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DEFINING A ROLE FOR DIFFERENT ONCOGENES IN BLADDER NEOPLASIA USING AN *IN VIVO* RECONSTITUTION MODEL

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ABSTRACT

In this thesis, I report a novel assay system for the detection of H-ras oncogene activation in transitional cell carcinomas using Western blot analysis. This approach exploits the aberrant migrational properties of altered ras p21 proteins in SDS-PAGE gel electrophoresis and provides information concerning the relative level of expression of H-ras proteins in human bladder tumours. This work demonstrates the occurrence of H-ras oncogene activation in approximately 16% of bladder tumours and extends previous reports of preferential activation of this ras gene member in transitional cell carcinomas.

Much of the previous work on oncogenes has been performed using established cell lines where neoplastic progression is related to cell growth, behaviour and tumorigenic potential. For the cell biologist these have proved to be good criteria, but to the clinician their relationship to in-vivo pathology remains unclear. In an attempt to define a role for ras and other oncogenes implicated in bladder cancer I have devised a reconstitution model for urothelium in which to address these issues. Using two in vivo sites, the subrenal capsule and the denuded bladder, I have demonstrated the feasibility of reconstituting urothelial elements in vivo without the requirement for tumorigenic potential. In this assay immortalized, non-tumorigenic urothelial cell lines have been shown to behave in a benign fashion in vivo, similar to that observed with normal mucosa.

Tumorigenic cell lines grow well at both sites and the removal of tumour bearing kidneys has enabled us to extend this model to study the metastatic potential of urothelial cell lines harbouring a ras oncogene.

The in-vivo maintenance of normal and preneoplastic urothelium beneath the renal capsule enables us to study early events in neoplasia prior to tumour formation. In addition this environment allows both proliferation and differentiation to occur, facilitating the investigation of altered differentiation events in bladder neoplasia. In preliminary experiments we have demonstrated the application of this system in defining the role of different oncogenes in bladder carcinogenesis at both early and late stages of neoplastic progression including metastasis.

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The experimental section of this thesis was undertaken at the Laboratory of Cancer Biology, in the Department of Surgery, New England Deaconess Hospital, Harvard Medical School, Boston USA and was made possible by the financial support of the Department of Surgery under the chairmanship of Dr. Glenn Steele Jr.

The proposed work to define a role for different oncogenes in bladder neoplasia using an in-vivo reconstitution model is wholly original and the sole work of the author.

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No portion of this work has been submitted in support of an application for any other degree or qualification of this or any other university or institute of learning.

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CHAPTER I

BLADDER CANCER - an overview

Urothelial cancer is the second most common genito-urinary malignancy after carcinoma of the prostate and accounts for about 6% of all male cancers (Silverberg and Lubera, 1980). In Scotland, an incidence of 23 patients per 100,000 population has been reported (WHO 1985), similar to those figures reported for North America. The incidence is variable both in geographical and racial distribution but overall the rates are highest in the white population with a sex ratio of three or four males to one female, and urban levels are higher compared to rural areas. Although most patients present in the sixth decade, cases have been reported below the age of 40 years (Fitzpatrick et al 1986) where the progression of the disease is no different. Aetiological factors include cigarette smoking, certain occupations and phenacetin abuse (Morrison 1984).

In Europe and North America, approximately 90% of all bladder tumours are transitional cell carcinomas with the remainder being squamous cell or rarer adenocarcinomas. In areas with endemic Schistosoma haematobium, 40 % of the tumours are squamous cell in origin. Bladder tumours are usually localised at the time of presentation with only 15% showing evidence of regional or distant metastases (Silverberg 1981). However, 75% of new patients will develop recurrent tumours and in 30% of these the behaviour of the disease alters with increased invasiveness and malignant potential (Heney et al 1983).

Cynics may argue that little has changed in the management of urothelial cancer. The conventional treatment remains endoscopic resection with long term cystoscopic review. Multifocal disease often necessitates the use of adjuvant cytotoxic intravesical therapy, and, despite the pharmacological armamentarium available, few of the recent compounds offer any significant advantage over those agents that have been available for the past 20 years.

It is against this background that research has been directed towards earlier identification of those patients who ultimately will progress to muscle invasion, and of the high grade lesion that rapidly disseminates to distant metastases. Even so, it is only by understanding the epidemiology and molecular biology of the events involved in urothelial carcinogenesis that future progress can hope to improve on these matters.

EPIDEMIOLOGY OF BLADDER CANCER

Like many cancers, the incidence of bladder tumours was found to be increased in certain geographical areas following the arrival of new chemical industrial sites. Epidemiological studies have implicated certain high risk factors and potential causes for the development of urothelial cancer (Morrison 1984). Rehn's initial observations, in Germany, (1895), amongst workers in the dye industry, implicated aniline as the carcinogenic agent involved in the high incidence of bladder tumours found amongst workers. However, later work has shown that naphthylamines, 4-amino-biphenyl (xenylamine), arising as impurities during the manufacture of fuchsin, were responsible for initiating the subsequent development of tumours (Hueper 1934).

A major epidemiological survey reported in 1954 by Case and his colleagues demonstrated that the incidence of bladder cancer amongst factory workers was thirty times more frequent than in the control group and that B-naphthylamine and certain Benzidine related compounds were potent bladder carcinogens. Case demonstrated that the risk varied with the chemical agent, the degree of exposure and the age of the individual at entry to the industry (Case 1954 a.b.). Later, 4 amino-biphenyl (xenylamine) was identified by Mellick (1955) as a potent urothelial carcinogen derived from the reduction of paranitrophenyl, but this has never been manufactured in the UK.

As a result of Case's survey and as a consequence of the use of aromatic amines in other settings, many different occupations have been studied as possible risk factors in the aetiology of bladder cancer. Using the same approach, Case and Hosker (1954) found a moderate elevation of bladder cancer risk in the rubber industry in England and Wales, but it was difficult to associate this exposure to any specific chemical carcinogen.

Occupational case controlled studies have the advantage that many possible risk factors many be investigated simultaneously, but the disadvantages are that the small numbers involved may result in an imprecise estimation with difficulty in identifying those exposed to a specific carcinogen. Occupations credited with an elevated risk are the paint and dye industry, metal machinists, leather, textiles and hairdressing (Table I.1) (Cartwright 1983, Cole et al 1972, Decoufle 1979).

Cigarette Smoking:

Less than 10% of human bladder cancer is attributable to exposure to B-naphthylamine or the Benzidine compounds, and the major risk factor today for urothelial cancer appears to be cigarette smoking, with a disease incidence two to three times greater than in non smokers (Morrison et al 1984). In general, the period between first exposure and onset of the disease is aproximately 25 years but there is considerable variation both in susceptibility and latent period, consistent with the concept of a multistage process occuring in urothelial cancer.

TABLE I.1: OCCUPATIONS INVOLVING A RISK OF DEVELOPING BLADDER CANCER:

A: Occupations where exposure to urothelial carcinogens has occurred at certain times:

Manufacture of Rubber, & Rubber Products.

Cable manufacturing industry.

Manufacture of Dyestuffs.

Manufacture of Organic Chemicals.

Gasworks and Coke ovens.

Rodent extermination.

Sewage works.

Manufacture of firelighters/patent fuels.

Laboratory work.

B. Occupations with a suspicion of an excess of bladder cancer where exposure may have occurred but careful investigation is needed:

Leather work.

Medical and Nursing.

Textile and Printing.

Hairdressing.

Aluminium refining and smelting.

Security printing.

Drivers and transport workers.

C. Occupations where an excess of bladder cancer might be expected but not yet observed:

Manufacture of Plastics.

from Br J Urol (1988) 61, 183-191.

The main reasons for establishing a cause relationship between smoking and bladder cancer are that the association has been described in both sexes in North America, Europe and Japan. Also similar results have been obtained from both case controlled and follow up studies (Cartwright et al 1983). The risk has been shown to increase with the frequency of consumption and the degree of inhalation (Cole, 1971, Morrison 1984). These findings indicate that the results are unlikely to be explained by observation bias or any other unidentified factor. In fact, since people tend to underestimate their smoking history the risk may be even greater than that observed.

However there are certain inconsistencies that deserve mention, duration of smoking has not been satisfactorily related to risk and although ex-smokers have generally been found to have a lower risk of developing the disease, a true risk relation between reduced risk with time has not been observed. Also those who have stopped smoking may have been relatively light smokers with a reduced risk anyway.

Analgesics:

Phenacetin containing analgesics have been implicated in the aetiology of urothelial tumours after a series of case reports from Sweden describing tumours ocurring in the renal pelvis of patients with chronic phenacetin abuse (Bengtsson et al 1968.) Subsequently confirmed by groups in other countries where use of the drug was popular (McCredie et al 1983). Essentially it is estimated that

patients who have taken in excess of one kilogramme cumulative dose have a 13 times relative risk compared to the normal population. Phenacetin is an aromatic amide with a metabolite that is structurally related to naphthylamine which may be important in the pathogenesis of the disease but as some studies have mentioned there is uncertainty as to which chemical component is causative. This is because chronic analgesic abusers have often been exposed to several different compounds and the composition of the preparations tend to alter with time.

Bilharzia:

In Egypt, where approximately 85% of the population have been infected with Schistoma at some time, a highly significant association has been shown with squamous cell carcinoma of the bladder. Hashem (1961) reported that from 4447 autopsies, 6.6% of chronic bilharzial bladders show malignant changes, and that 83% of all bladder tumours occured in individuals with a history of bilharzia. The disease shows no sex preponderance and tends to be seen in younger patients compared to transitional cell tumours. A possible mechanism of carcinogenesis has been postulated by Hicks and her colleagues. In experimental animals, simple infection of the bladder with S. haematobium results in extensive inflammation and hyperplasia but not urothelial carcinoma. Whereas, initiation of the urothelium with N-butyl-N-(4-hydroxybutyl)nitrosamine, at a low dose prior to infection, results in tumour formation in 40% of cases

(Hicks et al, 1980). It is known that nitrosamine derivatives have been found in the urine of bilharzia patients possibly due to bacterial degradation of nitrates. This, in conjunction with the increased cell turnover secondary to the chronic inflammatory response, may explain the pathogenesis of such tumour formation.

Section 1

CONCEPT OF MULTISTAGE CARCINOGENESIS

The development of a fully malignant tumour involves complex interactions between several factors both environmental and endogenous (e.g. genetic or hormonal) and the concept that carcinogenesis is a multistep process derives from the epidemiological and pathological studies detailing the time course of the disease (for review see Foulds 1975). The statistical analysis, suggesting several independent events for human epithelial cancers (Peto 1977), and the increasing evidence of cytogenetic defects, that arise during tumour growth and progression, enhances our present understanding of experimental multistage carcinogenesis which stems from the original two stage, initiation and promotion model developed by Berenblum (1940). This concept was proposed to explain the observation that mouse skin, previously exposed to a low subthreshold dose of carcinogen (Benze(a)pyrene), results in a significantly increased yield of papillomas when skin

is painted with croton oil. Evidence of a similar concept has accumulated for several organ systems and for the existence of many different promotors (Slaga 1983).

Initiation in the mouse skin model, involves the interaction between the carcinogen and the cellular DNA producing conformational or functional changes, such as interference with base pairing. This produces a heritable modification in the genetic material without overt alteration of the cellular phenotype, either by the activation of proto-oncogenes or more complex interactions involving loss of tumour suppressor genes or disturbances in regulatory sequences (Weinstein 1988). This event is dose related, for at higher levels the carcinogen may be directly mutagenic, and characteristically should affect only a small proportion of the target cell population. If the initiating event has involved one of the basal stem cells, then the damage may be considered permanent unless rapidly repaired (Berenblum 1974). Subsequent exposure of the initiated cell to promotional agents permits the expression of the altered phenotype otherwise there is no recognisable histopathological difference between the initiated and normal epithelium.

Promotion has been classified into two stages (Boutwell 1964). A specific early phase (stage 1), notably induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), which renders the cell susceptible to growth stimulation prior to a less specific stage 2, where other agents could replace the activity of TPA, resulting in the selective proliferation of these cells to form a benign

hyperplastic tumour (Marks and Furstenberger 1986).
The biochemical processes underlying stage 1 and 2 promotion are entirely different. Biochemically, a stage 1 promotor can be defined as an agent capable of binding to and activating a specific protein kinase in the cell membrane so interfering with cell function. Receptors for TPA have been identified in many tissues (Weinstein 1982), including skin, and via the activation of protein kinase C, TPA is able to regulate the activity of various hormones and growth factors.

Stage 2 promotion involves prolonged stimulation of the tissue resulting in hyperplasia. Thus these agents tend to induce enzymes which accelerate cellular division, for example Ornithine Decarboxylase (ODC). ODC is the rate limiting enzyme in polyamine synthesis an important modulator in the synthesis of DNA, RNA and proteins (Gaugas 1980). Many stimuli may act as stage 2 promotors but unless they can promote stage 1 they will be unable to promote tumour formation in initiated skin. Similarly, the type of modification evoked by the initiation process is thought to determine the class of agents that act as promotors in a particular system (Cerutti 1988).

In the mouse skin model, both benign and malignant lesions, can be initiated with dimethylbenz(a)anthracene (DMBA) followed by promotion with TPA. Using this system Balmain and colleagues (1984) were able to demonstrate that primary papillomas induced by DMBA contain an activated H-ras gene. Over 85% of the papilloma DNA's exhibited transforming ability, in transfection assays,

whereas only 5% progressed to carcinomas suggesting that, in this model, ras gene activation is an early mutational event. Many of the premalignant papillomas are promotor-dependent and regress with removal of the agent. Although the number of events leading to malignancy are unknown, the final conversion appears to require, at least, a second genetic event which may involve the activation of other proto-oncogenes, which may modify the cells response to other regulatory factors (Quintanilla et al 1986).

Following these results it is informative to view bladder carcinogenesis within this framework to analyse the different mechanisms involved in multistage carcinogenesis.

NEOPLASTIC PROGRESSION IN THE URINARY BLADDER

In attempting to study neoplastic progression in human urothelial cancer one is hampered by the lack of available tissue, both normal and malignant, especially in the early stages of neoplasia where cells cannot be maintained in culture or harvested in sufficient numbers for biochemical analysis. As a result three main categories of animal models have been derived for the study of bladder carcinogenesis utilising, strains with a high spontaneous rate of bladder tumour production, carcinogenic or viral agents. Chemical carcinogen derived tumours have had a prominent role in the study of urothelial cancer and in each of these models, a clear picture of the pathogenesis

of the induced disease has emerged, consistent with the concept that neoplastic progression in the bladder involves two or more stages. Attempts to reproduce the human situation, in animal models, has been singularly disappointing. B-naphthylamine, Benzidene and 4-aminobiphenyl are carcinogenic in dogs but no tumours were observed in rats after oral administration of these chemicals (Boyland et al 1964), possibly due to species difference in metabolism of these compounds or the relatively short lifespan of the animals. A suitable experimental model must satisfy certain requirements. That is, the carcinogen must be bladder specific; be reproducible (producing tumours in over 90% of animals with a reasonably short latent period), and reflect the human situation as far as is possible.

Rodent models, using different chemical carcinogens to initiate neoplasia, have been more successful and studies of the rat urinary bladder show many similarities with the mouse skin model. Effective carcinogens at initiating rodent urothelial tumours are N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) (Druckrey et al 1964), N-methyl-N-nitrosourea (NMU) (Hicks and Wakefield 1972) and N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) (Ertuk 1967) but in general, in order to produce a high yield of tumors the carcinogen has to be administered at a particular dose and over a protracted time period (Table I.2).

TABLE I.2: CHEMICAL CARCINOGEN DERIVED TUMOURS

Carcinogen	Species	Latent Period	Authors
FANFT	Mouse	28 weeks	Ertuk et al, 1970
BBN	Mouse	30 weeks	Bertram & Craig, 1972
FANFT	Rat	24 weeks	Ertuk et al, 1967
BBN	Rat	28 weeks	Hashimoto et al, 1972
MNU	Rat	30 weeks	Hicks & Wakefield, 1972.

N-butyl-N-(4 hydroxybutyl) nitrosamine (BBN): is a urinary specific carcinogen resulting in the sequential development of urothelial tumours. In rats the compound induces multiple, superificial, papillary lesions with invasion seen only occasionally in the larger tumours. Ohtani and colleagues (1986) were also able to demonstrate sequential neoplastic changes in mouse bladder epithelium after varying concentrations of BBN and although this is a good model for invasive disease, the lack of metastases make it non representative of the human situation.
Herman (1985) also noted that BBN induced tumours showed marked quantitative and qualitative differences from human transitional cell carcinomas with increased predominance of squamous morphology in the tumours. Using this model Bertram and Craig (1972) demonstrated that sexual differences may play a role in the response of the urothelium to the carcinogen, for the increased susceptibility of males can be abolished by castration or conversely by treating the females with testosterone. Thus hormonal balances seem to have a direct effect on the genesis of bladder tumours in this model.

N-methyl-N-nitrosourea (NMU): A second model extensively studied involves instillation of NMU directly into the rat urinary bladder, where a single low dose is sufficient to induce both transitional and squamous neoplastic changes.

Working with this model Hicks and her colleagues demonstrated that the effect of exposure to repeated low doses of carcinogen, (MNU and BBN), resulted in a higher

tumour production than was observed with a single exposure to a much larger dose of carcinogen. Manipulation of the doseage and timing of exposure of the urothelium to the carcinogen enables investigators to generate bladder material representative of the different stages of neoplastic progression.

FANFT: A third model has employed N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) as a carcinogen in both mice and rats producing transitional cell tumours, after a short latent period, which are both invasive and metastatic, although the potential is less than in humans (Crabtree 1983).

The effects of FANFT and BBN are reversible if administered for only a few weeks suggesting that there is a degree of decay in the number of initiated cells, whereas the initiating effect of MNU tends to persist for at least 25 weeks (Severs et al 1982).

These rodent models have been used to study neoplastic progression and the effects of certain tumour promotors on initiated urothelium (Table I.3). The results show considerable similarity between the rat bladder and the mouse skin models.

TABLE I.3: RODENT MODELS OF BLADDER CARCINOGENESIS

Carcinogen	Promoter	Author
MNU	Sodium Cyclamate	Hicks et al, 1975
	Sodium Saccharin	Hicks et al, 1975
BBN	Sodium Saccharin	Nakanishi, 1980
	Phenacetin	Nakanishi, 1978
FANFT	Sodium Saccharin	Cohen et al, 1979
	L-Tryptophan	Cohen et al, 1979

ODC activity in urothelium is inducible by FANFT, and similar induction of activity has been confirmed using three urothelial cell lines treated in vitro with MNU and BBN. ODC activity can be induced by subsequent treatment with either Saccharin or a metabolite of tryptophan, both promote tumour growth in vivo after initiation of the bladder with a low dose of carcinogen. However the most effective inducer, consistent with the multistage theory, is TPA. Physical and chemical agents have been shown to induce urothelial hyperplasia; for example chronic urothelial damage by calculi or the ova of S. haematobium. Other implicated inducers of urothelial hyperplasia are vitamin A deficiency, Testosterone and cyclophosphamide, these are unlikely to be true promotors, but appear to act later in the carcinogenesis pathway (RM Hicks 1983).

Section 2

ONCOGENES:- WITH PARTICULAR REFERENCE TO THE ras GENE

The field of human oncogenes started in 1981 with the discovery that NIH/3T3 cells, incubated with DNA from a tumorigenic cell line, would produce foci of morphologically altered cells which had acquired the transformed phenotype. This technique became known as NIH/3T3 transfection. The first human DNA reported active in this assay was derived from a bladder carcinoma cell line, EJ/T24 (Shih et al 1981, Krontiris and Cooper 1981). Altered foci of cells, arising at a low frequency, were identified in the primary transfection but this frequency was greatly enhanced when DNA from these transformed NIH/3T3 cells was taken through a second round of transfection in the same assay. These results were consistent with the concept of transference of a genetic element necessary for the maintenance of the transformed phenotype. Using a probe which specifically recognised repetitive sequences in human DNA, investigators were able to demonstrate the presence of a discrete human DNA fragment in the mouse cells transformed in this assay, in Southern blot analysis.

Isolation of the EJ transforming DNA fragment, using molecular cloning techniques, revealed homology to the known ras transforming gene in an oncogenic retrovirus, the Harvey sarcoma virus (Parada et al 1982, Der et al 1982). Subsequent sequencing of this human transforming gene (oncogene) and its normal cellular counterpart (the

proto-oncogene) demonstrated a single nucleotide base change in the coding sequence (point mutation), substituting a G for T, which results in the replacement of the amino acid glycine by valine at codon 12 of the ras oncogene (Tabin et al 1982, Reddy et al 1982). Further studies using chimaeric molecules, between the cloned oncogene and the ras proto-oncogene, demonstrated that this point mutation was sufficient to activate the gene in NIH/3T3 transfection assays. In human tumours, ras gene activation, as detected by transfection assays, was found to occur in 10-20% of all tumour types (Pulciani et al 1982), whereas non-tumour DNA from the same patient showed no evidence of ras activation (Murray et al 1983, Santos et al 1984). More recently, research suggests a higher frequency of ras activation in certain tumour types (see point mutation).

It is from this early work that the concept of human oncogenes and their possible role in neoplastic progression has flourished. Screening of DNA from many human tumours has resulted in the characterisation of a large number of cellular oncogenes (Table I.4) whose activation can occur via three main routes as outlined in the next section:

I. Chromosomal translocation, recombination or deletion.

II. Gene amplification

III. Point mutations

TABLE I.4: CLASSIFICATION OF ONCOGENE PRODUCTS WITH RESPECT TO THEIR BIOLOGICAL FUNCTION:

Nuclear proteins		fos	myb
		C-myc	ski
		L-myc	erbA
		N-myc	ceal
Protein kinase			
	a) Tyrosine specific	abl	kit
		erbB-1	met
		erbB-2	onc D
		fgr	ros
		fms	src
		fps	yes
	b) Serine/Threonine specific	mos	
		raf	
		pks	
	c) G protein	H-ras	
		K-ras	
		N-ras	
	d) Growth factor related	erbB-1	fms
		erbB-2/neu	fgf
Unknown		dbl	ret
		mef2	rel

CHROMOSOMAL ABNORMALITIES

Translocations or rearrangement are common in many tumour types and in some cases specific abnormalities were identified, many years ago, in classical cytogenetic studies. With the advent of molecular biology techniques it became feasible to more closely characterise the breakpoints involved in known chromosomal translocations and in some cases it has been possible to identify the genes associated with this event and to demonstrate the outcome of recombination in tumour material (Yunis 1983).

In Burkitts lymphoma, a characteristic translocation occurs between the distal regions of chromosome 8 and chromosome 14. Alternative recipients involved in this translocation being chromosome 2 or 22 (Bernheim et al 1981). In the human, the myc proto-oncogene is located at the point of translocation on chromosome 8 and the translocation places the gene in the promotor regions of the immunoglobulin genes (14- lg heavy chain, 2- and 22- lg light chains). In this case there is a close correlation between the site of translocation and the abnormal immunoglobulin produced by the tumour (Lenoir et al 1982). The question of how translocation of the c-myc gene alters the status quo of the cells is still unresolved. Sequence data on human c-myc genes have demonstrated a variety of alterations in the first exon with possible loss of controlling elements which may result in altered expression of the gene product in Burkitts lymphoma.

Similarly in 85% of patients with chronic myeloid leukaemia the Philadelphia Chromosome is associated with a complex translocation involving chromosome 9 and chromosome 22. Again, a human proto-oncogene, c-abl, has been mapped to the break regions implicating the activation of this gene by phosphorylation of Tyrosine (Heisterkamp et al 1983). Although exactly how these proteins with tyrosine kinase activity effect cellular transformation is still unclear.

In certain hereditary tumours, especially retinoblastoma and Wilm's tumour, there is evidence that recessive events, resulting in the loss of suppressor proteins which may regulate the expression of particular oncogenes, play a role in the progression of the disease. In retinoblastoma there are two forms of the disease. The hereditary bilateral form presenting at an early age where individuals are at high risk for developing a second primary malignancy, carrying a germinal mutation (Rb+/rb-) on chromosome 13q14. The unilateral, non-hereditary cases present later in life and do not carry the mutation. It has been postulated that the tumour is a consequence of two mutational events (Knudson 1971) and the loss or inactivation of both alleles of the retinoblastoma gene (rb-/rb-) appears to be a primary mechanism in the development of this tumour (Murphree and Benedict 1984). In Wilm's tumour a putative recessive mutation is located on chromosome 11p13, and like retinoblastoma, there are hereditary, non-hereditary and deletion forms (Yunis 1983).

GENE AMPLIFICATION

Experimental studies have demonstrated that over expression of different proto-oncogenes can result in transformation of NIH/3T3 cells (Alitalo K 1984, Marx YL 1984). Elevated levels of gene expression can be achieved either by altered regulation of the gene or by increasing the copy number within the genome. A number of examples of gene amplification have been reported and a few cases have been described in primary human tumours, although this does not appear to be a frequent event in human neoplasia.

In neuroblastoma, one member of the myc gene family, N-myc, has been found in multiple copies in DNAs from both cell lines and primary tumours (Schwab et al 1983). The correlation of gene amplification in advanced tumour with poor prognosis is purportedly evidence for a role of N-myc in the malignant progression of the disease (Brodeur et al 1984, Seeger et al 1985). Interestingly, tumours at stage IVS of the disease, which are associated with a better prognosis, show no elevation in the number of N-myc gene copies. Such evidence at best suggests a role for this gene in neoplastic progression at worst a good indicator of clinical progression.

More recently, Slamon and colleagues (1987) demonstrated that the c-erbB-2 oncogene is amplified in human breast carcinoma. This gene is related to, but distinct from, the epidermal growth factor receptor gene and codes for a 185 kdalton protein product.

Amplification of the c-erbB-2 gene was found in 30% of breast tumours examined and was a significant predictor both of overall survival and time to relapse in patients. Moreover, c-erbB-2 amplification was of greater prognostic value than the currently available indicators. In this case identification of gene amplification of c-erB-2 defines a subpopulation of patients with poor prognosis and offers a better indicator of potential progression of the disease.

Amplification of ras proto-oncogenes has been reported in a small number of cases in a variety of tumours, but does not appear to represent a common event in human neoplasia. Barbacid's group screened 70 primary tumours for evidence of ras gene amplification and detected only one case where elevated levels of K-ras occurred in a lung tumour (Pulciani et al 1985). A similar amplification event has been reported in a single urinary tract tumour (Fujita et al 1985).

POINT MUTATION

The third main route of proto-oncogene activation is best exemplified by the ras gene family, although it is not the only oncogene activated via this route. Transfection studies have revealed three ras genes implicated in human neoplasia, c-Ha-ras-1, c-K1-ras-2, and N-ras, which represent members of an extended family, some of which are pseudogenes (for review see Barbacid M 1987). As

previously mentioned, the first ras gene identified was isolated from a bladder carcinoma cell line where a point mutation, within codon 12, was demonstrated to be responsible for the activation of this gene in transfection assays. Subsequent screening of human tumours has demonstrated the presence of altered ras genes in a variety of tumours, where activation has been shown to occur within three codon hotspots, 12,13 and 61 (Tabin et al 1982, Yuasa et al 1983, Bos et al 1985, Der et al 1986). Site directed mutagenesis experiments, using transfection assays as an endpoint, revealed that activation of the proto-oncogene is obtained at position 12 by substitution of glycine by any other amino acid except proline at codon 12 (Seeburg et al 1984) (Table I.5). Similar results were obtained with the substitution at codon 61 where glutamine is normally found (Der et al 1986). Not all the permutations generated in this experimental system have been reported in human tumours, as activation was also found to occur, by point mutational events, at codons 59, 63, 116 and 119, none of which as yet, have been reported to occur in human tumours.

Screening of human malignancies in NIH/3T3 focus assays has revealed a 10-20% incidence of ras gene activation by a somatic point mutation (Marshall 1985), although a higher frequency has been reported in animal models following the administration of chemical carcinogens (Guerrera et al 1984, Zarbl et al 1985, Quintanilla et al 1986). Recently, using more sensitive molecular techniques, the frequency of ras activation has

TABLE I.5:

H-Ras Point Mutations

12		**13**		**61**	
GGC	**Glycine**	**GGT**	**Glycine**	**CAG**	**Glutamine**
C	Arginine	C	Arginine	G	Glutamic Acid
A	Serine	A	Serine	A	Lysine
T	Cysteine	T	Cysteine	T	Terminator
GGC		**GGT**		**CAG**	
C	Alanine	C	Alanine	G	Arginine
A	Asp. Acid	A	Asp. Acid	C	Proline
T	Valine	T	Valine	T	Leucine
GGC		**GGT**		**CAG**	
G	Glycine	A	Glycine	C	Histidine
A	Glycine	C	Glycine	A	Glutamine
T	Glycine	G	Glycine	T	Histidine

been found to be significantly elevated in some human tumour types where 90% of exocrine pancreas carcinomas, 40% of colorectal cancer and 25% of patients with AML or adenocarcinoma of the lung display altered ras genes (Almoguera et al 1988, Forrester et al 1987, Bos et al 1987, Farr et al 1988, Rodenhuis et al 1987). Interestingly, these studies revealed preferential activation of one member of the ras gene family, where K-ras activation was found almost exclusively in pancreas, colon and lung tumours, whilst N-ras was associated with AML.

ONCOGENES AND BLADDER CANCER

Although the first human oncogene identified in an NIH/3T3 transfection assay was isolated from a human bladder carcinoma cell line, early reports suggested that ras activation was an infrequent event in human bladder tumours (Feinberg et al 1983, Fujita et al 1985). More recent reports, using a modified NIH/3T3 assay, scoring for tumorigenicity in nude mice, have identified altered ras genes in 16% of transitional cell carcinomas, irrespective of tumour grade and stage (Visvanathan et al 1988). Recent research reports have shown that true values for the frequency of ras gene activation is best assayed using enzymatic amplification (polymerase chain reaction or PCR Technique) of tumour DNA or RNA, prior to analysis with oligonucleotide probes (Scharf et al 1986). As shown by the recent reports from Forrester (1987) and Bos et al

(1987) on the increased frequency of ras gene activation in human colorectal cancer.

Significantly in Visvanathan's study, of four ras genes from human bladder carcinomas, all were shown to be c-Ha-ras-1 a finding consistent with that reported by Fujita studying urinary tract tumours. This frequency of Ha-ras activation contrasts with the rarity of H-ras involvement in other tumours, lending emphasis to the preferential activation of c-Ha-ras-1 in transitional cell neoplasia. Although other members of the ras gene family have been detected in established human urothelial cell lines (Tabin et al 1982, Reddy et al 1982, Valenzuela and Graffen 1986, Bos et al 1986) no K-or N-ras oncogenes have been detected in the primary tumours from this organ.

Of the seven H-ras oncogenes detected in urothelial tumours, four have been shown to carry a point mutation at codon 12, two at codon 61 and one at codon 13 (the first reported H-ras activation at this site in a solid human tumour). The four cases with a codon 12 mutation have a glycine to valine substitution, a common event for many bladder carcinogens (Svenson and Kadlubar 1981), suggesting a possible carcinogenic pathway in these cases.

Section 3

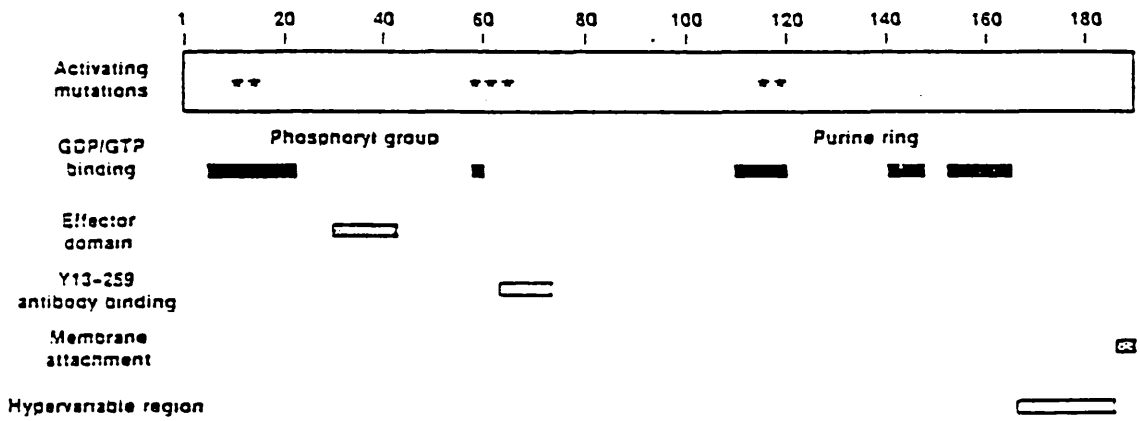
THE ras GENE

Mammalian ras genes have been highly conserved during evolution with only a 1% divergence in the protein sequence between mouse and human (Brosman and Liu 1987). The human ras gene is organised with a coding region of four exons and three introns (Capon et al 1983), and codes for a 21 kilodalton guanosine triphosphate binding protein (p21) which is localised to the inner surface of the cell membrane and related to the G proteins, suggesting a role in growth-signal transduction (for review: Barbacid M 1987). The Ha-ras gene encodes for a single protein but alternative splicing patterns can generate two Ki and possibly two N-ras genes (McCoy and Weinberg 1986).

Comparison of the deduced amino acid sequences between the mammalian ras gene proteins has helped to define four domains within the 188 amino acid p21 protein molecule (Figure I.1). The first domain encompasses one third of the amino terminal and is highly conserved where the first 85 amino acids of the mouse and human ras genes are identical. The next 80 residues show 85% homology. The rest of the molecule is highly variable and deletion of this region in activated p21 does not affect their transforming activity (Willumsen et al 1985). The final domain is the carboxy terminal sequence, including a conserved cysteine 186 residue, which is responsible for the attachment of p21 to the cell membrane.

FIGURE 1.1:

SCHEMATIC REPRESENTATION OF THE STRUCTURAL AND FUNCTIONAL DOMAINS DEFINED WITHIN MAMMALIAN p21 RAS PROTEINS:



(Barbacid M 1987)

The processing of p21 to the mature form, as detected by the increased migration rate in SDS-PAGE, involves the acylation of Cys^{186} by palmitic acid (Fujiyama and Tamaroi 1986) and research has shown that this processing is a prerequisite for the biological function of the ras protein. Mutants lacking the Cys^{186} residue remain in the cytosol and are unable to transform NIH/3T3 cells (Weeks et al 1985).

THE ras p21 PROTEIN

Before the finding of activated ras genes in human tumours, it was already known from research, into the Harvey and Kirsten murine sarcoma retroviruses that all cells express endogenous ras proteins. Indeed, much of the intrinsic biochemistry on the ras p21 was already known. The p21 protein is synthesised as a cytoplasmic precursor of 23 kilodaltons before being processed into the membrane bound mature form. In particular, it was known that the viral ras p21 encoded for a protein localised to the inner surface of the cell membrane, bound the guanine nucleotides, GTP and GDP, and in addition, possessed intrinsic GTPase activity (Shih et al 1980). Activation of the cellular gene has been shown not to affect nucleotide binding or membrane attachment, although the mutant proteins do possess altered electrophoretic mobility on SDS gels, reasons for which remain unclear (Finkel et al 1984, Der and Cooper 1983). However, the intrinsic GTPase activity is severely reduced in the

mutant proteins activated at codon 12 or 61 (Gibbs et al 1984, McGrath et al 1984, Marre et al 1985).

G proteins are activated by the binding of GTP and are inactivated by the hydrolysis of GTP to GDP (Gillman 1984). It is therefore an attractive hypothesis that ras proteins, as they are structurally similar, bind GTP in the biologically active state and because of their reduced intrinsic GTPase activity, in the mutant p21, continue to remain active by failing to return to the inactive GDP bound state (Gibbs et al 1984, Barbacid M 1987). Although this model is still without proof, the experimental evidence suggests that inhibition of GTPase activity may be the consequence of ras gene activation.

Site directed mutagenesis generating all possible substitutions at codon 12 demonstrates a close correlation between transforming potential and the inability to hydrolyse GTP (Seeburg et al 1984). However similar experiments at codon 61 shows that reduced GTPase activity does not necessarily result in cell transformation (Der et al 1986).

A recent article describing the determination of the three dimensional structure of the c-Ha-ras p21 by crystallographic techniques has generated intense interest by providing a structural basis to the understanding of the biological function of ras (Vos et al 1988). This analysis is helping to identify the amino acid residues that participate in the binding and cleaving of GTP by p21. The crystal structure reveals nine looped regions

exposed at the surface of the protein, four are used for GTP and GDP binding and hydrolysis.

Most of the transforming proteins have single amino acid substitutions at one of a few key positions in three of the four loops (Figure I.2). A monoclonal antibody Y13-259 has been shown to inhibit morphological transformation, after microinjection, induced by activated ras proteins (Mulcahy et al 1985). The antibody recognition site on loop L4 is almost opposite the GDP binding site, thus the antibody binding may induce a conformational change in the structure resulting in a neutralising effect. Amino acid substitutions in the region between residues 35-40 reduce the transforming potential, without altering the GTPase activity or nucleotide binding (Sigal et al 1986), suggesting that this region is involved in the effector activity of the ras protein.

Exciting recent work has reported the isolation of a soluble protein called GTPase Activating Protein or GAP, the first molecule known to bind to p21 (Trahey and McCormick 1987). GAP associates with p21 in vivo and dramatically increases the GTPase activity, in vitro, of normal p21 but not that of the activated p21 proteins. Point mutational activations at codon 12 or 61 may result in the inability to bind GAP, so preventing GTP hydrolysis and the return to the inactive GDP bound state. (Adam et al 1988, Cales et al 1988).

FIGURE I.2.

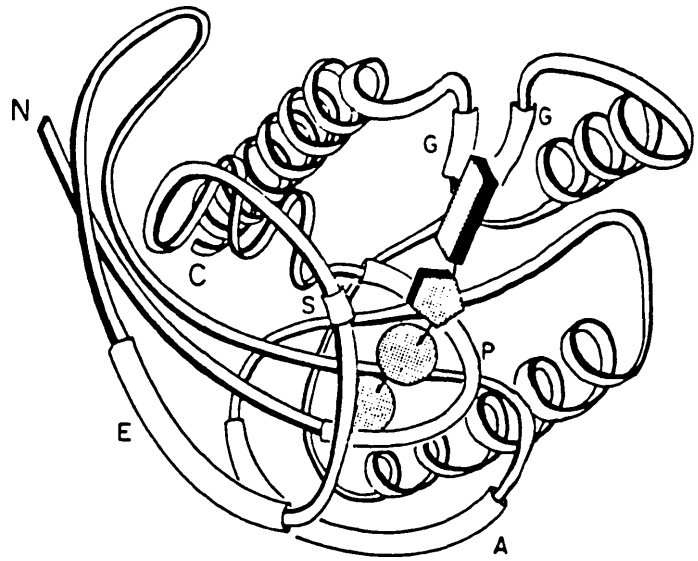
THE MOLECULAR STRUCTURE OF THE HUMAN c-Ha-ras p21 PROTEIN:

G: Guanine binding region,

E: Effector region,

A: Y13-259 neutralising antibody binding site,

C & N: Carboxyl- and Amino- terminals respectively.



From Science vol 239 p.892.

THE BIOLOGICAL FUNCTION OF ras ONCOGENES

The cellular function of mammalian ras genes is poorly understood, but current work suggests that activated p21 mimics the normal activity of the cellular protooncogene, rather than interacting with novel intracellular components, but evades a regulatory step involving the hydrolysis of GTP to GDP. The ability of oncogenic p21 to induce both neoplastic transformation and in some cases, terminal differentiation, suggests a possible role in both of these processes, which may be determined by the cell of origin.

Alternative approaches to transfection in studying the functionality of ras proteins has involved the microinjection of either purified p21 proteins or antibodies which have been shown to interact with the molecule in its native form. Early research demonstrated that microinjection of purified activated p21 proteins into mouse NIH3T3 cells induced morphological transformation, (Stacey and King 1984), demonstrating the ability of this protein alone, to interact with the normal cellular machinery. Conversely, microinjection of anti-p21 monoclonal antibodies, were able to induce transient reversion of the malignant phenotype of ras transformed rodent cells (Feramisco et al 1985). Using this approach a number of investigators have defined a role of ras p21 in cell cycle control demonstrating the requirement of cells for p21 to enter the S phase and progress through the cell cycle.

Microinjection of an antibody, specifically recognising p21, into cells stimulated to divide by the addition of serum resulted in their inability to enter the S phase of the cell cycle, thus inhibiting DNA synthesis and proliferation (Mulcahy et al 1985). From these studies it was concluded that ras gene products were an essential element of housekeeping genes involved in cell division.

The localization of p21 products to the inner surface of the plasma membrane raised questions as to its role in inducing alterations in surface membrane topography previously described in viral transformed cells. Microinjection studies reveal that both normal and mutant p21 produce surface ruffling and pinocytosis of rat embryo fibroblasts (Bar-Sagi and Feramisco 1986). The activity of normal p21 is short lived, whereas oncogenic p21 maintains its effect for several hours. Ruffling and pinocytosis are two characteristic membrane responses to certain hormones and mitogenic compounds. In preneoplastic urothelium the appearance of pleiomorphic microvilli and loss of the asymmetric membrane are representative of early changes in the carcinogenic process (Jacobs et al 1976) and have been observed in transfected rodent urothelial cell lines following the introduction of a ras oncogene (I.C. Summerhayes- personal communication).

The multistage model of carcinogenesis was realised at the molecular level with the discovery that an activated ras oncogene was insufficient to transform normal rat embryo fibroblasts in a one step process.

However, repetition of this experiment using immortalised fibroblasts or epithelial cells, considered preneoplastic, resulted in full transformation by the introduction of a ras oncogene (Land et al 1983, Ruley et al 1983, Newbold and Overell 1983). Conversion of primary embryo fibroblasts to tumorigenic cells was only obtained following co-transfection of a nuclear oncogene with the ras or polyoma middle T oncogene. These results suggest that at least two distinct functions are required in the transformation of primary cells, involving oncogenes that are localised to the nucleus and confer immortalisation, and those that are membrane bound and confer morphological transformation. Thus neoplastic progression requires cooperation of, at least two oncogenes, one from each group (Land et al 1983).

It is interesting to note, however, that in Land's original paper, transformation of primary cells by a combination of myc and ras resulted in tumours which grew to 1 cm but did not progress to kill the animal. In contrast, a polyoma large T and ras combination produced progressively growing tumors, suggestive of a third component necessary for full malignant transformation in the former case. One report by Spandidos and Wilkie (1984) describes the acquisition of tumorigenicity in primary hamster embryo fibroblasts following the introduction of an altered ras gene ligated to a strong transcriptional enhancer. This study stands alone, possibly reflecting the genetic instability of the system used, whereby secondary events can be elicited due to chromosomal

rearrangements from the transfection technique alone. Work with human cells furthers the concept of multiple events in carcinogenesis, since neither cellular nor retroviral ras oncogenes have been able to induce stable transformation of normal human fibroblasts (Sager et al 1983).

From the rodent studies it can be seen that an important prerequisite for transformation is the acquisition of an indefinite lifespan. The oncogene involved in this event cannot fully convert these cells to tumorigenicity but can cooperate with a second class of oncogenes to achieve this endpoint.

Immortalisation of human cells has been achieved using the SV40 virus or ^{60}Co g rays (Rhim et al 1985, Namba et al 1986). Transformation in vitro of human cells by SV40 routinely imparts many of the phenotypic traits associated with transformed cells including altered morphology, karyotypic changes, immortalization and anchorage independent growth (Tooze 1981). After an initial period of accelerated growth, human epithelial cells and fibroblasts, transformed by SV40, enter a crisis phase, with extensive cell lysis and death from which recovery is variable (Girardi et al 1965, Sack 1981).

Recently, normal human urothelial cells were transformed by SV40 in vitro resulting in a nontumorigenic immortalised cell line (Christian et al 1987). This line has great potential as a model for studying human urothelial neoplastic progression and the role of additional oncogene activations.

In addition to their transforming properties, ras activation can also induce terminal differentiation of a rat phaechromocytoma cell line (PC12). PC12 cells can be induced to differentiate into neurone-like cells after microinjection of oncogenic p21 and this effect can be inhibited by microinjection of anti-ras antibodies (Bar-Sagi and Feramisco 1985, Noda et al 1985). These results are similar to those induced by Nerve Growth Factor (NGF) and it seems likely that the ras oncogene activity may involve a signalling pathway shared by NGF.

Information on the mechanism of action of oncogenes has suggested that ras p21 proteins may act through changes in inositol lipid metabolism, which precedes cell proliferation. The normal pathway commences with the activation of cell surface receptors and the subsequent breakdown of phosphatidylinositol-4,-5 diphosphate (P1P2) to 1,2,deacylglycerol (DAG) and inositol triphosphates (Berridge and Irvine 1984). DAG activates protein kinase C which directs the phosphorylation of serine and threonine moieties in a variety of cell proteins, which, in turn, control many normal cell functions. Ras transformed fibroblast cell lines demonstrate a x2 fold increase in DAG and triphosphate levels compared to their untransformed counterparts, implicating p21 in the phosphoinositide metabolism signalling pathway (Fleischman et al 1986).

Similarly Wakelam et al (1986) demonstrated that normal N-ras p21 encodes a protein which couples bombesin and other growth factor receptors to inositol phosphate

production suggesting that ras p21 might be the G protein of phospholipase C stimulation.But the whole question has been thrown open by the loss of stimulation of phospholipase activity in ras transformed cells (Benjamin et al 1987) and that direct measurement of phospholipase C activity in ras cells does not support the earlier associations made in previous studies (Yu et al 1988).

IN VIVO MODELS OF RAS ACTIVATION

Although ras genes are the most frequently identified oncogenes in human cancer. There is controversy as to whether ras activation is an early event in neoplastic regression or a secondary and later consequence of tumour development. To address these questions several in vivo animal models have been utilised which result in reproducible activation of ras genes (Table I.6).

Previous work showing activation of the ras proto-oncogene by a chemical carcinogen in vitro demonstrates that the reaction of an ultimate carcinogen with DNA alone, can lead to the induction of mutations (Vousden et al, 1986). This concept has been extended to the animal model whereby the administration of a chemical carcinogen results in ras gene activation via base changes consistent with the known chemical action of the carcinogen.
In these cases, analysis of the neoplastic lesions show the same activating event in the majority of the tumours with preferential activation of one ras gene member.

TABLE I.6:

ACTIVATION OF ras ONCOGENES IN CARCINOGEN-INDUCED ANIMAL TUMOURS:

Species	Tumour	Carcinogen	Oncogene	Incidence
Rat	[1] Mammary	NMU	Ha-ras-1	100%
		DMBA	Ha-ras-1	23%
	[2] Kidney	DMN	Ki-ras-2	40%
Mouse	[3] Skin	DMBA	Ha-ras-1	90%
	[4] Mammary	DMBA	Ha-ras-1	100%
	[5] Lymphoma	NMU	N-ras,K-ras-2	81%
		X rays	N-ras,K-ras-2	57%
	[6] Hepatic	HOAAF	Ha-ras-1	100%
	[7] Bladder	DMBA	Ki-ras-2	90%

[1] Zarble et al 1985

[2] Sukumar et al 1986

[3] Quintanilla et al 1986

[4] Dandekar et al 1986

[5] Guerrero et al 1984

[6] Wiseman et al 1986

[7] Brookes et al 1988

One of the best characterised models exemplifying these points is that of breast carcinoma in Buf/N rats induced by a single dose of Nitrosomethylurea (Zarble et al 1985). In this system an altered H-ras gene was identified in all the tumours studied, activated by a codon 12 point mutation changing glycine to glutamic acid. In contrast, no such mutations were observed in mammary carcinomas induced by Dimethylbenz(a)anthracene (DMBA) in the same strain.

However, in the reported cases of ras activation, different carcinogens can clearly induce tumours by different routes of transformation. Similarly, the same chemical carcinogen may act through alternative routes in different organs of the same species. In addition activation of the same ras gene member is repeatedly observed in these models.

Dimethylbenz(a)anthracene in the mouse skin system initiates the specific activation of the H-ras-1 gene by an A - T transition in the second base of codon 61 (Quintanilla et al 1986), and the frequency of this mutation was dependent upon the initiating agent, not on the promotor, indicating that the mutation occurs at the time of initiation. DMBA in the mouse bladder results in activation of a K-ras gene, in contrast to the skin system, illustrating that the same carcinogen in the same animal but acting on a different organ can yield different results. Thus the complexity of these models is a significant problem.

Activation of ras oncogenes by initiating carcinogens implies that these oncogenes must participate in neoplastic progression, and the mouse skin model has been used to correlate ras activation with the timing of biological events in carcinogenesis. Balmain (1983) demonstrated that DNA, from squamous cell carcinomas, initiated with DMBA and promoted with TPA, resulted in the morphological transformation of NIH/3T3 cells due to an activated c-Ha-ras-1 gene. Subsequently he was able to show that the activated H-ras gene could be found in premalignant papillomas, induced by DMBA, as detected in transfection assays (Balmain et al 1984). In this case, most of the papillomas are polyclonal and promotor-dependent, regressing if treatment is interrupted. Hence ras seems to be the neoplastic initiator since even if promotion occurs after a long latent period, then papillomas will develop within four weeks (Brown et al 1986). These results support the concept that ras can initiate abnormal growth but that tumorigenicity seems to require further mutational events.

Using an alternative approach, Roop (1986) introduced the viral Ha-ras gene into cultured keratinocytes via a retroviral vector, selecting for cells expressing the exogenous gene and grafting these cells onto the backs of recipient nude mice. these grafts produced benign tumours after several weeks, in all cases where a positive graft take occurred. In a similar study, Yuspa's group, (1988), showed that grafts of immortalised keratinocyte cell lines, containing an activated ras gene with a c61

mutation, resulted in papilloma growth. In the human, they reported H-ras activation at c61, in a benign skin tumour (keratoacanthoma) which is able to undergo complete spontaneous regression, furthering the concept that ras oncogenes are contributory but not sufficient in maintaining the neoplastic phenotype (Leon et al 1988).

Transgenic Mice:

Clearly, in not all animals is it possible to perform reconstitution experiments, such as those outlined in the previous section, with the added advantage, in the skin of, monitoring events without recourse to surgery. A second, much more demanding approach, has been developed involving the production of transgenic animals. In vitro experiments have been useful both in evaluating the activity of known genes and in identifying novel oncogenes, but they preclude the normal physical and biological interactions that occur in vivo, controlling growth, cellular differentiation and tissue organisation. To overcome these limitations, researchers have turned to the transgenic model whereby cloned genes are microinjected into fertilised eggs resulting in offspring bearing the gene in all somatic and germinal cells (Cuthbertson and Klintworth 1988).

Gene expression is usually controlled by ligating the gene to a tissue specific promotor or enhancer.
This approach has proven particularly useful in studying the role of oncogenes in neoplastic progression. Initial experiments, demonstrated that transgenic mice bearing the

c-myc oncogene, ligated to a lymphoid specific immunoglobulin heavy chain enhancer, developed lymphomas in 13 of 15 animals within 3 months of birth (Adams et al 1985). Leders group (1986) looked at the consequences of an activated c-myc gene, under the control of a mammary tumour virus promotor, being expressed in a variety of tissues. Results showed that the deregulated gene contributed to the stochastic development of tumours in a variety tissues (mammary, testicular, lymphocytic and mast cell origin) but as transformation was not seen in all tissues expressing the fusion gene, additional events appear necessary. Especially as the mean age of tumour development in the animal was fourteen months.

Since these tumours appear to arise in a manner consistent with the recruitment of additional oncogenes, Sinn et al (1987) tested the activity of c-myc with that of v-Ha-ras, in view of the complementation seen in the rodent primary fibroblast model (Land et al 1983). Transgenic mice carrying the v-Ha-ras oncogene under the control of an MMTV promotor developed benign hyperplasia of the Harderian gland of the orbit. Subsequently in these animals, malignant mammary tumours arose stochastically over a 6 to 12 month observation period. Similarly a companion transgenic strain expressing the MMTV/c-myc gene developed stochastic tumours after a greater latency period. If the MMTV/v-Ha-ras and MMTV/c-myc strains are crossed to yield hybrid mice then there is a dramatic and synergistic acceleration of tumour development. 50% of the female animals develop mammary

tumours within 46 days and no animal is tumour-free beyond 160 days. Even so, the fact that adjacent non-malignant tissue expresses both the oncogenes and the stochastic nature of the tumours, suggest that other somatic events are still necessary for their full malignant progression. This approach has now been extended to include a number of different oncogenes, a review of which is to be found in Cuthbertson and Klintworth (1988) but is beyond the scope of this thesis.

RAS ACTIVATION: A Late Event in Neoplastic Progression

The previous section has laid emphasis on ras activation in the early stages of carcinogenesis, playing a role in the generation of benign lesions in human and mouse skin. One of the early studies on the role of the ras oncogene in neoplasia demonstrated that ras activation was associated with the acquisition of metastatic potential in a rodent lymphoid cell line (Vousden and Marshall 1984). In contrast Albino et al (1984) reported that, of five cell lines derived from a patient with metastatic melanoma only one contained an activated ras gene suggesting that ras activation may not be involved in tumour progression. Hence raising questions as to the timing of ras activation in neoplastic progression and the answer to this dilemma is still unresolved but probably is true for both events.

Until recently it has not been possible to systematically induce metastatic behaviour in cells. However, the ras oncogene can induce such behaviour for

although the H-ras oncogene was originally identified by inducing cellular transformation and tumorigenicity, it has been shown in many experiments to induce the metastatic phenotype. Thus, allowing important insight into the mechanisms of metastatic progression.

It has been difficult to determine suitable in vivo assays for metastatic behaviour, but essentially there are two main types (for review see Nicholson and Poste 1983). The spontaneous assay, involves s.c. injection of cells, tumour formation, and at a later date the animals are evaluated for metastasis. In the experimental metastasis assay, the cells are injected i.v. and the resultant lung nodules are "indicative" of the cells metastatic potential. As metastasis involves both the exodus of cell from the primary tumour and the formation of secondary tumours at distant sites, it may appear that the experimental assay has little in common with the actual mechanism of metastasis. In fact the results often, but not always, correlate with the results obtained in the spontaneous assay (Stackpole 1981). These two assays have been the main mechanisms for evaluating metastasis in animal models.

Research has repeatedly shown the independence of metastatic potential from tumorigenicity, thus implicating a separate role for the ras gene at different stages in neoplastic progression. Initial experiments showed that cloned H-ras oncogenes transfected into NIH/3T3 cells can result in metastases in nude mice (Thorgeirrson et al

1985). Muschel et al (1985) isolated clones of NIH/3T3 cells transformed with the EJ gene where all were metastatic in the experimental assay. These results were confirmed by Egan et al (1987) who demonstrated that metastatic potential, in both the spontaneous and experimental assays, was proportional to the level of p21 in the transformed cells. Elevated levels of the normal protooncogene do not result in cellular transformation, but linking the proto-oncogene to the long terminal repeat of the Moloney leukaemia virus, a powerful promotor and transcriptional enhancer, could transform NIH/3T3 cells (Chang et al 1982). These transfectants were tumorigenic, with greatly elevated levels of normal p21, but were non-metastatic in these assays (Muschel et al 9185). Thus metastatic potential seems to correlate with the level of ras expression and the threshold level of H-ras required for tumorigenicity appears to be less than the p21 level necessary for metastasis. Further confirmation from Egan was shown by linking the H-ras oncogene to a steroid responsive promotor. Pretreatment with Dexamethasone more than doubled the number of metastases (Egan et al 1987).

As a consequence of their susceptibility to transformation, their aneuploidy and other features, it is felt that results with NIH/3T3 cells may not be extrapolated to other cell types (Muschel and Liotta 1988). Rat embryo cells co-transfected with an H-ras oncogene and, either a dominant selectible marker (pSV2neo) or by linking to an SV40 enhancer, are also highly metastatic in both assays (Pozzati et al 1986).

However if the H-ras gene is co-transfected with the adenoviral oncogene, EIA, the resultant cells were never metastatic.

One explanation is that the intrinsic capacity of the cells to secrete Type IV collagenase has been altered (Muschel et al 1986). Type IV collagenase degrades Type IV collagen in the basement membrane, and this activity has been implicated in metastasis (Liotta et al 1986). Metastatic cells secrete elevated levels of this enzyme and the co- transfected rat embryo cells of high metastatic activity secrete easily detectable amounts of this enzyme, whilst cells co-transfected with the H-ras and EIA virus secrete only baseline levels, correlating with their low metastatic potential.

Further confirmation of the ability of the H-ras oncogene to induce metastatic behaviour has derived from experiments using tumorigenic but non-metastatic cell lines. Vousden et al (1986) introduced an activated ras oncogene into a mouse mammary carcinoma cell line with an increase in the metastatic potential compared to the parent line. More convincingly they isolated the metastases and one was discovered to have lost the expression of the oncogene. Retesting these cells gave results indistinguishable from the parent line, confirming that the activated ras gene was necessary for the acquisition of metastatic potential. It is evident from a number of studies that human cells differ from their rodent counterparts in their response to cloned oncogenes (Sager et al 1985) both in their tumorigenic potential and

in the production of different growth factors.

As yet, the role of ras involvement in the development of human metastases is unclear. The data suggests that ras may play a role but that other events appear to be necessary in the development of metastatic potential (Muschel and Liotta 1988).

Section 4

OUTLINE OF QUESTIONS AND APPROACHES

Ras gene activation has been reported in a wide variety of human tumours including 90% of lung adenocarcinomas, 40% of colon tumours and 27% of acute myeloid leukaemias. In addition, each of these studies demonstrates preferential activation of one member of the ras gene family, where K-ras activation is found almost exclusively in pancreas, colon and lung tumours, whilst N-ras activation is associated with acute myeloid leukaemia. The timing of ras activation has been shown to be an early event in both colon and skin tumours having been detected in preneoplastic lesions in these organs. In contrast, experimental studies have demonstrated a role of ras onco genes in metastasis, enhancing the escape of cells from primary tumours at later stages of carcinogenesis. Although these two findings may seem contradictory, they may both be correct, demonstrating a different role of the activity of the same gene dependent upon the timing of the activation. Even though large numbers of tumours have now been screened for ras activation, no clear picture has emerged regarding the timing and consequence of ras activation in different tumour types. The finding of preferential activation of one ras gene member in particular tumours, is intriguing and suggestive of an alternative role for individual ras gene members in different epithelial cell types. From the limited data

available it would appear that preferential expression of one ras gene member in different epithelial cell types is not the case, eliminating the simplest explanation of the molecular events reported. It is evident from this synopsis that the molecular and cellular events linked to ras gene activation remain unclear, and it is extremely difficult to extend any current observations to a clinical level. However, it is clear that ras gene activation can contribute to neoplastic progression, and as studies continue with larger tumour numbers, the possible clinical significance of this event may be determined.

My aim in this thesis is to try and address some of the aforementioned questions, with specific reference to bladder cancer, by developing models and techniques which will prove useful in elucidating the role of c-Ha-ras-1 in bladder cancer and are also applicable to other genes which may be implicated in the future. With this in mind we have addressed the following questions:

1. Is there a suitable screening assay to detect c-Ha-ras-1 gene activation in human urothelial tumours: Chapter 3.
2. Can one devise a suitable urological model for the in-vivo behaviour of urothelial cell lines: Chapters 4 and 5.
3. Can c-Ha-ras-1 confer metastatic potential or human urothelial cell lines: Chapter 6.
4. Do Oncogenes play a role in urothelial neoplastic progression: Chapter 7.

CHAPTER II

GENERAL MATERIALS AND METHODS

All the work described in this thesis was carried out in the Laboratory of Cancer Biology, Harvard Medical School, Boston, USA. In this chapter only the general materials and methods are detailed as the specific methodology is contained in the relevant chapters.

Mice: 2 types were used in the in-vivo model experiments and obtained from Taconic, Germantown NY.

a) C57/Bl6 males 8 - 10 weeks.

b) Athymic Swiss Nu/Nu males 8 - 10 weeks.

The mice were kept in plastic cages in a room maintained at 21°C and fed and watered ad libitum.

Cell Culture:

A panel of murine urothelial cell lines, established following exposure to different chemical carcinogens (Summerhayes and Franks 1979, Summerhayes and Chen 1982) were used in the in-vivo model experiments.

DMBA induced: MB 331
MB 48A
MB 48B
MB 49
MB 49B

BBN induced: BBN3
SH 264

All cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island NY) supplemented with 7.5% fetal calf serum (Hazleton, Denver PA), Penicillin and Streptomycin (Gibco).

Antibodies: Antibodies to total keratins, Type IV collagen and fibronectin were the generous gifts of Dr. M.J. Warburton, Ludwig Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey. Antibody D66 stains basal epithelial cells and previous studies have shown an increase in the basal cell compartment in early neoplastic lesions (Summerhayes and Chen 1982). All these antibodies were raised in rabbits and used at 1/1000 dilution in phosphate buffered saline (PBS).

Lipofection Assay:

A wide variety of methods have been used to facilitate the entry of DNA into cells, all of which suffer from one or more problems related to either cellular toxicity, poor reproducibility, or inefficiency of DNA delivery. The Lipofection technique described by Felgner et al (1987) utilises a cationic lipid that forms liposomes which interacts with DNA, fuses with tissue culture cells, and facilitates the delivery of functional DNA into the cell. This technique is simple, highly reproducible, and more efficient than certain other, commonly used proceedures.

Lipid-DNA complex:

A typical transfection used 1ug of DNA in 1.5ml serum free media with 100ug of lipid in 1.5 ml serum free media, according to the following protocol:

Treatment of Cells:

2x100mm tissue culture plates (confluent)

Wash x2 PBS

3ml lipid-DNA complex

Incubate 5 hours

Add 5ml DMEM + 10% FCS (16hrs)

Replace with fresh media

48 hours post transfection, passage the cells, and grow in selective medium for Neomycin resistance (0.2mg G418/ml)

It is important that the cells are just attaining confluency when transfection occurs, to avoid problems with toxicity from the Lipid-DNA complex.

CHAPTER III

H-ras ACTIVATION IN HUMAN BLADDER CANCER:

Screening by Western Blot Analysis.

H-ras ACTIVATION IN HUMAN BLADDER CANCER

H-ras p21 protein expression was investigated in bladder and colonic tumour tissue using an H-ras specific antibody in Western blot analysis. The specificity of this antibody to H-ras proteins was established using NIH\3T3 transfectants expressing oncogenic counterparts of the different ras gene family members. Use of this antibody to detect altered H-ras proteins was demonstrated using a panel of transfectants bearing different mutated H-ras genes and established cell lines previously characterised in transfection assays. Extension of this technique to the direct analysis of human tumour material confirmed previous observations of H-ras activation within a group of bladder tumours and identified three more urothelial tumours expressing altered H-ras proteins. The altered migrational properties of these three were suggestive of point mutational events in 12 (1-case) and 61 (2-cases) codon hotspots. This study extends previous observations on the preferential activation of H-ras in urinary tract tumours and provides a rapid technique for evaluating the status of H-ras proteins in human tumour tissue.

Since the report of the first ras oncogene, activation of different members of the ras gene family have been found in almost all tumour types studied (for review see Nishimura and Sekiya 1987). Ras activation represents the most frequently reported genetic alteration associated with human tumours. However, despite the detailed molecular and biochemical knowledge of the ras gene members and their protein products, the role of

altered ras genes in human neoplasia remains unclear (Nicholson 1987). It is known that ras gene activation can occur following a point mutational event within codon 12, 13 or 61 resulting in an amino acid change which confers transforming potential on the protein product.

In this way the altered 21,000 dalton protein (p21) displays decreased GTP'ase associated activity (McGrath et al 1984, Sweet et al 1984, Gibbs et al 1984, Temeles et al 1985, Der et al 1986), a characteristic felt to be causative in the ras transformation pathway. The normal p21 protein has been shown to be a guanine triphosphate-binding protein, related to the G proteins which are involved in cellular pathways of signal transduction. Recent reports suggest that the binding of GTP to p21 is mediated by an intermediary protein termed GAP (Trahey and McCormick 1987, Adari et al 1988, Cales et al 1988) and that the decreased hydrolysis of GTP to GDP results in a prolonged association of p21 and GTP maintaining the stimulation of an, as yet, unidentified signalling channel.

Point mutations at different codon hotspots are localised to specific regions of the protein involved with aspects of GTP binding (McCormick et al 1985). Ras oncogenes were initially identified using the NIH/3T3 transfection assay, a labour intensive technique which has recently been succeeded by a procedure utilizing oligonucleotide probes to amplify and characterize ras gene alterations directly from tumour DNA. Using this approach different investigators have shown the frequency

of ras activation to be higher than was originally shown in transfection assays, reporting altered ras genes in 90% of exocrine pancreatic carcinomas (Almoguera et al 1988), 50% of lung adenocarcinomas (Rodenhuis et al 1987), 40% of colon tumours (Bos et al 1987, Forrester et al 1987) and 27% of acute myeloid leukemias (Farr et al 1988).

In studies of colon cancer, ras activation was found in premalignant lesions suggestive of an early event in neoplastic progression. In addition each of these studies demonstrates preferential activation of one member of the ras gene family where K-ras activation was found almost exclusively in pancreas, colon and lung tumours, whilst N-ras activation was associated with acute myeloid leukaemia. Using both transfection and the polymerase chain reaction techniques we have recently reported preferential activation of H-ras in bladder carcinomas (Visvanathan et al 1988), consistent with previous reports involving urinary tract tumours (Fujita et al 1985, Fujita et al 1984). Using this same group of tumours we have assessed the feasibility of identifying altered ras proteins in Western blot analysis directly from tumour lysates, combined with the use of an H-ras specific polyclonal antibody. This approach exploits the altered migrational properties of mutated p21 proteins in SDS-PAGE electrophoresis (Der and Cooper 1983, Fasano et al 1984, Seeburg et al 1984, Srivastava et al 1985). This study confirms previous results obtained using transfection assays and reports on three additional H-ras activations in bladder tumours.

MATERIALS AND METHODS

Preparation of tumour specimens:

Tumour samples from forty-five patients were randomly selected and snap frozen in liquid nitrogen prior to storage at -70°C. Frozen tissue was embedded in OCT compound and sections were cut to review the tumour content of the material. Carcinomatous areas were identified, excised from the block and spun in phosphate buffered saline prior to lysis. Approximately 0.1 gm. of tumour was homogenised in cell lysis buffer (0.01M sodium phosphate pH 7.4, 5mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 0.1M sodium chloride, 0.2mM PMSF, 0.1M leupeptin), spun in a microfuge for ten minutes followed by removal of the supernatant. A 10ul aliquot of the supernatant material was taken to determine the protein concentration using the BSA protein assay (Pierce, Rockford, Ill.) and to the remaining lysate one fifth the volume of GSD (33% glycerol, 20% SDS, 0.3M dithiothriotol) was added. The lysates were frozen on dry ice and stored at -70°C. Total cell lysates from each tumour were standardised for protein concentration and 100ug of each was loaded into separate lanes of a 12.5% polyacrylamide gel.

Western blot proceedure:

Gels were run overnight at 50 volts and continued the full length of a 12.5% polyacrylamide gel measuring nineteen centimetres. Prestained molecular weight markers of 12,400 (cytochrome c) and 43,000 (ovalbumin) were used

to monitor the progress (Diversified Biotech). At the completion of the run, gels were equilibrated in deionized water prior to overnight transfer to nitrocellulose. Gels were cut at the 43,000 marker and only the lower half was transferred. Successful transfer of low molecular weight proteins was gauged by the visual presence of the prestained 43,000 MW marker on the nitrocellulose.

Following transfer, the nitrocellulose was initially blocked in TBS buffer (10mM Tris, 150mM NaCl pH 7.5) containing 1% gelatin for one hour. Each blot was incubated with the H-ras specific peptide antibody (Oncor, Gaithersburg Md.) at a 1 in 100 dilution in TBS-Tween 20 (0.5% v/v), overnight at room temperature. The nitrocellulose was washed in several changes of TBS-Tween 20 and then incubated with a phosphatase labelled second antibody. Following further washes in TBS-Tween 20, the blots were developed using the BCIP/NBT phosphatase substrate system (Kirkegaard and Perry Labs. Gaithersburg Md.). Each Western blot was run with a normal and altered H-ras control to standardise variations observed between different electrophoresis runs.

RESULTS

A total of forty-five human tumours have been used in this study, thirty bladder and fifteen colon carcinomas. Eighteen of the bladder tumours were previously screened in a modified NIH\3T3 transfection assay in which four were shown to contain a mutated H-ras proto-oncogene. In all cases, frozen sections of stored material were assessed and lysates were prepared directly from tumour material excised from the block. All lysates tested in Western blot analysis represent a comparable level of protein material from tumour tissue.

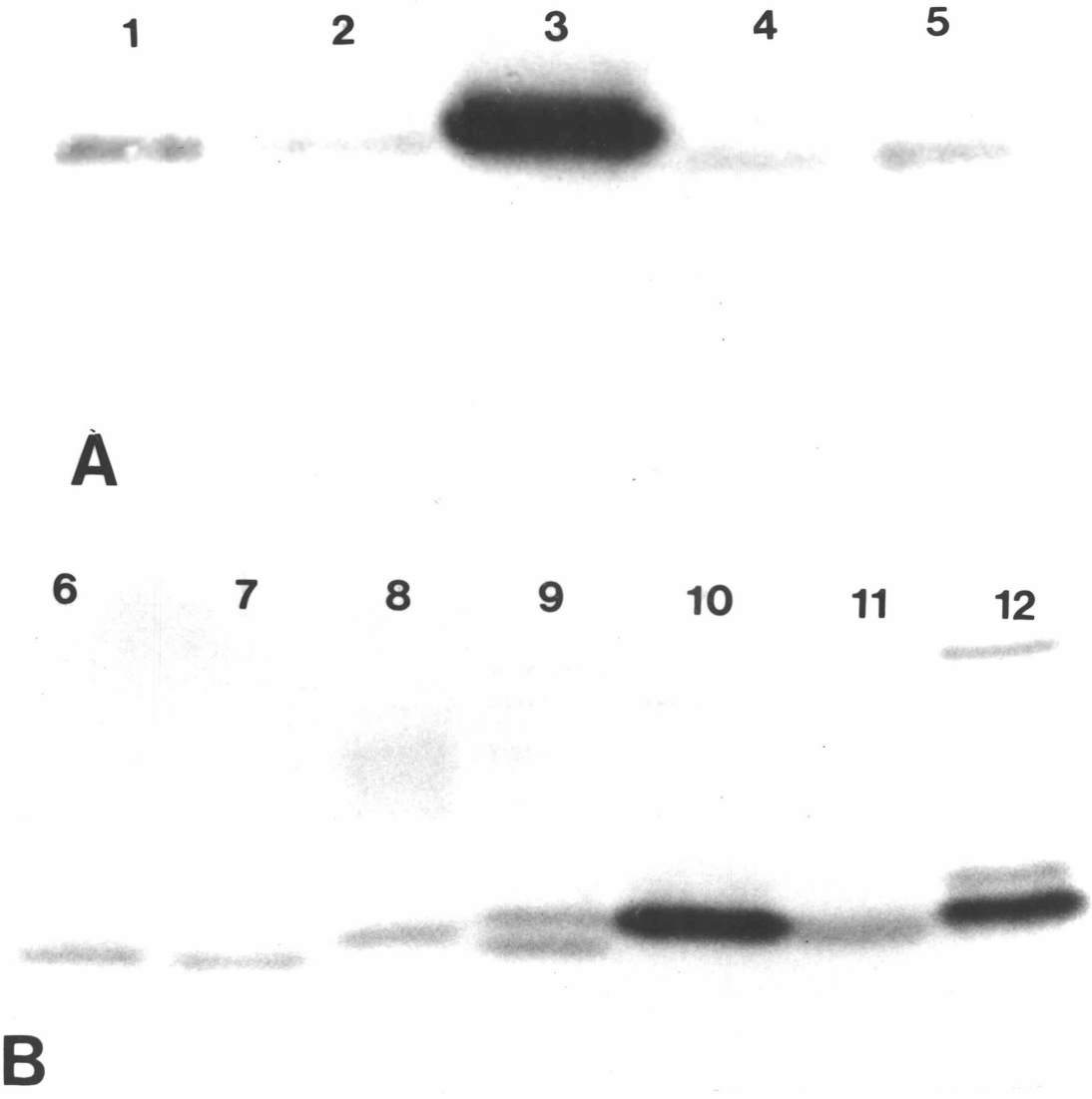
Antibody specificity to H-ras proteins:

Although extensive sequence homology exists between different members of the ras gene family, significant divergence has been reported within a variable region near the carboxy-terminus of the protein. Peptide sequences derived from this region and unique for particular ras gene members have been synthesized and used to raise antibodies designed to recognize a specific individual member of the ras protein family. One such antibody, raised to an H-ras specific peptide, was used in this study. The specificity of the antibody was initially confirmed in Western blot analysis using lysates from NIH/3T3 transfectants expressing human ras proteins from H,K and N-ras oncogenes. Figure III.1A shows one such Western blot where the mutated H-ras p21 (lane 3) is discernible from the endogenous H-ras protein (lane 4) by it's slower migration in this assay.

FIGURE III.1:

SPECIFICITY OF THE H-ras ANTIBODY IN THE DETECTION OF THE ALTERED H-ras p21 PROTEIN PRODUCT:

Normal H-ras codes for Glycine (c.12) and Glutamine (c.61)



Lanes 1-12 show Western Blots of NIH/3T3 transfectants expressing ras genes with known coding sequences. Lane 1. normal H-ras (gly-codon 12); 2. K-ras (val-12); 3. H-ras (val-12); 4. NIH/3T3 endog-enous H-ras; 5. N-ras (lys-61); 6. H-ras (leu-61); 7. H-ras (arg-61); 8. H-ras (his-61); 9. H-ras (lys-61); 10. H-ras (gly-12); 11. NIH/3T3 endogenous H-ras; 12. H-ras (arg-12).

The K-ras oncogene in this experiment is activated by the same mutational event as the H-ras gene and yet a slower migrating band was not apparent (lane 2). The N-ras oncogene product migrates more rapidly than normal p21 and yet also remained undetected by this antibody probe (lane 5). The p21 bands observed in the other lanes represent endogenous levels of H-ras p21 with a normal migratory pattern.

The potential use of this antibody to detect altered ras proteins in SDS-PAGE was initially evaluated using 3T3 transfectants expressing known H-ras oncogenes, activated by point mutations at either codon 12 or 61.

Figure III.1B shows a Western blot with such a panel of transfectants displaying p21 proteins with aberrant migrational properties compared to the normal p21 protein (lanes 1,4 and 11). Consistent with previous reports, p21 proteins resulting from codon 12 mutations migrate more slowly than the normal (3 and 12) and codon 61 more rapidly (lanes 6 - 9). Migrational differences between altered p21 products generated by mutations within a particular codon can be discerned in this gel system.

These experiments demonstrate the specificity of the antibody in recognizing H-ras proteins and the practicality of detecting altered p21 proteins in this system.

H-ras proteins in established cell lines:

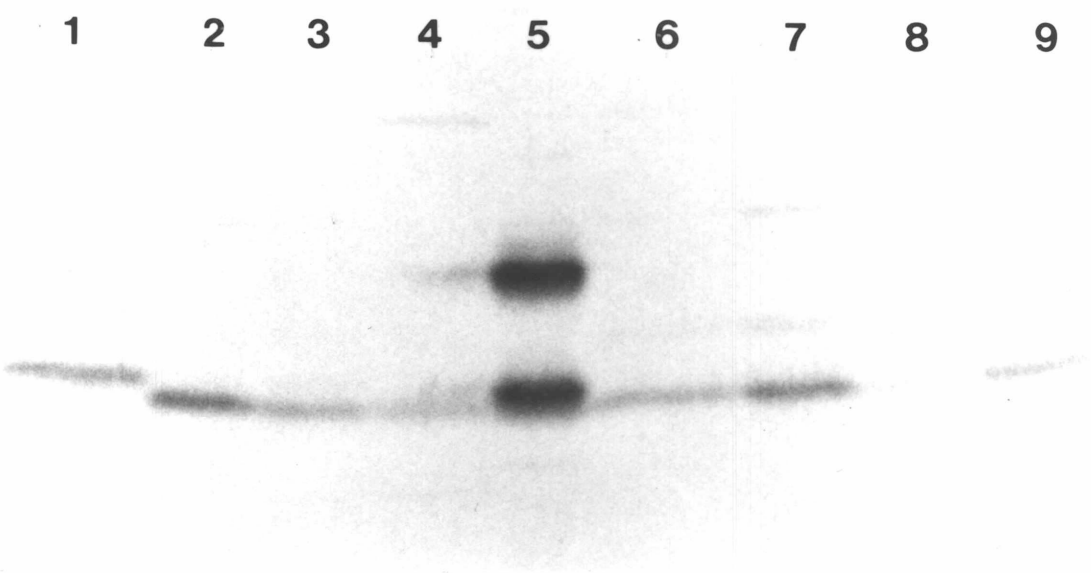
Since the isolation and characterization of the first altered human ras gene was reported from a bladder cell line(EJ/T24) many urothelial cell lines have been screened in transfection assays. In nearly all cases no activated transforming gene was detected, with the exception of HT1197, prompting the conclusion that ras activation is an infrequent event in bladder neoplasia. In contrast, recent reports have demonstrated the occurrence of K-ras mutations in 40%-50% of colon carcinomas studied, confirming early trends observed in established lines from colon tumours.

The expression of ras oncogenes in 3T3 transfectants is frequently elevated over that observed in the original cell where the mutated gene can sometimes be found to be heterozygous at this locus. In addition it is apparent from experimental studies that only small amounts of the altered p21 product is required to elicit the transformed phenotype.

Since these factors will play a role in the required sensitivity of the system we have studied a panel of tumorigenic cell lines, all of which have been previously screened in transfection assays where three contained activated ras genes. Figure III.2 shows an example of eight cell lines in Western blot analysis, with EJ (lane 1) displaying a slow migrating p21 characteristic of a p21 product derived from a ras gene with a codon 12 mutation. One bladder (HT1197) and one colon cell line (SW480) have altered N-ras (codon 61) and K-ras (c.12) respectively.

FIGURE III.2:

REPRESENTATIVE PANEL OF HUMAN BLADDER AND COLON CELL LINES PROBED IN WESTERN BLOT ANALYSIS WITH AN H-ras SPECIFIC ANTIBODY.



Lane 1. EJ; 2. HT1197; 3. RT4; 4. PS-1; 5. NIH/3T3-EJ-RAS control; 6. SW-480; 7. RT112; 8. DLD-2; 9.CCL-220.

These genes are not detected by this antibody (Fig: III.2 lane 2 & 6) but can be visualised on replica gels probed with a pan-reactive ras antibody (data not shown). The specificity and sensitivity of this assay is clearly sufficient to detect low level expression of H-ras proteins in this system.

Screening of human tumour tissue

Extension of this study to human tumour tissue required an assessment of the tumour content of stored material prior to the preparation of lysates. In most cases of bladder cancer >90% of the tissue was tumour, however, in those cases where substantial mesenchymal components were present (i.e. colon cancers), carcinomatous regions were excised and a range of protein quantities were analysed.

In the first instance we studied eighteen bladder tumours, from a group previously analysed in a modified 3T3 transfection assay, where four cases had been identified as having activated c-H-ras-1 genes (Visvanathan et al 1988) (Fig. III.3). In tumour material from two of these cases altered H-ras p21 products were detected as migrating more slowly than the normal p21 band, consistent with the genetic data derived from these tumours showing substitution of a glycine with a valine at codon 12. In a third tumour, known to harbour the same change, no altered p21 species was observed. Further analysis of this tumour lysate, using higher protein concentrations, did not result in the resolution of an

FIGURE III.3:

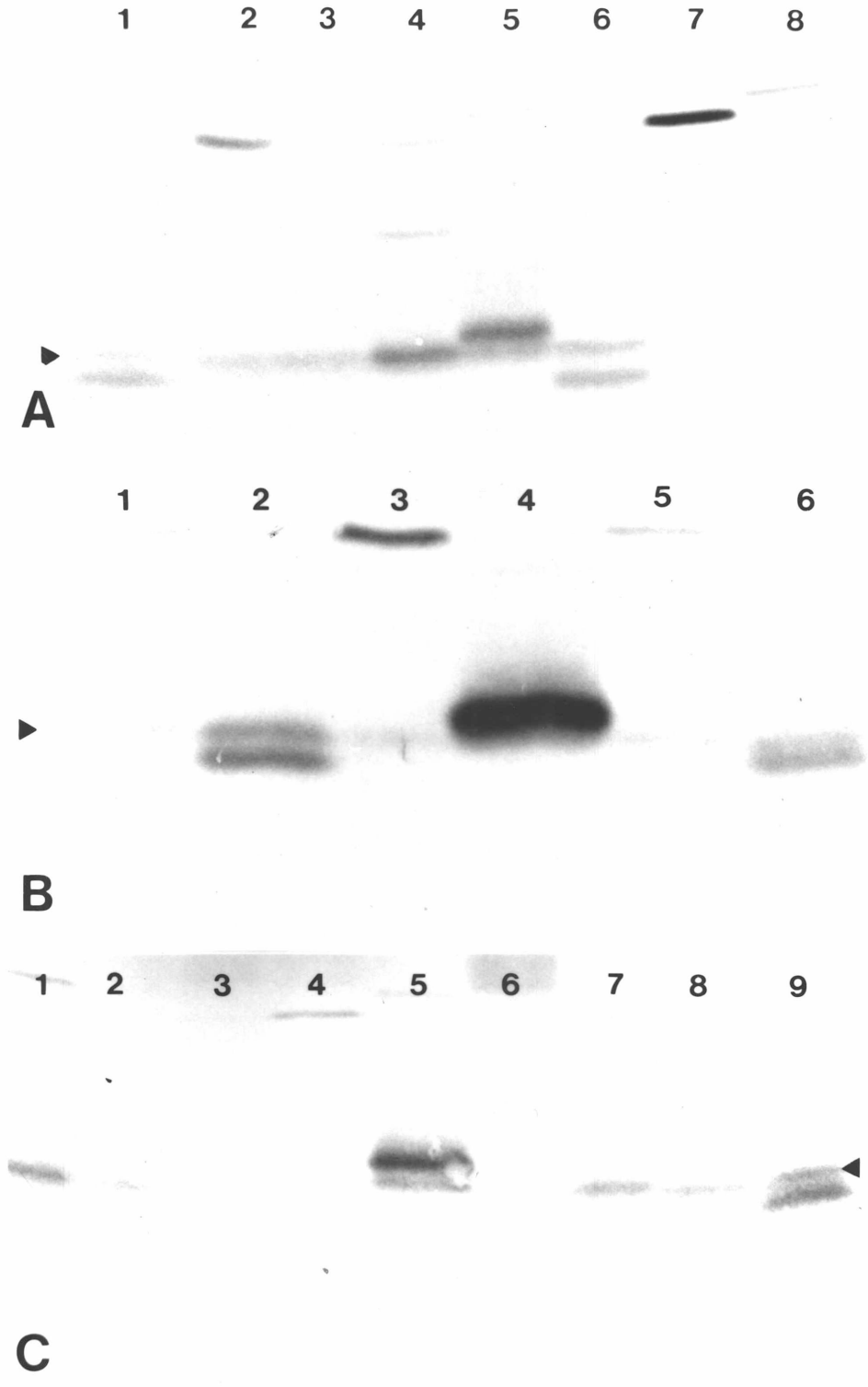
EXPRESSION OF H-ras PROTEINS IN BLADDER TUMOUR LYSATES

Panels A, B, and C, represent 20 Tumours in Western Blot analysis demonstrating aberrantly migrating p21 species in five cases; 3A-lanes 5 and 6; 3B-lanes 2 and 4; 3C-lane 5.

Lane 1 in panel A, 6 in panel B and 9 in panel C represent control cell lysate where both the normal protein (>) and the codon 61 mutated product (below) can be resolved. H-ras proteins with a codon 12 mutation migrate more slowly (3A-lane 5; 3B-lane 4; 3C-lane 5) than the normal (e.g. 3A - lane 4) whilst codon 61 mutated products migrate more rapidly (3A - lane 6; 3B - lane 2).

FIGURE 3.3

EXPRESSION OF H-ras PROTEINS IN BLADDER TUMOUR LYSATES



aberrantly migrating p21 species but a consistently high level of expression of p21 with a normal migratory pattern. The fourth tumour, previously characterised as having a H-ras oncogene activated by a cysteine mutation at codon 13, revealed p21 with a migratory pattern indiscernible from the normal, consistent with our previous results using 3T3 transfectants expressing this gene. The remaining fourteen tumours displayed H-ras p21 protein products, expressed at comparable levels in all tumours, migrating with the normal endogenous p21.

A survey of eleven additional bladder tumours in this assay resulted in the identification of three more cases of H-ras gene activation resulting in aberrant p21 proteins resolved in SDS-PAGE. In two of these cases the altered p21 band was observed migrating more rapidly than than the endogenous p21, indicative of an activation event in codon 61 of the c-H-ras-1 gene. The third revealed a p21 species migrating more slowly than the normal, consistent with a codon 12 activation. The histopathology of tumours with altered ras gene products is summarized in Table III.1. Analysis of sixteen colon carcinomas in Western blots revealed no case where an altered H-ras protein was detected (Figure III.4), although lysates from all colon tumours showed comparable levels of H-ras expression to that seen in the bladder samples (compare Figures IVA and IVB). This result was as expected from previously reported molecular data and demonstrates the reliability of this assay in specifically detecting altered H-ras proteins in tumour material.

TABLE III.1:

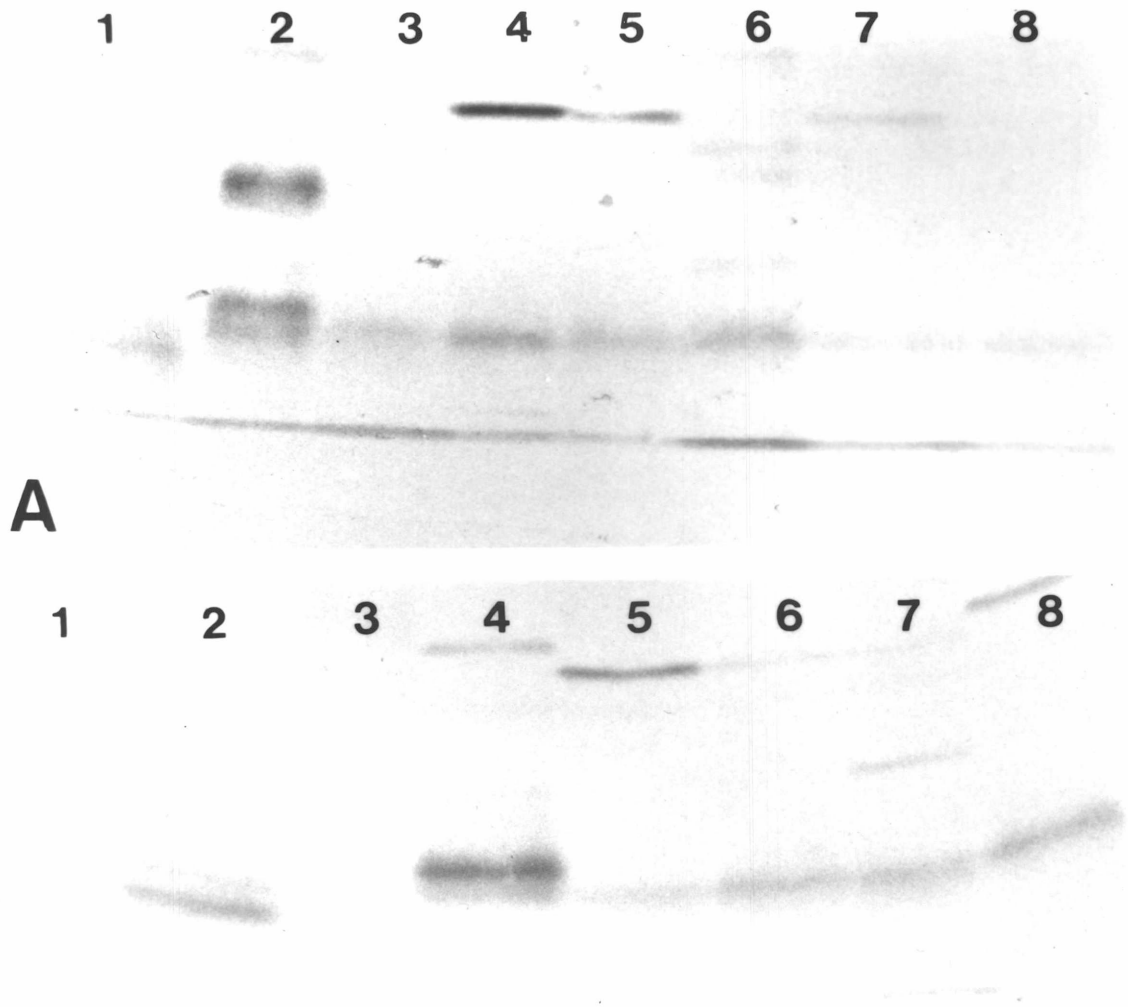
HISTOPATHOLOGY OF BLADDER TUMOURS EXPRESSING ALTERED H-ras PROTEINS:

Patient No.	Grade/Stage	Codon Mutation
3	G1/pTa	12 (gly-val)
4	G3/pTa	12 (gly-val)
5	G2/pT1	12 (gly-val)
6	G3/pT2	13 (gly-cys)
10	G3/pT3	12 (UD)
12	G3/pT4	61 (UD)
24	G2/pT1	61 (UD)

Stage and Grade of Bladder Lesions was according to the Union Internationale. (UD = Undetermined)

FIGURE III.4:

Comparison of H-ras Expression in Colon and Bladder Tumour Lysates.



Panel A: Colon Tumours, Panel B: Bladder Tumours.

All tumours display a normal migrating p21 H-ras species. Panel A, lanes 1 and 2, panel B lanes 2 and 4 represent control lysates of H-ras proteins with codon 61 and 12 mutations respectively, for each blot. Each lane was loaded with 100ug of total tumour protein facilitating comparable levels of H-ras expression.

DISCUSSION

The detection of ras oncogenes in human tumour material was originally achieved using NIH/3T3 transfection assays (Marshall 1985) and more recently has been succeeded by the introduction of the polymerase chain reaction (PCR) and the use of oligonucleotide probes. In this study we describe a complimentary approach which relies upon the aberrant migrational properties of altered ras gene products in SDS-polyacrylamide gel electrophoresis. The results obtained in Western blot analysis, with a H-ras specific antibody, parallel and extend those previously achieved using a modified NIH/3T3 transfection assay and present a rapid method for identification of altered H-ras proteins in tissue specimens.

Since the early stages of ras characterization there have been numerous reports of the altered migrational properties of p21 products from different cell lines expressing ras oncogenes. Although individual members of the ras gene family could not be identified by these criteria, the slower migration of p21 derived from codon 12 mutations and the faster migration of ras proteins from codon 61 mutations relative to the normal endogenous product, were well established. The generation of antibodies which identify a single protein member of the ras gene family now facilitates the investigation of the expression of individual ras gene products in different epithelial cell types. Use of one such antibody, directed against the H-ras protein, has revealed altered H-ras p21

products in three more bladder carcinomas raising the total number of ras activation events reported in this tumour type to ten.

A retrospective study in Western blot analysis of tumours used previously in transfection studies, confirms the expression of a slow migrating p21 H-ras protein in two tumours, consistent with the molecular data where a mutation at codon 12 substituted a valine for the normal amino acid glycine. In a third tumour, where the same mutational event had been found using the PCR technique, we failed to detect an altered p21 product even when higher concentrations of protein were analysed in this system. In this case use of the PCR technique on the original tumour DNA resulted in a strong normal gene signal and only a very weak signal from the mutated gene, a result originally interpreted as due to extensive mesenchymal components in the tumour tissue. In this study, sectioning of tumour tissue revealed a high content of carcinomatous material and yet no altered p21 was resolved, leading us to the conclusion that only a minor population of cells within the tumour bear the altered ras gene. The elevated levels of normal H-ras p21 observed in Western blots with this tumour suggest the possibility of over-expression of the normal allele contributing to the neoplastic lesion. As would be expected from our previous studies, mutation events occurring in codon 13 of the c-H-ras-1 gene do not result in p21 proteins with discernible aberrant migrational properties in this assay, however, the identification of three more bladder tumours

expressing altered ras proteins is at a frequency consistent with that previously reported using a sensitive transfection assay.

The disparity reported between the frequency of ras activation in colon tumours, detected by transfection or the PCR technique, are largely due to the size of the K-ras gene involved and the difficulty in maintaining such a large gene intact from the tumour DNA preparations. The H-ras and N-ras genes are considerably smaller and we consider that the frequency of H-ras activation reported in bladder tumours, using transfection and Western blot analysis, is a fair representation of ras mutation events in this organ. Although this assay will not identify all altered H-ras p21 products i.e. codon 13 mutations, the most frequently found mutational activations at codon 12 and 61 can be detected in this manner. The absence of any altered H-ras proteins in sixteen colon tumours studied is consistent with all previous reports where no case of H-ras activation has been found and demonstrates the absence of false positives in this assay. The development of a K-ras specific antibody will provide a similar tool for the rapid assessment of this protein member in colon tumours.

The advantages of this technique is its rapidity and simplicity in identifying altered ras gene products. The concomitant expression of the normal p21 product, whether from carcinoma cells heterozygous at the H-ras locus or from residual mesenchymal in the tumour, greatly facilitates the characterization of aberrant p21 migration in Western blots. Since only small protein concentrations

are required, this technique enables us to analyse biopsy material from early stages of neoplastic progression and to ascertain the timing of ras gene activation.

This approach is technically less demanding than the PCR technique and focuses on the expression of the mutated protein, the effector molecule. In addition analysis of ras products provides comparative data as to the levels of expression of H-ras proteins between different tumours of varying grades and stage. The frequency of H-ras activation is between 15-20% in bladder carcinomas and although the sample is small, ras activation was found in a number of non-invasive tumours representing different grades. In order to establish whether tumours expressing altered ras proteins represent a subpopulation with a particular clinical significance, larger numbers will have to be evaluated and followed with respect to their recurrence and progression. This approach provides a simple procedure for screening large numbers of tumours providing information as to the expression of normal and mutated ras proteins in tumours. With the inevitable production of antibodies recognizing individual members of the ras family, information concerning the expression of each in different epithelial cell types and differentiation pathways will help resolve questions concerning the possible different roles of these family members. Clearly from the study of colon cell lines and tumours, expression of the endogenous H-ras p21 is in evidence in a tumour population where K-ras activation has been reported in 50% of tumours.

In summary this work adds to the number of H-ras activation events reported in bladder carcinomas presenting a rapid method for identifying altered p21 products derived from mutations at two major codon hotspots. The analysis of tissue following resection or biopsy will enable us to study the timing of ras activation and its significance within the clinical setting.

CHAPTER IV

TRANSPLANTATION OF IMMORTALISED BLADDER EPITHELIAL CELL LINES IN DENUDED MOUSE BLADDER

INTRODUCTION

The regenerative capacity of bladder epithelium is well documented (Liang 1962, Tucci and Haralambidis 1963, Locher and Cooper 1970) where mucosal denudation of the bladder has been used as a form of treatment in patients with bladder lesions (Lund 1969, Harada et al 1965). We describe an approach involving stripping the bladder mucosa in mice and transplanting an intact bladder epithelial sheet on to the remaining stromal element. In this report, fluorescently labeled mucosal grafts were shown to persist for the duration of the experiment (20 weeks) offering a possible alternative approach to the treatment of early bladder cancer. This technique also has applicability to the experimentalist to try and resolve questions concerning the *in vivo* phenotype of different carcinogenesis stages defined by *in vitro* parameters and to study the role of epithelial-stromal interactions in neoplasia. In this chapter the temporal reorganization of different components of the mouse bladder following surgical stripping are described and report the transplantation of established epithelial cell lines into denuded mouse bladders.

The temporal and spatial regeneration of denuded mouse bladders was characterized using antibody markers to mucosal and submucosal elements in bladder tissue. Mechanical stripping of bladder mucosa resulted in a plane of cleavage in the submucosa leaving the muscle layers intact. Fully regenerated bladders were observed after

14 - 21 days although submucosal elements showed abnormal vacuolation. Implantation of immortalized epithelial cell line BBN3 into denuded bladders resulted in solid tumour formation whilst, in contrast, transplantation of MB331 showed cystic structures with no indication of invasion. Confirmation of the epithelial and implanted origin of cells lining the lumina of implanted bladders were shown using different keratin antibodies. The benign behavior of MB331 *in vivo* is suggestive of a cell line representing a preneoplastic stage in carcinogenesis and demonstrates an approach to assess the *in vivo* phenotype of cell parameters established *in vitro.*

MATERIALS AND METHODS

Cell culture:

Cell lines MB33I and BBN3 were maintained in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal calf serum, penicillin and streptomycin.

Antibodies:

Antibodies to total keratins, type IV collagen, fibronectin were generous gifts from Dr.M.Warburton. Antibody D66 has been described previously (Summerhayes and Chen 1982). All antibodies were raised in rabbits and used at 1/100 dilution in phosphate buffered saline (PBS).

Urothelial stripping:

The mice were anesthetized with barbiturate (10% Sagatal in saline), 0.1ml/10g body weight i.p. and a small mid-line incision over the supra-pubic area allowed the bladder to be exteriorized. Using iridectomy scissors, the muscle was incised at the dome of the bladder to expose the underlying intact urothelium. This was held with a pair of fine, blunt ended, forceps and the muscle layer gently detached with fine curved watchmakers' forceps. The total intact exposed urothelium was then excised from the base of the bladder. The muscle layer was restored to its original conformation, and a single ligature of polyglcolate used to seal the defect. The abdomen was closed in two layers and the mice recovered in a warm box maintained at 30 C.

Implantation of bladder epithelial lines:

Cell lines BBN3 and MB33I were established from C57BL/6J mice after exposure to N-butyl-N(4-hydroxybutyl)-nitrosamine (Summerhayes and Chen 1982) and Dimethylbenz(a)anthracene (Summerhayes and Franks 1979) respectively. For implantation into the denuded bladder, cells were washed in serum-free Dulbecco's modified Eagles medium and harvested from the dishes using a rubber policeman. Cell sheets were cut into small segments (approx 1-2 x 10^5 cells) and placed inside denuded bladders using a pair of watchmakers forceps. No attempt was made to orientate the cell sheets. Following this, the bladder dome was sutured and the mouse abdomen closed.

Histology and Immunofluorescence:

Mice were sacrificed by cervical dislocation and their bladders dissected to include the prostate for future orientation. These were immediately snap-frozen in n-Hexane (grade; free from aromatic hydrocarbons) at -70^{o} C in an alcohol/dry-ice bath. In all experiments, triplicate serial 6u transverse sections were cut using a cryostat with a knife temperature maintained at -30 C and sections were picked up on three 10-well test slides (Flow Labs). After each group of three sections had been cut the tissue was advanced by 400u before further sections were cut. Adopting this procedure, 9 and 10 sections were obtained from each bladder giving serial and adjacent samples through the whole organ. Sections were air-dried for about 20 minutes before processing for cyto-chemistry. Sections on one slide were stained with Haematoxylin and Eosin and adjacent slides were processed for immuno-flourescence.

Briefly, sections were incubated with first antibody for 40 minutes at room temperature in a humidified chamber, washed three times in PBS for a total of 15 minutes and subsequently overlaid with Goat anti-rabbit IgG-FITC (Miles) at a 1 in 20 dilution. Incubation with the second antibody was for 40 minutes followed by three washes in PBS before mounting in Aquamount (National Diagnostics, New Jersey). Immunofluorescence preparations were viewed using a Zeiss photomicroscope with epifluorescent illuminations.

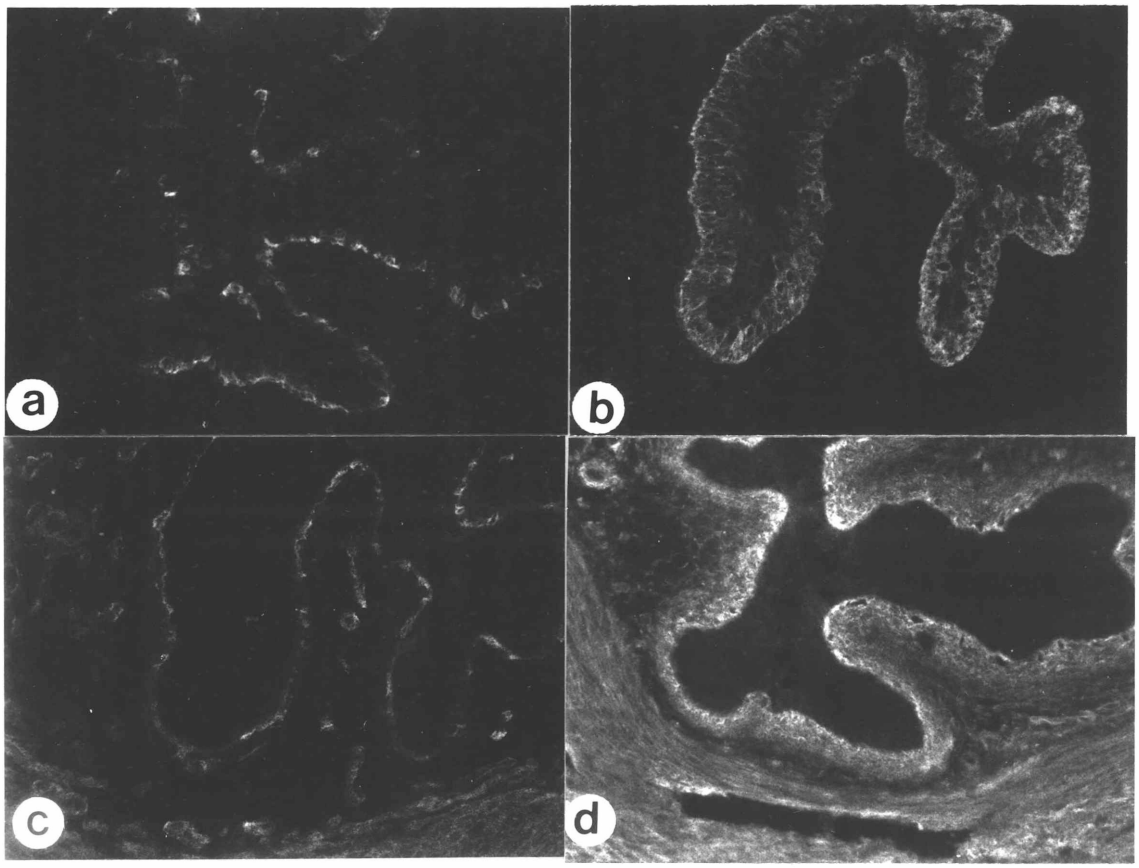
RESULTS

Epithelium was stripped surgically from an incision at the dome of the bladder leaving some mucosa in the bladder neck. Removal of the mucosa throughout the neck frequently resulted in death of the animal, probably as a result of extensive damage to the musculature in this region. Intact epithelium in the neck of stripped bladders served as an internal control in each experiment. Animals were sacrificed at regular intervals following mucosal stripping and bladders were processed for frozen sections. In each experiment sections were prepared from different levels throughout the bladder, section 1 being at the neck and section 10 at the dome. Sections from each level were assessed by conventional histological staining and by immuno-fluorescence using antibodies which identify different components of normal bladder (figure IV.1).

Immediately following mucosal stripping, the bladders were processed for histological evaluation where the plane of separation was found to be in the submucosa above the muscle layer (Data not shown). The bladder lumen had filled with fibrous material and blood but in some areas intact blood vessels could be seen surrounded by residual mesenchymal cells. Imunofluorescence staining with keratin antibodies were negative throughout sections 3-10 but showed positive staining in sections 1 and 2 where urothelium remained unstripped in the neck of the bladder.

FIGURE IV.1:

IMMUNOFLUORESCENCE MICROGRAPHS OF FROZEN SECTIONS THROUGH NORMAL MOUSE BLADDER:



Micrographs show the distribution of staining using the antibody panel: (a) D66; (b) total keratin antibody; (c) type IV collagen; (d) fibronectin. Sections a,d and b,c represent neighbouring sections, x200.

Post-operative mortality was uncommon and usually associated with injury to the bladder neck and ureteric orifices. Mice were sacrificed at time points 4,5,7,14 and 21 days post-stripping when sections were processed for immuno-fluorescence.

Epithelial regeneration in denuded bladders:

Unstripped bladder in sections 1 and 2 displayed normal staining with the antibody panel, at all stages (Figure IV.1). The timing of epithelial regeneration, as characterized by the presence of keratin positive cells in different bladder sections, is documented in Table IV.I. Re-epithelialization occurs from both the cell population remaining in the bladder neck and from migration of epithelial cells down the ureters into the bladder lumen.

Figures IV.2 (a-d) show representative staining of re-epithelialized areas in sections 5-6, seven days post-stripping. Submucosal elements displayed an intact basement membrane underlying regenerated mucosa (figures IV.2 a and b) with the presence of dilated vasculature deep in the submucosa and numerous small, newly formed, blood vessels abutting the basement membrane.

The thickened submucosal element displayed increased number of blood vessels and was rich in fibronectin (figure IV.2c). Incubation of neighbouring sections with D66 antibody revealed a mucosal element with an expanded basal cell compartment (figure IV.2d), characteristic of staining observed in mild hyperplasia.

TABLE IV.1:

TIMING OF RE-EPITHELIALISATION IN DENUDED MOUSE BLADDER:

Days post-stripping

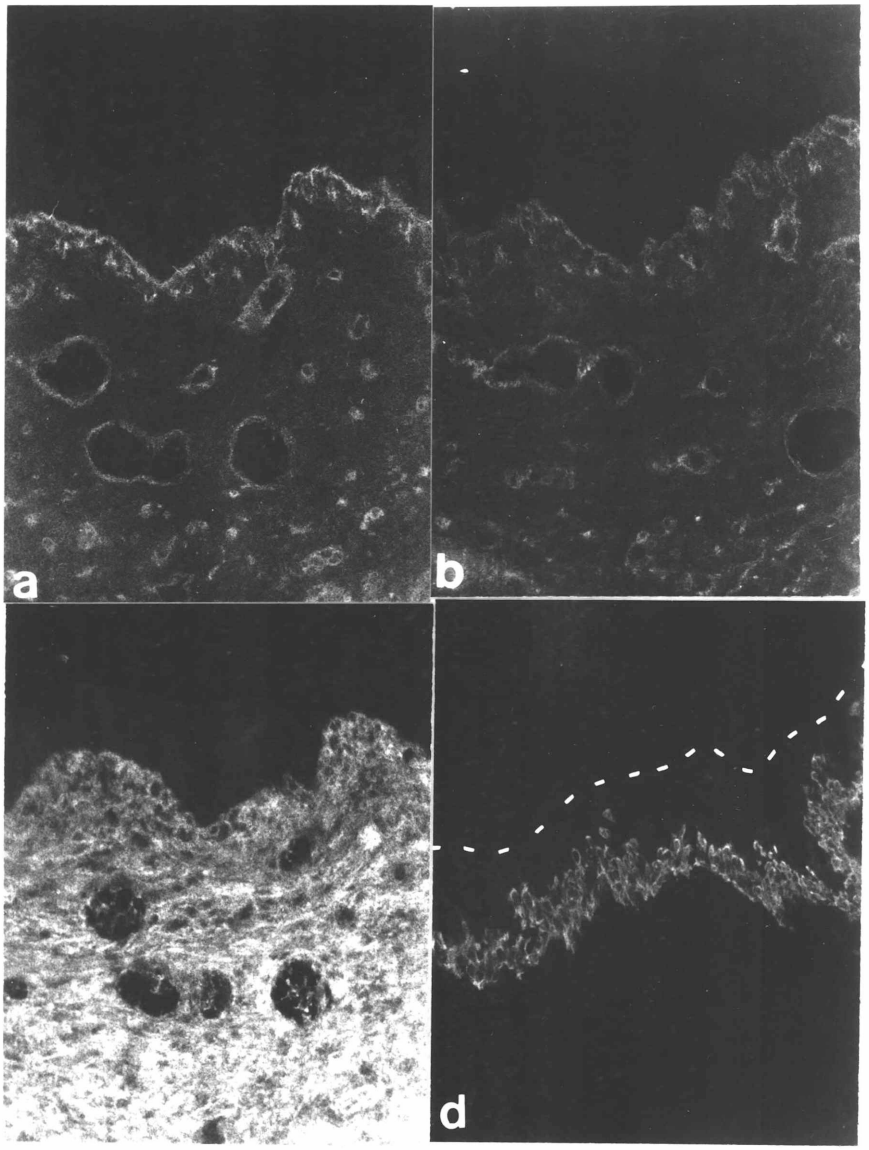
	Neck			Section Level						Dome
	1	2	3	4	5	6	7	8	9	10
0	*	*								
4	*	*	*	*						
8	*	*	*	*	*	*	*			
14	*	*	*	*	*	*	*	*	(*)	(*)

Level of re-epithelialization determined by the presence of keratin-positive cells in serial sections.

( ) represent 80% of mice (16/20) showed complete regeneration of the bladder.

FIGURE IV.2:

IMMUNOFLUORESCENCE MICROGRAPHS OF FROZEN RE-EPITHELIALISED BLADDER SECTIONS 7 DAYS POST STRIPPING.



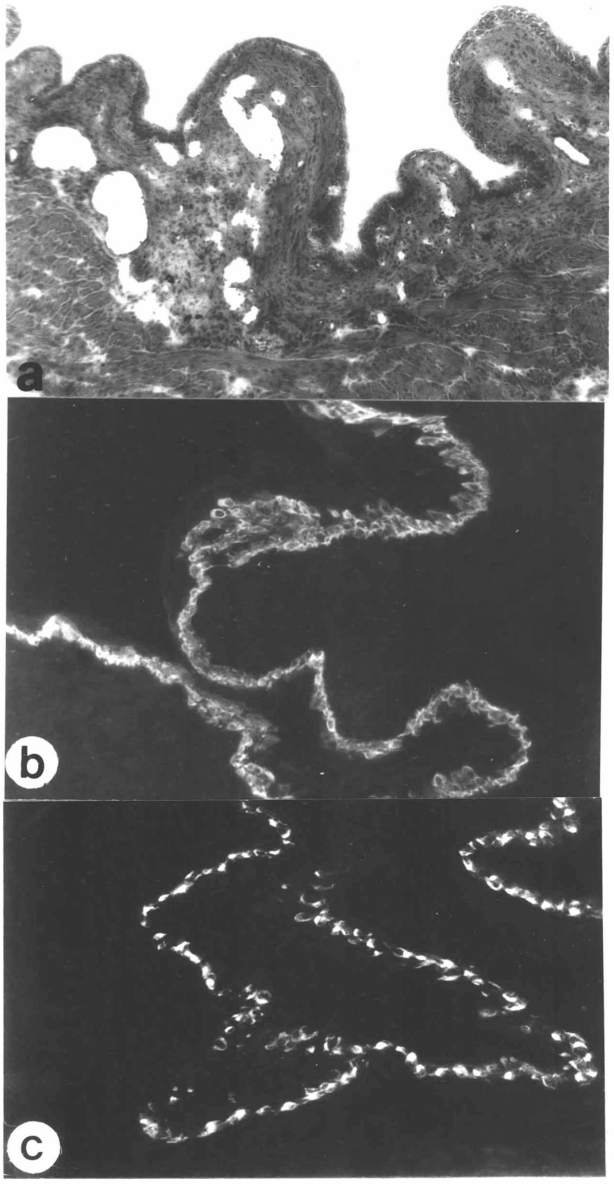
Sections incubated with antibodies to: (a) Type IV collagen, (b) Laminin, (c) Fibronectin, (d) D66/section 7, dotted line represents the luminal layer of unstained epithelial cells. Note the distended vasculature in the submucosa stained with basement membrane antibodies (a and b). All micrographs x200.

Three days later (day 10 post-stripping) the same sectioned level in the bladder appears normal, both in histological and immuno-fluorescence studies, thus re-epithelialization and differentiation occur as close temporal events. At later stages (14-20 days post-stripping) re-epithelialization of denuded areas was complete in a majority of mice (85%) demonstrating an intact mucosa 14 days post-stripping. Histology of regenerated bladders revealed an intact mucosa with a continued thickened submucosa displaying numerous vacuolations and dilated blood vasculature (Figure IV.3a). Incubation of regenerated bladders with extracellular matrix antibodies confirmed these observations (data not shown).

Although fully differentiated superficial cells were observed lining the lumen of regenerated bladders, incubation of sections with D66 antibodies revealed an expanded basal cell compartment in recently regenerated bladder regions (Fig IV.3):

FIGURE IV.3:

LIGHT AND FLUORESCENCE MICROGRAPHS OF BLADDERS 16 DAYS POST DENUDATION.



(a) light micrograph showing apparent normal mucosa with a vacuolated submucosa. (b and c) sections incubated with D66 antibody represent different regions within the same bladder. Section (b) shows regenerated mucosa, with normal histological features, displaying increased basal cells population - section level 9. (c) staining of basal cell compartment - section level 5. H & E x200.

Transplantation of established epithelial cell lines:

Mouse bladder epithelial cell lines BBN3 and MB33I were derived from C57BL/6J mice and have been described previously (Summerhayes and Chen 1982, Summerhayes and Franks 1979). Both lines are regarded as immortalized but display no anchorage independent growth or tumorigenic potential when injected subcutaneously in to nude or syngeneic hosts (Summerhayes et al 1986). Subconfluent cultures of BBN3 and MB33I were harvested from dishes by scraping and the resulting cell sheet was cut in to small segments (approx. 10^5 cells/segment) for introduction in to denuded bladders of syngeneic hosts. Epithelial cells were implanted through an incision at the dome of the bladder which was then closed by suturing. Animals were sacrificed after one month, or prior to this if sick. Two of four mice receiving BBN3 cell implants were killed at 21 days when histology revealed the presence of a solid carcinoma occluding the bladder lumen and invading the muscle layers (Figure IV.4a). Explant cultures of bladders resulted in mixed cultures of normal and implanted material, from which cells surviving after one month retained the same morphology and inability to grow in an anchorage independent manner, characteristic of cells in the original inoculum. The remaining two mice were sacrificed at 28 days and had apparently normal regenerated bladders with no evidence of transplanted material.

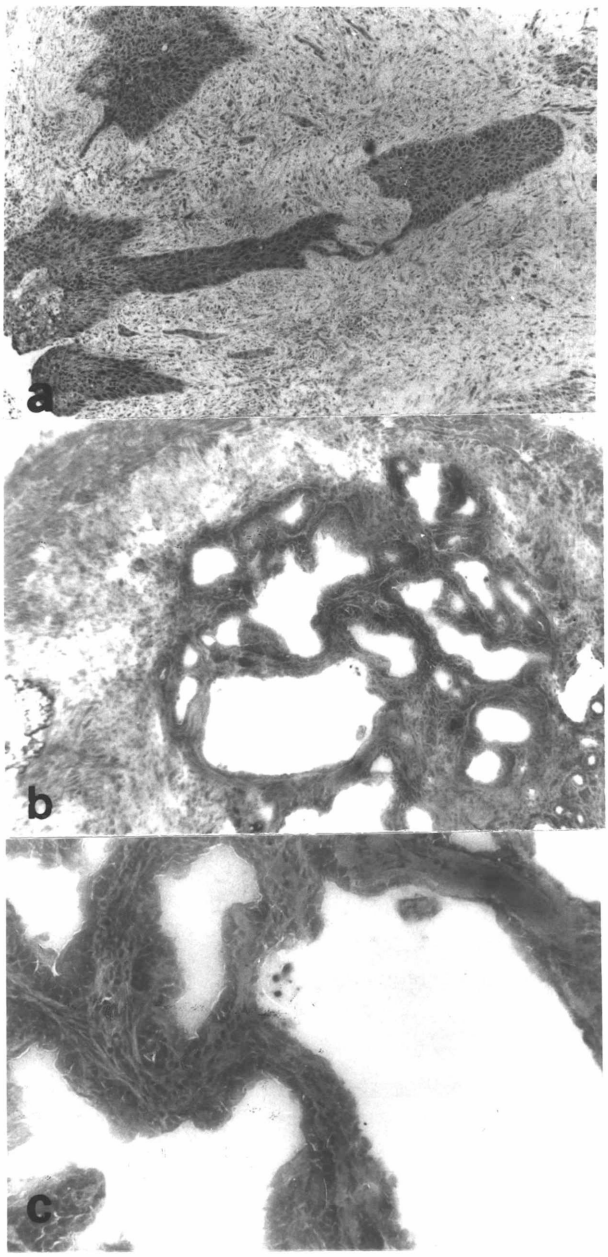
Four mice transplanted with the cell line MB33I all survived to 28 days and showed grossly normal bladders on

resection. Histological survey of sections from these bladders revealed an abnormal tissue organization located near the dome of the bladder where epithelial grafts had seeded. Figure IV.4b shows a photomicrograph of the bladder dome consisting of various lumina, which at higher power appeared lined by epithelial cells and surrounded by stromal elements (Figure IV.4c). Confirmation of the epithelial origin of luminal cells was shown following incubation of sections with total keratin antibodies which stained all the cells lining the lumina in this region (Figure IV.4d). To distinguish between implanted MB33I cells and regenerated normal urothelium we exploited the differential staining observed between these cells using antibody D66.

Lumina lined with regenerated urothelium displayed basal cell staining, characteristic of normal bladder, whilst lumina lined by implanted MB33I cells stained through all layers with D66. Figure IV.4e shows adjacent lumina within an implanted bladder displaying differential staining of cells lining the cyst, with basal cells staining in the regenerated region and staining of all cell layers of the lumen lined by implanted MB33I cells.

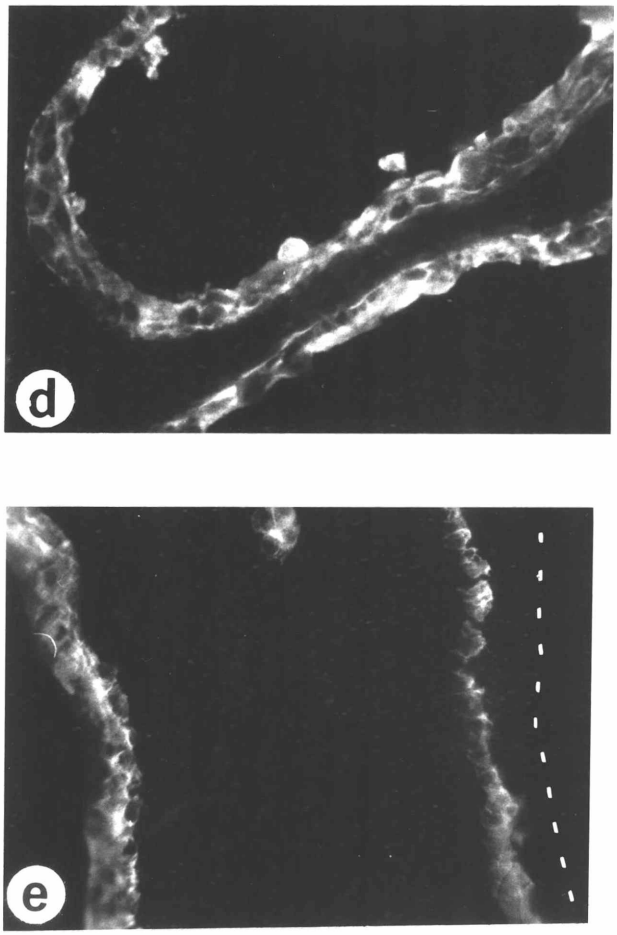
FIGURE IV.4 A-C:

LIGHT AND IMMUNOFLUORESCENCE MICROGRAPHS OF TRANSPLANTED MOUSE BLADDERS.



(a) solid tumour formation after implantation of cell line BBN3. H & E. x200. (b) section of bladder implanted with MB 33I showing cyst formation in the implant near the dome. H & E. x200. (c) HP of previous section showing epithelioid cells lining luminal surfaces. H & E. x500.

FIGURE IV.4 D-E:



(d) adjacent lumina in transplanted bladder incubated with anti-keratin antibodies showing the epithelial origin of the cells lining the cysts. (e) section of implanted bladder incubated with D66 antibody showing adjacent lumina displaying differential staining. Left represents lumen lined with MB 33I cells and stained throughout. Right lumen lined by normal regenerated host urothelium showing only basal cell staining. Dotted line represents luminal surface of mucosa. x500

DISCUSSION

We have exploited the technique of bladder denudation to study the behavior of immortalized epithelial cell lines *in vivo* in an attempt to correlate carcinogenesis stages, as defined by *in vitro* parameters, with events in neoplastic progression as characterized by histopathology. Since denudation of bladders results in rapid re-epithelialization, where grafted cells will compete with regenerating host epithelium, we have characterized the temporal and spatial reorganization of denuded bladders using a panel of antibody markers.

Urothelial stripping of mouse bladders results in a plane of cleavage in the submucosa leaving an intact muscle layer and fibrous material with scattered blood vessels lining the bladder lumen. Although denudation has a dramatic effect on the cytoarchitecture of the bladder, survival was 100% provided residual mucosa was left in the neck of the bladder. In the few cases where animals died following mucosal stripping, histology revealed damage to the muscle layers in the bladder neck.

Regeneration of mucosa was completed in nearly all animals after 14 days when a complete epithelial coverage was observed throughout the bladder and fully differentiated superficial cells lined most of the bladder lumen. Prior to this stage regenerating mucosa was shown to consist of numerous cell layers expressing keratin components, characteristic of basal cells in the normal bladder, and representing a hyperplastic mucosa.

Regions close to the leading edge on the same section, showed luminal cells which did not stain with basal cell characteristics but were obviously not the fully differentiated superficial cells found in normal bladder. These cells represent an intermediate stage of differentiation and indicate that re-epithelialization and differentiation, within regenerating bladders, occur as closely linked temporal events. The expansive superficial cells found in normal bladders were observed in regenerated regions within 3 days of coverage.

No discernible basement membrane was observed in bladder regions lacking an epithelial coverage, indicating the necessity for epithelial-stromal interactions for the assembly of a coherent lamina. Since residual extracellular components remained in the bladder lumen following stripping, the reorganization of submucosa was difficult to decipher against this background. However, incubation with all submucosa-specific antibodies highlighted the presence of grossly dilated blood vessels and the formation of new ones abutting the regenerated mucosa.

In bladders denuded 21 days previously, incubation with antibodies to basement membrane components demonstrated the elevated numbers of blood vessels and the large submucosal vacuoles which were found in all bladders at this stage. It is possible that longer periods following urothelial stripping are required before a normal tissue architecture is restored.

Transplantation of immortalized epithelial cell lines

into denuded bladders resulted in the production of a solid invasive carcinoma with the cell line BBN3. In two of four animals transplanted. The remaining two showed no evidence of implanted cells on serial sections and it was assumed that cells failed to seed in these cases. More recent experiments, using the same cell line implanted subcutaneously on a collagen gel support, confirms the tumorigenic potential of BBN3 and prompts us to re-evaluate parameters used for defining immortalized epithelial cell lines. Although BBN3 showed no anchorage independent growth and did not produce tumours when injected subcutaneously in a cell suspension, it clearly has tumorigenic potential in an anchorage dependent environment.

As with keratinocytes, it seems probable that an anchorage independent situation triggers terminal differentiation in these cells (Green 1977, Rheinwald and Green 1977) explaining our previous observations with these cells (Summerhayes et al 1986). Unlike most immortalized mesenchymal cell lines, differentiation events may be a determining factor in epithelial cell behavior in vitro. In contrast, MB33I produced cystic structures in 4 out of 4 transplantations displaying no evidence of invasion. Cysts lined by MB33I cells were distinguished from regenerated normal urothelium by the differential staining of these two cell populations using antibody D66. In all four mice transplanted with MB33I no evidence of solid tumour formation was observed and the benign behavior of these cells in vivo is suggestive of an

intermediate stage in bladder carcinogenesis prior to the acquisition of tumorigenic potential.

The application of transplanting epithelial cell lines into denuded bladders is straightforward and reproducible enabling us to correlate *in vitro* parameters with *in vivo* pathology. In addition, with appropriate markers for identifying implanted cells, the interaction of epithelial and stromal elements can be studied along with the role of different genes in neoplastic progression and differentiation.

CHAPTER V

THE SUBRENAL CAPSULE: AN ALTERNATIVE SITE FOR THE MAINTENANCE OF NORMAL AND PRENEOPLASTIC UROTHELIUM

INTRODUCTION

In the previous chapter we described an implantation technique whereby urothelial cell lines were seeded into denuded recipient bladders and contributed to the regenerating mucosa of the bladder (Summerhayes et al 1988). This approach enabled us to evaluate the in vivo behavior of immortalized cell lines with a view to studying the role of different genes in neoplastic progression. However, material implanted in the denuded bladder competes against a rapidly regenerating normal mucosa and different cell lines tested vary in their ability to become established in this environment.

Since early events in bladder carcinogenesis may present as subtle phenotypic changes in vivo, it is important to establish an assay in which such events can be detected. In this chapter we describe the use of the subrenal capsule as an alternative site for the implantation and maintenance of normal and preneoplastic urothelium in vivo.

Implantation of normal bladder mucosa beneath the kidney capsule of a syngeneic animal, results in a heterotopic bladder lined by a 1-3 cell layer of urothelium. This cystic structure can be maintained for greater than 90 days and displays many of the differentiation characteristics of a normal bladder, both at the light and electron microscopy level. Preneoplastic urothelial cells behave in a similar manner recreating a heterotopic bladder lined by urothelium displaying

aberrant differentiation and surface morphology.

All tumorigenic cell lines tested in this model produced solid tumour growth within a few weeks. The subrenal capsule thus represents an alternative site to the bladder model for the reconstitution of urothelial material at different stages of neoplastic progression.

MATERIALS AND METHODS

Cell Culture:

Urothelial cell lines MB331 and MB49B were established from C57Bl/6J mice after exposure of urothelium to 7,12 Dimethylbenz[a]anthracene in culture (Summerhayes and Franks 1979). All lines were maintained in Dulbecco's modified Eagles medium supplemented with 7.5% fetal bovine serum, penicillin and streptomycin.

Urothelial stripping:

C57Bl/6J mice aged between 6 and 10 weeks were fed and watered ad libitum. The donor mice were anaesthetized with Nembutal (5.55 mg/ml), 0.1 ml/10g body weigh i.p. and a small midline incision over the suprapubic area allowed the bladder to be exteriorized. Using iridectomy scissors, the muscle was incised at the dome of the bladder to expose the underlying intact urothelium. This was held using a pair of fine, blunt-ended forceps and gently detached from the muscle layer with fine curved watchmakers forceps. The total intact urothelium was then excised from the base of the bladder above the level of

the trigone, subsequently the muscle layer was restored to its original conformation and the defect sealed with a purse string suture of chromic catgut. The abdomen was closed in two layers and the mice recovered in a warm box maintained at 30^{o}C.

Subrenal Capsular Implants:

The mucosa is divided such that complete stripping of a single bladder provided sufficient material for three subrenal capsular implants. The mucosa was maintained in sterile phosphate buffered saline (PBS) until recipients were ready. The recipient kidney was exteriorized through a small flank incision and the capsule incised over the lower pole using iridectomy scissors, then the mucosa was inserted beneath the kidney capsule using watchmaker's forceps and gently manipulated to the superior aspect of the upper pole of the kidney. The kidney is returned to the abdomen and the wound closed in two layers.

Implantation of urothelial cell lines was performed using the above technique except that the cells were washed in serum-free Dulbecco's modified Eagle's medium and harvested from the dishes using a rubber policeman. Cell sheets are then implanted beneath the renal capsule using a 20G teflon intravenous cannula as a trocar.

In each experiment, 10 mice were included in each group and the experiments were repeated on a minimum of two occasions (Table V.1).

Histology and Immunofluorescence:

At intervals after one month the mice were killed by cervical dislocation and the respective kidneys removed for further evaluation. Kidneys were either fixed in buffered formalin for conventional histological staining, or snap-frozen in OCT over dry ice. For immuno-fluorescence, serial 5u sections were cut using a cryostat with a knife temperature maintained at -20^{o}C, a minimum of 20 sections per kidney were picked up onto frosted microslides (Curtin Matheson Scientific, Inc.) and stored at -20^{o}C. The sections were air dried for 30 minutes before processing. Adjacent sections were selected and processed using conventional H & E staining to confirm histological orientation.

Briefly, sections were rehydrated in PBS for 10 minutes, then incubated with the first antibody, D66 (1:30 dilution) for 1 hour at 37^{o}C in a humidified chamber. After washing three times in PBS, the sections were incubated with a goat anti-rabbit IgG-FITC (Miles) for a further hour. Following three washes in PBS, the preparations were mounted in Glycerol Gelatin (Sigma) and viewed using a Ziess photomicroscope with epifluorescent illuminations.

Electron microscopy:

Excess tissue was trimmed and the specimen fixed overnight at $4^{o}C$ in 2.5% glutaraldehyde buffered in 0.1M Cacodylate at pH 7.4. The relevant areas were diced and further fixed in fresh 2.5% glutaraldehyde at room temperature for one hour. After washing in Sabatini's buffer, the specimens were post fixed for 1 hour at $4^{o}C$ using 1% osmium tetroxide with 1% potassium ferricyanide in 0.1M cacodylate buffer. Following further washing in Sabatini's the tissue was dehydrated in graded ethyl alcohol, and then processed through propylene oxide before embedding in Epon 812. Ultrathin sections were cut on a Sorvall MT2, stained with uranyl acetate and lead citrate and viewed on a Phillips 300 electron microscope.

RESULTS

Normal Bladder Mucosal Implants:

Bladder mucosa surgically stripped from donor animals is characterized by an intact epithelial layer on a continuous basement membrane with varying amounts of submucosal components. Complete stripping of a single bladder provided material sufficient for three subrenal capsular implants. Following insertion of mucosal elements beneath the kidney capsule animals were closed and left for a minimum of one month. After this period, greater than 90% of animals displayed cystic structures at the site of implantation on the kidney apex (Figure V.1) (Table V.1).

Table V.1:

EXPERIMENT 1:

		OUTCOME (Cyst)			
	C57Bl/6	1st Round		2nd Round	
	Mice	30(d)	90(d)	30(d)	90(d)
Normal Mucosal Implant	10	9/10	-	8/10	-
	10	-	8/10	-	8/10

Success was clearly related to volume of tissue implanted.

EXPERIMENT 2:

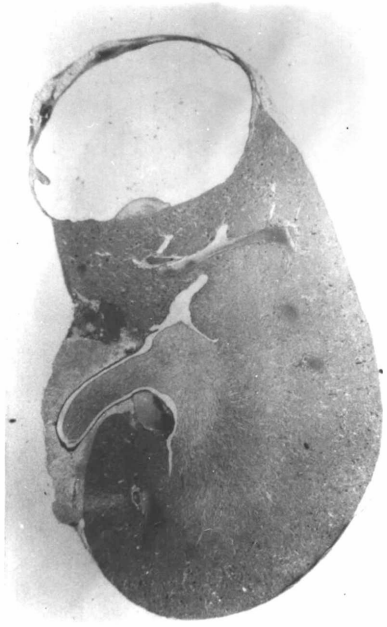
		OUTCOME	
IMPLANT	MICE	1st Round (30d)	2nd Round (90d)
Mucosa	10	Cyst-9/10	
Mucosa	10	-	Cyst-8/10
MB331	10	Cyst-6/10	
MB331	10	-	Cyst-5/10[a]
MB49B	10	Tumour-100%	

a - even on leaving the cysts until 150 days, no evidence of tumour formation was detected.

All experiments were performed at least twice.

FIGURE V.1:

LIGHT MICROGRAPH OF KIDNEY IMPLANTED WITH NORMAL BLADDER MUCOSA.



One month following implantation, a large cyst is found at the apical pole forming a heterotopic bladder lined by normal urothelium. H & E stained x20.

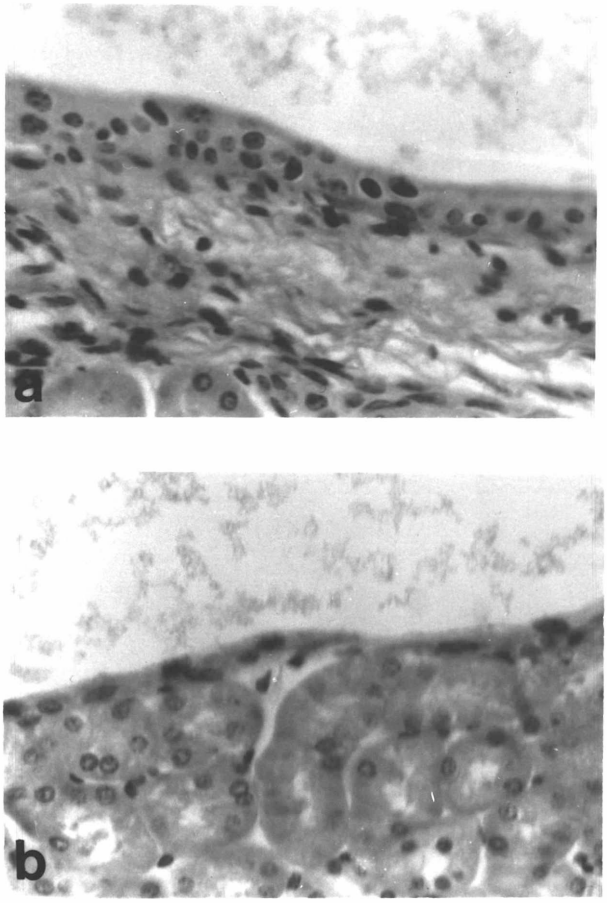
Histological studies revealed each cyst to be lined by an epithelial layer (Figure V.2a), which varied in thickness throughout the cyst. The bladder and epithelial origin of cells lining the cyst was demonstrated using a polyclonal antibody D66 which recognizes keratin components in bladder but not kidney epithelium (Figure V.3a).

Where more than one cell layer was present lining the cyst, staining was found in only the basal layer adjacent to the kidney tubules (Figure V.3a). This is consistent with staining observed in normal bladder mucosa as the antibody recognizes only basal epithelial cell components (Summerhayes and Chen 1982). The absence of staining in luminal cells of the cyst is suggestive of urothelial differentiation in these regions.

Confirmation of differentiation events associated with the epithelial lining was apparent in electron micrographs where cytoplasmic fusiform vesicles, characteristic of intermediate and superficial cells of normal bladder (Hicks 1975, Walker 1960), were found in cells lining the lumen of the cyst (Figure V.4). These cells also displayed tight junctional complexes between cell borders and indicated the formation of an angular luminal membrane (Figure V.4) characteristic of the fully differentiated superficial cells of the normal bladder (Walker 1960).

FIGURE V.2:

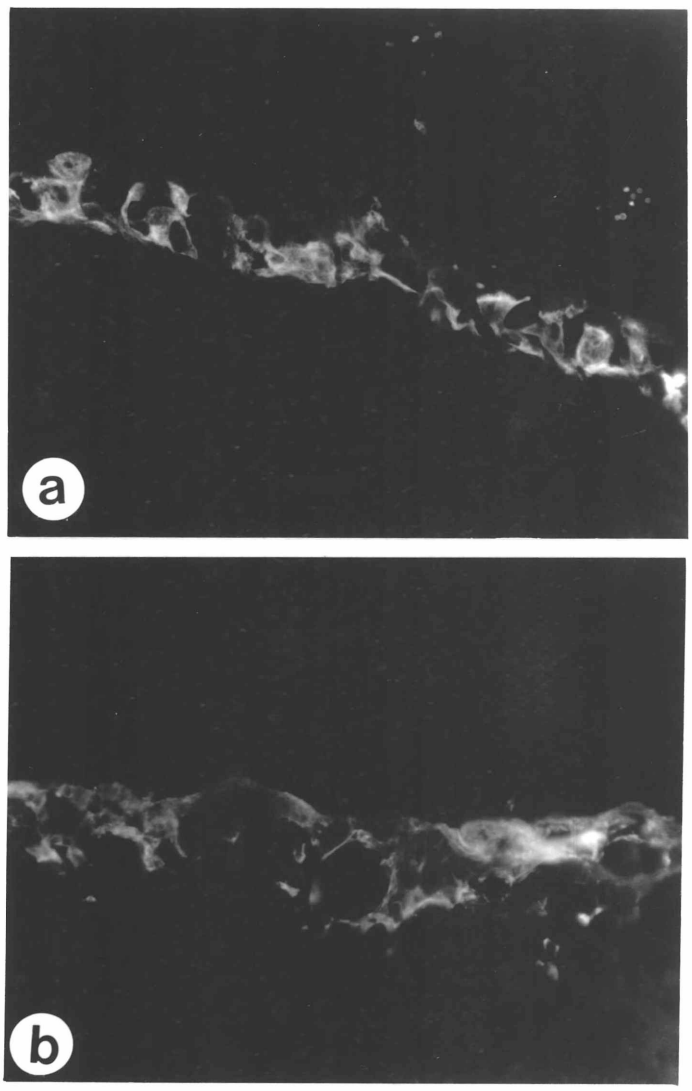
LIGHT MICROGRAPHS OF SUBRENAL CAPSULAR CYST LINING FOLLOWING IMPLANTATION.



Compare normal urothelium(a) and MB33I(b). (a) - note multilayered epithelial lining with larger superficial cells at the luminal surface. Underlying the epithelial layer is a stromal element implanted with the mucosa. (b) cyst lined by MB33I showing urothelial layer abutting the kidney tubules with no stromal element apparent. H & E stained x 320.

FIGURE V.3:

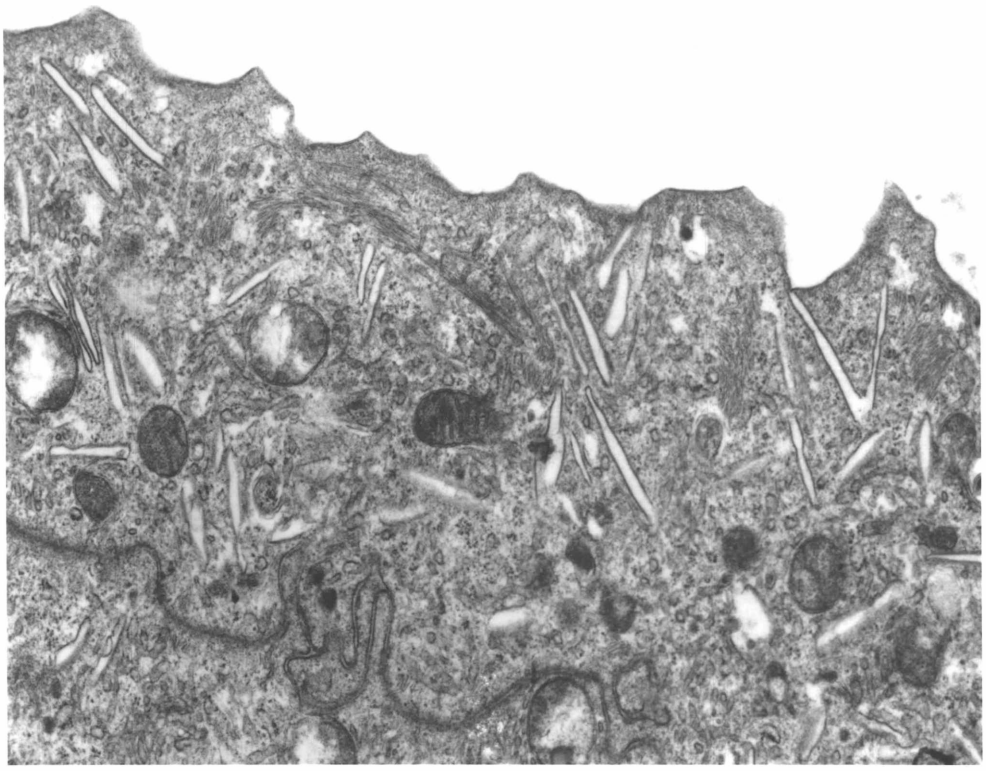
IMMUNOFLUORESCENCE MICROGRAPHS OF CAPSULAR CYSTS IN FIG. V.2 STAINED WITH ANTIBODY D66.



(a) - normal bladder implant showing staining in the basal cells adjacent to the kidney. Cells of luminal layer show no staining with this antibody. (b) - implants of MB33I stain throughout all urothelial layers following incubation with D66. Note in both sections no staining of kidney tubules is observed with this antibody. x 550.

FIGURE V.4:

ELECTRON MICROSCOPY OF THE UROTHELIAL CELL LAYER LINING THE SUBRENAL CAPSULAR CYST:



Section shows numerous layers with junctional complexes present between adjacent epithelial cells. The superficial cells display an angular luminal membrane and fusiform cytoplasmic vesicles lined by an asymmetric membrane, a feature characteristic of fully differentiated cells in normal bladder. x22,610.

Mice maintained for extended periods (>90 days) retained an inflated kidney cyst lined by an extensive urothelial component. Many epithelial areas within older cysts appeared as normal bladder mucosa on histological examination, but rarely was this differentiation state observed throughout the cyst.

Preneoplastic Urothelial Implants:

A previously characterized series of immortalized urothelial cell lines (Summerhayes et al 1986) have been shown to consist of both anchorage dependent tumour lines and cells which are truly preneoplastic (Summerhayes et al 1988). One preneoplastic cell line (MB33I), found to behave in a benign manner in bladder reconstruction experiments, was harvested as sheets from dishes in culture and implanted via a trocar, beneath the kidney capsule of a syngeneic animal.

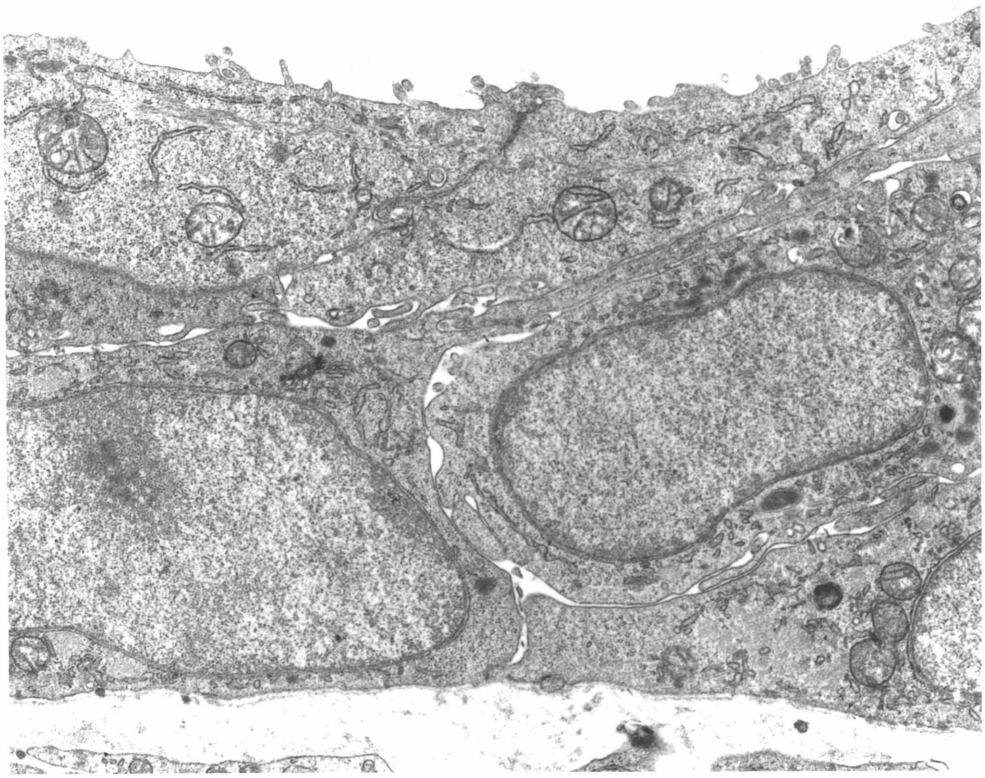
Animals left for one month displayed large kidney cysts at the site of implantation similar to that observed with normal mucosal implants (Figure V.5). Serial sections through one cyst revealed a continuous cellular lining consisting of 1-3 epithelial cell layers (Figure V.2b). Confirmation of the urothelial nature of these cells was shown by the positive staining of luminal cells following incubation with the D66 antibody (Figure V.3b). Electron microscopy studies revealed a multilayered epithelial cell lining to the cyst (Figure V.5a) with junctional complexes and an organization similar to normal urothelial implants.

However, in contrast to normal urothelium, no specialized asymmetric membrane was observed in the cytoplasmic vesicles or at the plasma membrane lining the luminal surface (compare Figs V.4 and V.5b). Most notable was the presence of pleiomorphic microvilli on the surface of cells at the cyst lumen (Figure V.5b), a feature previously described as characteristic of preneoplastic urothelium (Hodges et al 1976).

A basement membrane was evident in all preparations studied (Fig.V.5b) although the continuity of such as structure was often doubtful. Mice bearing kidney implants of MB33I have now been maintained for periods up to 150 days when no solid tumour formation was found. Tumorigenic cell lines implanted at the kidney site as cell sheets, or single cells, produced solid tumours after only a few weeks. No cystic formation was found with any cell line previously established as tumorigenic.

FIGURE V.5A:

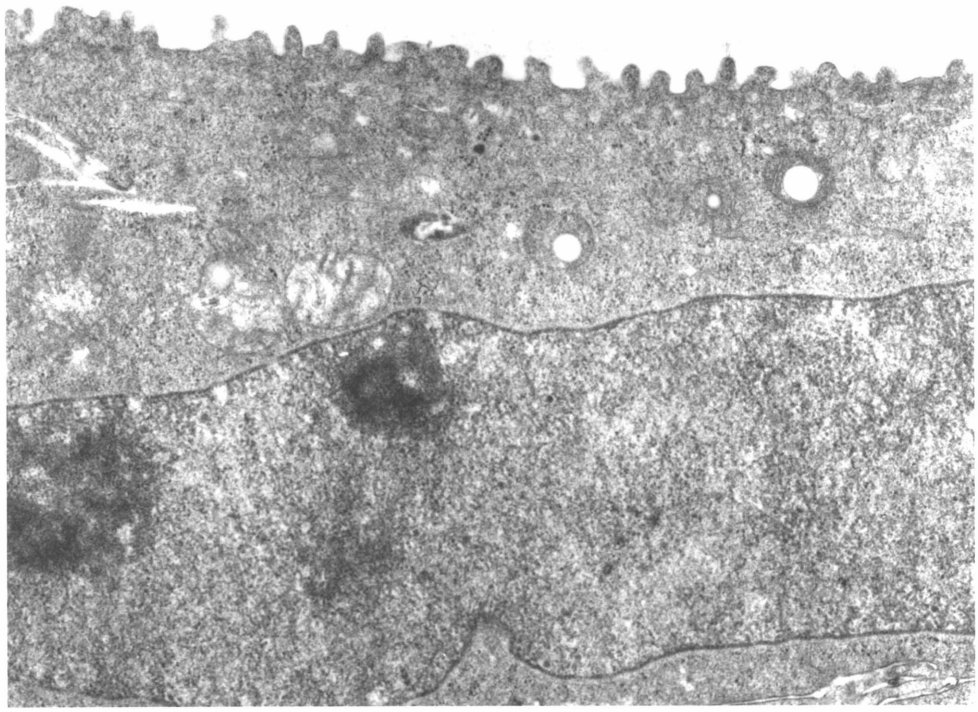
ELECTRON MICROGRAPHS OF THE UROTHELIAL LAYER DERIVED FROM IMPLANTATION OF MB331 BENEATH THE SUBRENAL CAPSULE.



(a) low power micrograph showing multilayered urothelial cell lining. x 7775; Note the absence of the cytoplasmic vesicles, the asymmetric membrane and the characteristic presence of stubby microvillous structures at the luminal surface.

FIGURE V.5B:

ELECTRON MICROGRAPHS OF THE UROTHELIAL LAYER DERIVED FROM IMPLANTATION OF MB331 BENEATH THE SUBRENAL CAPSULE.



(b) Higher power of MB33I cells lining the subcapsular cyst demonstrating the presence of a basement membrane, a lack of structural differentiation markers, and the presence of microvilli at the luminal surface, x22,610.

DISCUSSION

In a previous report we have described a transplantation technique useful in the evaluation of different urothelial cell lines in reconstitution experiments in vivo (Summerhayes et al 1988). Extended studies using this approach have produced varying success with different urothelial cells, due in part to the range in ability of cell lines to compete with a rapidly regenerating normal mucosa. All tumorigenic cell lines tested, attach and grow rapidly in the denuded bladder, however, with slower growing urothelial cells only a small percentage (10%) of transplantation experiments result in cells successfully seeding in this environment.

The subrenal capsule has been used extensively in the past as a site for the growth of tumour material (Bennett et al 1985, Fingert et al 1987) and we have exploited this rich vascular bed for the maintenance of normal urothelium. Implantation of normal bladder mucosa results in the formation of a heterotopic bladder in 9/10 implants. The capacity of bladder epithelium to form a cyst beneath the kidney capsule is not a characteristic common to other mucosal elements and is likely due to the ability of urothelium to rapidly line denuded surfaces (Tucci and Haralambadis 1963). Submucosal elements implanted with normal urothelium appear not to be essential for cyst formation since the preneoplastic epithelial cell line MB33I develops a large heterotopic bladder in the absence of any implanted mesenchyme.

However, electron micrographs reveal mesenchymal-like elements underlying these implanted cells, possibly recruited from within the kidney. The cystic behavior of implanted MB33I cells is similar to that of normal urothelium and provide further confirmation of the preneoplastic nature of these cells.

The subrenal capsule represents an alternative site for the study of normal and preneoplastic urothelium in vivo and offers some advantage over the denuded bladder in reconstitution experiments. Implantation of cells beneath the kidney capsule provides a non-competitive environment for cell proliferation enabling slow growing cells to become established over long periods in vivo. In addition, the maintenance of normal and preneoplastic urothelium at this heterotopic site greatly facilitates the monitoring of early events in neoplasia, as implanted material is easily distinguished against a kidney background. The technique is less demanding than that previously described in the bladder and results in a higher percentage of successful implants in all cell lines tested.

Clearly, however, the subrenal capsule does not provide a complete environment for normal bladder as we have not observed a cyst with a complete fully differentiated urothelial lining. Preneoplastic cell lines at this site rapidly produce a cyst and display a urothelial lining 2-3 cells layers. Although the cyst enlarges over time, the epithelial lining remains well organized and does not develop into a solid tumour growth.

The transition from this growth pattern to that of a solid tumour in vivo is of great interest and use of this model system enables the monitoring of such transition following manipulation of cells in culture. The ability to maintain urothelium at different stages of neoplastic progression in a subrenal capsular site, will enable us to study the early events in neoplastic progression. Experiments are in progress to try and extend the approach to normal human urothelial implants in the kidney capsule of athymic mice.

CHAPTER VI

METASTATIC POTENTIAL OF MURINE UROTHELIAL CELLS EXPRESSING A *ras* ONCOGENE FROM A SITE BENEATH THE RENAL CAPSULE

INTRODUCTION

Recent reports using urothelial cell lines in metastatic assays have demonstrated the importance of the anatomic site in primary tumour growth for the assessment of metastatic potential. These have included a comparison of intramuscular, subcutaneous, footpad and intravenous sites for the introduction of murine cells (Klaunig and Barut 1988) and the differential metastatic behavior of human urothelial cell lines seeded directly into the bladder of a nude mouse (Aherling et al 1987). Although the bladder presents the most desirable site for the growth of primary urothelial tumours we have found that many established cell lines, both human and mouse, grow rapidly to occlude the bladder lumen causing death after only a few weeks. In these cases it is not clear whether an absence of secondary tumour growth is a function of time or an inability to metastasize. In this study we have extended our previous observations using the subrenal capsule as the primary site for the growth of urothelial cells (Joyce et al 1988) in a syngeneic murine urothelial model where a nephrectomy can be performed at a later stage facilitating the true assessment of metastatic potential. The high success rate of tumour take and the ease of removal of the primary tumour, following nephrectomy, resulted in the extended lifespan of the animal enabling the establishment of secondary tumour growth. To test this model, and to evaluate the possible role of ras oncogenes in urothelial cell metastasis, we have used a panel of cell lines which

constitutively, or following transfection, express ras oncogene products. In addition we have included murine urothelial cell lines, constitutively harbouring a K-ras oncogene or transfected cells expressing a human H-ras or N-ras oncogene, to assess the role of this gene family in the metastatic behavior of urothelial cells.

Expression of altered ras proteins following transfection was demonstrated using an H-ras specific antibody in Western blot analysis. All tumorigenic urothelial cell lines expressing ras oncogenes (K, H, or N-ras) were metastatic in this assay. This study demonstrates the potential involvement of ras oncogenes in urothelial cell metastasis and the application of the subrenal capsule model as a metastatic assay.

MATERIALS AND METHODS

Cell Culture:

Urothelial cell lines were established from C57BL/6J mice and were maintained in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum, penicillin and streptomycin.

Transfection assay:

BBN3 and SH264 were seeded at 10^6 cells/60mm culture dish 24 hours prior to the transfection experiment. Plasmid DNA, pSV2neo or pSV2neo-EJ, were applied at 1ug/plate essentially by the method outlined by Felgner et al.(1987). Following a 4 hour incubation with the DNA, in

the absence of serum, cultures were fed with the same volume of complete medium. Drug resistant colonies were selected in G418 (400ug/ml) and individual clones were picked after two weeks and tested for the expression of the altered p21 product.

Western blot analysis:

Gels were run over night at 50 volts and continued the full length of a 12.5% polyacrylamide gel measuring nineteen centimeters. Prestained molecular weight markers of 12,400 (cytochrome c) and 43,000 (ovalbumin) were used to monitor the progress (Diversified Biotech). At the completion of the run, gels were equilibrated in deionized water prior to overnight transfer to nitrocellulose. Gels were cut above the 43,000 marker and only the lower half of the gel was transferred. Successful transfer of low molecular weight proteins was gauged by the visual presence of the prestained 43,000 molecular weight marker on the nitrocellulose. The nitrocellulose was initially blocked in TBS buffer (10mM Tris, 150mM NaCl pH 7.5) containing 1% gelatin for one hour. Each blot was incubated with the H-ras specific peptide antibody (Oncor, Gaithersburg, MD) at a 1 in 100 dilution in TBS-tween 20 (0.5% v/v), overnight at room temperature. The nitrocellulose was washed in several changes of TBS-tween 20 and then incubated with a phosphatase labelled second antibody. Following further washes in TBS-tween 20, blots were developed using the BCIP/NBT phosphatase substrate system (Kirkegaard and Perry Labs, Gaithersburg, MD).

Each Western blot analysis was run with a normal and altered H-ras control to standardize variations observed between different electrophoresis runs.

Metastatic assay:

Subconfluent cells grown in culture were harvested with a rubber policeman, whilst a duplicate dish was trypsinized and counted to determine the total cell number. An approximation of 2 x 10^6 cells was cut from the cell sheet and drawn into a 1/2cc syringe ready for implantation. C57BL/6J recipient mice were anaesthetized and the kidney exteriorized through a left loin incision. Cells were implanted beneath the renal capsule using a 20G teflon intravenous cannula as a trocar.

Mice were left for 2 weeks before a single mouse from each experiment was reassessed, under anaesthesia, for the growth of the primary tumour. When at least one third of the kidney had been replaced by tumour, a nephrectomy was performed and the animal allowed to recover. Mice were maintained for two months or until moribund, at which time autopsies were performed.

RESULTS

Characterization of urothelial cell lines and transfectants:

A panel of mouse bladder epithelial cell lines, established following exposure to different chemical carcinogens, have previously been tested in NIH/3T3 transfection assays for the presence of active transforming genes (Brookes et al 1988). Four tumorigenic cell lines established following exposure of primary cultures to Dimethylbenz(a)anthracene (DMBA) (Summerhayes and Franks 1979) were found to be active in this assay. Three of which were characterized as having a K-ras oncogene activated by a point mutational change at codon 12 resulting in a substitution of serine for glycine. The fourth tumour contains a non-ras related transforming sequence which has not yet been characterized (Brookes et al 1988). DNA from cell lines established after exposure of urothelium to N-butyl(4-hydroxybutyl)Nitrosamine (BBN) (Summerhayes and Chen 1982) consistently scored negative in repeated transfection assays, irrespective of their tumorigenic capacity. It would appear from these experiments that BBN-induced transformation occurs via a route not involving ras gene activation, a finding consistent with previous reports from other groups (Fujita et al 1988). We have used one non-tumorigenic cell line (BBN3), and a tumorigenic non-metastatic cell line SH264, as recipients in transfection with the cloned H-ras gene isolated from the human bladder cell line EJ/T24.

Introduction of the H-ras oncogene into these cells resulted in the acquisition of anchorage independent growth and the ability to form tumours in syngeneic hosts (Summerhayes et al 1986). Confirmation of expression of the oncogene product was obtained using Western blot analysis, by probing with a H-ras specific polyclonal antibody. The cloned c-Ha-ras-1 oncogene used in this part of the study (pEJ) harbours a point mutational change at codon 12, substituting a valine for a glycine at this position. The resultant p21 protein encoded by this gene has previously been shown to migrate more slowly than the normal endogenous H-ras protein and can be distinguished in this way by Western blot analysis (Figure VI.1A-B) following SDS-polyacrylamide gel electrophoresis (Fasano et al 1984, Seeburg et al 1984, Srivastava et al 1985). Only transfected clones expressing the altered p21 protein were used in the metastasis assay.

Table VI.1 summarizes the properties of the different cell lines used in this study.

In vivo metastatic assay:

Two approaches were used for establishing primary tumour growth beneath the subrenal capsule. Cell sheets (approximately 2 x 10^6 cells) were introduced via a trocar or a small tissue piece (1mm^3), excised from a tumour grown subcutaneously in a donor animal, was inserted beneath the subrenal capsule using a pair of fine forceps. Both approaches proved successful in establishing tumour growth.

TABLE VI.1: CHARACTERISATION OF MOUSE UROTHELIAL CELL LINES AND TRANSFECTANTS:

Cell lines/ Transfectants	Tumorigenicity[a]	Activated ras oncogene[b]
DMBA induced		
MB48A	5/5	K-ras
MB48B	5/5	K-ras
MB49	5/5	K-ras
MB49B	5/5	N.D.[c]
BBN-induced		
BBN3	0/10	N.D.
SH264	8/10	N.D.
Transfected		
BBN3-neo	0/10	N.D.
BBN3 pEJ-neo	10/10	H-ras
BBN3 Nras-neo	9/10	N-ras
SH264-neo	8/10	N.D.
SH264 pEJ-neo	9/10	H-ras

DMBA = Dimethylbenz(a)anthracene; BBN = N-Butyl(4-hydroxybutyl)Nitrosamine; N.D. = not detected

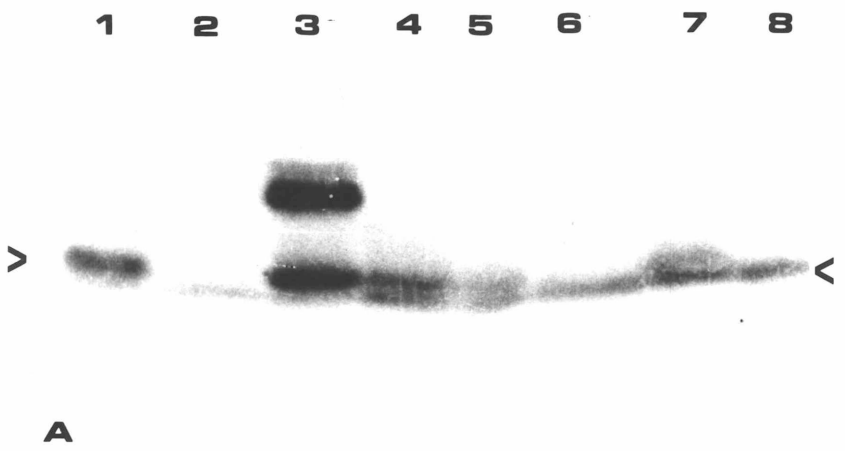
(a): Established in both subrenal capsular and subcutaneous site by implantation of 2 x 10^6 cells.

(b): Presence of a ras oncogene determined following transfection of DNA on NIH/3T3 recipients.

(c): Transforming gene, as detected in NIH/3T3 transfection is not a member of the ras gene family

FIGURE VI.1A:

WESTERN BLOT ANALYSIS OF TOTAL LYSATES PROBED WITH AN H-ras SPECIFIC ANTIBODY:



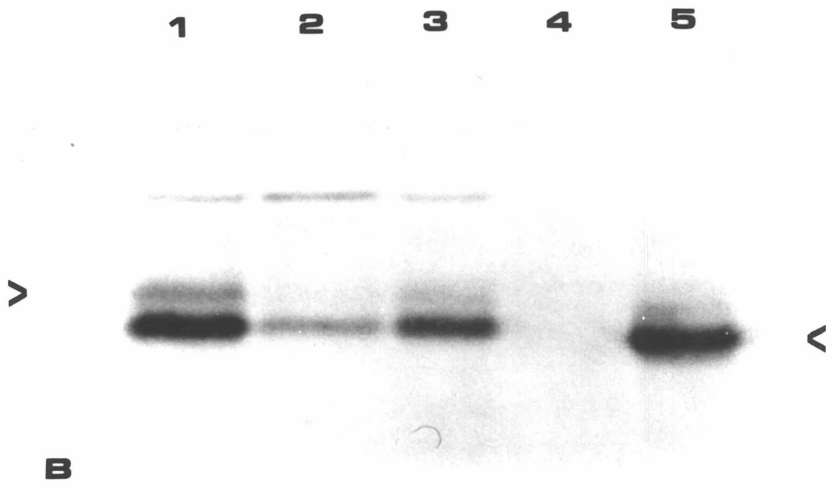
A

A: cell lysates of primary transfectants, lane (1) SH264 pEJ-CL5; (2) SH264 neo-CL4; (3) control 3T3/Wit, transfectant expressing the pEJ oncogene; (4) BBN3 pEJ-CL6; (5) BBN3 pEJ-CL7; (6) BBN3 neo-CL2; (7) SH264 pEJ-CL10 (8) control EC807, transfectant over-expressing the normal H-ras proto-oncogene.

In both panels (A & B), the arrow on the right indicates the migrational level of the normal endogenous H-ras protein and the left arrow the slower migrating mutated p21 oncoprotein.

FIGURE VI.1B:

WESTERN BLOT ANALYSIS OF TOTAL LYSATES PROBED WITH AN H-ras SPECIFIC ANTIBODY:



B: cell lysates from lung secondaries, lane (1) BBN3 pEJ-CL6; (2) BBN3 pEJ-CL7; (3) SH264 pEJ-CL5; (4) SH264 neo-CL2 where the normal p21 is not resolved at this concentration; (5) EC807.

Malignant nodules were detected by palpation after 2-3 weeks and animals were left for varying periods (Table VI.2) until at least one third of the kidney had been replaced by tumour. At this stage animals were anaesthetized and the tumour bearing kidney was removed. All tumours studied represented poorly differentiated carcinomas. Mice were maintained for a further two months or until moribund, when autopsies were performed.

Table VI.2 shows the results of implanting different cell lines at the kidney site with subsequent colonization in the lungs. All tumorigenic urothelial cell lines expressing a ras oncogene (K, H, or N-ras), whether constitutively or following transfection, were metastatic in this assay. One tumorigenic cell line (SH264) remained non-metastatic following transfection with the pSV2-neo gene but displayed metastatic behaviour after transfection with the ras oncogene. Dissociation of cells from lung tissue in these experiments, resulted in the isolation of epithelial cell lines displaying anchorage independent growth and the morphological characteristics of the implanted urothelial cell line (data not shown).

In the case of H-ras transfectants, Western blot analysis confirmed the origin of cells isolated from lung preparations, since all isolates displayed the altered p21 product expressed in tumours from the primary implant (Figure VI.1B). Most notable in secondary isolates, was the increased expression of the oncogenic p21 protein product relative to endogenous p21.

TABLE VI.2 METASTATIC POTENTIAL OF UROTHELIAL CELL LINES FROM A PRIMARY SITE BENEATH THE RENAL CAPSULE

Cell Lines/ Transfectants.	Period to Nephrectomy (d)	No. animals with lung mets/No. animals inoc.	
		1st round	2nd round[a]
MB48A	15	4/4	4/4
MB48B	15	4/4	4/4
MB49	22	4/4	N.D.
MB49B	16	4/4	4/4
BBN3 pEJ-CL6	16	6/6	N.D
BBN3 pEJ-CL7	16	6/6	4/4
BBN3 Nras-CL2	20	4/6	N.D.
BBN3 Nras-CL8	19	5/6	4/4
SH264 neo-CL2	20	0/8	-
SH264 neo-CL4	22	0/8	-
SH264 pEJ-CL5	24	6/6	N.D.
SH264 pEJ-CL10	18	4/6	N.D.

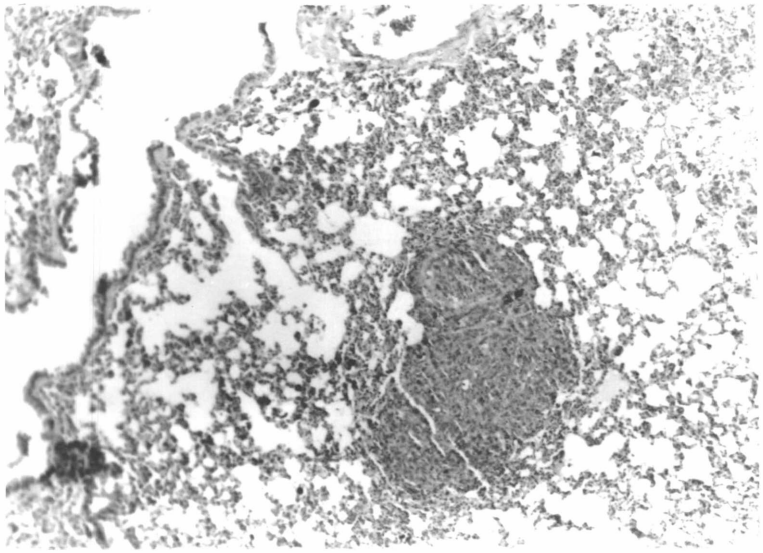
2 x 10^6 cells were introduced beneath the renal capsule via a trocar. Nephrectomies were performed when tumour replaced one third of the kidney. Animals were evaluated after 2 months for metastases or when moribund.

(a): Second round experiments were conducted using carcinoma cells isolated from the lungs of 1st round animals. N.D. = Not done

This contrasts with the Western blot profile of transfectants used to establish the primary tumour growth (compare Figures VI.1A and VI.1B). The band migrating more slowly than the altered p21 product (Figure VI.1B lanes 1 and 3) represents the unprocessed H-ras protein of 23,000 daltons. One cell line from the DMBA-series (MB49B), which does not have a detectable ras oncogene in transfection assays, was highly metastatic in this assay. In contrast SH264, established after exposure to BBN, was non-metastatic in repeated experiments despite the comparable growth rate of the primary tumour to other metastatic lines. Following introduction of an H-ras oncogene via transfection, tumours were established at the kidney site over the same time period as control cells containing the neomycin resistance gene, however, only the ras transfectants displayed metastatic potential in this assay. In all cases of secondary lung tumours the histopathology revealed poorly differentiated islands of cells (Fig. VI.2) comparable to the pathology of the primary tumour. In a number of cases cell lines isolated from dissociated lung tissue were retested in a second round in vivo (Table VI.2). In all cases studied, the metastatic ability of these cells was reproduced with no significant difference in the timing or frequency of secondaries observed in the lungs.

FIGURE VI.2:

LIGHT MICROGRAPH OF MICROSCOPIC LUNG METASTASES.



Microscopic secondary lesions in the lung of an experimental animal showing the poorly differentiated nature of the epithelial island. H & E stained x100.

DISCUSSION

There are numerous reports of the enhanced metastatic ability of NIH/3T3 cells following the introduction of a ras oncogene (Bondy et al 1985, Greig et al 1988, Thorgeirsson et al 1985). One report has extended these observations to a mouse mammary epithelial cell line, where differential metastatic potentials of transfected pairs was observed after intramuscular injection but not intravenous inoculation of paired cell lines (Vousden et al 1986). Conclusions from this study suggest that ras oncogenes enhanced the capability of cells to escape from the primary tumour but have little effect on the ability of cells to seed and become established at a secondary site, a finding corroborated by Aherling et al.(1987) using human bladder cell lines tested in intravenous injections and after seeding in the urothelium of nude mice. Our experience has been that the success in seeding cell lines into a denuded or intact bladder is wide ranging and for this reason we have previously reported the use of the subrenal capsule as a primary site for urothelial cell implantation (Joyce et al 1988). The bladder carcinoma cell line MB48A will become established and grow rapidly in the bladder of a syngeneic mouse, however, we have failed to detected secondary tumours in the lungs of these animals because the primary tumour growth was so rapid the animal died before microscopic lung colonies could become established. Using the subrenal capsule as the primary site for tumour growth,

followed by removal of the tumour bearing kidney, we have been able to identify secondary tumour growth in the lung after longer periods and have extended this study to a panel of well characterized mouse urothelial cell lines to further assess the potential of this model. Metastatic spread of human renal cell carcinomas, following implantation beneath the subrenal capsule, has previously been reported (Naito et al 1986). The advantage of the subrenal capsule as a primary site for tumour growth is twofold; firstly there is a higher success rate of tumour take than following inoculation of cells into the bladder; secondly, in cases of rapidly growing tumours a nephrectomy can be performed extending the lifespan of the animal and ensuring the time period required for the establishment of secondary tumours. In the syngeneic model reported here all tumorigenic cell lines expressing a K, N or H-ras oncogene displayed metastatic potential with secondary tumours appearing in the lungs after relatively short periods. Confirmation of H-ras p21 oncogene expression in lung secondaries demonstrated the derivation of these malignant colonies from transfectants of the primary tumour. Interestingly, the relative levels of endogenous and oncogenic p21 products expressed in lung secondary isolates were altered compared to cells of the original inoculum, displaying elevated levels of the aberrant p21 species in Western blot analysis. This is suggestive of a selection for cells expressing high levels of the oncoprotein although second round metastatic assays, using these cells, revealed no significant

difference in the timing or frequency of secondary lesions in the lungs of recipient animals.

One cell line derived from the BBN-series (SH264) showed no secondary tumour growth in repeat experiments and no detectable ras oncogene in transfection assays. Introduction of an H-ras oncogene into this cell line resulted in the acquisition of metastatic ability whilst the parental cell line, transfected with the pSV2-neo gene, displayed no enhanced metastatic ability. These results demonstrate that cells expressing ras oncogenes are capable of metastasizing in syngeneic animals and that this ability is not as a consequence of transfection techniques. In addition ras activation is clearly not an absolute prerequisite, since MB49B displayed metastatic potential without the presence of an oncogene related to the ras gene family.

We have previously reported preferential activation of H-ras proto-oncogenes in human transitional cell carcinomas (Visvanathan et al 1988) and have characterized seven ras activation events in bladder tumours over a wide range of grade and stage. Interestingly, many of these represent non-invasive lesions, raising the issue of the relevance of ras gene activation in urothelial metastasis. Previous studies have reported that the introduction of a ras oncogene into immortalized cell lines does not always result in the acquisition of tumorigenic potential (Franza et al 1986) or growth phenotypes associated with the transformed state (Summerhayes et al 1986). The action of a ras oncogene in tumorigenicity and metastasis is

presumably complemented by other genetic events involved in the immortalization step.

Hence, immortalized cells in culture should not be considered to universally represent the same stage in neoplastic progression in different cell lines. There is growing evidence that ras gene activation can occur at early stages of neoplastic progression both in human (Bos et al 1987, Forrester et al 1987, Leon et al 1988)and animal model systems (Balmain et al 1984, Zarbl et al 1985, Roop et al 1986, Strickland et al 1988) and may elicit different phenotypes dependent upon the timing and epithelial system studied. However, in this study and the introduction of a ras oncogene has been shown to be capable of inducing metastatic potential in animal model systems and clearly has this potential at later stages of progression. Urothelial cells containing different ras oncogenes are metastatic in this model, however, the mechanism by which ras acts to enhance metastatic potential is still unclear although many different parameters are presently being investigated. The accommodating nature of the subrenal capsular site for the growth and maintenance of urothelial tissue, from all stages of neoplastic progression, now enables us to assess the role of different genes in neoplasia without the prerequisite of tumour induction for in vivo studies and to extend these observations to the metastatic potential of implanted material.

CHAPTER VII

THE ROLE OF DIFFERENT ONCOGENES IN UROTHELIAL NEOPLASTIC PROGRESSION

INTRODUCTION

The development of cancer as a multistage process is now generally accepted although the number of events involved is, as yet, undetermined but probably a minimum of three. The biological transitions involved in carcinogenesis remain unclear but may be, in part, a consequence of the activation of cellular proto-oncogenes. Although ras genes are the most frequently identified transforming gene in human cancer, the role of this oncogene in neoplastic progression has yet to be elucidated. One very productive experimental approach to the study of ras genes in tumour initiation and progression has been the mouse skin model. In this system, initiation is accomplished by administering a single topical treatment of a low dose carcinogen (Dimethylbenz(a)anthracene) whilst tumour development is dependent upon repeated treatment with a promotional agent (TPA). Early work in this model system revealed the presence of an activated H-ras oncogene in preneoplastic papillomatous lesions, and subsequent work, using transfection and transplantation techniques, has demonstrated a causative link between H-ras protooncogene activation and the appearance of papillomas. Interestingly, many of the papillomas containing a ras oncogene regress in this model yet remain as initiated cells in the skin, responsive to the action of promotional agents at a later stage.

A second approach has been the development of transgenic animals where oncogene activation can be

assessed in a host capable of mounting a full biological and physiological response to tumour formation. In this system, cloned oncogenes are introduced into the zygotes of mice followed by implantation of eggs into a receptive foster mother who brings the litter to term. The siblings harbour the exogenous gene sequence, the expression of which can be targeted to different organs by use of a tissue specific promotor or enhancer. This approach has proven to be particularly useful in studying the role of oncogenes throughout neoplastic progression. Initial experiments with the c-myc oncogene, under the control of a mammary tumour virus promotor (Leder et al 1986), demonstrated that the deregulated gene was necessary but not sufficient for mammary tumour formation, as tumours appeared with a T_{50} of 325 days. Similar experiments using the viral Ha-ras oncogene with the same promotor resulted in benign hyperplasia of the Harderian gland and mammary tumour formation, T_{50} of 168 days. As malignant transformation occurred in both studies after a lengthy period, additional events were deemed necessary for tumorigenicity. However, if the two strains were crossed, to yield hybrid mice expressing both genes, then there is a dramatic and synergistic acceleration of tumour development with a T_{50} of 46 days.

Since early events in bladder carcinogenesis may present as subtle changes <u>in vivo</u>, we have previously described a model using the subrenal capsule whereby such events may be detected. As an alternative to the transgenic model, we have adopted a different approach to

study the sequence of early changes involved in urothelial carcinogenesis. This utilizes a viral vector construct as a vehicle to introduce oncogenes into normal murine urothelial cells. Preliminary experiments are described, using the myc/src viral construct, which are designed to establish the feasibility of this approach with the intention of extending this model to include the c-Ha-ras-1 oncogene, and the sequence of changes involved in urothelial neoplastic progression.

The src gene product is a protein kinase that attaches phosphate ions specifically to the aminoacid Tyrosine. This is unusual in that most protein kinases phosphorylate serine or threonine (Ingebritsen and Cohen 1983). Promotion of tyrosine phosphokinase activity is also associated with the mode of action of certain growth factors, i.e. PDGF, EGF, and insulin (Heldin and Westermark 1984). The myc gene product is a phosphorylated protein localized to the nucleus and binds to DNA and may regulate the rate of entry into the S phase of cell division. The involement of the c-myc oncogene in neoplastic transfomation has been established by the frequent DNA rearrangements in tumour cells, yet the contribution of these lesions to the malignant phenotype is still controversial (Cole MD 1986).

Urothelial stripping:

C57Bl/6J mice aged between six and ten weeks were fed and watered ad libitum. The donor mice were anaesthetised with nembutal (5.55mg/ml), 0.1ml/10g. body weight i.p. and a small midline incision over the suprapubic area allowed the bladder to be exteriorised. Using iridectomy scissors, the muscle was incised at the dome of the bladder to expose the underlying intact urothelium. This was held using a pair of fine, blunt-ended forceps and gently detached from the muscle layer with fine curved watchmakers' forceps. The total intact urothelium was then excised from the base of the bladder above the level of the trigone, and incubated with the viral supernatant.

Viral harvest and titres:

Producer cell lines of both the myc/src and pSV2-neo control virus were fed at subconfluence and left overnight with fresh medium. The following morning, after approximately 14 hours, the medium was harvested and filtered through a 0.45 micron filter. Viral titres were assayed on NIH/3T3 focus assays ($2x10^5$ colony forming cells/60mm dish) for the myc/src virus and by selection in G418 (200ug/ml) for the control virus. Equivalent viral titres were used within a single experiment. Mucosal elements, stripped from C57BL/6J mice as above, were incubated for 4 hours in viral supernatant containing polybrene at 8 ug/ml., following incubation, tissue

elements were washed in DMEM and implanted beneath the renal capsule of recipient animals.

Subrenal capsular assay:

Five recipient mice were sacrificed at 2, 4 and 6 weeks post implantation; similarly control animals, containing only the PSV2-Neo construct, were sacrificed at these time points and the implanted kidneys removed for further evaluation. The kidneys were snap frozen in OCT over dry ice. For histological and immunofluorescence studies serial 5u sections were cut using a cryostat with a knife temperature maintained at -20C. The sections were taken and processed for either staining with hematoxylin and eosin or for immunoflourescence using D66 antibody (Summerhayes and Chen 1982), which recognises keratin elements in bladder tissue, as previously described (chapter 4 and 5). Immunofluorescence preparations were viewed using a Zeiss photomicroscope with epiflourescent illuminations.

Preparation of tissue lysates:

In order to confirm the presence of the virus, by the expression of the onco-proteins in the urothelial implants, Western Blot assays were performed to detect the pp60 src gene product. To prepare lysates solely from the implant urothelium, the frozen tissue embedded in OCT compound was sectioned to confirm the implant and the normal kidney material then excised from the block. Cell lysates were then prepared using the technique described

in chapter III.

Total cell lysates were standardised for protein concentration and a 100ug of each were loaded into separate lanes of a 15% polyacrylamide gel.

Confirmation of expression of oncogene products:

This was established using the Western Blot procedure, similarly described in chapter III, utilising an src monoclonal antibody which recognises the endogenous gene product. Thus the viral src gene is detected by the increased tyrosine kinase activity above the normal. Each blot was run with a normal control to standardise any variations observed between different electrophoresis runs.

RESULTS

To determine the logistics and validity of the ideas outlined in the introduction we have selected a viral vector harbouring two different classes of oncogenes. These oncogene classes have been shown to act in a cooperative fashion following transfection into primary fibroblastic cells in culture, conferring tumorigenic potential on these cells (Land et al. 1983). In addition, the viral constructs used are competent for replication allowing continued infection of surrounding mucosal and submucosal elements following implantation. This was considered a desirable feature as confirmed by the recent report by Dotto et al. (1988), which suggests a possible

regulatory role for mesenchymal elements in a keratinocyte reconstruction model. The overall aim of this section of work was to establish the parameters for infection and implantation with future work directed towards studying single oncogenes in bladder neoplastic progression using non-productive infection protocols.

The viral construct used for this preliminary study is diagramed below, the control virus used also encompasses a bacterial gene (PSV2-Neo) which confers on mammalian cells resistance to geneticin (G418), a neomycin analogue:

LTR		tk promoter		LTR
lXXXl	v-myc	lXXXXXXXXXXl	v-src	lXXXl

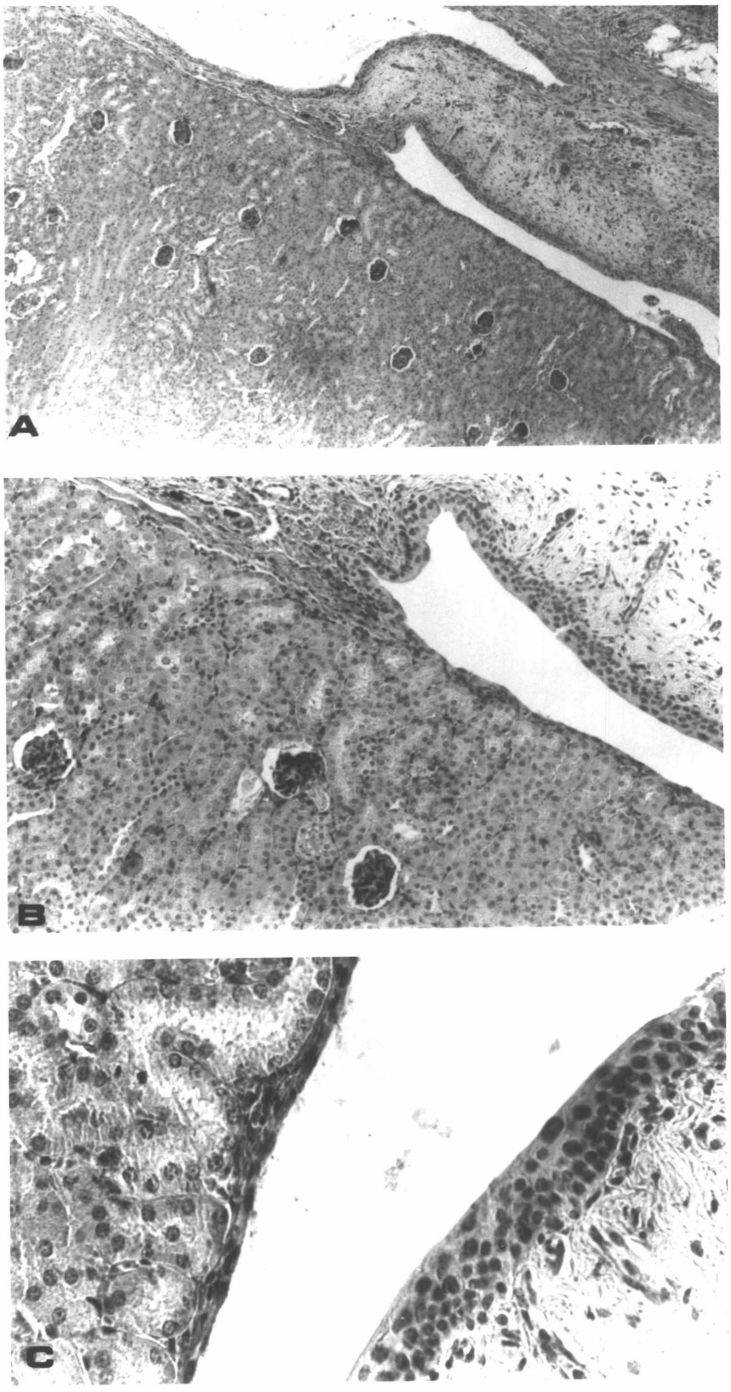
All mucosal elements were exposed to viral medium at a multiplicity of 0.5-1.0 for a period of four hours in culture. The viral titres were assayed in NIH/3T3 focus assays prior to infection. Five animals were sacrificed at 2, 4 and 6 weeks post implantation. Control animals sacrificed at these time points revealed, in all cases, the formation of a heterotopic bladder similar to that previously reported by us using uninfected normal bladder mucosa (Joyce et al. 1988). Histological evaluation revealed the cyst to be lined by a 1-2 cell layer of epithelium (see chapter II) which displayed fully differentiated characteristics in some regions. Immunofluorescence studies of frozen sections from these kidney implants, confirmed the urothelial origin of these implants using an antibody (D66) which recognizes keratin

elements in bladder and not renal tissue. Five animals left for a period of 90 days showed no change in the mucosa lining the cyst although the maturation and differentiation of epithelial elements was more pronounced on histological examination.

At two weeks, the experimental implants exposed to the myc/src construct showed no macroscopic evidence of cyst formation beneath the renal capsule. However, histological evaluation reveals a viable bladder mucosal element with a lumen displaying the features of fully differentiated urothelium (figure VII.1). The differentiation status is reflected in the mucosal thickness on the mesenchymal surface (3-4 cell layers) together with large superficial cells characteristic of normal urothelium. The organisation of such urothelial elements contrasts with the thin mucosal layer observed in the cysts of control animals, but is more consistent with the mucosal layer of normal bladder. Given that these cells are infected with the myc/src construct there is no evidence of malignant transformation, thus additional events may be necessary.

After a further two weeks (4 weeks post implantation) the urothelial implants were even more extensive, presenting as proliferative nodules at the kidney apex. Histological sections showed an increase in the urothelial components consistent with a benign hyperplastic state (figure VII.2). The organization and differentiation of such elements appeared normal, using the criteria of epithelial cell layer number and the presence of fully differentiated superficial cells at the luminal surface.

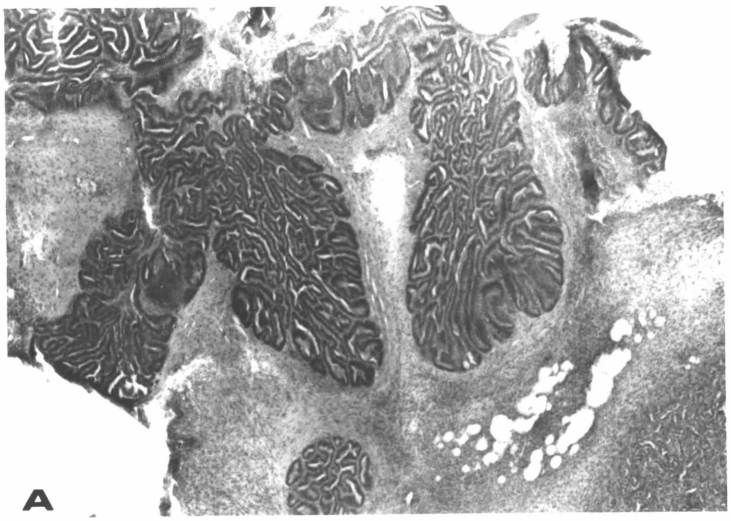
FIGURE VII.1 LIGHT MICROGRAPHS OF EXPERIMENTAL IMPLANT AT TWO WEEKS.



(A) lumen surrounded by a multilayered mucosal lining, especially on the mesenchymal aspect (B), containing superficial cells characteristic of fully differentiated urothelium (C). H & E stained x320

FIGURE VII.2:

LIGHT MICROGRAPH OF EXPERIMENTAL IMPLANT AT FOUR WEEKS



Micrograph demonstrates an increase in the urothelial components consistent with a benign hyperproliferative state. H & E stained x320

Some areas were suggestive of malignant transformation, however we were unable to establish any cell lines from these implants enabling further confirmation of the gene product and subsequent manipulation of the cells in-vivo behaviour.

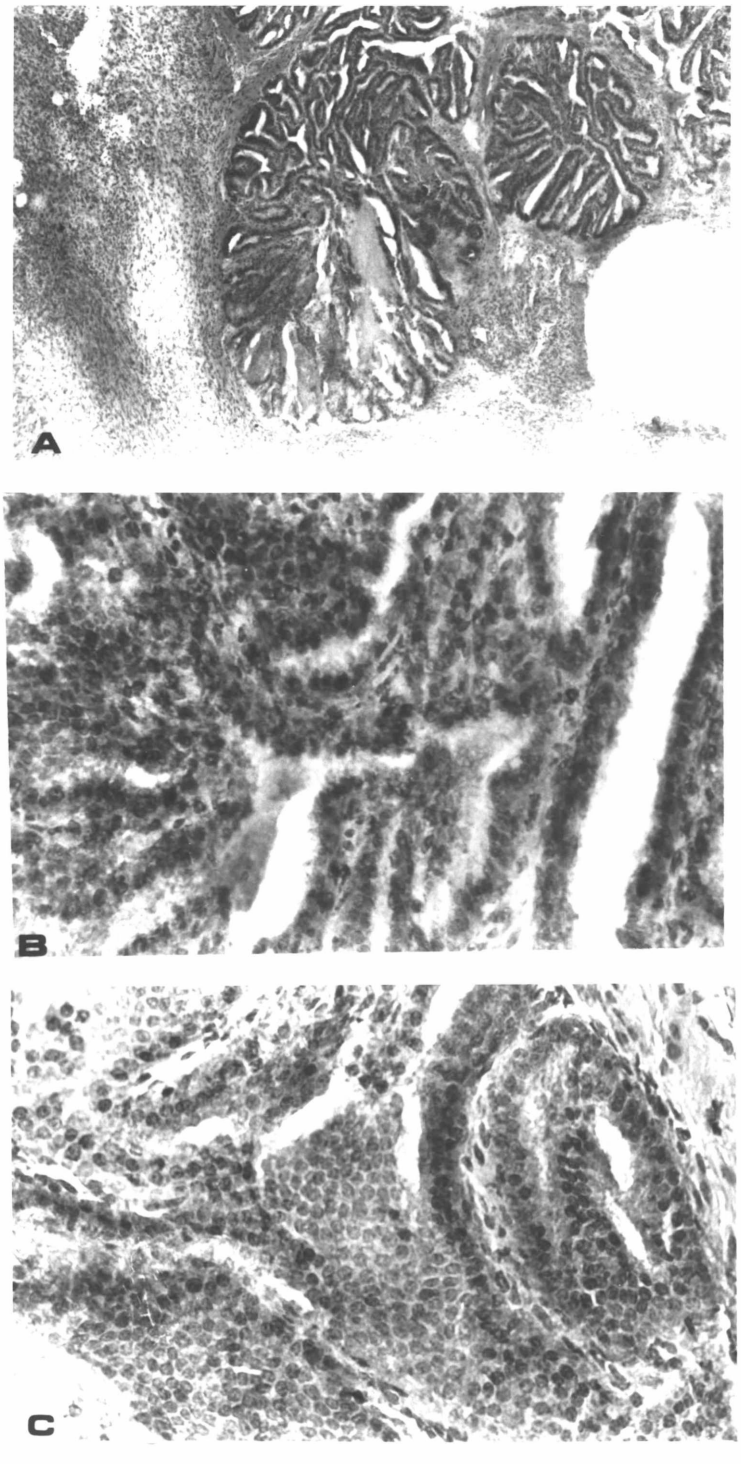
Six weeks post implantation reveals a similar histological appearance with most areas of the implant demonstrating a well organized pattern (figure VII.3), but there are definite areas consistent with a well-differentiated carcinoma with increased cellular layers but regular nuclei.

Confirmation of expression of oncogene products:

To establish the presence of virus and expression of onco-proteins in urothelial implants immunoprecipitation assays were performed to detect the pp60 src gene product. Since the src monoclonal antibody used also recognizes the endogenous gene product, the viral src gene is detected by the increased tyrosine kinase activity compared to the normal endogenous level (figure VII.4). Thus it was necessary to prepare lysates directly from the implant urothelium to prevent dilution of the signal by contaminating kidney material. To achieve this, we prepared a frozen section from implanted kidneys and identified the urothelial implant following staining with haematoxylin and eosin. From this we were able to excise the normal kidney material from the block and prepare cell lysates directly from the sectioned implanted material.

FIGURE VII.3:

LIGHT MICROGRAPHS OF EXPERIMENTAL IMPLANT AT SIX WEEKS



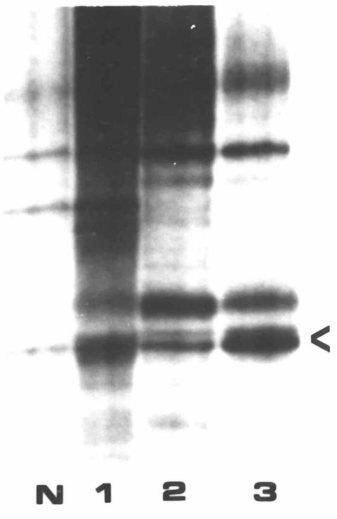
H & E

x320

Micrographs demonstrate a similar histological appearance to earlier implants (A), but note the increased cell layers (B) and the regular organisation of the nuclei (C).

FIGURE VII.4:

WESTERN BLOT ANALYSIS CONFIRMING THE PRESENCE OF THE VIRAL src GENE PRODUCT



Western blot illustrating the increased tyrosine kinase activity associated with the implants (lanes 1-3) compared to a normal subrenal implant (lane N). The increase in kinase activity reflects the greater phosphorylation associated with the increased expression of the viral src gene product.

Figure VII.4 shows a tyrosine kinase assay from control urothelial implants and those exposed to the myc/src construct. All lysates were standardized for protein content, prior to immunoprecipitation, thus the differentials observed in kinase activity reflect the increased phosphorylation associated with the expression of the viral src gene product.

DISCUSSION

Although this section of work is incomplete with respect to the full characterization of events in this model, it does demonstrate the validity of this approach in studying the role of different oncogenes in bladder carcinogenesis. It is interesting to note that the first indication of a compromised mucosa is reflected in the inability of such urothelial implants to form a cyst in this assay system. This has been a good indicator of the successful introduction of different oncogenes in this and subsequent studies. Up to the six week period, we have not observed a frank carcinomatous growth, although regions within implants appear to be progressing from the hyper-proliferative state observed at earlier time points. This observation is not inconsistent with the original work done by Land et al. (1983) in culture, or with results in transgenic mice using oncogenes from the two different classes we have employed. Land showed progressive tumour growth in cooperative experiments with the polyoma large T and ras oncogenes but when myc was co-transfected with

ras, only limited tumour growth was observed. In the case of transgenic mice, Leder's group have demonstrated a synergistic effect of myc and ras in oncogenesis but the timing of the appearance of tumours in such animals suggests that the action of these oncogenes maybe necessary, but alone are not sufficient, for neoplastic progression. It is not surprising therefore that, in such a short study period (6 weeks), we have not observed tumours using the v-myc and v-src oncogenes. Our observations at early time points following infection suggest that the action of the myc/src construct is to promote proliferation without resulting in disorganization of the urothelium or disruption in differentiation. This continues up to six weeks and possibly creates a state whereby a third event is more likely to occur, enabling progression to the tumorigenic state. Such animals are being left for longer periods to determine whether tumour progression occurs. Confirmation of the presence of the src gene product in implants established the presence of the functional virus in compromised urothelial tissue. A second aspect of this study to be considered is the permissive nature of this infection. A recent report by Dotto et al.(1988), using a reconstruction model for mouse keratinocytes, demonstrated the potential for mesenchymal elements to play a regulatory role in epithelial phenotypes in vivo. Using the Harvey sarcoma virus in permissive and non-permissive infection protocols, this work demonstrates a marked difference in the phenotypes scored following grafting onto the backs of recipient

animals. In addition, this work conflicts with previous reports of H-ras oncogenes eliciting papilloma in skin grafting experiments (Roop et al. 1986). However, there are a number of differences between these studies which could account for the disparity and which may be relevant for future evaluation of this model. The conclusion from Dotto's study is that mesenchymal elements can regulate the behavior of epidermal cells expressing an H-ras oncogene and, as such, is consistent with the early work by Balmain's group who showed that direct application of H-ras virus to the backs of mice resulted in no change until that same area was treated with a promoting agent (TPA). The requirement for a promoting agent before papillomas were observed can be interpreted as an event which released the initiated H-ras infected cells from surrounding regulatory control, enabling proliferation of these cells to form papillomatous growths. In the model described in this chapter we have used a permissive infection protocol which would presumably lead to deregulation of epithelial elements following infection of mesenchymal cells involved in the implant. The work in this chapter demonstrates the application of this model in studying the role of different genetic elements in bladder neoplasia. The advantage of this approach over transgenic animals in the ease of manipulation and the ability to introduce a number of different genes without the requirement for a tissue specific promotor. The fact that urothelial tissue at all stages of neoplastic progression remains viable in the subrenal capsular site enables us to

study the early stages of neoplastic progression *in vivo* without the necessity for tumorigenic potential. The work in this section is incomplete with respect to the full characterization of growth and differentiation events associated with myc/src infected mucosa and requires additional controls of myc and src genes in separate viral constructs. However, it does complete the overall plan of this thesis in designing a working model for studying the role of different oncogenes in bladder carcinogenesis.

CHAPTER VIII

SUMMARY AND FUTURE PERSPECTIVES

It is now widely accepted that most tumours are clonal in origin, arising from a single altered cell and that tumour progression frequently results from a series of acquired genetic events within this neoplastic clone. Neoplastic progression involves not only the initiation and promotion of the target cell, but also the ability to invade and metastasize. Thus, it is important to try and define the timing and consequence of oncogene activation within this sequence and to determine its' significance within the clinical setting. The work presented in this thesis has been directed towards resolving some of these issues with respect to bladder carcinogenesis.

Reports on the incidence and frequency of ras mutations on a wide variety of human tumours are accumulating rapidly. Even so, although the ras gene family was first identified in studies with oncogenic viruses, there is still only a limited understanding of the role of these genes in human neoplasia as detailed in the literature review in chapter 1, sections 3 and 4.

H-ras Activation in urothelial tumours:

Our previous results, together with those of Fujita, have suggested the preferential activation of one member of the ras gene family the c-Ha-ras-1 oncogene, in urothelial carcinogenesis. This oncogene was originally identified in NIH/3T3 transfection assays, a labour intensive technique, which has recently been superseded by the PCR (Polymerase Chain Reaction) technique, utilizing oligonucleotide probes to amplify and characterize ras

gene alterations directly from tumour DNA. In an effort to determine a simpler, but sensitive assay to identify altered ras proteins directly from human tumour tissue, we have exploited the different migrational properties of mutated p21 proteins in Western blot analysis. This approach using an H-ras specific antibody provides a rapid, sensitive and specific assay in screening for altered p21 protein products derived from mutations at two major codons hotspots, codons 12 and 61. In addition it provides information as to the levels of protein expression in different lesions.

In our small sample, ras activation was found to occur in tumours representing a range of grade and stages. In order to establish whether these urothelial tumours, expressing altered ras proteins, represent a subpopulation with a particular clinical significance, larger numbers will have to be evaluated with respect to their recurrence and tumour progression.

Biological events in neoplastic progression:

Much of the work on oncogenes has been performed using established cell lines in culture where neoplastic progression is related to cell growth, behavior and tumorigenic potential. These criteria have provided good guidelines in carcinogenesis for cell biology studies, but to the clinician their relationship to in vivo pathology is unclear. In an attempt to resolve this issue the work in this thesis presents a progressive model for reconstituting normal, preneoplastic and tumorigenic

urothelial material in vivo, to study the organization, differentiation and metastatic ability of both cell lines and tissue in this environment. Two sites for reconstituting urothelial elements have been described and both have different advantages for future experimental approaches. As we have demonstrated, the subrenal capsule is an accommodating site for the study of different oncogenes in bladder carcinogenesis, whilst the denuded bladder provides a situation where one can study the interplay of genes and bladder specific carcinogens. By conducting reconstruction experiments at both sites with an immortalized urothelial cell line, we have demonstrated the benign behavior of these cells in vivo leading to the formation of a heterotopic bladder beneath the renal capsule with an organized 2-3 cell mucosal layer.
Fully transformed cell lines produce tumours after implantation at either site and removal of the tumour bearing kidney, demonstrates this to be a useful model for the study of metastatic behavior of urothelial cells. We envisage that the subrenal capsular site in this model will have greater application in future experiments.

The initial goal of this thesis was to define a role for the c-Ha-ras-1 oncogene in bladder neoplasia. However, I have managed only to set the stage for these studies to progress. It is clear from our earlier studies, and those of others, that ras activation can be an early event in neoplasia and yet animal models, including work in this thesis, demonstrate a potential role for this gene in metastasis.

This may seem contradictory and yet it need not be if we consider the timing of ras activation to be important in the phenotype it elicits. It is clear, in either event that activation of a ras proto-oncogene is not the only route by which to achieve metastatic potential. However, we have not been able to address this question fully in these studies, although the reconstitution model described now enables us to do these experiments. As with nearly all scientific studies the experiment is never as straightforward as was originally envisaged, as we have found from preliminary experiments not reported in this thesis. Incubation of normal mucosa with a H-ras virus in a non-productive infection, results in a normal heterotopic bladder following implantation beneath the renal capsule. The same experiment conducted with helper virus in a productive infection, results in the formation of larger cysts with a normal mucosal lining. The preliminary results contrast with those of Dotto et al. and may be explained in a number of ways;

1) the ras oncogene does not elicit a pathological phenotype but may act as an initiation event;
2) ras oncogene does not play a role in bladder carcinogenesis;
3) that mesenchymal elements play a regulatory role in non-productive infections;
4) that the immunocompetence of the syngeneic animals controls the spread of virus in productive infection enabling other regulatory factors to prevail.

Some of these issues can be resolved by using immunodeficient mice but the regulatory role of different tissue elements is more difficult and will undoubtedly be of great interest in future studies.

The role different oncogenes play in neoplasia is still unclear and in fact, whether they play a role at all is still questioned by some. However, the weight of evidence suggests they do have a part to play and this may vary in different organs depending upon the timing of activation and possibly the cast of genetic elements appearing with them. It is clear we are still a long way from resolving many of these issues but the work described in this thesis represents a concerted effort to develop a system that will enable us to address some of the more fundamental questions concerning oncogenes and bladder carcinogenesis. With the possible exception of the neu-oncogene it is evident that a single oncogene will not elicit the fully transformed phenotype in vivo. When one considers this in the light of the regulatory controls mentioned, together with further evidence that loss of genetic material is necessary for tumour formation, some comfort can be drawn from the knowledge that it is a tortuous route to becoming a cancer. On the other hand it is a very complex system to unravel, considering that there is clearly more than one route to achieve tumorigenic potential and we have not touched upon the production of growth factors or any of the numerous other elements implicated in this process.

From the clinicians point of view, characterization of genetic elements important in the tumour progression may lead to the identification of patient cohorts at high risk and possibly, in the long term, to therapies designed specifically to deal with type of tumour. This may involve interferance with transforming gene products or blocking of growth factors required in the maintenance of the transformed phenotype. Whatever the outcome of future research in this field, this approach provides for exciting scientific discoveries relating to cancer and relies upon the identification of different elements involved in a specific cancer, around which possible curative therapies may be designed.

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