Generation of \textit{Ren-2/SV40 TstAg} Transgenic Mice

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Except where stated all the work for this thesis was carried out by myself. I would like to thank Davy Fettes for the RT-PCR results and Stewart Fleming for cutting and viewing the first kidney sections produced from the transgenic founder animals. I would also like to thank Steve Morley for all the advice given over the years and all the other members of John Mullins' lab for their help, advice and patience.
Abbreviations

aa - afferent arteriole
AA - arcuate artery
ACE - angiotensin 1 converting enzyme
Ala - alanine
AMPS - ammonium persulphate
Arg - arginine
Asn - asparagine
BPB - bromophenol blue
BSA - bovine serum albumin
cAMP - cyclic adenosine monophosphate
cDNA - complementary DNA
CsCl - caesium chloride
DAB - diaminobenzidine
DEPC - diethyl pyrocarbonate
dNTP - deoxy nucleotide triphosphate
ddNTP - dideoxy nucleotide triphosphate
EDTA - diaminoethanetetra-acetic acid
EtBr - ethidium bromide
FAC sorting - fluorescent activated cell sorting
G3PDH - glyceraldehyde 3-phosphate dehydrogenase
dH$_2$O - distilled water
HCl - hydrochloric acid
H & E - haemallum and eosin
IA - interlobular artery
IC - inner cortex
IFN - interferon
Ig - immunoglobulin
JG - juxtaglomerular
Kac - potassium acetate
kb - kilobases
KKL - klenow kinase ligase
Leu - leucine
Lys - lysine
mA - milliAmperes
MC - middle cortex
mg - milligrammes
MgCl₂ - magnesium chloride
MgSO₄ - magnesium sulphate
ml - millilitres
mRNA - messenger RNA
NaCl - sodium chloride
NaOH - sodium hydroxide
ng - nanogrammes
NLS - nuclear localization sequence
O.D. - optical density
PBS - phosphate buffered saline
p.c. - post coitum
PCI - phenol/ chloroform/ isoamyl alcohol 25:24:1
PCR - polymerase chain reaction
PolyA - polyadenylation
Rb - retinoblastoma
RB - retinoblastoma gene product
RER - rough endoplasmic reticulum
ROP - reverse osmosis purified
rpm - revolutions per minute
SDS - sodium dodecyl sulphate
Ser - serine
ShAP - shrimp alkaline phosphatase
SMG - submaxillary gland
SV40 - simian virus 40
TAE - Tris acetate EDTA
Tag - T Antigen
TBE - Tris Borate EDTA
TE - Tris EDTA
TEMED - N, N, N', N' tetramethylethylethylene diamine
TESPA - 3-aminopropyl-triethoxy-silane
TLES - Tris HCl, LiCl, EDTA, SDS
tRNA - transfer RNA
UV - ultraviolet
V - volts
Val - valine
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Chapter 1 - Introduction

1.1. Renin.

Renin is an aspartyl protease with a molecular mass of 42000 daltons, which is a key regulator of mammalian blood pressure homeostasis (1). The only known substrate for renin is angiotensinogen, which is cleaved into the decapeptide angiotensin I. A further two amino acids are cleaved by angiotensin I converting enzyme (ACE) to produce the active peptide angiotensin II, (2) which exerts a range of physiological effects (3) (Figure 1.1). These include vasoconstriction and the stimulation of aldosterone and prostaglandin production. Although renin is classically considered to be synthesized by the JG cells of the kidney and secreted into the circulation, a number of studies in mice have identified renin and its mRNA in additional tissues including the adrenal gland, brain, ovary, spleen, testis and uterus (4).

![Renin Angiotensin System](image)

**Figure 1.1:** Renin Angiotensin System. Showing cascade of reactions involved and the effects that the active angiotensin II has on various systems.

Inbred laboratory strains of mice have one of two identified renin genotypes (5). Strains such as C57BL/6 and CBA/CA contain a single renin gene, denoted *Ren-1C*, while strains such as DBA/2J contain two
genes Ren-1\textsuperscript{d} and Ren-2\textsuperscript{d}. The two genes are separated by approximately 21kb and have the same transcriptional orientation with Ren-2\textsuperscript{d} being upstream of Ren-1\textsuperscript{d} (Figure 1.2) (6).

![Diagram of Ren-1 and Ren-2 genes](image)

**Figure 1.2** - Structure of Renin Locus showing positions of exons and transcriptional orientation of both renin genes.

*Ren-2\textsuperscript{d}* is believed to have arisen by a duplication of the renin locus occurring by recombination (7). The coding regions of Ren-1\textsuperscript{c} and Ren-1\textsuperscript{d} are 99% identical (8) and those of Ren-1\textsuperscript{d} and Ren-2\textsuperscript{d} 97% identical (9). At the amino acid level the three encoded proteins are approximately 97% identical (10) with one significant difference being the loss of potential asparagine linked glycosylation sites in Ren-2\textsuperscript{d} (11). Significant differences in the sequences of the 5' and 3' flanking regions of the genes have been observed, (12) including the identification of repetitive elements (13)(14). The differences seen outside the coding regions may account for the different expression patterns observed for the three genes (15).

The expression pattern for the three genes in the mouse varies between different tissues (Table 1.1). In the kidney and the foetal adrenal gland all three genes are expressed at approximately similar levels.
Table 1.1: Showing relative expression levels of the three renin genes in different tissues and the cell types in which expression is seen.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell Type</th>
<th>Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney (15)</td>
<td>JG cells</td>
<td>Ren-1c = Ren-1d = Ren-2d</td>
</tr>
<tr>
<td>SMG (18)(15)</td>
<td>Granular Convoluted Tubule</td>
<td>Ren-2d &gt;&gt; Ren-1c &gt;&gt; Ren-1d</td>
</tr>
<tr>
<td>Foetal Adrenal (23)</td>
<td>Throughout developing cortex</td>
<td>Ren-2d = Ren1d = Ren-1c</td>
</tr>
<tr>
<td>Adult Adrenal (24)</td>
<td>X-zone, Zona fasciculata. Cycling phenotype in females.</td>
<td>Ren-2d = Ren-1d &gt;&gt; Ren-1c</td>
</tr>
<tr>
<td>Testes (15)</td>
<td>Interstitial Leydig cells</td>
<td>Ren-1d &gt; Ren-2d = Ren-1c</td>
</tr>
<tr>
<td>Ovary (25)</td>
<td>Theca, corpus luteum</td>
<td>Ren-2d = Ren-1d = Ren-1c</td>
</tr>
<tr>
<td>Anterior Prostate (24)</td>
<td>Glandular epithelium</td>
<td>Ren-1c &gt;&gt; Ren-2d = Ren-1d</td>
</tr>
<tr>
<td>Foetal Subcutaneous (26)</td>
<td>Specific population of fibroblasts</td>
<td>Ren-1c = Ren-1d &gt; Ren-2d</td>
</tr>
</tbody>
</table>

In the adult adrenal gland the pattern of expression seen differs between mouse strains containing one or two renin genes. Ren-2d and Ren-1d are expressed at equivalent levels whereas Ren-1c mRNA is undetectable in this tissue. Mouse renin exists in two zones of the adrenal gland, the zona glomerulosa and the X zone (16), with the phenotype cycling in relation to the oestrus cycle in female mice (17). In the submaxillary gland the expression of Ren-2d is approximately two orders of magnitude greater than that seen for Ren-1c in single gene mice (18). Expression is regulated by androgens and thyroxine with a higher
expression level seen in male mice after puberty due to the presence of testosterone (19)(20)(21). Treatment of female mice with testosterone will increase the level of expression to that seen in male mice (22).

1.2. Juxtaglomerular Cells

JG cells are modified smooth muscle cells which are localized in the proximal portion of the afferent arterioles of the kidney, adjacent to the glomerulus (27) (Figure 1.3). These cells are characterized by cytoplasm containing renin secretory granules (28)(29) and a well developed Golgi apparatus and rough endoplasmic reticulum (RER) (30)(31). The more distal portions of the afferent arterioles contain vascular smooth muscle cells which are apparently similar in structure to those of other resistance vessels (32). No secretory granules are seen in these cells and the Golgi apparatus and RER are poorly developed (33).

![Diagram of renal juxtaglomerular apparatus](image)

**Figure 1.3**: Juxtaglomerular Apparatus - Diagram of renal juxtaglomerular apparatus showing position of the granulated JG cells in relation to the glomerulus.

The smooth muscle cells of the afferent arteriole have the ability to reversibly differentiate into JG cells and *vice versa* (metaplastic transformation) causing the length of the renin staining portion of the afferent arteriole to vary under different physiological conditions (34). After prolonged periods of hypertension the majority of cells in the afferent arteriole have the characteristics of vascular smooth muscle cells, resulting in a reduction in the length of the renin antibody staining region. The length of the renin antibody staining region of the afferent
arteriole is increased after long periods of hypotension with more cells displaying the JG cell phenotype. *In situ* hybridization studies for renin mRNA also show a similar result indicating that the effect is not due to uptake of renin by these cells (35). Potential intermediate cells have been identified with the electron microscope, where the secretory granules have been sequestered in a vesicle and are undergoing autolytic digestion (36). The effect of an increased renin antibody staining region during hypertension can also be observed after treatment with ACE inhibitors where the length of renin positive region of the afferent arteriole is increased, and in some cases stretches back into the interlobular arteries (23). This reflects a pattern of staining for renin seen during ontogeny of the kidney.

The distribution of the renin positive cells in the kidney during development differs from the adult pattern (Figure 1.4) (37). Renin transcripts are first identified between 14.5 and 15.5 days post coitum (p.c.) and are found in the walls of the largest intrarenal branches of the renal artery. At day 16.5 p.c. immunoreactive renin has been identified in the walls of the interlobular arteries and afferent vessels. Renin transcripts are progressively restricted from the arcuate and interlobular arteries as the kidney matures until they are restricted to the afferent arterioles (38). In the normal adult kidney this is further restricted to the region just adjacent to the glomerulus. It is unclear if the cells mature to a non-renin expressing phenotype or whether the renin expressing cells are replaced by a non-renin expressing cell population. In light of the fact that it is possible for some of these cells to express renin under varying physiological conditions the former possibility appears more likely.

One problem with isolating JG cells for culture is that they only comprise approximately 0.01% of the cells in the kidney (18) and there are presently no selectable markers which would enable their isolation by, for example, fluorescent activated cell (FAC) sorting. Another problem is that cells in primary culture tend to loose their cell specific characteristics (40) and JG cells in primary culture rapidly loose their storage granule phenotype (41).
14.5 days p.c.  
16.5 days p.c.  
18.5 days p.c.

Newborn  
Postnatal-4 days  
Adult

Figure 1.4: Renin Expression in Developing Mouse Kidney. - Black regions show sites of renin expression during development of the kidney. AA - arcuate artery; IA - interlobular artery; aa - afferent arteriole; IC - inner cortex; MC - middle cortex. Adapted from Gomez et al (1986) (39).

One approach to overcoming the problem of isolation of JG cells would be the introduction of a gene, whose product is capable of immortalizing cells, under the control of the renin promoter which would result in the selective immortalization of only those cells which express renin. This would have the advantage that when culturing tissue from the kidney only the JG cells would be immortalized and this would allow the cells to be isolated from the other cells which should not proliferate.

1.3. SV40 T Antigen.

One such gene used to obtain cell lines is the SV40 large T Antigen (SV40 TAg) gene (42). SV40 T Antigen is one of the proteins produced during the early phase of infection during which a quiescent infected cell is activated to produce the enzymes necessary for DNA synthesis (43)(44)
and viral replication (45). This is believed to be achieved by forcing the cells to progress from G0 or G1 into S phase and this induction of S phase is thought to underlie the immortalizing ability of this gene (46). In the infection of the SV40 virus the expression of SV40 TAg is autoregulated (47)(48) and once a certain threshold has been reached the gene is switched off allowing the late phase of viral infection to proceed (49). This will not occur in constructs where the SV40 TAg gene is linked to a particular promoter, allowing the expression of the SV40 TAg gene to continue as long as this promoter is active. It is possible, however, for the T Antigen to bind within the promoter region and increase the expression of cellular genes (50). T Antigen has the ability to both immortalize and transform cells. Immortalization is defined as "the acquisition of unlimited growth potential by cells, without the characteristics usually associated with transformation" (51). These cells can grow indefinitely in culture but still maintain their differentiated phenotype, such as growth factor requirements. Immortalization can be seen as one step on the path towards transformation of the cell. Transformation is a multistep process (52) which is associated with changes in the expression of tumour suppressor genes as well as activation of transforming oncogenes such as ras. Transformed cells are also capable of indefinite growth in culture but these cells lose their growth factor requirements and tissue-specific gene expression is lost. There is often a change in morphology in these cells as well as chromosomal deletions and rearrangements.

DNA binding is not essential for transformation by T Antigen (53). This is best demonstrated by one of the temperature sensitive so-called "supertransforming" mutants which is completely unable to bind DNA but transforms transfected cells at a very high frequency (54). Both carboxy (55) and amino terminals (56)(57) have been found to be essential for efficient transformation by SV40 and these regions are involved in binding cellular proteins. The amino terminal is required for binding to retinoblastoma (Rb) (58) and a more central region is required for binding to p53. The retinoblastoma gene product (RB) has been implicated as a tumour suppressor gene by the observation that when introduced into cell cultures derived from RB deficient tumours the growth rate of the
cells is slowed and by the fact that it can inhibit tumour development in nude mice (59)(60). Rb binds the transcription factor E2F during G1 phase of the cell cycle (61). This complex inhibits the function of E2F and dissociates before the shift into S phase releasing E2F which can stimulate the growth promoting genes required for cell proliferation (62). T Antigen binds to the same region of the retinoblastoma protein as E2F and this binding will dissociate E2F allowing progression of the cells into S phase (63). The p53 protein acts as a tumour suppressor but the mechanism by which it acts is still unclear (64). However, non-specific DNA-protein interactions (65) and DNA sequence-specific interactions (66) may be involved. It was postulated that the T Antigen may inhibit the p53 binding to specific DNA sequences by competing for the same position. However, p53 binding to DNA sequences which do not contain SV40 T Antigen binding sites is still inhibited suggesting that the interaction is between the T Antigen and the p53 protein directly (67).

The aim of producing an immortal cell line is to obtain cells which display as completely normal a differentiated phenotype as possible but grow indefinitely in culture. However the introduction of immortalizing genes can alter the normal phenotype of the cells reducing the use of these cells. This is especially a problem in the case of an immortalizing gene such as SV40 T Antigen which has transforming activity (68). One way to attempt to overcome this problem is to use mutants of the immortalizing gene to produce a temperature sensitive protein that can be switched from an active to an inactive form by changing the temperature at which the cells are grown. Temperature sensitive forms of SV40 T Antigen are available and these include those which have an altered amino acid structure at one (tsA58) (69) or two (tsU19) (53) positions. At 33°C the protein is functional, allowing cells expressing the protein to grow indefinitely. When the temperature is increased to 39°C, the body temperature of rodents, the protein is rapidly denatured and degraded allowing the cells to return to a more normal, differentiated phenotype (70). It has been demonstrated in rat hepatocytes immortalized with a temperature sensitive SV40 TAg that the cells secrete low levels of characteristic liver proteins when grown at 33°C. When the temperature is raised to 39°C normal hepatocyte functions are restored with an
increase in the secretion level and a normal hepatocyte response to glucagon being observed (71)(72).

1.4. Generation of Transgenic Mice.

Microinjection allows the introduction of foreign DNA into the fertilized egg which is then cultured in vitro for a short time before being returned to a foster mother (Figure 1.6). Fertilized eggs are obtained from a CBA/CA - C57BL/6 cross both of which are Ren-1c homozygotes. These strains are used since they produce a large number of eggs when superovulated. DNA is injected into the larger male pronucleus of fertilized eggs which are cultured in vitro for several hours before transferring to a pseudopregnant female. A percentage of the eggs which survive contain the introduced DNA, integrated into the genome. In most cases the DNA integrates randomly as a concatamer to produce a tandem array of many copies at a single locus. This array can be in varying arrangements the most common being a head - tail array (Figure 1.5).

![Head - tail array after transgene integration - showing the most common transgene arrangement where transgenes are aligned in the same orientation, 5' to 3'.](image)

Where integration occurs before the division of the fertilized egg all of the cells of the animal will contain the transgene. In some cases integration occurs at a later stage to produce a mosaic animal in which a smaller percentage of cells contain the transgene. A higher proportion of
the progeny of mosaics will be transgene negative and therefore more offspring may be required before a positive transgenic is detected. In order to detect these mosaics a screen such as PCR must be designed to have the sensitivity to identify mosaic positives. An arbitrary lower level of sensitivity has been set in this laboratory which requires detection of positives where only one tenth of the cells contain the integrated transgene.

Figure 1.6: Generation of Transgenic Mice by Microinjection - showing stages involved in this process. Adapted from Watson et al. (73).

1.5. Ren2(4.6)TAg Transgenic Mice

One approach which has been used successfully to isolate a transformed JG cell line was to produce transgenic mice containing a
construct consisting of 4.6kb of the Ren-2d 5' flanking region (-4600 - +6) upstream of the SV40 T Antigen structural gene (Figure 1.7) (74). This promoter was shown to confer a Ren-2d pattern of tissue specific expression (4). Eight founder mice were produced, five of which produced tumours within 8 months of age. These tumours were found in a number of sites including, kidney, adrenal gland, subcutaneously and in the testes, corresponding to sites of renin synthesis. Renin expression was seen in tumour sections by in situ hybridization as well as by Northern blot. The majority of the renin in renal tumours was present as active renin since treatment of the tissue with trypsin did not significantly increase the amount of active renin present.

Cell lines established from renal tumors were analysed for renin activity in the culture media and cell extracts. The cells contained predominantly active renin whereas the media contained at least ten times more prorenin than active renin. This suggested that the cells were constitutively secreting prorenin into the media. To determine if the cells were capable of regulating the secretion of active renin from the secretory granules 8-bromo-cAMP (75) was added to the culture. This resulted in a 2 - 5 fold increase in the secretion of active renin suggesting that these cells were capable of regulating the secretory mechanisms involving renin. The cells exhibit a fixed granulated morphology which would prevent the use of these cells in the study of factors involved in metaplastic transformation. A more recent paper (99) disagrees with the above result of regulation of Ren-1 secretion suggesting that the previous result was due to activation of expression of the renin gene since the addition of 8-bromo-cAMP was over four hours and not secretion from the granules. The result obtained by Laframboise et al (99) showed no
activation of active Ren-1 secretion on addition of 8-bromo-cAMP. An interesting result however was that As4.1 cells that were transfected with a ren-2 expressing construct did show activation of active Ren-2 secretion on addition of 8-bromo-cAMP. The lack of activation of Ren-1 could be explained by the lack of the appropriate processing enzyme, possibly from the family of prohormone/proprotein convertases (PCs). A member of this family previously shown to be capable of activating Ren-2 protein, PC5 (100), was shown to be present in the As4.1 cells but expression of other members of this family, PC1 and PC2, was not detected which could explain the lack of activation of Ren-1.

Prior to the studies of Sigmund et al (74) it was not predicted that subcutaneous tumours would be seen in these animals since renin had not been identified in this site. Since this occurred in more than one line of transgenic mice it was not thought to be an artifact of the integration site of the transgene (26). Northern blots using RNA isolated from subcutaneous tumours from these transgenic animals identified endogenous Ren-1C expression as well as expression of the T Antigen. In situ hybridizations for renin mRNA combined with T Antigen immunocytochemistry demonstrated a colocalization to the neoplastic cell population. mRNA from normal, nontransgenic mice was then tested and renin mRNA was detected. The mRNA was detectable in eviscerated foetuses and newborn but could not be detected at 6 and 9 days of age or in the adult tissue. At present it is not known whether the reason it is not detected later is due to the loss of renin expressing cells or the dilution due to the rapid growth of other cell types.

1.6. H2-K tsA58 Transgenic Mice

Temperature sensitive forms of SV40 T Antigen have been used to establish immortalized cell lines from transgenic mice expressing the T Antigen gene from SV40 strain tsA58 under the control of the mouse major histocompatibility H2-K (b) promoter (76). This promoter is active at various levels in different tissues and can be induced to higher levels by exposure of the cells to murine γ-interferon (γ-IFN) (77). Thirty four founders were produced after microinjection of this H2-K tsA58 construct
of which only one was suitable to produce conditionally immortal cell lines due to variation in the level of expression of T Antigen between founder animals varying the conditionality of the cells obtained. This demonstrates the lack of tight control possible with a temperature sensitive T Antigen. The transgenic founder animals were tested for their suitability in producing conditionally immortal cells by testing the growth of fibroblasts at 33°C with IFN-γ (permissive conditions), 33°C without IFN-γ or 39.5°C with IFN-γ (semi-permissive conditions) and 39.5°C without IFN-γ (non-permissive conditions). Cells taken from different founders showed varying responses to the conditions, some being able to grow at only the permissive conditions, others at both permissive and semi-permissive conditions and others that were still capable of growth even in the non-permissive conditions. These responses directly correlated with the amount of expression of T Antigen with those able to grow at the higher temperatures having the higher levels of expression. This illustrates one important consideration in using this approach, the level of T Antigen produced is critical for obtaining completely conditional cell lines. In the above example a line of transgenic mice was established from one of 34 original founders where fibroblasts in culture showed relatively low levels of SV40 TAg expression at 33°C and showed optimal growth at 33°C in the presence of IFN-γ. These cells were able to grow indefinitely in culture at 33°C but rapidly ceased proliferation when the temperature was switched to 39.5°C or even semi-permissive conditions. A temperature rise to 37°C was found to be sufficient to prevent growth of the cells. An interesting point to note is when using SV40 TAg temperature sensitive mutants when cells are first switched to 39.5°C the cessation of growth is reversible. After culture at this temperature for longer than 48 hours cells returned to 33°C will no longer proliferate (70). One problem found with all the H2-K tsA58 animals was thymic hyperplasia which began between two and twenty weeks of age. Dissociated cells from these hyperplasias did not produce tumours when transplanted into recipient mice. Hyperplasia was also seen in some cases in peripheral lymphoid tissues. These H2-K tsA58 animals have enabled a wide variety of cell types to be immortalized in culture including osteoclast precursors (78) and colonic epithelial stem cells (79).
1.7. Ren2tsTAg Transgenic Mice

This project sought to utilize the above approaches by targeting the temperature sensitive T Antigen expression using the Ren-2\textsuperscript{d} promoter to obtain conditionally immortalized cell lines expressing the renin genes. The temperature sensitive SV40 TAg used contained three point mutations, one being the tsA58 mutation as used in the H2-K construct and the other two comprising the tsU19 mutation (Figure 1.8). The combination of the two mutations was thought to increase the conditionality of the SV40 TAg although there was no direct evidence to support this. One reason why the combination of two mutations could increase the conditionality could be that because there are two regions of thermolability within the protein this could increase the chances of the protein structure altering sufficiently to prevent its normal function when the temperature is increased.

![Diagram](image)

**Figure 1.8.** Positions of SV40 Temperature Sensitive Mutations - numbering refers to amino acid residue number. NLS - nuclear localization sequence, B. - binding. TsU19 mutation - Paucha et al. (53), tsA58 mutation - Ray et al. (69)

Transgenic mice using this construct containing the temperature sensitive TAg should not produce the tumours described in the
Ren2(4.6)TAg transgenic mice since the SV40 TAg used is inactive at 39°C. This method would lead to the ability to isolate JG cells since in a culture of kidney cells only these will contain an active renin promoter and be immortalized. The fact that the current cell lines isolated from the Ren2(4.6)TAg transgenic mice are not capable of regulating the secretion of active renin may be due to the cells being transformed in the presence of the SV40 TAg protein and losing this characteristic of their differentiated phenotype. The use of a temperature sensitive form of SV40 TAg may allow a more "normal" phenotype to be maintained.

Although it was believed that the combination of tsA58 and tsU19 mutations would increase the conditionality, other information suggested that the A58 mutation alone may be more efficient in rodent cells. There is as yet no direct evidence that the U19 mutation does decrease the conditionality and a second construct containing the A58 mutation alone was designed in order to address this question. This will allow a direct comparison of the two mutations to be made since the promoter region used in each construct is the same. If a reduction in conditionality does occur with the presence of the U19 mutation then the generation of mice containing the Ren2tsA58 transgene may allow the isolation of the conditionally immortal cell lines required.

This thesis describes the construction and generation of transgenic mice containing the renin promoter and the tsA58/tsU19 or tsA58 SV40 T Antigen mutants and analysis of the transgenic mice containing both mutations. These mice may provide a resource for the isolation of JG cell lines.
2.1 Standard Protocols.

2.1.1. Gel Electrophoresis.

Unless stated DNA samples were run on a 0.8% agarose (FMC Bioproducts SeaKem LE agarose 50004) gel buffered with 0.5x TAE and containing 0.5mg/ml ethidium bromide. The two formats used for running the agarose gels were either the 75ml Pharmacia minigel format or the 300ml Pharmacia format. Before loading, 6x Maniatis type IV loading buffer (0.25% bromophenol blue, 40% (w/v) sucrose, 60mM EDTA, pH8.0) was added to the DNA solutions to give a final concentration of 1x buffer. Gels were usually run at 80mA until the bromophenol blue loading dye was approximately halfway down the gel.

2.1.2. Enzyme Digests.

The basic rules set out in Sambrook et al. (80) were followed when setting up restriction digests. DNA was digested in a final concentration of 0.1 - 0.5µg/µl with not more than 10% (v/v) enzyme in the solution. A 4x enzyme/time excess was used, usually 2x enzyme and 2x time. After the required incubation time, 0.5µg of DNA was run on an agarose gel with 6x Maniatis type IV loading buffer to check if digestion was complete. An exception to this was in the case of digests of miniprep DNA, when the whole 10µl digest was run on the gel. Digests were stopped by the addition of 10mM excess of EDTA, pH8.0 and incubation at 65°C for fifteen minutes.

2.1.3. DNA Markers.

2.1.3.1. Bluescript Sau3A Markers.

These markers were used to run on 2% agarose gels with PCR samples and have a range of fragment sizes from 1000 to 78bp. The digest was set up using either Bluescript SK- or Bluescript KS- DNA (Stratagene). An overnight digestion was set up using 100µl plasmid
DNA (1mg/ml) with 28 units of Sau3A enzyme at 37°C. After checking on a gel to ensure digestion was complete, the reaction was stopped by the addition of 10mM excess of EDTA and incubated at 70°C for ten minutes. The DNA was then precipitated with ammonium acetate and isopropanol before resuspending in 200µl TE. Once resuspended, 200µl of 2x Agarose Gel Loading buffer (50% (w/v) glycerol, 2% (w/v) Ficol, 2x TBE, 20mM EDTA, 0.04% bromophenol blue) was added. This solution was then aliquoted and stored at -20°C for use as DNA markers.

2.1.3.2. λHindIII/EcoRI Markers

An overnight digest of 50µg of lambda DNA was set up with 25 units of HindIII and 25 units of EcoRI using Boehringer buffer B, since both enzymes work at maximum efficiency in this buffer. After checking that digestion was complete, the reaction was stopped by the addition of a 10mM excess of EDTA and precipitated with isopropanol and ammonium acetate. The DNA was resuspended in 200µl TE and 200µl 2x Agarose Gel loading buffer was added. This was then stored at -20°C for use as DNA markers.

2.1.4. Phenol/Chloroform Extraction

The phenol/chloroform extraction is used to stop enzyme reactions and remove protein from DNA solutions, the protocol below can be scaled up to the desired volume. An equal volume of buffered phenol was added to a 50µl reaction volume containing 1-10µg DNA and vortexed briefly. This was then centrifuged for a few seconds in an Eppendorf centrifuge 5415C at maximum speed (14000rpm) before adding 50µl of chloroform/isoamyl alcohol (24:1) and vortexing briefly. After centrifugation in the Eppendorf centrifuge for two minutes at maximum speed to separate the phases, the aqueous phase was transferred into a 1.5ml Sarstedt tube, avoiding the interphase. To achieve the maximum DNA recovery a back extraction of the remaining organic phase was usually carried out. This involved addition of one volume of TE, pH8.0, centrifugation for two minutes and pooling the two aqueous phases. 100µl of chloroform/isoamyl alcohol (24:1) was added to the pooled supernatants and vortexed briefly. The phases were then separated by centrifuging for two minutes and the aqueous supernatant was transferred to a 1.5ml Sarstedt tube avoiding the interphase. The DNA in
the aqueous supernatant was precipitated by standard sodium acetate-ethanol precipitation methods and the DNA resolubilized in an appropriate volume of TE, pH8.0 assuming 95% recovery.

2.1.5. EtOH/NaAc Precipitation.

One tenth volume of 3M sodium acetate, pH5.5 and two volumes of ethanol were added to the DNA solution and incubated for one hour to overnight at -20°C. The DNA was pelleted by centrifugation at maximum speed in the 5415C eppendorf centrifuge for fifteen minutes. The supernatant was removed and the pellet washed in 70% ethanol and recentrifuged for five minutes. The supernatant was again removed, the pellet air dried for ten minutes and the DNA resuspended in the appropriate amount of TE, pH8.0.

2.1.6. Isopropanol/Ammonium Acetate Precipitation.

One half volume of 6M Ammonium acetate and 2x final volume isopropanol were added to the DNA solution and incubated at room temperature for fifteen minutes. The DNA was then treated in exactly the same manner as for sodium acetate/ethanol precipitation.

2.1.7. DNA Fluorimetry.

DNA fluorimetry was carried out on a Hoefer Minifluorometer TKO-100. This needed to be switched on at least 30 minutes before use to allow the lamp to warm up sufficiently to give a steady reading and the scale knob turned to 50% sensitivity, as suggested in the manufacturer's protocol. To obtain a zero reading, 2ml of the 1x TNE + dye mix was mixed in the cuvette, placed in the fluorimeter and the zero knob adjusted as required. This step was repeated until consistent readings were obtained (within +/- 5 units). To set the DNA standard, 2ml of 1x TNE + dye mix was placed in the cuvette, 2μl of 250ng/μl of calf thymus DNA was added and mixed well by inverting the cuvette. The reading was taken and the scale knob adjusted to read 250. This step was repeated until consistent readings were obtained as above. The standardization was arranged so that the reading given corresponded to the DNA sample concentration in ng/ml. Readings were then taken from the DNA samples by adding 2μl of DNA to 2ml of 1x TNE + dye mix as above.
2.2 Plasmid Preparations.

2.2.1. Alkaline Lysis.

Large scale preparations of plasmid DNA were carried out by a modification of the alkaline lysis method of Birnboim and Doly (1979) (81). A single bacterial colony was inoculated into 5ml of L-broth (10g/l Difco Bacto-tryptone, 5g/l Difco Bacto Yeast Extract, 10g/l NaCl, pH 7.2) containing 0.2% (w/v) glucose and the appropriate antibiotic and grown to saturation at 34°C for eight hours, or overnight, in an orbital shaker at 250rpm. This culture was then inoculated into 500ml L-broth containing 0.2% (w/v) glucose and the appropriate antibiotic and grown for 16-24 hours at 34°C in an orbital shaker at 250rpm. The culture was transferred to 250ml Sorval centrifuge bottles and centrifuged for ten minutes at 4°C, 4000rpm in a Heraeus Omnifuge 2.0RS centrifuge. The cell pellet was resuspended in 4ml of glucose/Tris/EDTA solution (25mM Tris HCl, pH 8.0, 10mM EDTA, pH 8.0 and 50mM glucose) and transferred to 50ml Sorval round bottomed tube. After addition of 2ml of 10mg/ml fresh lysozyme (Sigma Chemical Co. Ltd. L7651) solution this was mixed and left on ice for ten minutes before adding 10ml of freshly prepared NaOH/SDS solution (0.2M NaOH, 1% (w/v) SDS). This was mixed by stirring gently with a 2ml plastic pipette and the solution left on ice for ten minutes before addition of 7.5ml potassium acetate solution pH 5.5 (3M potassium acetate, 1.18M formic acid). The solution was left on ice for ten minutes and then centrifuged for thirty minutes at 4°C and 16000rpm using an SS34 rotor in a Sorval RC5C centrifuge. The supernatant was decanted into a 50ml Corning conical tube avoiding any floating white material and the DNA precipitated with one volume of isopropanol at -20°C for thirty minutes. The precipitated DNA was then centrifuged at 4°C and 4K for ten minutes in a Heraeus Omnifuge 2.0RS centrifuge and the supernatant removed. The DNA pellet was resuspended in 9ml of TE, pH 8.0 by warming to 37°C with gentle shaking for thirty minutes. Any remaining insoluble material was removed by centrifugation at 4K, 20°C for five minutes in the Heraeus Omnifuge 2.0RS. The supernatant could then be used in the caesium chloride (CsCl) gradient purification protocol.
The method used for isolation of plasmid DNA using a CsCl/ethidium bromide equilibrium centrifugation was similar to that described in Sambrook et al. (80). To achieve a CsCl density of 1.1g/ml, 9ml of the supernatant from the alkaline crude lysate preparation was transferred to a 15ml Falcon 2059 tube containing 9.9g of CsCl (Sigma Chemical Co. Ltd. E8751). The CsCl was dissolved by warming briefly to 37°C and once dissolved 0.45ml of ethidium bromide (10mg/ml) was added before transferring the solution to 11ml Beckmann polyallomer Quickseal tubes. The tubes were filled to the top with a TE/CsCl/EtBr solution (9ml TE, pH 8.0, 9.9g CsCl and 0.45ml 10mg/ml ethidium bromide) and balanced to within 0.02g with light mineral oil (Sigma Chemical Co. Ltd. M3516). These were sealed and centrifuged in a Beckman L-60 or L-7 ultracentrifuge using a NVT-65.1 rotor for sixteen hours at 20°C and 55K, brake set to slow. After centrifugation the plasmid was removed from the gradient with a 19G needle and 2.0ml syringe under long wave U.V. light (Figure 2.1).

This procedure usually resulted in the recovery of approximately 2ml of solution containing the plasmid DNA. This was transferred to a new Quickseal tube and the method repeated. After removing the plasmid DNA from the second centrifugation it was transferred to a 15ml
Falcon 2059 tube and made up to a volume of 2.5ml TE, pH 8.0. This was then loaded on to a NAP-25 column (Pharmacia Biotech - 17-0852-02), previously equilibrated with TE, pH8.0, allowing the solution to run completely into the column bed and discarding the flow through. The plasmid was eluted into a 15ml Falcon 2059 tube by addition of 3.5ml of TE, pH8.0 to the column. To remove the intercalated ethidium bromide, the plasmid solution was extracted with one volume of phenol (Fisons P/2318/05)/chloroform repeating the extraction if the aqueous phase remained pink. This was followed by extraction with one volume of chloroform/isoamyl alcohol 24:1. After extraction the DNA was precipitated with 0.1 volumes 3M sodium acetate, pH5.5 and two volumes of isopropanol for one hour at -20°C. The DNA was pelleted by centrifugation for ten minutes in a Heraeus Omnifuge 2.0RS centrifuge at 4K and 4°C. After removal of the supernatant the pellet was air dried for ten minutes before resuspending in 200μl TE, pH8.0 and transferring to a 1.5ml Sarstedt tube. The DNA concentration was determined by measuring the OD260/280 on a spectrophotometer and the final concentration adjusted to 1mg/ml. The identity of the plasmid was checked by the appropriate restriction digests and the DNA stored at -20°C.

2.2.2. Qiagen Maxipreps.

This method involves alkaline lysis followed by binding of plasmid DNA to the resin in QIAGEN columns. Contaminating RNA and proteins can then be removed during a medium salt wash resulting DNA quality equivalent to that which has undergone two rounds of CsCl/ethidium bromide gradient purification (82)-(83). The protocol can be used to prepare up to 500μg plasmid DNA using a QIAGEN-tip 500 (Qiagen 12163). A single bacterial colony was inoculated into 5ml of L-broth containing 0.2% (w/v) glucose and the appropriate antibiotic and grown for eight hours in an orbital shaker at 37°C and 250rpm. This culture was then inoculated into 250ml of L-broth containing 0.2% (w/v) glucose and the appropriate antibiotic and grown overnight at 34°C in the orbital shaker at 250rpm. The culture was then transferred to a 250ml centrifuge bottle and centrifuged for ten minutes at 4°C, 4000rpm in a Heraeus Omnifuge 2.0RS centrifuge. This pellet was sufficient for two QIAGEN-tip 500 columns and therefore the pellet resuspended in 20ml of buffer P1 (100mg/ml RNase A, 50mM Tris-HCl, 10mM EDTA, pH8.0) and
split between two 50ml Sorval centrifuge tubes. For each tube, 10ml of buffer P2 (200mM NaOH, 1% SDS) was then added, mixed and incubated at room temperature for five minutes. After this time 10ml of buffer P3 (2.55M KAc, pH4.8), prechilled to 4°C, was added, mixed and incubated on ice for twenty minutes. The mix was then centrifuged at 16000rpm using a Sorval SS-34 rotor for thirty minutes at 4°C in a Sorval RC5C centrifuge. The supernatant was then removed immediately into a 50ml Corning tube and added to a QIAGEN-tip 500, previously equilibrated with 10ml of buffer QBT (750mM NaCl, 50mM MOPS, 15% ethanol, pH7.0, 0.15% Triton X-100). After allowing the solution to enter the gel bed the QIAGEN-tip 500 was washed with 2x 30ml of buffer QC (1.0M NaCl, 50mM MOPS, 15% ethanol, pH7.0). The DNA was eluted with 15ml buffer QF (1.25M NaCl, 50mM MOPS, 15% ethanol, pH8.2) into a 30ml Corex tube. The eluate was then precipitated by addition of 0.7 volumes of isopropanol at -20°C overnight as recommended in the manufacturer’s protocol. The DNA was pelleted by centrifugation at 8500rpm in the Sorval SS34 rotor for thirty minutes. The DNA was then washed in 70% ethanol, air dried for five minutes, redissolved in 200μl of TE, pH8.0 and transferred to a 1.5ml Sarstedt tube. The DNA was then quantitated on the spectrophotometer and diluted if required to give a 1mg/ml stock solution. Where smaller amounts of DNA were required the plasmid DNA was recovered using Qiagen-tip 100 columns (Qiagen 12143) in a midiprep protocol. This was suitable for recovery of up to 100μg DNA from 25ml cultures of high copy number plasmids. The basic method was the same as for the maxiprep using Qiagen-tip 500 with the volumes being scaled down to one third of their volume in the maxiprep protocol.

2.3. Cloning Protocols.

2.3.1. Electroelution.

Fragments of DNA (10-100μg) were recovered from agarose gels by electroelution using the ISCO “Little Blue Tank” electroelution apparatus. DNA fragments were isolated on a 0.8% agarose (FMC Bioproducts SeaKem GTG agarose 50072) gel by slicing the region of gel containing the DNA under long wave (366nm) U.V. light (UVP Inc. - Model UVGL-58). Prior to use, the electroelution tank, elution chambers, collars and screens were soaked in 3% (w/v) hydrogen peroxide for a minimum of fifteen
minutes before rinsing in nuclease free water. The ISCO "Little Blue Tank" was then set up as shown in Figure 1.2. This involved 3M sodium acetate/1x ISCO buffer (50mM Tris HCl, pH7.7, 0.2mM EDTA) placed on the positive side of the tank, 2x ISCO buffer placed between the bridge and the screen and 10x ISCO buffer placed between the screen and the negative electrode. The two buffers on the negative side of the tank were poured together to prevent leakage through the screen.

![Diagram of ISCO "Little Blue Tank" setup](image)

**Figure 2.2:** Set up of ISCO "Little Blue Tank" for Electroelution.

Presoaked dialysis membrane (Gibco BRL) was cut to a size slightly larger than the bottom of the wells of the electroelution chamber so that a small amount was left visible once the collar was put in place. The electroelution chamber was assembled by inverting and attaching the dialysis membrane on top of the well by pushing an appropriately sized collar down onto the outside of the well. The chamber was then rinsed in nuclease free water to ensure that there were no leaks in the membrane and 0.1x ISCO/0.05% SDS buffer was then dripped into the narrower well to above the level where the screen sat (Figure 2.3), making sure that no air bubbles were trapped underneath. Pieces of agarose containing the DNA were placed on the screen and 0.1x ISCO/0.05% SDS buffer was dripped around the gel slices until they were covered. The chamber was filled with this solution from the larger well until the depth in the middle of the chamber was a third the height of the wall.
The underside of the chamber was then checked to ensure that it was dry to prevent the formation of short circuits. This was then placed in the electroelution tank with the smaller well towards the positive electrode. If running more than one chamber in the tank at one time each chamber was checked individually for the ability to conduct current. The DNA was electroeluted at 10-15 mA per chamber for two hours. At the end of the elution period the current was reversed for fifteen seconds. The 0.1x ISCO/0.05% SDS buffer was removed with a plastic Pasteur pipette down as far as the grid, the agarose gel slices and grid were removed and the gel slices were checked under U.V. to ensure that the DNA had been eluted. The precipitate under the grid was usually visibly pink due to the ethidium bromide and this was resuspended in the remaining buffer and transferred to a 1.5ml Sarstedt tube. The lower chamber was then rinsed with a further 200μl of 0.1x ISCO/0.05% SDS which was added to the Sarstedt tube. This precipitate was then extracted with 400μl buffered phenol/400μl chloroform; isoamyl alcohol (24:1)/40μl water saturated isobutanol to remove the contaminating SDS and ethidium bromide. The phases were separated by centrifugation in the 5415C eppendorf centrifuge at maximum speed for one minute and the aqueous phase transferred to a 1.5ml Sarstedt tube. This was then re-extracted with chloroform/isoamylalcohol (24:1) as for the standard phenol/chloroform extraction protocol. The DNA was then precipitated with 200μl 6M ammonium acetate and 600μl isopropanol overnight at -20°C. The precipitated DNA was treated as for standard precipitation protocols (2.1.6.) and resuspended in 25μl TE, pH8.0. To check the amount of DNA recovered, 0.5μl of this was run on a 0.8% agarose gel.
2.3.2. QIAEX.  

This method is based on the solubilization of agarose with sodium perchlorate, followed by selective adsorption of DNA onto the QIAEX particles (Qiagen-20020). The protocol described was suitable for DNA fragments from 50bp up to 5Kb from agarose gels and was used in the majority of fragment isolations except those for preparation of microinjection fragments. DNA fragments were excised from 0.5x TAE buffered 0.8% agarose gels in the same manner as that described in the electroelution protocol. The gel slice was transferred to a 1.5ml Sarstedt tube and the weight of the slice determined. For each 100mg of gel slice 300μl of solubilization buffer QX1 (3M NaCl, 4M sodium ClO₄, 10mM Tris HCl, pH7.0, 10mM sodium thiosulphate) was added. The QIAEX suspension was then vortexed vigourously for approximately one minute until a homogeneous solution was obtained and 10μl of QIAEX added for every 5mg of DNA. The solution was then vortexed and incubated at 50°C for ten minutes, vortexing for a few seconds every two minutes to keep the QIAEX in suspension. After ten minutes the sample was centrifuged in the eppendorf centrifuge at maximum speed for thirty seconds in an Eppendorf centrifuge to pellet the QIAEX containing the bound DNA. The supernatant was removed with a pipette and 500μl of QX2 wash buffer (8M sodium ClO₄, 10mM Tris HCl, pH7.0) added. The pellet was resuspended by vortexing and then centrifuged, as above, to pellet the QIAEX. This step was repeated once before adding 500μl of QX3 wash buffer (70% ethanol, 100mM sodium chloride, 10mM Tris HCl, pH7.5) to the pellet and treating as for QX2, again repeating the wash step. After the supernatant from the second QX3 step had been removed, the pellet was centrifuged for a further thirty seconds and any traces of remaining ethanol removed with a pipette. The pellet was then left to air dry for ten minutes. To elute the DNA from the QIAEX, 20μl TE, pH8.0 was added and the pellet was resuspended by vortexing. This was incubated at room temperature for five minutes, vortexing periodically to keep the QIAEX in suspension. The QIAEX was then pelleted by centrifugation for thirty seconds as above and the supernatant removed to a clean 1.5ml Sarstedt tube. A second elution step was carried out and the two supernatants pooled.
2.3.3. Shrimp Alkaline Phosphatase (ShAP) Treatment.

Shrimp alkaline phosphatase (USB 70092) was used instead of the calf alkaline phosphatase because of the ease of inactivation of this enzyme. The enzyme will work in most restriction buffers and is completely and irreversibly inactivated by heating for fifteen minutes at 65°C in Tris buffers. 2.5 units of ShAP were added to the enzyme digest reactions for the final thirty minutes. To inactivate the enzyme this was then heated to 75°C for fifteen minutes in the presence of 10mM excess EDTA.

2.3.4. Blunt Ending of DNA Fragments.

T4 DNA polymerase was used to blunt end DNA fragments containing 3' or 5' single stranded overhangs after digestion with restriction enzymes. This enzyme has a 3' to 5' exonuclease activity, which will cut back single stranded 3' overhangs (84), while a single stranded 5' overhang will be filled in by the polymerase action of the enzyme (85). Two reactions were carried out, one where the product would be used in the cloning steps and the other where [α^32P] dCTP was used in order to check that the reaction had worked. The first reaction mix used 5μg DNA resuspended in 37μl of TE, pH 8.0 after purification by QIAEX. To the DNA 5μl 10x T4 DNA polymerase buffer, 2.5μl 2mM dNTPs, 1μl T4 DNA polymerase (5U/μl) were added and the reaction volume made up to 50μl with ROP water. The reaction was then mixed and incubated at 37°C for one hour.

The labelled reaction was prepared in a similar manner the only difference being that 2.5μl 2mM dNTPs - dCTP were added and 1μl of stock 3000Ci/mole dCTP was added separately. A 2μl aliquot of this reaction mix was taken before addition of the enzyme as a time=0 sample and diluted fifty fold. The reaction was incubated for one hour at 37°C before a time=end aliquot was removed and diluted fifty fold. These samples were used to determine the amount of incorporation of radioactivity by the CTAB precipitation method. After it was determined that the reaction had worked the non labelled reaction was phenol extracted and precipitated by standard methods. The precipitated DNA was resuspended in a suitable volume for the ligation of the linkers, typically 13μl.

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2.3.5. Addition of Linkers.

The ligation reaction for the addition of linkers was set up in a similar manner to that recommended in Sambrook et al. (80). To the 13μl of blunt ended fragment 2μl of 10x ligation buffer, 5μl of appropriate linkers and 5μl T4 DNA Ligase (2.5U/μl) was added. The reaction was then mixed and incubated at 4°C overnight. The reaction was then diluted to 40μl to stop the ligation reaction and digested with the appropriate restriction enzyme by addition of 5μl 10x buffer, 2μl restriction enzyme (10U/μl), 3μl ROP water and incubation at 37°C for two hours. After this time a sample of the fragment was checked on an agarose gel and the digest left to continue during this time. Once it had been shown that the digestion was complete the fragment was gel purified to remove any additional linkers before cloning.

2.3.6. Ligation.

Ligations were set up with a insert:vector molar ratio of 10:1 in a similar manner to that recommended by Sambrook et al. (80), e.g. a 200bp insert ligated to a 2000bp vector results in a 100:1 insert:vector ratio in mg. For each ligation 50ng of vector was used in a total reaction volume of 20μl. In the above example this would result in a reaction mix as follows:-1μl (50ng/μl) vector DNA, 5μl (1mg/ml) insert DNA, 2μl 10x ligation buffer (Boehringer - 660mmol/l Tris HCl, 50mmol/l MgCl2, dithiothreitol 10mmol/l, 10mmol/l ATP; pH7.5), 1μl ROP water and 1μl Boehringer T4 ligase (1U/μl). A parallel reaction was set up ligating vector alone to determine if the vector used was capable of self ligation, thereby potentially reducing the numbers of positive clones on the vector + insert ligation plates. The reactions were incubated at room temperature for a minimum of four hours or overnight before using 10μl of the reaction for transformation.

2.3.7. Competent Cell Method.

This was carried out in a similar manner to that of Chung et al. (86). A 1ml sample of fresh overnight bacterial culture was inoculated into 100ml LB broth supplemented with 10mM MgSO4, 0.2% (w/v) glucose and the appropriate antibiotics. This was incubated at 37°C, 225rpm until an O.D.600 of 0.2-0.3 which took approximately two hours.
The cells were then cooled by swirling culture flasks in ice-water and care was taken that the remaining steps were carried out at 4 °C to achieve maximum competence. The cells were transferred to 250ml sterile Sorval centrifuge bottles and centrifuged for ten minutes at 1000g, 4°C using a precooled swing out rotor in a Heraeus Omnifuge 2.0RS. The supernatant was decanted and the cell pellet resuspended with 10ml ice cold Miller transformation solution (L-Broth containing 10% w/v PEG, 5% w/v DMSO, 20-50mM Mg²⁺). The cells came up in a ‘cloud’ on pipetting and resuspension could be completed by swirling gently. The competent cells were then aliquoted into 1.5ml Sarstedt tubes, snap frozen in a dry ice-ethanol bath and stored at -80°C until required.

2.3.8. Transformation.

Frozen aliquots of competent cells were thawed in an ice-water bath and used immediately. Once thawed, 100μl of cells was pipetted into a cold Falcon 2059 tube containing 1-10μl (100-1000ng) of plasmid DNA and mixed by swirling gently. Alternatively, minitransformations were carried out in 1.5ml Sarstedt tubes using 20μl cells and appropriate volumes of the other solutions. The cells were then incubated for thirty minutes at 4°C before adding 0.9ml prewarmed SOC medium (2% bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM Glucose) and incubating at 37°C, 225rpm for one hour. When transforming the cells with the product of a ligation reaction a control transformation of 50ng pBluescript SKII+ was also carried out to ensure transformation was occurring efficiently. Transformants were then selected by plating 200μl of the transformation solution onto each L-agar plate containing the appropriate antibiotic for selection. The competence was expected to be in the range 10⁷-10⁸ transformants/μg DNA

2.3.9. Minipreparations of Plasmid DNA.

This small scale preparation of plasmid DNA was carried out by a modification of the alkaline lysis method of Birnboim & Doly (81) and Ish-Horowicz & Burke (87). A single bacterial colony was inoculated into 2ml of L-broth containing 0.2% (w/v) glucose and the appropriate antibiotic and grown to saturation overnight. A 1.5 ml aliquot of these cells were transferred to a 1.5ml Sarstedt tube and the remaining solution
stored at 4°C. The 1.5ml aliquots were centrifuged for twenty seconds in an Eppendorf 5415C centrifuge at 14000rpm to pellet the cells and the supernatant aspirated using a Pasteur pipette connected to a water pump. The cell pellet was resuspended in 100μl lysis buffer (25mM Tris HCl, pH8.0, 10mM EDTA, 10% (w/v) glucose, 2mg/ml lysozyme) and left for ten minutes at room temperature when 200μl fresh sodium hydroxide/SDS solution was then added, mixed by tapping the side of the tube with a finger and left on ice for five minutes. After this time 150μl potassium acetate, pH4.8 was added and each tube vortexed at high speed for two seconds to mix before placing on ice for five minutes. The tubes were spun for one minute at full speed in an Eppendorf centrifuge to pellet the cell debris and chromosomal DNA. The supernatant was transferred to a fresh 1.5ml Sarstedt tube and the DNA precipitated by addition of 0.9ml isopropanol and incubation at -20°C for fifteen minutes. The DNA was pelleted by centrifuging for two minutes at maximum speed in the Eppendorf centrifuge, the supernatant removed and the DNA pellet resuspended in 40μl TE, pH8.0. The DNA was then reprecipitated with 20μl 6M ammonium acetate and 120μl isopropanol, incubating for fifteen minutes at -20°C. The DNA was then pelleted by centrifugation for two minutes in an Eppendorf centrifuge. The pellet was washed in 180μl 70% (v/v) ethanol, air-dried for five minutes and redissolved in 20μl TE containing 50mg/ml DNase-free RNase. This gave approximately 1-5μg plasmid DNA which could then be use in restriction analysis.

2.4. PCR to Obtain SV40 PolyA Sequence.

All PCR reactions were carried out in a 50μl volume on a Hybaid PCR machine. Oligonucleotide primers were obtained from Oswell DNA service (Medical and Biological Sciences Building, University of Southampton), the sequences of which are shown in Table 2.1. PCR amplification was carried out using 100ng of DNA and specific oligonucleotide primers directed against the appropriate regions of the SV40 polyA sequence of pBS α1ATRen2 (Chapter 3 - Figure 3.2). Reaction mixtures and conditions were essentially as described previously (88) except after the initial denaturation at 94°C for 5 minutes, templates were subjected to 30 cycles of denaturation for 1 minute 94°C, annealing at one
of three temperatures 58°C, 55°C or 52°C for 1 minute, and extension at 72°C for 1.5 minutes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence - 5' to 3'</th>
<th>Tm</th>
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</thead>
<tbody>
<tr>
<td>SV40 (F)</td>
<td>CTA AGA TCT AAG GTA CCT TTT ACT TGC TTT AAA AAA C</td>
<td>48°C</td>
</tr>
<tr>
<td>SV40 (R)</td>
<td>CTA GCC GGC TTG CTA GCG ACA TGA TAA GAT ACA TTG A</td>
<td>52°C</td>
</tr>
<tr>
<td>Ren2P250 (F)</td>
<td>GTA GGG TAA CTG TGG GGA GG</td>
<td>64°C</td>
</tr>
<tr>
<td>TsA58 (R)</td>
<td>CTG GAA TAG CTC AGA GGC CG</td>
<td>64°C</td>
</tr>
<tr>
<td>G3PDH (F)</td>
<td>ACC ACA GTC CAT AGA GCC ATC AC</td>
<td>62°C</td>
</tr>
<tr>
<td>G3PDH (R)</td>
<td>TCC ACC ACC CTG TTG CTG TA</td>
<td>62°C</td>
</tr>
</tbody>
</table>

Table 2.1: Sequences of oligonucleotide primers used for PCR amplification of SV40 polyA sequences and mouse tail DNAs.

2.5. Klenow Kinase Ligase Reaction (KKL).

The KKL reaction allows efficient cloning of PCR products by ligating the products together to form large concatamers (89). These can then be digested with appropriate restriction enzymes. DNA from the PCR reaction was first precipitated by standard method of ammonium acetate/ethanol precipitation. The precipitated DNA was resuspended in 23μl ROP water and 3μl 10x KKL buffer (300mM Tris HCl, pH7.9, 100mM-MgCl2, 100mM-DTT, 5mM-ATP), 1.5μl 4mM dNTPs, 1.0μl Klenow polymerase (Boehringer, 5U/μl), 0.5μl T4 polynucleotide kinase (BRL 104 531, 10U/μl), 2μl T4 DNA ligase (Pharmacia 27-0985, 1U/μl) were added. The reaction was mixed and incubated at room temperature overnight. A 1μl aliquot of this reaction mix was removed to another Sarstedt tube to give a time=0 sample. The remainder was diluted to double volume in 1x restriction buffer and digested with the appropriate restriction enzymes. After digestion a further 1μl aliquot was taken for time=end and the samples from after the PCR reaction, time=0 and time=end were run on a gel. The digested product was then gel purified by the QIAEX method.
2.6. Sequencing.

2.6.1. T7 (gene 6) Exonuclease Treatment.

Treatment with T7(gene 6) exonuclease produces a single stranded template for sequencing (90) and also removes the possibility of reannealing of the template by digestion of the complementary DNA strand to the template (Figure 2.4). The first step involved linearization of the plasmid by digestion of 4-6µg of DNA with the appropriate restriction enzyme. In an ideal situation a unique restriction site is used which is required to be 5' of the primer recognition site with respect to the template strand. Once digestion was complete, 5 units of T7 (gene 6) exonuclease (USB 70025) was added and incubated at 37°C for a further thirty minutes before stopping the reaction by incubation at 70°C for fifteen minutes. The DNA was precipitated with two volumes of 100% ethanol and 0.1 volumes 3M sodium acetate, pH 5.5 at -70°C for thirty minutes. This was pelleted by centrifugation for thirty minutes in the Eppendorf 1415C centrifuge at maximum speed, the pellet washed in 70% ethanol and resuspended in 7µl ROP water before continuing with the Sequenase Version 2.0 kit (USB 70770).

2.6.2. Sequencing Reaction.

Dideoxy chain termination sequencing (91) was carried out using the single stranded template from the T7 (gene 6) exonuclease reaction. This was added to the annealing mixture containing 2µl 5x Sequencing buffer (100mM MgCl₂, 200mM Tris HCl, pH 7.5, 250mM NaCl) and 1µl of the appropriate primer (0.5 pmol) to give a final reaction volume of 10µl. The mixture was then annealed by heating to 65°C for two minutes and then cooling slowly by removing the 65°C hot block onto the bench and allowing it to cool to below 35°C. The mix was then chilled on ice before addition of the reagents whose preparation is described below. While the tube containing the annealing
Figure 2.4: T7 (gene 6) Exonuclease Reaction. Plasmid DNA is digested with an enzyme which cuts 5' of the primer recognition site and leaves a single stranded overhang. This is digested by the 5' to 3' exonuclease to leave single stranded DNA molecules which cannot reanneal during the sequencing reaction.

mix was cooling, 2.5μl of each Termination mix (G, A, T and C - 80μM of each dNTP, 50mM NaCl, 8μM of the appropriate ddNTP) was added to 1.5ml Sarstedt tubes and placed at 37°C. During this time the labelling mix (1.5mM of dGTP, dCTP and dTTP) was diluted 1:5 and enough Sequenase Version 2.0 enzyme was diluted in ice cold Enzyme Dilution Buffer (1:8) (10mM TrisHCl, pH7.5, 5mM DTT, 0.5mg/ml BSA) to provide for all the templates. Where dITP was being used instead of dGTP, 0.33mg pyrophosphatase (40mg/ml in 10mM Tris HCl, 0.1mM EDTA, 50% glycerol) was added to each ml of the enzyme dilution. Once the above
solutions were prepared and the annealing reactions had cooled to below 35°C the following reagents were added: 1μl 0.1M DTT, 2μl diluted labelling mix, 0.5μl (35S) dATP, 2μl diluted Sequenase Version 2.0. This was mixed and left at room temperature for five minutes before adding 3.5μl of these labelling reactions to each of the termination tubes. These were then mixed and incubated at 37°C for 5 minutes. The reactions were stopped by addition of 4μl of Stop Solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FX). Immediately before loading the samples were heated to 75°C for two minutes and then transferred to ice.

2.6.3. Sequencing Gels.

The plates used for sequencing were prepared in a similar manner to that described in Sambrook et al. (80) by cleaning with phosphate free detergent before use and rinsing well. One of each pair of plates was siliconized before use by wiping Repelcote (BDH 63239) over the plates three or four times. Both plates were rinsed with distilled water and ethanol and left to air dry before taping. The long sides of the plates were taped first, using tissue to smooth down the tape and then the bottom was taped twice to ensure a good seal. The acrylamide solutions were made using the amounts shown in Table 2.2 and were left to dissolve overnight. The solutions were then filtered, degassed and could be stored at 4°C for up to six weeks.

<table>
<thead>
<tr>
<th></th>
<th>0.5x TBE (500ml)</th>
<th>4.0x TBE (100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide</td>
<td>75.0ml</td>
<td>15.0ml</td>
</tr>
<tr>
<td>10x TBE</td>
<td>25.0ml</td>
<td>40.0ml</td>
</tr>
<tr>
<td>Urea</td>
<td>230.0g</td>
<td>46.0g</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>-</td>
<td>2.0ml</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>5.0g</td>
</tr>
</tbody>
</table>

Table 2.2: Amounts of reagents required for acrylamide gels.

The gels run were 600mm buffer gradient gels with a 0.5 - 4.0x TBE gradient (92). To make these gels, 8.0ml of 4.0x TBE and 60.0ml of 0.5x TBE were placed in separate beakers with stirrers, AMPS (ammonium persulphate) and TEMED (N, N, N', N'...
tetramethylethylethylenediamine) added as set in Table 2.3 and the solutions mixed thoroughly.

<table>
<thead>
<tr>
<th></th>
<th>8.0ml - 4.0x TBE</th>
<th>60ml - 0.5xTBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% (w/v) AMPS</td>
<td>16μl (2x8)</td>
<td>120μl (2x60)</td>
</tr>
<tr>
<td>TEMED</td>
<td>8μl</td>
<td>50μl</td>
</tr>
</tbody>
</table>

Table 2.3:- Reagents required for addition immediately prior to making acrylamide gels.

After addition of TEMED the gels begin to polymerize and therefore it was required to work quickly. 7ml of the 0.5x TBE solution was pipetted using a 10ml glass pipette followed by 8ml of the 4.0x TBE solution and a gradient formed by introduction of four or five bubbles into the pipette. This solution was poured carefully down the edge of the plates, lowering gently to the horizontal position to slow the flow down and the gel filled with the remaining 0.5x TBE solution. The gel was placed upright to settle the bottom gradient before gently lowering. The comb was then placed in the top of the gel with the teeth facing out of the gel and the gel clamped round the sides and bottom before leaving to polymerize for one hour. The remaining 0.5x TBE solution was left in the beaker for this time to give an indication of when the gel had polymerized.

Once polymerized the tape from the bottom of the plates was removed and the excess gel around the comb cut away. This region was rinsed under the tap to clean off any excess pieces of gel still in place and the comb removed. The gel was placed in the apparatus (Gibco BRL, Model SA 31096-068) with 0.5x TBE in the upper and lower troughs with the large plate facing out. The top of the gel was cleared with a plastic Pasteur pipette, the comb repositioned with the teeth facing down and the lanes marked on the glass leaving an uneven orientation. Two 2.5μl samples were loaded in the order T, C, G, A and then the next four lanes were cleared with the Pasteur pipette before loading. This was repeated until all the lanes were loaded and the gel then run at 2000V for approximately six hours until the bromophenol blue loading dye had almost reached the bottom of the gel. After this time the gel was removed, after draining the top tank of the apparatus, and placed small plate facing up. The tape was removed and a scalpel used to separate the
two plates. The small plate was removed and the larger plate with gel attached was placed gently in fixing solution (10% methanol, 10% acetic acid). This was left for fifteen minutes and then if the gel was firmly stuck, the plate was stood up vertically to drain. The plate was then laid, gel side down, onto Whatmann 3mm paper, pressed firmly down and the gel peeled off onto the paper. This was then placed in the gel drier, covered with a piece of Saran wrap and dried for two hours at 80°C. The dried gel was then exposed to Kodak XOMAT film at room temperature overnight.

2.7. Isolation of Microinjection Fragments.

2.7.1. Dialysis Tubing Preparation.

Dialysis tubing was prepared as described in Sambrook et. al. (80). The tubing was cut into 10-20 cm lengths and rinsed thoroughly in distilled water before boiling for ten minutes in 2% (w/v) sodium bicarbonate and 1mM EDTA, pH8.0. After allowing to cool the dialysis tubing was stored in 1mM EDTA, pH8.0 at 4°C. Before use the tubing was washed inside and out with distilled water.

2.7.2. Isolation of Injection Fragments.

Injection fragments were isolated from plasmids by digestion with two restriction enzymes. Digestion of 50μg of DNA was carried out using 25 units of the appropriate restriction enzyme, in a final volume of 100μl, and incubation for four hours. After incubation 1μl was run on an agarose gel to check that digestion was complete. The DNA was then digested overnight with 25 units of the second enzyme. Next morning the reaction was spiked with a further 10 units of the second enzyme and digested for a further two hours at 37°C. To check that digestion was complete 2μl were run on an agarose gel after which 5μl 0.4M-EDTA, pH8.0 was added to the reaction mix and heated to 65°C for fifteen minutes.

To purify the injection fragment 20μl of 6 x Maniatis loading buffer was then added to the 100μl reaction and loaded onto a minigel which had been cast with a one slot comb. The gel was then run at 10mA/cm until the bromophenol blue loading dye was about halfway down gel.
The fragment was then excised under long wave U.V. light and divided between two electroelution traps.

The DNA was then electroeluted by the standard 'ISCO' protocol, followed by phenol:chloroform:isobutanol extraction and precipitation for at least one hour at -20°C with 200μl 6M Ammonium acetate and 600μl isopropanol. After centrifugation the pellet was washed with 70% ethanol, air dried and resuspended in 20μl of TE; pH8.0 (10mM Tris-HCl pH8.0 containing 1mM EDTA) and 0.5μl checked on a gel.

The DNA sample in 20μl TE, pH8.0 was added to 2.4μl TE containing 2.7g CsCl. This solution was then placed in a 5ml Beckmann polyallomer centrifuge tube and centrifuged in a SW50.1 rotor at 40K for 48 hours at 20°C. The samples were then removed from the rotor and clamped in a retort stand and the bottom of the tube punctured with a 19g needle collecting 4-drop samples into 1.5ml Sartstedt tubes as they were released by gravity. A 3μl aliquot from each sample was run on a gel to check which fractions contained the DNA. These fractions were pooled, placed in dialysis tubing and dialyzed in TE for 48 hours at 4°C changing the buffer eight times during this time.

A 5μl sample of the dialyzed material was run directly on a 0.8% gel with lambda markers and the O.D. checked with the fluorometer. The fragment was diluted to 1ng/μl in Injection Buffer (10mM Tris; pH7.4, 0.1mM EDTA) and stored at -20°C ready for injection. A 2μl sample of the diluted fragment was run on a gel to check the DNA concentration. At the correct concentration the DNA should be visible as a faint band on the gel.

2.8. Preparation of DNA from Tail Samples.

Mouse tails were clipped and sealed with Vetbond by Gillian Brooker and DNA was prepared from these clips. Tail clips (~1cm) were first minced in 600μl of tail buffer (50mM Tris-HCl pH8.0, 100mM EDTA, 100mM NaCl, 1.0% SDS) and 35μl 10mg/ml Proteinase K (Boehringer 161519) added to each sample. These were then incubated overnight at 55°C. The tubes then had 20μl of 20mg/ml DNase free RNase (1119915)
added before being incubated at 37°C for one hour. Subsequently 600μl phenol was added and the tubes placed on a vertical rotator for fifteen minutes. The phases were then separated by centrifugation for fifteen minutes at maximum speed in an eppendorf centrifuge. After removing the aqueous phase to a clean 1.5ml Sarstedt tube using a cut off blue pipette tip, 300μl of phenol and 300μl of chloroform/isoamyl alcohol (24:1) was added and the tubes rotated. The phases were then separated by centrifugation for five minutes and the aqueous phase and interphase removed as above, 600μl of chloroform/isoamyl alcohol added and the tubes rotated for five minutes. After centrifugation for five minutes the aqueous phase was removed to a clean tube without taking the interphase. The DNA was precipitated with 600μl isopropanol for fifteen minutes at room temperature. The pelleted DNA was then resuspended overnight in 200μl of TE, pH8.0 and reprecipitated with 0.5 volumes 6M ammonium acetate and two volumes of isopropanol. This was then centrifuged to pellet the DNA as above and the pellet washed in 70% ethanol. The DNA was finally resuspended overnight in 200μl TE.

2.9. PCR Analysis of Tail DNAs.

The PCR block was preheated to the denaturing temperature of 95°C and paused until ready to load the samples. Each 0.5μl sample of tail DNA was pipetted into a 0.5ml Sartstedt tube and placed on ice. The reaction mix was made up in bulk in a 1.5 ml Sarstedt tube on ice and 24.5μl then aliquoted into each tube containing the DNA. For each reaction the following was added 19.2μl ROP water, 2.5μl 10x PCR buffer (500mM KCl, 100mM TrisHCl pH9 at 25°C, 1% TritonX-100), 0.5μl 10mM dNTPs, 0.5μl 50mM Ren2P250 forward primer, 0.5μl 50mM tsA58 reverse primer, 0.1μl 50mM G3PDH forward primer, 0.1μl 50mM G3PDH reverse primer, 1.5μl magnesium chloride (25mM stock, Promega A351) and 0.141 Taq DNA polymerase. Details of the primers are shown in Table 2.1. After the reaction mix was aliquoted 50μl of light mineral oil was added to each tube and the tubes left on ice until all were ready to be transferred to the PCR block. The conditions for the PCR reaction involved an initial denaturation step at 95°C for ten minutes, followed by thirty cycles consisting of, denaturation - 95°C, 1 minute; anneal - 64°C, 1 minute and
extension - 72°C, 1.5 minutes. The thirty cycles were followed by one cycle at 72°C for ten minutes followed by one cycle at 25°C for one minute.

After completion of the cycles 50μl of chloroform/isoamylalcohol (24:1) was added to each tube, vortexed and briefly centrifuged. The aqueous phase containing the amplified product could then be easily pipetted from the tube without having to pass the tip through a layer of light mineral oil. A 10μl sample of the product was then run on a 2% agarose gel.

2.10. Southern Analysis.(93)

2.10.1. Restriction Digests for Southern Blots.

Restriction digests were set up using 12μg of tail in a 50μl reaction volume with 2x excess of the appropriate restriction enzyme and incubated overnight at 37°C. To check if digestion was complete 1μl samples were run on a 0.8% agarose gel and the remainder of the reactions spiked with more enzyme and allowed to digest further. Once it had been shown that digestion was complete, 8μl of 6x Maniatis IV loading buffer was added and the digests loaded onto a 0.8% agarose gel with a 2x 14 slot, wide comb format. Digested mouse DNA, negative for the transgene, and 1, 10 and 50 copy controls were also loaded on the gel along with lambda HindIII/EcoRI DNA markers. The gel was then run at 60V for approximately five hours, photographed and then cut to size before blotting.

2.10.2. Southern Blotting.

The DNA was depurinated by soaking the gel with gentle agitation for 10 minutes in 0.2M HCl and then rinsed in ROP water. The gel was then soaked in denaturing solution (1.5M sodium chloride, 0.5M sodium hydroxide) for 45 minutes with gentle agitation. The gel was again rinsed briefly in distilled water before soaking in neutralizing solution (1M Tris HCl, pH7.4, 1.5M sodium chloride) for thirty minutes with gentle agitation. The neutralizing solution was then changed and the gel soaked for another fifteen minutes. Boehringer nylon membrane (1417240) and three sheets of Whatmann 3mm paper were cut to the size of the gel to be blotted. A Pyrex dish containing 20x SSC with a glass plate over the top
was set up with three sheets of Whatmann 3mm paper acting as a wick into the 20x SSC solution. The gel was then inverted and placed on the Whatmann papers over the dish, smoothing out to prevent any bubbles. The membrane was prewetted in 6x SSC and using millipore forceps placed on top of the gel. Any air bubbles that could be seen were smoothed out and a glass pipette rolled over the gel to remove them. The Whatmann papers cut to the same size as the gel were individually wetted in 6x SSC and placed on the gel in the same manner as the membrane. The gel was surrounded with Saran wrap and a stack of Kleenex paper towels placed on top. A glass plate was then placed on top of the towels, a full 500ml bottle placed on top of this and the stack left blotting overnight. After blotting the membrane was removed after marking the positions of the wells from the gel and U.V. treated in a Stratagene U.V. Stratalinker 1800, 2x AUTO setting, to bind the DNA.

2.10.3. Hybridization of Southern Blots.

Southern Blots were wetted by floating it in a tray of 6x SSC and then submerging it for 5 minutes once completely wetted. This was then placed in a warm Techne hybridization bottle containing 20ml of prewarmed prehybridization solution (5x SSC, 5x Denhardt's solution, 0.5% (w/v) SDS, 1mM EDTA and 100mg/ml heat denatured sheared salmon sperm DNA) at 65°C for a minimum of 4 hours. After prehybridization a heat denatured random primed probe was mixed with the solution along with 200μl 10mg/ml heat denatured salmon sperm DNA and hybridized overnight at 65°C. On completion of hybridization the probe solution was discarded and the blot washed as follows:

   i) 2x rinse, room temp. in 2x SSC, 0.1% (w/v) SDS,
   ii) 2x 15 mins, 65°C in 2x SSC, 0.1% (w/v) SDS,
   iii) 2x 10 mins, 65°C in 1x SSC, 0.1% (w/v) SDS,
   iv) 2x 30 mins, 65°C in 1x SSC, 0.1% (w/v) SDS.

The blot was briefly air dried on a piece of Whatmann 3mm paper. While still damp it was wrapped in Saran wrap and placed in a cassette for autoradiography using Kodak XOMAT XAR-5 film and two enhancement screens. The film was exposed for 24 hours before
developing and then re-exposed if required for a suitable amount of time to detect the required signal.

2.11. Labelling Probes.

2.11.1. Random-primed Probes.

The probes were made in a similar manner to that described by Feinberg and Vogelstein (94)-(95). The 1μl template DNA (50ng/μl), 2.5μl random primer (100ng/μl) and 6.5μl TE, pH 8.0 were mixed together and denatured at 100°C for 5 minutes before placing on ice. After brief centrifugation the remaining reagents were added in the following order; 5μl oligo labelling buffer (0.5M Tris HCl pH6.9, 0.1M MgSO₄, 1mM DTT, 1mM dATP, 1mM dGTP, 1mM dTTP), 5μl (32P)-dCTP (10mCi/ml), 24μl sterile ROP water, 1μl nuclease free BSA (10mg/ml), 5μl Klenow enzyme (1U/μl). This was then incubated at 22°C for one hour before spotting a 1μl sample onto a DE81 filter disc to be used to determine the incorporated radioactivity. The reaction was then stopped with a 10mM excess of EDTA. The 50μl probe solution was then made up to a 500μl 6x SSC solution by addition of 150μl 20x SSC and 300μl ROP water and applied to a NAP-5 column (Pharmacia) which had been equilibrated with 6x SSC solution. This was eluted into a 1.5ml screw capped eppendorf tube with 1ml 6x SSC. The probe was denatured by splitting into two aliquots and heating at 100°C for 10 minutes before quenching on ice.

2.11.2. NAP-5 Columns.

Pharmacia NAP-5 columns are prepacked disposable columns containing Sephadex G-25 medium of DNA grade. These were used to remove unincorporated nucleotides from the random-primed labelling reactions. The columns were first equilibrated with 20ml 6x SSC and the equilibration buffer allowed to completely enter the gel bed. The 50μl sample from the random-primed reaction was then made up to a volume of 500μl using 150μl 20x SSC and 300μl ROP water to give a final concentration of 6x SSC. The 500μl sample was then added to the column and allowed to completely enter the gel bed. The DNA was then eluted with the addition of 1ml of 6x SSC and collected in a 1.5ml screw capped
eppendorf tube. This was then split between two screw-capped eppendorf tubes and heat-denatured as required for hybridization.

2.11.3. CTAB Precipitation.

This method was used to check the incorporation of radioactively labelled nucleotides. To obtain a time=0 reading 2μl samples were taken before addition of the enzyme and diluted fifty fold in a 15ml Corning tube with ice-cold TE, pH8.0. A time=end sample was taken after incubation and treated in a similar manner. To determine the total number of counts in the samples 2μl was taken and spotted onto a Whatmann glass fibre filter which was left to air dry for five minutes. To the remainder of the solution the following reagents were added in order:- 1ml 4% (w/v) CTAB, 0.2ml yeast tRNA solution. This was vortexed and left to stand at room temperature for ten minutes. The precipitate was then collected on a Whatmann glass fibre filter by suction into a waste flask. The tube was then rinsed with five changes of 2ml ROP water and the rinses also filtered in a similar manner. The glass fibre filters containing the precipitate were then dried in the microwave for one minute on power level 4. The filters were placed individually in vials and 2.5ml of non-aqueous scintillation fluid (Ultima Gold) added. These were then placed in the scintillation counter to determine the amount of radioactivity present in each sample.

2.11.4. Cerenkov Counting of Labelled Probes.

To determine the incorporated activity a two 1μl samples were taken after incubation of the reaction and spotted onto Whatmann DE81 filters which were then left to air dry for five minutes. One filter was then placed in a scintillation vial and 2.5ml scintillation fluid added before counting the total radioactivity present, in the scintillation counter. The second filter was then rinsed with 0.5M Na2HPO4 for approximately five minutes and this continued until the discarded solution showed a background level of radioactivity. The filter was then rinsed in two changes of ROP water and then two changes of 100% ethanol before leaving to air dry for five minutes. The amount of radioactivity on this filter was then determined in the scintillation counter as above to give an indication of the incorporated counts.
2.11.5. RNA Probes.

This method has approximately ten times greater sensitivity than the random priming labelled probes and involves transcription of a single stranded RNA probe corresponding to the antisense of the RNA of interest. This can be transcribed from the T7 or SP6 promoters present in the vector sequence of a linearized plasmid. This reaction works most efficiently for transcribed products between 300 and 1000bp and therefore the plasmid was linearized between 300 and 1000bp downstream of the promoter used. The linearized plasmid was gel purified by standard methods (Section 2.3.1) ensuring that the digested plasmid could be distinguished from any uncut plasmid remaining. The gel slice was then electroeluted, phenol/chloroform extracted and precipitated overnight. The DNA was resuspended to give a final concentration between 0.2 and 1mg/ml.

Cold Transcription.

To ensure that the DNA template can be used to transcribe the required RNA the reaction is first carried out with cold NTPs rather than incorporating radioactively labelled CTP. The reagents for this reaction are added in the order shown below:-

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (0.2 - 1mg/ml)</td>
<td>1.0μl</td>
</tr>
<tr>
<td>DTT (100mM)</td>
<td>1.5μl</td>
</tr>
<tr>
<td>BSA (2mg/ml)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>NTPs (25mM each)</td>
<td>1.25μl</td>
</tr>
<tr>
<td>RNAsin (Promega)</td>
<td>1.0μl</td>
</tr>
<tr>
<td>5x Transcription Buffer (Promega)</td>
<td>3.0μl</td>
</tr>
<tr>
<td>ROP water</td>
<td>5.25μl</td>
</tr>
<tr>
<td>T7 RNA Polymerase (Promega)</td>
<td>1.0μl</td>
</tr>
</tbody>
</table>

The reaction was incubated at 37°C for two hours before a sample was run on a 0.8% agarose gel. If the transcription reaction worked two bands were seen on the gel, one corresponding to the DNA fragment used and the other to the transcribed product.
Transcription with Radioactively Labelled CTP.

The reagents were added as for the cold transcription with the cold NTPs being replaced with the solutions below and the RNAsin being added between the cold and labelled NTPs as shown:

\[
\begin{align*}
\text{ATP/UTP/GTP (33mM each)} & \quad 1.0\mu l \\
\text{RNAsin} & \quad 1.0\mu l \\
\text{\{\(\alpha\-^{32}P\) CTP} & \quad 6.25\mu l \\
\end{align*}
\]

After incubation for two hours the volume was made up to 100\(\mu l\) with DEPC water and then phenol/chloroform extracted once. The aqueous phase from the extraction was precipitated with 100\(\mu l\) 4M Ammonium Acetate and 200\(\mu l\) isopropanol and centrifuged immediately. The pellet was then resuspended in 100\(\mu l\) DEPC water and 2\(\mu l\) of a 1:100 dilution of this used with 3ml of Ultima Gold in the scintillation counter to determine the incorporated radioactivity.

2.12. RNA Preparation and Analysis.

2.12.1. DEPC Treatment of Solutions.

All solutions were where possible treated with diethyl pyrocarbonate (DEPC) to remove any contaminating RNases. This was not possible for any solutions containing Tris and also SDS which were therefore made up with DEPC treated water. To each litre of solution 100\(\mu l\) DEPC solution (Sigma Chemical Co. Ltd.) was added. This was then shaken and left in the fume hood overnight before shaking again and autoclaving to destroy the DEPC.

2.12.2. RNA Preparation.

The following method was appropriate for small samples of tissue such as one quarter of a submaxillary gland or one half of a kidney. Tissues were frozen in liquid nitrogen and stored at -80°C prior to use. Before use the homogenizer head was treated with a 3% (w/v) hydrogen peroxide solution for fifteen minutes to ensure that it was nuclease free and then rinsed in DEPC water. The tissues were homogenized in 15ml Falcon 2059 tubes containing 3ml PCI (phenol/ chloroform/ isoamyl alcohol 25:24:1) and 5ml TLES (0.2M-Tris HCl, pH9.0, containing 0.1M-
LiCl, 25mM-EDTA, pH8.0, 0.1% (w/v) SDS) using an Ultraturrex with the smallest head until no obvious tissue fragments were visible. The homogenate was centrifuged at 3200rpm in a Heraeus varifuge for twenty minutes at 25°C. If the phases were not separated at this time the sample was divided between two 15ml Falcon tubes and 2ml TLES added to each tube before centrifugation for another twenty minutes under the same conditions. The aqueous phase was then removed to a fresh 15ml Falcon 2059 tube and 2ml PCI added. This was homogenized and centrifuged for ten minutes under the same conditions. This solution could then be stored at -80°C or used immediately. The Beckman SW50.1 Ti rotor holding 5ml polyallomer tubes was used to centrifuge the RNA through a caesium chloride pad. 1.4ml 5.7M CsCl, 0.1M EDTA, pH7.5 (DEPC treated) was placed in each polyallomer tube using a Gilson pipette. This pad was overlaid with 3.5ml RNA sample using a Gilson pipette being careful not to disturb the interface. The tubes were then filled to just below the top edge and balanced with light mineral oil. The tubes were placed in the rotor and centrifuged at 40K for 5.5 hours at 25°C. The brake on the centrifuge was set to slow to prevent mixing of the gradient as the centrifuge was stopped. The supernatant was aspirated off to just below the aqueous/CsCl boundary and then the tube inverted so the remaining solution drained while leaving an RNA pellet at the base of the tube. Keeping the tube inverted, the bottom of the tube containing the RNA pellet was cut off from the remainder of the tube and placed on ice. The pellet was resuspended in 200μl DEPC water by pipetting up and down and scratching the bottom of the tube. The resuspended RNA was transferred to a 1.5ml Sarstedt tube and precipitated with 22μl 3M sodium acetate, pH5.5 and 450μl ethanol, overnight at -20°C. The precipitated RNA was then pelleted by centrifuging at 4°C for ten minutes in a microfuge. The supernatant was removed, the pellet washed in 70% ethanol and resuspended in 200μl DEPC water. The O.D. of the RNAs was then determined and the quality of the RNA checked on a gel before storing at -80°C.

2.12.3. Northern Method.

The method for electrophoresis of RNA samples was adapted from that of McMaster and Carmichael (96). Samples of total kidney RNA containing 20μg were precipitated with 1/10th volumes of 3M-sodium
acetate, pH 5.5 and three volumes of EtOH on dry ice for twenty minutes. DNA size markers and copy number controls were processed similarly. The precipitate was centrifuged in an Eppendorf centrifuge at maximum speed for fifteen minutes at 4°C, washed in 70% ethanol, air dried and resuspended in 3.7ml DEPC water. Once fully resuspended 12.3μl of fresh "Glyoxal mix" (80μl spectroscopy grade DMSO, 16μl 0.1M sodium phosphate buffer, pH7.0, 27μl deionized glyoxal) was then added and the samples denatured by incubating at 50°C for one hour. After one hour the samples were cooled on ice, 2μl of gel loading buffer (10mM sodium phosphate stock, pH7.0, 50% (v/v) glycerol, 0.4% (w/v) bromophenol blue) was added and then immediately loaded on a 200ml 1.2% agarose gel, appropriate for the Biorad apparatus, containing 10mM sodium phosphate buffer, pH7.0 (from 0.1M stock solution containing 78ml 0.5M NaH2PO4, 122ml 0.5M Na2HPO4 made up to volume of 1 litre) and no ethidium bromide. The gel was then run overnight in 10mM-sodium phosphate; pH7.0 at a constant current of 30mA, with recirculation of the sodium phosphate buffer, until the bromophenol had migrated approximately 10 - 12cm. The recirculation of the buffer was obtained by placing the electrophoresis apparatus on a pair of magnetic stirrers so that each chamber was fully mixed and also using a peristaltic pump so that the buffer was mixed between chambers. Once run, the gel was sliced 2cm below the bromophenol blue and the RNA transferred to nylon membrane. The transfer was set up in the same manner as that used for the Southern transfer. After transfer the position of the gel wells were marked on the filter and one corner cut off to mark the orientation of the gel. The blot was then rinsed briefly in 6x SSC. The filter was then U.V. treated in the same manner as the Southern filters before baking at 80°C for thirty minutes to deglyoxylate the RNA.

2.12.4. Hybridization of Northern Blots.

The filter was prewetted in 6x SSC before placing in the Techne hybridization bottle containing the prehybridization solution (5x SSC, 5x Denhardt's solution, 0.5% SDS, 1mM EDTA, pH8.0) heated to 68°C. The filter was prehybridized for a minimum of four hours at 68°C before changing the solution, adding 200μl of 10mg/ml heat denatured salmon sperm DNA, the heat denatured probe and hybridizing overnight at 68°C.
After hybridization the probe solution was discarded and the filter washed in the following solutions:

i) 2x rinse, room temp. in 2x SSC, 0.1% (w/v) SDS,
ii) 2x 15 mins, 65°C in 2x SSC, 0.1% (w/v) SDS,
iii) 2x 10 mins, 65°C in 1x SSC, 0.1% (w/v) SDS,
iv) 2x 30 mins, 65°C in 1x SSC, 0.1% (w/v) SDS.

The filter was then air dried for five minutes and while still damp was wrapped in Saran wrap. This was placed in a cassette for autoradiography using Kodak XOMAT XAR-5 film and two enhancement screens. The film was exposed for 48 hours and then put down for longer, if necessary, to get the required signal.

2.12.5 SV40 TAg RT-PCR Methodology.

The reagents were added to a 0.5ml thin-walled PCR tube in the following order before denaturing at 65°C for ten minutes using the Hybaid Omnigene:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA (1mg/ml)</td>
<td>1µl</td>
</tr>
<tr>
<td>d(NTP)6 (50pM/µl)</td>
<td>1µl</td>
</tr>
<tr>
<td>DEPC water</td>
<td>10µl</td>
</tr>
</tbody>
</table>

After denaturation the reagents were cooled on ice and the solutions added in the following order:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expand RT buffer (5x)</td>
<td>4µl</td>
</tr>
<tr>
<td>dNTP (10mM)</td>
<td>2µl</td>
</tr>
<tr>
<td>DTT (100mM)</td>
<td>2µl</td>
</tr>
<tr>
<td>Expand RT (50U/µl)</td>
<td>1µl</td>
</tr>
</tbody>
</table>

The reaction mix was then incubated for ten minutes at 30°C followed by forty-five minutes at 42°C using the Hybaid Omnigene and cooled on ice immediately. This was then used in a PCR reaction using the Expand enzyme to determine if there was any SV40 TAg transcribed DNA present. Two mixes were made up as follows and mix 1 then added to mix 2 and overlaid with 30µl of mineral oil. The sequences of the SV40 TAg primers are described in Table 2.4.
Mix 1. (prepare on ice)

<table>
<thead>
<tr>
<th>Sterile water</th>
<th>up to 25μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTP (10mM)</td>
<td>1.75μl</td>
</tr>
<tr>
<td>TAg Primer (JJM-71)</td>
<td>300nM</td>
</tr>
<tr>
<td>TAg Primer (JJM-72)</td>
<td>300nM</td>
</tr>
</tbody>
</table>

Mix 2. (prepare on ice)

<table>
<thead>
<tr>
<th>Sterile water</th>
<th>up to 25μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT reaction</td>
<td>5μl</td>
</tr>
<tr>
<td>Expand enzyme</td>
<td>0.75μl</td>
</tr>
</tbody>
</table>

The overlaid mixtures were then incubated in the PCR reaction described below using the Hybaid Omnigene set to a simulated probe and a calibration factor of 80. The samples were first heated at 94°C for two minutes before entering ten cycles of 94°C, 10 seconds; 60°C, 30 seconds; 68°C, 45 seconds. Twenty cycles of 94°C, 10 seconds; 60°C, 30 seconds; 68°C 45 seconds + 5 seconds increment were then carried out before finishing with an incubation at 68°C for seven minutes. Samples of the PCR reaction were then electrophoresed on a 2% agarose gel using pBluescript Sau3A markers. The expected sizes were 580bp for unspliced RNA (& DNA), 510bp for small t Antigen and 240bp for large T Antigen.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence- 5' to 3'</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>T antigen-1 (JJM-71)</td>
<td>GGC ATT CCA CCA CTG CTC C</td>
<td>55°C</td>
</tr>
<tr>
<td>T antigen-2 (JJM-72)</td>
<td>GAA AGG AGT GCC TGG GGG</td>
<td>57°C</td>
</tr>
</tbody>
</table>

Table 2.4: Sequences of primers used in RT-PCR to determine if SV40 TAg RNA was present.

2.13. Histology.

2.13.1. Preparation of Tissue for Paraffin Sectioning.

After the tissue had been removed from the animal it was placed in formal saline for a minimum of 24 - 48 hours. After this time the tissues were processed in a Citadel 2000 (Shandon Southern Products Ltd.) processor using the program described below.
<table>
<thead>
<tr>
<th>Solution</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>1</td>
</tr>
<tr>
<td>80% ethanol</td>
<td>1</td>
</tr>
<tr>
<td>90% ethanol</td>
<td>1</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>2</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>2</td>
</tr>
<tr>
<td>Histoclear</td>
<td>1</td>
</tr>
<tr>
<td>Histoclear</td>
<td>2</td>
</tr>
<tr>
<td>Histoclear</td>
<td>2</td>
</tr>
<tr>
<td>Wax</td>
<td>3</td>
</tr>
<tr>
<td>Wax (under vacuum)</td>
<td>4</td>
</tr>
</tbody>
</table>

After processing the tissues were embedded in wax blocks using the Blockmaster III embedder (Raymond A. Lamb - Cat. No. E/66.2). The tissues were then sectioned at 7μ thickness using a microtome. The sections were placed onto TESPA coated slides (Section 2.13.2) and baked in the oven at 50°C overnight. After this treatment they could then be used for staining with haemallum and eosin (Section 2.13.3) or antibody staining (Section 2.13.4.).

### 2.13.2. Preparation of TESPA Coated Slides.

Glass microscope slides were coated with 3-aminopropyl-triethoxysilane (TESPA, Sigma Chemical Co.) using the method described below. The slides were racked in metal slide racks and passed through the following solutions:-

- 10% HCl in 70% ethanol: 10 seconds
- Water: 10 seconds
- 95% ethanol: 10 seconds
- Dry slides, 150°C: 5 minutes, then allow to cool.
- 2% TESPA in acetone: 10 seconds
- 100% acetone: 10 seconds
- 100% acetone: 10 seconds
- Water: 10 seconds

48
The slides were then dried at 42°C before being wrapped in foil and placed in the 'fridge. They were ideally used within four days of being made.

2.13.3. Haemalum and Eosin Staining.

This staining method was used to determine if the morphology in the kidneys of transgenic positive mice from the two constructs differed from normal, non-transgenic mice. Histoclear was first used to clear the sections of paraffin wax before they were placed in a series of graded alcohols to dehydrate the sections before staining. The protocol for this procedure is as follows:-

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histosol</td>
<td>5 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>80% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>dH2O</td>
<td>5 min</td>
</tr>
<tr>
<td>Haemalum</td>
<td>5 min</td>
</tr>
<tr>
<td>dH2O</td>
<td>Change until clear</td>
</tr>
<tr>
<td>Eosin</td>
<td>2 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>Rinse</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>2 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>5 min</td>
</tr>
<tr>
<td>Histosol</td>
<td>5 min</td>
</tr>
</tbody>
</table>

After this time the slides were left to dry before addition of mounting media (DPX mountant - 36029) and coverslips.

2.13.4. Immunohistochemistry Using Paraffin Sections.

After the slides had been left to cool they were dewaxed and rehydrated in a similar manner to that used for H&E staining:-

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histoclear</td>
<td>5 min</td>
</tr>
</tbody>
</table>
100% ethanol 5 minutes
95% ethanol 5 minutes
80% ethanol 5 minutes
70% ethanol 5 minutes
50% ethanol 5 minutes
dH₂O 5 minutes

After rehydration the slides were placed in a moist box and washed in 1x PBS for five minutes. The solution was removed by aspiration and the wash step repeated for a further five minutes. To block any non-specific binding of the antibody the sections were then incubated for twenty minutes with normal goat serum, diluted 1:5 with 1x PBS. After removal of the goat serum the sections were incubated overnight at 4°C in the presence of the primary antibody. This was a rabbit anti-T Antigen antibody obtained from F. Brewster (CRC Cell Transformation Research Group, University of Dundee) and used at a dilution of 1:2000, diluted with 1x PBS. After incubation overnight the sections were washed twice in 1x PBS for five minutes before incubating for thirty minutes with the secondary antibody. The secondary antibody used was a biotinylated goat anti-rabbit IgG at a 1:100 dilution, diluted with 1x PBS. While this was incubating the "Vectastain ABC reagent" (Elite ABC Peroxidase Kit, Vector Lab, pK 6100) was made up as follows since this needed to prepared thirty minutes before use.

<table>
<thead>
<tr>
<th>1x PBS</th>
<th>1ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;A&quot; solution</td>
<td>10μl</td>
</tr>
<tr>
<td>&quot;B&quot; solution</td>
<td>10μl</td>
</tr>
</tbody>
</table>

After incubation with the secondary antibody the sections were washed twice in 1x PBS for five minutes before addition of the Vectastain ABC reagent which would bind the Avidin conjugate (Avidin) to the antibody complex (Rabbit anti-SV40 TAg/Goat biotinylated peroxidase antirabbit IgG). The "Vectastain ABC" system produces conjugates of avidin and horse radish peroxidase reagents which can then bind to the biotinylated secondary antibody during the incubation on the sections. This increases the sensitivity of the detection system compared to using DAB directly after a horse radish peroxidase conjugated secondary
antibody since the biotinylated antibody can bind more than one avidin conjugate. The sections were incubated for thirty minutes with the Vectastain ABC reagent before being washed in 1x PBS for five minutes. This wash step was repeated twice to ensure that any unbound Vectastain ABC reagent had been removed. The sections were then incubated with the diaminobenzidine (DAB) reagent for ten to fifteen minutes until the brown colouration appeared (Dako Dab chromogen (Dako ref. S3000) - 1mg/ml-diaminobenzidine tetrahydrochloride, 0.09% (v/v) -hydrogen peroxide) prepared as follows: 10ml 1mg/ml-diaminobenzidine, 30µl 30% (w/v) hydrogen peroxide). The sections were then washed twice in water for five minutes before counterstaining and dehydrating as follows:

<table>
<thead>
<tr>
<th>Stain</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin</td>
<td>5 minutes</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>Rinse</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Histoclear</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

The slides were then air dried for a minimum of thirty minutes and then mounted and coverslipped using 3-4 drops of DPX Xylene mountant (BDH 36029). The mountant was then allowed to dry completely by leaving the slides overnight at room temperature.

2.13.5. Preparation of Tissues for Frozen Sections.

After the tissues had been removed from the animals they were placed in 30% sucrose in 1x PBS to cryoprotect the solutions and left at 4°C overnight. After incubating overnight the tissues were placed in foil cups half filled with OCT and then the remaining volume of the cup filled. The cups were placed in dry ice until completely frozen and then placed in the -70°C freezer until required.
3.1. Cloning pR2TsTAGPA

The first construct which was generated consisted of the 4.6kb \textit{Ren-2d} promoter sequence previously shown to give tissue specific expression of the wild type SV40 T Antigen structural gene (4), linked to a sequence encoding a temperature sensitive form of the SV40 T Antigen. The SV40 T Antigen sequence used contained two mutations, the tsA58 and the tsU19 mutations which confer temperature sensitive properties to the encoded protein. This was provided in a plasmid, pZiptsU19 (Figure 3.1.), by Ron McKay (Massachusetts Institute of Technology, Cambridge, U.S.A.), and lacked the polyadenylation (polyA) sequences and transcription termination site of SV40.

Figure 3.1: - Diagram of pZip tsU19, a plasmid containing a temperature sensitive form of the SV40 T Antigen gene in the pZip vector.

3.1.1. PCR Amplification of the SV40 Polyadenylation Sequence.

The first step in cloning was to PCR amplify the SV40 polyA sequence from a construct available in the laboratory, pBS α1ATRen2 (Figure 3.2.). This construct contained the human α1 antitrypsin promoter fused to the mouse \textit{Ren-2d} cDNA and SV40 polyA. A fragment containing the SV40 polyA sequence was available as a 205bp fragment after digestion with \textit{HindIII}/\textit{XhoI}.

Figure 3.2: - Diagram of pBS α1ATRen2, containing the SV40 polyA sequence used in cloning.

1: Construct contains two separate temperature sensitive mutations, tsA58 (containing one point mutation) and tsU19 (containing two point mutations) as shown in Figure 1.8
The primers used for the PCR amplification of the SV40 polyA sequence incorporated 20bp of perfect match with 5' tails containing restriction sites, allowing later cloning steps (Figure 3.3.). The restriction sites added to the primers were designed such that they were not found in the Ren-2d promoter or TsTAg sequence. Two restriction sites were incorporated into each primer with a 2bp spacer between the sites. At the 5' end of the primer 3bp were added to prevent problems with cleavage near the end of DNA molecules.

\[
\text{Sequence Of Primers}
\]

**Forward Primer**

\[
5' \text{CTA AGA TCT AAG GTA CCT TTT ACT TGC TTT AAA AAA C} 3'
\]

**Reverse Primer**

\[
5' \text{CTA GCC GGC TTG CTA GCG ACA TGA TAA GAT ACA TTG A} 3'
\]

Figure 3.3: - Sequences of Primers for PCR Amplification of SV40 PolyA Fragment.

The forward primer for the PCR reaction had a \(T_m\) of 48°C and the reverse primer a \(T_m\) of 52°C. Annealing temperatures of 52°C, 55°C and 58°C were used with 100ng or 10ng of DNA per reaction. The PCR was set up as described previously (Section 2.4.) and the products of the reaction were electrophoretically separated over a 1.5% agarose gel. In all lanes a fragment of the predicted size, 226bp, was present but at the lower annealing temperatures a fainter, higher molecular weight fragment of approximately 400bp was seen (Figure 3.4.). The two samples from the 58°C annealing reactions were then used for the Klenow Kinase Ligase reaction.
Figure 3.4: PCR products obtained by amplification of the SV40 polyA sequence from pBS α1 AT Ren2. Lanes 2 - 4 amplify 10ng plasmid DNA at varying annealing temperatures and Lanes 5 - 7 amplify 100ng plasmid DNA. Lane 1: pBluescript Sau3A DNA molecular weight standard, Lanes 2 & 5: Anneal 52°C, Lanes 3 & 6: Anneal 55°C, Lanes 4 & 7: Anneal 58°C, Lane 8: λ HindIII/EcoRI DNA molecular weight standard.

Figure 3.5: PCR product, amplified from 100ng pBS α1 AT Ren2, before the KKL reaction (Lane 3), after the KKL reaction (Lane 2) and after digestion of the KKL reaction products (Lane 1). Lane 4: pBluescript Sau3A DNA molecular weight standard.
The KKL reaction allows the blunt ending and ligation of PCR amplification products to produce concatamers. These concatamers can then be digested with the appropriate restriction enzymes to isolate a fragment for cloning. The concatamers of PCR product were digested with *NaeI* and *BglII* to obtain the 226bp SV40 polyA fragment with sticky ends suitable for cloning. Samples of the PCR amplification, concatamerized and restricted products were electrophoretically separated (Figure 3.5.). Lane 2 shows that after incubation in the KKL reaction the 226bp PCR product was reduced in intensity and fragments of increasing size could be seen, representing a 226bp ladder of concatamerized product. Digestion with *NaeI* and *BglII* resulted in the reduction of these fragments to the 226bp SV40 polyA fragment, although one higher molecular weight fragment was still present. Because of the presence of this fragment it was decided to gel purify the 226bp SV40 polyA fragment using QIAEX.

### 3.1.2. Cloning of SV40 PolyA into pR2P2.2.

The original plan was to clone the SV40 polyA fragment into a plasmid already containing the *Ren-2d* promoter, pR2P2.2 (Figure 3.6.). This plasmid was digested with *NaeI* and *BglII* to allow cloning of the SV40 polyA fragment.

![Diagram of pR2P2.2 showing positions of restriction sites available in the polylinker.](image-url)

A problem anticipated in using these two enzymes to cleave the vector was that the two restriction sites were both inefficient at cleaving DNA near
the ends of molecules and lay adjacent to one another in the polylinker. This would result in inefficient cleavage with the second restriction enzyme used. In an attempt to determine the optimal cleavage of the DNA, two parallel reactions were prepared with \textit{BglII} or \textit{NaeI} being the first enzyme to cleave the DNA. In the last half hour of digestion shrimp alkaline phosphatase (ShAP) was added to the reaction to prevent the recircularization of any plasmid molecules which were not cleaved by the second restriction enzyme. The reaction mix was then incubated at 75°C in the presence of 10mM EDTA which inactivated the ShAP and which would also release the first restriction enzyme from the DNA, where it could have prevented binding of the second enzyme. The DNA was ammonium acetate/isopropanol precipitated before the next digestion. The two reactions were carried out in parallel and a small sample was run on a gel after the first digestion. At this point, an extra 300bp fragment was seen in the \textit{BglII} digestion (Figure 3.7.) suggesting that there was a second \textit{BglII} site within the \textit{Ren2d} promoter, not predicted from the GenBank sequence data (GenBank Accession Number: MUSREN2G). For this reason the cloning strategy was altered to clone the SV40 polyA fragment into pSP72, poly2 (Section 3.1.4.). The SV40 T Antigen fragment would then be cloned into this construct before finally ligating in the promoter fragment isolated from pR2P2.2. The final cloning strategy used is shown in Figure 3.8.

![Figure 3.7](image)

\textbf{Figure 3.7:-} Sample of pR2P2.2 after digestion with the first restriction enzyme. Lane 1: \textit{\lambda\ HindIII/EcoRI} DNA molecular weight standard, Lane 2: pR2P2.2 digested with \textit{BglII} showing the unpredicted 300bp fragment, Lane 3: pR2P2.2 digested with \textit{NaeI}.
PCR Amplified SV40 polyA Sequence.

Digest vector with BgIII and Nael. Ligate with SV40 polyA fragment.

Digest pP2PA with BgIII and ligate to 2.5kb SV40 TAg Fragment digested with BamHI.

Digest pP2PA + TAg intermediate with Ascl and Sall. Ligate with ren2d Promoter Fragment.

**Figure 3.8.** - Cloning Strategy For pR2TsTAgPA.
3.1.3. Cloning SV40 PolyA into pSP72, poly2.

Parallel digestions were set up for pSP72, poly2 in a similar manner to that described in Section 3.1.2. and the vector fragment gel purified by QIAEX after digestion with the second enzyme. Transformation of the ligation reactions showed no significant difference between the number of colonies from the vector self-ligation and vector+insert ligation suggesting that the vector digestion had not been complete. It was possible that one arm of the SV40 polyA fragment was ligated to the vector and was unable to ligate to the other end of the vector because this had not been digested by the second enzyme. A similar result was obtained on a second transformation with no DNA samples, miniprepped from colonies, containing the polyA fragment and therefore an alternative strategy was used to enable cloning of this fragment into pSP72, poly2.

Since the problem with ligation of the SV40 polyA fragment probably lay in the fact that neither of the two enzymes cut efficiently near the end of DNA molecules, it was decided to ligate a small fragment at the BgIll site of the vector to separate the second restriction site from the end of the molecule. The intermediate could then be linearised with NaeI before cleaving with BgIll, permitting the isolation of the required vector fragment with a higher probability of the restriction sites having been efficiently cut. The 300bp BgIll fragment obtained from digestion of pR2P2.2 was gel purified and ligated to the pSP72, poly2 vector, previously digested with BgIll and treated with shrimp alkaline phosphatase. The intermediate vector obtained containing the 300bp insert, pP2-300Ren, was prepared for the cloning of the SV40 polyA fragment by sequential digests of 10μg DNA with NaeI followed by BgIll. DNA miniprepped from colonies after transformation of the ligation reaction gave the predicted pattern of 1.7kb and 958bp after digestion with BamHI and HindIII, indicating the presence of the SV40 polyA fragment (Figure 3.9.) and therefore a maxiprep culture was grown from this sample. Since unique KpnI and NheI sites had been added during the PCR amplification of the SV40 polyA fragment, these enzymes were used to confirm that the construct contained a correctly inserted SV40 polyA fragment. Double digests were carried out using either KpnI or NheI in combination with PvuI. The digests gave the correct pattern of fragments, 1512bp and 1175bp for the NheI/PvuI digest and 1717bp and 970bp for the KpnI/PvuI digest, indicating that this clone, pP2PA, contained the SV40 polyA fragment inserted into pSP72, poly2 (Figure 3.10).
Figure 3.9:- Samples of DNA, miniprepped from colonies after transformation of the ligated pSP72, poly2 and SV40 polyA fragment, digested with BamHII/HindIII to determine if the SV40 polyA fragment is present. Lane 10 shows the correct pattern of restriction fragments indicating the presence of the SV40 polyA sequence. Lane 11: λ Hin dIII/ EcoRI DNA markers.

Figure 3.10:- Samples of miniprepped DNA, from colonies after transformation of the ligated pSP72, poly2 and SV40 polyA fragment, digested with PvuI & Nhe I (Lanes 4 - 7) or PvuI & KpnI (Lanes 8 - 11) to determine if the SV40 polyA fragment was correctly inserted. Lanes 6 and 11 show the digest of the clone containing the SV40 plyA fragment correctly inserted. Lanes 1 & 12: λ Hin dIII/ EcoRI DNA molecular weight standard, Lane 2: Undigested plasmid DNA.
The entire amplified 200bp polyA sequence was sequenced and this verified that no mutations had been introduced during the PCR amplification (Figure 3.11).

DNA from the plasmid pP2PA was digested with BglII and treated with ShAP prior to cloning of the SV40 T Antigen fragment. The SV40 T Antigen fragment was isolated from pZiptsU19 as a BamHI fragment and contained the SV40 T Antigen sequence from HpaII to BglII (Positions 2666 to 5234, GenBank Release 81: Accession Number: J02400) to which BamHI linkers had been added for cloning into the pZip vector. The pZiptsU19 vector was found to yield very low amounts of DNA after maxipreps using alkaline lysis and therefore in addition to preparing the SV40 T Antigen fragment for cloning into the pP2PA construct it was also cloned into pBluescript SKII+ (Stratagene), to allow its easy isolation on other occasions. The SV40 T Antigen fragment was isolated by the BamHI digest and gel purified using electroelution.

Ligations were set up with the pP2PA construct and the SV40 TAg fragment and DNA was miniprepped from a number of the colonies obtained from transformation of the ligation reaction. The DNA was digested with BamHI/HindIII and this showed a number of clones containing the SV40 T Antigen inserted in the correct orientation (Figure 3.12. - Lanes 3 and 6).
Figure 3.12: BamHI/HindIII digests of DNA, miniprepped after ligation of the TAg fragment into pP2PA. Lanes 3 & 6 show clones where the TAg fragment has inserted in the correct orientation.

Figure 3.13: PvuII digests of DNA, miniprepped after ligation of the Ren-2d promoter fragment into the intermediate construct containing the SV40 T Antigen and polyA sequence. Lanes 2 - 10 show clones containing the Ren-2d promoter sequence.
3.1.6. Cloning the Ren-2 \textsuperscript{d} Promoter Fragment.

The plasmid DNA containing the SV40 TAg insert in the correct orientation was then maxiprepped using QIAGEN columns and digested with \textit{AscI} and \textit{SalI} to allow cloning of the promoter fragment. The plasmid pR2P2.2 was also digested with \textit{AscI} and \textit{SalI} to isolate the promoter fragment and both were gel purified using QIAEX. DNA from colonies from the vector+insert ligation was miniprepped and digested with \textit{PvuII} to determine if any contained the Ren-2\textsuperscript{d} promoter insert (Figure 3.13.). DNA from one positive colony was then maxiprepped, using alkaline lysis, and the DNA used for preparation of the microinjection fragment and for sequencing the cloning junctions to ensure no mutations had been introduced during cloning. The fragment for microinjection was isolated using a \textit{NaeI} - \textit{NotI} digest.

3.2. Cloning of pRen2tsA58.

The temperature sensitive SV40 T Antigen sequence containing the tsA58 mutation alone was available in the plasmid pUCtsA58 (Figure 3.14.), consisting of the pUC18 vector and a \textit{BamHI} - \textit{KpnI} fragment of SV40 sequence (Positions 2533 to 294 on GenBank SV40 Sequence J02400). This included the SV40 polyA sequences and could be isolated from this construct by a \textit{BglII} - \textit{BamHI} digest but this did not provide suitable sites for cloning into the available vectors in the laboratory. It was therefore decided to blunt end the \textit{BglII} - \textit{BamHI} fragment before adding \textit{SalI} linkers, which would allow cloning into pR2P2.2.

![Figure 3.14:- Diagram of Plasmid pUCtsA58 containing a KpnI - BamHI Fragment from SV40 ligated to vector pUC18. The SV40 T Antigen Sequence is contained within the BglII - BamHI Fragment.](image-url)
3.2.1. Blunt-Ending the SV40 T Antigen Fragment from pUCtsA58.

The pUCtsA58 plasmid was digested with BgII - BamHI and the fragment gel-purified using QIAEX. The blunt-ending reaction was performed using T4 DNA polymerase, which removed the single-stranded 3' overhang produced by the BgII digestion using the 3' to 5' exonuclease activity of the enzyme. The 5' single-stranded overhang produced by BamHI digestion was filled in by the 5' to 3' polymerase action of the enzyme.

Two blunt-ending reactions were set up in parallel, one to be used to produce the fragment for cloning and the other using radioactively labelled dCTP to determine if the reaction had worked. The incorporation of radioactivity was determined by CTAB precipitation and showed a 16 fold increase after the reaction compared to before, indicating that the reaction had worked. Since the position of the incorporated dCTP was at the end of the DNA fragment, this increased the certainty that the reaction was complete and the fragment was completely blunt-ended. Following this step, SalI linkers were ligated to the blunt-ended fragment and the reaction mix then digested with SalI for 2 hours. A sample of the reaction was run on a gel, which showed a fragment at the correct size for the blunt-ended TsTAg DNA but a fainter higher molecular weight fragment was also present (Figure 3.15.). This was thought to be due to ligation of two blunt-ended fragments which had not been digested to completion with the SalI enzyme. The correctly sized DNA was therefore gel purified using QIAEX.

3.2.2. Cloning the SV40 T Antigen Fragment into pSP72, poly2.

It was possible to clone the tsA58 fragment, after digestion with SalI and BamHI, into pSP72, poly2 since the BamHI site originally used to isolate the tsA58 fragment was recreated during the blunt ending and ligation of SalI linkers. The vector and tsA58 fragments were prepared with the appropriate digestion and QIAEX gel-purification before ligation. DNA was miniprepped from colonies obtained after transformation of the ligation reaction. After digestion with BamHI and HindIII the pattern of fragment sizes expected if the insert was present was obtained (Figure 3.16.). DNA from one of these positive colonies was maxiprepped using alkaline lysis and the DNA used for cloning the promoter fragment into this construct and for sequencing the cloning junctions.
Figure 3.15: T Antigen fragment after ligation of SalI linkers and digestion with SalI. Lane 1: T Antigen fragment showing higher molecular weight fragment. Lane 2: 1 HindIII / EcoRI DNA molecular weight standard.

Figure 3.16: DNA, miniprepped from colonies after ligation of the T Antigen fragment, digested with BamHI & HindIII. Lane 3 shows digest of clone containing the T Antigen fragment. Lane 5: 1 HindIII / EcoRI DNA molecular weight standard.
3.2.3. Cloning the Ren-2\textsuperscript{d} Promoter Fragment.

The promoter fragment was introduced using an *AscI* - *SalI* fragment as for pR2TsTAGPA (Section 3.1.6.). DNA from the colonies obtained after vector+insert ligation and transformation was miniprepped and digested with *Pvu*\textsubscript{II} to determine if the Ren-2\textsuperscript{d} promoter insert was present. One of the positive DNA samples obtained was maxiprepped by alkaline lysis and used for sequencing and microinjection preparation as described previously. The fragment for microinjection was again isolated by a *NaeI* - *NotI* digest. The final cloning strategy used is shown in Figure 3.17.
Digest pUC tsA58 with BglII and BamHI. Blunt end with T4 DNA polymerase and add Sall linkers. Digest with Sall and BamHI. 

Digest with BglII and BamHI. 

Blunt end with T4 DNA polymerase and add Sall linkers. Digest with Sall and BamHI. 

pUC tsA58 

Digest with BglII and BamHI. 

Blunt end with T4 DNA polymerase and add Sall linkers. Digest with Sall and BamHI. 

Digest with Sall and BamHI. 

Blunt end with T4 DNA polymerase and add Sall linkers. Digest with Sall and BamHI. 

pUC tsA58 

Digest with BglII and BamHI. 

Blunt end with T4 DNA polymerase and add Sall linkers. Digest with Sall and BamHI. 

Digest with BglII and BamHI. 

Blunt end with T4 DNA polymerase and add Sall linkers. Digest with Sall and BamHI. 

Digest with BglII and BamHI. 

Blunt end with T4 DNA polymerase and add Sall linkers. Digest with Sall and BamHI. 

Figure 3.17: Cloning Strategy for pRen2tsA58.
4.1 Amplification of DNA by the Polymerase Chain Reaction.

The polymerase chain reaction (PCR) allows the amplification of a specific region of DNA using two flanking oligonucleotide primers approximately 17 - 30bp in length. The first step in the reaction is the denaturation of the target DNA followed by a decrease in temperature to allow the primer pairs to anneal to opposite strands of the DNA template. At this point a small amount of DNA synthesis takes place which stabilizes the primer binding, allowing it to remain annealed to the DNA when the temperature is increased to the extension temperature of 72°C. During this extension step the region of DNA between the two primers is synthesized by the DNA polymerase enzyme. Repeated cycles of denaturing, annealing and extension result in an exponential amplification of the target DNA sequence, with the products being available as templates in the subsequent amplification cycles. One important aspect of the PCR is the ability to amplify very small amounts of DNA. This not only leads to a high degree of sensitivity but can lead to problems with contamination. These contamination problems can be addressed by preparing the reaction mixes in a room where plasmids containing the template DNA are not present. Other precautions include cleaning the pipettes regularly in 1M hydrochloric acid for thirty minutes before rinsing in sterile water.

4.1.1. Polymerase Enzymes Used in PCR Amplification.

The most commonly used polymerase is the thermostable Taq polymerase, from a strain of thermophilic bacteria, Thermophilus aquaticus. This replicates DNA at 72°C and can survive the heat denaturation step in each cycle of the PCR reaction, therefore only needing to be added once to the reaction mix. This enzyme lacks a 3' to 5' proofreading exonuclease function which can result in the incorporation of an incorrect nucleotide during the PCR amplification. Where the reaction is being used to identify positive transgenics, by amplification of
a fragment of specific size, this misincorporation is not important but where cloning of the PCR product is required it is important to sequence the amplified region to ensure that mutations have not been incorporated. To reduce the number of incorporated mutations alternative DNA polymerases to Taq can be used. Other polymerases that are available, containing a 3' to 5' proofreading exonuclease function, include Pfu from the thermophilic bacteria Pyrococcus furiosus, which has a twelve fold greater fidelity of DNA replication than that of Taq.

4.2. Considerations for Primers.

When amplifying tail DNAs, primers of 18 - 20 nucleotides are commonly used. The GC content of these primers is approximately 50% to give a higher T_m, the temperature at which the primer is expected to dissociate from the DNA. A rough estimate of the T_m can be obtained by adding 4°C for each G or C nucleotide present and 2°C for each T or A nucleotide. To increase the stability of the primer - DNA template complex, the two bases at the 3' end of the primer should be G or C and runs of more than four of the same nucleotide avoided. The primers should not contain regions of complementarity to each other or a high degree of secondary structure. In the design of the second primer pair these last two questions were addressed by use of the computer programs Align and Prime Mate.

4.3. Original Strategy for the PCR Assay.

The original plan in designing a PCR screening strategy was to use a tripartite reaction, using one forward primer complementary to a region of the Ren-2d promoter sequence, one reverse primer complementary to a region of Ren-2d intron A and one reverse primer complementary to a region of the SV40 T Antigen sequence (Figure 4.1.). The PCR reaction was designed to produce an internal control that amplified in all tail DNA samples and a lower molecular weight positive product, seen only in those animals where integration of the transgene had occurred. The positive amplification product was the smaller of the two in order to ensure that it competed favourably with the amplification of the control fragment in the PCR reaction. Ideally the size of the positive fragment
would be approximately 300 - 400bp and the control fragment approximately 700bp. The main problem initially in using this approach was identifying a potential primer sequence with the correct $T_m$ in the first exon of the SV40 T Antigen sequence since this region is relatively AT rich. The second problem encountered was finding a region of intron A. Little sequence is known in the intron, but that available on GenBank Release 81 (Accession Number: MUSREN2G) proved sufficient to design a primer for this region. The chosen primers were expected to amplify a control fragment of 480bp and a positive fragment of 318bp which could be separated on a 2% agarose gel.

**Control Amplified Product**

- ren2 d Promoter
- Exon1
- Intron A
- 480bp

**Positive Amplified Product**

- ren2 d Promoter
- T Antigen
- 318bp

**Sequences of Primers Used.**

1. ren2 d promoter - 5' GGA GGA GCC AAG CAG CAG CC 3' Position -268 to -252
2. Intron A - 5' GGA CTG GAT GAC CCA ACG 3' Position +209 to +192
3. T Antigen - 5' GGA ATA GCT CAG AGG CCG 3' Position +41 to +24

*Figure 4.1:* Diagram of original Tripartite Screening Strategy for Amplification of Mouse Tail DNAs showing sizes of expected PCR products. Positions and sequence of the primers refers to GenBank Release 81: Accession numbers:- Ren-2d- MUSREN2G, SV40 T Antigen-J02400.
4.3.1. Verification of Primers.

The primers were synthesized by Oswell DNA service (Medical and Biological Sciences Building, University of Southampton) without HPLC purification. On receiving the primers an aliquot was diluted to 50μM and both this and the undiluted stock stored at -20°C. The primers were first tested with samples of negative mouse DNA titrated with varying concentrations of pR2tsTAgPA plasmid DNA.

Samples used in titration reaction.
Negative DNA alone
Negative DNA + spike 10^{-13} g plasmid DNA
Negative DNA + spike 10^{-12} g plasmid DNA
Negative DNA + spike 10^{-11} g plasmid DNA
Negative DNA + spike 10^{-10} g plasmid DNA
Negative DNA + spike 10^{-9} g plasmid DNA

Using the rough estimate, the T_m of the primers was calculated to be 58°C. It is usual to be able to anneal at temperatures slightly higher than this and for this reason the original annealing temperatures used were 60°C and 62°C. The 0.5μl tail DNA sample plus spike was pipetted into 0.5ml Sarstedt tubes. The mix for all reactions was made up on ice as follows, per PCR tube 40.75μl ROP water, 5μl 10x PCR buffer, 1μl 10mM dNTPs, 1μl 50μM forward primer, 1μl 50μM reverse primer 1, 1μl 50μM reverse primer 2, 0.25μl Taq polymerase. Once aliquoted, the tubes were left on ice until ready to load into the PCR machine. To decrease the chances of non-specific annealing of the primers, the PCR block was preheated and held at the denaturing temperature before addition of the PCR tubes. The PCR reaction was then allowed to run as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time (min.)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>95°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>60°C/62°C</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>72°C</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>18°C</td>
<td>HOLD</td>
<td></td>
</tr>
</tbody>
</table>

70
The reaction produced a number of non-specific products when amplifying lower concentrations of positive DNA. When tail DNAs were amplified under the same conditions as above, one positive showed up clearly but a large background of non-specific fragments were seen, including one at a similar size to the positive amplified product (Figure 4.2.). To determine which primer pair was causing the high background, PCR amplification was carried out with either the positive primer pair alone or the control primer pair alone. The reaction was also carried out at a higher annealing temperature to determine if this reduced the amount of non-specific amplification.

4.3.2. Reaction to Determine Which Primer Pair Causes the High Background.

Transgenic positive DNA, negative DNA and water controls were amplified with positive primer pair alone at 63°C, control primer pair alone at 63°C or positive pair alone at 64°C (Figure 4.3).

At 64°C amplification products were no longer seen suggesting that the primers cannot anneal at temperatures higher than 63°C. In the reactions with the positive primers alone a few non-specific amplification products were seen in lanes not containing the positive DNA. These fragments were also seen in the control reaction, one of which was approximately the same size as the positive product and could therefore cause problems in the identification of weakly positive, mosaic animals.

One possibility at this stage was to use a different primer pair to amplify a control fragment. A primer pair available in the laboratory amplified mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) DNA to produce a 452bp product and had a T_m of 62°C, compatible with the positive primer pair. The G3PDH primers were tested on DNA positive and negative for the transgene, using water as a control. One potential problem with the addition of a fourth primer was that this could cause interference between the primers and resulting in a higher background of non-specific products. This was not found to be the case when amplifying the above DNAs and the available tail DNAs were then amplified using the positive and G3PDH primers (Figure 4.4.).
Figure 4.2: Amplification of tail DNAs using the tripartite PCR reaction involving one forward primer, complementary to a region of the Ren2d promoter, one reverse primer complementary to a region of the Ren2d intron A sequence and one reverse primer complementary to a region of the TAg sequence. Lane 8: Shows positive amplification product indicating the presence of the transgene. Lane 11: pBluescript Sau3A molecular weight standard.

Figure 4.3: Showing amplification of sample DNAs using the positive primer pair alone at anneal temperature 64°C (Lanes 1 to 5), control primer pair alone at anneal temperature 63°C (Lanes 7 to 11) and positive primer pair alone at anneal temperature 63°C (Lanes 13 to 17). pBluescript Sau3A markers are shown in Lanes 6, 12 and 18. DNA samples are negative transgenics in lanes 2, 4, 5, 8, 10, 11, 14, 16, 17; positive transgenic in lanes 3, 9 and 15. Water controls are shown in lanes 1, 7 and 13.
Figure 4.4: PCR amplification of tail DNAs using the positive primer pair and the G3PDH control primers pair. Lane 3: Contains amplified products from DNA previously shown to be positive, (Figure 4.2 - Lane 8). Lane 7: Shows amplified products indicating the presence of the transgene. Lane 13: pBluescript Sau3A molecular weight standard.

Figure 4.5: PCR amplification of tail DNAs with the positive primer pair and the G3PDH control primer pair using Promega Taq (Lanes 8 - 12) or an alternative Taq (Lanes 2 - 6). Lanes 2, 3, 8 & 9: Contain positive tail DNA, Lanes 4, 5, 10 & 11: Contain negative tail DNA, Lanes 6, 7 & 12: Contain water as a control.
The reaction with the positive primers and G3PDH control primers did not produce non-specific fragments but the intensity of the amplification product from the known positive was very low compared to previous reactions with the other primer pairs (Figure 4.4. - Lane 3). One possibility for the faint positive is that there are more potential templates available for the G3PDH primers, since they amplify pseudogenes present in the genome, and compete with the positive primer pair for the nucleotides in the reaction. In order to determine that no positives were missed, due to the lower intensity of the product, DNA amplifications were also carried out using only the positive primers. This practice was continued for tail DNAs from founder animals where it was possible that there would be low intensity fragments due to mosaics. Once amplifying tail DNAs to determine the G1 animals and no positives were being missed by the tetrameric reaction, the positive primers alone reaction was omitted.

4.4. Amplification of Tail DNAs from G2 Transgenic Animals.

When using the above conditions for these amplifications, problems started occurring with the appearance of non-specific products. These fragments were not present during each amplification of the DNA but did appear relatively frequently. At this point the enzyme used had been changed to a different Taq polymerase from the Promega enzyme previously used. One possibility for the extra amplification products was thought to be the efficient amplification of the DNA by this enzyme in combination with fresh primer aliquots. This was tested by setting up reactions in parallel with the two polymerases. Two positive DNAs, two negative DNAs and water samples were amplified under standard conditions (Figure 4.5.). The results showed a faint positive fragment in the positive DNA tracks but little evidence of extra non-specific amplification.

To attempt to overcome the problem of non-specific amplification products a titration was set up with varying concentrations of magnesium chloride present in the reaction mix, 1mM, 1.5mM, 2mM, 2.5mM, 3mM and 3.5mM.
Figure 4.6: PCR amplification of tail DNAs with the positive primer pair and the G3PDH control primer pair. Positive tail DNAs (Lanes 1, 4, 7, 10, 13 & 16), Negative tail DNAs (Lanes 2, 5, 8, 11, 14 & 17) and water controls (Lanes 3, 6, 9, 12, 15 & 18) were amplified at varying Magnesium chloride concentrations. Concentrations used were 1mM (Lanes 1 - 3), 1.5mM (Lanes 4 - 6), 2mM (Lanes 7 - 9), 2.5mM (Lanes 10 - 12), 3mM (Lanes 13 - 15) and 3.5mM (Lanes 16 - 18).

Figure 4.7: PCR amplification of tail DNAs with the positive primer pair and G3PDH control primer pair. Positive tail DNAs (Lanes 1, 4, 7, 10, 13 & 16), negative tail DNAs (Lanes 2, 5, 8, 11, 14 & 17) and water controls (Lanes 3, 6, 9, 12, 15 & 18) were amplified using differing Magnesium Chloride concentrations and cycle lengths. Lanes 1 - 9: 30 cycles, Lanes 10 - 18: 32 cycles. Magnesium Chloride concentrations used were 1mM (Lanes 1 - 3 and 10 - 12), 1.25mM (Lanes 4 - 6 and 13 - 15) and 1.5mM (Lanes 7 - 9 and 16 - 18).
The result from this titration showed an increase in the non-specific amplification as the concentration of magnesium chloride increased (Figure 4.6.). Concentrations of 1mM and 1.5mM appeared to give the best amplification without non-specific products being present. The problem of lower intensity of the positive fragment was addressed by increasing the number of cycles in the PCR reaction. The cycle length was first changed to 35 but this produced visible contamination products and therefore 32 cycles were used. This had no significant effect on the intensity of positive product and still produced a degree of contamination (Figure 4.7.).

At this point it was decided that the primers being used in this reaction were not likely to produce a consistent result and the possibility of other primer pairs was considered. Two problems needed to be overcome, the problem of the high intensity control fragment when using the G3PDH primers and the problem of non-specific amplification products. The first problem could be overcome by changing the control primers used to others available in the laboratory which amplified the mouse major histocompatibility H2-K (b) gene, which do not amplify pseudogenes. The H2-K primers had a Tm of 64°C and therefore a primer pair which would amplify the positive transgene DNA and be compatible with the H2-K pair was designed. The design of the positive primer pair was such that the option of returning to the G3PDH primers was available if the H2-K primers were found not to be suitable.

The problem of amplification of non-specific products was addressed by designing primers with a higher Tm, to allow an annealing temperature to be used where the non-specific annealing should not take place. A primer specific to the SV40 T Antigen sequence, with a Tm of 64°C, was already available in the laboratory and therefore alternative regions in the Ren-2d promoter sequence were identified which were used to design primers. The SV40 T Antigen primer was tested in an amplification with mouse genomic DNA since it had not previously been used in a mouse genomic DNA environment. Mouse genomic DNA was spiked with a plasmid containing the 21OH promoter linked to a temperature sensitive SV40 T Antigen and amplified using a forward primer homologous to a region of the 21OH promoter and the TsA58
reverse primer. This primer pair was found to work consistently in the mouse genomic DNA background confirming the validity of using this tsA58 primer in the mouse environment.

4.4.1. Design of a Second Ren-2\textsuperscript{d} Promoter Primer.

It was required that the primer in the Ren-2\textsuperscript{d} promoter sequence was positioned to allow amplification products from the transgene to be separated from either the H2-K amplified product or the G3PDH product. This gave a total number of five potential primers, the sequences of which were entered in the computer programs Align and PrimeMate. Align allowed any regions of complementarity between the primers, which could cause them to anneal to each other rather than the target sequence, to be identified. PrimeMate was used to identify any potential hairpin structures or dimerization of the primers, which would prevent annealing to the target sequence. This screening reduced the number of potential primers to four. One other of the primers was discarded as a first choice, since it used the same region of homology in the target sequence as that of the original Ren-2\textsuperscript{d} promoter primer and may have resulted in the same problems of non-specific amplification. The chosen primer, Ren2P250 had the sequence 5' GTA GGG TAA CTG TGG GGA GG 3' (position -219 to -200 in the Ren2\textsuperscript{d} promoter sequence) and when used in conjunction with the tsA58 primer would amplify a fragment of approximately 250bp.

4.5. Verification of the Ren2P250 Primer.

4.5.1. Amplification with Positive Primers, with and without Control Primers.

The first requirement was to test the ability of the positive primer pair to amplify the DNA in the presence or absence of a control primer pair, either G3PDH or H2-K control primers. Negative mouse genomic DNA was used for the amplification spiked with 100, 10, 1, 0.5, 0.2, 0.2 copies of transgene with controls of negative mouse DNA and water.
Figure 4.8: PCR amplification of negative tail DNA spiked with varying concentrations of pR2TsTAgPA plasmid DNA equivalent to 100 copies of the transgene per genome (Lanes 2, 10 & 18), 10 copies per genome (Lanes 3, 11 & 19), 1 copy per genome (Lanes 4, 12 & 20), 0.5 copies per genome (Lanes 5, 13 & 21), 0.2 copies per genome (Lanes 6, 14 & 22) and 0.1 copies per genome (Lanes 7, 15 & 23). Lanes 8, 16 & 24 contained a negative DNA control and Lanes 9, 17 & 25 contained a water control. Amplification reactions were carried out with the positive primer pair alone (Lanes 2 - 9), positive primer pair and H2-K control primer pair (Lanes 10 - 17) and positive primer pair and G3PDH control primer pair (Lanes 18 - 25).

Figure 4.9: PCR amplification of negative tail DNA spiked with varying concentrations of pR2TsTAgPA plasmid DNA equivalent to 100 copies of the transgene per genome (Lanes 1, 9 & 17), 10 copies per genome (Lanes 2, 10 & 18), 1 copy per genome (Lanes 3, 11 & 19), 0.5 copies per genome (Lanes 4, 12 & 20), 0.2 copies per genome (Lanes 5, 13 & 21), 0.1 copies per genome (Lanes 6, 14 & 22). Lanes 7, 15 & 23 contain amplification products of negative DNA and Lanes 8, 16 & 24 contain water controls. PCR amplifications were carried out at three different annealing temperatures: 64°C (Lanes 1 - 8), 65°C (Lanes 9 - 16) and 66°C (Lanes 17 - 24).
PCR conditions were similar to those described previously (Section 4.3.1.) with the differences being an anneal temperature of 64°C and the temperature being held at 25°C after the ten minute incubation at 72°C.

The amplification with the positive primer pair alone allowed detection of the positive product in the sample containing 0.1 copies per genome (Figure 4.8.). Sensitivity with the H2-K primers was similar but the control fragment did not amplify. With the G3PDH primers the control fragment amplified well but the sensitivity for detection of the positive product was reduced, with 0.5 copies the lowest copy number detected.

4.5.2. Amplification with Positive and G3PDH Primers at Varying Anneal Temperatures.

To attempt to overcome the lack of sensitivity, amplification of the same controls with both primer pairs were performed at annealing temperatures of 64°C, 65°C and 66°C. Since the Tm of the G3PDH primers was 62°C and the Tm of the positive primers 64°C it was thought that it should be possible to increase the temperature of the annealing step to a level where the G3PDH primers were no longer annealing at the maximum efficiency whereas the positive primer were still annealing efficiently. This should reduce the intensity of the control fragment compared to the positive product. It was found with this amplification however that the positive primers did not work at annealing temperatures above 64°C whereas the G3PDH were still amplifying efficiently at 65°C (Figure 4.9.).

4.5.3. Amplification with Positive and H2-K Primers.

The control DNAs were amplified under the same conditions described in Section 4.5.1. The amplification was sensitive enough to identify 0.1 copies and the H2-K control product was amplified although a number of non-specific amplification products were identified (Figure 4.10.). Because of these non-specific products and the unreliability of the reaction, it was decided to concentrate on overcoming the sensitivity problem of the amplification with the G3PDH control primers. Two possible solutions were to increase the cycle length or to decrease the amount of G3PDH primers compared to positive primers.
4.5.4. Amplification at Different Cycle Lengths and Concentrations of G3PDH Primers.

Three different concentrations of G3PDH primers were used in PCR reactions with three different cycle lengths, 30, 33 and 36 (Figure 4.11.). The concentrations of G3PDH used were 1μM, 0.2μM and 0.04μM. The DNA templates in this reaction omitted the one copy control. At 30 cycles and 1μM concentration of the G3PDH primers, the positive amplification product was only detectable to a concentration of 0.5 copies per genome. At 30 cycles and 0.2μM concentration the sensitivity increased to detect 0.2 copies while at 0.04μM concentration the control fragment did not amplify. When the cycle length was increased to 33, a similar pattern was seen with the sensitivity increasing to 0.1 copies detectable where a 0.2μM concentration of G3PDH primers was used. At 36 cycles the sensitivity of the reaction was sufficient to identify 0.1 copies but amplification of contaminating DNA began to present a problem. From these results it was decided to use the concentration of 0.2μM G3PDH primers with 33 cycles for the amplifications. This was tested further to ensure that the reaction was robust enough to work consistently. This appeared to be the case and therefore was used on all further tail DNA samples. When amplifying the tail DNAs a titration reaction was also carried out in parallel to ensure that the reaction was still working to a sufficient sensitivity.
Figure 4.10: PCR amplification of negative DNA spiked with varying concentrations of pR2TsTAgPA plasmid to give the equivalent of 100 copies of the transgene per genome (Lane 2), 10 copies per genome (Lane 3), 1 copy per genome (Lane 4), 0.5 copies per genome (Lane 5), 0.2 copies per genome (Lane 6) and 0.1 copies per genome (Lane 7). PCR amplifications were carried out using the positive primer pair and the H2-K control primer pair. Lane 1: pBluescript Sau3A DNA molecular weight standard.

Figure 4.11: PCR amplification of negative tail DNA spiked with pR2TsTAgPA plasmid DNA equivalent to 100 copies of the transgene per genome (Lanes 1, 8 & 15), 10 copies per transgene (Lanes 2, 9 & 16), 0.5 copies per genome (Lanes 3, 10 & 17), 0.2 copies per genome (Lanes 4, 11 & 18), 0.1 copies per genome (Lanes 5, 12 & 19). Lanes 6, 13 & 20 contained negative tail DNA controls and Lanes 7, 14 & 21 contained water controls. PCR amplification was carried out using the positive primer pair and the G3PDH control primer pair at varying cycle lengths as indicated in the figure. The concentration of the G3PDH primer pair compared to the positive primer pair was also varied with 1x concentration (Lanes 1 - 7), 0.2x concentration (Lanes 8 - 14) and 0.04x concentration (Lanes 15 - 21) being used.
5.1. Generation and Identification of Founder Animals.

At 4 weeks of age the mice were weaned and approximately 1cm of tail taken to obtain DNA for PCR and Southern analysis. Mice were originally identified as transgene positive using the PCR reaction described in Chapter 4. Table 5.1 shows details of the positive transgenic founders obtained.

<table>
<thead>
<tr>
<th>Number of Eggs Injected</th>
<th>R2TsTAgPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice born</td>
<td>106</td>
</tr>
<tr>
<td>Percent survival</td>
<td>9.6%</td>
</tr>
<tr>
<td>Positive transgenics</td>
<td>13</td>
</tr>
<tr>
<td>Percent transgenic</td>
<td>12.3%</td>
</tr>
</tbody>
</table>

Table 5.1: Number of transgenic mice generated.

5.1.1. Generation Of Transgenic Lines from Founder Mice.

The thirteen founders generated from the R2TsTAgPA construct, are shown in Table 5.2. with the status of the line. These mice were then bred with CBA/CA mice to produce the transgenic lines.

<table>
<thead>
<tr>
<th>Founders</th>
<th>History of Mice and Lines Generated.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4719</td>
<td>Subcutaneous tumour at ~3 months. No line generated.</td>
</tr>
<tr>
<td>4723</td>
<td>Died during operation at 5 weeks. No line generated.</td>
</tr>
<tr>
<td>4856</td>
<td>Dead line. Subcutaneous tumours in founder, G1 and G2.</td>
</tr>
<tr>
<td>4590</td>
<td>Continuing line. Subcutaneous tumours seen in founder and G1.</td>
</tr>
<tr>
<td>4592</td>
<td>Dead line. One G1 positive never produced live offspring.</td>
</tr>
</tbody>
</table>

Table 5.2: History of Founder Mice Generated. Continued on next page.
<table>
<thead>
<tr>
<th>Founders</th>
<th>History of Mice and Lines Generated</th>
</tr>
</thead>
<tbody>
<tr>
<td>4847</td>
<td>Subcutaneous tumour at ~3 months. No line generated.</td>
</tr>
<tr>
<td>4930</td>
<td>Continuing line. No incidence of tumours.</td>
</tr>
<tr>
<td>4933</td>
<td>Continuing line. Subcutaneous tumours seen in founder and G1.</td>
</tr>
<tr>
<td>4944</td>
<td>Dead line - positive G1 by PCR bred from and found negative by Southern blot.</td>
</tr>
<tr>
<td>4959</td>
<td>No line generated.</td>
</tr>
<tr>
<td>5256</td>
<td>Continuing line. Subcutaneous tumour seen in G1.</td>
</tr>
<tr>
<td>5257</td>
<td>Subcutaneous tumour seen at 2 months. No line generated.</td>
</tr>
</tbody>
</table>

Table 5.2:- History of Founder Mice Generated. Continued from Previous Page.

5.1.2. Tumour Development in Transgenic Mice.

Tumour development had previously been observed in lines of transgenic mice containing mutations of the SV40 TAg gene, which confer temperature sensitive properties on the protein, under the control of a tissue specific promoter. This meant there could be a problem with tumour development in the transgenic mice generated containing the R2TsTAGPA construct. Originally it was thought that the supposed higher conditionality of the SV40 T Antigen mutations used would reduce the risk of tumours compared to mice generated with a tsA58 TAg construct. When tumours were first seen a number of mice had to be sacrificed and the potential transgenic line from these animals lost. Although this was not ideal, it was probable that any line that was producing tumours at a very early age would not respond to the temperature control required to obtain conditionally immortalized cells and would not be the most useful lines to pursue in the project.

The tumours which were not identified early on were often approximately 6g when dissected from the animal. All were subcutaneous in origin therefore showing a similar pattern, but not time scale, to the first tumours seen with the mice generated containing the
Ren2(4.6)TAG construct. Most of the subcutaneous tumours occurred in the hindleg region but one was found on the head area. A pathology report on the tumour from 4847 showed features consistent with an undifferentiated sarcoma (Dr C.J. Clark, Royal Dick Veterinary College, Edinburgh).

5.2. Development of a Southern Screening Strategy.

Southern analysis is used to confirm the results obtained from the PCR amplification of tail DNAs and to provide more information on the integration of the transgene. The technique involves the immobilization on a nylon filter of DNA, previously digested with appropriate restriction enzymes and electrophoretically separated. The immobilized DNA can then be hybridized to a labelled probe complementary to a region of the transgene. The patterns and sizes of the restriction fragments hybridizing to this probe will give information on the arrangement of the transgene array and the number of transgene insertion sites present in the genome. A comparison of hybridization signals to DNA copy number controls will allow an estimate of the number of transgene copies present within the array. Where multiple insertion events have occurred the inheritance in the offspring can be followed, using the Southern technique, to identify those animals which contain only one of the transgene insertion sites.

5.2.1. Choice of Restriction Enzymes and Probe Fragment.

The enzymes chosen for digestion of genomic DNA must not have a bias against cutting methylated, genomic DNA and must have little star activity, to allow the reactions to continue overnight with no problems of degradation of the DNA. The enzymes chosen had to satisfy these requirements and cleave at one point within the SV40 T Antigen sequence. The renin promoter contains a number of repetitive elements which could result in a high background hybridization signal if a probe containing these regions was used. For this reason the fragment chosen to hybridize to the filters containing the Southern blotted DNA was the SV40 T Antigen fragment used in the pR2TsTAGPA construction (Figure 5.1.).
4.6kb ren2 Promoter

SV40 TAg Sequence

SV40 polyA

646bp

2.5kb TAg Fragment Used for Random-Primed Probe.

**Figure 5.1:** Showing the Region of Transgene Used as a Probe in the Hybridization of Southern Blots.

Two candidate restriction enzymes identified, *Pvu*II and *Pst*I which were used to digest the founder genomic DNAs. The patterns expected from the possible transgene arrangements are shown in Figure 5.2.

HHH

HHT

HTH

TTH

**Key**

- *Pst*I Restriction Site
- *Pvu*II Restriction Site
- 3' Flanking Fragment
- Region Hybridizing with Probe.

**Figure 5.2:** Showing simple transgene arrangements and the pattern of hybridization signals expected with hybridization using a probe to the SV40 TAg sequence used in cloning pR2TsTAgPA.
Figure 5.3a: PvuII digest of founder tail DNAs confirming PCR positives. Lane1: 4856, Lane2: 4930, Lane3: 4933, Lane4: 4590, Lane5: 5256, Lane6: 4944, Lane7: 4723, Lane8: 4847, Lane9: 4592, Lane10: 4719, Lane11: 4959, Lane12: 5257, Lane13: 4590, Lanes 14 & 16: Blank, Lane15: Negative DNA, Lane17: 1 copy control, Lane18: 10 copies control, Lane19: 50 copies control.

Figure 5.3b: PstI digest of founder tail DNAs confirming PCR positives. Lane1: 4856, Lane2: 4930, Lane3: 4933, Lane4: 4590, Lane5: 5256, Lane6: 4944, Lane7: 4723, Lane8: 4847, Lane9: 4592, Lane10: 4719, Lane11: 4959, Lane12: 5257, Lane13: 4590, Lanes 14 & 16: Blank, Lane15: Negative DNA, Lane17: 1 copy control, Lane18: 10 copies control, Lane19: 50 copies control.
5.2.2. Format Used for Southern Analysis.

DNA from founders, including those transgenic positive mice which did not generate lines, was digested with \textit{PvuII}, electrophoretically separated and Southern blotted (Figure 5.3a). A similar blot was carried out with DNA digested with \textit{PstI} (Figure 5.3b). The format for the gel electrophoresis used a 22 slot comb but this did not give the required resolution. Although the results from these Southerns confirmed the PCR results for these animals, little more information could be determined.

5.3. Results from Line 4590.

This and the remaining lines were run on a Southern format described in Chapter 2 to prevent the problems seen with resolution in the founder blots. The results from this line, when digested with \textit{PvuII}, showed a single insertion site present (Figure 5.4a). The copy number of the transgene array was estimated to be ten copies. The sizes of the internal fragments detected by the SV40 T Antigen probe were \(~2.1\text{kb}\) and \(~1.4\text{kb}\) indicating that this array had a standard head - tail arrangement. No flanking fragment was seen although the one copy control was visible suggesting that the 3' flanking fragment which could hybridize to the SV40 TAg probe was not detectable in the size range possible.

5.4. Results from Line 5256.

This line gave similar results to those of line 4590 when digested with \textit{PvuII} (Figure 5.4b). There was a variability in the DNA quantity loaded but an estimate of the copy number was achieved by using the lanes of DNA most closely reckoned to be \(10\mu\text{g}\) and comparing this with the intensity of the copy number controls. The copy number was therefore estimated to be eight copies. The fragment sizes were similar to those found with line 4590 suggesting that the array was arranged in a head - tail manner. No flanking fragment was seen with this family although the one copy control was detectable, suggesting that again the 3' flanking fragment was not visible in this size range.

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5.5. Results from Line 4930.

This line gave a different fragment pattern to that seen from lines 4590 and 5256, when digested with \textit{PvuII}. The sizes of the fragments were approximately 2.4kb and 2.6kb and the copy number was between two and five. The fragment pattern was not predicted in any of the simple
arrangements and further digests would be necessary to determine the organization of the transgene array. No flanking fragment was seen although the one copy control was visible, again suggesting that in the possible range this was not detectable.

5.6. Results from Line 4933.

The pattern of the hybridization signals seen when DNA from this line had been digested with PvuII before Southern blotting suggested the presence of two integration events (Figure 5.5a). In the founder and a number of the offspring three hybridizing fragments were present, the sizes of the two outer fragments were 2.7kb and 1.7kb and at a higher intensity than the 2.1kb inner fragment. In the founder and the G₁ offspring all three fragments were present. In the G₂ three different patterns were seen, those which were identical to the founder and G₁ parent, those in which three fragments were present but without the stronger intensity of the outer fragments and those in which only the two outer fragments were seen. It was thought that this pattern represented two insertion sites, both of which contained a head - tail transgene array, with the central fragment postulated to be a 3' flanking fragment from one array. The flanking fragment from the other array was not visible. Where both transgene arrays were present the two outer fragments were at a higher intensity due to them being the internal fragments from both head - tail arrays. In the G₂ offspring this transgene arrangement had segregated, resulting in some containing only one of the transgene arrays. The arrangements seen were designated pattern a, for that containing only the two outer fragments, and pattern b, for that containing the three fragments. From these it was possible to estimate the copy number being approximately ten for pattern a and five for pattern b. The animals containing single insertion sites were then bred separately as lines 4933a and 4933b, with a view to determining if both transgene insertion sites were able to express SV40 T Antigen. It was known that at least one of the sites was expressing SV40 T Antigen since the founder had developed a tumour, although this had occurred after thirteen months. The pedigree of this line is shown in Figure 5.5b and details the known inheritance of the two transgene insertion sites.
Figure 5.5a: PvuII digests of tail DNAs from line 4933. Lane1: 4933, Lane2: 6179, Lane3: 6181, Lane4: 6453, Lane5: 6183, Lane6: 6457, Lane7: 6458, Lane8: 6459, Lane9: 8274, Lane10: 8277, Lane11: 8276, Lane12: 8278, Lane13: 8276, Lane14: 8280, Lane15: 8281.

Figure 5.5b: Pedigree of first two generations generated from founder 4933. Southern hybridization results are shown in Figure 5.5a. Notation of a and b refers to the two different integration sites present in these transgenic animals. Pattern a refers to that producing only two fragments when using a PvuII digest. Pattern b refers to that producing three fragments when using a PvuII digest.
5.7. Results from Southern Blot of Founder Animals From the Ren2tsA58 Transgene.

Tail DNA from four of the potential founder animals identified by PCR to contain the Ren2tsA58 transgene were digested with PvuII and Southern blotted as described previously. This showed that three of these animals were positive for the transgene (Figure 5.6.). The one animal which displayed a weak PCR positive signal did not produce a positive signal on the Southern blot and therefore the PCR signal was thought to be contamination. These four founders have now been bred to generate lines of transgenic animals and have transmitted the transgene to their offspring.

![Figure 5.6: PvuII digests on tail DNAs from founder animals containing the Ren2tsA58 transgene. Lane1: 9864, Lane2: 9079, Lane3: 9080, Lane4: 9087, Lane5: 9090, Lanes 6 & 7: Blank, Lane8: Negative DNA, Lane9: 1 copy control, Lane 10: 10 copies control, Lane11: 50 copies control.]

No tumours have been seen in any mice from the four transgenic lines produced from these founders. This is not due to lack of expression of the transgenes in at least two of the founders. RNA from founder 9087 produced a positive signal in the RT-PCR carried out by Davy Fettes (Figure 6.3). It is also known that there was expression of the transgene in founder 9079 since a positive result was obtained when SV40 TAg immunohistochemistry was carried out on sections from the kidney of the founder.
Kidneys were dissected from the mice and halved to provide samples for RNA, cryosections and paraffin sections. Half a kidney was used for both the RNA and cryosection samples and two halves were used for paraffin sections. The paraffin sections were used to look at the morphology of the kidneys and also for immunohistochemistry to determine if the SV40 T Antigen protein was present. The cryosections were originally taken for the immunohistochemistry since the SV40 TAg antibody available would not work with fixed, paraffin sections but the morphology of these sections was not ideal. A different antibody was later found which would allow SV40 TAg immunohistochemistry to be carried out on paraffin sections.


One Northern was carried out on earlier RNA samples taken from founder animals. This was carried out using 40µg of total kidney RNA on a MOPS RNA gel and a different method of hybridization. A sample from one of the surviving lines, 4590, was loaded on this gel and produced a faint positive signal (Figure 6.1.). Much stronger hybridization signals were seen in those lanes containing kidney RNA from the founders which developed tumours early and did not survive to breed. This Northern had poor resolution and for this reason the RNA gel and hybridization conditions were changed.

6.1.1. Hybridization Using the Southern SV40 TAg Probe and a G3PDH Probe.

RNA gel electrophoresis was carried out using 20µg total kidney RNA from each line of transgenic animals, using the conditions described in Section 2.12.3. Control samples included on the gel were 20µg total kidney RNA from a negative transgenic mouse and 20µg total RNA from a tumour.
Figure 6.1: Result from original Northern run. 20\mu g total RNA from founder and negative animals was electrophoresed using a MOPS buffer system. The blotted RNAs were hybridized SV40 Tag probe. Lane 1, 4723 SMG RNA; Lane 2, 4592 SMG RNA; Lanes 3 & 7, negative transgenic RNA; Lane 4, 4592 kidney RNA; Lane 5, 4719 kidney RNA; Lane 6, 4723 kidney RNA; Lane 8, 4854 kidney RNA; Lane 9, 4593 kidney RNA; Lane 10, 4590 kidney RNA.

The tumour RNA was obtained from a line of transgenic rats containing the 21 hydroxylase promoter fused to the SV40 T Antigen gene containing the tsA58 mutation and had previously been shown to give a positive signal on a Northern blot when hybridized to a SV40 T Antigen probe. The construct in this transgenic line was 21OHtsA58 and was constructed by Isabelle Veniant in the Mullins laboratory. The RNA was then Northern blotted onto nylon membrane and hybridized to a random-primed SV40 T Antigen probe derived from the same region used for the hybridization of Southern blots. No hybridization signal was seen after autoradiography although the sample RNA track stained with methylene blue showed that there was RNA present on the nylon membrane. To confirm that transfer of the RNA to the nylon membrane had taken place the blot was hybridized to a random-primed G3PDH probe which showed RNA present in equal quantities in each track.
(Figure 6.2). The lane containing 20μg of tumour RNA produced a smeared hybridization signal when using the G3PDH probe.

![Image of Northern blot](image)

6.1.2. Hybridization Using a Smaller SV40 TAg Probe Fragment.

Since the problem with detection of SV40 T Antigen gene expression was not caused by a lack of RNA on the blot it was decided to change the probe fragment used in the hybridization. The probe chosen was a HpaI fragment from SV40 T Antigen that had been used in the laboratory to probe Northern blots and had been shown to give a positive signal with the tumour RNA used as a positive control on this blot. When using this fragment as a probe a positive hybridization signal was seen for the tumour RNA but even after two weeks exposure no signal was seen from the kidney RNA samples (Data not shown).

6.2. SV40 TAg RT - PCR

When no positive hybridization signal was seen on a repeat Northern, carried out by Davy Fettes, it was decided that an RT-PCR experiment should be carried out to determine if the TAg expression could be detected in any of the lines known to be expressing from the tumour or immunohistochemical data. This was also carried out by Davy Fettes and produced a positive product for one of the transgenic lines,
9087, containing the Ren2tsA58 construct (Figure 6.3). The figure also shows a positive signal from a subcutaneous tumour RNA sample obtained from line 4933 (Lane 1), this is the tumour sample shown positive in the immunohistochemistry in figure 6.6E. The size of this product is slightly lower than that seen in the positive control but this could be due to the fact that it is a tumour sample where aberrant splicing is more likely to occur. The positive signal was obtained from the kidney RNA from line, 9087 (Lane2), which has so far not produced a positive signal with the immunohistochemistry. This is probably due to the number of samples taken from each line when the immunohistochemistry was carried out. Approximately 6 to 9 sections only were taken from each line to be used for immunohistochemistry and therefore the sample number is very small when expecting to find a positive signal from a lowly expressed protein.

![Image of RT-PCR result](image-url)

**Figure 6.3:-** showing the result of the RT-PCR using total RNA from tumour (Lane1), kidney from 9087 (Lane 2), kidney from 4930 (Lane 4), kidney from negative transgenic (Lane 5). Lane 3 shows a positive control sample. Lane 6 contains pUC/HpaI DNA markers and Lane 7 contains pBluescript Sau3A DNA markers. Expected sizes of product are as follows:- Unspliced RNA (and also DNA) 580bp, Small t Antigen RNA 510bp and Large T Antigen RNA 240bp.
6.3. Analysis of Kidney Morphology.

From the information provided by the Ren2(4.6)TAyg transgenic mice there was reason to believe that there may be changes in the morphology of the JG apparatus, even if no outward signs of tumours were visible. This was investigated in the R2TsTAygPA animals by paraffin sectioning of kidney samples and staining with haemallum and eosin (H&E). Sectioning of kidney samples from founder animals was carried out by Stewart Fleming and gave the same results as those later obtained when sectioning G2 animals from the R2TsTAGPA transgenic lines.

Paraffin sections from the different lines of transgenic mice were cut at 5μm and stained with haemallum and eosin. These sections showed varying degrees of dysplasia and hyperplasia in the kidney between the lines. These effects were seen at the position of the JG cells. This indicated that the tissue specificity of the promoter was intact.

Figure 6.5 shows examples of the morphology of the kidney sections from the R2TsTAygPA G2 transgenic animals. In most cases at low power magnification there were no obvious differences between the normal control animals (6.5A) and the positive transgenic animals (6.5B). One exception to this is shown in Figure 6.5C where the glomerulus was found to be greatly enlarged compared to normal glomeruli. A higher magnification of this glomerulus and a normal comparison is shown in Figure 6.6.

The most common form of abnormal glomerulus is shown in Figure 6.5D where a large cluster of cells is seen at the vascular pole. This hyperplasia was seen in all the R2TsTAygPA transgenic lines sectioned and is presumed to arise from overgrowth of the JG cells expressing SV40 TAg although this will have to be demonstrated. Other examples of abnormal glomeruli are shown in Figure 6.5 E and F. Only three Ren2TsA58 animals, one from three different transgenic lines have had kidneys taken for sectioning. From these no gross abnormalities of the glomeruli have been seen although one of these lines has shown expression of SV40 TAg from the immunohistochemistry results (Section 6.4).
Figure 6.5: Morphology of kidney sections stained with H & E from R2TsTAGPA transgenic lines. A - Low power (10x magnification) section of negative transgenic mouse to compare with low power from positive transgenic (B). C - shows a low power section from a transgenic mouse with an abnormally enlarged glomerulus. D - higher power (25x magnification) section of an abnormal glomerulus from line 5256 showing a cluster of cells at the vascular pole. E - 25x magnification of section from a G2 animal of line 4933B showing another type of abnormal glomerulus seen. F - gives another example of an abnormal glomerulus seen in a G2 animal from line 4933A (25x).
Figure 6.6: A shows a 40x magnification of abnormal glomerulus from Figure 6.5 C compared to a more normal glomerulus at the same magnification (B).
6.4. Results from SV40 T Antigen Immunohistochemistry.

Figure 6.7 shows the results from the SV40 T Antigen immunohistochemistry. Within some kidney sections brown DAB staining was seen in the region of the JG apparatus where the JG cells are present. Some light staining was seen over the erythrocytes and within the glomerulus, at positions which could relate to red blood cells, in sections from both positive animals and negative controls suggesting that this was background staining. This staining was also seen in the sections stained with non-immune serum again suggesting that this was background staining. The positive staining of the JG cell regions of the glomeruli was never seen in sections from negative transgenic animals or in sections from positive animals treated with non-immune serum.

Sections from subcutaneous tumours were included in the immunohistochemistry to give a positive control. The results from these sections are shown in Figure 6.7E with brown DAB staining in the nuclei of the cells. When incubated with non-immune rabbit serum there was no staining seen in the cells indicating that the brown stain was due to the presence of SV40 T Antigen protein (Figure 6.7F).
Figure 6.7: Results from immunohistochemistry using an antibody against SV40 T Antigen and staining with DAB. A - shows 25x magnification of a kidney section from a positive G2 animal of Ren2TsA58 transgenic line 9079 showing a positive signal in the glomerulus at the position of the JG cells. B - shows a section from a positive G2 transgenic animal from R2TsTAg PA transgenic line 4930 again showing staining at the position of the JG cells. C - shows a kidney section from a negative transgenic incubated with immune serum which gives the same result as shown in D where a section from a positive transgenic animal was incubated with non-immune serum. E - shows a positive control using a section from a subcutaneous tumour from line 4933 incubated with immune serum compared with a section from the same tumour incubated with non-immune serum (F).
Chapter 7 - Discussion.

This project has generated two constructs using temperature sensitive mutants of the SV40 T Antigen under the control of the Ren-2d promoter which have been used to obtain lines of transgenic mice the two constructs being Ren2TsTAgPA (where the TAg contains both the tsU19 and tsA58 temperature sensitive mutations) and Ren2tsA58 (where the TAg contains the tsA58 mutation alone). The mice generated from the two constructs will allow the effect of the two mutations on the conditionality of the TAg to be investigated as well as allowing the eventual isolation of JG cell lines.

7.1. Generation of R2TsTAgPA Transgenic Animals.

A number of the original founder mice generated with the R2TsTAgPA construct containing both mutations developed tumours at an early age and did not therefore produce a line of transgenic animals (Table 5.1). The problem of tumour development in transgenic animals has been seen previously where a temperature sensitive SV40 TAg gene is placed downstream of a tissue-specific promoter, a problem not encountered with the H2-K tsA58 transgenic mice where a ubiquitous promoter was used. This is probably due to the higher expression in these tissues, leading to a loss of conditionality. This expression level dependent ability to escape the conditionality was demonstrated in the H2-K tsA58 mice, although this was still not high enough to lead to tumour development in the animals themselves. Fibroblast cultures from the different founder animals were grown at permissive, semi-permissive and restrictive conditions. The ability to grow in these conditions was directly related to the amount of SV40 TAg expression, with the higher expressing lines being able to grow in semi-permissive and even restrictive conditions.

7.1.1 Identification of positive transgenic mice by PCR and Southern Analysis.

The PCR strategy originally decided upon for identification of transgenic positive animals was a simple tripartite reaction involving
one common primer complementary to Ren-2d promoter sequence with a primer complementary to the SV40 TAg transgenic sequence producing a positive PCR product and a primer complementary to a region of Ren-2d intron A sequence providing a control PCR product amplified in all individuals. Unfortunately this strategy broke down in practice when it was shown that however carefully a primer pair is designed it may still not produce the exact result required. The presence of the intron A primer produced a large number of non-specific products, one of which was the same size as the positive amplified product and therefore could not be used. The control amplification product was then produced by the GAP primers which amplify the GAP gene and pseudogenes present in the genome, a point which will be discussed later. This proved satisfactory enough to identify the founder animals but in a lot of cases the amplifications were repeated with the positive primer pair alone due to the high intensity of the GAP product due to the presence of the pseudogenes. When identifying the G1 positive animals the duplicate PCR strategy was abandoned after no extra positive offspring were identified with the PCR reaction involving only the positive primer pair.

The PCR reaction proved problematic when amplifying for identification of G2 positive animals. At this time it was decided that the best course of action was to design a new Ren-2d promoter primer since changing conditions such as magnesium concentration could not totally eliminate the problem of non-specific products. This was to be used in conjunction with a SV40 TAg primer already available in the laboratory after testing this primer to ensure that it worked in a mouse genomic environment. The Ren-2d primer design came up with five possibilities which were then tested on computer programs to determine if any problems could occur due to hairpin loops or primer dimerization. This testing eliminated one of the primers and another was eliminated on the principle that the sequence to which it was complementary ran over the region of the original primer. One of the remaining three primers was then synthesized and used in the PCR in conjunction with the new SV40 TAg primer. This was found to overcome the problem of the non-specific amplification products which had been occurring but left a problem with the faintness of the positive amplified product in comparison to the control product due to the use of the GAP primers for
this amplification. An alternative primer pair for the control amplification proved ineffective since the reaction conditions did not always produce a reliable result. It was thought that the GAP primers should no longer be amplifying exponentially and therefore one possibility would be to increase the number of cycles in the PCR reaction. This should increase the intensity of the positive product by a greater proportion than the GAP product since the amount of positive product should still be increasing exponentially. This in combination with a reduction in the amount of GAP primer concentrations, to a fifth of that used for the positive primer pair, eventually produced a satisfactory result which has now been reliably reproduced on a large number of occasions. This experience has been very useful in identifying those aspects of the PCR reaction which are most likely to produce an effect when trying to optimize reactions.

The founder mice identified by PCR were shown to be positive on Southern blots and those which produced lines of transgenic mice were blotted with G₁ and G₂ offspring. The original Southern method using a 22 slot comb format proved ineffective at giving the resolution required to size the bands accurately, possibly due to the amount of DNA, 10µg, which was loaded in each lane. The alternative method used with a 14 slot comb format proved much better at producing the resolution required despite the fact that the gels were electrophoresed for a shorter time. This actually led to more samples being able to be run on a gel since two rows of 14 lanes were available. The probe chosen to hybridize to the Southern blots was a fragment of the SV40 TAg sequence available. This did not include sequence from the Ren-2ᵈ promoter since regions of this promoter contain repetitive elements which would have resulted in background hybridization. This would result in one of the flanking fragments not being identified using this probe and the PvuII digest used since PvuII cleaves the promoter at a number of sites. PvuII was decided upon as the restriction enzyme since it would produce cleaved products at a range which was easily identifiable and is known to cleave genomic DNA well under the conditions of Southern digests. Since the main aim was to identify the positive transgenic animals and trace the inheritance of the transgene through their offspring to ensure that no loss of transgene occurred this was not thought to be a problem. The PvuII
digest could identify the common arrangements of transgenes and further investigation of arrangements and flanking fragments will require cleavage with different restriction enzymes. The Southern analysis showed three of the four lines contained a single transgene insertion site with the transgene array in two of these being in the standard head-tail arrangement. The line 4930 will require digestion with different restriction enzymes to identify the arrangement since the pattern was not predicted from any of the simple arrangements shown in Figure 5.2. The results from the fourth line, 4933 suggest the presence of two integration sites which results in the potential of two lines from this founder. Mice from this line with only one of the integration sites present have been bred to generate separate lines. RNA samples have been taken from the two separated lines to determine if the transgene is being expressed at both integration sites.

7.1.2 SV40 TAg expression in the transgenic lines

Evidence suggests that the tumour development in the founder animals could be related to the amount of SV40 TAg expression present, as seen in other transgenic constructs. The original Northern blot using total kidney RNA from founder animals showed a strong hybridization signal in the lane containing RNA from an animal which developed a tumour at ten weeks of age. Hybridization signals from founder animals which did produce transgenic lines were extremely faint on this blot. The hybridization signal was not detectable on later Northerns when using a random-primed probe to identify SV40 TAg where only the tumour sample showed a positive hybridization signal. Since the lines of mice present on the blot showed incidences of tumours or had some abnormalities at the histological scale, the lack of positive signal using the random primed probe was not thought to reflect a lack of expression of the transgene but a lack of sensitivity of the probe used. Some of these lines have also now produced a positive signal with SV40 TAg immunohistochemistry.

One method which was attempted to overcome the problem with the Northern sensitivity was to hybridize the Northerns using a single stranded RNA probe instead of the random primed probe. One of the plasmids constructed in the cloning steps contained the SV40 TAg coding
sequence in a position which allowed an antisense SV40 TAg probe to be transcribed from the T7 promoter in the vector sequence. The digested plasmid was purified and used in a cold transcription to determine if the reaction would work. Once the cold transcription reaction was demonstrated to work a hot transcription using $^{32}$P labelled CTP was carried out. The labelled riboprobe was first hybridized to an old Northern blot to ensure that it did not hybridize to ribosomal RNA and after confirmation of this was hybridized with the stripped Northern previously hybridized with the random primed probe. Unfortunately this method, although approximately ten times more sensitive than a random primed probe hybridization still did not produce a positive signal for any lane other than the positive tumour control. The problems of detection of the SV40 TAg RNA are likely to be due to the low proportion of cells from the kidney which will be expressing this RNA. One method which will now be used to try and detect the SV40 TAg RNA will be to extract polyA+ RNA from the total RNA, therefore increasing the proportion of the SV40 TAg RNA present per µg. As discussed in chapter 6 RT-PCR has now been used and identified a transgenic line expressing SV40 TAg previously negative on Northern blots.

7.1.3. Morphology of kidney sections

A possible problem with the R2TstTAGPA transgenic animals is suggested in the evidence from the histology on G1 animals. This shows degrees of hyperplasia and dysplasia in the kidneys of these animals even where no incidence of tumours had occurred mirroring the result seen in the Ren2(4.6)TAG transgenic animals. The evidence of hyperplasia suggests that cells isolated from these animals may not be ideal for use in isolating a conditionally immortal JG cell line since they have escaped "normal" growth regulation at 39°C in the animals. Although the pattern of hyperplasia and dysplasia seen reflects that seen in the Ren2(4.6)TAG construct, suggesting that it is the JG cells that are affected, this has yet to be demonstrated directly.
7.1.4. SV40 TAg Immunohistochemistry.

When originally attempting SV40 TAg immunohistochemistry the only SV40 TAg antibody available to us was one which could not be used with fixed paraffin sections. Therefore samples of kidneys were frozen in OCT and used to produce cryosections. Before attempting immunohistochemistry with these kidney samples, sections were stained with haemallum and eosin to determine if the sections were suitable for this. In all the sections cut no obvious glomeruli were seen suggesting that the morphology in these sections was nowhere near as good as that seen in paraffin sections. What little immunohistochemistry that was done at this time did not produce a positive result with any of the transgenic lines, even those which had died of tumours at an early age and had had enough expression to be detectable on a Northern blot. Later an antibody to SV40 TAg became available which was able to be used with paraffin sections. This was first used in conjunction with a method reported to increase the likelihood of detecting a lowly expressed protein, microwave antigen retrieval method (97). This method involves boiling the slides containing the sections in citrate buffer for approximately ten minutes in the microwave which is thought to relax the antigen protein structure to allow the antibody to bind to it. This was not found to be the case in my hands where too many of the sections were dislodged during the boiling treatment and those that did stay did not produce any more positive a signal than an equivalent section which had not undergone this treatment. Those sections which did survive microwaving were also damaged so that the morphology was not as clear in these as in those untreated sections.

7.2. Generation of Ren2tsA58 Transgenic Animals.

The cloning of pRen2tsA58 and the generation of mice containing this construct will allow the direct comparison of the two temperature sensitive SV40 TAg mutations since the promoter fragment used in both constructs is the same. Four founder animals were identified by PCR and these have been bred to generate lines of animals. Northern blots containing RNA from these animals did not show a positive hybridization signal for SV40 TAg RNA for the same reasons as stated for the R2TsTAgPA transgenic animals since SV40 TAg
immunohistochemistry has shown that SV40 TAg is being expressed in at least one of the lines. An encouraging point with the histology from these animals is that no gross abnormalities in glomerular structure have been seen although they are expressing the T Antigen. This may mean that cell lines established from these animals are more useful since they have not escaped the conditionality of the temperature sensitive SV40 TAg.

7.3. Intentions for Further Analysis of the Transgenic Lines Generated.

7.3.1. Further Investigation of Transgene Integration Events by Southern Analysis.

Southern blots of DNA cleaved with other restriction enzymes are required to fully investigate the arrangement of the transgene at the sites of insertion into the genome. This is especially required for investigation of the line 4930 which did not exhibit any pattern expected from the simple arrangements shown in Figure 5.2. A probe fragment from a region of Ren2d promoter not containing repetitive elements, can be used to identify other flanking fragments not identified with the PvuII digested DNA hybridized to the T Antigen probe.

7.3.2. Analysis of Transgene expression in a Range of Tissues.

It was expected that the promoter fragment used would give a tissue specific pattern of expression since it gave such a pattern when fused to the wild type T Antigen gene. The pattern of tumours seen in these animals reflects the pattern seen in the transgenic animals generated using the Ren2(4.6)Tag construct. This and the kidney histology results, suggest that the promoter fragment does give a tissue specific pattern of expression. RNA samples have been taken from sites of known renin expression as well as those where renin expression is not expected which can be used to demonstrate this directly. The RNA samples obtained from G1 male and female animals and will allow the tissue specificity of this construct to be confirmed.

7.3.3. Renin Antibody Staining of Kidney Paraffin Sections.

The morphology of the kidney sections reflects that seen in
transgenic Ren2(4.6)TAg animals which did not develop renal tumours. This suggests that the hyperplastic cells seen in these sections are JG cell in origin but again this needs to be confirmed. Antibodies against renin are available in the laboratory which can be used to determine if this is the case. It is expected that the hyperplastic cells will be expressing renin and therefore be stained using an antibody against renin. It would be useful to confirm that the SV40 TAg protein is seen in the same cell type as that expressing renin. This can be determined using antibodies available against SV40 T Antigen.

7.3.4. Comparison of the two temperature sensitive T Antigen genes.

As stated previously there is as yet no direct evidence to support the hypothesis that the U19 mutation reduces the conditionality of the temperature sensitive T Antigen. The generation of two constructs which use the same promoter fragment differing only in the T Antigen mutations used will allow this comparison to be made. If expression levels in the Ren2tsA58 animals are found to be at equivalent levels, or greater, than that seen in the R2TsTAgPA animals without development of tumours this will suggest that the tsU19 mutation does indeed reduce the conditionality of the temperature T Antigen. If this is correct then it would be expected that at an equivalent level of expression the R2TsTAgPA lines of mice would be expected to have a greater level of dysplasia and hyperplasia in the kidney than the Ren2tsA58 lines of mice.

7.3.5. Isolation of an Immortal JG Cell Line.

If it is found that the U19 mutation does reduce the conditionality then isolation of JG cells from the Ren2tsA58 animals may allow a conditionally immortal cell line to be established. Ideally a line of mice which exhibit no abnormal characteristics in their kidney morphology and yet still express the SV40 TAg would be required. This level of expression may still be sufficient to immortalize the cells in culture but would allow them the immortalization to be controlled by temperature. Animals which display mild hyperplasia in the kidney will still be useful in the establishment of JG cell lines. Although the cells may still grow at the restrictive temperature it is likely that these cells are not fully transformed by the T Antigen and therefore may still display a more "normal" phenotype than transformed JG cell lines available at the
When good candidate cell lines have been isolated and shown to express both SV40 T Antigen and renin then electron microscopical studies can be carried out to determine if the cells have the granulated morphology characteristic of JG cells. Renin antibody staining could also be performed at this level to determine if granules present in these cells contained renin.

7.4. Applications of an Immortal JG Cell Line.

The isolation of these cells will allow mechanisms involved in renin secretion to be investigated. Current cell lines available cannot regulate their renin secretion but this may be related to a loss of tissue-specific characteristics due to transformation by SV40 T Antigen. Established cell lines can be used to identify regions of the promoter involved in tissue-specific expression using the promoter-deletion constructs available in the laboratory.

If isolation of JG cells from these animals proves difficult then it may be necessary to enrich for the afferent arteriole portion of the disaggregated kidney before culturing the cells. A method for enrichment used by Chatziantoniou (98) has been to perfuse the kidney with a magnetized ferric oxide suspension which results in the afferent arterioles being filled with this suspension while larger vessels are partially filled. After disaggregation of the kidney the afferent arteriole portion can be obtained by separation using a magnetic field and various sized sieves.

Such cells may also be used to understand the mechanism by which metaplastic transformation takes place in response to physiological stimuli. Isolation of cells at different time points in development may permit the identification of factors involved in the differentiation of putative JG cells. It is hoped that these transgenic animals will provide a resource for the successful future immortalization of these important cells.
References


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