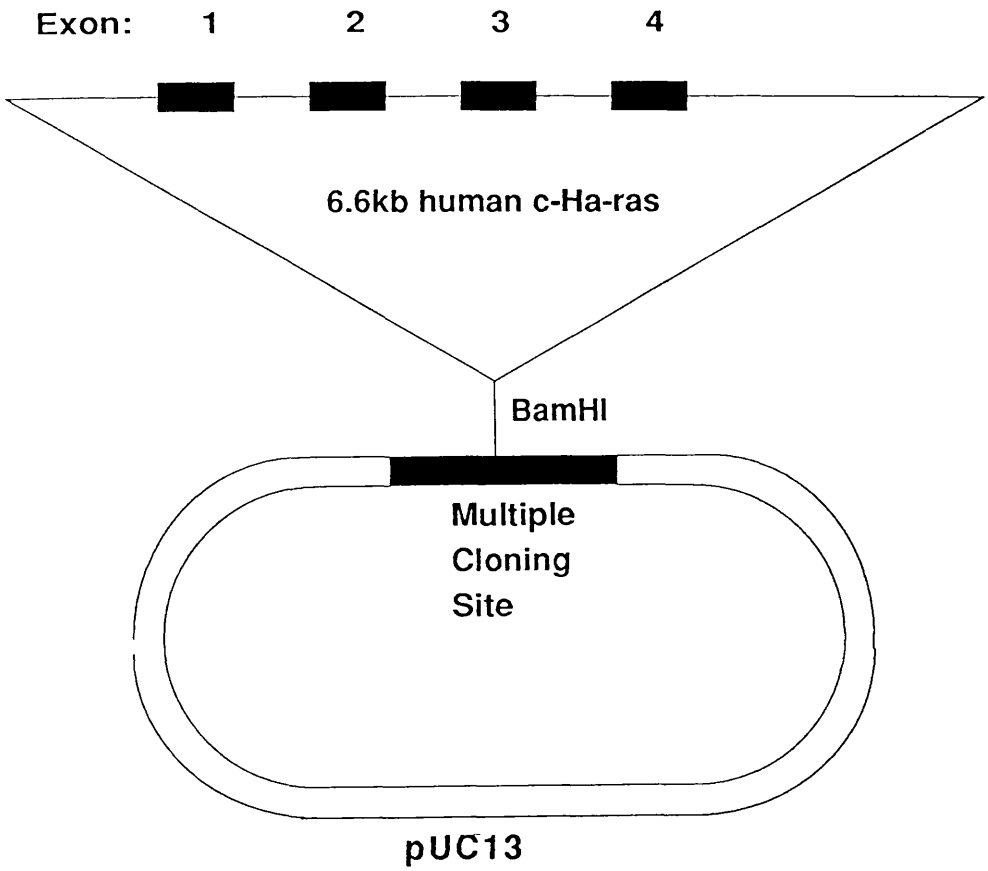
Alternatively, expression of the transforming function(s) of BPV4, even in the presence of an activated **c-Ha-ras** gene, may not be sufficient for cellular transformation in this assay. To try to

distinguish between these possibilities, two separate strategies were followed: firstly, the cloning of subgenomic fragments of BPV4 into expression vectors in order to determine whether any BPV4 genes are **capable** of cooperating with activated proto-oncogenes in the transformation of Pal cells; and secondly the use of neomycin resistance assays to try to detect transforming gene(s) within the context of the complete BPV4 genome.

This second assay allows the growth of clonal cell lines at low density in the absence of surrounding untransformed cells: since such untransformed cells have in certain cases been shown to exert inhibitory effect on focal growth of transformed cells (Dotto *et al*. (1988)), it might be the case that this assay would be more sensitive than that of the focus assay.

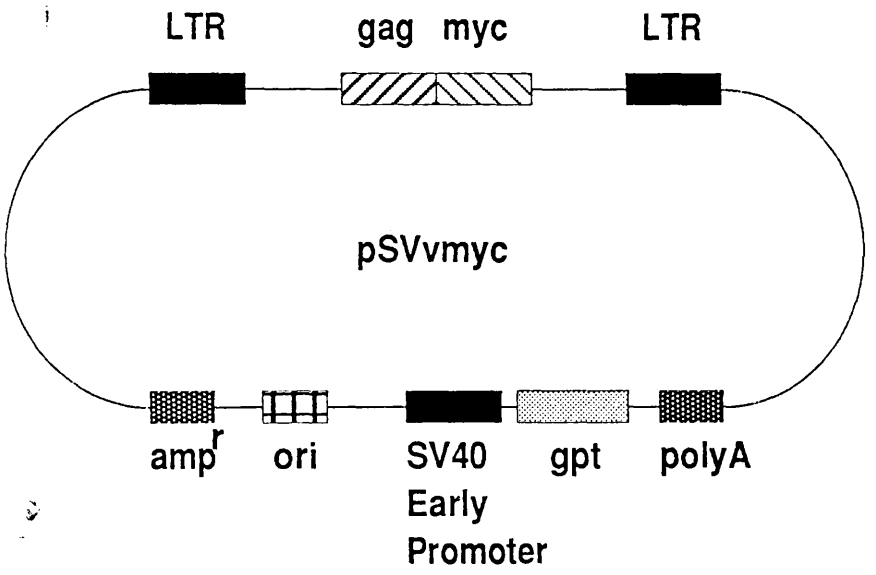
At this stage, technical problems concerning the calcium phosphate coprecipitation method of eukaryotic cell transfection were overcome. Since this technique requires 10- to 20-fold fewer cells per experiment as compared to the electroporation method, it was far more suited to the requirements of Pal cell growth, which exhibit low cell density at confluence. As a result, all further experiments described used the calcium phosphate coprecipitation technique in preference to that of electroporation.

Figure 3.4: Structure of the pT24 plasmid



The 6.6kb insert was isolated as DNA from the human bladder carcinoma T24 by virtue of its ability to transform NIH3T3 cells to the tumorigenic phenotype (Santos *et al* .(1982)). It was originally cloned in pBR322 and subsequently transferred to pUC13 (M. O'Prey (unpublished results)).

Figure 3.5: Structure of the pSVvmyc plasmid



Legend: The gag-myc fusion protein expressed from this construct is the transforming protein of the acutely transforming retrovirus MC29. It has been shown to be active in the malignant transformation of primary Rat Embryo Fibroblasts in cooperation with the plasmid pT24 (described in Figure 3.6).

LTR:	Long Terminal Repeat.
gpt:	Hypoxanthine phosphoribosyl transferase gene.
ori:	Origin of plasmid replication in E. coli.
amp^r:	Ampicillin resistance gene.
SV40:	Simian Virus 40.
poly(A):	Polyadenylation signal.

Plasmid first described in Land *et al* . (1983).

Figure 3.6: Structure of pBV4 BI and pBV4 SstI plasmids

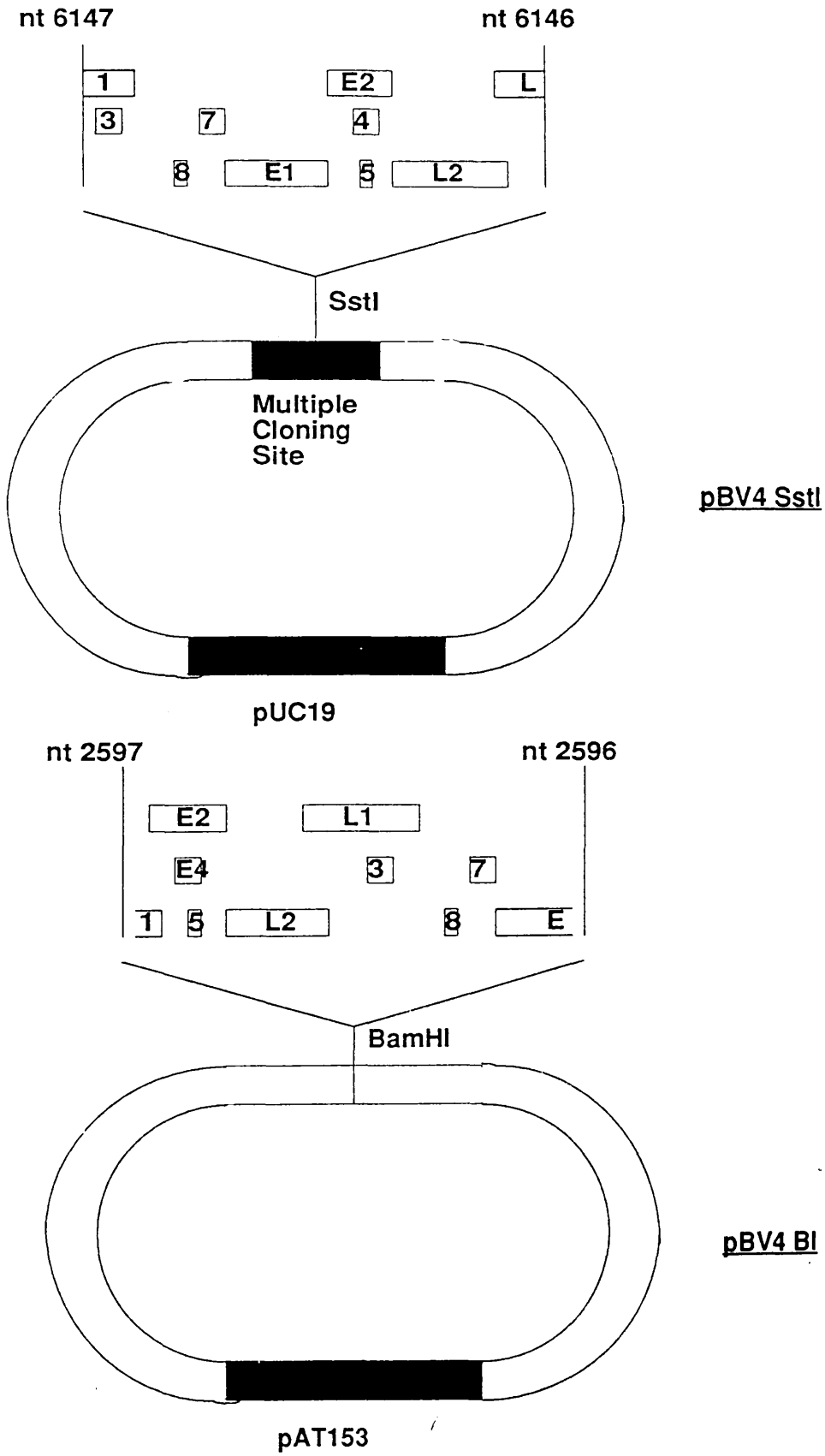


Table 3.3: Focus formation assays of Pal cells electroporated with BPV4 and/or activated proto-oncogenes

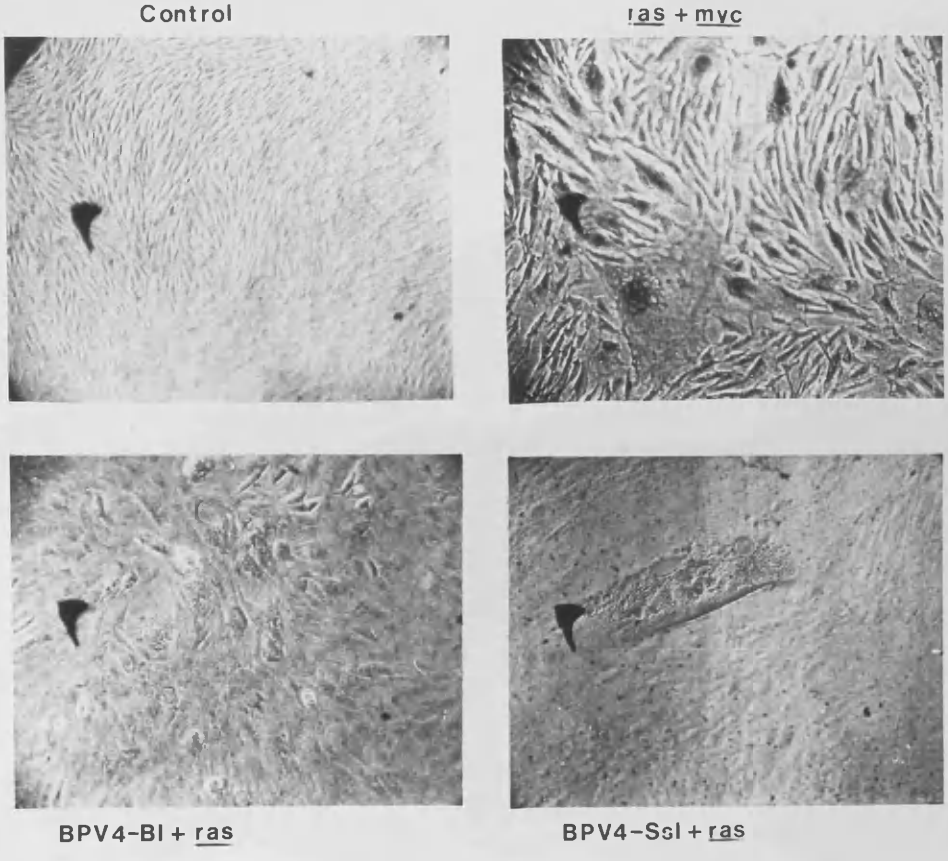
Cells were electroporated as described in Table 3.2, 20µg of each linearised plasmid being used where appropriate.

DNA added	Foci
Calf Thymus	0
BPV4 BI	0
BPV4 SI	0
pT24 B1 (ras)	0
pSVvmyc E1	0
BPV4 BI + ras	12
BPV4SI + ras	10
BPV4 BI + myc	0
BPV4 SI + myc	0
ras + myc	15

Legend: BI/SI/EI: Prior digestion with BamHI/SstI/EcoRI

Foci expressed per 10^7 cells.

Figure3.7: Foci and giant cells induced after electroporation of Pal cells with BPV4 and ras



Magnifications:

Control: x18

ras + myc: x45

BPV4-BI + ras: x45

BPV4-SstI + ras: x45

3.1.4: Use of neomycin coselection assays in the analysis of BPV4 transforming functions

3.1.4(i): Cotransfection of pal cells with BPV4, **ras** and a neomycin resistance gene

In these experiments involving coselection with a neo^r marker gene, the plasmid pSV2neo (Southern and Berg (1982)) was used (a map of the plasmid is shown in Figure 3.17). This plasmid has been shown to confer neomycin resistance to fibroblastic cells of both human and murine origin and was thus considered likely to be active in bovine cells as well.

5 x 10^5 Pal cells were plated in T75 flasks and incubated overnight at 37°C. DNA-calcium phosphate coprecipitates were prepared the next day: in all cases, 5µg of pSV2neo was used; 10µg of linearised pBV4 BI and/or pT24 was used where appropriate and the DNA content of each precipitate was normalised to 25µg using pIC20H as a filler plasmid. 48hrs after addition of the precipitates the cells were split 1:6 and were grown for 21 days in medium containing 500µg/ml G418. The results are shown in Table 3.4.

Using only the neomycin resistance plasmid, several colonies were visible after 14 days, whereas after 21 days, only a few, very small colonies survived: these all showed a flat morphology and none of these could be established in 24-Multiwell plates. Similar results were obtained when cotransfecting the activated **c-Ha-ras** gene. Upon cotransfection of linear BPV4, however, ≈ 20 flat colonies greater than 5mm in diameter (subsequently called 'macrocolonies') per 5 x 10^5 cells could be readily visualised after 21 days selection. Representative colonies are shown in Figure 3.8a

and -b. Cotransfection of both BPV4 and the activated **c-Ha-ras** gene gave results essentially similar to those for BPV4 alone: the great majority of colonies formed had a diameter >5mm and all showed a flat phenotype (see Figure 3.8c and-d).

These results may indicate that in the presence of G418, the Pal cells are undergoing senescence in the period between 14 and 21 days after the beginning of selection, a process which can be delayed by the presence of BPV4. The results do not address the question of immortality: such a question requires continuous passaging of cells for greater than twelve months.

The results also indicated that even in the absence of surrounding normal cells, cotransfection of BPV4 and the activated **ras** gene is not sufficient to induce a morphologically transformed phenotype. This experimental protocol cannot distinguish whether this is due to inefficient expression of the transfected genes or simply due to the inability of this combination of genes to transform bovine fibroblasts. This question has been addressed in subsequent sections.

These results are similar to those of Pirisi *et al* . (1987) and Matlashewski *et al* . (1988), obtained after transfection of HPV16-containing plasmids into human genital fibroblasts. Firstly, the papillomavirus DNA, when transfected in the presence of a neo^r plasmid, is able to prolong the proliferative capacity of the recipient fibroblastic cells in the presence of G418. Secondly, cotransfection of papillomaviral- and activated **ras** DNA is <u>not</u> sufficient to induce a non-contact-inhibited phenotype, even in the absence of surrounding normal cells.

[Since it appears that BPV4 shows similar properties to HPV16 in homologous fibroblastic systems, it would perhaps be of interest to

determine whether BPV4 is similar to HPV16 in transformation assays using Rat Embryo Fibroblasts as recipients, since in these cells the complete tumorigenic phenotype may be induced by HPV16 in cooperation with an activated **ras** gene (Chesters and McCance (1989)). This project was, however, concerned with the development of an homologous cell system and this question was thus not addressed.]

Table 3.4: Neo^r colony formation after cotransfection of Pal cells with pSV2neo and linear BPV4 and/or activated c-Ha-ras DNA

Exponentially growing Pal cells were harvested and plated at 5 X 10^5 /T75 flask. 24hrs later, calcium phosphate-DNA coprecipitates were added dropwise and left on the cells at 37°C overnight. Cells were then washed and refed. 48hrs after addition of the precipitates, cells were harvested, replated at a 1:6 dilution in the presence of 500µg/ml G418. Cells were refed twice weekly for 21 days, when colony formation was scored.

Plasmid DNA	Macrocolonies/ total colonies			% morphologically transformed
	Experiment			
	1	2	3	
pSV2neo	0/3	0/0	0/2	0
pSV2neo + pT24 BI	0/3	0/1	0/0	0
pSV2neo + BPV4 BI	20/20	17/18	24/26	0
pSV2neo + pT24 BI + BPV4 BI	20/24	11/12	17/18	0

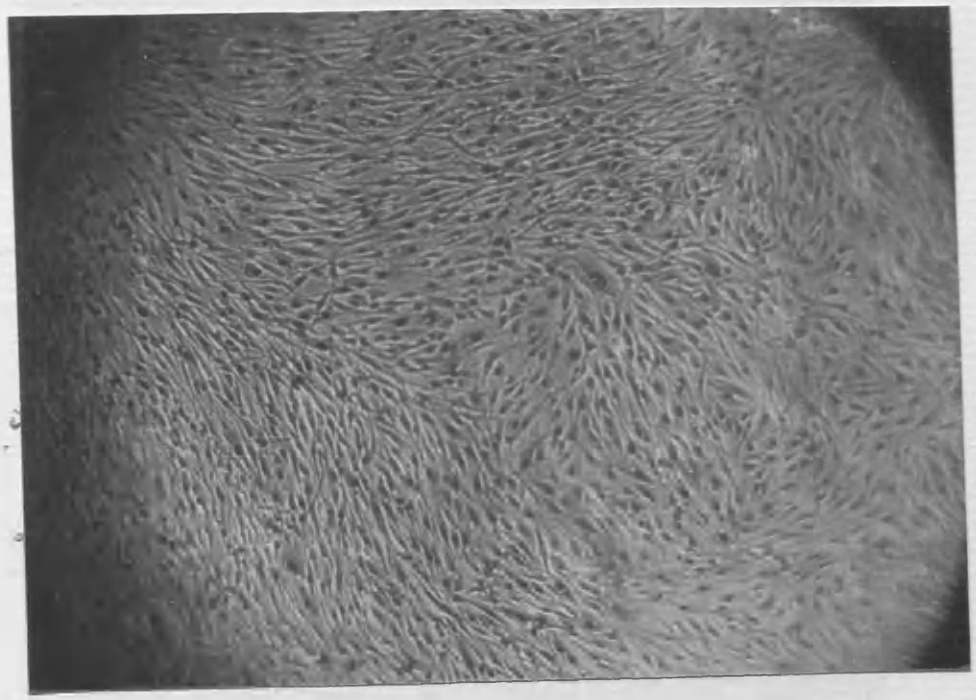
Legend: 'Macrocolony': colonies of diameter > 5mm

BI: prior digestion of DNA with BamHI

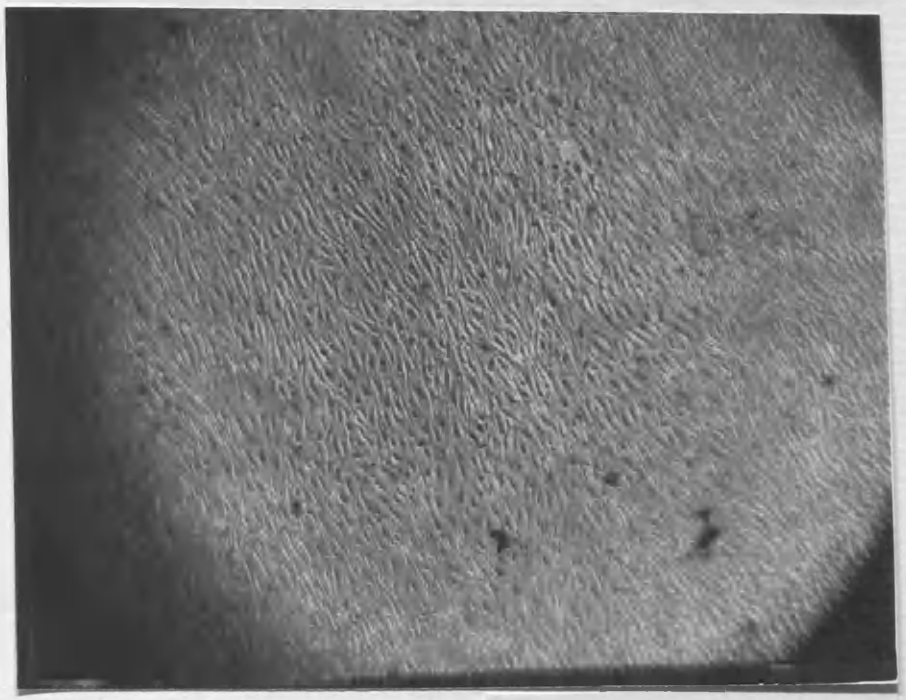
Macrocolony formation expressed per 5 X 10^5 cells.

Figure 3.8: 'Macrocolonies' produced after cotransfection of pSV2neo and linear BP4 with or without activated ras.

a:

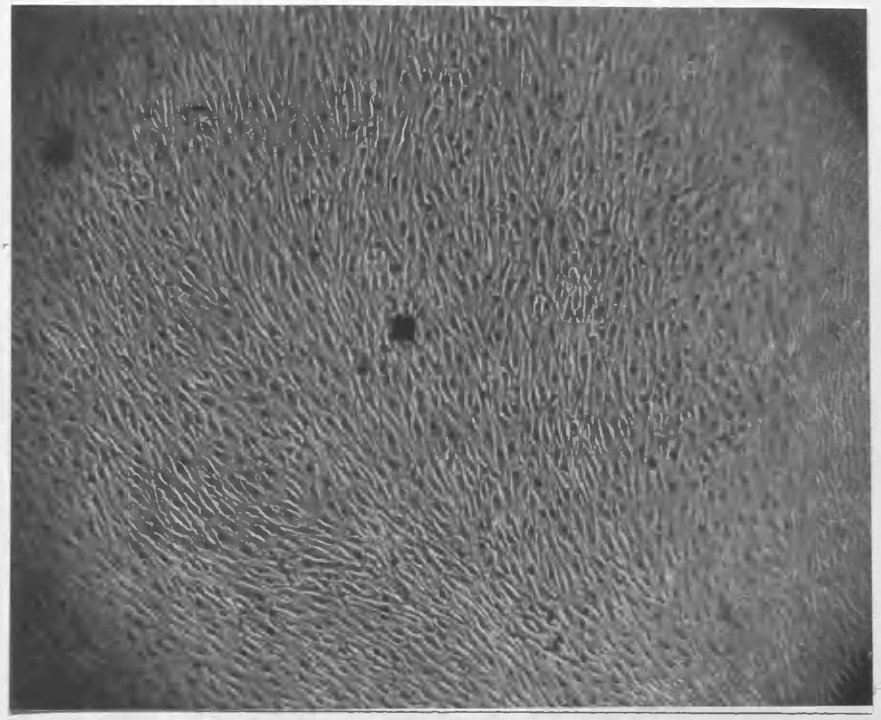


b:

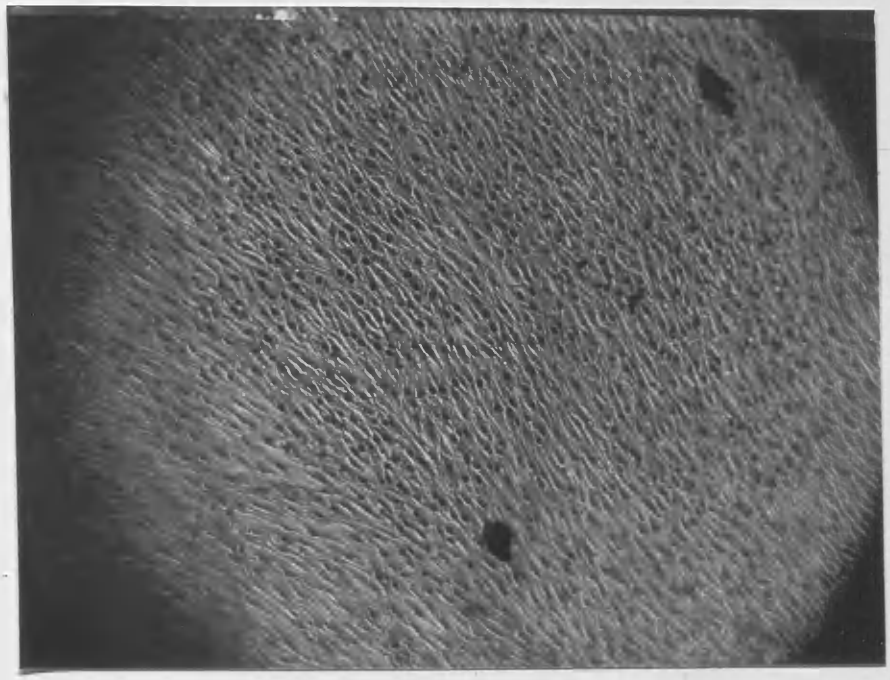


Magnification: x60

c:



d:



Legend: a,b: pSV2neo + BPV4 BI

c,d: pSV2neo + BPV4 BI + pT24 BI

Magnification: x60

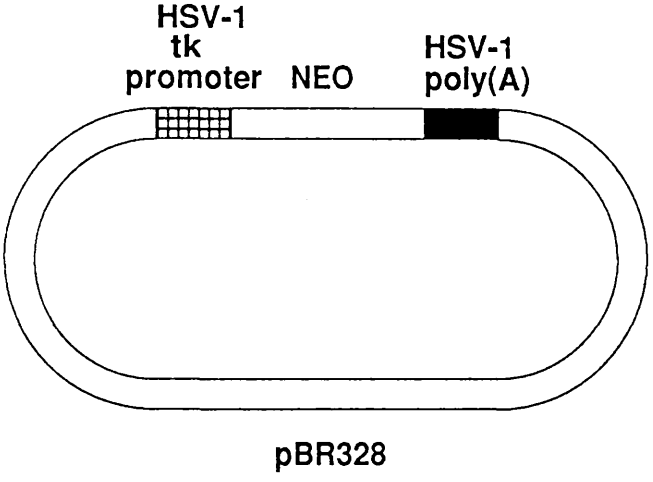
3.1.4(ii): The effect of TPA on BPV4-mediated transformation in neomycin resistance assays

Although TPA was unable to induce focus formation in BPV4-transfected Pal cells, it was considered possible that TPA-mediated phenotypes might be observed upon coselection with neomycin resistance plasmid. The protocol used was identical to that in Section 3.4(i), adding 20ng/ml TPA throughout the selection process where appropriate. In such assays, all cells treated with TPA died even more quickly than controls receiving no neomycin plasmid but not exposed to TPA: such control cells took 14 days to die, whereas all cells treated with TPA were dead within one week.

One possible reason for this is that downregulation of **neo** gene expression might occur. The action of TPA has been shown to be mediated by modulation of gene transcription, which has been further localised to a control of the activity of a protein complex term AP-1, which binds to the DNA consensus sequence 5'-TGACTCA. Since such sequences exist within the SV40 early promoter, it was possible that TPA induced a downregulation of **neo** expression. A second neo^r-encoding plasmid was therefore tested: this plasmid, pMS-2, utilises the Herpes Simplex Virus (HSV) thymidine kinase promoter to drive **neo** expression; the activity of this promoter has been reported to be stimulated by addition of TPA when used in $LATK^-$ cells (Campo and Roe (1986)). The structure of pMS-2 is shown in Figure 3.9. Nevertheless, again all the cells died faster than the untreated controls.

Another possible explanation of the phenomenon is that the treatment sensitises the cells to the effects of neomycin. However,

Figure 3.9: Structure of the plasmid pMS-2



The plasmid was originally constructed by M.S.Campo (unpublished).

Abbreviations: poly(A): polyadenylation signal
NEO: neomycin resistance gene from Tn5
HSV-1: Herpes Simplex Virus Type 1

in the absence of observable phenotypes, the approach was not carried further.

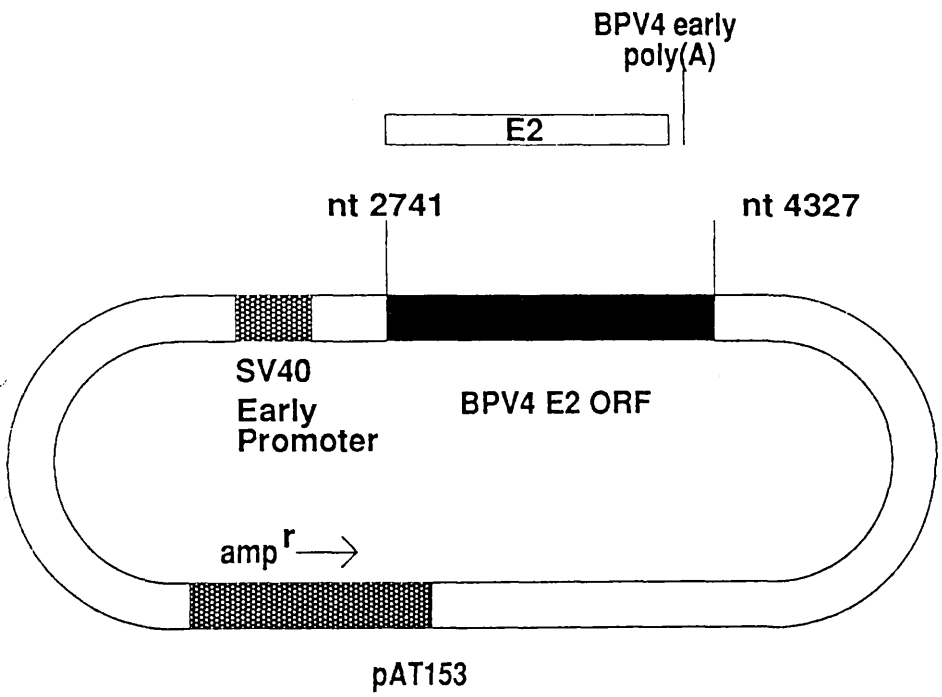
3.1.4(iii): The effect of the E2 ORF on BPV4-associated transformation of Pal cells

It has recently been shown that stimulation of morphological transformation of primary rat epithelial cells induced by cotransfection of HPV16 and activated **ras** genes was enhanced by the presence of the HPV16 E2 gene product (Lees *et al* . (1990)).

In order to examine whether this was the case for BPV4, the plasmid pSVE2B was used, the structure of which is shown in Figure 3.10. This plasmid contains the E2-E5 region of the BPV4 genome expressed under the control of the SV40 promoter, and has been shown to induce a 3-fold increase in transcriptional activity of the BPV4 early promoter in Pal cells in transient CAT assays (M. Jackson, Beatson Institute, pers. comm.), a level of stimulation similar to that seen with the analogous constructs of HPV18 on its own early promoters (Thierry and Yaniv (1987)).

Cells were transfected with 5μg of pSV2neo along with 10μg of BPV4, pT24 and pSVE2B, using all possible permutations of these three plasmids. No obvious effect of E2 could be discerned. In focus assays a single area of more densely growing cells was identified in the flask transfected with BPV4, pT24 and pSVE2B, but upon picking this colony and allowing further growth, the phenotype appeared indistinguishable from the parental cells and showed no increased cell density at confluence. It was noted that all neo^r colonies formed after cotransfection of BPV4 and pSVE2B were smaller than those formed by BPV4 alone, but the number of

Figure 3.10: Structure of the plasmid pSVE2B



The plasmid was constructed by M. Jackson (Beatson Institute) (unpublished results).

colonies was too small to be of real significance. Again, in the absence of readily scorable phenotypes it was decided not to follow up this line of enquiry.

3.1.5: Cloning of BPV4 subgenomic fragments into expression vectors

To determine whether inefficient expression of putative transforming genes of BPV4 was the reason for the lack of appearance of fully morphologically transformed cell after transfection of Pal cells with BPV4 and activated **ras**, it was decided to clone individual regions of the BPV4 venome into two plasmids: pSV2neo (Southern and Berg (1982)) and pZIPneoSV(X1) (Cepko *et al* . (1984)). The former requires the use of BPV4 promoter, but contains the 72bp repeat of the SV40 early promoter which acts as a strong enhancer (see Figure 3.17). The latter contains two Moloney Murine Leukaemia Virus (Mo-MuLV) Long Terminal Repeats (LTRs) flanking the cloned sequences (see Figure 3.18): since these contain both enhancer and promoter functions, the use of BPV4 promoters is not necessarily required for expression of BPV4 functions. It was predicted therefore that all BPV4 ORFs cloned into pZIPneoSV(X1) should be expressed efficiently, whereas cloning of fragments into pSV2neo should lead to levels of expression similar to that of the full genome, unless transmodulation by virally-encoded factors occurs to a significant degree in the context of the full viral genome. Both plasmids encode the neomycin resistance (neo^r) gene, thus allowing selection both for focus- and colony formation.

The restriction enzyme XhoII conveniently cleaved BPV4 DNA at three sites (see Figure 3.11): two regions are of interest:

E7/8 (nt 6487 - 1275 - **designated 2.0**)

and

E2-E5 (nt 2597 - 6487 - **designated 3.9**).

These could simply be cloned straight into the BamHI sites of the two vectors since the 5' overhangs generated by XhoII- and BamHI digestion are identical.

A third recombinant plasmid was also produced: this was identical to the E7/8 construct, except that it lacked a 233bp EcoRI fragment (nt906 - 1139), thus interrupting the E7 ORF. (**This was designated 1.8**). This construct was predicted to be informative, since digestion of BPV4 DNA with Eco RI abolishes transforming activity in NIH3T3 cells (M.S. Campo (pers. comm.)).

The E7/8 fragment was isolated from pIC20R.H3-H3 3.6 (shown in Figure 3.12), the analogous fragment lacking E7 was isolated from pBV4 AB (shown in Figure 3.13) and the E2-E5 fragment was isolated from pBV4 BI. After purification of fragment and ligation to dephosphorylated linear vector DNA, E.Coli strain DH5-a was transformed and grown on L-amp plates overnight. The results are listed in Tables 3.5 and 3.6.

The efficiency of transformation by the supercoiled vectors pSV2neo and pZIPneoSV(X1) was 10-fold less than that claimed for pUC19 by the suppliers: this is not surprising since it is a commonly known phenomenon that the transformation efficiency of competent bacteria decreases as the molecular weight of the plasmid increases (5.7kb and 10.1kb respectively as compared to 2.7kb of pUC19). Routine use of pUC19 as a control in early experiments consistently gave transformation frequencies between 8×10^7 and 2×10^8: this indicated that the observed decreases in

transformation efficiency were not likely to be due to poor experimental technique.

Religation of linearised, non-phosphatased vectors showed a 10-20 fold decrease in efficiency as compared to supercoiled vector control: this could be due firstly to the presence of protein (DNA ligase), but could also be due to possible non-linearity of colony formation with increasing DNA concentration and/or near saturation of plates with ampicillin^r colonies. Since, however, a decrease in transformation efficiency of greater than two orders of magnitude was observed after prior treatment of linearised vectors with Calf Intestinal Phosphatase (denoted by DP in the Tables), it was considered that ligase activity was more than satisfactory for the current purpose. The lack of transformants after ligation of inserts in the absence of vector sequences also indicate that such inserts had been satisfactorily separated from any possible residual vector DNA.

The relatively large number of colonies seen on the experimental plates thus indicated that the vast majority of transformants were likely to be recombinants: Grunstein-Hogness screening procedures were unlikely to be necessary and they were thus screened by miniprep. analysis.

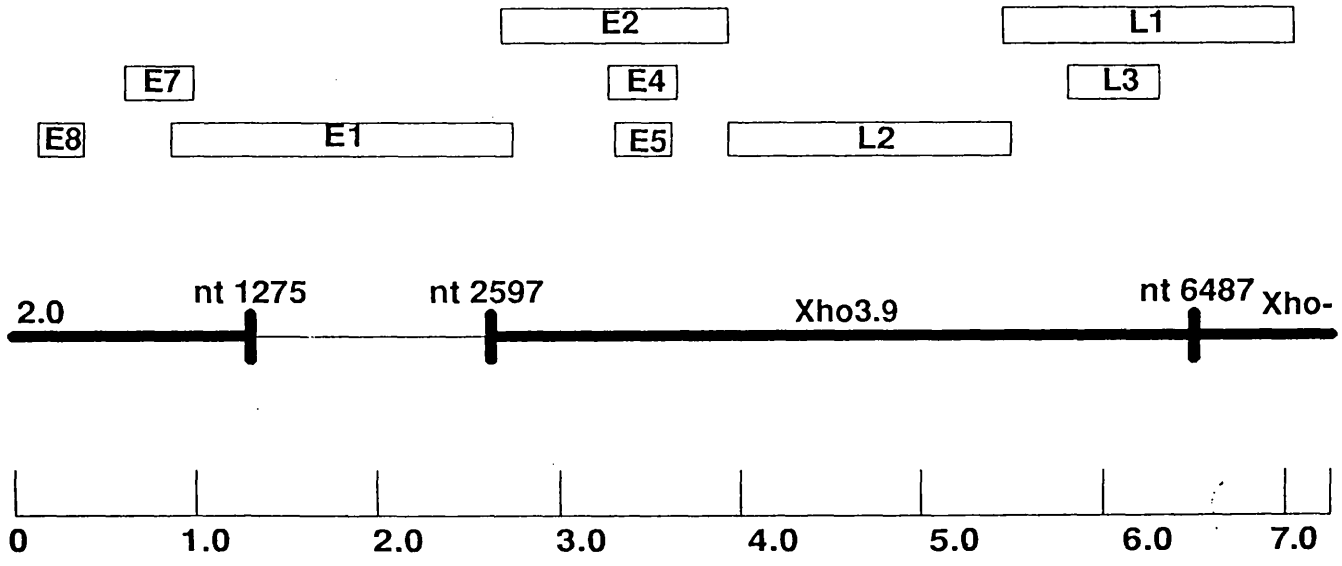
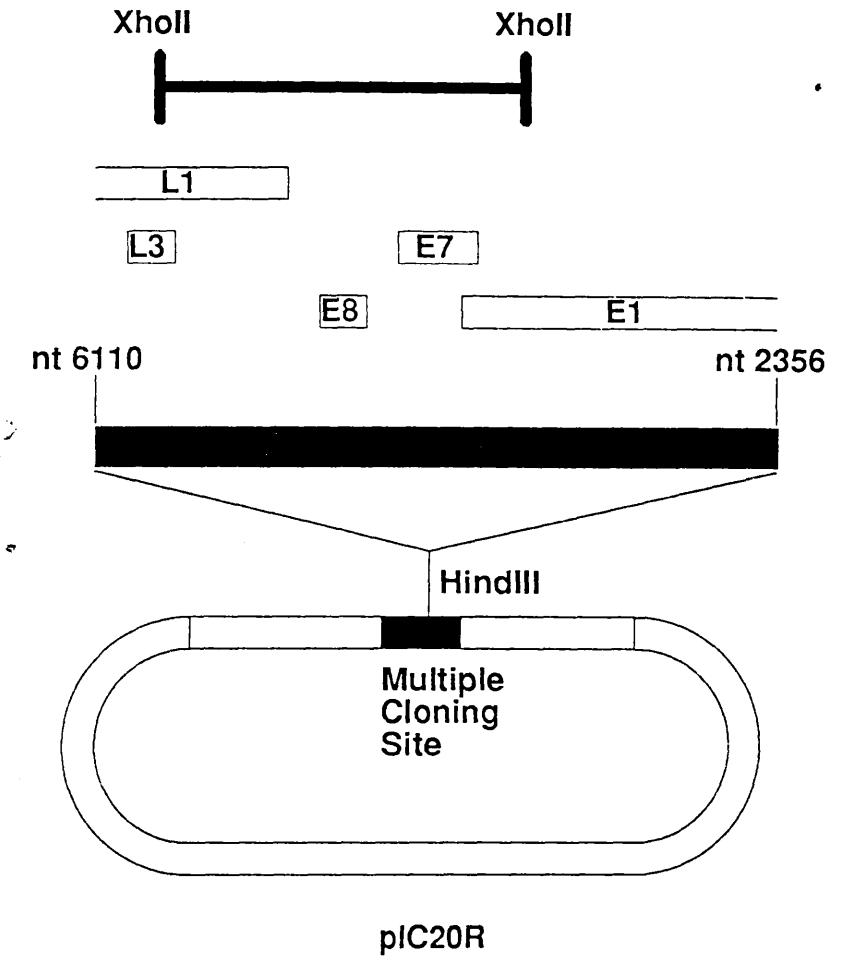


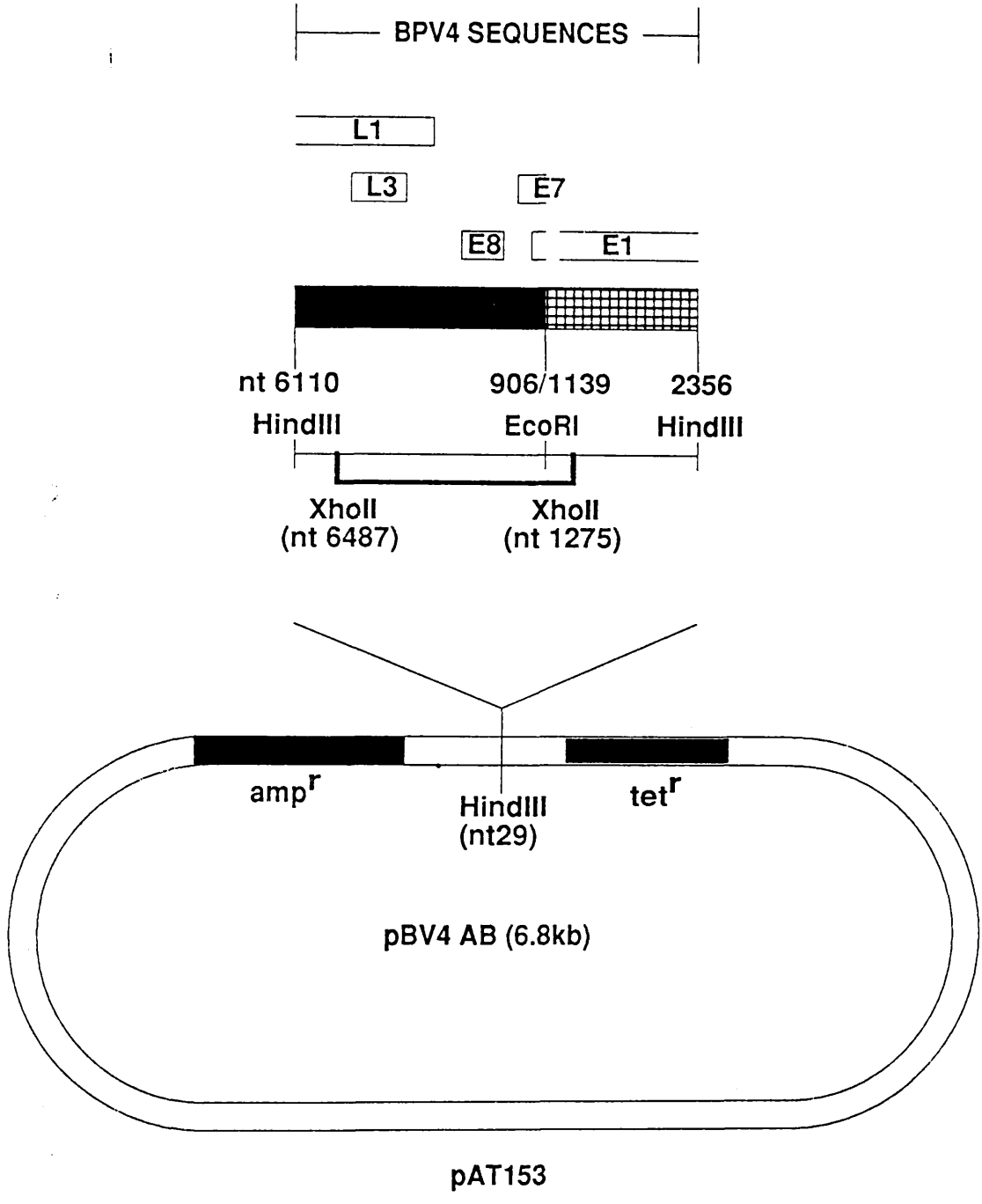
Figure 3.11: XhoII sites within the BPV4 genome

Figure 3.12: Structure of the plasmid pIC20R.H3-H3 3.6



The plasmid was constructed by I. Doherty (Beatson Institute) (unpublished results).

Figure 3.13: Structure of the plasmid pBV4 AB.



The plasmid was constructed by M.S. Campo (unpublished results).

Table 3.5: Transformation of E. coli DH5-a by pSV2neo-derived clones.

200ng of linearised, dephosphorylated vectorDNA and a threefold molar excess of insert were ligated overnight at 14°C in a volume of 10µl. The reaction mix was diluted 5-fold and 1µl was used to transform 50µl of freshly thawed, competent E. coli strain DH5-a. The bacteria were selected overnight on agar plates containing 50µg/ml ampicillin and scored the next day.

DNA in transform-ation reaction	Colonies/ plate	Colonies/ ug DNA
pSV2neo 50pg	214	2.1×10^7
pSV2neo BI self 4ng	>500	$>6.2 \times 10^5$
pSV2neo BI DP self 4ng	2	2.5×10^3
BPV4 XhoII 2.0 self 4ng	0	$< 2.5 \times 10^3$
BPV4 XhoII 3.9 self 8ng	0	$< 2.5 \times 10^3$
pBV4AB XhoII 1.8 self 4ng	0	$< 2.5 \times 10^3$
pSV2neo B1DP + BPV4 XhoII 2.0	112	1.4×10^5
pSV2neo BI DP+ BPV4 XhoII 3.9	103	1.3×10^5
pSV2neo BI DP + pBV4AB XhoII 1.8	56	8.0×10^4

For legend see foot of Table 3.6 overleaf.

Table 3.6: Transformation of E. coli DH5-a by pZIPneo-SV(X1)derived clones.

200ng of linearised, dephosphorylated vector DNA and a three-fold molar excess of insert were ligated overnight at 14°C in a volume of 10µl. The reaction was diluted 5-fold and 1µl was used to transform 50µl of freshly thawed, competent E. coli strain DH5-a. The bacteria were selected overnight on agar plates containing 50µg/ml ampicillin and scored the following day.

DNA in transform-ation reaction	Colonies/ plate	Colonies/ ug DNA
pZIPneoSV(X1) 50pg	84	8.4×10^6
pZIPneoSV(X1) BI self 8ng	>500	$> 3.1 \times 10^5$
pZIPneoSV(X1) BI DP self 8ng	0	$< 1.3 \times 10^2$
pZIPneoSV(X1) BI DP + BPV4 XhoII 2.0	104	6.5×10^4
pZIPneoSV(X1) BI DP + BPV4 XhoII 3.9	86	5.7×10^4
pZIPneoSV(X1) BI DP + pBV4 AB XhoII 1.8	120	7.5×10^4

Legend: BI: Digestion with BamHI prior to ligation

DP: Treatment with Calf Intestinal Phosphatase prior to ligation

self: ligation in the absence of heterologous DNA molecules

Note: the colonies/µg quoted refers to µg of vector DNA.

3.5(i): Analysis of pSV2neo-derived recombinants

The enzyme EcoRI was useful in this analysis: it cuts asymmetrically within all the inserts, and at a single site within the parent vector. These are shown in Figure 3.14. The predicted size bands for parental vector and each recombinant are listed below: (the + and - refer to the relative direction of transcription of BPV4 ORFs with respect to the direction of transcription of the neo^r gene).

pSV2neo: **5.7kb**

pSV2neo2.0(+): **6.7kb, 0.8kb and 0.2kb**
pSV2neo2.0(-): **5.1kb, 2.4kb and 0.2kb**

pSV2neo1.8(+): **6.7kb and 0.8kb**
pSV2neo1.8(-): **5.1kb and 2.4kb**

pSV2neo3.9(+): **5.4kb and 4.2kb**
pSV2neo3.9(-): **8.3kb and 1.3kb**

Recombinants were identified and prepared in bulk. These were digested with EcoRI to confirm their identity. A photograph of the gel is shown in Figure 3.15.

Figure 3.14: Location of EcoRI sites within XhoII fragments of BPV4

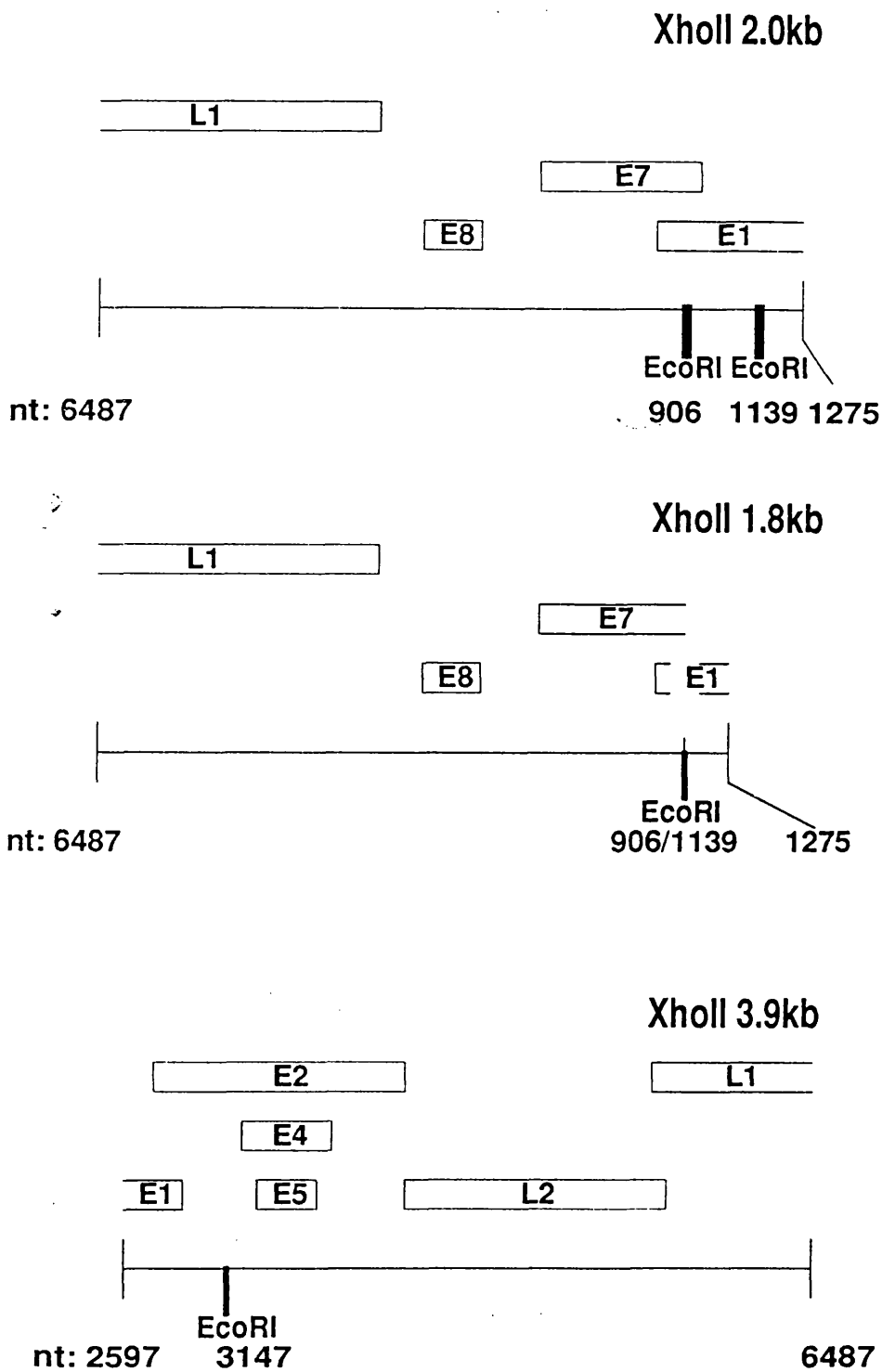
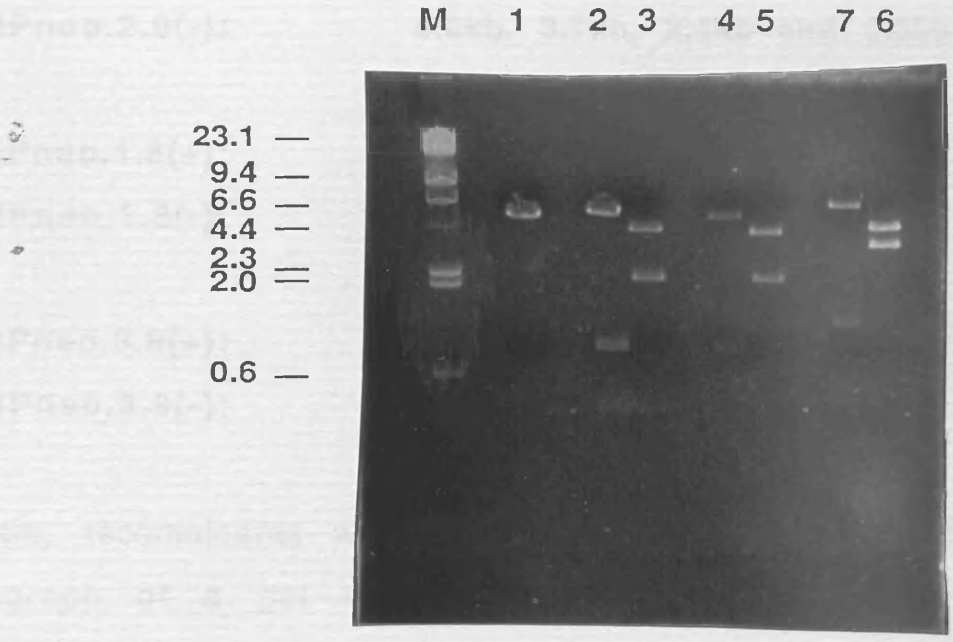


Figure 3.15: EcoRI digests of pSV2neo-derived BPV4 recombinant clones

2µg of purified plasmid were digested with 20U EcoRI. for 1hr at 37°C. The samples were then separated by electrophoresis through a 1% TAE agarose gel at 4V/cm., using HindIII-digested bacteriophage lambda as markers. The gel was stained for 15mins with ethidium bromide and visualised using a UV transilluminator.



Legend: M: HindIII-digested bacteriophage lambda markers

1: pSV2neo

2,3: pSV2.0(+), pSV2.0(-)

4,5: pSV1.8(+), pSV1.8(-)

6,7: pSV3.9(+), pSV3.9(-)

3.5(ii): Analysis of pZIPneoSV(X1)-derived recombinants

The enzyme EcoRI was again used to identify the desired recombinants: two sites exist in the parent vector, but again relative orientations of inserts could be determined. The expected restriction fragment sizes are:

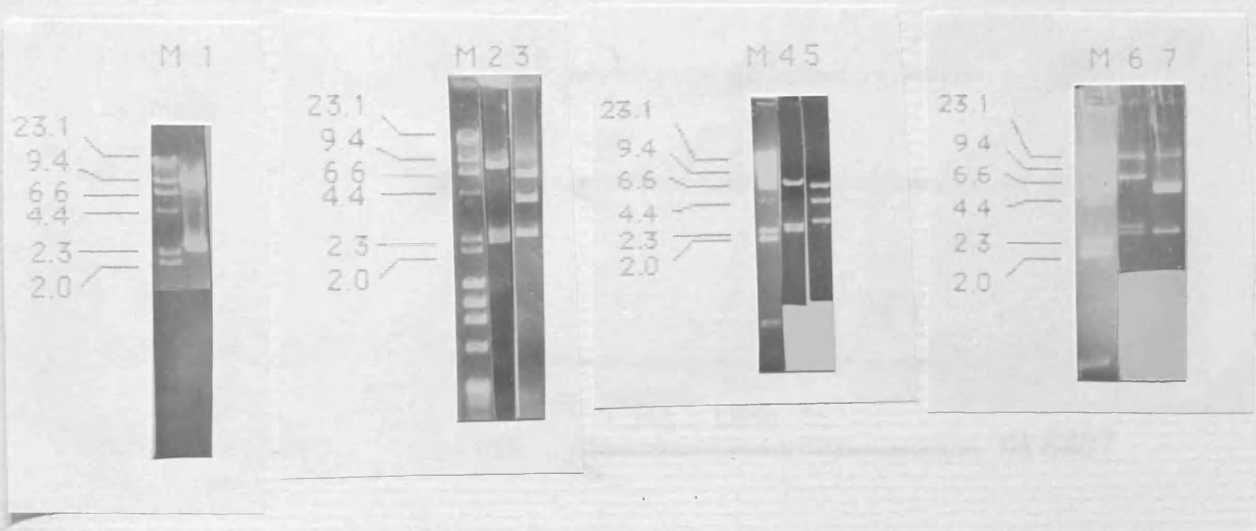
pZIPneoSV(X1):	**7.7kb and 2.4kb**
pZIPneo.2.0(+):	**7.4kb, 2.4kb, 2.1kb and 0.2kb**
pZIPneo.2.0(-):	**5.8kb, 3.7kb, 2.4kb and 0.2kb**
pZIPneo.1.8(+):	**7.4kb, 2.4kb and 2.1kb**
pZIPneo.1.8(-):	**5.8kb, 3.7kb and 2.4kb**
pZIPneo.3.9(+):	**6.3kb, 5.3kb and 2.4kb**
pZIPneo.3.9(-):	**9.0kb, 2.6kb and 2.4kb**

Again, recombinants were identified and purified in bulk. A photograph of a gel showing the EcoRI digests of these recombinants is shown in Figure 3.16.

These plasmids could then be used in cooperativity assays with the activated **ras** gene in Pal cells. The physical map of the recombinant plasmids is shown in Figures 3.17 and 3.18.

Figure 3.16: EcoRI digests of pZIPneoSV(X1)-derived BPV4 recombinant clones

2µg of purified plasmid were digested with 20U EcoRI. for 1hr at 37°C. The samples were then separated by electrophoresis through a 1% TAE agarose gel at 4V/cm., using HindIII-digested bacteriophage lambda as markers. The gel was stained for 15mins with ethidium bromide and visualised using a UV transilluminator.



Legend: M: HindIII-digested bacteriophage lambda markers

1: pZIPneoSV(X1)

2,3: pZIP2.0(+), pZIP2.0(-)

4,5: pZIP1.8(+), pZIP1.8(-)

6,7: pZIP3.9(−), pZIP3.9(+)

Figure 3.17: Summary of BPV4 recombinants cloned into pSV2neo.

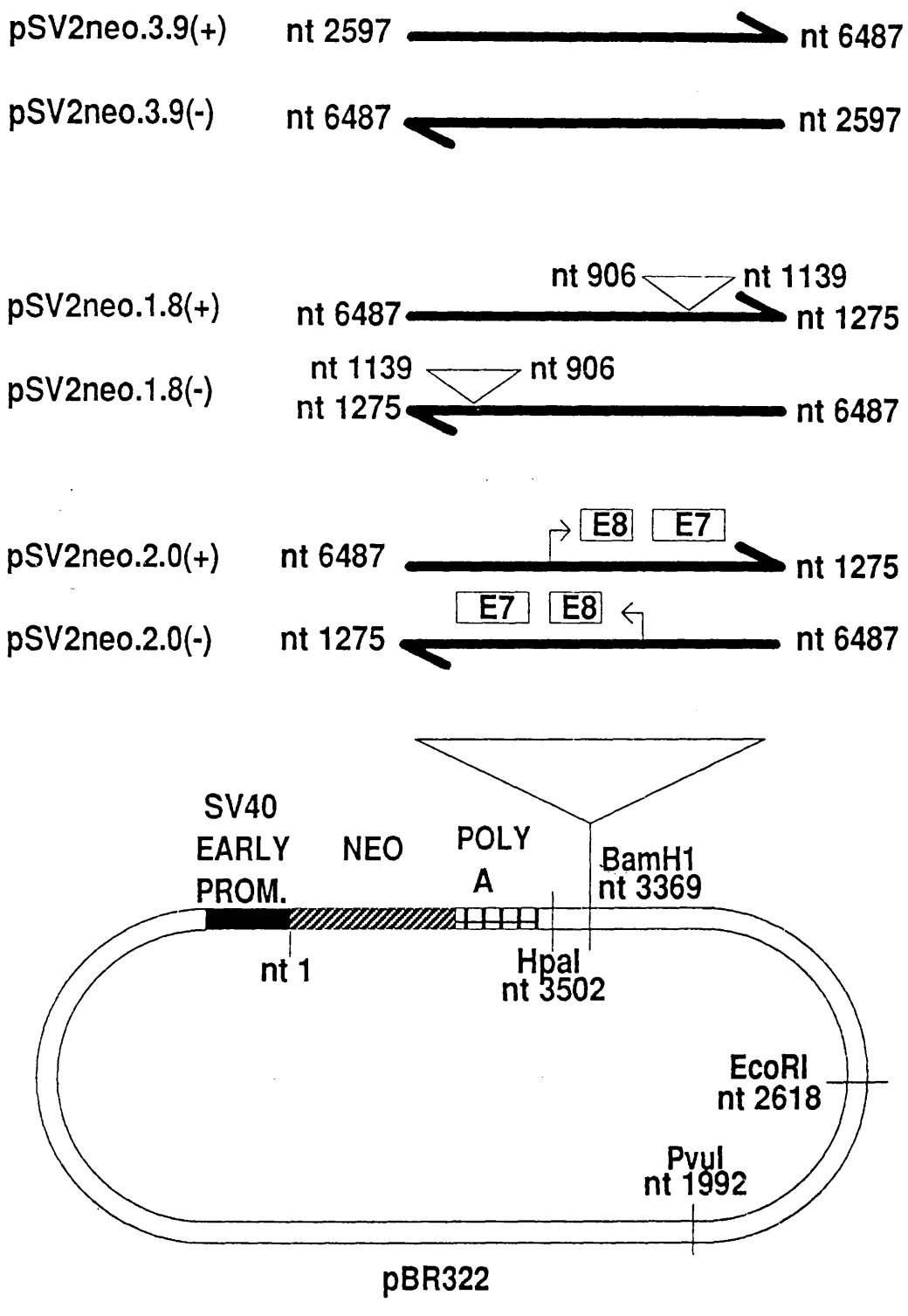
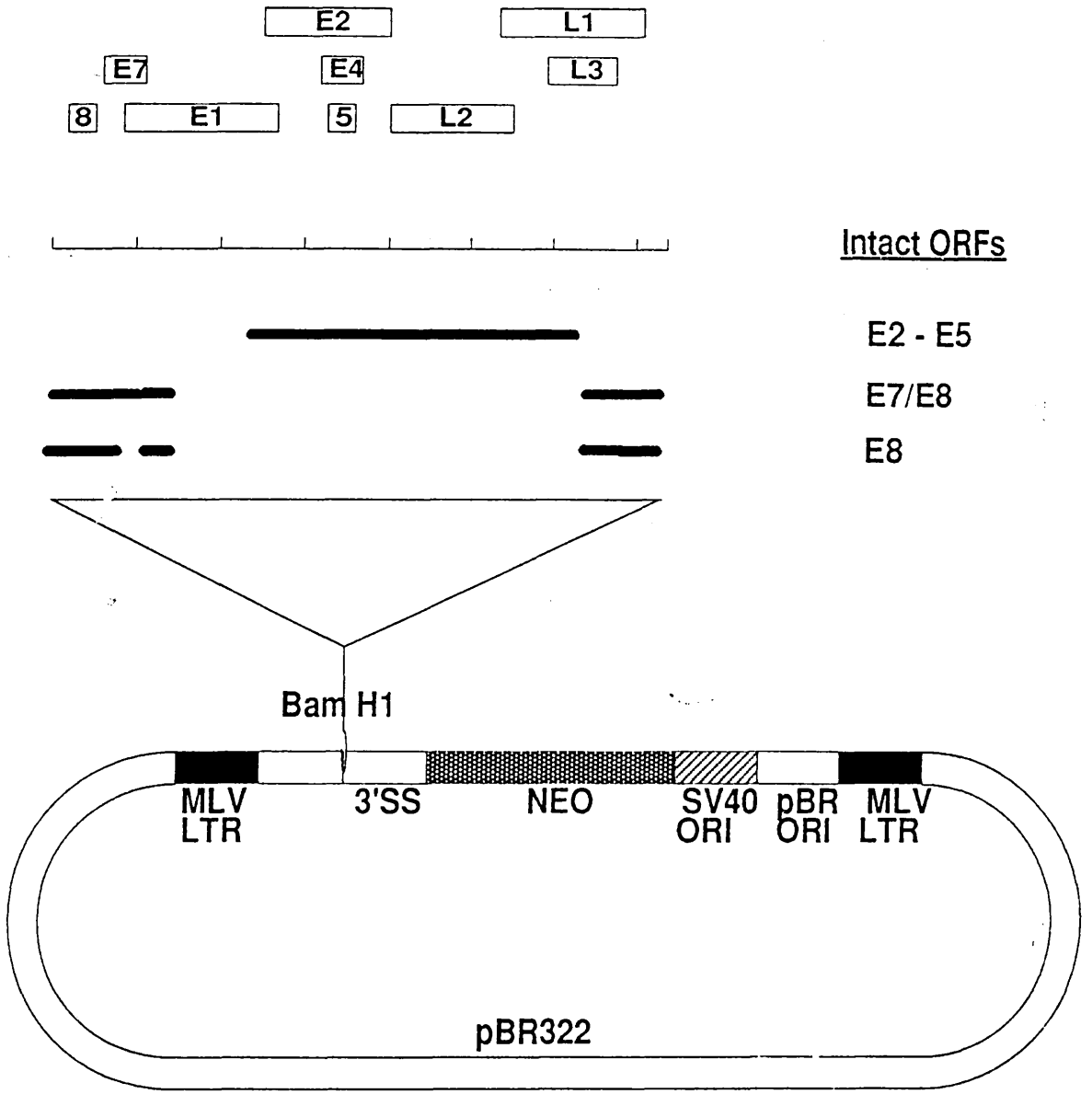


Figure 3.18: Summary of BPV4 recombinants cloned into pZIPneoSV(X1)



Legend:

SS:	Splice site.
MLV LTR:	Moloney Murine Leukaemia Virus Long Terminal Repeat.
ORI:	origins of DNA replication.
NEO:	Neomycin resistance gene.

3.6 Activity of pSV2neo-derived clones in transfection assays

3.6(i): Transfection of C127 murine fibroblasts

Since transfection of primary cell lines is inherently less efficient than when using established lines, it was decided to test experimental technique and the purity of plasmids using the established C127 murine fibroblast cell line, since this is known to be transfected efficiently using the calcium phosphate coprecipitation procedure. (At this stage, the pSV3.9 clones had not been obtained: when they were, confidence in the transfection procedure meant that such C127 control experiments were not important).

3×10^5 cells were plated in T25 flasks and transfected with 10μg of appropriate plasmid DNA. Two days after addition of the DNA-calcium phosphate coprecipitate, the cells were split: half were plated at 2×10^3 cells/cm^2 in medium containing 1.2mg/ml G418. The medium was changed weekly and after 21 days, the number of neo^r colonies were scored by Giemsa staining. The other half were plated at 5×10^3 cells/cm^2 in growth medium containing 10% FCS, grown to confluence and maintained for 4 weeks with changes of medium twice a week, after which they were scored for focus formation. (This is the Protocol 2 described by Smith and Campo (1988)). The results are shown in Table 3.7a.

In the experiments involving neomycin selection, the colony-forming efficiency was between 5×10^{-4} and 10^{-3}. This indicated that the transfection system was working adequately and that any negative results obtained using Pal cells would not be due

to any trivial technical problems. All colonies showed a flat morphology, similar to that obtained by cotransfection of pSV2neo with linear BPV4 genome (K.T. Smith (pers. comm.)).

The lack of any detectable focus-forming activity was unexpected: even the positive control of linear BPV4 DNA failed to induce foci after maintenance at confluence for up to 5 weeks. The reasons for this were unclear: the possibility that the digestion process had resulted in impure DNA could at this stage not be discounted, but use of the same sample in experiments involving Pal cells (see Section 3.4) indicated that this was not the case. In the cases other than the positive control, one possible reason could involve the use of circular DNA: BPV4-induced transformation of C127 cells has been shown to be inhibited when using DNA covalently linked to pAT153 (Smith and Campo (1988)).

It was thus decided to separate the BPV4 DNA from the pSV2neo vector sequences. since, during the cloning step, the joining of XhoII- to BamHI ends resulted in the regeneration of a hexanucleotide sequence recognised by no restriction enzyme, the best available linearisation strategy involved cutting with two restriction enzymes, PvuI and HpaI, which cut on either side of the BPV4 sequences while maintaining an intact **neo** gene (at nts 1992 and 3502 of pSV2neo repectively). The position of the restriction enzyme sites within pSV2neo are shown in Figure 3.17. Transfection of linearised plasmids into C127 cells was performed as before and the results are shown in Table 3.7b. Once again, no focus formation was discernible, although again, neomycin-resistant colonies appeared, albeit at a reduced frequency, as compared to the circular DNA. Either, therefore, the sequences of pSV2neo still linked to the BPV4 DNA inhibited transformation, or the E8 + E7 sequences were

not sufficient for focus formation, or some unidentified parameter concerning the susceptibility of the C127 cells to BPV4-induced morphological transformation was affected. At this stage, however, this point was not pursued further, as the transformation of C127 cells was not central to the body of work in this thesis.

Since the transfection technique appeared to be working satisfactorily, it was decided to proceed to the transfection of Pal cells both with the BPV4 recombinants alone as well as in cooperation with the activated **ras** gene.

Table 3.7a: Transfection of C127 cells with pSV2neo-derived clones.

DNA	Foci	Neo colonies	% transformed
pSV2neo	0	>100	0
pSV2.0(+) (E7/E8)	0	>100	0
pSV2.0(-)	0	>100	0
pSV1.8(+) (E8)	0	>100	0
pSV1.8(-)	0	>100	0
BPV4 B1	0	0	-

Table 3.7b: Transfection of C127 cells with linearised pSV2neo-derived clones.

DNA	Foci	Neo colonies	% transformed
pSV2neo	0	67	0
pSV2.0(+) (E7/E8)	0	79	0
pSV2.0(-)	0	45	0
pSV1.8(+) (E8)	0	39	0
pSV1.8(-)	0	54	0

Legend: neo^r colonies expressed per 2 X 10^5 cells

DNAs linearised with PvuI and HpaI

3.6(ii): Transfection of Pal cells with pSV2neo-derived clones in cooperation with an activated human **c-Ha-ras** gene

The ability of the subgenomic fragments of BPV4 cloned in pSV2neo to cooperate with the activated **c-Ha-ras** gene encoded by the plasmid pT24 in the transformation of Pal cells was examined using calcium phosphate-mediated transfection procedures. In all cases only the (+) clones (those containing BPV4 sequences whose putative direction of transcription is the same as that of the neomycin resistance gene) were used.

Two days after transfection, the cells were trypsinised and split into two halves: one half was diluted 1:6 and grown for 21 days in normal growth medium containing 500µg/ml G418, whereupon flasks were scored for neo^r colonies and the morphological appearance of such colonies. The other half was diluted 1:2, grown to confluence and maintained for 3 weeks, again in normal growth medium and scored for the formation of foci. In both cases, medium was changed three times a week. The results are shown in Tables 3.8a and 3.8b.

In the focus formation assays, no foci were induced during the (extended) 6 week period of observation of the cells at confluence. In flasks transfected with the activated **ras** gene, the elongated cells were observed as before, but none developed into the small colonies found in the initial cotransfection experiment with BPV4 full genome plus **ras** (see Section 3.4). In the case of cotransfecting pSV2.0(+) and **ras**, a single area of cells showing greater density (but not piling up) was observed. This was picked and grown on 10% serum. Upon return to confluence, the cell density was identical to

Table 3.8a:Transforming activities of pSV2neo-derived clones of BPV4 in Pal cells.

Cells were transfected as described in Table 3.4, except that 10μg of all neo^r-encoding plasmids were used.

Plasmid DNA	Foci	Macrocolonies/ total colonies			Colony morphology
		Experiment 1	2	3	
pSV2neo	0	0/3	0/1	0/0	NA
pSV2neo + BPV4 BI	0	20/20	17/18	24/26	Flat
pSV2.0(+) (E7/E8)	0	16/16	23/24	13/14	Flat
pSV1.8(+) (E8)	0	0/3	0/1	1/3	NA
pSV3.9(+) (E2 - E5)	ND	ND	ND	13/20	Elongated, criss-cross

Legend: ND: not done

NA: not applicable

Focus- and macrocolony formation expressed per 5 X 10^5 cells.

Table 3.8b:Transforming activities of pSV2neo-derived clones of BPV4 in cooperation with the activated c-Ha-ras gene in Pal cells.

Plasmid DNA	Foci	Macrocolonies/ total colonies			Colony morphology
		Experiment			
		1	2	3	
pSV2neo + pT24	0	0/1	0/2	0/0	NA
pSV2.0(+) + pT24	0	20/23	16/18	17/19	2 colonies piling up; others flat
pSV1.8(+) + pT24	0	0/0	0/0	2/2	Criss-cross, but not piling up
pSV3.9(+) + pT24	ND	ND	ND	6/10	Various(see text)

Legend: ND: not done

NA: not applicable

Focus- and macrocolony formation expressed per 5 X 10^5 cells.

that of the parental cells, but further analysis was prevented due to fungal contamination of the nascent cell line.

In the neomycin resistance assays, novel observations were made which will be described in two parts (for comments on the control plates, see Section 3.4).

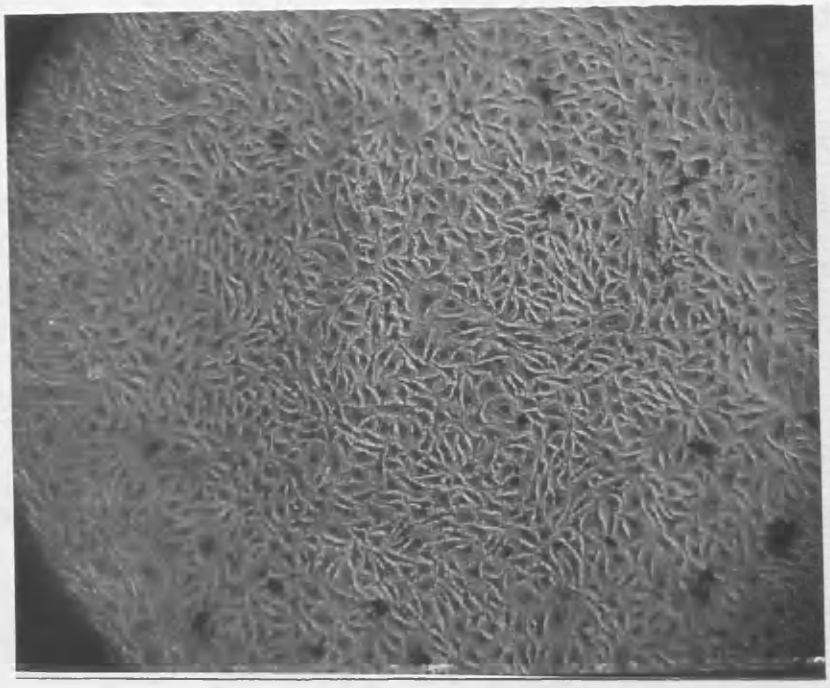
3.6(ii)A: Comparison of activity of pSV2.0(+) and pSV1.8(+)

Transfection of Pal cells with pSV2.0(+) led to macrocolony formation at a frequency of ≈ 20 per 5 x 10^5 cells, similar to those observed with complete, linear BPV4. Two representative colonies are shown in Figure 3.19a and -b. In two independent experiments, such a phenotype was absent when pSV1.8(+) was used: the results using this latter plasmid were identical to transfection with pSV2neo or cotransfection of pSV2neo with pT24. A more recent experiment, however, yielded a single colony of significant diameter (≈5mm) whose morphology appeared to be different from that of cells transfected with pSV2.0(+), in that the cells showed a more elongated criss-cross pattern, in contrast to the tight, more square-like shape of cells found in macrocolonies induced by BPV4 or pSV2.0(+). Attempts to expand this colony, however, were unsuccesful: presumably, therefore, continued growth of the cells also requires the presence of the E7 gene product.

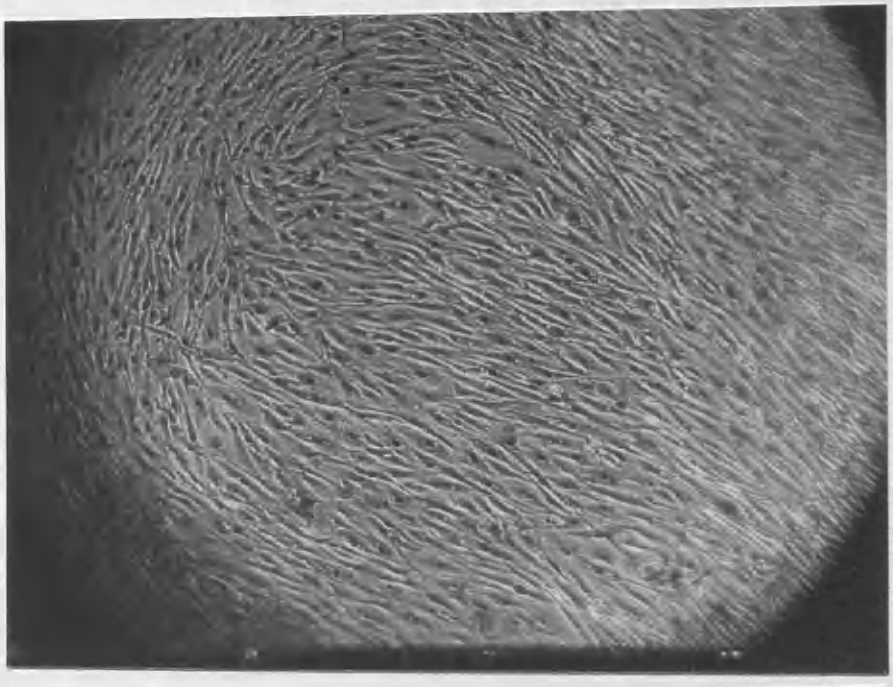
Comparison of these results indicated two things: firstly, the macrocolonies induced by the complete BPV4 genome could be reproduced by the region of the virus encoding the E7 and E8 ORFs; and secondly, the presence of the E7 ORF was required for the generation of macrocolonies: in the absence of functional E7, E8

Figure3.19: Neo^r colonies produced after transfection of Pal cells with pSV2.0(+)

a:



b:



Magnification: x60

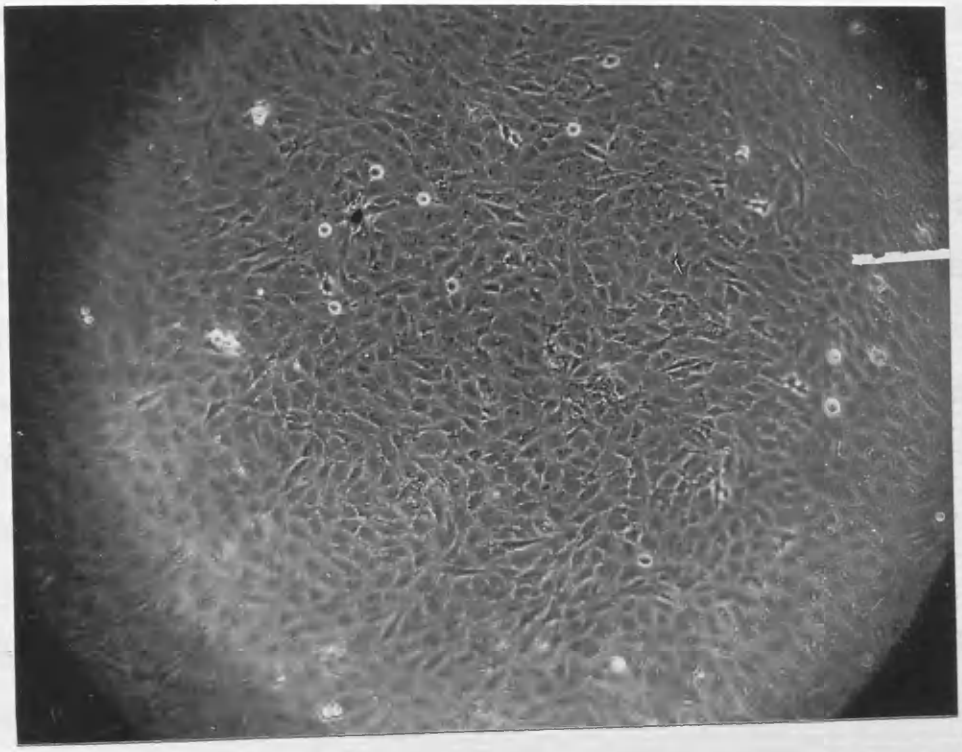
could not induce macrocolony formation. Also, since the macrocolonies induced by the complete BPV4 genome could not be induced by any other plasmid but pSV2.0(+), it can be predicted that expression of E7 is able to occur within the context of the full genome. This prediction could be tested by the reconstruction of a recombinant BPV4 genome lacking the 233bp (nts 906 - 1139) deleted in pSV1.8(+). Should such a linearised construct prove defective in macrocolony formation, the above hypothesis would be proven.

These data do not prove that the E7 ORF is sufficient for macrocolony formation: in the background of the pSV2neo vector, such a question could be addressed by making nonsense mutations within the E8 ORF and assaying the activity of the resulting recombinants for their ability to induce macrocolony formation. Cloning the E7 ORF alone into pSV2neo might not be useful, since it is presumed that E7 transcripts initiate near the as yet poorly mapped promoter 5' to the E8 ORF (TATAA box at nt 115); such E7 constructs might thus lack a promoter to drive expression in Pal cells.

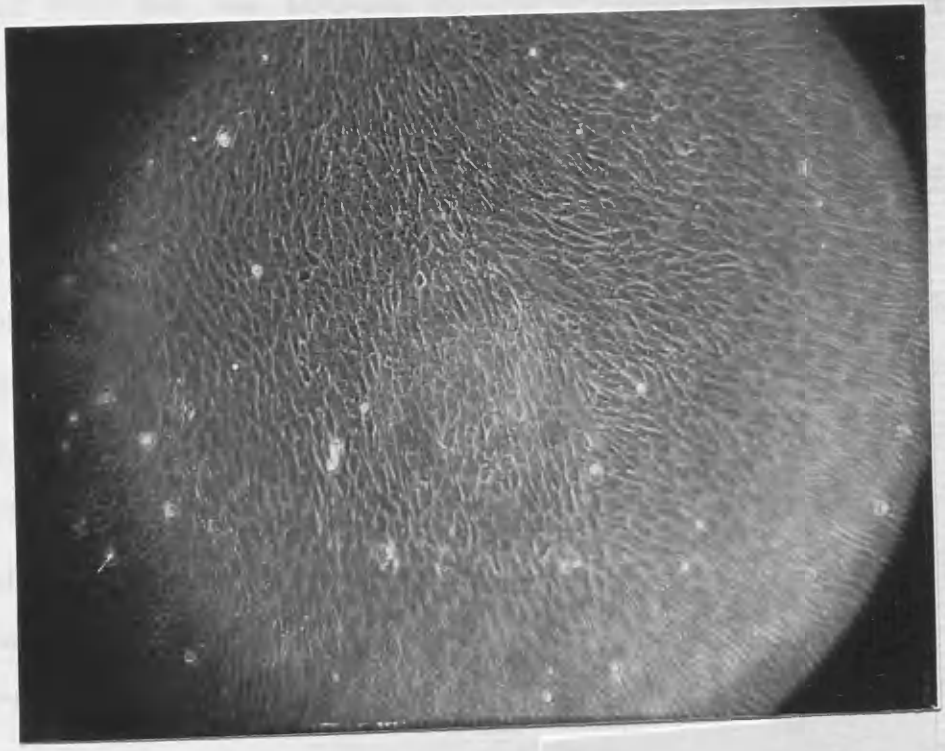
Upon cotransfection with the **ras** oncogene, a few further novel phenotypes became apparent. Cotransfection of pSV2.0(+) with pT24 led to large colonies at a frequency similar to that found for pSV2.0(+) alone (see Table 3.8b). Sometimes, however, these were of obviously higher cell density than the analogous colonies induced by pSV2.0(+) alone and occasionally the colonies displayed criss-cross patterns at their edge (see Figure 3.20a and -b). Two colonies showed cells obviously piling up highly, but the rarity of

Figure 3.20: Neo^r colonies produced after cotransfection of Pal cells with pSV2.0(+) and activated ras

a:



b:



Magnification: x90

such a finding (2 in over 100 colonies screened) indicated that a rare additional event had occurred rendering the cells less contact-inhibited and more overtly transformed than the majority of other colonies. The failure of **ras** alone to induce any measurable phenotype in neomycin resistance assays indicates that the primary requirement to elicit such measurable effects in this assay is that induced by the BPV4-encoded function. In the presence of such a function, however, the **ras** oncogene may enhance in subtle ways the effect with regard to the morphological phenotype of surviving colonies. The rarity of induction of overtly non-contact-inhibited morphology indicated that in these cases, rare secondary changes had occurred.

Macrocolonies resulting from cotransfection of pSV2.0(+) and activated **ras** were readily expanded into cell lines and two (those derived from the two colonies shown in Figure 3.20a and -b) were frozen down ≈ 25 population doublings after transfection.

Cotransfection of pSV1.8(+) with **ras** in two consecutive experiments led to no development of colonies at all at the 21 day stage. In the third experiment, 2 colonies of ≈5mm diameter were apparent, each showing the criss-cross morphology seen for the single pSV1.8(+) colony; no additional phenotype due to the **ras** oncogene could be seen and again, such colonies could not be expanded succesfully.

Comparison of these two experiments again indicates that the macrocolony formation observed in cotransfections of linear BPV4 and **ras** requires the presence of the E7 ORF. Furthermore, comparing the results obtained when cotransfecting pSV2.0(+) with **ras** with those of complete BPV4 genome and **ras** indicate that E7

gene expression in Pal cells is not overtly affected by the presence either of an activated **ras** gene or the remaining 5.2kb of viral genomic DNA.

3.6(ii)B: Activity of pSV3.9(+) in transfections of Pal cells

[The results in this section concern only a single experiment and and should thus be interpreted with care. The fact, however, that no background colonies have ever been seen in transfections of pSV2neo alone or upon cotransfection with an activated **ras** gene, would indicate that the effect is not artefactual.]

Transfection of Pal cells with pSV3.9(+) gave rise to ten colonies (equivalent to 20 colonies/5 x 10^5 cells) after 21 days of selection. Of these, three were appearing to be dying, three were small, flat and sparse (<5mm diameter) whereas four showed a criss-cross pattern and were large in size (8 - 10mm diameter). A representative colony is shown in Figure 3.21a.

This phenotype induced by the E2-E5 region plus part of the late region of BPV4 was not observed when the complete linear BPV4 genome was used upon screening of ≈ 100 colonies: this indicates that sufficient expression of whichever gene mediates this phenotype cannot normally be achieved after transfection of the full viral genome under the conditions used. This inhibition might be due to a virally- or cellularly-encoded transrepressor acting on an unknown viral promoter or enhancer sequence, or the lack of a required transactivator. Alternatively, the phenotype seen might be

the result of the expression of a single domain of a protein, due to the loss of exons normally expressed in intact viral DNA. Further analysis would be necessary to distinguish between the above possibilities.

Cotransfection of pSV3.9(+) with **ras** gave three obvious phenotypes: one was that of a small colony (<5mm diameter) of flat, sparse phenotype which was presumed to be senescing, another was that of large colonies (8 - 10mm diameter) similar to those of pSV2.0(+) + **ras**, of obviously high cell density, but not piling up overtly, whereas in a single colony, a very obvious piling up of criss-crossed cell (see Figure 3.21b) was observed.

All attempts to expand such colonies, including the one displaying piled-up cells, were unsuccesful, but from this single experiment, no firm conclusions can be drawn with regard to the extended lifespan of the surviving colonies.

What the mechanism of induction of the observed phenotypes induced by pSV3.9(+) might be is unclear, but alteration of cellular gene expression by transmodulation is an obvious possibility. The region certainly encodes putative DNA-binding proteins: the E2 ORF has been shown in several PVs to encode multiple transmodulators of viral (and possibly cellular-) gene expression (Spalholz *et al* (1987)) and the expression vector pSVE2B, which expresses the BPV4 E2 ORF from the SV40 early promoter, is able to activate the BPV4 NCR in transient reporter gene assays (Maria Jackson, Beatson Institute (pers. comm.)), while the E4 ORF of BPV4 has been predicted recently to contain a 'leucine zipper' motif (M.S. Campo (pers. comm.) similar to those found in several well-characterised

DNA-associated proteins (for review, see Abel and Maniatis (1989)).

Although no mapping of BPV4 promoters used in the generation of the full-length E2 gene product has been performed to date, the presence between nts 2686 and 2692 of a TATA box-like element (5'-TATTAAT-3') with two CCAAT box-like elements ≈ 30nts upstream (5'CCAAT-3' (between nts 2658 and 2662) and 5'-CAAAT-3' (between nts 2647 and 2651)) may indicate a site for transcript initiation of the full-length E2 protein mRNA within pSV3.9(+).

Two initiation sites for a putative E2 repressor-encoding mRNA have previously been mapped to nts 3069 and 3093 (Stamps and Campo (1988)). This message is expressed at very low levels in virally-induced papillomas, but could possibly, in the context of pSV3.9(+), allow macrocolony formation by some unknown means.

The BPV4 E4 ORF is predicted from transcriptional analysis to be expressed as an E1-E4 fusion protein in papillomas and the 1kb transcript which encodes this protein is the major BPV4-encoded message found in such lesions (Stamps and Campo (1988)). As yet, nothing is known about the role of the short leader peptide derived from the E1 ORF in the functioning of this protein, so were the E4 ORF to be transcribed from pSV3.9(+), it would not be possible to exclude possible artefacts due to the absence of this leader peptide. The fusion protein is presumed to subvert the keratin expression programme within normal keratinocytes, possibly via an interaction with cytokeratin promoters. Since in fibroblasts keratins are not expressed, presumably alterations in expression of other genes would have to be postulated to explain any action of the E4 protein in such a scheme. Obvious candidates are the other cytoskeletal

elements, since the cellular morphology of Pal cells transfected with pSV3.9(+) is clearly different from those transfected with pSV2.0(+) or BPV4.

The overtly transformed appearance of the single colony induced by pSV3.9(+) and **ras** was a completely novel phenotype: in repeated cotransfections of linear BPV4 + **ras**, no such cells were ever seen in ≈ 100 colonies screened. This is further evidence that the functions expressed in the context of the pSV3.9(+) plasmid are not expressed in the context of the full viral genome in Pal cells. No direct comparison with other papillomaviruses is possible in this case, however, since no phenotypes attributable to the E2-E5 region of other PV genomes have been reported in primary cells. A weak transforming activity of the E2-E5 region of HPV16 has, however, been reported in NIH3T3 cells (Vousden *et al* .(1989)). One analogy which can be drawn is the transfection of an activated **ras** gene into SV40-transformed human fibroblasts: morphologically transformed foci developed but resulting cell lines senesced more rapidly than non-transfected controls (O'Brien *et al* . (1986)). The apparently transformed colony induced by pSV3.9(+) and **ras** could not be expanded: it is possible therefore that BPV4 encodes a second transforming function, which does <u>not</u> efficiently prolong the lifespan of bovine cell *in vitro* .

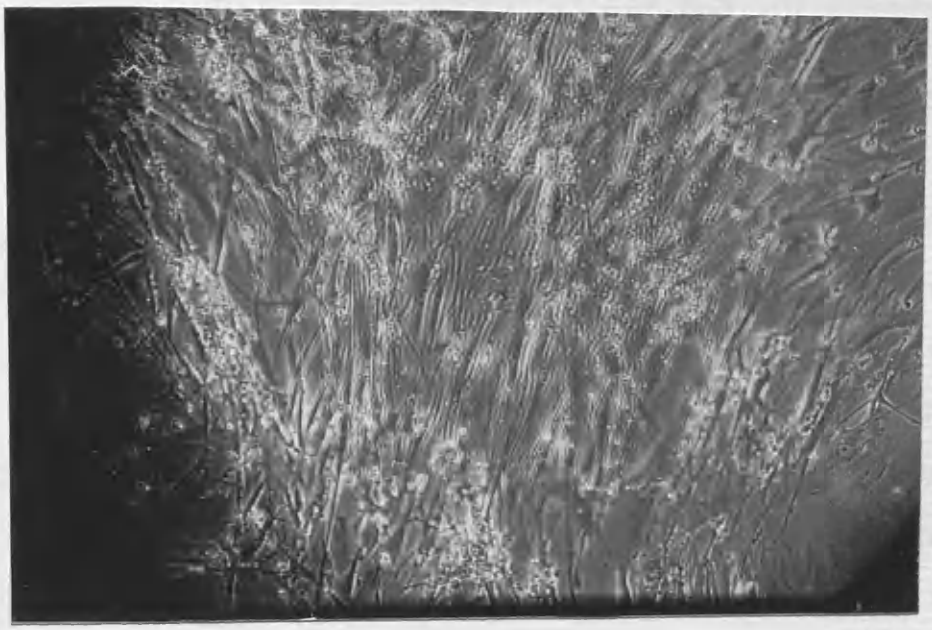
A possible explanation of these facts may be put forward using analogies with recent results obtained with HPV16 (T. Crook (pers. comm.)). Using primary Baby Mouse Kidney (BMK) epithelial cells, it was found that HPV16 encoded a **myc**-like function which, when overexpressed, could cooperate with an activated **ras** gene in the tumorigenic transformation of the recipient cells. Cotransfection of

such overexpressing HPV16 constructs with an activated **myc** gene, however, led to no observable phenotype. However, cotransfection of the linear viral genome (which does not express its products at high levels within cells) with an activated **myc** gene did lead to tumorigenic transformation. If one were to postulate that the 3.9kb XhoII fragment of BPV4 contained a **ras**-like function, then one could predict that cotransfection of two **ras**-like functions could lead to the piled-up phenotype observed upon cotransfection of pSV3.9(+) and **ras**. The lack of a **myc**-like function capable of extending the life-span of the cells *in vitro* could explain the failure to expand such colonies into cell lines.

If the hypothesis that the 3.9kb fragment encodes a **ras**-like function is correct, then overexpression of such a gene should induce focus formation in NIH3T3 cells. Lack of time prevented such experiments being performed. Clearly, such theories would need to be tested using a larger body of data than that which was obtained in this study. If this is indeed the case, identification of the ORF responsible for inducing such a phenotype could be pursued by engineering specific STOP codons into individual ORFs and assaying the resultant plasmids in Pal cells for colony formation.

Figure 3.21: Neo^r colonies produced after transfection of Pal cells with pSV3.9(+) in the absence- or presence of activated ras

a: pSV3.9(+)



b: pSV3.9+) + **ras**

Magnification: x90

3.7: Analysis of pZIPneoSV(X1)-derived clones in transfection assays

All transfections were done using identical protocols to those described in 3.6 and these will thus not be repeated again here.

3.7(i): Transformation of C127- and NIH3T3 cells by pZIPneoSV(X1)-derived clones

The results of this transfection are shown in Table 3.9a: once again, no foci could be induced after transfection with any of the constructs, but the results from the flasks which underwent selection with neomycin showed again that the transfection procedure was working efficiently. However, pZIP3.9(+) gave no neo^r colonies at all. This was also shown to be the case in NIH3T3 cells (see Table 3.9b), indicating that it was not a cell-line-specific effect. Two possible reasons were considered: firstly, that expression of the **neo** gene was inefficient or absent: this could be due to a small alteration in the DNA sequence of the plasmid undetectable by the restriction mapping used in the identification of the recombinant, or due to some inhibitory effect of the 3.9kb sequence of DNA. The possibility of this latter occurrence, although rare, was reported by the authors who originally described the pZIPneoSV(X1) plasmid (Cepko *et al* . (1984)); the second possibility considered was that overexpression of some gene in the 3.9kb fragment was lethal to cell growth *in vitro* .

Table 3.9a: Transfection of C127 cells with pZIPneo-SV(X1)- derived clones.

Plasmid DNA	Foci	Neo colonies	% transformed
pZIPneoSV(X1)	0	81	0
pZIP2.0(+) (E7/E8)	0	123	0
pZIP1.8(+) (E8)	0	68	0
pZIP3.9(+) (E2-E5)	0	0	-

Table3.9b:Transfection of NIH3T3 cells with pZIPneoSV-(X1)- derived clones.

Plasmid DNA	Foci	Neo colonies	% transformed
pZIPneoSV(X1)	-	73	0
pZIP2.0(+) (E7/E8)	-	90	0
pZIP1.8(+) (E8)	-	33	0
pZIP3.9(+) (E2-E5)	-	0	-

Legend: Neo^r colonies are expressed per 2×10^5 cells

Since it was found that the pSV3.9(+) clone was able to induce measurable phenotypes in Pal cells using a neo^r assay, it was decided to try to distinguish between these two possibilities by cotransfecting pSV2neo and pZIP3.9(+) into Pal cells; it was known that pSV2neo alone was unable to induce colonies in Pal cells (see Section 3.4), and from the lack of neo^r colony formation after transfection of either C127- or NIH3T3 cells with pZIP3.9(+), it was predicted that pZIP3.9(+) would also be unable to induce neo^r colony formation in Pal cells. It was thus reasoned that if any neo^r colonies were induced after cotransfection of both plasmids, it would have to be assumed that pZIP3.9(+) was inducing the phenotype, and could not therefore be lethal to cells. The results of such a transfection experiment are shown in Table 3.10.

The formation of 13 colonies/5 x 10^5 cells upon cotransfection of pSV2neo and pZIP3.9(+) indicated that the reason for the lack of neo^r colony formation after transfection of pZIP3.9(+) into C127- or NIH3T3 cells was not due to lethality of the pZIP3.9(+) construct, but rather to the lack of **neo** gene expression in this construct. Two representative colonies are shown in Figure 3.22.

Once again, since transfections into established cell lines was relatively efficient, the analagous experiments in Pal cells were carried out.

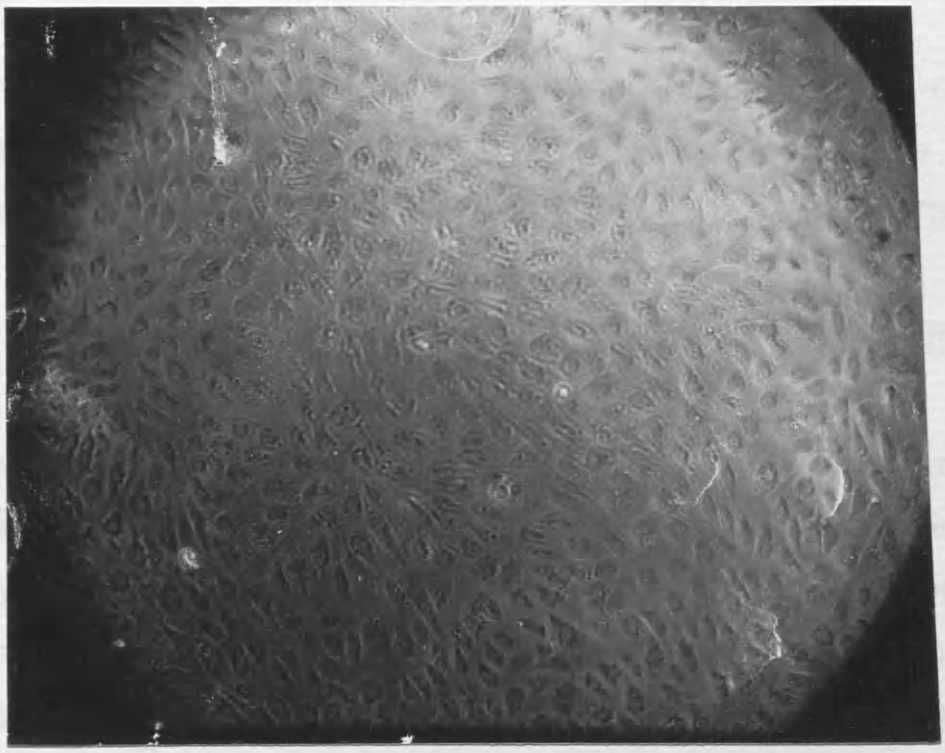
Table 3.10: Transfection of Pal cells with pSV2neo and/or pZIP3.9(+).

Plasmid DNA	Foci	Neo colonies	% transformed
pSV2neo	-	0	-
pZIP3.9(+) (E2-E5)	-	0	-
pSV2neo + pZIP3.9(+)	-	13	0

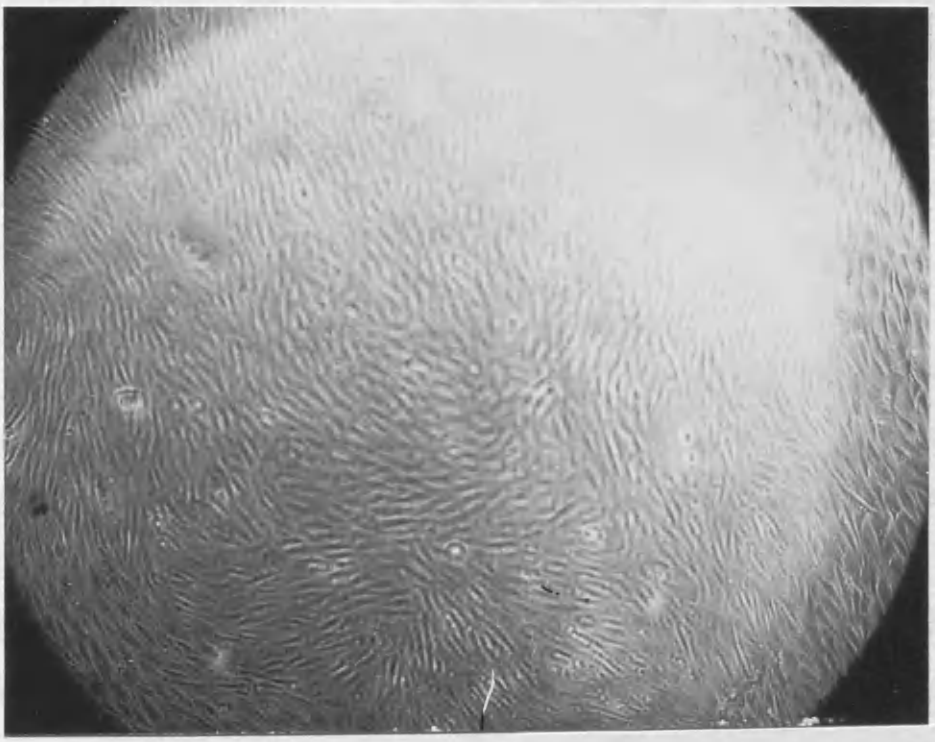
Legend: Neo^r colonies are expressed per 5 X 10^5 cells

Figure 3.22: Neo^r-colonies produced after transfection of Pal cells with pSV2neo and pZIP3.9(+).

a:



b:



Magnification: x90

3.7(ii): Transformation of Pal cells by pZIPneoSV(X1)-derived clones

The results of transfection of the various pZIPneoSV-(X1)-derived clones into Pal cells are shown in Table 3.11a.

The results of cotransfections of the pZIPneoSV(X1)-derived clones with the activated **ras** genes are shown in Table 3.11b: discussion of the results will again be dealt with in two parts.

3.7(ii)A: Comparison of the activities of pZIP2.0(+) and pZIP1.8(+)

Once again, as in the case of the pSV2neo-derived clones, the presence of the E7 ORF was required for colony formation in neo^r assays. Whilst pZIP2.0(+) induced colonies at a frequency of >100 colonies/ 5 x 10^5 cells, pZIP1.8(+) was unable to induce any colonies. Similar to the case of pSV2.0(+), all colonies were flat, with morphologies identical to those shown for pSV2.0(+) in Figure 3.19. The frequency of colony formation induced by pZIP2.0(+) was 3-6-fold greater than with either pSV2.0(+) or linear BPV4 genomic DNA, and may reflect more efficient expression of BPV4 ORFs in the retroviral vector environment.

Upon cotransfection of pZIP2.0(+) with **ras**, ≈ 60% of neo^r colonies were morphologically transformed., Some representative colonies, stained with Giemsa are shown in Figure 3.23. Also focus formation could be induced when cotransfecting pZIP2.0(+) and pT24 at a frequency ≈ 3-fold less efficient than that of neo^r colony formation. Representative foci are shown in Figure 3.24. No such foci were obtained in the pZIPneoSV(X1) + **ras** control, indicating

Table 3.11a:Transforming activities of pZIPneoSV(X1)-derived clones of BPV4 in Pal cells.

DNA added	Foci		Neo colonies		% transformed
	Experiment		Experiment		
	1	2	1	2	
pZIPneoSV(X1)	0	0	0	0	NA
pZIPneoSV(x1) + ras	0	1	0	0	NA
pZIP2.0(+)	0	0	>100	>100	0
pZIP1.8(+)	0	0	0	0	NA
pZIP3.9(+)	0	0	0	0	NA

Legend: NA: not applicable

ND: not done

Foci and neo^r colonies expressed per 5 X 10^5 cells.

Table 3.11b: Transforming activities of pZIPneoSV(X1)-derived clones of BPV4 in cooperation with the activated c-Ha-ras gene in Pal cells.

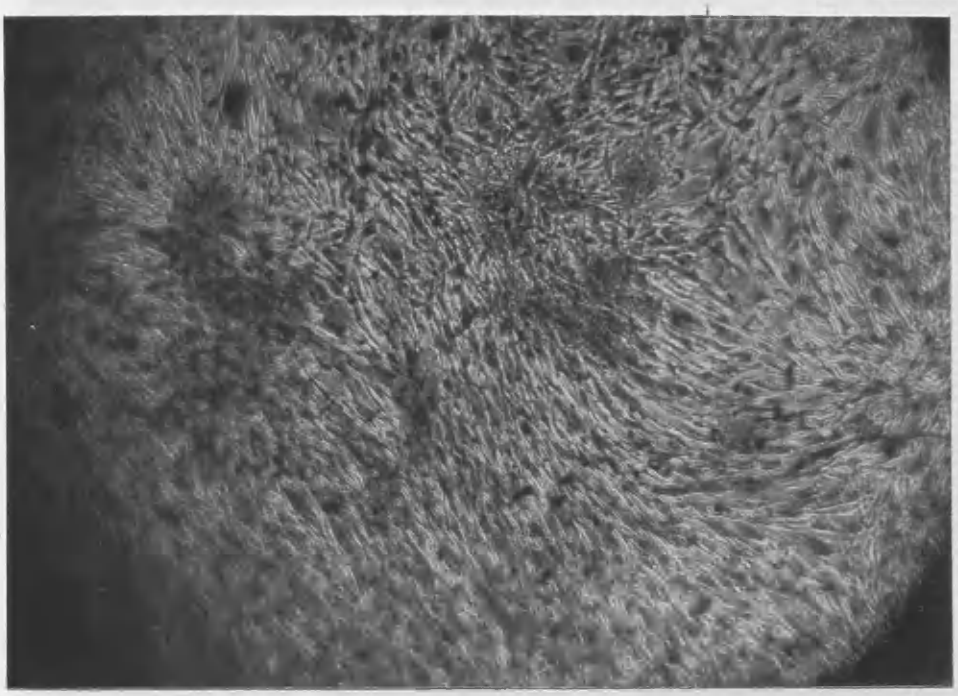
DNA added	Foci		Neo colonies		% transformed
	Experiment		Experiment		
	1	2	1	2	
pZIP2.0(+) + ras	18	6	>100	74	60
pZIP1.8(+) + ras	0	0	0	0	NA
pZIP3.9(+) + ras	ND	0	ND	0	NA

Legend: NA: not applicable
ND: not done

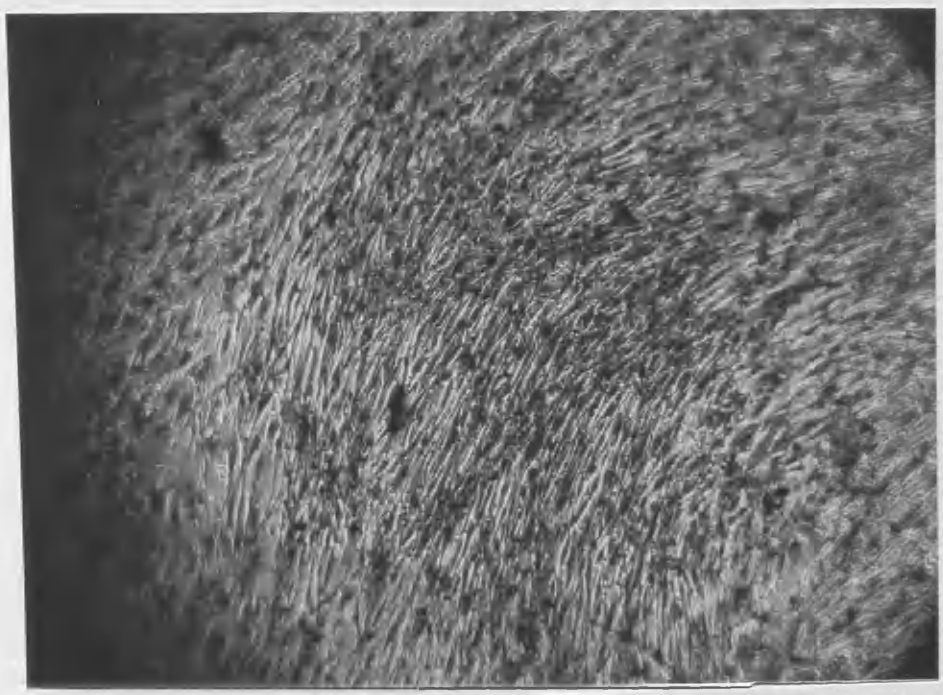
Foci and neo^r colonies expressed per 5 X 10^5 cells.

Figure 3.23: Neo^r colonies produced after transfection of Pal cells with pZIP2.0(+) and activated ras

a:



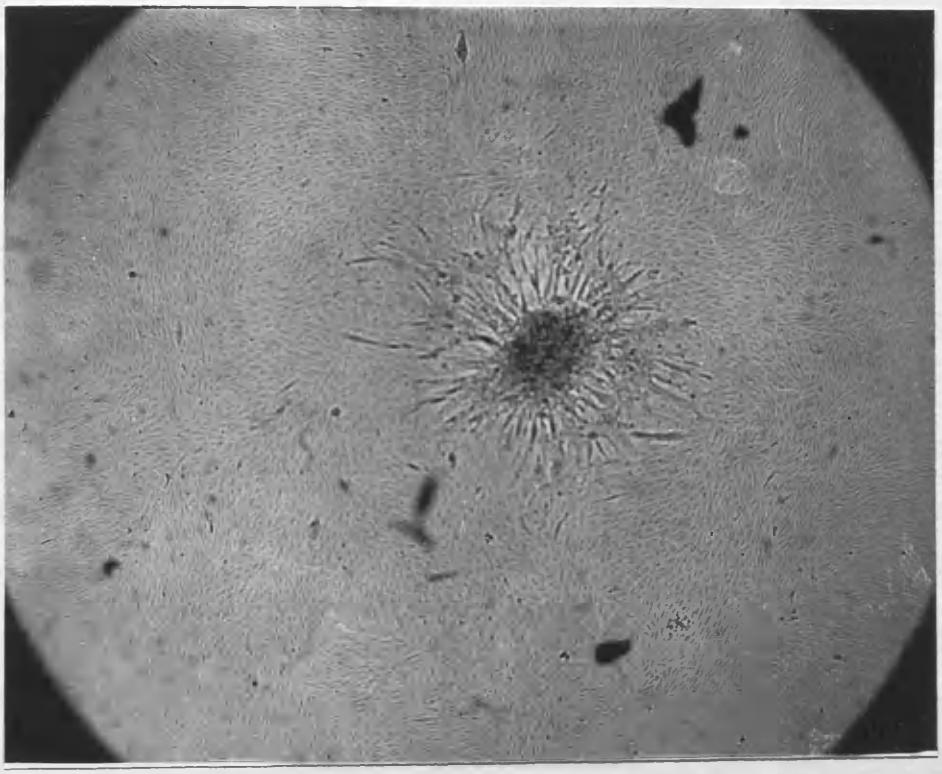
b:



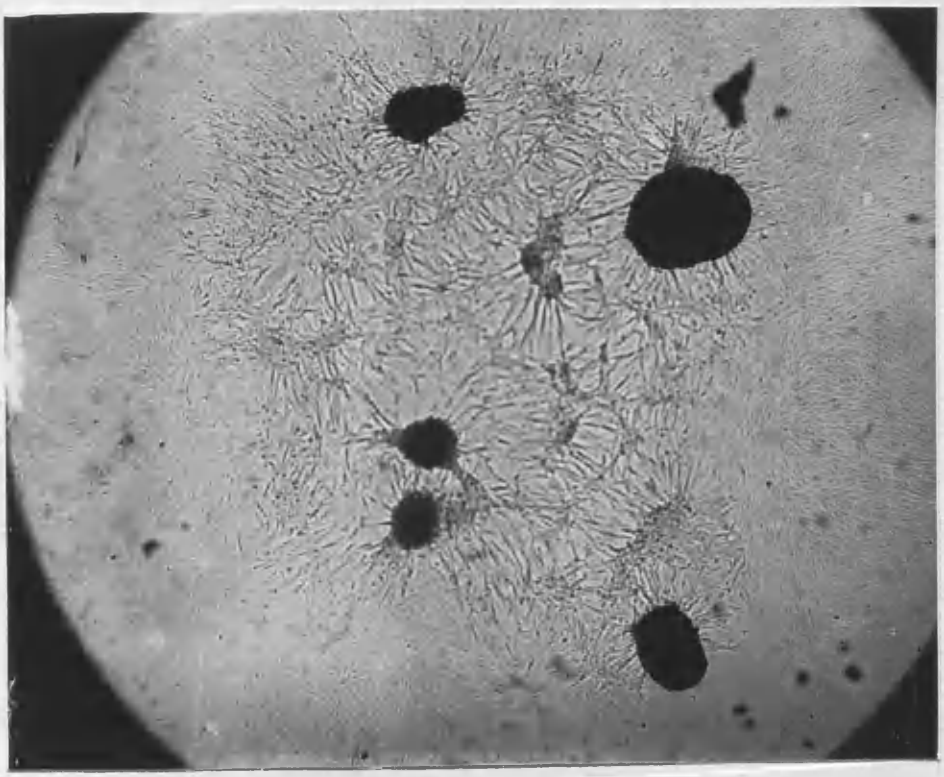
Magnification: x60

Figure 3.24: Foci induced after transfection of Pal cells with pZIP2.0(+) and activated ras

a:



b:



Magnification: x30

that it was unlikely that such foci arose from recombination events leading to overexpression of activated **ras** genes from a retroviral LTR. Furthermore, none were induced after cotransfection of pZIP1.8(+) with **ras**, which implicates a requirement for the presence of the E7 ORF for focus formation. In this case, indeed, it was noted that in both transfections, the cell died faster than cells transfected simply with carrier DNA.

Comparing the activities of pZIP2.0(+) and pSV2.0(+) upon cotransfection with **ras** into Pal cells (from the Results in Section 3.7) implies that somehow the retroviral vector environment of the 2.0kb fragment in pZIP2.0(+) enhances the transforming activity of this region of BPV4. The most likely explanation to be put forward is that the presence of retroviral LTRs leads to more efficient expression of E7 and/or E8 and that this, in the presence of an activated **ras** gene, is sufficient to drive the cells to a non-contact-inhibited phenotype. Similar results have been obtained in cotransfections of HPV16 and **ras** (Matlashewski *et al* . (1987b)), with the major difference that such experiments were performed in epithelial cells rather than fibroblasts.

3.7(ii)B: Activity of pZIP3.9(+)

As expected, no colonies were obtained when transfecting the pZIP3.9(+) construct. Also, in the analagous selections for focus formation, no transformed foci were ever observed. In hindsight, selection with neomycin after cotransfection of pZIP3.9(+) + **ras** was superfluous, since it had by then been shown that no **neo** gene expression was possible from pZIP3.9(+). To assess any possible

phenotypes attributable to pZIP3.9(+) in the context of a neomycin resistance assay, pZIP3.9(+) and pT24 could be cotransfected with pSV2neo, as previously described for pSV2neo and pZIP3.9(+) alone. Due to lack of time, however, no data on this were obtained.

3.8: The BPV4 E7 ORF: its possible functions in cells grown *in vitro*

The results described in 3.6(ii)A and 3.7(ii)A indicated that the BPV4 E7 ORF was required for Pal cell macrocolony formation. Similar results have been obtained using HPV16 in human fibroblast cells *in vitro* (Watanabe *et al* . (1989)). In the case of HPV16, the E7 gene product has been shown to bind to the 105kDa product of the tumour suppressor gene Rb1 (Dyson *et al* .(1988)). It was thus of interest to analyse the putative amino acid sequence of the BPV4 E7 gene product to determine whether this protein might have a similar function. A computer alignment of the two putative amino acid sequences (starting at the first methionine within the respective ORFs)using the ALIGN function of 'MicroGenie' (Queen and Korn (1984)) revealed three well-conserved motifs, as shown in Figure 3.25:

Firstly, a pair of cys-x-xcys motifs towards the C-terminal region. These have been proposed to be 'zinc fingers', and mutation of such cys residues in HPV16 E7 expression vectors leads to loss of transforming activity in NIH3T3 cells (Edmonds and Vousden(1989)).

Secondly, a region of 5 amino acids, four of which are negatively charged glutamic acid residues.

Thirdly, a series of conserved hydrophobic residues (mainly leucine, but also isoleucine and valine) found in the C-terminal third of the putative protein sequences.

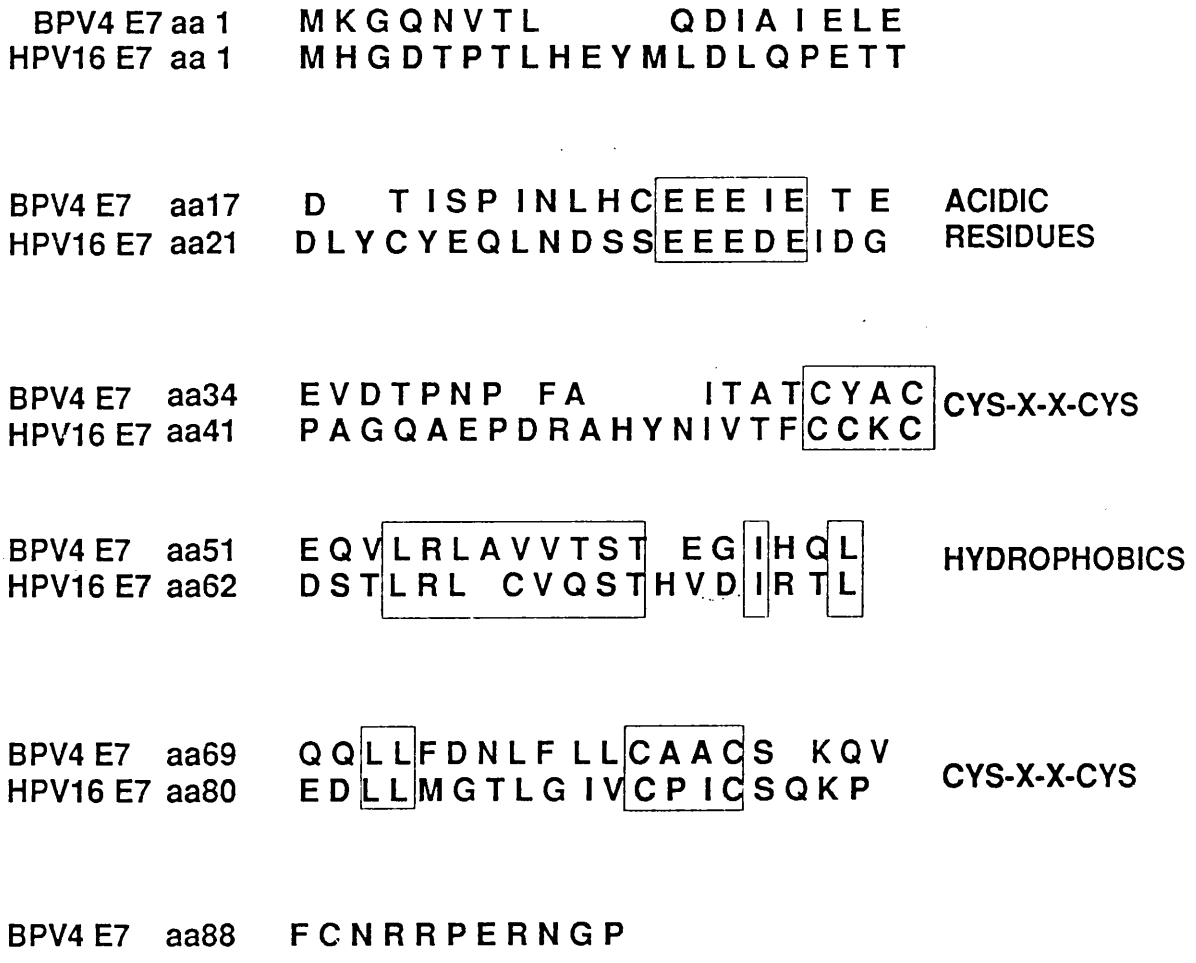
What of the regions of HPV16 E7 predicted to be required for Rb1 binding? In the region of HPV16 (amino acids 1-26) concerned, BPV4 E7 showed little homology using this programme with the proteins lined up from the first methionine. By visual inspection, however, it was possible to identify within the BPV4 E7 sequence the two groups of residues absolutely conserved between HPV16 E7, adenovirus E1a and Sv40 large T antigen, all of which have been shown to bind to $pp105^{Rb}$ *in vitro* . These are shown in Figure 3.26. In the case of adenovirus E1a, the regions denoted as 1 and 2 have also been shown to be <u>required</u> for Rb binding.

Two points should however be made. Firstly, region 1 and region 2 (the two domains required for the Rb binding function of E1a) are not separated at all in BPV4 E7, whereas in the other 3 proteins the two are separated by at least 6 amino acids. Secondly, region 2, namely leu-x-cys-x-glu, is found completely separated from the run of acidic residues in HPV16, adenovirus E1a and the large T antigen of SV40, whereas in BPV4, the two motifs merge with one another.

Neverthelesss, it is likely, on the basis of this analysis, that the <u>structure</u> of the BPV4 E7 protein shares similar domains to those of HPV16 E7, adenovirus E1a and the large T antigen of SV40 and it may therefore be predicted that one of the functions of the protein

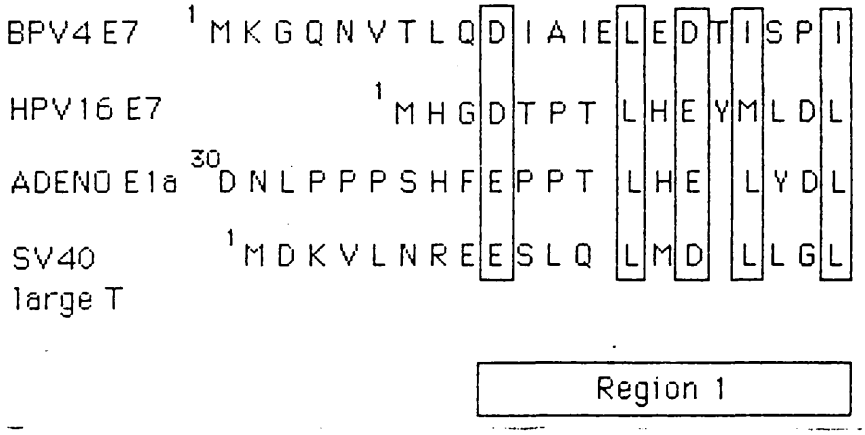
is an interaction with the prouct of (a) bovine tumour suppressor gene(s). With the development of both prokaryotic- and eukaryotic expression vectors for the E7 ORF as well as anti-E7 antibodies (G.J. Grindlay, Beatson Institute (pers. comm.)), it should in future be possible to analyse any protein-binding activities of the BPV4 E7 protein using immunoprecipitation assays, just has been done for the E7 proteins of the oncogenic HPVs (Dyson *et al*. (1989)).

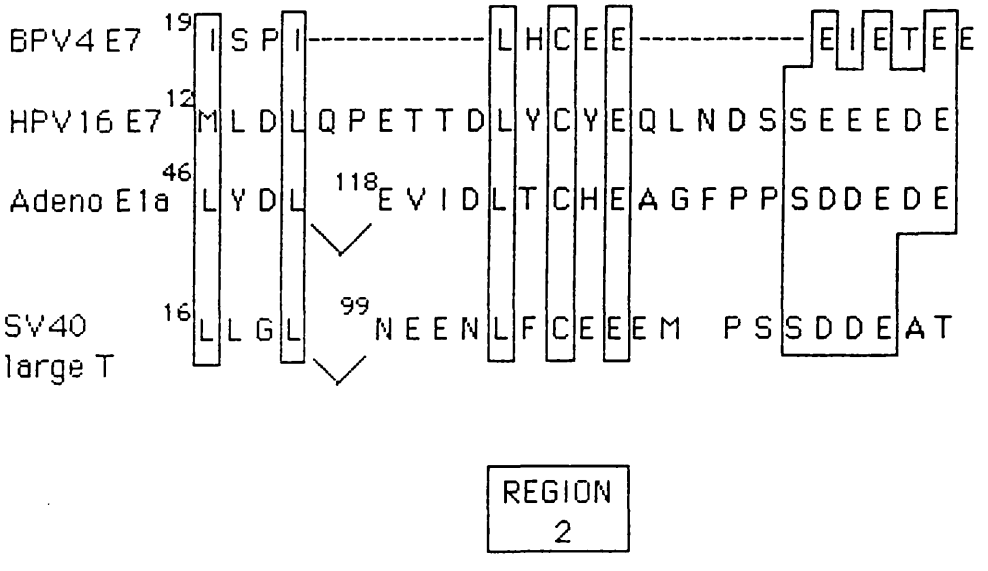
Figure 3.25: Amino acid sequence homology between the E7 ORFs of BPV4 and HPV16.



The alignment of amino acid sequences was performed using the ALIGN function of 'Microgenie' (Queen and Korn (1984)).

Figure 3.26: Homology of BPV4 E7 to the Rb binding domains of various DNA tumour virus oncogenes.





3.9 Screening of bovine UAC tumours for altered EGF-receptor gene activity

As described in Section 1.11.2, preliminary experiments using the cell line 88529C (derived from a carcinoma of the UAC) indicated very high levels of EGF binding to the cell surface of these cells. In order to analyse the reasons for this, it was thus desirable to analyse the nucleic acid levels of tumours for evidence of any gene amplification.

3.9.1 Identification of a suitable EGF-receptor probe

Since no bovine EGF-receptor probe was available, it was necessary to identify a suitable heterologous probe for the analysis. The murine genomic probe pME2.0 (generously provided by M. Hung and described in Hung *et al* .(1986)), as shown in Figure 3.27, was found to be of use in the analysis of bovine DNA.

20µg of calf thymus DNA was digested with either BamHI or EcoRI, separated on a 0.8% agarose gel, transferred to nylon membrane and hybridised under stringent conditions to radiolabelled 2.5kb EcoRI insert purified from pME2.0. As a positive genomic DNA control, NIH3T3 cell DNA was used. In addition, 25pg and 50pg of pure plasmid DNA (equivalent to 1 and 2 genome equivalents) were also used. The result is shown in Figure 3.28.

The strong hybridisation to the bovine DNA (lanes 6 and 7) indicated that the probe was indeed of use as a cross-species probe. The fact that the hybridising fragment was also similar to that of

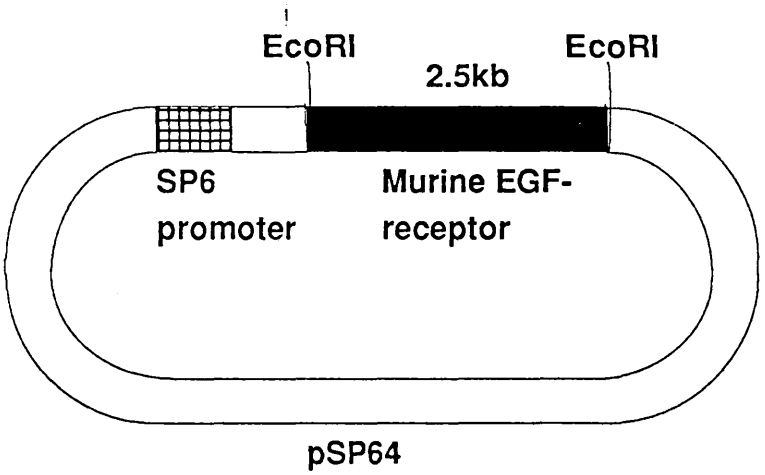
mouse was a further confirmation of the evolutionary conservation of this region of the EGF receptor gene. The additional weak hybridising bands may be other related genes, possibly other members of the family of transmembrane tyrosine kinases, of which the EGF receptor is perhaps the best characterised member at present.

3.9.2 Use of pME2.0 to screen bovine UAC tumours for EGF receptor gene amplification.

Since pME2.0 appeared to be capable of hybridisation to bovine sequences, tumour DNAs were screened for any evidence of amplification. 34 DNAs were screened in total, but no evidence for gene amplification could be established. A typical Southern blot tumour DNA is shown in Figure 3.29.

The screening of 88529C cell line DNA could not be done as the line was lost due to freezer malfunction, and thus the role of gene amplification in the high level of EGF binding remains unclear.

Figure 3.27: Structure of the plasmid pME2.0

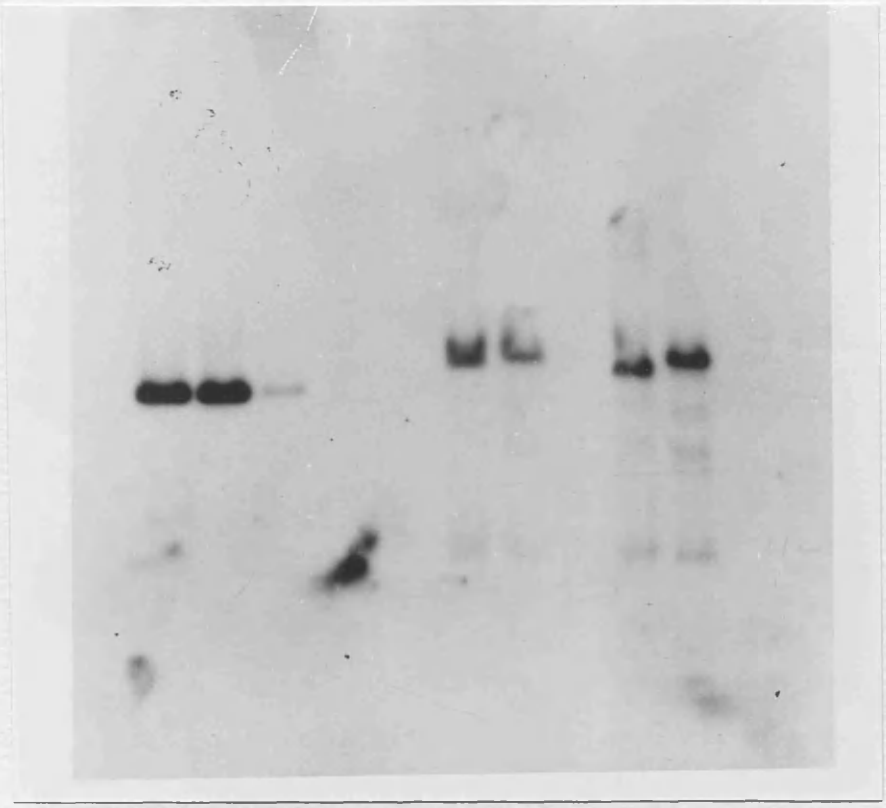


Legend: SP6 promoter: The DNA sequence recognised by the RNA polymerase encoded by the bacteriophage SP6 directing the initiation site for mRNA transcription.

The plasmid was kindly donated by M. Hung and was originally described in Hung *et al* . (1986), where its usefulness as a cross-species probe was first reported.

Figure 3.28: Cross-hybridisation of pME2.0 to bovine DNA

20µg of NIH3T3 (murine) or calf thymus (bovine) DNA were digested with EcoRI or BamHI, separated on a 0.8% agarose gel, transferred to Pall membrane and hybridised O/N under stringent conditions to the 2.5kb insert (labelled to a specific activity of 2 x 10^8 dpm/µg). 0.4, 1 and 2 genome equivalents of purified plasmid DNA was run as a positive control. The membrane was washed under stringent conditions (0.1 X SSC, 0.5% SDS at 65°C for 1hr) and exposed to X-Ray film for 11 days.



Lane: 1 2 3 4 5 6 7

Lanes: 1-3, 4, 6: digested with EcoRI

5, 7: digested with BamHI

1-3: pME2.0 (50pg, 25pg and 10pg)

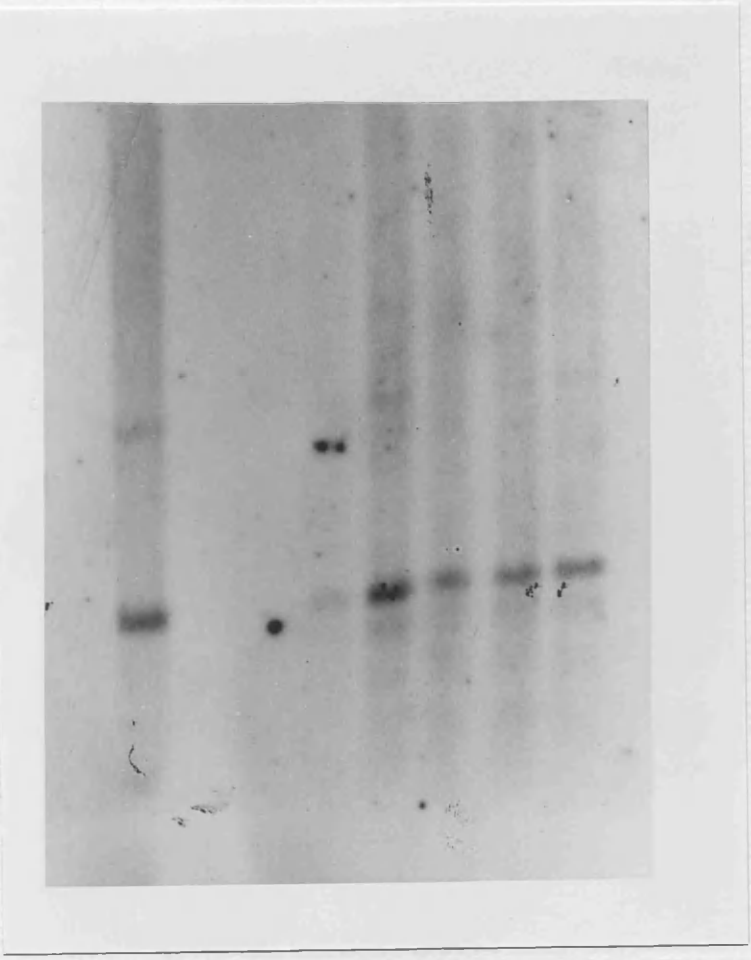
6,7 : NIH3T3

: calf thymus

4,5 :

Figure 3.29: Hybridisation of pME2.0 to bovine UAC tumour DNA

20μg of calf thymus and various tumour DNAs were digested with EcoRI, separated on a 0.8% agarose gel, transferred to Pall membrane and hybridised O/N under stringent conditions to the 2.5kb insert of pME2.0 (labelled to a specific activity of 1.6 X 10^8 dpm/μg). The membrane was washed under stringent conditions (0.1 X SSC, 0.5% SDS at 65°C for 1hr) and exposed to X-ray film for 11 days.



Lanes: 1 2 3 4 5 6 7 8

Lanes: 1: Calf thymus

2-8: UAC tumours

SECTION 4

SUMMARY AND CONCLUSIONS

4. Summary and conclusions

The broad aims at the beginning of this study were to develop a bovine *in vitro* system for the analysis of BPV4-mediated alterations in cellular proliferation, the identification of the viral gene products mediating those alterations and to analyse the means by which such changes were brought about.

A tissue culture system utilising non-established bovine fibroblasts derived from the palate of a bovine foetus has been developed. Using coselection with the dominant selectable marker **neo**, an assay system sensitive to BPV4-encoded functions has been devised and used to identify two regions of the genome capable of inducing measurable phenotypes. These regions were shown to cooperate with the activated **ras** gene in the induction of more transformed phenotypes, but the system appeared to differ from the primary rodent fibroblast systems previously described in the literature, in that cotransfection of activated **ras** and **myc** oncogenes did not appear to induce efficient focus formation.

The analysis of EGF-receptor genes in BPV4-associated tumorigenesis was limited to the identification of suitable non-homologous probes and the screening of tumour DNA, which indicated that amplification of the gene did not take place in the thirty samples screened and is not therefore a frequent event.

The results obtained will now be discussed as a whole and placed into context in the overall view concerning BPV4-associated carcinogenesis; finally, suggestions will be made for future work.

4.1 Bovine fibroblasts as a tissue culture system for the analysis of BPV4 functions

A new assay system for the analysis of BPV4-encoded transforming functions has been developed. The cells used, namely non-established bovine fibroblasts derived from the palate of a bovine foetus, display two distinct advantages as compared with the previously used established murine fibroblast cell lines C127 and NIH3T3: firstly, the cells are derived from the species which the virus infects; and secondly, the cells are not converted to a tumorigenic phenotype after transfection with BPV4, just as *in vivo* , BPV4 infection does not lead directly to carcinomatous development. The cells are not, however, of epithelial origin, and thus wil not ultimately be the best model system for the study of BPV4 *in vitro* .

The BPV4-bovine fibroblast system shows many similarities to analogous systems involving HPV16 and human embryonic fibroblasts (Pirisi *et al* . (1987)) and in future years, comparisons of this system with epithelial cell systems may lead to a greater understanding of the tissue specificity of BPV4 action.

The bovine cells appear to display differing susceptibility to conversion to a non-contact-inhibited phenotype when compared to their rodent counterparts: cotransfection of activated **ras** and **myc** oncogenes does not lead to stable morphological transformation. This correlates with the increased latency period of malignant progression observed in cattle as compared to mice and hamsters - in the UAC, carcinomas take a minimum of seven years to develop, a time greater than the complete lifespan of an average mouse. Whilst

this difference makes for more subtle changes in morphological phenotype, in time these systems may be of much use in the analysis of the events occurring in human tumour progression, the ultimate aim of all cancer research.

4.2 Identification of transforming activity of BPV4 *in vitro*

The use of a neomycin resistance coselection assay (see Section 3.4) showed that BPV4, but not an activated **ras** oncogene, is able to prolong the lifespan of Pal cell *in vitro* . The formation of large macrocolonies of > 5mm diameter after 3 weeks selection in G418 was only observable when BPV4 DNA was present in the transfection. The fact that results were essentially identical upon cotransfection with an activated **ras** gene indicated that expression of the BPV4 transforming genes was unaffected by the presence of such an activated oncogene. Furthermore, bovine cells were apparently not susceptible to morphological transformation to a non-contact-inhibited phenotype after transfection of papillomaviral- and activated **ras** DNAs within the selection period described. Such results are similar to those obtained by Matlashewski *et al* . (1988) upon transfection of HPV16 and activated **ras** into human cervical fibroblasts.

As yet, no reports exist of direct cotransfection of PV DNA and activated **ras** into human epithelial cells, so the relative susceptibilities of the two human cell types to PV-associated transformation cannot be assessed. Nevertheless, in rodent systems, both fibroblastic (Chester and McCance (1989)) and epithelial cells (Matlashewski *et al* (1987b)) appear to be equally

susceptible to conversion to a non-contact-inhibited phenotype using such a transfection protocol.

4.3 Identification of the E7 ORF as a transforming function of BPV4

Using this same neomycin resistance assay, subclones of BPV4 DNA were assayed for their activity in the prolonging of Pal cell growth *in vitro* . The activity induced by BPV4 could be reproduced by a 2.0kb XhoII fragment (nts 6487 - 1275), which contains the 3' end of L1, the NCR and the complete E8 and E7 ORFs, when cloned into either pSV2neo or pZIPneoSV(X1). This activity was lost in analagous constructs lacking a 233bp sequence (nts 906 - 1139), which caused an interruption in the E7 ORF. This implied that the E8 ORF alone was unable to induce macrocolony formation, but that the presence of both the E7 and E8 ORFs was sufficient. The E7 ORF of BPV4 was shown to contain motifs within its amino acid sequence identical to those present within the adenovirus E1a protein which are responsible for binding to the product of the tumour suppressor gene Rb1. Such motifs are highly conserved within the E7 ORFs of the oncogenic HPVs. It could be predicted therefore that the binding of E7 to an unknown bovine tumour suppressor gene product may well be necessary for the prolonging of Pal cell growth *in vitro* .

The lack of activity in this assay of the clones containing only the E8 ORF indicate that any transforming activity of this protein is masked by the presumed senescence of Pal cells in the absence of E7. Since the E8 protein has been predicted to show structural homology with the BPV1 E5 ORF, which appears to sensitise cells to

growth factors, it may be the case that the E8 gene product may also contribute to BPV4-mediated hyperproliferation *in vivo* . Novel assays will have to be developed in order to study this further.

4.4 A novel transforming function of BPV4 within the E2-E5 region

The colony-forming activity of pSV3.9(+) or the combination of pSV2neo and pZIP3.9(+) in neomycin resistance assays after transfection into Pal cells indicated that a second activity capable of prolonging the lifespan of Pal cells upon selection in medium containing neomycin was present within the BPV4 genome. Such cells, in a single experiment, were incapable of being further expanded. The ORF(s) responsible for this phenotype have not been identified.

The characterisitics of this (as yet) unidentified function differed slightly from those encoded by the E7/E8 region. Firstly, transfection of Pal cells with pSV3.9((+) alone lead to large colonies of elongated cells, a phenotype never seen with pSV2.0(+). Secondly, cotransfection of pSV3.9(+) with **ras** led to rare colonies of elogated and piled-up morphology, a phenomen on never seen upon cotransfection of pSV2.0(+) with **ras**.

The data are consistent with the hypothesis that the 3.9kb XhoII fragment of BPV4 encodes a **ras**-like function, which upon cotransfection with activated **ras** could lead to the piled-up phenotype. The lack of a **myc**-like function capable of significantly extending the life-span of the cells *in vitro* would explain the failure to expand such colonies into cell lines.

Two likely candidates for the ORF responsible for the transforming properties of the 3.9kb fragment are E2 and E4. The transactivator properties of the BPV4 E2 ORF indicate that, like the E1a gene products of adenovirus, modulation of cellular gene expression may play a role in the transforming activity of the 3.9kb fragment. The BPV1 E2 ORF has been shown to activate the promoter of the haemopoietic growth factor Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) in transient CAT assays performed in lymphoid cells (Miyatake *et al* . (1988)), but as yet, no data concerning cellular gene activation by E2 gene products of any PV in more natural cell systems has been obtained.

The E4 ORF has no known molecular function as yet but is presumed to be involved in the alteration of keratin gene expression found in PV-induced papillomas (Breitburd *et al* .(1987)). It is predicted to contain the 'leucine zipper' motif found originally in the transcription factor C-EBP (Landschulz *et al* . (1988)). This motif has been shown to be necessary for the dimerisation of many transcription factors, either as homodimers or in combination with distinct proteins (such as the fos-jun complex, which binds to the AP-1 element responsible for responsiveness to TPA (for review, see Vogt and Tjian(1988)). It may be the case, therefore, that interaction of E4 with other DNA-binding proteins leads to an alteration of cellular gene expression resulting in altered cell morphology.

4.5 Further work

The development of an *in vitro* model system for the transformation of primary bovine fibroblasts has opened a new avenue for studies on BPV4-mediated transformation. Much work can now be undertaken using such assays. This section will firstly propose some direct experiments to continue the strategies employed in this project and secondly look in a more long-term way at the development of studies into the BPV4-associated carcinogenesis of the UAC.

The role of the E7 ORF in the extension of *in vitro* lifespan of Pal cells could be further examined by the reconstruction of a recombinant BPV4 lacking the 233bp deleted in pSV1.8(+) and analysis of its neo^r colony-forming activity upon cotransfection with pSV2neo into Pal cells. The requirement for the E8 ORF could also be examined by the introduction of STOP codons within the E8 coding sequence, either in pSV2.0(+) or within BPV4 itself. With the cloning of a sequence containing only the E7 ORF into pZIPneoSV(X1) (G.J. Grindlay, Beatson Institute (pers. comm.)), a direct test of whether E7 is sufficient for the induction of neo^r macrocolonies can now be performed without further ado. This construct could also be used to test whether cotransfection of E7 and **ras** will induce full morphological transformation in the absence of the E8 ORF. The system also offers possiblilities for a structural analysis of the E7 ORF by Site-Directed Mutagenesis (SDM).

The induction of neo^r macrocolonies obtained after transfecting the E2-E5 region (encoded by pSV3.9(+)) should be repeated and confirmed. Should the data prove to be repeatable, the ORF

responsible could be identified by the introduction of STOP codons into each of the possible transforming ORFs.

The reasons for the lack of neo^r colony formation in any cell line after transfection of pZIP3.9(+) could be further investigated by two experiments in C127 cells. Firstly, pZIP3.9(-) could be transfected into C127 cells. Secondly, pSV2neo and pZIP3.9(+) could be cotransfected at a molar ratio of perhaps 1:20. Should both these experiments yield neo^r colonies, then one could deduce that the presence of the 3.9kb fragment of BPV4, within the context of the pZIPneoSV(X1) plasmid, was not lethal to C127 cells. Since it has been shown that these sequences are apparently not lethal in Pal cells, it could then be deduced that the reason for no neo^r colony formation in any cell line after transfection with pZIP3.9(+) would be due to the lack of **neo** gene expression in this construct.

With a reliable assay system capable of generating neo^r cell lines now available, a correlation between cellular physiology and viral gene product expression will now be possible. In particular, the question as to whether the rare incidence of non-contact-inhibited cell lines upon cotransfection of BPV4 or pSV2.0(+) with **ras** is due to poor expression of transfected genes, lack of maintenance of (a) transfected gene(s) or inability of these genes to fully transform primary bovine fibroblasts is of importance. A start can be made with a detailed analysis, at the levels of DNA and RNA, of BPV4 E7/E8 and **ras** gene expression in the two cell lines generated after transfection of Pal cells with pSV2.0(+) and **ras**.

This primary bovine fibroblast system represents an intermediate step towards the development of the ideal tissue culture system for the study of BPV4-associated carcinogenesis. With the

ever-increasing standardisation of epithelial cell culture, it would appear that the time is now ripe for the development of such a system for the bovine papillomaviruses and in particular BPV4. With the development of such a system, the relative susceptibilities of fibroblasts and epithelial cells could be analysed using assays similar to those described in this project. In particular, it would be of interest to know whether introduction of BPV4 and **ras** into keratinocytes could lead, in contrast to the case of fibroblasts, to the development of a fully non-contact-inhibited phenotype.

The recently developed collagen raft system could provide a means for analysing the effect of BPV4 upon bovine keratinocyte differentiation, and the plasmids constructed during this study could act as useful tools in the elucidation of specific ORF functions in such a process.

5. References

Abel, T. and Maniatis, T. (1989):
Nature **341**, 24

Amtmann, E., Muller, H. and Sauer, G. (1980):
J. Virol. **35**, 962

Amtmann, E. and Sauer, G. (1982):
J. Virol. **43**, 59

Androphy, E.J., Schiller, J.T. and Lowy, D.R. (1985):
Science **230**, 442

Androphy, E.J., Lowy, D.R. and Schiller, J.T. (1987):
Nature **325**, 70

Avery, O.T., MacLeod, C.M. and McCarthy, M. (1944):
J. Exp. Med. **79**, 137

Baker, C.C. and Howley, P.M. (1987):
EMBO J. **6**, 1027

Barbosa, M.S., Lowy, D.R. and Schiller, J.T. (1989):
J. Virol. **63**, 1404

Berg, L., Lusky, M., Stenlund, A. and Botchan, M.R. (1986a):
Cell **46**, 753

Berg, L., Singh, K. and Botchan, M.R. (1986b):
Mol. Cell. Biol. **6**, 859

Bergman P., Ustav, M., Sedman, J., Moreno-Lopez, J., Vennström, B;. and Pettersson, U. (1988): Oncogene **2**, 453

Birnboim, H.C. and Doly, J. (1979):
Nuc. Ac. Res. **7**, 1513

Boshart, M., Gissmann, L., Ikenberg, H., Kleinheinz, A., Scheurlen, W. and zur Hausen, H. (1984): EMBO J. **3**, 1151

Boyle, J., MacKie, R.T., Briggs, J.D., Junor, B.J.R. and Aitchison, T.C. (1984): The Lancet **i**, 702

Breitburd, F., Croissant, O. and Orth, G. (1987):
Cancer Cells **5**, 115

Broker, T.R. and Botchan, M.R. (1986):
Cancer Cells **4**, 17

Burnett, S., Kiessling, U. and Pettersson, U. (1989):
J. Virol. **63**, 2215

Campo, M.S., McCaffery, R.E., Doherty, I., Kennedy, I.M. and Jarrett, W.F.H. (1990): Oncogene **5**, 303

Campo, M.S. (1989):
Br. J. Cancer **60**, 970 (Erratum published due to misprint in 1988 article (Br. J. Cancer **58**, 80))

Campo, M.S., Moar, M.H., Jarrett, W.F.H. and Laird, H.M. (1980)
Nature **286**, 180

Campo, M.S. and Spandidos, D.A. (1983):
J. Gen. Virol. **64**, 549

Campo, M.S., Moar, M.H., Sartirana, M.L., Kennedy, I.M. and Jarrett, W.F.H. (1985): EMBO J. **4**, 1819

Campo, M.S. and Roe, F. (1986):
Ann. Inst. Pasteur **137B**, 27

Campo, M.S. and Jarrett, W.F.H. (1987):
In: 'Molecular Basis of Virus Disease', p215 (Cambridge Uni. Press).

Cepko, C.K., Roberts, B.E. and Mulligan, R.C. (1984):
Cell **37**, 1053

Chesters, P.M. and McCance, D.J. (1989):
J. Gen. Virol. **70**, 353

Choe, J., Vaillancourt, P., Stenlund, A. and Botchan, M.R. (1989):
J. Virol. **63**, 1743

Choo, K.-B., Pan, C.-C. and Han, S.-H. (1987):
Virology, **161**, 259

Chu, G., Hayakawa, H. and Berg, P. (1987):
Nuc. Ac. Res. **15**, 1311

Cole, S.T. and Danos, O. (1987):
J. Mol. Biol. **193**, 599

Croissant, O., Breitburd, F. and Orth, G. (1985):
Clin. Dermatol. **3**, 43

Crook, T., Storey, A., Almond, N., Osborn, K. and Crawford, L. (1988):
PNAS **85**, 8820

Crook, T., Morgenstern, J.P., Crawford, L. and Banks, L. (1989):
EMBO J. **8**, 513

Curran, T. and Franza, B.R. (1988):
Cell **55**, 395

DiMaio, D., Guralski, D. and Schiller, J.T. (1986):
PNAS **83**, 1797

Dotto, G.P., Weinberg, R.A. and Ariza, A. (1988):
PNAS **85**, 6389

Dürst, M., Gissmann, L., Ikenburg, H. and zur Hausen, H. (1983):
PNAS **80**, 3812

Dürst, M., Dzarlieva-Petrusevska, R.T., Boukamp, P., Fusenig, N. and Gissmann, L. (1987): Oncogene **1**, 251

Dyson, N., Howley, P.M., Münger, K. and Harlow, E. (1988):
Science **243**, 934

Dvoretzky, I., Shober, R., Chattophadhyay, S.K. and Lowy, D.R. (1980):
Virology **103**, 36

Edmonds, C. and Vousden, K. (1989):
J. Virol. **63**, 2650

Engel, L.W., Heilman, C.A. and Howley, P.M. (1983):
J. Virol. **47**, 516

Feinberg, A.P. and Vogelstein, B. (1983):
Anal. Biochem. **132**, 6

Franke, W.W., Moll, R., Achtstätter, T. and Kuhn, C. (1986):
Banbury Report **21**, 273

Freshney, R.I. (1987): Culture of Animal Cells:
A manual of basic technique. (Alan R. Liss, Inc.)

Gaukroger, J., Bradley, A., O'Neil, B., Smith, K.T., Campo, M.S. and Jarrett, W.F.H. (1989): Vet. Record **125**, 391

Giri, I. and Danos, O. (1986):
TIG **2**, 227

Gius, D. and Laimins, L.A. (1989):
J. Virol. **63**, 555

Graham, F.L. and van der Eb, A.J. (1973):
Virology **52**, 456

Horwitz, B.H., Burkhardt, A.L., Schlegel, R. and DiMaio, D. (1988):
Mol. Cell. Biol. **8**, 4071

Hubbert, N.L., Schiller, J.T., Lowy, D.R. and Androphy, E.J. (1988):
PNAS **85**, 5864

Hung, M.-T., Thompson, K.L., Chiu, I.-M. and Rich Rosner, M. (1986):
Biochem. Biophys. Res. Comm. **141**, 1109

Jarrett, W.F.H. (1985):
In: 'Papillomaviruses: Molecular and Clinical Aspects', p299 (Alan R. Liss, Inc.)

Jarrett, W.F.H., McNeil, P.E., Grimshaw, W.T.R., Selman, I.E.and McIntyre, W.I.M. (1978a): Nature **274**, 215

Jarrett, W.F.H., Murphy, J., O'Neil, B.W. and Laird, H. (1978b):
Int. J. Cancer **22**, 323

Kaur and McDougall, J.K. (1988):
J. Virol. **62**, 1917

Kidd, J.G. and Parsons, R.J. (1936):
Proc. Soc. Exp. Bio. Med. **35**, 438

Kidd, J.G. and Rous, P.R. (1940):
J. Exp. Med. **71**, 813

Klinger, H.P. (1982):
Cytogenetics and Cell Genetics **32**, 68

Koi, M., Morita, H., Yamada, H., Satoh, H., Barrett, J.C. and Oshimura, M. (1989): Mol. Carcinogenesis. **2**, 12

Kopan, R., Traska, G. and Fuchs, E. (1987):
J. Cell. Biol. **105**, 427

Kreider, J.W. and Bartlett, G.L. (1981):
Advances in Cancer Research **35**, 81 (Academic Press).

Kreider, J.W., Howett, M.K., Wolfe,S.A., Bartlett, G.L., Zaino, R.J., Sedlacek, T.V. and Mortel, R. (1985): Nature **317**, 639

Lacy, M., Alpert, S. and Hanahan, D. (1986):
Nature, **322**, 609

Lambert, P.F., Spalholz, B.A. and Howley, P.M. (1987):
Cell **50**, 69

Lambert, P.F. and Howley, P.M. (1988):
J. Virol. **62**, 4009

Land, H., Parada, L.F. and Weinberg, R.A. (1983):
Nature **304**, 586

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1988):
Science **240**, 1759

Law, M.-F., Lowy, D.R., Dvoretsky, I. and Howley, P.M. (1981):
PNAS **78**, 2727

Lee, W.-H., Shew, J.-Y., Hong, F.D., Wery, T.W., Doonoso, L.A., Young, L.-J., Bookstein, R. and Lee, E.Y.-H.P. (1987): Nature **329**, 642

Lees, E., Osborn, K., Banks, L. and Crawford, L. (1990):
J.. Gen. Virol. **71**, 165

Lindgren, V., Sippola-Thiele, M., Skowronski, J., Wetzel, E., Howley, P.M. and Hanahan, D. (1989): PNAS **86**, 5025

Lusky, M. and Botchan, M.R. (1984):
Cell **36**, 391

Lusky, M. and Botchan, M.R. (1985):
J. Virol. **53**, 955

Lusky, M. and Botchan, M.R. (1986a):
PNAS **83**, 3609

Lusky, M. and Botchan, M.R. (1986b):
J. Virol. **60**, 729

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982):
Molecular Cloning: A Laboratory Manual. (Cold Spring Harbour Laboratory).

Martin, P., Vass, W.C., Schiller, J.T., Lowy, D.R. and Velu, T.J. (1989):
Cell **59**, 21

Matlashewski, G., Osborn, K., Murray, A., Banks, L. and Crawford, L. (1987a): Cancer Cells **5**, 195

Matlashewski, G., Schneider, J., Banks, L., Jones, N., Murray, A. and Crawford, L. (1987b): EMBO J. **6**, 1741

Matlashewski, G., Osborn, K., Banks, L., Stanley, M. and Crawford, L. (1988): Int. J. Cancer **42**, 232

Matthias, P., Böger, U., Danesch, U., Schütz, G. and Bernard, H.-U. (1986): J. Mol. Biol. **187**, 557

McBride, A.A., Byrne, J.C. and Howley, P.M. (1989): PNAS **86**, 510

McCaffery, R.E., Coggins, L.W., Doherty, I., Kennedy, I.M., O'Prey, M., McColl, L. and Campo, M.S. (1989): Oncogene **4**, 1441

McCance, D.J., Kopan, R., Fuchs, E. and Laimins, L.A. (1988): PNAS **85**, 7169

McKay, J.A., Marshall, C.J., Cales, C. and Hall, A. (1986): EMBO J. **5**, 2617

Meselson, M. and Stahl, F.W. (1958): PNAS **44**, 671

Miller, J., McLachlan, A.D. and Klug, A. (1985): EMBO J. **4**, 1609

Miyatake, S., Nishida, J., Abe, E., Yokota, T., Shlomai, J., Arai, K.-i. and Arai, N. (1988): Nuc. Ac. Res. **16**, 6547

Moar, M.H., Campo, M.S., Laird, H.M. and Jarrett, W.F.H. (1981): Nature **293**, 749

Münger, K., Phelps, W.C., Bubb, V., Howley, P.M. and Schlegel, R. (1989): J. Virol. **63**, 4417

Neary, K. and DiMaio, D. (1989):
J. Virol. **63**, 259

O'Brien, W., Stenman, G. and Sager, R. (1986):
PNAS **83**, 8659

Ocadiz, R., Sauceda, R., Cruz, M., Graeg, A.M. and Gariglio, P. (1987):
Cancer Res. **47**, 4173

Orth, G., Jeanteur, P. and Croissant, O. (1971):
PNAS **68**, 1876

Orth, G., Favre, M., Breitburd, F., Crossaint, O., Jablonska, S., Obalek, S., Jarzabek-chorzelska, and Rzesa, G. (1980):
In: 'Viruses in Naturally Occuring Cancers', p259 Ed. M. Essex, G. Todaro and H.zur Hausen, Cold Spring Harbour Lab., New York.

Patel, K.R., Smith, K.T. and Campo, M.S. (1987):
J. Gen. Virol. **68**, 2117

Pater, M.M., Hughes, G.A., Hyslop, D.E., Nakshatri, H. and Pater, A. (1988): Nature **335**, 832

Pecoraro, G., Morgan, D. and Defendi, V. (1989):
PNAS **86**, 563

Phelps, W.C., Yee, C.L., Münger, K. and Howley, P.M. (1988):
Cell **53**, 539

Pirisi, L., Yasumoto, S., Feller, M., Doniger, J. and DiPaolo, J. (1987):
J. Virol. **61**, 1061

Ptashne, M., Jeffrey, A., Johnson, A.D., Mauer, R., Meyer, B.J., Pabo, C.O., Roberts, T.M. and Sauer, R.T. (1980): Cell **19**, 1

Prakash, S.S., Horwitz, B.H., Zibello, T., Settleman, J. and DiMaio, D. (1988): J. Virol. **62**, 3608

Queen, C. and Korn, L.J. (1984):
Nuc. Ac. Res. **12**, 581

Rigoni-Stern, D. (1842):
G. Servire Progr. Pathol. Terap. **2**, 507

Riou, G.F., Barrois, M., Dutronquay, V., and Orth, G. (1985): in Papillomaviruses: Molecular and Clinical Aspects, p47
(Alan R. Liss, Inc.)

Riou, G.F., Barrois, M., Le, M.G., George, M., Le Doussal, V. and Haie, C. (1987): Lancet **ii**, 761

Riou, G.F., Barrois, M., Sheng, Z.-M., Duvillard, P. and Lhomme, C. (1988): Oncogene **3**, 329

Rösl, F., Dürst, M. and zur Hausen, H. (1988):
EMBO J. **7**, 1321

Rous, P.J. (1911):
J. Exp. Med. **13**, 397

Rous, P.J. and Beard, J.W. (1935):
J. Exp. Med. **52**, 523

Rous, P.J. and Kidd, J.G. (1936):
Science **83**, 468

Santos, E., Tronick, S., Aaronson, S., Pulacini, S. and Barbacid, M.:
(1982): Nature **298**, 343

Sarver, N., Rabson, M.S., Yang, Y.-C., Byrne, J.C. and Howley, P.M.
(1984): J. Virol. **52**, 377

Saxon, P.J., Srivatsan, E.S. and Stanbridge, E.J. (1986):
EMBO J. **5**, 3461

Schiller, J.T., Vass, W.C. and Lowy, D.R. (1984):
PNAS **81**, 7880

Schlegel, R., Wade-Glass, M., Rabson, M.S and Yang, Y.-C. (1986):
Science **233**, 465

Schlegel, R. and Wade-Glass, M. (1987):
Cancer Cells **5**, 87

Schlegel, R., Phelps, W.C., Zhang, Y.-L. and Barbosa, M. (1988):
EMBO J. **7**, 3181

Schneider-Maunoury, S., Croissant, O. and Orth, G. (1987):
J. Virol. **61**, 3295

Settleman J. and DiMaio, D. (1988):
PNAS **85**, 9007

Shope, R.E. (1935):
Proc. Soc. Exp. Biol. Med. **32**, 830

Smith, K.T., Campo, M.S., Bradley, J., Gaukroger, J. and Jarrett, W.F.H. (1987): Cancer Cells **5**, 267

Smith, K.T. and Campo, M.S. (1988):
Virology **164**, 39

Smith, K.T. and Campo, M.S. (1989):
Oncogene **4**, 409

Smits, H.L., Raadsheer, E., Rood, I., Mehendale, S., Slater, R.M., van der Noordaa, J. and ter Schegget, J. (1988): J. Virol. **62**, 4538

Southern, E.M. (1975):
J. Mol. Biol. **98**, 503

Southern, P.J. and Berg, P. (1982):
J. Mol. Appl. Genet. **1**, 327

Spalholz, B.A., Yang, Y.-C. and Howley, P.M. (1985):
Cell **42**, 183

Stamps, A.C. (1987):
Ph.D. Thesis, University of Glasgow.

Stamps, A.C. and Campo, M.S. (1988):
J. Gen. Virol. **69**, 3391

Stanbridge, E.J., Flandermeyer, R.R., Daniels, d.W. and Nelson-Rees, W.A. (1981): Somatic Cell Genetics **7**, 699

Stenlund, A., Zabielski, J., Ahola, H., Moreno-Lopez, J. and Pettersson U. (1985): J. Mol. Biol. **182**, 541

Storey, A., Pim, D., Murray, A., Osborn, K., Banks, L. and Crawford, L. (1988): EMBO J. **7**, 1815

Storey, A., Almond, N., Osborn, K. and Crawford, L.(1990):
J. Gen. Virol. **71**, 965

Sundberg, J.P., O'Banion, M.K. and Reichmann, M.E. (1987):
Cancer Cells **5**, 373

Sugden, B. (1989):
Cell **57**, 5

Thierry, F. and Yaniv, M. (1987):
EMBO J. **6**, 3391

Thorner, L., Bucay, N., Choe, J. and Botchan, M.R. (1988):
J. Virol. **62**, 2474

Tidy, J., Vousden, K.H., Mason, P. and Farrell, P.J. (1989):
J. Gen. Virol. **70**, 999

Tilbrook, P.A., Greenoak, G.E., Reeve, V.E., Canfield, P.J., Gissmann, L., Gallagher, C.H. and Kulski, J.K. (1989): J. Gen. Virol. **70**, 1005

Vogt, P.K. and Tjian, R. (1988):
Oncogene **3**, 3

von Knebel-Döberitz, M., Oltersdorf, T., Schwarz, E. and Gissmann, L. (1988): Cancer Research **48**, 3780

Vousden, K.H., Androphy, E.J., Schiller, J.T. and Lowy, D.R. (1989a):
J. Virol. **63**, 2340

Vousden, K.H., Doniger, J., DiPaolo, J.A. and Lowy, D.R. (1989b):
Oncogene Research **3**, 167

Watanabe, S., Kanda, T. and Yoshiike, K. (1989):
J. Virol. **63**, 965

Watson, J.D. and Crick, F.H.C. (1953):
Nature **178**, 737

Weinberg, R.A. (1988):
Scientific American **259** No. 9, p34

Woodworth, C.D., Bowden, P.E., Doniger, J., Pirisi, L., Barnes, W., Lancaster, W.D. and DiPaolo, J.A. (1988): Cancer Res. **48**, 4620

Woodworth, C.D., Doniger, J. and DiPaolo, J.A., (1989):
J. Virol. **63**, 159

Yang, Y.-C., Spalholz, B.A., Rabson, M.S. and Howley, P.M. (1985):
Nature **318**, 575

Yokata, J., Tsudada, Y., Nakajima, T., Gotoh, M., Shimosato, Y., Mori, N., Tsunodawa, Y., Sugimura, T. and Terada, M. (1989):
Cancer Res. **49**, 3598