Expression of the Rat Calcium-Binding Protein p9Ka in Transgenic Mice

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Abstract

Breast cancer, and in particular the metastatic spread of mammary neoplasia, is a major cause of death amongst women in the developed world, but, despite much effort, little progress has been made in combating this disease. By exploiting the techniques of molecular biology, and in particular the production of transgenic animal models of mammary oncogenesis, it is hoped that new targets for drug therapy can be developed that might help combat primary onset and secondary spread of tumours of the breast.

One gene that has been implicated in the metastatic progression of mammary tumours in rodent models is p9Ka. This S-100-related, calcium-binding protein is also of interest since its pattern of expression would appear to be different in rat and mouse tissues. By producing mice transgenic for the rat p9Ka gene it was hoped that both the expression of p9Ka and its contribution to mammary neoplasia could be investigated.

Two DNA constructs were thus produced, based on a 10.3 kilobase fragment of rat genomic DNA harbouring the p9Ka gene. The transgene constructs were different, in that one contained an insert designed to code for an additional antigenic epitope to which an antibody was available, there being no antibody available with which to study p9Ka protein expression when the project was started. Transgenic mice containing multiple copies of either transgene were produced and analysed, both for expression of the transgene mRNA and protein, and for any phenotypic trait associated with either expression or insertional mutagenesis caused by the transgene.

Differences in both p9Ka mRNA and protein expression between rat and mouse were confirmed and transgene expression was seen in all lines of transgenic mice produced, in a wide range of tissues. Independent transcriptional control of expression is apparently conferred by the transgene construct, since p9Ka-transgene expression in terms of both tissue specificity and absolute level is more akin to the rat than to the mouse, and is dependent on transgene copy-number. The implications of the expression patterns of the p9Ka transgene with respect to control of p9Ka expression are discussed.

Apart from a dwarfism seen in one line of transgenic mice, which was investigated further, there was no evidence of a phenotypic or oncogenic effect of transgenesis by the p9Ka gene. Nevertheless, considering that p9Ka is believed to be involved in progression to the metastatic phenotype rather than in primary oncogenesis, and bearing in mind the multi-step nature of tumorigenesis, mice expressing enhanced levels of p9Ka may provide an invaluable resource for the development of an animal model of metastatic spread of breast cancer. Since transgene p9Ka expression is not limited to the mammary gland, models of other metastatic diseases may also be produced by mating of the mice described here with mice exhibiting enhanced levels of primary neoplasia.

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List of Abbreviations

cDNA	complementary DNA
DAB	diaminobenzoate
DMBA	7,12-dimethylbenzanthracene
DTM	"dwarf transgenic mice" (Chen *et al.*, 1991)
EDTA	ethylenediaminetetra-acetic acid
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
hGH	human growth hormone
kbp	kilo-base pairs
L-broth	Luria Bertani medium (also referred to as LB both)
MMTV	Mouse Mammary Tumour Virus
MMTV-LTR	Mouse Mammary Tumour Virus long terminal repeat
MOPS	3-[N-morpholino]propane-sulfonic acid
mRNA	messenger RNA
NGF	nerve growth factor
PCR	polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TGFα	transforming growth factor α
Tris	Tris(hydroxymethyl)methylamine
Tween-20	polyoxyethylenesorbitan monolaurate
WAP	whey acidic protein

1. Introduction

1.1 Breast Cancer and Metastasis

Breast cancer is a major cause of death among females in the Western world, accounting for 20% of deaths from malignant disease whilst afflicting, in one form or another 8% of all women (Vorherr, 1980). Despite a large investment in research and clinical treatment, there has been little progress in successful treatment of this disease (Carter, 1980; Baum, 1985, Harris *et al.*, 1992). The ætiological factors of breast cancer are largely unknown, although environmental agents, heredity, hormonal exposure and diet (Mant and Vessey, 1991), as well as reproductive history (Boyle, 1988), are believed to constitute risk factors (Harris *et al.*, 1992).

There is substantial evidence that cancer is a multi-step phenomenon associated with the cumulative selection of somatic mutations (Foulds, 1958; Nowell, 1976; Poste and Fidler, 1980a; Seemayer and Cavenee, 1989; Weinberg, 1989; Bishop, 1991) and this would seem to be true of human breast cancer (Groner *et al.*, 1988; Callahan and Campbell, 1989; Van de Vijver and Nusse, 1990). The mutations involved may result in gain or loss of function of particular genes, and may involve gene amplification, gene deletion, gene translocation or point mutation (Weinberg, 1989). The involvement of individual genes (oncogenes) in tumorigenicity, and the nature of their normal cellular counterparts (proto-oncogenes), have been intensively studied and reviewed (Bishop, 1987, 1989, 1991; Varmus, 1989; Weinberg, 1989). Some studies into breast cancer have implicated oncogenes in the establishment of this disease (Groner *et al.*, 1988; Callahan and Campbell, 1989; Van de Vijver and Nusse, 1990; Harris *et al.*, 1992) and growth factors are also involved (Groner *et al.*, 1988; Van de Vijver and Nusse, 1990; Dickson *et al.*, 1992; Harris *et al.*, 1992). There is some evidence for amplification of c-*myc*, c-*erb*B2 and *int*-2-related genes (Callahan and Campbell, 1989) and expression of *ras* oncogenes (Callahan, 1987) in primary human breast tumours. Loss of heterozygosity is believed to be responsible for unmasking mutations in recessive oncogenes, for example the retinoblastoma gene (T'ang *et al.*, 1988; Varley *et al.*, 1989) and p53 (Nigro *et al.*, 1989), but also as yet unidentified genes.

The most life-threatening aspect of breast carcinogenesis, as with many other cancers is the metastatic spread of the disease (Fidler *et al.*, 1978; Fidler, 1991). Clues to the involvement of oncogenes in the malignant progression of breast cancer have come from correlations of amplification and/or expression of particular oncogenes in relation to clinical outcome of patients (Callahan and Campbell, 1989). The study of prognostic indicators of human breast disease has yielded few, if any adequate markers of metastatic potential. One indicator of a positive prognosis would seem to be expression of the"anti-metastatic" gene nm23 (Henessy *et al.*, 1991). The primary candidates for negative correlations (i.e. those oncogenes which are linked with poor clinical outcome and therefore may contribute to the metastatic phenotype) are amplifications of c-*myc*, *int*-2 and c-*erb*B2 (Callahan and Campbell, 1989), but reports vary as to whether or not such amplifications are significantly linked to poor prognosis and there are questions raised as to whether such indicators are useful in the clinic (Callahan, 1989).

The obvious importance of the metastatic spread of primary tumours in determining clinical outcome is not, unfortunately, reflected in our knowledge of how this process operates. Metastasis is a complex process involving many individual stages, and our understanding is inhibited by the lack of a good model of this phenomenon as part of breast cancer. The "metastatic cascade" includes several distinct steps: invasion of surrounding tissue, blood vessels and/or lymphatics, aided by the ability to degrade the extracellular matrix; survival in the circulation, including avoidance of immune surveillance; adhesion to the vascular endothelium, aggregation, or entrapment in the microvasculature; extravasation from the bloodstream or lymphatic vessel; and, growth in the secondary site including, when necessary, angiogenesis. Investigators have concentrated on modelling individual aspects of this multi-step phenomenon using tissue culture and whole animal systems. The results leading to the identification of the metastatic cascade and implicating specific factors in the ability of primary tumours to metastasise have been the subject of many good reviews and editorials (Fidler *et al.*, 1978; Poste and Fidler, 1980b; Fidler and Hart, 1982; Nicolson, 1982; Liotta *et al.*,1983; Klein and

Klein, 1985; Schirrmacher, 1985; Nicolson, 1987; Nicolson, 1988; Hart *et al.*, 1989; Fidler and Radinsky, 1990; Egan *et al.*, 1991; Evans, 1991a; Evans, 1991b; Fidler , 1991; Hart and Easty, 1991; Liotta *et al.*,1991; Evans, 1992; Steeg, 1992).

Much evidence for the involvement of oncogenes in breast cancer, and metastasis, has come from the use of cultured cell-lines and animal model systems. The ability to grow cells derived from carcinogen-induced, virally-induced or spontaneous tumours in tissue culture has allowed not only comparison of mRNA and proteins expression, *in vitro* and *in vivo* studies of oncogene and proto-oncogene function, but also the possibility of studying causative roles for oncogenes in tumour formation and metastasis, via transfection and introduction of these cells into nude mice or syngeneic hosts. One animal model system that has shed some light on the complexity of mammary neoplasia is that of infection of susceptible mouse strains with mouse mammary tumour virus (MMTV; Nusse, 1988; Callahan, 1989; Van de Vijver and Nusse, 1990). Perhaps the most interesting and potentially the most powerful tool for the study of mammary neoplasia is that by which oncogenes are expressed in transgenic mice. The use of whole animal systems is a necessity if we are to fully understand the complex series of events leading to establishment of secondary tumours via metastasis.

1.2 Transgenic Mice

From the time of the initial success of microinjection of embryos with cloned genes to produce transgenic mice (Gordon *et al.*, 1980), the technique has been widely used for a number of different purposes, and new and exciting techniques have become available which allow targeted disruption of specific genes. I would therefore like to limit this introduction and concentrate particularly on three areas (gene expression, neoplastic breast disease and metastasis) and to refer to several reviews and books dealing with not only the technology (Brinster *et al.*, 1985; Hogan *et al.*, 1986; Palmiter and Brinster, 1986) but also its applications (Cuthbertson and Klintworth, 1988; Jaenisch, 1988; Hanahan, 1989; Westphal, 1989; Grosveld and Kollias, 1992), in particular to models of neoplastic disease

(Groner *et al.*, 1987; Hanahan, 1988; Pattengale *et al.*, 1989; Adams and Cory, 1991; Berns, 1991; Dickson *et al.*, 1991; Muller, 1991; Kioussis, 1992).

Since microinjection of single-cell mouse embryos is the technique of choice in most laboratories producing transgenic mice, it is worth considering some of the factors that influence the production of these mice and expression of the transgenes in their tissues (Brinster *et al.*, 1985; Hogan *et al.*, 1986; Kollias and Grosveld, 1992). Techniques of DNA preparation, microinjection and implantation, and the strains of mice used (both for embryo production and pseudopregnant foster mothers) vary between laboratories and account, in part, for the various efficiencies of transgenic mouse production. Skill also plays a major part, as does the quality of the embryos and of the foster mothers. Over 90% of injected embryos can survive injection with 20% surviving to term, and of these as many as 25% can be found to carry the transgene. If transgene integration does occur, it is usually at a single, random chromosomal locus, but can be at two or more positions, and usually the transgene is inherited in a simple Mendelian fashion. The number of copies inserted varies from one to over one hundred, and multiple copies often integrate in a head-to-tail array. The size of the DNA used seems only to be dependent on the size which can be cloned, produced cleanly in relatively large amounts, and injected without significant shearing (caused by microinjection). Since inclusion of prokaryotic DNA in some transgenes has been shown to inhibit or interfere with transgene expression (Chada *et al.*, 1985; Townes *et al.*, 1985; Shani, 1986), it is now common to exclude such DNA from transgene constructs. Although the use of a cDNA- or genomic DNA-based construct is dependent upon the particular experiment to be performed, it is now considered wise to include introns within the transgene construct, in order to achieve its enhanced expression (Palmiter *et al.*, 1991).

1.3 Transgenic Mice in the Study of Gene Expression

One major use of transgenic mice is in the study of gene regulation, and

whenever a new gene is used to produce transgenic mice this deserves some consideration. Transgenic mice offer some distinct advantages, over *in vitro* and *in vivo* cell culture techniques, which stem mainly from the fact that the gene of interest is present (or should be) in every cell type from the single-cell embryo stage. This enables gene regulation to be studied in cell types which are not available in culture, or which are not easily transfected. Since the gene is subject to the chromatin changes (methylation as well as conformation) that accompany development and cellular differentiation prior to expression, rather than being presented to the cell as naked DNA, the influence of these factors can be studied. Also there is no need to introduce a second gene as a selectable marker (as is common in transfection experiments), a technique which selects for integration into an "active" region of host cell chromatin.

Although the advantages of using transgenic mice in the study of gene regulation are considerable, it is worthwhile to consider the disadvantages. The most obvious disadvantage to anyone wanting to set up such experiments is the cost in terms of time and of money. Given that equipment can be expensive and the time needed to train personnel, to produce and maintain animal stocks, and, once microinjections have been performed, to complete the breeding and screening for a full round of experiments, is a matter of months, if not years, these experiments require a great deal of time and effort, even before any results can be obtained. On a more fundamental note, it could be argued that transgenic mice are best used to study the developmental and chromatin organisational factors effecting gene expression, whereas promotor-enhancer interactions and local control regions can be studied in stable transfection systems, and promotor function be can satisfactorily investigated in *in vitro* experiments (Sippel *et al.*, 1992).

Technical aspects of transgene production with respect to expression have already been discussed (section 1.2) and reviewed (Brinster *et al.*, 1985; Hogan *et al.*, 1986; Palmiter and Brinster, 1986). There is usually little or no correlation between the number of copies of a transgene inserted into the embryo and the level of its expression in tissues. This can be explained in two ways: either the transgene

does not contain all the necessary regulatory regions, or there is a "positional effect" on expression due to the site at which integration has occurred (for review see Lima-de-Faria, 1983; Hogan *et al.*, 1986). In the case of the latter, it is thought that local chromatin structure, or the presence of regulatory elements proximal to the transgene, can have either a positive or negative effect on expression. It is possible to achieve position independence and copy-number dependence in transgenic mice, as exemplified by the β-globin gene (Grosveld *et al.*, 1987). A region of DNA 50-60 kbp upstream of the β-globin gene has been shown to confer these traits not only to β-globin, but to the α-globin gene (Hanscombe *et al.*, 1989; Ryan *et al.*, 1989), the *Thy*-1 gene and the Herpes virus thymidine kinase promotor (Blom van Assendelft *et al.*, 1989; Talbot *et al.*, 1989). The mechanisms by which such regions (now know as locus control regions) function and their relationship to other chromosomal functional domains has been discussed in detail elsewhere (Kollias and Grosveld, 1992; Sippel *et al.*, 1992) and will not be considered here.

1.4 Transgenic Mice in the Study of Mammary Gland Neoplasia

Transgenic mice offer a means to study the *in vivo* effects of oncogenes in a system which is not prone to many of the pitfalls of *in vitro* techniques involving tissue culture. Whilst the primary culture of cells from tissues does not rely on any abnormal growth properties which may contribute to a neoplastic phenotype, the same cannot be said to be true for the use of established cell-lines. Such cell-lines, merely by their ability to grow, divide and, in some cases, undergo changes similar to those seen in differentiation, outside of the normal environment of the body, must be considered to be altered in some way. Such alterations parallel those believed to be involved in tumorigenesis, and indeed cells which have undergone known tumorigenic transformation are often those most likely to be established in culture (be this transformation virally-induced, chemically-induced or spontaneous). Since tumorigenesis is believed to be a multi-step phenomenon (Weinberg, 1989), it cannot be ruled out that uncharacterised neoplastic factors contribute to studies utilising established cell-lines. Indeed, cell-lines in culture may carry additional transforming

activities to those used to establish them initially, since they can be genetically unstable (Hart and Easty, 1991). In the case of lines established from spontaneous lesions, even the primary transforming event is unknown. Nevertheless *in vitro* studies provide much of the initial evidence to implicate oncogenes in neoplastic disease by means of correlations between expression and phenotype, and can be used to investigate the effects of oncogene expression by transfection (Weinberg, 1989; Hart and Easty, 1991).

Whilst the transfection of oncogenes into cell lines suffers from the problems mentioned here, the insertion of oncogenes into mice does not, since the mice are not artificially altered in any other way. Unlike some other animal model systems, transgenic mice usually have an intact immune system and hence allow the study of immune system modulators in disease progression. Also, tumour formation in transgenic mice is not usually dependent on the administration of chemical agents whose non-specific pharmacological actions can have a bearing on experimental data analysis. Another advantage of using transgenic mice is that, unlike cell culture experiments, they can reveal the role of humoral or cell-mediated factors in development or oncogenic transformation. The genetic background of transgenic mice can lead to some variation in the effect of a particular oncogene, since some strains of mice are predisposed to certain types of tumour, but often this can be used to the advantage of the investigator.

The use of transgenic mice to study both the tissue-specific expression and effect of oncogenes has been widespread (for reviews see: Groner *et al.*, 1987; Hanahan, 1988, 1989; Pattengale *et al.*, 1989; Adams and Cory, 1991; Berns, 1991; Dickson *et al.*, 1991; Muller, 1991; Kioussis, 1992). By expressing oncogenes, under their own or other control promotors/enhancers, the role of these genetic elements in neoplastic disease has been investigated. Also the function of proto-oncogenes in the cell-cycle, cellular differentiation, transcription and embryonic development has been probed. The literature contains several excellent reviews on the use of transgenic animals to probe this area of intense interest (see

above). Here I shall concentrate on a few examples of oncogenesis in the mammary gland of transgenic mice, this being of prime interest given the implication that p9Ka expression in mammary epithelia may contribute to malignancy of breast tumours (Ebralidze *et al.*, 1989; Davies *et al.*, 1993), as discussed later.

The first observation of mammary neoplasia in transgenic mice was produced by expression of a c-*myc* transgene (Stewart *et al.*, 1984), and since that time many other transgenic mouse models of mammary tumorigenesis have been produced (as reviewed here). Mostly these arise from the expression of activated oncogenes, or growth factors, from promotors which predominantly (but not exclusively) express in mammary epithelia. The promotor elements used are usually mouse mammary tumour virus long terminal repeat (MMTV-LTR, more commonly referred to simply as MMTV promotor; Ross and Solter, 1985; Ross.*et al.*, 1990) or whey acidic protein promotor (WAP; Hobbs *et al.*, 1982). The range of oncogenes and growth factors used is quite wide: *myc* has been expressed under the control of both MMTV (Stewart *et al.*, 1984) and WAP promotors (Schöenenberger *et al.*, 1988); c-Ha-*ras* has been expressed under the control of the WAP promotor (Andres *et al.*, 1987); both v-Ha-*ras* (Sinn *et al.*, 1987; Tremblay *et al.*, 1989) and N-*ras* (Mangues *et al.*, 1990) have been expressed under the control of the MMTV promotor; as have *ret* (Iwamoto *et al.*, 1990), *wnt*-1 (*int*-1) (Tsukamoto *et al.*, 1988), *int*-2 (Muller *et al.*, 1990), *int*-3 (Jhappan *et al.*, 1992), TGF-α (Matsui *et al.*, 1990), human c-*erb*B2 (Suda *et al.*, 1990), activated, rat c-*erb*B2 (c-*neu*) (Muller *et al.*, 1988; Bouchard *et al.*, 1989; Lucchini *et al.*, 1992), SV40 large T antigen (Choi *et al.*, 1987) and polyoma virus middle T antigen (Guy *et al.*, 1992). Some strains of these mice exhibited abnormalities in their mammary glands ranging from hyperplasia, with relatively low tumour incidence, in MMTV-*int*-1 (Tsukamoto *et al.*, 1988), MMTV-*int*-2 (Muller *et al.*, 1990) and MMTV-TGF-α mice (Matsui *et al.*, 1990), spontaneous, stochastic development of mammary tumours of differing incidence and latency in MMTV-*ras* (Sinn *et al.*, 1987), MMTV-*myc* (Stewart *et al.*, 1984), Wap-*myc* (Schöenenberger *et al.*, 1988), Wap-*ras* (Andres *et al.*, 1987), MMTV-*int*-3 (Jhappan *et al.*, 1992), MMTV-*ret* (Iwamoto *et al.*,

1990), and MMTV-c-*erb*B2 mice (Bouchard *et al.*, 1989), to short latency, polyclonal tumours with almost 100% incidence in MMTV-c-*erb*B2 mice (Muller *et al.*, 1988; Lucchini *et al.*, 1992) and widespread transformation with rapid production of multifocal mammary adenocarcinomas followed by metastatic dissemination in MMTV-polyoma virus middle T antigen mice (Guy *et al.*, 1992).

The appearance of multifocal tumours of high incidence with both activated c-*erb*B2 and polyoma virus middle T antigen has been interpreted as suggesting single step oncogenesis, which is not in keeping with the generally accepted model of multi-step carcinogenesis (Muller *et al.*, 1988; Cardiff *et al.*, 1991; Guy *et al.*, 1992; Lucchini *et al.*, 1992). It has been suggested that both of these genes code for proteins which may play a multifunctional role through their involvement in signal transduction pathways (through tyrosine kinases) and that above a threshold level they can contribute to more than one oncogenic pathway (Guy *et al.*, 1992).

A lower level of transgene expression may be responsible for the disparate results obtained with a different MMTV-c-*erb*B2 construct by Bouchard *et al.* (1989). As opposed to multifocal tumours encompassing the whole of the mammary tissue and arising relatively early (Muller *et al.* , 1989; Lucchini *et al.*, 1992), these mice exhibited a stochastic tumour incidence with a later onset. One subtle difference between the two MMTV-c-*erb*B2 constructs may contribute to the differences seen in tumorigenesis (Lucchini *et al.*, 1992). The construct of Muller *et al.* (1989), also used by Lucchini *et al.* (1992), contains a 600 bp fragment of DNA derived from Harvey murine sarcoma virus, which separates the MMTV promotor from the c-*erb*B2 cDNA, and may give rise to a slightly longer transgene protein product, or, by some unknown mechanism, to enhanced tumorigenesis. [N.B. in Van de Vijver and Nusse (1991) the insert is erroneously described as being in the MMTV-c-*erb*B2 construct (Bouchard *et al.*, 1989) which gave the lower tumour incidence.] Alternatively, there may be additional factors involved, but not detected, in the "single-step" models of mammary tumorigenesis. These factors could be due to "insertional effects" (not likely since more than one strain, having ostensibly different integration loci, gave the same phenotype), due to the mouse strain used (different

genetic backgrounds contribute to differences in susceptibility to various cancers) or due to some other unknown mechanism.

Differences in tumorigenic potential of *myc* and *ras* oncogenes in different lines of transgenic mice can be seen using different promotors. The MMTV and WAP promotors operate under different conditions, and hence oncogene expression in the different lines occurs under different circumstances, which probably contribute to the tumour incidence and latency observed (Dickson *et al.*, 1991). Under control of an MMTV promotor, c-*myc* gave rise to tumours in multiparous females at 5-6 months of age, in connection with their second or third pregnancies (Stewart *et al.*, 1984); whereas, when driven by a WAP promotor, a higher incidence of tumours (80%) was seen at 2-3 months after initial onset of lactation (Schöenenberger *et al.*, 1988). The MMTV-LTR-driven expression of v-Ha-*ras* gave rise to a high incidence of tumours, even in virgin mice (Sinn *et al.*, 1987; Tremblay *et al.*, 1989). Multifocal, stochastic mammary adenocarcinomas were seen as early as 1 month and in 10/13 founder lines by Sinn *et al.* (1987) and at 4-10 months in all four founder lines by Tremblay *et al.* (1989). Under control of the WAP promotor c-Ha-*ras* was less potent, with mammary tumours seen in only 1% of mice and with a latency of approximately one year (Andres *et al.*, 1987). This low tumour incidence in WAP-ras transgenic mice has been attributed more recently to low expression of the oncogene, since a higher tumour incidence (72%) was seen in a mouse line exhibiting a higher *ras* expression (Andres *et al.*, 1991). A higher incidence of mammary tumorigenesis was also seen in progeny of the low-expressing WAP-*ras* mice (Andres *et al.*, 1991) produced by mating with transgenic mice expressing increased levels of human growth hormone (hGH; Andres *et al.*, 1991; Bchini *et al.*, 1991). Expression of growth hormone in these mice caused inhibition of mammary gland regression, and this was suggested as the event which contributed to the induced transformation in these mice (Andres *et al.*, 1991).

In keeping with the multi-step model of tumorigenesis, the appearance of

tumours in the *myc* and *ras* transgenic mice is always stochastic, and, even when these lines of mice are mated to give doubly transgenic (bitransgenic) animals, the tumours still occur monoclonally and randomly (Sinn *et al.*, 1987; Andres *et al.*, 1988). However, the tumorigenicity of the bitransgenic animals is generally greater than that of the original *ras* or *myc* transgenic mice (Sinn *et al.*, 1987; Andres *et al.*, 1988). In the case of the MMTV-LTR-driven transgenes, tumour incidence occurred at a rate synergistic with respect to the rate of incidence with either transgene alone (Sinn *et al.*, 1987). Although synergistic effects on differentiation, and a high incidence of neoplastic foci, were observed in bitransgenic WAP-*myc*/WAP-*ras* mice (Andres *et al.*, 1988), the actual incidence or rate of tumour formation could not be said to be significantly different from the WAP-*myc* transgenic mice. This is perhaps not surprising, when it is noted that the WAP-*ras* transgenic mice used have the lowest incidence and longest latency of the *myc* or *ras* lines mentioned here (Andres *et al.*, 1987).

As well as these bitransgenic mice, there are reports of other bitransgenic mice (*ras*/c-*erb*B2 and *myc*/c-*erb*B2) and "tritransgenic" mice (*myc*/*ras*/c-*erb*B2) in which all oncogenes are expressed under the control of the MMTV promoter (Cardiff *et al.*, 1991). The tritransgenic animals had a 100% incidence and the shortest latent period; the *myc*/c-*erb*B2 mice had a higher incidence and shorter latent period than the *myc* or *ras* but not the c-*erb*B2 animals; but, the *ras*/c-*erb*B2 mice had a reduced incidence and longer latent period than either individual transgenic line. It should be pointed out that the c-*erb*B2 line used in this study is the one which gives a high and polyclonal incidence of neoplasia (Muller *et al.*, 1988). Another study carried out by the same group provided evidence that tumour phenotype could predict genotype. Despite not conforming to standard nomenclatures, the type of tumours produced in each line of mice were apparently related to the (single) transgene present (Cardiff *et al.*, 1991)

As well as the activated *ras* oncogenes already mentioned, the proto-oncogene was also used in transgenic mouse studies of breast neoplasia. The MMTV-LTR-directed over-expression of the N-*ras* proto-oncogene gives rise (stochastically) to

tumours not only in the mammary gland but also in Harderian glands, salivary glands and small intestine (Mangues *et al.*, 1992). These tumours, which are apparently invasive locally and capable of forming distant metastases, were originally thought to be due to the expression of activated N-*ras*, mutated at codon 61 (Mangues *et al.*, 1990).

The *wnt*-1 (*int*-1), *int*-2 and *int*-3 oncogenes are related by virtue of being transcriptionally activated by the insertional mutagenesis of MMTV upon viral induction of tumours in mice (Nusse, 1988). While their relevance in human breast disease is questionable, they do provide some insight into tumorigenesis and have been expressed in transgenic mice either individually (Tsukamoto *et al.*, 1988; Muller *et al.*, 1990; Jhappan *et al.*, 1992) or together (Kwan *et al.*, 1992) under the control of the MMTV promoter. The expression of *wnt*-1 is accompanied by mammary gland hyperplasia and a relatively high incidence of neoplasia in both male and female transgenic mice, but the kinetics of this incidence suggests other events are required for the development of mammary tumours in these mice (Tsukamoto *et al.*, 1988). In these mice the MMTV-LTR is used in the opposite transcriptional orientation to the *wnt*-1 gene, in order to better mimic the naturally occurring allele from an MMTV-induced tumour (Tsukamoto *et al.*, 1988). The developmental abnormalities induced by *wnt*-1 are apparently limited to those (transgenic) mammary epithelial cells which exhibit expression, and are independent of the action of endocrine growth factors (Lin *et al.*, 1992). Whilst *int*-2 transgenic mice develop mammary gland hyperplasia, this is only seen in breeding females, and is only rarely accompanied by neoplasia with a long latent period (Muller *et al.*, 1990). As with *wnt*-1, the effects of *int*-2 expression are apparently only autocrine or "ultra-short-range" (Ornitz *et al.*, 1992). Expression of both *wnt*-1 and *int*-2 oncogenes leads to an enhanced level of tumour incidence and a shorter latent period, most notably in the male mice (Kwan *et al.*, 1992). The expression of *int*-3 in transgenic mice not only causes an increased incidence of mammary and salivary gland adenocarcinomas, but also leads to retardation of alveolar-lobular development and fat-pad penetration of the mammary gland (Jhappan *et al.*, 1992).

The mitogenic peptide transforming growth factor α (TGFα) not only gives rise to mammary hyperplasia, and to a lesser extent neoplasia, when expressed under control of the MMTV promotor (Matsui *et al.*, 1990; Halter *et al.*, 1992), but exhibits some effects on mammary gland development and neoplasia when expressed under the control of a metallothionein promotor (Jhappan *et al.*, 1990; Sandgren *et al.*, 1990).

The reported mammary adenocarcinomas in MMTV-*ret* were accompanied by salivary gland tumours, and in both cases development was stochastic and closely related to high levels of transgene expression (Iwamoto *et al.*, 1990).

Mammary adenocarcinomas have also been reported in mice expressing increased levels of growth hormone (Törnell *et al.*, 1991). Female transgenic mice, which contained a hGH genomic sequence driven by a mouse metallothionein I promotor, and which exhibited elevated plasma levels of hGH, were predisposed to a high incidence of papillary adenocarcinomas of mammary origin (Törnell *et al.*, 1991). Interestingly, the hGH expressing mice used by Andres *et al.* (1991) to investigate the role of post-lactational regression in *ras*-induced transformation were not reported to suffer from mammary tumours (Bchini *et al.*, 1991). However, it would seem that the dramatic increase in tumorigenicity of low-level *ras* expression in the bitransgenic (*ras*/hGH) mice (Andres *et al.*, 1991) is, in-part, due to the actions of hGH. Growth hormone is firmly established as being of fundamental importance in normal somatic cell growth. These results would seem to confirm that over-expression of growth hormone can predispose the mice to tumour formation, and support the idea that growth hormone may play a role in pathological growth (i.e. neoplasia). In relating this mouse model to the human disease, it is worth noting that forty percent of breast cancer patients have elevated hGH levels (Emerman *et al.*, 1985).

The role of recessive oncogenes in breast cancer has not been investigated thoroughly in transgenic mouse models. Mice have been produced, by homologous recombination in embryonic stem cells, which are totally deficient for p53

(Donehower *et al.*, 1992) but these succumb to a diverse range of neoplasms (including one case of mammary adenocarcinoma) and cannot easily be used to study mammary oncogenesis.

Whilst the MMTV-LTR has been used to direct expression of oncogenes to the mammary gland with the aim of producing mammary tumours (see above), developmental and (or) neoplastic abnormalities are not always obvious in mice bearing MMTV-LTR-hybrid transgenes. Mice transgenic for MMTV-LTR-driven normal human c-*erb*B2, as opposed to activated, rat c-*erb*B2, displayed no evidence of mammary neoplasia (Suda *et al.*, 1990). However, these mice did suffer from a variety of other tumours including lung and Harderian gland adenocarcinomas and lymphomas (Suda *et al.*, 1990). Since no attempt was made to assess the expression of human c-*erb*B2 in the mammary gland of these transgenic mice, it is not clear whether the absence of a recognised phenotype is due to low levels of transgene expression, or to other factors (e.g. an inability of the human c-*erb*B2 receptor to bind the rat ligand). The SV40 large T gene, under control of the MMTV-LTR is reported to give rise to adenocarcinomas of the lung and kidney, but only rarely of the mammary gland (Choi *et al.*, 1987).

As already highlighted for N-*ras*, *int*-3, *ret*, non-mutated c-*erb*B2 and SV40 large T antigen, the MMTV-LTR-driven expression of oncogenes is not limited to mammary epithelium and neither is the tumorigenic effect. That is to say that expression of the oncogenes was seen in other tissues, and some of these tissues exhibited an increased tumour incidence. Other examples of adverse pathology at sites other than the mammary gland include expression of activated-*ras* leading to hyperplasia of the Harderian gland (Sinn *et al.*, 1987; Tremblay *et al.*, 1989; White *et al.*, 1990) and salivary gland tumours (Nielsen *et al.*, 1991) and deregulated c-*myc* expression leading to tumours of testicular, lymphocytic (B-cell and T-cell) and mast cell origin (Leder *et al.*, 1986). The various MMTV-c-*erb*B2 mice produced had a range of adverse pathologies from hypertrophy of salivary gland and epididymis (Muller *et al.*, 1988) and epithelial hyperplasia of epididymis, seminal vesicles and salivary gland, with occasional salivary gland tumours (Bouchard *et*

al., 1989), to multifocal tumours of Harderian gland, salivary gland and epididymis (Lucchini *et al.*, 1992). Whilst both expression and adverse pathology were seen in tissues other than the mammary gland, the range of tissues which expressed some level of oncogenic transgene was somewhat greater than that in which neoplasia was observed. This suggests that the action of the oncogenes used relies somewhat on the nature of the tissue in which they are expressed.

Whilst the use of transgenic mice as *in vivo* models of tumorigenesis have distinct advantages over *in vitro* models which rely on tissue culture, there are many questions left unanswered or unaddressed. As well as the question of what additional events are involved in cases of stochastic tumour incidence, it remains unclear why some oncogenes, in the hands of certain investigators, apparently lead to one-step induction of tumours. The question of the same oncogene giving rise to different effects dependent on the use of different promotor elements (believed to be due to the different temporal, developmental and hormone dependence of the promotors) has been only partially resolved. The identification of a better mammary-specific promotor with inducible expression may go some way to answering the questions raised. Since the temporal and developmental expression of oncogenes seems to play a role in the hyperproliferative or neoplastic effects of the oncogene, it may be better to express potential oncogenes under the same conditions as in the human disease. Whilst this is in itself unclear, one approach is merely to mimic amplification (as seen with some oncogenes) by inserting multiple copies of potential oncogenes under their own expression control.

Perhaps the most important question to ask is what relevance the various transgenic mouse models have to human breast cancer. Transgenic mouse models which are based on clinically important oncogenes will be the most useful in testing drugs against not only primary breast disease, but also against the malignant spread of the disease. However, the transgenic mouse models produced so far do not always relate well to the human disease.

Whilst the c-*erb*B2 gene can be seen to be amplified in up to 20% of human tumours, and this change is implicated in poor prognosis of patients with mammary

lesions, the gene is not mutated (Callahan and Campbell, 1989). Conversely, a role of mutated c-*erb*B2 in mammary neoplasia of transgenic mouse models is obviously strong (Muller *et al.*, 1988; Bouchard *et al.*, 1989), but no evidence of mammary neoplasia was seen in similar mice harbouring the non-mutated version of the human gene (Suda *et al.*, 1990). The *wnt*-1, *int*-2 *int*-3 and polyoma virus middle T antigen models of mammary neoplasia would seem to be predominantly relevant to rodents, since the role of viral activation of these oncogenes, or direct oncogenesis by viral antigens, as major contributing factors in human breast cancer is uncertain (Callahan, 1987; Dalgleish, 1991; Van de Vijver and Nusse, 1991). Similarly, the role of the *ret* oncogene in human breast cancer is not well understood. Consistent with the results in transgenic mice (Halter *et al.*, 1992), the role of TGFα in human breast is apparently limited to normal development and relatively early stages of tumorigenesis (Dickson *et al.*, 1992). Better candidates for models of the human disease are provided by c-*myc* and the *ras* oncogenes. C-*myc* can be seen to be amplified in up to 30% of human tumours, a change which is implicated in poor prognosis (Callahan and Campbell, 1989), and the expression of this gene in the mouse leads to mammary neoplasia (Stewart *et al.*, 1984; Schöenenberger *et al.*, 1988). There is some evidence, albeit controversial, for involvement of *ras* in human breast neoplasia (Slamon *et al.*, 1984; Callahan, 1987) and this oncogene would seem to be important in carcinogen-induced rodent models (Russo and Russo, 1987) or transgenic mice (Andres *et al.*, 1987; Sinn *et al.*, 1987; Tremblay *et al.*, 1989; Mangues *et al.*, 1992). However, from the stochastic nature of tumour incidence in *myc* and *ras* transgenic mice, or in bitransgenic mice (Sinn *et al.*, 1987; Andres *et al.*, 1988), it is obvious that other changes are involved and these are as yet unidentified. The relative relevance of the transgenic breast cancer models so far produced, and of future models, will depend somewhat on the interpretation of clinical data with regard to oncogenes involved in breast cancer and metastasis.

Despite the many valuable insights into mammary development and neoplastic disease that the various mammary-based transgenic mouse models have provided,

there is little evidence in the literature of the utility of these mice in testing potential anticancer drugs. Recently, one line of Wap-*ras* transgenic mice harbouring the transgene on their Y-chromosome, and exhibiting mammary gland and salivary gland tumours in male mice (Nielsen *et al.*, 1991), have been used to study the effects of several clinically relevant cytotoxins (Nielsen *et al.*, 1992). The results of these experiments apparently indicate that transgenic mice will indeed be relevant to the discovery of novel therapeutics.

1.5 Transgenic Mice in the Study of Metastasis of Breast Cancer

Many lines of mice exhibiting increased incidence of breast neoplasia have been reported (as reviewed above), but few specifically mention what incidence of metastatic disease was seen. This is understandable, since the chief use of these mice was in trying to understand the nature of the primary disease. Nevertheless, it is the metastatic spread of breast cancer which causes most deaths from this disease, and an animal model which can be used to address this phenomenon would be useful. Whilst such a model has recently been produced by using a MMTV-polyoma virus middle T antigen transgene (Guy *et al.*, 1992), it cannot be considered to mimic fully the human disease. Not only is neoplasia and progression apparently induced in a single step, but the causative agent is not one which is considered to be important in the human disease. Whilst providing some interesting insights into tumour progression, such mice cannot be considered to form a sensible basis for drug design or evaluation. The metastatic spread reported in mice over-expressing the N-ras oncogene (Mangues *et al.*, 1992) is also unlikely to form a sensible assay system, since the metastases are derived from numerous different tumour types and are highly sporadic.

It is likely that a better model of human breast disease with metastatic spread will be the product of many years of hard work. Current wisdom dictates that such a model would arise from the production of mice carrying multiple oncogenes. Not only should the oncogenes used be shown to play a causative role in either initiation, promotion or progression in the human disease but also their expression should

mimic that observed in human tumours. It is not necessarily the case that all contributing oncogenes should be subject to targeted expression, and it may be possible to limit targeted expression to the primary initiating oncogene(s). Since tumours arise clonally, it may be better to initiate tumours in a clonal manner, but at present clonal expression of transgenes is not possible. Nevertheless, the stochastic nature of tumours in transgenic mice (presumably due to mutational events in other oncogenes) does allow the study of tumours which arise in a manner not dissimilar to those thought to occur in the human disease.

1.6 Genes Involved in Metastasis

The complex nature of metastasis, and the diverse properties that metastatic cells require to form tumours at secondary sites, suggest that as well as the classical oncogenes involved in tumorigenesis through growth control, additional classes of "metastatic oncogenes" are involved (Fidler *et al.*, 1978; Evans, 1991; Fidler, 1991; Liotta *et al.*, 1991; Steeg, 1992). These metastatic oncogenes may be involved in cell motility, cell adhesion or proteolysis, all of which are necessary, along with proliferation, for invasion, dissemination and secondary growth of malignant tumours. Control of these functions, via cellular communication, is important, and the signal transduction mechanisms involved are possible targets for the action of other "metastatic oncogenes".

Various genes with known functions in cell motility (e.g. autocrine motility factor and scatter factor), cell adhesion (e.g. E-cadherin, CD44 and the integrins) or proteolysis (e.g. cathepsin D, urokinase-type plasminogen activator and matrix metalloproteinases) have been implicated in metastasis, as have some of the factors involved in controlling these processes (Fidler *et al.*, 1978; Fidler, 1991; Liotta *et al.*, 1991; Steeg, 1992). Changes in such genes may be more important in metastasis than the classical oncogenes. Other genes with no defined function have also been implicated in progression of particular tumours to a metastatic phenotype and one such gene is that for p9Ka. A historical perspective of p9Ka, evidence of a role for p9Ka in metastasis of mammary tumours, and other reasons for wanting to

express rat p9Ka in transgenic mice are given here.

1.7 Identification and Characterisation of p9Ka

The isolation, characterisation, structure, and expression of p9Ka has recently been reviewed (Barraclough and Rudland, 1991) and only relevant data will be summarised here.

The rat protein p9Ka was first identified using two-dimensional polyacrylamide gel electrophoresis as being differentially expressed in two rat mammary cell lines (Barraclough *et al.*, 1982). The epithelial cell line isolated from a benign rat mammary tumour, Rama 25 (Bennett *et al.*, 1978), yielded elongated cells at low frequency (1-3 %) and one cell line derived from these elongated cells was designated Rama 29 (Bennett *et al.*, 1978). Examination of the protein expression patterns of these cell lines, on 2-dimensional gels, revealed an acidic, low-molecular-weight protein which was expressed at relatively high levels in the elongated cell line (Rama 29) but virtually absent from the parental epithelial Rama 25 cells (Barraclough *et al.*, 1982). The protein had an apparent molecular weight of 9.0 ± 0.5 kDa and an acidic isoelectric point of 5.5 ± 0.3 (Barraclough *et al.*, 1984a) and was thus called p9Ka.

The elongated cell line Rama 29 has been characterised as being myoepithelial-like in terms of ultrastructure and immunocytochemical staining with defined myoepithelial cell markers, by comparison with primary mammary cell cultures (Warburton *et al.*, 1985) and rat mammary gland (Ormerod and Rudland, 1982; Warburton *et al.*, 1982). The myoepithelial cells are those which surround the epithelial cells lining the mammary ducts and are characterised by their smooth muscle-like myofilaments and basement membrane on their basal surfaces (Hughes and Rudland, 1990). Myoepithelial-like cells can also be seen to be derived from epithelial cells of mouse mammary tumours (Sanford *et al.*, 1961; Dexter *et al.*, 1978; Hager *et al.*, 1981) and normal rat mammary glands (Ormerod and Rudland, 1985). Although p9Ka expression is seen to increase as the epithelial cells progress

towards a myoepithelial cell type, and is approximately 16-fold higher in the myoepithelial-like cells than in epithelial cells (Barraclough *et al.*, 1984a), it is not clear whether p9Ka plays a part in this *in vitro* "differentiation" or is merely a result of it (Barraclough and Rudland, 1989).

The cDNA corresponding to p9Ka mRNA has been cloned (Barraclough *et al.*, 1984b) and used, in Northern blot analysis, to investigate the expression of the p9Ka mRNA in different cell lines and in selected rat tissues. The expression of p9Ka mRNA follows the same pattern as the protein in the mammary cell lines studied, and at levels consistent with the p9Ka expression being predominantly transcriptionally regulated (Barraclough *et al.*, 1984b; Barraclough and Rudland, 1989). The expression pattern of p9Ka mRNA in normal rat tissues has not been extensively studied, but p9Ka mRNA was expressed in mammary gland, spleen and uterus with variable levels in the liver (Barraclough and Rudland, 1991).

The p9Ka cDNA (Barraclough *et al.*, 1984b) was used to screen a genomic library and obtain an 18 kbp rat DNA clone containing the p9Ka gene (Barraclough *et al.*, 1987). The coding regions of the p9Ka gene were seen to be located on two sub-clones of 5.0 and 5.3 kbp which were produced from the initial 18 kbp clone of rat genomic DNA by digestion with *Eco*RI (Barraclough *et al.*, 1987). Although initially it was believed that the p9Ka gene consisted of two coding exons and a single intron (Barraclough *et al.*, 1987), it has more recently come to light that the gene also includes an additional intron and non-coding 5´ exon (Barraclough and Rudland, 1991). Hence the gene consists of two introns (1172 bp and 675 bp) which exhibit intron/exon boundaries conforming to the consensus (Mount, 1982), and three exons (a short 37 bp exon which is untranslated and two protein-coding exons of 156 bp and 295 bp)(Barraclough and Rudland, 1991). The gene has a TATAAA sequence 32 bp upstream of the transcription start-site, which probably acts as a TATA box (Breathnach and Chambon, 1981), but contains no clearly-defined CAAT-like sequence (Benoist *et al.*, 1980).

The protein sequence derived from the open reading frame of the p9Ka gene (Barraclough *et al.*, 1987) is a 101 amino acid protein which is homologous to a

family of S-100 and S-100-related proteins (Barraclough *et al.*, 1987; Kligman and Hilt, 1988; Heizmann and Hunziker, 1990). Such proteins are present in a variety of eukaryotic cells and consist of a polypeptide chain which contains two EF-hand-like potential calcium-binding sites (Kligman and Hilt, 1988; Heizmann and Hunziker, 1990). The protein sequence of p9Ka conforms well to the general structure of this protein family (Barraclough *et al.*, 1987; Barraclough and Rudland, 1991) in having two helix-loop-helix (EF-hand) regions separated by a linker and followed by a C-terminal "tail". The gene structure of p9Ka is essentially the same as that described for other S-100-related calcium-binding proteins, such as human calcyclin (Ferrari *et al.*, 1987), MRP-8 and MRP-14 (Lagasse and Clerc, 1988). All these genes have a short, 5´, non-coding exon and two coding exons, each of which contains the coding region for a single EF-hand (Barraclough and Rudland, 1991).

The EF-hand motifs of p9Ka conform well to the EF-hand test of Moews and Kretsinger (1975), and are highly homologous to those described for carp parvalbumin (Kretsinger, 1980) and bovine, intestinal calcium-binding protein (Szebenyi *et al.*, 1981; Szebenyi and Moffat, 1986). Homologies with known calcium-binding proteins are predominantly within the calcium-binding loops of these motifs, particularly for the C-terminal EF-hand of p9Ka (Barraclough *et al.*, 1987). In this case, 11 out of 14 amino acids are identical between p9Ka and bovine, intestinal calcium-binding protein (Barraclough *et al.*, 1987), including the five residues containing carboxylic acid derivatives believed to be involved in calcium-ion binding (Szebenyi and Moffat, 1986). Although not all members of the calcium-binding family bind calcium ions [e.g. the annexin-binding protein p11 (calpactin light-chain) does not; Klee, 1988], p9Ka has been shown to do so (Barraclough *et al.*, 1990). From the structure of the N-terminal EF-hand it would be expected that this site is less likely to bind calcium ions, and this may explain why apparently only a single, high affinity binding site has been shown for the p9Ka protein (Barraclough and Rudland, 1991). It may be that calcium-binding at the N-terminal site is of low affinity and not detected in the assay used, as would seem to be the case for calcyclin (Kuzniki and Filipek, 1987).

It is possible that p9Ka and other S-100-related proteins act as transducers of stimulatory signals in the calcium second-messenger system. Whilst many of the reported calcium affinities are not in the physiological range of the calcium signal, the calcium-binding activity of some of these proteins apparently varies according to whether they are in a dimeric or monomeric form (the dimers having a greater affinity). It is also possible that subcellular distribution of the proteins and subcellular variations in the calcium signal allow this family of proteins to participate in signal transduction. It is clear that some physiochemical properties are altered when these proteins bind calcium and these changes expose hydrophobic domains of the protein to the solvent (Baudier and Gerard, 1983; Moore, 1988). Such changes may be involved in the interaction of S-100 and S-100-related proteins with their target molecules.

Although it was originally observed that epithelial cell-lines derived from normal rat mammary glands (Ormerod & Rudland, 1985) or benign mammary tumours (Bennett *et al.*, 1978) fail to express p9Ka mRNA or protein (Barraclough *et al.*, 1982; Barraclough *et al.*, 1984b), one rat mammary epithelial cell line, Rama 800 (Dunnington *et al.*, 1984) expressed p9Ka mRNA and protein at high levels. Interestingly, the Rama 800 cell-line, but not other mammary epithelial cell lines studied, have a high metastatic potential (Dunnington *et al.*, 1984). This expression of p9Ka in a metastatic mammary epithelial cell-line was later corroborated by studies on the mouse homologue of p9Ka (*mts*1, see below) which indicated that an increased expression of p9Ka in mammary epithelial cells correlates with metastatic potential (Ebralidze *et al.*,1989).

1.8 p9Ka mRNA or Protein Homologues Isolated from Different Sources

The protein, or more commonly the cDNA, first identified as p9Ka has been isolated by a number of laboratories and has subsequently been given several different names.

An mRNA named 18A2, which codes for the murine homologue of p9Ka, was

identified due to its increased abundance in quiescent mouse fibroblasts which had been stimulated to grow by addition of serum to their culture medium (Jackson-Grusby *et al.*, 1987). This mRNA was expressed in the uterus of non-pregnant mice and in placenta (Jackson-Grusby *et al.*, 1987). The placental expression has given rise to the suggestion that 18A2 (p9Ka) be renamed *Capl* (for *ca*lcium-binding protein of *pl*acenta); a suggestion which, given the broad range of postulated expression patterns and activities, this author does not consider to be appropriate. The gene for 18A2 has been mapped to the same region of chromosome 3 in the mouse as p11, and shown to be within 8 kbp of the gene for calcyclin (Dorin *et al.*, 1990). In the human genome all these genes have been mapped to chromosome 1q21-25 (Ferrari *et al.*, 1987; Dorin *et al.*, 1990). Interestingly, structural and numerical changes in chromosome 1 have been found in human carcinomas (Kakiti *et al.*, 1977; Sozzi *et al.*, 1990) and may be involved in neoplastic progression (Atkin and Pickthall, 1977).

The mRNA for the mouse homologue of p9Ka has also been identified due to its increased abundance in established mouse cell lines (Balb/c3T3), when compared to primary cultures of mouse embryo fibroblasts (Goto *et al.*, 1988), and was named pEL98. The amount of this transcript was also seen to be increased in NIH3T3 transformed with the activated Ha-*ras* gene and in C3H10T1/2 cells transformed with methylcholanthrene. A homologous mRNA was present in two human cancer lines (human stomach cancer cells and human cervical cancer cells) but not in a third cell-line (human renal cancer cells) (Goto *et al.*, 1988). Several mouse tissues (brain, small intestine, kidney and liver from a 5 week old Balb/c mouse) apparently showed no detectable levels of expression (Goto *et al.*, 1988).

The rat mRNA designated 42A (Masiakowski and Shooter, 1988) is the same as p9Ka and was identified due its induced expression when a pheochromocytoma cell line (PC12) was stimulated with nerve growth factor (NGF). Treatment of this cell line with NGF induces neuronal differentiation, cessation of cell division and expression of not only p9Ka (42A) but an mRNA termed 42C (which is apparently the rat homologue of p11) (Masiakowski and Shooter, 1988).

Perhaps the most interesting differential expression pattern seen for p9Ka, with regard to this thesis, was that described for the mouse mRNA homologue *mts*1 (Ebralidze *et al.*,1989). A differential screening of mRNA expression in two closely-related mouse mammary carcinoma cell lines, independently isolated from the same spontaneous tumour, revealed that *mts*1 expression was associated with the metastatic phenotype. The *mts*1 transcript was seen to be more abundant in the metastatic variant (giving rise to a 100% incidence of lung metastasis when inoculated into mice) than in the variant with low metastatic potential (less than 10% incidence in the same assay), and a cell-line of intermediate metastatic potential expressed an intermediate level of *mts*1 mRNA (Ebralidze *et al.*,1989). The single copy gene for *mts*1 has been cloned (Tulchinsky *et al.*,1990) and has the same structure as that described for the rat p9Ka gene (see above). Cross-hybridising genes were found in pig, chicken and human as well as rat, by Southern blot hybridisation with an *mts*1 cDNA probe (Ebralidze *et al.*,1989). The *mts*1 (p9Ka) mRNA has also been shown to be more highly expressed in a series of human tumours (four melanomas, three sarcomas and one adrenal carcinoma) than in their non-metastatic counterparts (Ebralidze *et al.*,1990). In normal mouse tissues *mts*1 mRNA is reportedly only expressed in lymphoid organs (spleen, thymus and bone marrow) and blood-stream lymphocytes (Ebralidze *et al.*,1989).

The most recent observation of rat p9Ka expression was in rat kidney cells, shown to express increased levels of a 10 kDa protein, identified as p9Ka/42A, upon viral-induced transformation (De Vouge and Mukarjee, 1992). The v-K-*ras* gene of Kirsten murine sarcoma virus (Ellis *et al.*, 1981) induced the expression of several proteins including p9Ka, when it was used to transform normal rat kidney cells (De Vouge and Mukarjee, 1992). Thus the presence of p9Ka not only correlates with metastatic potential of mammary epithelial cells but also with the oncogenic transformation of kidney cells.

The most recent description of a p9Ka homologue is that of calvasculin (Watanabe *et al.*,1992a). This protein was isolated from an extract of bovine aorta by column chromatography with column-bound isoquinolinesulfonamide (W-66), a

vasorelaxant which also acts as a calmodulin-antagonist (Watanabe *et al.*,1992a). Using an antibody raised to this bovine p9Ka homologue, expression of calvasculin was detected in bovine aorta but not in bovine brain, lung, heart or testis. Further work seems to indicate that this protein can be released from smooth muscle cells in culture, and is associated extracellularly with a 36 kDa myofilament protein (Watanabe *et al.*,1992b).

Whilst all the homologues described here would seem to be essentially the same protein/mRNA/gene (Barraclough and Rudland, 1991; Hilt and Kligman, 1991), it is interesting that different groups have described expression in different tissues and under different circumstances. This may be due to limitations of the techniques employed, of the tissues studied, or of the interest of any one research group. In the case of different expression levels, and expression patterns, in different species it may be that these differences are real; if this is so then the apparent difference in function as inferred from the different expression pattern may be interesting.

1.9 A Role for p9Ka in Neoplastic Progression

Whilst the S-100 family of proteins and p9Ka itself seem to be expressed in a wide range of circumstances, and may play a part in diverse systems, the most intriguing role for p9Ka is that in malignant progression of breast cancer. In terms of trying to define a role for p9Ka, it is not hard to see why a possible role in neoplastic progression has been put forward. Given the possibility that p9Ka could be involved in signal transduction, it is plausible that in a number (if not all) of the varied conditions in which p9Ka is expressed, it may be more than just a passive component. Certainly calmodulin, another calcium-binding protein, plays a regulatory role in many diverse systems and is encountered in increased amounts in tumour cells (Means *et al.*, 1982; Cohen and Klee, 1988). As with p9Ka in mammary epithelial cells, expression of the S-100-related protein calcyclin (Calbretta *et al.*, 1986) has been correlated with metastatic behaviour of human melanoma cells

in nude mice (Weterman *et al.*, 1992). Nevertheless, it is not satisfactory to rely merely on correlations when postulating a role for p9Ka in neoplasia or metastasis. No firm conclusions can be drawn about a role for p9Ka without first performing a "transfection" experiment in order to address any causative role.

One approach which was used in our laboratory (Barry Davies, Ph.D. student; concurrent with the work described here), was to transfect the p9Ka gene into a benign rat mammary epithelial cell line, and introduce those cells into the mammary fat pad of completely syngeneic rats in order to assay for metastatic potential (Davies *et al.*, 1993). This work yielded some interesting results that would suggest that p9Ka can act as an progression factor in establishment of the metastatic phenotype. Non-transfected cells or cells transfected with control DNA both yielded primary tumours upon introduction into the mammary fat pad, but no metastatic spread was observed. The transfection of a construct containing the p9Ka gene yielded cells which exhibited a higher incidence of tumours, with a shorter mean latent period and a significant level of metastatic spread to the lymph nodes and lungs of the syngeneic host. These results, together with unpublished observations from similar experiments, will be considered in greater detail in the final discussion section (section 4).

Although providing good evidence of a causative role for p9Ka in metastasis, the experiments using benign cell lines and syngeneic rats represent a system in which other known and unknown factors may play a part in the final metastatic phenotype. It would therefore be attractive to transfect p9Ka into a system which does not possess any known innate neoplastic predisposition and observe the effects of p9Ka expression.

1.10 Aims of Expressing the Rat p9Ka Gene in Transgenic Mice

There are two principle aims in investigating the expression of the rat p9Ka gene in transgenic mice. These are to address questions arising from the apparently disparate expression of p9Ka between species and to study the effect of p9Ka expression on mammary neoplasia. As discussed, transgenic mice provide a means

to perform both such experiments

A great deal of work has been performed using transgenic mice to study gene expression and often this work makes use of reporter genes. In the case of p9Ka, since the same mice were to be used to assay the effect of gene expression, and possibly any pathological effect of over-expression, it was decided not to use a reporter gene but simply to observe p9Ka expression. Given possible differences in mouse and rat p9Ka gene expression patterns, it should prove interesting to determine whether these differences are due to local cis-acting controls, or to more complicated cis- and trans-acting elements.

Transgenic mice have provided a means of studying mammary gland neoplasia and a way to model breast cancer *in vivo*. It is hoped that the expression of p9Ka under its own expression control, but amplified due to the presence of multiple gene copies, may contribute to such a model, especially with respect to metastasis.

2. Methods

2.1 Manipulation of DNA

2.1.1 Extraction and Precipitation of DNA

Methods were adapted from those of Sambrook *et al.* (1989). The DNA solution to be extracted / precipitated was made up to a suitable volume (0.2 or 0.5 ml) with T.E. buffer (1 mM EDTA, 10 mM Tris.Cl, pH 8.0) and an equal volume of phenol / chloroform / isoamyl alcohol (25:24:1 v/v) added. Following a brief vortex, the tube was allowed to stand for 1 minute then spun in microcentrifuge at full speed (12,000 g_{max}) for 1 minute. The top (aqueous) layer was removed carefully to a fresh tube. (A second phenol / chloroform / isoamyl alcohol extraction was similarly performed if deemed necessary.) To the aqueous layer, an equal volume of chloroform / isoamyl alcohol (24:1 v/v) was added, the tube vortexed and centrifuged for 1 minute. Again the aqueous layer was removed to a fresh tube and one-tenth volume of 3M sodium acetate (pH 5.5) and 2 volumes of redistilled ethanol were added. After a brief vortex, the tube was incubated at -70°C for 20 minutes or at -20°C for 1 hour followed by a 20-minute centrifugation at full speed in a microcentrifuge (at 4°C if possible). The supernatant was removed and the pellet washed with 70% (v/v) ethanol then dried and redissolved in an appropriate volume of the required buffer.

2.1.2 Digestion of DNA with Restriction Enzymes

Restriction enzyme-digests were performed in conditions recommended by the manufacturers using buffers provided with the enzymes. Digestion was stopped by heat treatment (according to manufacturers' recommendations, if appropriate), by adding EDTA to a final concentration of 25mM, by addition of one-fifth volume of agarose-gel-loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 30% v/v glycerol in H_20) or by addition of 0.2 ml T.E. buffer (1 mM EDTA, 10 mM Tris.Cl, pH 8.0) followed by phenol / chloroform extraction (see above).

2.1.3 Treatment of DNA with Alkaline Phosphatase

Methods were adapted from those of Sambrook *et al.* (1989). The 5' terminal phosphate groups of restriction-enzyme-digested and heat-treated DNA were removed with alkaline phosphatase by addition of 1 unit (1 µl) calf intestinal alkaline phosphatase (Boehringer) per 20 µl reaction volume and incubation at 37°C for 30 minutes. Alternatively, DNA was extracted with phenol / chloroform, precipitated with ethanol and dissolved in 17 µl double-distilled (d.d.) H_2O and 2 µl 10x phosphatase buffer (10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM Tris.Cl, pH 8.3) provided with the enzyme, 1 µl enzyme (1 unit) was added and the reaction mixture incubated at 37°C for 30 minutes. The reaction was stopped by being incubated at 65°C for 15 minutes or by addition of agarose-gel-loading buffer (4 µl).

2.1.4 Electrophoresis of DNA on Agarose Gels

Methods were adapted from those of Sambrook *et al.* (1989). Agarose-gel electrophoresis was carried out in T.A.E. buffer (40 mM Tris.acetate, 1mM EDTA, pH 7.6) typically at 3-5 V/cm. Gels were 0.7 - 1.0% (w/v) agarose for plasmid DNA, or 1.5% (w/v) agarose for polymerase chain reaction (PCR) products. DNA was loaded following addition of one-fifth volume of agarose-gel-loading buffer. λ DNA digested with *Hind*III (Boehringer), ΨX174 DNA digested with *Hae*III (New England Biolabs) or pGEM markers (Promega) were used as DNA size markers for agarose-gel electrophoresis (the first-mentioned for digested plasmid or genomic DNA and the latter two for PCR products).

DNA was visualised on a u.v. transilluminator (302 nm) by inclusion of 500 ng/ml ethidium bromide in the gel, or alternatively (e.g. if DNA is to be extracted from the gel following electrophoresis) by immersing the gel for 5 minutes after electrophoresis in T.A.E. buffer containing 500 ng/ml ethidium bromide.

2.1.5 Extraction of DNA from Agarose Gels

Individual bands of DNA were excised from ethidium-bromide-stained gels on

a low power u.v. transilluminator (366 nm) using a scalpel and transferred to a pre-weighed tube. DNA was extracted from the agarose using glass-milk (GeneClean kit, Bio 101). Briefly, the agarose gel slice was dissolved in a "sodium iodide solution" at 50 - 55 °C, incubated with glass powder (supplied in the form of glass-milk) to which the DNA binds, the glass powder was pelleted and the pellet was washed three times in "wash buffer" before being resuspended in T.E. buffer at 50 - 55 °C to elute the DNA. Typically 5 µl of glass-milk was used and the DNA was extracted into 20 or 40 µl T.E. buffer (1 mM EDTA, 10 mM Tris.Cl, pH 7.6).

2.1.6 Ligation of DNA Fragments

Ligation reactions typically contained 50 ng vector DNA, 150 or 300 ng insert DNA, 2 µl 10x buffer (50 µg/ml bovine serum albumin,10 mM ATP, 100 mM $MgCl_2$, 200 mM dithiothreitol, 500 mM Tris.Cl, pH 7.8; provided with enzyme), d.d.H_2O to 19 µl and 1 µl T4 DNA Ligase (New England Biolabs) (Weiss *et al.*, 1968).

Two ligation reactions were performed (one having twice the insert / vector ratio of the other). Control ligations containing vector only, with and without enzyme, were also carried out. When transformed into competent *E. coli* cells these controls give an indication of the number of colonies expected due to religation of vector, uncut vector and/or other contaminating plasmid DNA. The reactions were incubated either overnight at room temperature or for 3 hours at 16°C.

2.1.7 Preparation of Competent Cells

Methods were adapted from those of Cohen *et al.* (1972). A single colony of DH5α *E. coli* from a freshly-streaked SOB plate (1.5% w/v bacto-agar, 2% w/v bactotryptone, 0.5% w/v bacto-yeast extract, 0.05% w/v NaCl, 2.5 mM KCl, 10 mM $MgCl_2$) was inoculated into 3 ml of L-broth (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% w/v NaCl, pH 7.0) and incubated with shaking overnight at

37°C. A 0.5 ml aliquot of this culture was used to inoculate 50 ml L-broth and the subsequent culture incubated with shaking at 37°C, until the optical density was 0.4 - 0.6 at 550 nm. 30 ml of culture were centrifuged in a polypropylene centrifuge tube at 1000 g_{max} for 10 minutes at 4°C and the cell pellet resuspended in 15 ml ice-cold, sterile 100 mM $CaCl_2$, then incubated on ice for 30 minutes. Again the cells were pelleted (1000 g_{max}, 10 minutes, 4°C) and the supernatant discarded. The pellet of cells was resuspended in 3 ml ice-cold, sterile 100 mM $CaCl_2$ and incubated on ice for at least 2 hours before use.

2.1.8 Transformation of Competent Cells

Methods were adapted from those of Cohen *et al.* (1972). Freshly-prepared competent cells (0.2 ml) were mixed with up to 20 µl of DNA-containing solution (typically half of a ligation) and incubated on ice for 30 minutes. The cells were then heated at 42°C for 2 minutes and cooled rapidly on ice before addition of 0.8 ml L-broth (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% NaCl, pH 7.0) and incubation at 37°C for one hour. The cells were pelleted by centrifugation (10 seconds in a microcentrifuge), 0.8 ml supernatant was removed and the cells resuspended in the remaining supernatant. This was then plated onto L-amp plates (1.5% w/v bacto-agar, 0.1 mg/ml ampicillin in L-Broth) and incubated at 37°C overnight. 1 ng uncut vector and "no DNA" were similarly transformed as controls for transformation efficiency and contamination respectively.

L-amp plates with colonies of potential interest were sealed and stored inverted at 4°C until required (but used within one month).

2.1.9 Preparation of Plasmid DNA From Bacterial Cultures

Methods were adapted from those of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). Single colonies were picked, inoculated into L-broth with 0.1 mg/ml ampicillin and grown overnight with shaking at 37°C. Typically 3 ml cultures were grown for small-scale DNA extraction and 100 ml cultures for

large-scale DNA extraction. Large-scale inoculation was alternatively performed using 0.1 ml of a 3 ml culture.

For small-scale DNA extraction, 1.5 ml of the overnight culture was transferred to a microcentrifuge tube, centrifuged for 10 seconds and the pellet resuspended by vortexing in 0.1 ml of freshly-prepared TGE / lysozyme [10 mM EDTA, 25% (w/v) sucrose, 50 mM Tris.Cl, pH 8.0 with 4 mg/ml lysozyme (Sigma)]. After being incubated at room temperature for 5 minutes the tube was vortexed again, 0.2 ml of NaOH / SDS [0.2 M NaOH, 1% (w/v) SDS, made fresh] added with vortexing and the tube incubated at room temperature for 10 minutes. After adding 75 µl of 5M potassium acetate (pH 5.2) and vortexing, the tube was left to stand for 2 minutes and then centrifuged in a microcentrifuge at maximum speed (12,000 g_{max}) for 10 minutes. The supernatant was removed and 0.35 ml isopropanol was added with gentle mixing (not vortexing). After incubating on ice for 5 minutes the tube was centrifuged at maximum speed in a microcentrifuge (12,000 g_{max}) and the resulting pellet washed with 70% (v/v) ethanol before being dried. The dried pellet was dissolved in 40 µl T.E. buffer (1 mM EDTA, 10 mM Tris.Cl, pH 7.6 or pH 8.0). Alternatively the pellet was resuspended in 20 µl T.E. buffer (pH 7.6 or pH 8.0) and 20 µl of 100 mM $MgCl_2$ added. The resulting precipitate (containing contaminating RNA) was removed by centrifugation, the DNA in the supernatant was precipitated with ethanol (see above) and resuspended in 40 µl T.E. buffer (pH 7.6 or pH 8.0).

Large-scale DNA extraction was performed in two ways. Either by alkaline-lysis followed by anion-exchange chromatography as specified in protocols provided with the Qiagen kit, or by an augmented small-scale method as described here. A 100 ml culture was divided between two centrifuge tubes and centrifuged at 1000 g_{max} for 10 minutes at 4°C. The cell pellets were resuspended in 5 ml TGE / lysozyme, incubated on ice for 5 minutes then vortexed again. After adding 10 ml NaOH / SDS the tubes were vortexed and incubated on ice for 10 minutes. After

adding 4.5 ml 5 M potassium acetate (pH 5.2) and vortexing, the tubes were left to stand for 2 minutes and then centrifuged at 1000 g_{max} for 10 minutes. The supernatants were mixed with 19 ml isopropanol and after 5 minutes on ice the tubes were centrifuged at 1000 g_{max} for 10 minutes at 4°C. The pellets were each resuspended in 0.4 ml T.E. buffer (pH 8.0), transferred to microcentrifuge tubes and the DNA was precipitated with 40 µl 3 M sodium acetate (pH 5.5) and 0.8 ml of redistilled absolute ethanol at -20°C for 20 minutes. Following microcentrifugation at maximum speed for 15 minutes the pellets were combined by redissolving in a total volume of 0.4 ml T.E. buffer (pH 8.0). The DNA preparation was treated by addition of DNase-free ribonuclease A (1 µl of 10 mg/ml) and incubation at 37°C for 30 minutes; SDS was added to a final concentration of 0.5% (w/v) and Proteinase K was added to a final concentration of 50 µg/ml and the tube returned to 37°C for a further 30 minutes. The DNA solution was extracted with phenol / chloroform and the DNA precipitated with ethanol (see above).

2.1.10 Polymerase Chain Reaction (PCR) to Verify Plasmids

PCR (Saiki *et al.*, 1988) was used to identify plasmids containing a specific arrangement of DNA. Single colonies of bacteria containing the plasmid of interest were picked and streaked onto fresh L-Amp plates. Individual samples of the bacteria were then washed off the picks into 10 µl aliquots of T.E. buffer (1 mM EDTA, 10 mM Tris.Cl, pH 7.6), the aliquots were then boiled to lyse the bacteria, centrifuged for 1 minute in a microcentrifuge at full speed (12,000 g_{max}) and 1 µl of the supernatants removed for use in PCR reactions. The DNA templates were over-layered with 50 µl of mineral oil, incubated for 10 minutes at 100°C and cooled on ice before addition of a reaction premix. PCR reactions were performed in a total volume of 25 or 50 µl and contained 0.2 mM of each deoxynucleoside triphosphate (dNTP), 2 mM $MgCl_2$, 1 µM of each primer, 1x reaction buffer (supplied with enzyme) and 1-2 units of Taq Polymerase (Amersham or Promega). Primers used were synthetic oligonucleotides (see **Figure 2.1**). Reactions were incubated on a

thermocycler (various models used) for 30 cycles of incubation (10 - 30 seconds at 94°C, 30 - 60 seconds at 65°C and 1 - 2 minutes at 72°C). Agarose-gel-loading dye was then added to the reactions (one-fifth volume) and each reaction (or a portion of it) analysed by agarose-gel electrophoresis (see above).

2.1.11 Nucleotide Sequence Analysis of DNA

The nucleotide sequence of double-stranded plasmid DNA was analysed using a Sequenase version 2.0 kit (USB). Primers used were synthetic oligonucleotides (see **Figure 2.1**). Approximately 3 μg of plasmid was used and the primer annealed as specified for double-stranded DNA sequencing in the Sequenase kit. The Sequenase kit utilises the chain termination method of Sanger *et al.* (1977) in which *de novo* synthesis of DNA is terminated by incorporation of dideoxy nucleotides to generate four nested series of oligonucleotides (each differing with respect to the terminating dideoxy nucleotide). The *de novo* synthesis of DNA also incorporates radiolabelled [^{35}S]dATP which permits detection of the individual oligonucleotides by autoradiography following polyacrylamide-gel electrophoresis.

Electrophoresis of DNA on polyacrylamide gels (Sambrook *et al.*, 1989) was performed by loading between 3 and 6 μl of the sequencing reactions on 0.4 mm thick, 6% denaturing polyacrylamide gels. The gels were prepared using a Sequagel kit (National Diagnostics) which contains most of the components of the gel in three solutions (concentrate, diluent and buffer), these were mixed in the appropriate proportions, TEMED (0.04% v/v) and ammonium persulphate (0.008% w/v) were added and the gels cast between two glass plates. Prior to loading, the gels were pre-run at 1500V for 30 - 60 min. and the reactions heated to 75°C for 2 min. Electrophoresis was carried out in a vertical apparatus with T.B.E. buffer (1mM EDTA, 45 mM Tris.borate, pH 7.6) at 1500 - 2000 V and at a temperature of 50 - 55°C.

Following electrophoresis, the polyacrylamide gel was treated for 30 - 60 min.

in 10% acetic acid, 12% methanol and dried onto Whatman 3MM paper under vacuum. Autoradiography was performed by exposing the gel to X-ray film (Fuji RX) either at room temperature or with an intensifying screen at -70°C. The DNA sequence was read by eye from the developed autoradiograhic films.

2.1.12 Preparation of Oligonucleotides

Oligonucleotides for use as sequencing primers, PCR primers or as a "tag" insert (see **Figure 2.1**) were prepared in-house both at Glaxo Group Research, Greenford and Biochemistry Department, University of Liverpool. Oligonucleotides were sythesised using β–cyanoethyl phosphoramidite chemistry on an Applied Biosystems Model 381A oligonucleotide synthesiser.

2.1.13 Annealing of "Tag" Oligonucleotides

In order to anneal the two oligonucleotides ("primers" Upper and Lower, see **Figure 2.1**) that code for the peptide "tag" to be inserted into the p9Ka gene, equimolar amounts were mixed and incubated in a 96°C waterbath for 1 minute. The waterbath was then turned off and allowed to cool slowly to room temperature.

Primer	Sequence (5' to 3')	Priming site	Strand
5'a	CACGGTGTCTGCAAGCCTTCCTGAG	1832 to 1856*	lower
5'b	GGCGTCTAACTGTACCTCTTCTACC	1872 to 1896*	lower
3'a	GCCATCTCTGCCACCACCCTCCCTC	2259 to 2235*	upper
3'b	AGGTCAACTTCCAGCCATCTCTGCC	2272 to 2248*	upper
Upper	AATTAATGGAGCAAAAGCTTATTTCTGAAGAGGACTTGAATA	tag sequence	lower
Lower	AATTTATTCAAGTCCTCTTCAGAAATAAGCTTTTGCTCCATT	tag sequence	upper
HPRT1	GATTACACATATGTGTCGCCA	HPRT gene	
HPRT2	AGCTACCAAGAAAGGAGAATCAC	HPRT gene	

* with respect to the published nucleotide sequence of p9Ka (Barraclough *et al*, 1987)

Primers	Length of DNA amplified (base pairs)	
	p9Ka	tagged-p9Ka
5'a & 3'a	428	470
5'a & 3'b	441	483
5'b & 3'a	388	430
5'b & 3'b	401	443
5'a & Lower	none	236
5'b & Lower	none	196
3'a & Upper	none	279
3'b & Upper	none	292

Figure 2.1

Identity of oligonucleotide primers and size of DNA fragments amplified by PCR with pairs of primers.

2.2 Production of Transgenic Mice

Transgenic mice were produced by microinjection of DNA into the pronuclei of single-cell mouse embryos (**Figure 2.2**). Both the materials used and the methodology employed closely followed those described in "Manipulating the Mouse Embryo - A Laboratory Manual" (Hogan *et al.*, 1986) and will be only briefly described here.

All animal work for production of transgenic mice was carried out using good laboratory animal practice, in a specific-pathogen-free facility which conformed to Home Office guidelines. Matings were set up and surgical procedures carried out by trained personnel under the appropriate Home Office licences. Surgical implantation of embryos into pseudopregnant foster mothers were performed by either Shirley Pease or Dr. Günter Schmidt.

2.2.1 Preparation of DNA for Microinjection

The cloned p9Ka transgene (see Results) was excised from the cloning vector using *Not*I; the cloned tagged-p9Ka transgene (see Results) was excised from the cloning vector using *Not*I or *Eco*RI. DNA for microinjection was prepared by extraction with glass-milk from agarose gels following restriction-enzyme digestion (see DNA manipulation). Finally, the DNA was dissolved at 3-10 µg/ml in injection buffer (0.1 mM EDTA, 10 mM Tris.Cl, pH 7.6).

2.2.2 Isolation of Single-Cell Mouse Embryos

Female B6D2F1 mice between 3 and 5 weeks old were treated with gonadotrophins (pregnant mare's serum to mimic follicle-stimulating hormone and human chorionic gonadotrophin to mimic luteinizing hormone) in order to induce superovulation. These females were mated with B6D2F1 males and checked for copulation by the presence of a vaginal plug. Mated females were then culled and the

oviducts removed into M2 medium (for maintenance at room temperature (Hogan *et al.*,1986)). The pre-implantation embryos were then harvested from the oviducts and treated briefly with hyaluronidase to removed associated cumulus cells. The single-cell embryos were then transferred to M16 medium [for maintenance at 37°C (Hogan *et al.*,1986)] in multidrop cultures under oil, and incubated in a humidified 37°C incubator with an atmosphere of 5% (v/v) CO_2 and 95% (v/v) air.

2.2.3 Microinjection of Single Cell Embryos

Embryos were transferred to M2 medium for injection. The injection apparatus consisted of an inverted microscope with image-erect optics (Zeiss Axiovert) to which two joystick-controlled micro-manipulators were attached. The left-hand joystick controlled a holding pipette (a blunt-ended, oil-filled glass pipette attached to a micrometer-controlled syringe) used to manipulate and hold the embryos for injection. The right-hand joystick controlled the injection pipette (a finely pulled, glass needle containing the DNA solution, attached to a pneumatic microinjection controller) was used to inject DNA into a single pronucleus of the embryo, whilst viewed under the microscope. Typically DNA was injected into the larger pronucleus (generally the male one) so as to produce a visible swelling. Following microinjection the embryos were returned to M16 microdrop culture until needed.

2.2.4 Implantation of the Embryos into Pseudopregnant Foster Mothers

Pseudopregnant foster mothers were prepared for acceptance of the injected embryos by mating female CRH mice with vasectomised stud males. Embryos were selected for implantation having survived injection and being either single cell (if injected the same day) or two-cell (if cultured overnight prior to implantation). Typically 10 - 15 embryos were surgically implanted into each oviduct of anaesthetised, pseudopregnant foster mothers. Mice were born approximately three weeks after implantation.

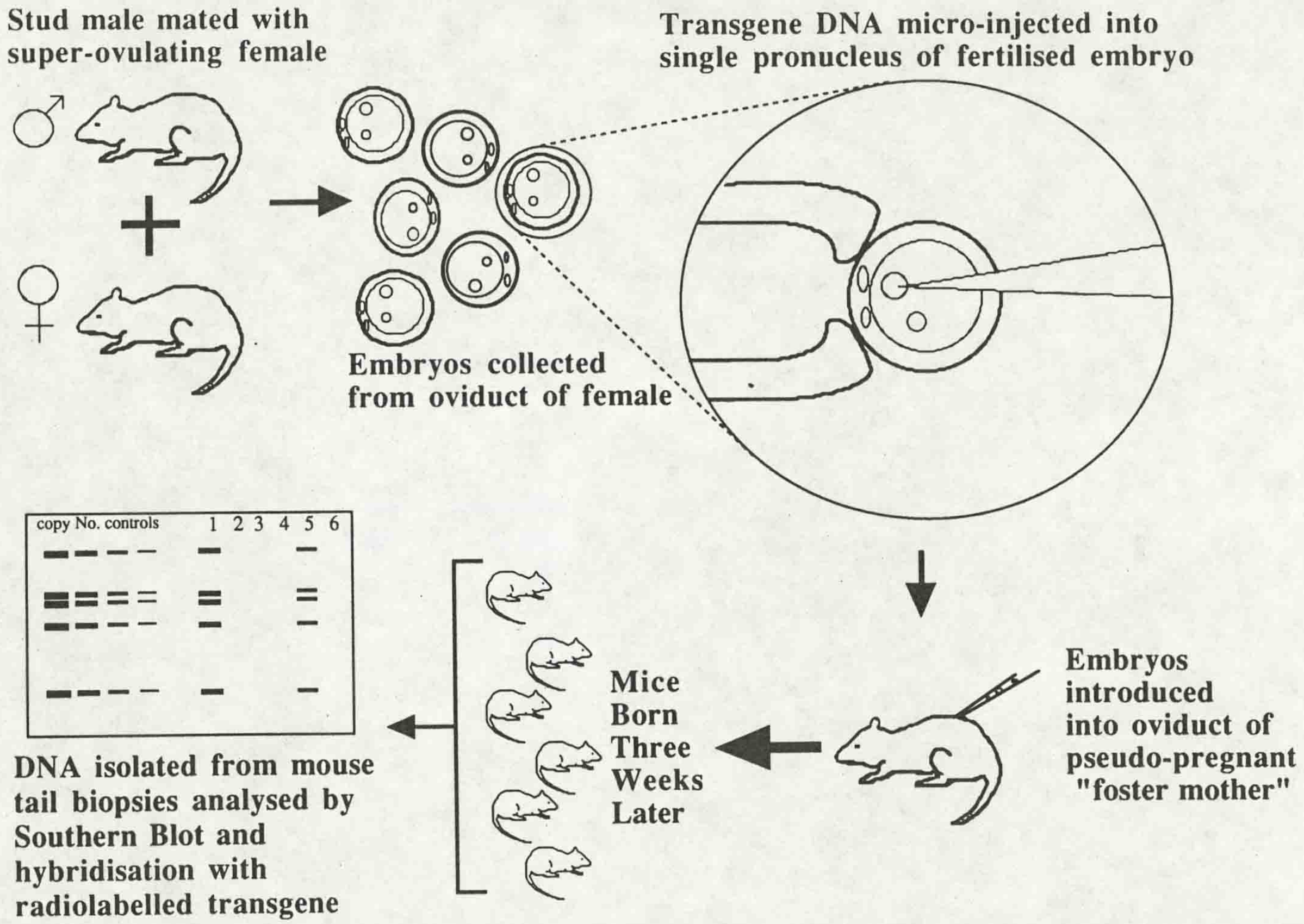

Figure 2.2 Production of Transgenic Mice by Pronuclear Microinjection of Single Cell Embryos

2.3 Analysis of Transgenic and Non-transgenic Rodents

Transgenic mice and non-transgenic rodent controls (mice and rats) were housed in animal facilities which conformed to Home Office guidelines. All procedures were carried out using good laboratory animal practice by trained personnel under the appropriate Home Office licences.

2.3.1 Tail-cutting and Labelling of Mice

Each litter born was weaned and at 5 weeks of age the mice were anaesthetised with i.p. avertin anaesthetic: 0.7 ml per 25 g body weight of 2% (w/v) 2,2,2-tribromoethanol (Aldrich), 2% (v/v) tertiary amyl alcohol (Aldrich) in sterile PBS (Ca^{2+} and Mg^{2+}-free phosphate buffered saline, pH 7.4). A small piece (1 - 2 cm) of tail was removed and placed into a labelled microcentrifuge tube and at the same time mice were given earmarks (if necessary). The tail was then cauterised and the mice were identified, by sex, coat-colour and number and position of earmark(s), and given an identifying number. All mice from embryos injected with the p9Ka gene-DNA construct were given the prefix PK; whilst those originating from injections with the tagged-p9Ka gene-DNA construct were given the prefix TPK.

2.3.2 Mating of Mice

Mice were mated with either non-transgenic B6D2F1 or DBA2 mice or with other transgenic mice. The nomenclature adopted for labelling the progeny was such that the transgenic parents were identifiable from the mouse number.

2.3.3 Isolation of High-Molecular -Weight DNA from Mouse Tail Biopsies

This method was adapted from that of Hogan *et al.*(1986). Mouse tail biopsies were obtained as above and incubated overnight at 37°C or 50°C in 0.5 ml of "Tail" buffer [1% (w/v) sodium dodecyl sulphate (SDS), 0.3 M sodium acetate,

1mM EDTA, 10 mM Tris.Cl, pH 7.9] containing 0.2 mg/ml Proteinase K with shaking. The tubes were centrifuged at full speed (12,000 g_{max}) in a microcentrifuge for 10 minutes and 0.45 ml of the supernatant removed to a fresh tube. This tail-digest was extracted once with phenol / chloroform / isoamyl alcohol, and once with chloroform / isoamyl alcohol, then the DNA was precipitated by the addition of one-thirtieth volume of 3 M sodium acetate (pH 5.5) and one volume of isopropanol. DNA was pelleted by centrifugation at full speed in a microcentrifuge (12,000 g_{max}) for 15 minutes (at 4 °C if possible) and the pellet washed with 70% (v/v) ethanol. All remnants of the ethanol were removed and the pellet dissolved in T.E. buffer (1 mM EDTA, 10 mM Tris.Cl, pH 7.6). The DNA concentration was determined by measuring the absorbance of a sample diluted 1/200 with d.d.H_2O at 260 nm against a d.d.H_2O blank (the extinction coefficient was taken as 50 $mg^{-1}.ml.cm^{-1}$).

2.3.4 Analysis of Mouse DNA by Southern Blot and Hybridisation with Radioactively-labelled DNA Probes

Methods were adapted from those of Sambrook *et al.* (1989). Mouse DNA (10 µg) was digested with a suitable restriction enzyme (usually *Eco*RI) by incubation with 4 µl of high concentration (40 - 60 units/µl) enzyme in 50 µl total reaction volume for 2 hours followed by addition of 1 µl of low concentration (10 - 20 units/µl) enzyme and incubation for a further hour. Agarose-gel-loading buffer was then added (one-fifth volume of sample) and the samples subjected to agarose-gel electrophoresis through a 0.8% (w/v) gel. Copy-number controls were also run on the gel; these consisted of restriction-enzyme-digested transgene plasmid diluted to correspond to a known copy number of transgene per mouse haploid genome.

The gel was stained briefly with ethidium bromide and viewed on a u.v. transilluminator to check for digestion, loading and absence of electrophoresis artefacts. If satisfactory, the gel was then blotted onto Hybond-N nylon membrane by capillary transfer (Southern, 1975). Protocols used for Southern blotting did not

vary significantly from those recommended by the manufacturer of the membrane (Amersham). DNA was fixed to the membrane by illumination on a calibrated u.v. transilluminator for 45 - 60 seconds [the calibration of the transilluminator for power output is important since too low or too high a dosage of u.v. irradiation will adversely effect the efficiency of subsequent hybridisation protocols (Sambrook *et al.*, 1989)].

After 3 - 5 hours pre-incubation in 20 - 40 ml of DNA Hybridisation Buffer {5 x SSPE [0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.4], 5 x Denhardt's buffer [0.1% (w/v) Ficoll-400 (Pharmacia), 0.1% (w/v) polyvinyl pyrrolidone (Sigma), 0.1% (w/v) bovine serum albumin (Fraction V, Sigma)] 0.5% (w/v) SDS, 50 µg/ml sonicated salmon sperm DNA} at 65°C in a Hybaid hybridisation oven, the membrane was incubated for approximately 18 hours at 65°C in 10 - 20 ml of DNA Hybridisation Buffer containing 25-35 ng of ^{32}P-labelled DNA (p9Ka genomic DNA or cDNA). The labelling of the DNA was performed by the method of random-primed synthesis (Feinberg and Vogelstein, 1983; 1984)using a "Random Primed DNA Labelling Kit" (Boehringer) to a specific activity of 0.5 -1 x 10^9 d.p.m./µg.

All filter washes were performed with shaking at 65°C in a waterbath, the volume of wash solution used was 100 - 400 ml. The filters were washed with 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% (w/v) SDS for at least an hour (with 2 - 3 changes of wash solution) for low-stringency hybridisation; and a further 1 - 2 hours with 0.1 x SSC, 0.01% (w/v) SDS (at least 3 changes of wash solution) for high-stringency hybridisation. The radioactivity which remained bound to the membrane was then visualised by autoradiography.

To remove any bound DNA (in order to perform a further hydbridisation of the blot) the membrane was washed at 45°C in 0.4 M NaOH for 30 minutes followed by incubation for a further 30 minutes at 45°C in 0.1 x SSC, 0.1% (w/v) SDS, 0.2 M Tris.Cl (pH 7.5), as stated by the manufacturers of the membrane (Amersham).

2.3.5 PCR analysis of Mouse DNA

PCR (Saiki *et al.*, 1988) was used to identify transgenic mice containing a specific p9Ka transgene. Either 1 µl of crude tail-biopsy digest, or 1 µl of a 1 in 100 dilution of prepared mouse DNA, were used in PCR reactions performed as for verification of plasmids (section 2.1.10). Four PCR primers (**Figure 2.1**) were used in each reaction, two of which annealled to the p9Ka or tagged-p9Ka transgene and two of which annealled to the endogenous, single copy, mouse hypoxanthine ribosyl transferase (HPRT) gene (S. Harris, personnal communication). The latter primers acted as a positive control for DNA viability and *Taq* polymerase activity. PCR products were analysed on a 1.5% (w/v) agarose gel (section 2.1.4).

2.3.6 Rodent Dissection

Mice or rats were culled by cervical dislocation or by asphyxiation with CO_2 and tissues dissected out carefully. Tissues were placed in pre-labelled cryovials and dropped into liquid nitrogen, or placed in Methacarn fixative [60% (v/v) methanol, 10% (v/v) acetic acid, 30% (v/v) Inhibisol (BDH)]. Any tissue samples frozen in liquid nitrogen were subsequently stored in liquid nitrogen until needed for RNA and / or protein extraction. Tissue samples to be fixed for histological and immunohistological analysis remained immersed in Methacarn fixative for at least 24 hours and were subsequently transferred to 70% (v/v) ethanol (for storage) or to paraffin blocks for histological examination. Samples containing bone were de-calcified by treatment with De-Cal (National Diagnostics) for at least 1 week prior to transfer to paraffin blocks.

Upon autopsy, great care was taken to observe all major organs for signs of abnormalities, to perform as thorough a dissection as possible (unfortunately mice found dead could not always be autopsied properly due to an advanced state of decomposition), to preserve the dissected tissues in methacarn fixative and subsequently to store them in 70% (v/v) ethanol. Any tissues seen to differ from the

norm were transferred to paraffin blocks and subjected to histological examination, as were all tissues of mice found dead and any spare tissues from those mice used for analysis of expression.

2.3.7 Transfer of Tissue Samples to Paraffin Blocks

Methods were adapted from those of Gordon (1990). Fixed tissue samples were transferred into histology cassettes (Miles) and processed on a Shandon 2L processor (Shandon Southern). The processing involved twelve individual steps, the aim of which was to dehydrate the tissue samples and impregnate them with paraffin wax. The steps consisted of sequential immersions: in 70% (v/v) ethanol for at least 1 hour; five times in absolute ethanol (2, 2, 1.5, 1.5 and 1.5 hours); once in 50% (v/v) ethanol / 50% (v/v) Inhibisol for 2 hours; three times in 100% Inhibisol (1.5, 1.5 and 1 hours); and, twice in molten paraffin (at 60°C) (2 and 2.5 hours, or until ready to proceed). The processed tissues were embedded in paraffin wax at 60°C on a Shandon Histocentre (Shandon Southern) and cooled to harden. The blocks were stored at room temperature, and if necessary (e.g. to realign the tissue sample for cutting in a different plane) paraffin wax blocks were melted and reset.

2.3.8 Cutting of Sections of Tissue Embedded in Paraffin Blocks

Pre-cooled blocks were sectioned at room temperature on an Anglia AS 300 rotary microtome (Raymond A. Lamb) using a microtome knife holder containing stainless steel disposable blades (Miles Inc.). Sections cut to 3µm thickness were floated on a 50°C water bath and subsequently transferred to glass microscope slides. The slides were placed in an incubator at 60°C for a minimum of 1 hour and then cooled to room temperature before storing in boxes.

2.3.9 Staining of Cut Tissue Sections with Haematoxylin and Eosin (H&E)

Methods were adapted from those of Steven (1990). The glass slides with

paraffin-embedded sections were placed in a rack and immersed in xylene for 3 minutes. They were then transferred to fresh xylene for 3 more minutes and this process repeated a further two times. The slides were rinsed three times in absolute ethanol, once in 70% (v/v) ethanol and once in water before being placed in Mayer's Haematoxylin [0.1% (w/v) haematoxylin, 5% (w/v) potassium aluminate, 0.02% (w/v) sodium iodate, 5% (w/v) chloral hydrate, 0.1% (w/v) citric acid, (Mayer, 1903)] for 10 minutes. They were rinsed in water, placed for five seconds in acid alcohol [70% (v/v) ethanol, 1% (v/v) conc. HCl] and again rinsed in water. The slides were then placed in saturated lithium carbonate solution for sufficient time for the dye to turn blue (usually a few seconds) and this process was monitored with a microscope. After a rinse in water, the slides were placed in eosin solution [0.5% (w/v) in 25% (v/v) ethanol] for five minutes. After a rinse in water, the sections were dehydrated by rinsing sequentially in 70% (v/v) ethanol, three times in absolute ethanol and four times in xylene (slides can be left in xylene for a few hours prior to mounting coverslips). Coverslips were mounted onto the slides using DPX mountant (BDH), the slides were labelled and left to dry on a flat surface.

2.3.10 Immunocytochemical Analysis of Tissue Sections

Immunocytochemical staining was carried out using an indirect method employing an antibody-complex containing horse-radish peroxidase (the ABC method of Hsu *et al.*, 1981, or a secondary antibody method adapted from that of Robson *et al.*, 1990). Histological sections were re-hydrated and incubated with PBS (Ca^{2+} and Mg^{2+}-free, phosphate-buffered saline, pH 7.4) for 15 minutes at 37°C. In order to block endogenous peroxidase activity, all sections were incubated for 30 minutes in methanol with hydrogen peroxide added to 0.05% (v/v) (Streefkerk, 1972). The sections were washed with PBS and incubated for approximately 2 hours in a moist chamber with an appropriate dilution of first antibody in PBS. The sections were washed three times in PBS, for 5 minutes each, and then incubated with the relevant second antibody at the appropriate dilution (1 in

200 for biotinylated donkey anti-rabbit IgG (Amersham) or 1 in 1000 for peroxidase-conjugated goat $F(ab')_2$ anti-mouse IgG (Pierce)) in PBS for 1 hour in a moist chamber. If a biotinylated secondary antibody was used, the slides were washed three times in PBS, for 5 minutes each, and sections were then incubated with a complex of avidin and biotinylated horse-radish peroxidase (Dakopatts) freshly prepared according to the manufacturers instructions. After this procedure, or immediately following incubation with primary antibody (if a peroxidase-conjugated secondary antibody was used), the sections were washed in PBS three times for 5 minutes each, and bound antibody was visualised by incubating with 0.05% (w/v) diaminobenzoate (DAB), 0.003% (v/v) hydrogen peroxide in 0.05 M Tris.Cl (pH 7.6) (Graham and Karnovsky, 1966). Sections were finally washed in distilled water, counterstained with Mayer's Haematoxylin, dehydrated and coverslips mounted with DPX.

2.3.11 General Precautions for Maintenance of a Ribonuclease-Reduced Environment

In order that RNA prepared from tissue samples was not degraded by contaminating RNases precautions must be taken against these pervasive and unusually stable enzymes. All pipette tips, flasks, centrifuge tubes and general laboratory wares were autoclaved prior to use (20 minutes at 15 p.s.i.) where possible. Electrophoresis tanks, gel trays, combs and plastic containers used for formaldehyde gels were treated for 30 minutes with 0.1% diethylpyrocarbonate (DEPC) and rinsed with d.d.H_2O. Solutions used for work with RNA are usually treated with 0.1% DEPC overnight before being autoclaved; when autoclaving is not possible the solution is made up with autoclaved d.d.H_2O and filter-sterilised. We did not find it necessary to treat d.d.H_2O with DEPC but chose only to autoclave it. All reagents for use with RNA were reserved for that purpose, and work was carried out whilst wearing gloves.

2.3.12 Isolation of RNA and Protein from Rodent Tissues

Methods were adapted from those of Glison *et al.* (1974), Chirgwin *et al.* (1979) and Coombs *et al.* (1990). Samples of mouse or rat tissue already frozen in liquid nitrogen were powdered by crushing them between a brass pummel and a brass vessel, both previously cooled to -70 °C and further cooled with liquid nitrogen. The powdered tissue was then homogenised for 1 minute at 19,000 r.p.m. in 7 to 8 ml of homogenisation buffer (4 M guanidine isothiocyanate, 50 mM Tris.Cl (pH 7.5), 25 mM EDTA, 0.05% (w/v) sodium lauryl sarcosine, 8% (v/v) 2-mercaptoethanol). The homogenate was centrifuged at 7,700 g_{max} at 4 °C and the supernatant layered onto a 3 ml caesium chloride cushion (5.7 M caesium chloride, 0.1 M EDTA) in a polyallomer centrifuge tube. The tubes were centrifuged at 32,000 rpm (140,000 g_{max}) in a swing-out rotor at 20 °C for 18-24 hours in either a Sorvall or Kontron ultracentrifuge.

Protein isolation: The upper layer (homogenisation buffer containing protein) was removed down to the level of birefringence which marks the presence of DNA, and placed in dialysis tubing pre-soaked in 100 mM ammonium bicarbonate. This protein solution was dialysed against two changes (1 litre total) of 100 mM ammonium bicarbonate for at least 24 hours at 4 °C. The dialysate was then stored at -70 °C until being freeze-dried (24 - 48 hours depending on sample size). It was then dissolved in protein sample buffer [0.1% (w/v) SDS, 25 mM Tris.Cl, pH 6.8] and stored at -70 °C. Protein concentrations were measured using Comassie reagent from Pierce or Biorad according to the manufacturers' instructions. This assay involves incubation of a protein sample, or a dilution thereof, with the reagent provided and the measurement of the absorbance of the mixture at the appropriate wavelength. By comparison with a standard curve (prepared from measurements of a bovine serum albumin standard diluted in protein sample buffer), the approximate concentration of the protein samples were quantified.

RNA isolation: RNA formed a pellet at the bottom of the caesium chloride cushion following centrifugation. The caesium chloride cushion was removed

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initially with a pipette but the last 0.5 ml was poured off and the tube inverted to drain the contents. The inside of the tube was dried with a paper tissue, taking care not to disturb the pellet. The pellet was resuspended in 180 µl 0.1% (w/v) SDS and transferred to a microcentrifuge tube containing 36 µl 2 M NaCl and 900 µl absolute ethanol. A further 180 µl 0.1% SDS was used to rinse the centrifuge tube and was transferred to the same microcentrifuge tube. The RNA was then precipitated overnight at -20 °C and centrifuged at 4 °C (18,300 g_{max}) in a microcentrifuge. The pellet was dried and resuspended in 50 to 100 µl T.E.buffer (1 mM EDTA, 10 mM Tris.Cl, pH 7.6). RNA concentration was determined by measuring the absorbance of a sample diluted 1/200 with d.d.H_2O at 260 nm against a d.d.H_2O blank (the extinction coefficient was taken as 40 $mg^{-1}.ml.cm^{-1}$).

2.3.13 Denaturing Agarose-Gel Electrophoresis and Northern Blotting

Aliquots of RNA were separated electrophoretically on agarose gels containing formaldehyde by standard methods (Sambrook *et al.*, 1989) with minor alterations. The "formaldehyde gels" [0.8% (w/v) agarose, 1 x formaldehyde-gel running buffer, 2.2 M formaldehyde (pH > 4.0)] were run in circulated 1 x formaldehyde-gel running buffer [40 mM sodium acetate, 5 mM EDTA, 0.1 M MOPS, pH 7.0] at 5 V/cm for 5 minutes prior to loading the RNA samples. These samples (containing up to 30 µg RNA) were treated in a total volume of 20 µl of 1 x formaldehyde-gel running buffer, 50% de-ionised formamide, 2.2 M formaldehyde (pH > 4.0), for 15 minutes at 65°C and cooled on ice. 2.5 µl of formaldehyde-gel loading buffer [33% (v/v) glycerol, 0.67 mM EDTA (pH 8.0), 0.17% (w/v) bromophenol blue, 0.17% (w/v) xylene cyanol FF, 0.33 mg/ml ethidium bromide] were added and after mixing they were loaded carefully into the slots of the formaldehyde gel. The gel was then run at 3 to 4 V/cm until the dye front was approximately $^3/_4$ of the way down the gel.

Where necessary RNA samples were freeze-dried and resuspended in

autoclaved, d.d. H_20 prior to electrophoresis. The formaldehyde-gel loading buffer used here differs from that described by Sambrook *et al.* (1989) in that, in order to visualise the RNA, this buffer was supplemented with ethidium bromide.

After electrophoresis the gel was treated with 10 mM NaCl, 50 mM NaOH for 45 minutes and then treated with 10 mM NaCl, 0.1 M Tris.Cl (pH 7.5) for 45 minutes. The gel was blotted overnight on to nylon membrane (Hybond-N, Amersham) by capillary transfer with 10 x SSC. RNA was fixed to the membrane by illumination for 3 minutes with u.v. light. During this u.v. illumination of the membrane, the ethidium-bromide-stained RNA on the membrane was monitored in order check for loading anomalies and degradation. Membranes were stored wrapped in Saranwrap (Genetic Research Instrumentation) at 4 ^oC until use.

2.3.14 RNA and DNA Dot Blots

RNA dot blots were performed as described for nitrocellulose filters (Sambrook *et al.*, 1989) but using nylon membranes (Hybond-N) in a 96 well vacuum manifold. The nylon membrane was soaked briefly in water and then for an hour in 20 x SSC. The vacuum manifold was rinsed in 0.1 N NaOH, placed in 0.1% (v/v) DEPC for 30 minutes and rinsed in d.d.H_2O. The apparatus was assembled with a sheet of Whatman 3MM (soaked in 20 x SSC) on the vacuum bore, and the pre-soaked nylon membrane positioned at the bottom of the wells, taking care to avoid bubbles and to clamp both parts together firmly. The wells were rinsed by passing 10 x SSC through the system before adding the pre-treated RNA samples to the wells. RNA was pre-treated by incubation for 15 minutes at 68 ^oC, in a solution of 50% (v/v) de-ionised formamide, 7% formaldehyde (pH > 4.0), 1 x SSC. It was then cooled on ice and 2 volumes of 20 x SSC added. After applying the vacuum to suck the RNA onto the membrane, the wells were filled with 0.25 ml 10 x SSC and this was also sucked through the manifold. After a second wash of 0.25 ml 10 x SSC, the vacuum was left on for 5 minutes and the membrane was removed to air dry. RNA was fixed to the membrane by illumination for 3 minutes with u.v. light.

DNA dot blots were performed as stated in the manual provided with the Hybond-N membrane (Amersham). Briefly this method consisted of treating the DNA samples by heating to 95 °C, cooling the solutions on ice and then adding an equal volume of 20 x SSC. The samples were then spotted onto a Hybond-N membrane (pre-soaked in 10 x SSC) and allowed to dry. In order to bind the DNA to the membrane, the DNA was denatured (by soaking the membrane in a buffer containing NaOH), neutralised , dried and then fixed by illumination with u.v. light.

2.3.15 Hybridisation of Radioactively-Labelled cDNA Probes to Northern and Dot Blots

The following methods were used for either Northern blots containing RNA, or dot blots containing either RNA or DNA. Blots were incubated for 1-5 hours at 42 °C in RNA Prehybridisation Buffer [5 x SSPE, 5 x Denhardt's buffer, 0.5% (w/v) SDS, 100 µg/ml sonicated salmon sperm DNA] and then incubated for approximately 18 hours at 42 °C in RNA Hybridisation Buffer [5 x SSPE, 5 x Denhardt's buffer, 0.5% (w/v) SDS, 200 µg/ml sonicated salmon sperm DNA, 10% (w/v) dextran sulphate] containing 25-35 ng of ^{32}P-labelled cDNA. The ^{32}P-labelling was performed by random-primed synthesis ("Random Primed DNA Labelling Kit", Boehringer) to a specific activity of 0.5 to 1 x 10^9 d.p.m./µg. The blots were washed at 65 °C with 1 x SSC, 0.1% (w/v) SDS for up to 30 minutes followed by 0.1 x SSC, 0.1% (w/v) SDS for up to an hour (with two changes of washing buffer). The radioactive probe which remained bound to the membrane was then visualised by autoradiography, as before.

Both actin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probes were used as constitutive controls to check for RNA loading and blotting anomalies.

2.3.16 Quantification of Autoradiography

In order to quantify the signal obtained upon autoradiography of Northern (2.3.14) and Southern (2.3.4) blots probed with radioactively-labelled DNA, the developed X-ray film was analysed on a Shimadzu CS9000 Flying Spot Scanner. For Kodak XAR-5 film a wavelength of 500 nm was used and transmission scans were performed. The auotoradiographs were scanned down the lanes with a linear beam size of 0.4 x 5 mm. Peak areas of the densitometric trace for any particular lane was taken as a measure of intensity of hybridisation, and thus of the amount of target DNA or RNA present.

In order to allow for loading anomalies between individual lanes of a Northern blot, peak area values from individual p9Ka exposures were corrected by first dividing by the constitutive probe peak area for that sample. This value was then multiplied by the average constitutive probe peak area for that tissue on any one blot. The average corrected peak area was taken as the average of the corrected peak areas for a single p9Ka exposure, there being multiple corrected values due to the use of two different constitutive probes (actin and G3PDH) and multiple exposures of constitutive probe blots. Error bars used on plots of average corrected peak areas are the standard deviation of the corrected peak areas for a single p9Ka exposure.

In order to verify that this methodology was appropriate, a Northern blot of a dilution series of two seperate samples of RNA was probed with radioactively-labelled cDNA for p9Ka, actin and G3PDH and all autoradiographs scanned in this way (see Results, section 3.2.3 and Figures 3.2.2 & 3.2.3).

2.3.17 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Protein

SDS-PAGE was carried out using the tricine gel method of Schagger and Von Jagow (1987) on vertical "mini-gel" apparatus (Biorad) with 1 mm spacers. Both stacking and running gels were prepared using a premixed acrylamide stock solution [30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide (Severn Biotech Ltd.)]. Running gel contained a 5:4:3 ratio of acrylamide stock solution, gel buffer [3 M Tris

base, 0.3% (w/v) SDS] and d.d. H_20; and was polymerized by adding TEMED (0.033% v/v) and ammonium persulphate (0.033% w/v). Stacking gel contained a 4:5:11 ratio of acrylamide stock solution, gel buffer and d.d. H_20; and was set by adding TEMED (0.125% v/v) and ammonium persulphate (0.05% w/v). The cathode buffer (top reservoir) consisted of 0.1 M Tris base, 0.1 M tricine, 0.1% (w/v) SDS (pH 8.25) and the anode buffer consisted of 0.2 M Tris base (pH 8.9). Prior to loading on the gel, the protein samples (freeze-dried if necessary) were dissolved in protein sample buffer and diluted with an equal volume of SDS-PAGE sample buffer [4% (w/v) SDS, 10% (w/v) sucrose, 0.125 M Tris.Cl (pH 6.8), 0.002% (w/v) bromophenol blue] to a maximum of 30 µl; one-tenth volume of 2-mercaptoethanol was added and the samples were boiled for 5 minutes. Molecular weight standards (Electran from BDH) were treated in the same way. Gels were run at 100V for approximately one hour or until the dye front was about 0.5 cm from the bottom of the gel.

In order to visualise protein in the gel (either instead of or after Western blotting) the gel was placed in gel-staining solution [0.25% (w/v) Coomassie blue R250, 50% (v/v) methanol, 7% (v/v) glacial acetic acid] for one hour and then transferred to de-staining solution [40% (v/v) methanol, 7% (v/v) glacial acetic acid] for a few hours with several changes of de-staining solution, until the background staining of the gel was acceptable.

2.3.18 Electroblotting of Size-Fractionated Protein to PVDF Membranes

Proteins were size-fractionated by SDS-PAGE (see above) and electoblotted (Western blotted) using a Biorad "Mini Trans-Blot" apparatus onto Immobilon polyvinylidene difluoride (PVDF) microporous membrane (Millipore). Following SDS-PAGE, the gel (running gel only) was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3) for 30 minutes. The membrane was immersed, in turn, in methanol for 1 to 3 seconds, d.d. H_2O for 1 to 2 minutes

and transfer buffer for up to 30 minutes. The "Trans-Blot" cassette was assembled according to the manufacturer's instructions (Biorad), with the membrane and gel sandwiched together between two sheets of Whatman 3MM paper, which had been presoaked in transfer buffer. Care was taken to avoid air bubbles. The assembly was slotted into the tank so that the gel was on the cathode side of the membrane, and pre-cooled (4 °C) transfer-buffer added together with a frozen cooling block. The transfer was performed at a constant 100 V for one hour. During this time the apparatus was monitored for generation of excessive heat, as denoted by an increase in current. If the current rose above 290 milliamps the transfer buffer was circulated so as to disperse the excess heat.

After the gel was blotted, it was stained with Coomassie as above to detect any protein which had failed to transfer, and the membrane was used immediately in immunochemical analysis.

2.3.19 Immunochemical Analysis of Protein Samples on Western Blots

Membranes, carrying Western-blotted proteins, were incubated for one hour at room temperature (20 to 25°C) in an excess of blocking buffer [2% (w/v) Marvel, 50 mM Tris.Cl, 20 mM NaCl, pH 7.5]. The membrane was then transferred to a polythene bag containing 10 ml of primary antibody at a suitable dilution in blocking buffer with, or without, 0.1% (v/v) Tween 20 (Sigma), and then incubated on an orbital shaker at 4°C overnight. After the membrane was washed for a total of thirty minutes with five changes of blocking buffer, it was placed in a polythene bag containing the second antibody at a suitable dilution in blocking buffer. The second antibody used for a rabbit polyclonal primary antibody was a peroxidase-conjugated, goat anti-rabbit IgG (Sigma) at a 1 in 200 dilution; for a mouse monoclonal antibody (e.g. anti-tag), a peroxidase-conjugated goat $F(ab')_2$ anti-mouse IgG (Pierce) was used at a 1 in 1000 dilution. After the membrane was incubated for 1 to 2 hours on an orbital shaker at room temperature (20 - 25°C), it was washed for a total of 15 minutes with three changes of blocking buffer followed by two rinses in d.d. H_2O.

minutes with three changes of blocking buffer followed by two rinses in d.d. H_2O. The presence of bound secondary antibody was then visualised by transferring the membrane to a solution of 0.5 mg/ml diaminobenzoate (DAB) in 0.03% (v/v) hydrogen peroxide, 10 mM imidazole, 100 mM Tris.Cl (pH 7.6). After 2 to 3 minutes, when the colour had developed, the reaction was stopped by washing the membrane in freshly prepared 0.02% (w/v) sodium azide. The membrane was allowed to dry between two sheets of Whatman 3MM paper and stored in the dark.

2.3.20 Biotinylation of Anti-Tag Monoclonal Antibody

The anti-tag monoclonal antibody Ab9E10 (Evan *et al.*, 1985; OM-11-908 from Cambridge Research Biochemicals) was biotinylated using ImmunoPure Biotin-LC-Hydrazide (Pierce) as described by the manufacturer. This reagent conjugates a biotin molecule via a spacer arm to aldehyde groups on the Fc portion of immunoglobulins, following mild oxidation of the antibody with sodium periodate.

2.3.21 Detection of Biotinylated anti-tag antibody on Western blots

After the Western Blot membrane was incubated with primary antibody in the usual manner (section 2.3.18), it was rinsed three times in PBS, for 5 minutes each time, and then incubated with a complex of avidin and biotinylated horse-radish peroxidase (Dakopatts) which had been freshly prepared according to the manufacturer's instructions (N.B. the avidin/horse-radish peroxidase conjugate was diluted 1 in 100 compared with that used in immunocytochemical staining of tissue sections). After this procedure, the membrane was washed three times in PBS, for 5 minutes each time, and bound antibody was visualised by incubating the membrane in 0.5 mg/ml diaminobenzoate (DAB) in 0.03% (v/v) hydrogen peroxide, 10 mM imidazole, 100 mM Tris (pH 7.6). After 2 - 3 minutes, when the colour had developed, the reaction was stopped by washing the membrane in freshly prepared 0.02% (w/v) sodium azide. The membrane was allowed to dry between two sheets of Whatman 3MM paper and stored in the dark.

3. Results

3.1 Production of Transgenic Mice

The aims of producing mice transgenic for p9Ka were two-fold, and the transgene constructs produced had to reflect the requisites of both these aims. Broadly the aims were to achieve expression of the transgene and then to study the nature of this expression (with regard to absolute level, tissue-specific expression, copy-number dependence and position independence), and at the same time to investigate any phenotypic effect of increased expression of p9Ka (particularly with the envisaged role of p9Ka in metastasis of breast cancer).

The expression of p9Ka with respect to its proposed role in neoplastic progression might have been achieved by using a p9Ka cDNA under the control of a promotor directed to the mammary epithelium (e.g. MMTV or WAP, see Introduction). In this way, levels of p9Ka expression significantly greater than normal might have been expressed in the appropriate cells, and thus produce a recognisable phenotype. However, the study of the expression of p9Ka could not have been achieved with such constructs. The use of a genomic-derived transgene is obviously an advantage in terms of studying the expression pattern of p9Ka under its own regulatory apparatus. Also the use of transgene constructs containing introns seems to result in greater expression of the gene of interest (Palmiter *et al.*, 1991), and the p9Ka introns, themselves, may contain elements necessary for expression of p9Ka. It should prove possible to express significantly high levels of p9Ka (compared to the endogenous mouse p9Ka) using genomic-derived transgenes, providing that multiple copies are integrated and that the construct contains the necessary elements to provide copy-number dependent, position-independent expression (Hogan *et al.*, 1986; Kollias and Grosveld, 1992; Sippel *et al.*, 1992). Such expression would not be limited to the specific cells defined by a targeted promotor, and this might be advantageous, since expression of p9Ka in a wider selection of cell-types may produce a more interesting phenotype. Expression at a particular developmental stage or in a specific cell lineage may be required for the

action of p9Ka to manifest itself, and such conditions may be imposed by the p9Ka promotor and (or) enhancer elements present in a genomic-derived, but not a cDNA-based transgene. Mice expressing p9Ka at elevated levels in a wider range of cell-types may also be used to study a wider range of neoplastic disorders than mice designed to express specifically in breast epithelium. Given that the use of heterologous promotors was not applicable to expression studies, but there was reason to believe that a genomic-derived transgene could fulfil both aims, this was the strategy of choice.

In order to analyse for the expression of p9Ka protein in the absence of a suitable antibody, a second DNA construct was designed which included a "tagging" epitope recognised by an available antibody. To be comparable to the first, this second construct was essentially the same except for the necessary insert.

Once designed and produced, these constructs were used to produce transgenic mice by microinjection of the construct DNA into single cell embryos. The transgenic nature of the resultant mice had then to be identified and the mice bred to produce individual lines of transgenic mice, not only for analysis of expression of the transgene, but also for assay of the phenotypic effect of the integration and expression of the p9Ka transgene.

3.1.1 Production of the p9Ka DNA Construct

The p9Ka DNA construct used as a "transgene" consisted of 10.3 kbp of cloned rat genomic DNA. This 10.3 kbp of DNA, containing the p9Ka gene, was isolated as two *Eco*RI fragments (5.0 & 5.3 kbp) from a sub-cloning of a 17.8 kbp genomic clone of DNA that had been derived from a partial *Eco*RI digest of normal rat DNA (Barraclough *et al.*, 1987). The gene for p9Ka has been shown to span the two fragments and the central *Eco*RI site, which divides the two fragments, is in the coding sequence of the p9Ka gene (Barraclough *et al.*, 1987). That these fragments contained a substantial amount of ancillary DNA in the form of 2 kbp upstream and 5 kbp downstream flanking regions was not considered a disadvantage. Such regions

might contain control elements necessary for the correct tissue-specific or developmental-specific expression. Since no mRNA transcripts other than p9Ka had been shown to be produced *in vitro* by these fragments (R. Barraclough, personal communication), and there is no real difficulty in producing transgenic mice with a 10.3 kbp DNA fragment (Hogan *et al.*, 1986), it was decided to join the two fragments together and use the reconstructed rat gene as the first p9Ka transgene construct.

The 5.0 and 5.3 kbp rat genomic DNA fragments were provided, cloned separately into the *Eco*RI site of vector pAT153 (designated pR5.0 & pR5.3, respectively, **Figure 3.1.1**). In order that the two p9Ka genomic DNA fragments could be orientated with respect to each other when in the reconstructed p9Ka gene, restriction enzyme sites were mapped. The *Xba*I sites were chosen for this purpose, since some information as to their location was already available. The resulting restiction enzyme maps are shown in **Figure 3.1.1**. The positions of the mapped *Xba*I sites proved to be useful in the orientation of the two rat genomic DNA fragments with respect either to each other or to the plasmid in which they were cloned, and they are indicative of the relative orientation of the two fragments upon reconstruction of the p9Ka gene.

To facilitate manipulation of the p9Ka gene-fragments and ultimately the construction of a 10.3 kbp gene-construct that could be cleanly isolated from vector sequences, the two *Eco*RI fragments were first sub-cloned individually into pPolyIII-I vector (herein called pIII; Lathe *et al.*, 1987). The excision of transgene DNA from vector DNA is important since such vector sequences can apparently interfere with expression of transgenes (Chada *et al.*, 1985; Townes *et al.*, 1985; Shani, 1986), and the pIII vector has the benefit of a large multiple cloning site with two sites for each of the rare-cutting restriction enzymes *Not*I and *Sfi*I which flank the *Eco*RI site. Individual clones that contained a single copy of either of the p9Ka DNA fragments were isolated separately (pIII5.0 & pIII5.3), with both clones having the *Xba*I site of the pIII plasmid at the 3' end with respect to the p9Ka gene

(**Figure 3.1.1**). It followed then that the *Sal*I site of the pIII vector multiple-cloning-site was also at the 3' end with respect to the p9Ka gene (**Figure 3.1.1**), and this was important in the strategy for the reconstruction of the p9Ka gene.

In order to reconstruct the 10.3 kbp of rat genomic DNA from the 5.0 and 5.3 kbp fragments, and thus the p9Ka gene, a cloning procedure was designed; this procedure is shown in **Figure 3.1.2** and described here. Digestion of the plasmids with *Sal*I and then partial digestion with *Eco*RI yields three fragments of different sizes, with different combinations of cohesive ends. The *Eco*RI-digestion conditions were selected so as to produce the best yield of the desired DNA fragments (i.e. 7.1 kbp for pIII5.0 and 5.3 kbp for pIII5.3). These fragments were isolated by electrophoresis through agarose gels, purified following excision from the gels, and the 7.1 kbp fragments of pIII5.0 were treated with phosphatase in order to inhibit religation of fragments digested only by *Sal*I. Upon ligation, only those fragments which have a *Sal*I and *Eco*RI overhang at opposite ends will ligate together to give transforming plasmids with a 10.3 kbp insert. Colonies were picked and screened by digestion of the resultant plasmid DNA with restriction enzymes *Eco*RI, *Sal*I and *Xba*I (**Figure 3.1.3**). The selected construct (designated pIII10.3, **Figure 3.1.4**) was then digested with *Not*I to confirm that this enzyme excised the cloned 10.3 kbp fragment (**Figure 3.1.3**). This result allowed the isolation of the p9Ka gene construct for use as a transgene. The construct was also checked by PCR analysis (**Figure 3.1.3**) using sets of DNA primers synthesised to anneal to DNA either side of the central *Eco*RI site (see Methods **Figure 2.1**). All sets of primers gave bands (visualised on agarose gels stained with ethidium bromide) which corresponded to the expected sizes (**Figure 3.1.3**). This analysis confirmed that the two p9Ka gene fragments had ligated in the correct orientation and that there were no significant deletions at the splicing junction. The PCR analysis also served to validate the PCR primers for use in later experiments. In order to check further the sequence at the central *Eco*RI site for possible abnormalities due to the cloning procedure, the nucleotide sequence of this region of the gene was

determined by DNA sequencing using the PCR primers designed to anneal to p9Ka genomic DNA. The accuracy of the ligation, which affected this site, was important, since the *Eco*RI site in question lies within the coding sequence of the p9Ka gene. No ligation-induced anomalies were detected, and the DNA sequence of this part of the p9Ka-coding region was identical to that of the natural gene. Only when the pIII10.3 construct had been thoroughly verified could it be used to produce the tagged-p9Ka construct, or to generate the DNA fragment used as the p9Ka transgene.

3.1.2 Design of a "Tagged-p9Ka" DNA Construct

In order to follow expression of the p9Ka transgene at the protein level, an epitope was added to the p9Ka to which a commercially-produced antibody was available. These so-called "tagging epitopes" can be used to follow the expression of appropriately manipulated proteins (Munro and Pelham, 1984; Vassar *et al.*, 1989).

The inclusion of a tag in the p9Ka gene was achieved by inserting an annealed pair of oligonucleotides encoding the epitope into the coding region. The epitope of choice was derived from human c-*myc* (**Figure 3.1.4**), and is recognised by the monoclonal antibody Ab9E10 (Evan *et al.*, 1985). The human c-*myc* epitope used was chosen largely on the advice of Dr. H. Pelham (MRC Laboratory of Molecular Biology, Cambridge) in whose laboratory similar studies had been performed (Munro and Pelham, 1987; Pelham, 1988) and the monoclonal antibody produced (Evan *et al.*, 1985). The antibody was raised to a synthetic peptide, the epitope is not found in murine c-*myc* and the antibody is reported not to detect the murine protein (G.I. Evan, personal communication).

The coding sequence for the tag was taken from the published human c-*myc* gene (EMBL genebank). In order to aid analysis of subsequent clones, this sequence was altered by replacing the last C of the first leucine's codon by a T to generate an extra *Hind*III site (**Figure 3.1.4**). This extra restriction enzyme site could be used to distinguish the tagged-p9Ka transgene from the non-tagged counterpart (**Figure**

3.1.3). It was advised (H.R. Pelham, personal communication) that the epitope should be flanked by a methionine at the N-terminal end and an asparagine at the C-terminal end.

Analysis of the known DNA sequence of p9Ka, in association with restriction enzyme digestion studies, revealed that there were few unique sites suitable for insertion of the tag. Thus the choice of site for the insertion of the tag was determined partly by the ease of manipulation of the 10.3 kbp p9Ka genomic DNA fragment. The simplest solution, from the point of view of methodology, was to insert the tag DNA into the central *Eco*RI site as this site not only lies in the coding sequence, but is rare within the p9Ka-gene construct (pIII10.3). In order to be able to be spliced into the *Eco*RI site, the tag DNA was lengthened further to include the necessary 5' and 3' overhangs, giving rise to codons for leucine (N-terminal end) and lysine (C-terminal end). These codons were designed to achieve an in-frame insertion whilst destroying the *Eco*RI site. The resulting tag construct and its putative translation product are shown in **Figure 3.1.4**.

Other important considerations that were borne in mind when designing the tagged construct and deciding on the insertion site were that no aberrant stop codons would be generated, that the insertion was in the same coding frame as that for p9Ka, and that the tag insert should have a minimal effect on the structure of the resultant protein. The first two points were easily addressed by an analysis of the resulting gene sequence and of the putative translation products. These analyses ensured that no spurious stop codons were included and that an in-frame insertion was achieved. The third point (i.e. that the protein structure should not be overtly altered) was addressed by analysing various protein structure and property predictions of the putative, encoded translation products of both the p9Ka gene and the tagged-p9Ka gene (**Figure 3.1.5**). These results are more subjective.

It would be unrealistic to expect not to alter the structure of a 101 residue protein by a 14 residue insertion. However, inserting the DNA sequence corresponding to the tag at the *Eco*RI site of the p9Ka gene placed the tag at the end

of the helix-loop-helix motif of the C-terminal EF-hand of the protein. Thus neither of the two potential Ca^{2+}-binding EF-hands would be expected to be disrupted directly. Nevertheless, there was no guarantee that more-distant events would alter the function of these sites in binding calcium ions. The effect of the insertion of the tag was to lengthen the α-helical region with a region that had a reasonably high surface probability, flexibility and hydrophilicity, and to displace the highly exposed C-terminal domain (**Figure 3.1.5**). The predicted antigenicity for the tag insert itself was also reasonably high, as would be hoped given its intended use. These results could not be relied upon to predict accurately any adverse effects of the insertion of the tag, but could probably be taken as an indicator that the tag might not have severe consequences on the expression, or overall structure, of the protein. Certainly, the alterations in protein structure are not so extreme as those which might be caused by insertion of a more "disruptive" tag in a more sensitive location. The types of predictions made in this analysis will, no doubt, become more precise and elaborate as more is understood about protein structure, particularly with regard to this family of small calcium-binding proteins. Until then, they serve only as tentative predictors of local protein structure.

Despite the apparent lack of any drastic effect on the potential local structure of the p9Ka protein, the tag might be expected to disrupt some aspect of the function of p9Ka. Indeed, it would be somewhat remarkable if the function of the p9Ka protein were not in some way impaired.

3.1.3 Construction of the Tagged-p9Ka DNA Construct

The plasmid pIII10.3 (**Figure 3.1.4**) was partially digested with *Eco*RI and the required 12.4 kbp fragment, respresenting linearised pIII10.3, was purified. The oligonucleotides used for tagging (**Figure 3.1.4**) were synthesised, purified and equimolar amounts were annealed together. These annealed oligonucleotides were then ligated in 100-fold excess with the prepared 12.4 kbp DNA fragment. Since three *Eco*RI sites were present, only a third of the recombinant clones would be

expected to contain an insertion in the correct (intragenic) site. However, because the oligonulceotides forming the tag DNA destroyed the *Eco*RI site and added a *Hind*III site it was possible to distinguish the insertion site of the tag DNA by restriction enzyme digestion (**Figure 3.1.3**). Selected cloned DNAs were isolated with the tag inserted in the correct site and were analysed further by PCR using primers to the p9Ka gene and with the tag oligonucleotides themselves. This analysis allowed the identification of cloned plasmids with the correct number of tags (i.e. one) as shown by the size of the bands generated by PCR with p9Ka primers; and the correct orientation as shown by the size of the bands generated by PCR with p9Ka primer(s) and one tag oligonucleotide (**Figures 3.1.3**). To confirm the selection of clones with a single tag in the correct orientation and to check that no spurious artefacts of cloning were produced at the insertion site, plasmid DNAs from the selected clones were sequenced using one of the p9Ka PCR primers. This analysis confirmed the results of the PCR and also indicated that the insertion was in frame with respect to the p9Ka gene and that the surrounding p9Ka coding sequences were unaffected by any artefacts of cloning. To confirm that the rest of the p9Ka genomic sequence was unaltered, the tagged-p9Ka plasmid construct was analysed by restriction-enzyme digestion with *Xba*I (diagnostic of correct reconstruction of the p9Ka gene), *Eco*RI (indicative of correct site of tag insertion) and *Hind*III (for which an extra site was generated by the tag) (**Figure 3.1.3**). The tagged-p9Ka clone was designated pIII10.3Tag. It was then used to generate a tagged-p9Ka "transgene" by isolation of the 10.3 kbp insert after it had been excised from the vector with either *Not*I or (since the design of the tag effectively destroyed the central *Eco*RI site but not the flanking *Eco*RI sites) with *Eco*RI.

3.1.4 Production of Transgenic Mice by Microinjection

The techniques of embryo isolation and culture together with those of microinjection and implantation have been developed and used by many laboratories (Hogan *et al.*, 1986), and are shown schematically in **Figure 2.2**. Single cell

embryos were microinjected with suitably prepared DNA from the p9Ka and tagged-p9Ka constructs described above. Three separate rounds of injections were carried out over a period of ten months. The numbers of mice born and the proportion of transgenic offspring that were obtained are summarised in **Figure 3.1.6**.

The procedures that were involved in production of transgenic mice by microinjection were complex (see Methods Section 2.2 and **Figure 2.2**), and each step was prone to various difficulties. The number of embryos available for injection and the proportion of suitably staged embryos depended on the age of the donor female mice, the dose of the hormones given to stimulate superovulation and the performance of the stud males used. Whilst tight control was imposed on these factors, there was considerable variation in both the numbers and the suitability of embryos for injection. Similarly, the suitability of the foster mothers for implantation was not totally reliable, and the added risk of surgery under general anaesthetic posed some minor problems. The quality of the DNA and the quality of the microinjection needles both affected the outcome of the microinjections. By using several preparations of DNA, and constantly monitoring and adapting the microinjection needles, successful injections were carried out. Probably the overwhelming factor in obtaining success with this procedure was the quality of the injection technique and of the techniques involved in manipulating the embryos in culture. That the early rounds of injections were not as successful as the later ones (**Figure 3.1.6**) reflected the time needed to master the techniques as much as any innate experimental variability.

3.1.5 Identification of Transgenic Progeny

Transgenic mice, carrying one of the p9Ka transgene constructs in their genome, were identified in two ways. PCR analysis of DNA prepared from tail biopsies (**Figure 3.1.7**) provided a relatively rapid means of identifying transgenic progeny. Positive control primers (HPRT1 and HPRT2) included in the reactions allowed any false negatives to be identified. These primers amplify a 224 bp region

of the single-copy mouse hypoxanthine ribosyl transferase (HPRT) gene and act as a control for the successful extraction of DNA and its amplification by PCR (S. Harris, personal communication). The two different transgenes could be identified by virtue of the different sizes of their PCR products (**Figures 3.1.3 & 3.1.7**). The transgene could be distinguished from the endogenous, single copy, mouse p9Ka gene, since the PCR primers were designed to anneal to intron regions of the p9Ka gene which are less homologous between rat and mouse than the coding (exon) regions of the two genes. For example, whilst having 100% identity with the rat gene, the 3´a primer (Methods **Figure 2.1**) has only 64% identity to the same region of the mouse gene.

The DNAs of all mice were subsequently screened by Southern blot analysis which had several distinct advantages over PCR. Firstly, by including copy-number controls on the Southern blots, an estimate of the number of transgene integrations could be gained. However, the copy-number shown for transgenic mice produced by microinjection are not always reliable since some of these mice can be chimeric (Hogan *et al.*, 1986). Southern blots also showed whether the complete transgene had integrated as opposed to a degradation product or a contaminating fragment. By digesting the tail biopsy DNA with *Eco*RI, it was possible to distinguish between the p9Ka transgene (giving 5.0 and 5.3 kbp bands) and the tagged-p9Ka transgene (giving a single 10.3 kbp band) (**Figure 3.1.7**). The ability to distinguish between mice containing the individual transgenes, by PCR or Southern blot analysis, was an advantage since it was possible that implanted embryos, or the foster mothers into which they were implanted and the subsequent litters of mice produced, could have been labelled incorrectly.

Seven transgenic offspring (the so called G_0 or founder transgenic mice) were identified amongst the litters of mice from the microinjected embryos. These mice were designated PK51, PK84, TPK6, TPK9, TPK40, TPK41 and TPK45. The PK or TPK referred to the p9Ka and tagged-p9Ka transgenes, respectively, the number being the identifier of the individual mouse. Since their transgene integrant was

present in only one copy of the host genome, all founder transgenic mice were deemed to be hemizygous, as opposed to heterozygous, which implies a corresponding allele is present in the other copy of that chromosome.

It is likely that, for each individual transgenic line, the site of integration of the DNA was different. Although this has not been formally shown in this case, it is usual for transgenic mice produced by microinjection to incorporate all copies of the transgene at a single locus, and for that locus to be different in each founder mouse (Hogan *et al.*, 1986).

3.1.6 Breeding of Founder Transgenic Mice to Produce Transgenic Mouse Lines

Each of the founder transgenic mice was mated with a non-transgenic mouse (or mice) to produce F_1 progeny, some of which would be expected to carry the transgene in their chromosomes. The transgenic status of all F_1 progeny was determined as for the founders.

Since the F_1 transgenic progeny cannot be chimeric, they can reliably be used to investigate the number of copies of the transgene present in the genome. For this purpose DNAs, from all transgenic lines and non-transgenic mice, were digested with *Eco*RI, a Southern blot was produced, probed with the p9Ka 10.3 kbp "transgene" and washed to high stringency [0.1 x SSC, 0.1% (w/v) SDS at 65 ^{o}C]. Several such blots were scanned with a microdensitometer, and a value for the number of integrated transgene copies was obtained. The number of integrated transgenes varied considerably. TPK41 had the highest copy number (approximately 66), followed by, in decreasing numbers of copies, PK51 (approximately 17), TPK9 and TPK40 (both approximately 4), PK84 (approximately 2) and TPK45 (apparently only a single integrated transgene).

Once transgenic offspring had been identified (except from TPK6 which did not exhibit germ-line transmission), some of the F_1 transgenic mice were used in further matings. Some of these hemizygous transgenics were mated with transgenic littermates to produce homozygous transgenics, i.e. with two copies of the transgene

integrant, one in each of the parental-derived genomes. The potential homozygous nature of these mice was investigated by Southern blot, and homozygotes identified due to a doubling of their transgene copy-number relative to the parental mice and to hemizygous littermates. Homozygous mice are useful not only as a breeding stock (all progeny of such mice are transgenic) but may exhibit additional phenotypic effects due to either an increased expression of the transgene or a double disruption at the chromosomal location of the transgene integrant.

As an example, the mating strategy for two of the transgenic lines are given in **Figures 3.1.8 & 3.1.9**. The numbers of offspring analysed from each mating is given along with the number of hemizygous and homozygous transgenic progeny. In most cases the transgene was passed on in a Mendelian fashion, although it was interesting to note that the proportion of PK51 female transgenic mice with regard to the total number of female offspring was very high. In the F_1 generation, 20 of the 23 female mice (87%) are transgenic and in the F_2 generation the number is 16 out of the 19 (84%). Since not all F_1 female offspring were transgenic, and some homozygous male mice were identified, this result was not due to an integration into the X chromosome. The numbers and type of transgenic progeny produced for each line are summarised in **Figure 3.1.6**.

3.1.7 Summary

Two p9Ka DNA constructs were produced for use as transgenes. One (p9Ka) consisted of 10.3 kbp of rat genomic DNA encompassing the known coding and non-coding exons, a 2 kbp upstream flanking region, 5 kbp downstream flanking region and minimal flanking plasmid sequence. The second (tagged-p9Ka) was essentially the same but for the inclusion of an insert in the p9Ka coding region designed to code for a human c-*myc*-derived epitope which is recognised by a commercially available antibody. These DNA constructs were successfully used in microinjections to produce a total of seven transgenic founders. Six of these founder animals were shown to exhibit germ-line transmission of the transgene, and were

used to produce a significant number of both hemizygous and homozygous transgenic offspring.

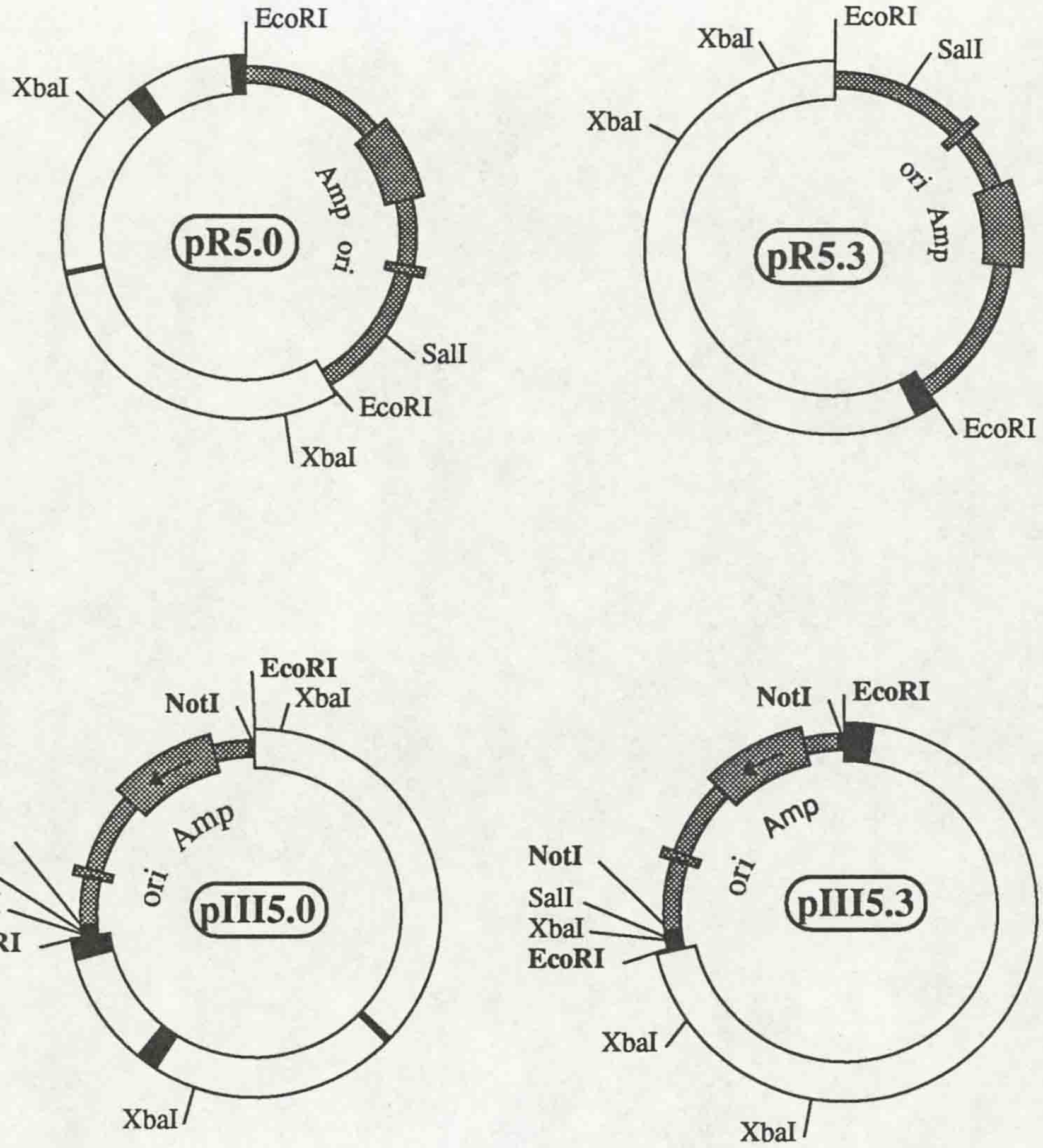


Figure 3.1.1

Maps of pR5.0 (pAT153 vector containing the 5.0 kbp p9Ka genomic fragment), pR5.3 (pAT153 vector containing the 5.3 kbp p9Ka genomic fragment), pIII5.0 (pPolyIII-I vector containing the 5.0 kbp p9Ka genomic fragment) and pIII5.3 (pPolyIII-I vector containing the 5.3 kbp p9Ka genomic fragment) plasmids.

Amp = Ampicillin-resistance gene
ori = origin of replication
= plasmid DNA
= multiple cloning site
= p9Ka-gene introns and flanking DNA
= p9Ka-gene exons

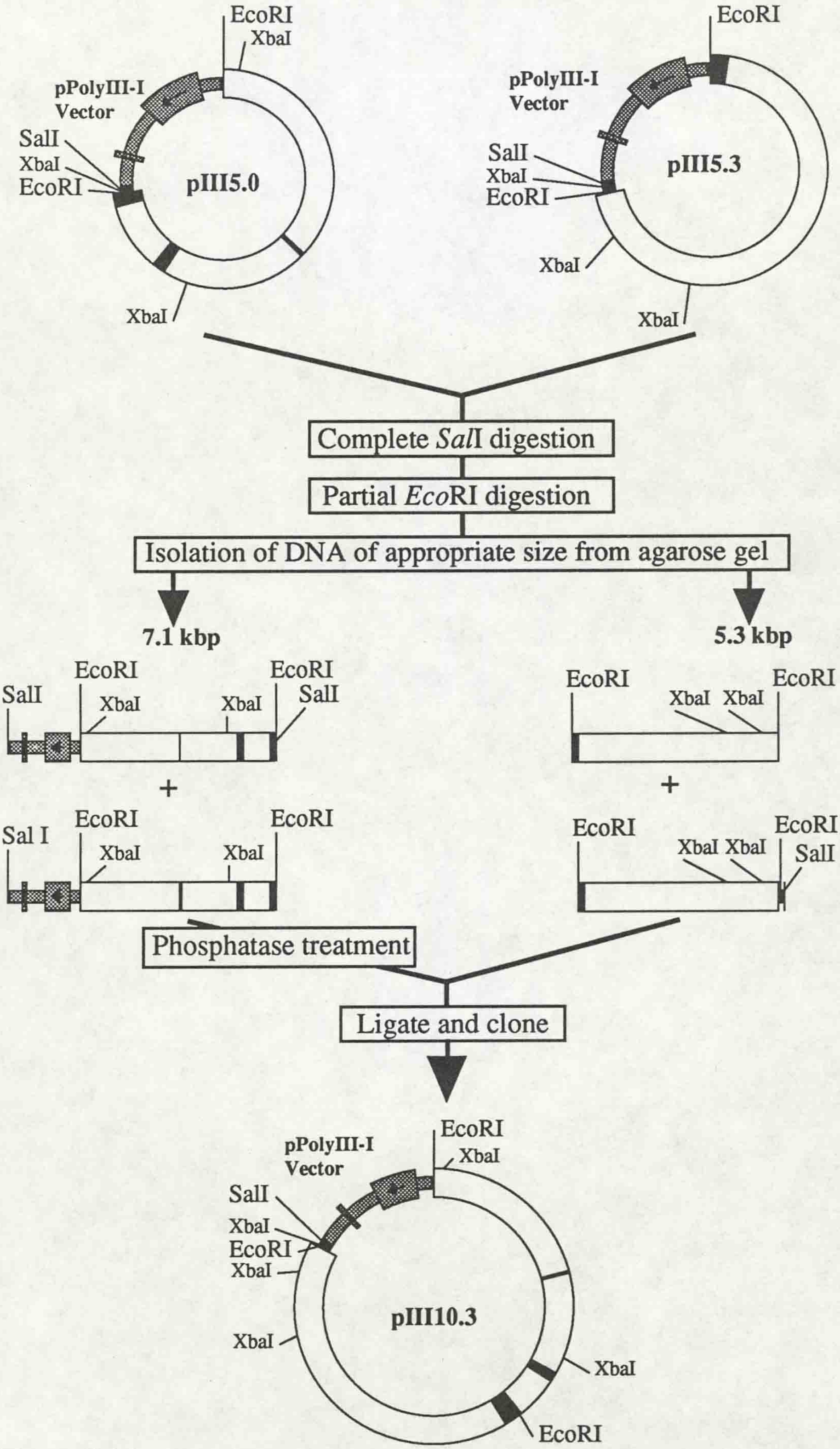


Figure 3.1.2
Strategy for construction of the 10.3 kbp clone of rat genomic p9Ka in pIII plasmid.

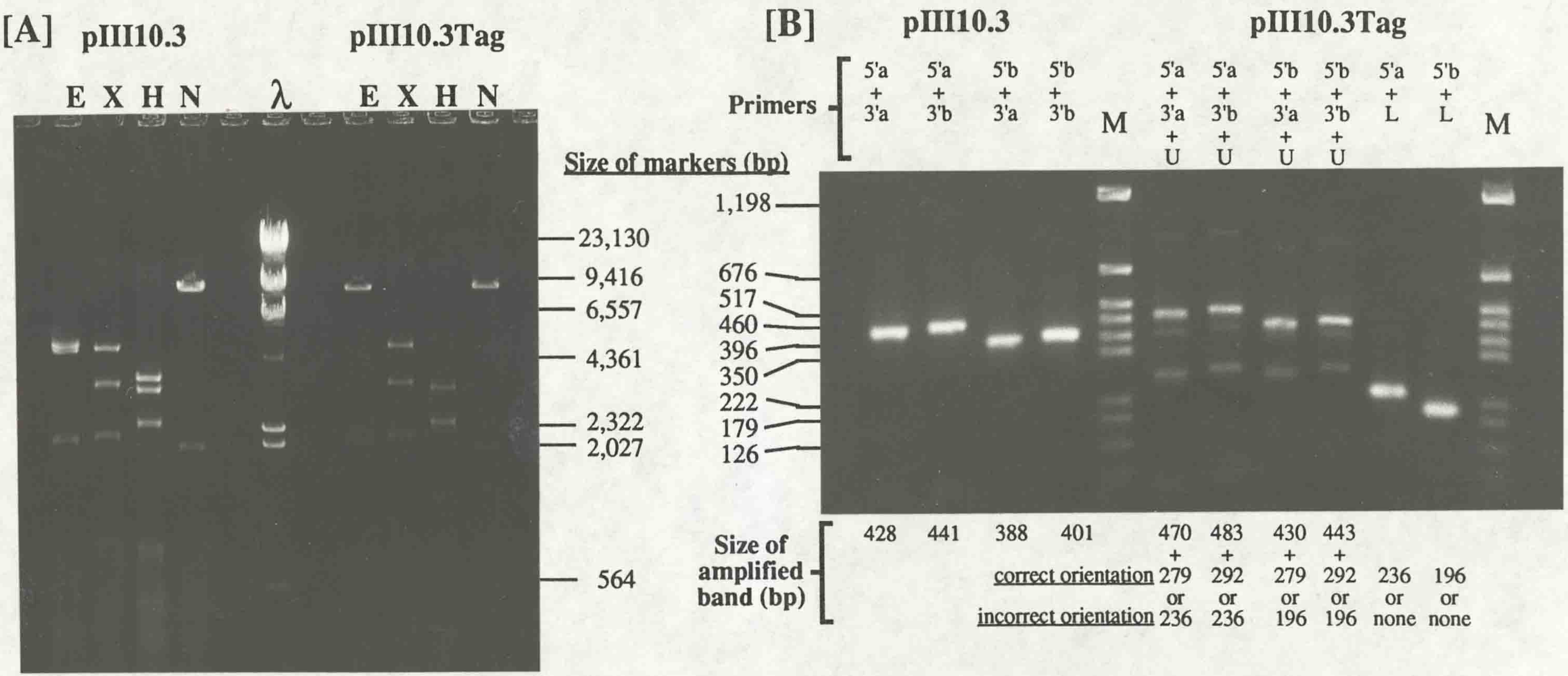


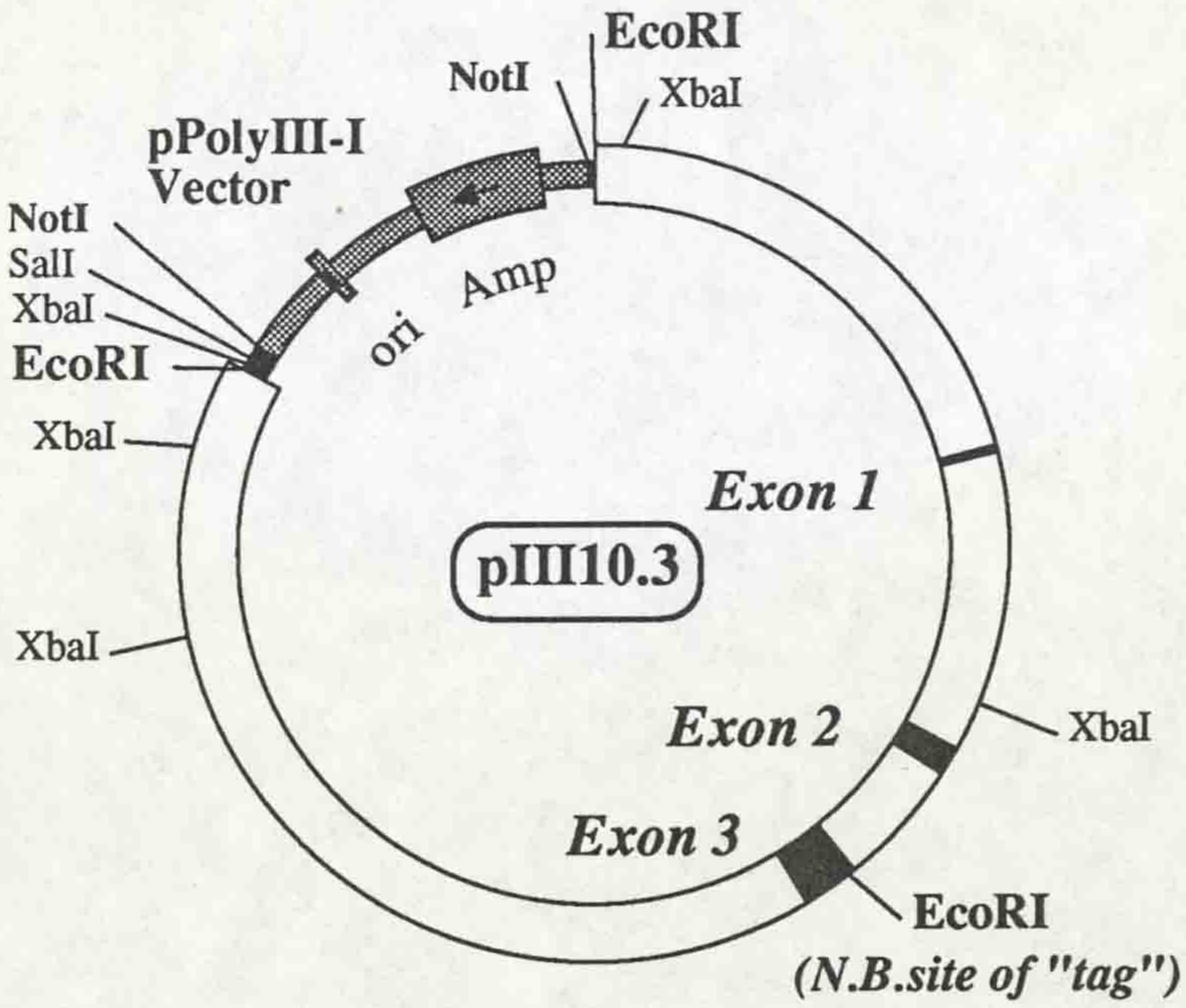
Figure 3.1.3

[A] Restriction-enzyme digest and [B] PCR analysis to confirm the plasmid constructs.

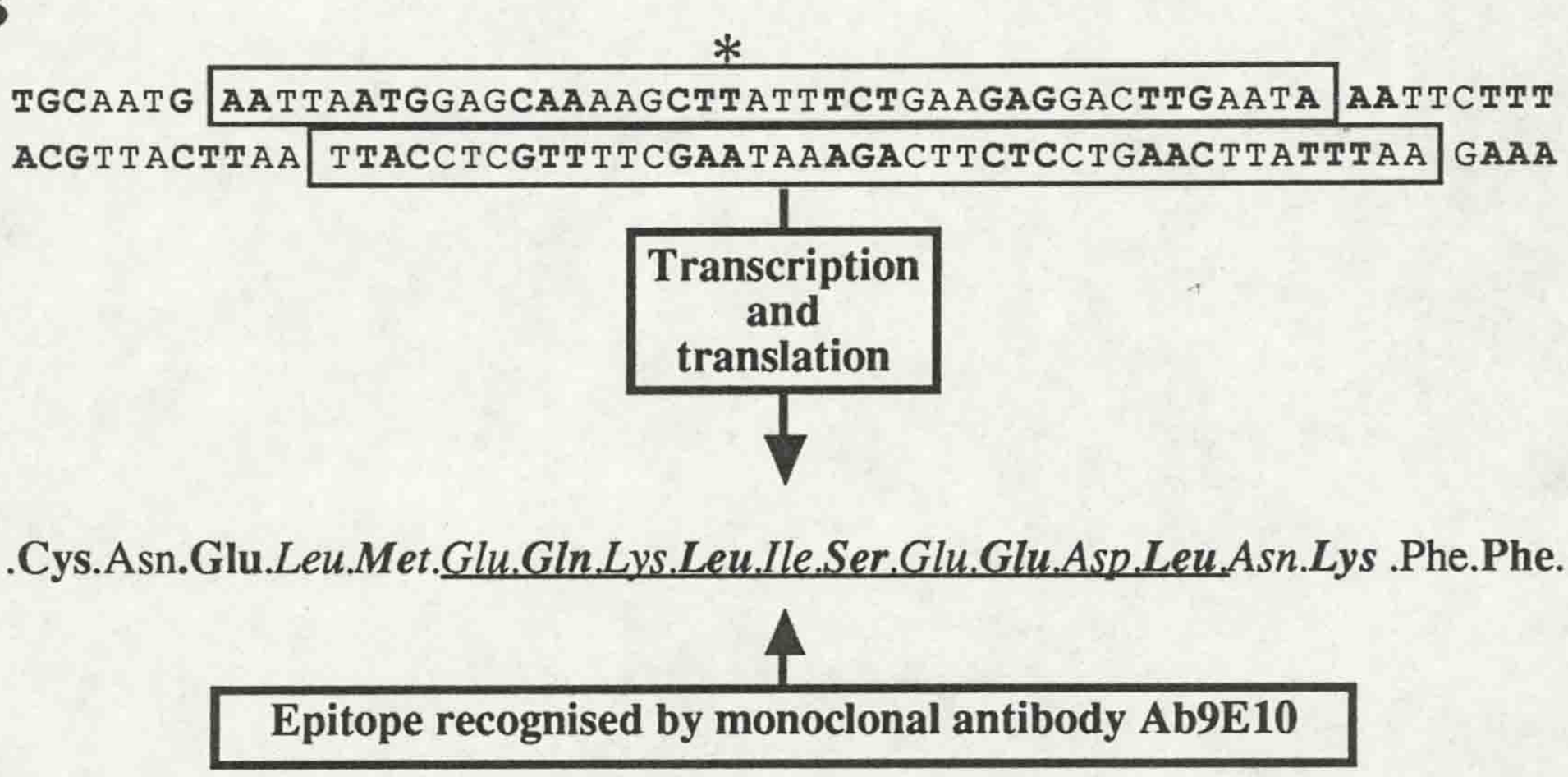
In [A]: λ = λ/*Hind*III DNA size markers; E= Plasmid digested with *Eco*RI; X= Plasmid digested with *Xba*I; H= plasmid digested with *Hind*III; N= Plasmid digested with *Not*I.

In [B]: M= pGem DNA size markers and primers indicated are those described in Figure 2.1.

A



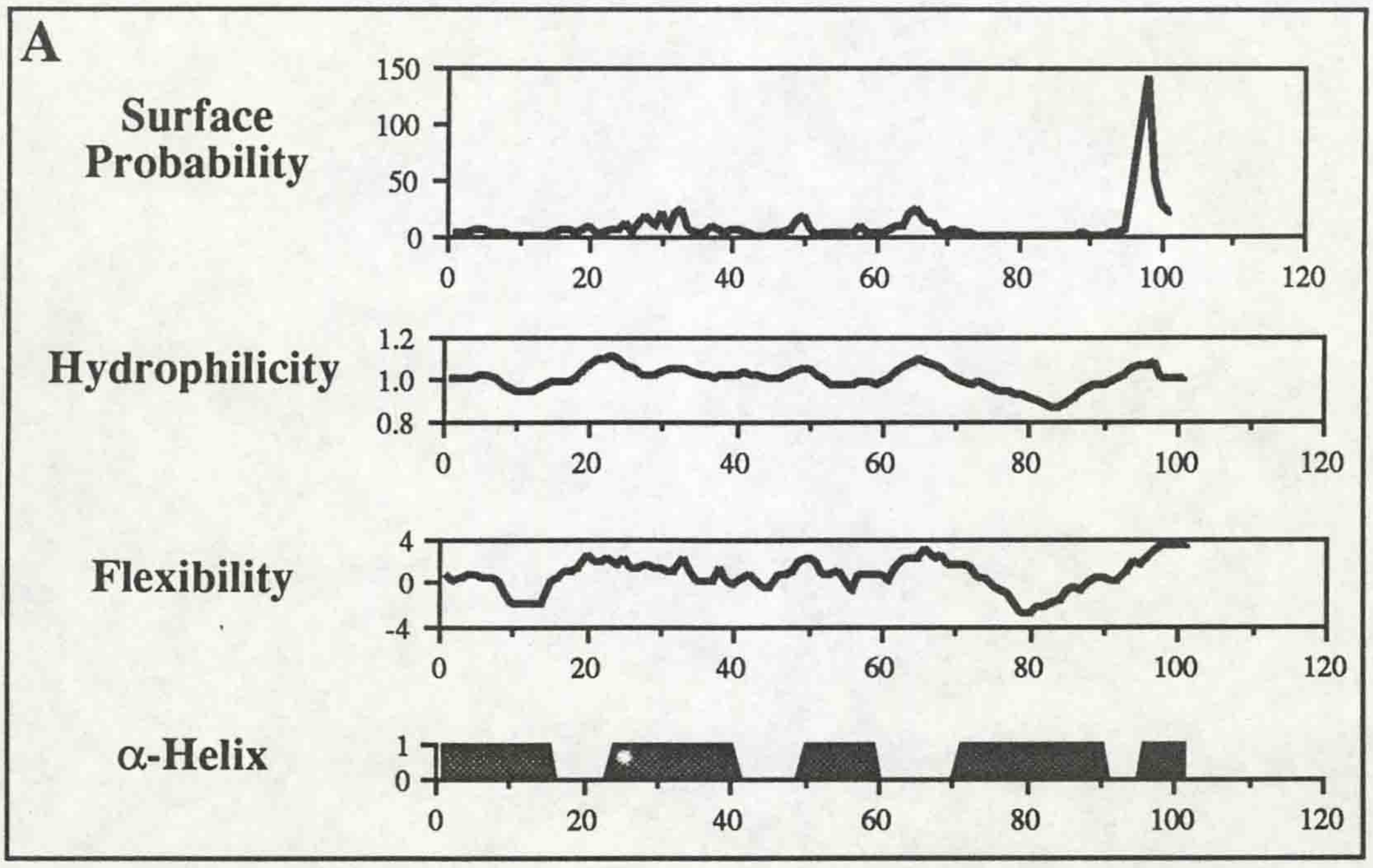
B



Figures 3.1.4

(A) Map of the plasmid pIII10.3 indicating the ampicillin-resistance gene (Amp), origin of replication (ori), plasmid DNA (▒▒), multiple cloning site (▬) and exons of the p9Ka gene (■).

(B) Diagram of "tag" inserted into p9Ka gene indicating nucleotide sequence (bold lettering defines codons, boxes indicate the tag oligonucleotides, asterisk marks nucleotide altered to produce *Hind*III site), protein sequence (bold amino acids correspond to bold codons, italics indicate residues coded for by tag), and tag epitope (defined by underlined residues).



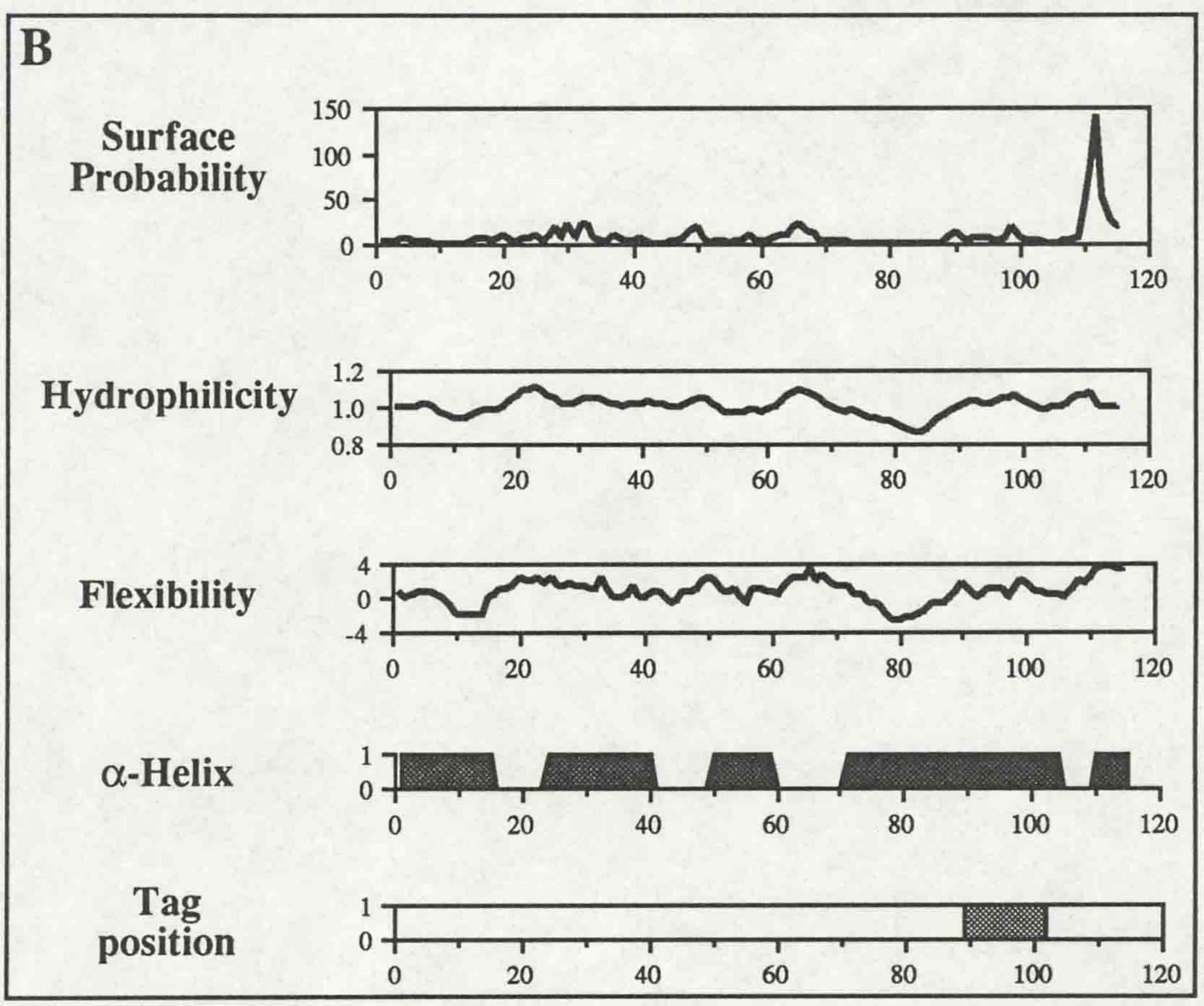


Figure 3.1.5

Protein Structure Predictions for (A) p9Ka and (B) Tagged-p9Ka as generated by the University of Wisconsin Genetics Group "GCG" software package.

Numbers along the x axis refer to amino acid position.

Helix = Garnier-Osguthorpe-Robson (Garnier *et al.*, 1978)

Hydophilicity = Kyte-Doolittle (Hopp & Woods, 1981)

(Chain) Flexibility = Karplus-Schulz (Karpus & Schulz, 1985)

Surface Probability = Emini (Emini *et al.*, 1985)

[A]

DNA construct		p9Ka (PK)			Tagged-p9Ka (TPK)	
Injection round		1	2	3	2	3
Injected embryos implanted		163	272	287	147	274
Foster mothers		9	12	13	7	13
Litters born		4	5	11	3	9
Mice born	*male*	17	16	15	6	49
	female	11	18	19	6	37
Transgenic mice	*male*	0	1	0	0	2
	female	0	0	1	0	3

[B]

	F_1		F_2			F_3		
MouseLine	Born	hemi	Born	hemi	homo	Born	hemi	homo
PK51	45	32	32	11	14	16	0	16
PK84	6	2	55	19	4	6	1	0
TPK6*	29	0						
TPK9	20	2	28	14	0	8	4	2
TPK40	33	17	36	17	3	22	11	0
TPK41	14	7	32	14	8			
TPK45	6	1	64	43	0	13	7	0

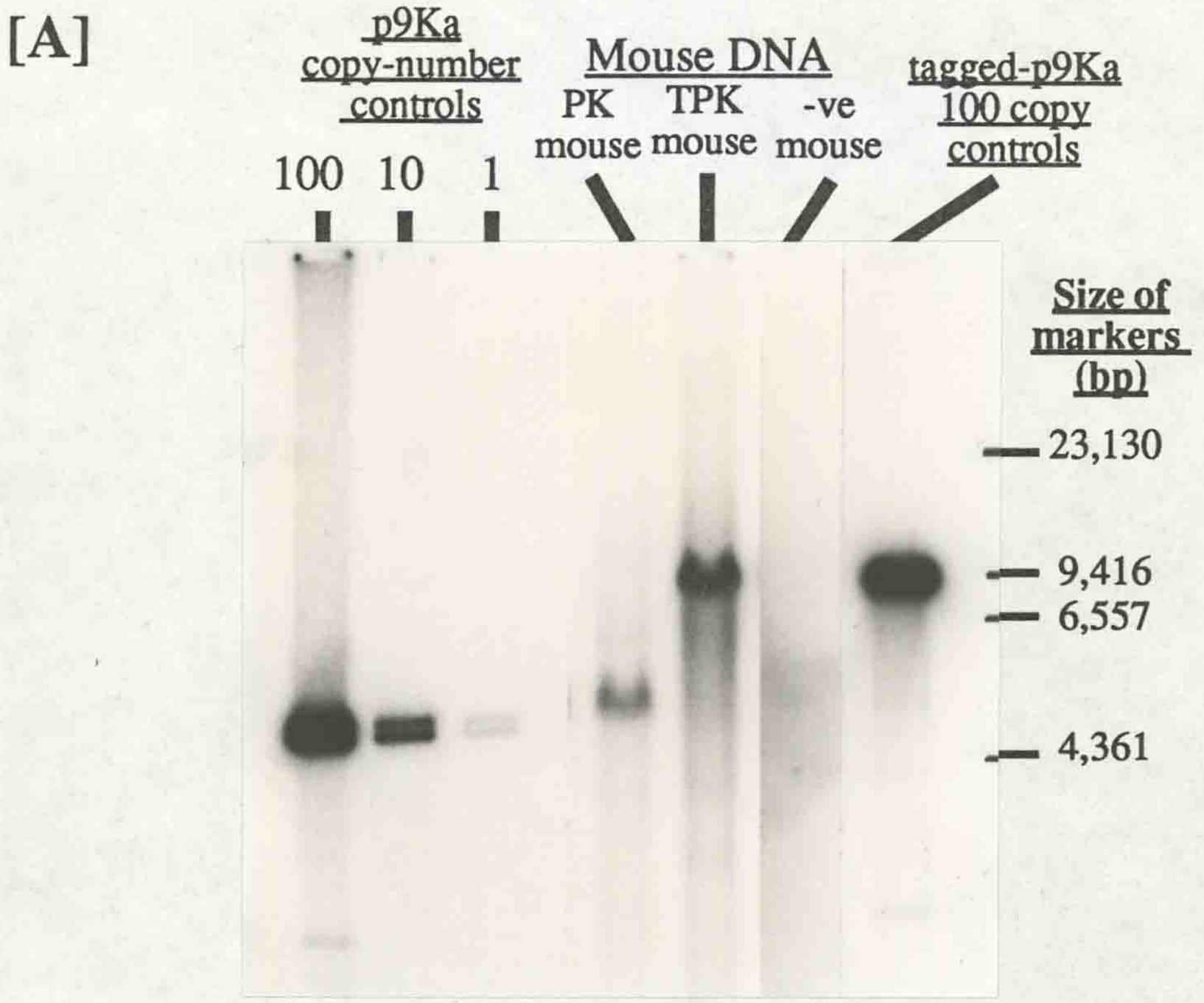
Figure 3.1.6

[A] Summary of embryo injections, implants into pseudopregnant foster mothers and resultant litters, including numbers of transgenic offspring.

[B] Summary of mice produced by mating.

Key for [B]

Born = number of mice born, weaned and screened for transgenic status
hemi = hemizygous offspring
homo = homozygous offspring
F_1, F_2, F_3 = generation number
N.B. * = the founder transgenic of this line did not exhibit germ-line transmission



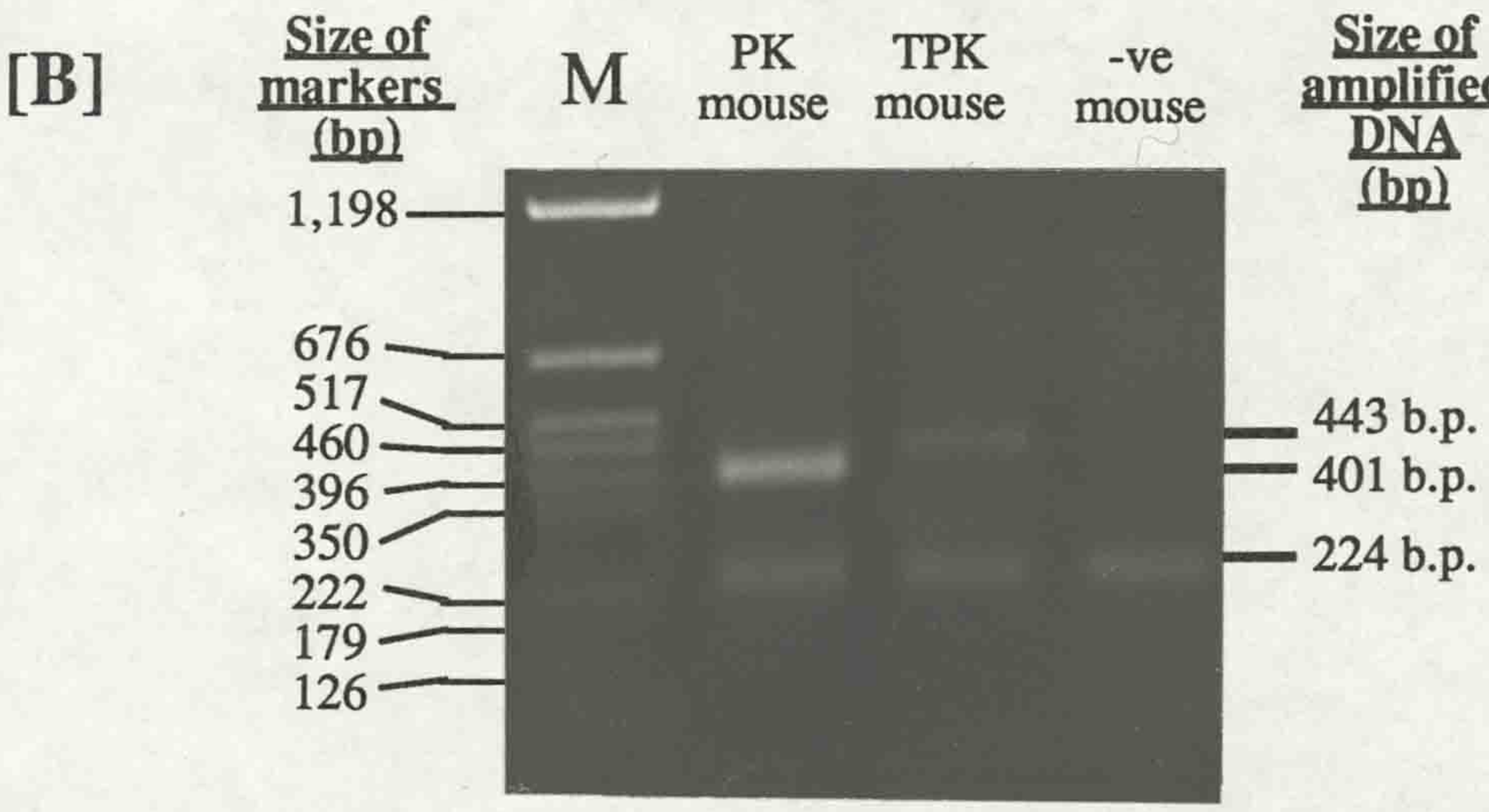


Figure 3.1.7

[A] Southern blot and [B] PCR analysis of transgenic mice.

In [A] 10 μg of genomic DNA, from either PK transgenic mouse, TPK transgenic mouse or non-transgenic mouse, was digested with *Eco*RI and analysed by Southern blot hybridisation with a rat genomic p9Ka probe. Also included were copy number controls, i.e. dilutions of *Eco*RI-digested plasmid DNA approximating to a specific number of copies of the transgene per haploid mouse genome (as indicated).

In [B] PCR was performed on crude digests of mouse-tail biopsies using primers 5'b, 3'b, HPRT1 and HPRT2 (see Figure 2.1); aliquots of the PCR products were run on a 1.5% (w/v) agarose gel and visualised using ethidium bromide and u.v. illumination; pGem DNA size markers (M) and the sizes of the PCR products are indicated.

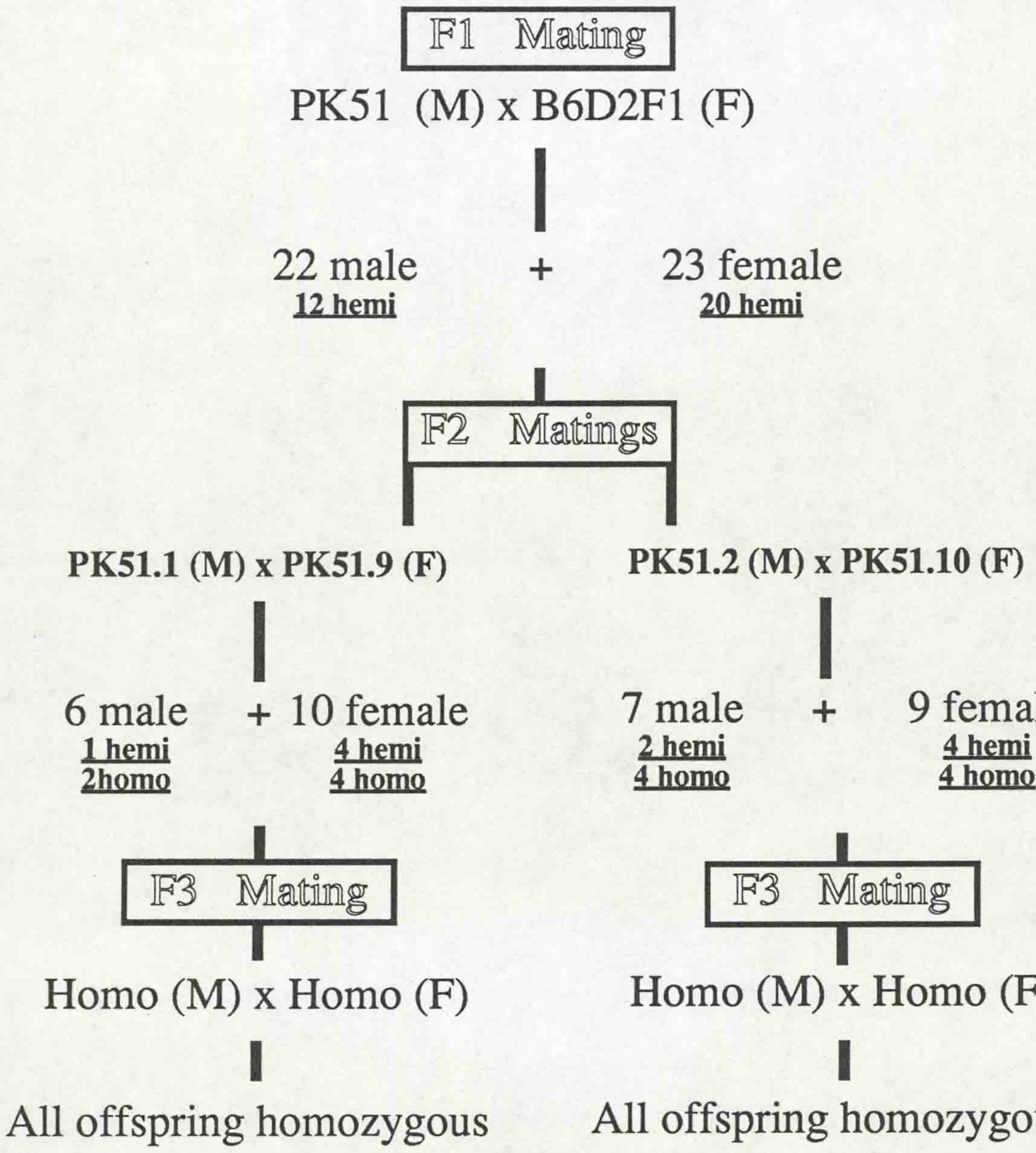


Figure 3.1.8

Flow diagram indicating the mating strategy for the PK51 transgenic mouse line. Total numbers of mice born and screened are given, together with numbers of hemizygous (hemi) mice and homozygous (homo) mice for each set of litters. Gender is indicated as male (M) or female (F) and numbers following line number indicate specific mice.

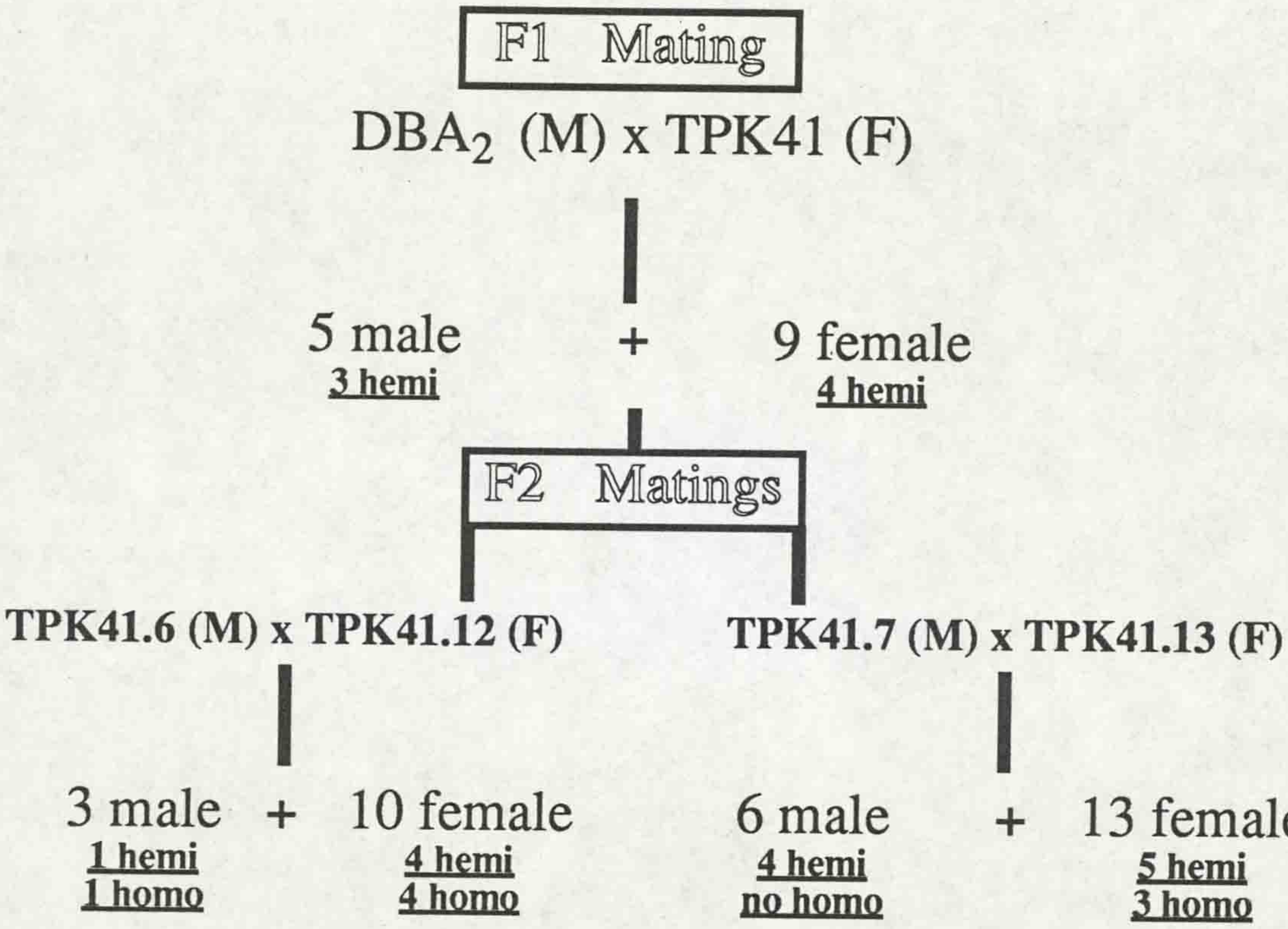


Figure 3.1.9

Flow diagram indicating the mating strategy for theTPK41 transgenic mouse line. Total numbers of mice born and screened are given, together with numbers of hemizygous (hemi) mice and homozygous (homo) mice for each set of litters. Gender is indicated as male (M) or female (F) and numbers following line number indicate specific mice.

3.2 Analysis of the Expression of p9Ka in Transgenic and Non-Transgenic Rodent Tissues

Once mice transgenic for the p9Ka and tagged-p9Ka genes had been produced, there were two main priorities. The first priority was to analyse the expression of the two transgenes in the individual transgenic mouse lines. The second priority was to maintain a significant number of mice in order to detect any phenotypic effect caused by the integration of the p9Ka or tagged-p9Ka transgene DNAs and/or by their expression. The former will be dealt with here, while the latter will be considered in the next chapter (3.3)

The first line of investigation was conducted at the level of mRNA expression, principally using Northern blot hybridisation. The next form of analysis was conducted at the level of protein expression, for which the tagged-p9Ka construct was specifically designed, both using Western blot and immunocytochemical means. During the course of this project a polyclonal antibody to rat p9Ka became available from Fiona Gibbs (Ph.D. student at the University of Liverpool), and facilitated direct analysis of the expression of p9Ka protein. The resultant analysis of the expression of p9Ka protein in rodent tissues is therefore novel, since this is the first time that such an antibody had become available.

Expression of the p9Ka transgenes can be compared and contrasted with the expression of the endogenous p9Ka gene in rat and mouse tissues. This analysis is particularly interesting due to the apparent differences in expression of p9Ka mRNA between species (see Introduction, section 1.8). The pattern of expression of p9Ka mRNA in the individual transgenic mouse lines is also of interest in itself, since it may be influenced by the copy number of the transgenes, their site of integration and the nature of the construct used. Since we had included a considerable amount of intron and flanking DNA in the transgene constructs, it was hoped that the expression of p9Ka mRNA and protein would be characteristic of the rat gene. The pattern of expression of the p9Ka protein in rat and mouse has not been well studied,

but the availability of an antibody to p9Ka, and the ability to detect the epitope inserted into the tagged-p9Ka transgene product, should also allow an investigation at this level. Differences in post-transcriptional events between rat and mouse may influence the pattern of expression of the rat gene in the transgenic mouse. Thus studies of the expression of the rat p9Ka gene in transgenic mice provide a way of investigating both transcriptional and post-transcriptional effects on expression of the rat gene in the mouse cells. Indeed the use of transgenic mice has distinct advantages over transfection of *in vitro* cell cultures, since the transgene is subject to the same developmental changes in chromatin structure that are believed to be involved in control of the expression of the normal gene. A further advantage is that the cells in which expression is being studied have not been subject to the processes of immortalisation and transfection which may affect gene expression (as discussed in the Introduction, section 1.3).

3.2.1 Expression of p9Ka mRNA in Rat Tissues

The distribution of expression of p9Ka mRNA in different tissues was first investigated in the rat by Northern blot analysis, and this mRNA was found to be more widespread than anticipated (**Figure 3.2.1**). A single, major band was detected upon autoradiography of Northern blots which had been hybridised with a radioactively-labelled p9Ka cDNA. The size of this p9Ka mRNA was the same as that seen for Rama 29 RNA and agreed well with that described previously (between 700 and 800 nucleotides; Barraclough *et al.*, 1984b). Occasionally larger bands (approximately 1400 and 2500 nucleotides) were observed in some lanes on blots probed with the p9Ka cDNA (**Figure 3.2.1**). The intensity of the bands of higher molecular mass is usually in proportion to the intensity of the major band of p9Ka mRNA, and their sizes agree well with these bands possibly representing preprocessed forms of p9Ka mRNA. That is to say that they were of the size expected for unspliced forms of p9Ka mRNA with intron sizes of 675 and 1172 nucleotides.

All tissues analysed showed some level of p9Ka mRNA (**Figure 3.2.1**). These tissues included lung, mammary gland, spleen, kidney, heart, brain, uterus, stomach, ovary, thymus, salivary gland and lymph node. As expected, the expression of p9Ka mRNA was less than that of the Rama 29 cell-line which was included as a positive control in all subsequent Northern-blot analysis.

3.2.2 Difference in Expression of mRNA Between Rat and Mouse p9Ka Genes

Differences in the expression of p9Ka mRNA were seen between rat and non-transgenic mouse tissues, both during analysis of levels of transgene mRNA between the transgenic lines (**Figures 3.2.5, 3.2.6 & 3.2.7**) and when comparing levels between rat and mouse p9Ka mRNA directly on a single Northern blot (data not shown). Despite the fact that levels of p9Ka mRNA in rat and mouse cannot be corrected for anomalies in loading (as discussed below), the raw data, when plotted as bar graphs (**Figure 3.2.2**), highlights a significant difference in the tissue specific expression between these two closely-related species. This difference in the pattern of expression was also seen when non-transgenic mouse and rat data from the "all line" blots (**Figures 3.2.5, 3.2.6 & 3.2.7**) was compared, and in this case the mouse data had been corrected for unequal loading, by comparison with the transgenic mouse samples.

The differences in expression of p9Ka mRNA between rat and mouse p9Ka genes are two-fold, distribution in different tissues and absolute level. Most obviously the expression of p9Ka mRNA in the spleen of the (non-transgenic) mouse was unusually high (**Figure 3.2.2**). This tissue was the highest expressing one in the mouse, whilst the other tissues exhibited a pattern of expression similar to that found for expression of the rat gene (in rat or in transgenic mice). The other major difference in the expression of p9Ka mRNA is that it was considerably lower in the normal mouse compared to that in rat or in transgenic mice (as measured on blots probed with a rat p9Ka cDNA, **Figures 3.2.5, 3.2.6 & 3.2.7**).

The inability to make corrections for unequal loading of RNA when comparing

expression of p9Ka mRNA between species was due to the fact that the differences in the levels of constitutive mRNAs between rat and mouse samples were too large to arise from a loading artefact (e.g. in the actin-probed blots, **Figures 3.2.5, 3.2.6 & 3.2.7**). The actin cDNA probe seems to anneal more effectively to rat mRNA, since for all tissues the intensity of the hybridisation band for the rat sample is greater than that for the same tissue from any of the mice analysed. There is no reason to suspect that expression of actin is consistently higher in rat. Differences in levels of the corresponding mRNAs were not so apparent when the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was used as a probe, and this result can be explained by the greater homology between rat and mouse G3PDH mRNAs than between rat and mouse actin mRNAs. The G3PDH gene is not only better conserved than that for actin, but the use of a human G3PDH cDNA effectively reduces the apparent differences between rat and mouse.

That the level of p9Ka mRNA expression was seen to be significantly lower in mouse compared to that in rat could also be due to differences in probe hybridisation between species. The 335 bp, partial, rat p9Ka-cDNA probe used shows a high (93.4%) level of homology with the murine p9Ka cDNA (Jackson-Grusby *et al.*, 1987). Such a minor difference in homology may not be expected give rise to the large difference in the level of hybridisation with rat and mouse mRNAs necessary to cause the observed disparity between the measured levels of rat and mouse p9Ka mRNA. To confirm that the observed differences in levels of expression were not an artefact of the probe used for hybridisation, the apparent difference between the levels of rat and mouse p9Ka mRNA was investigated further.

Firstly, the levels of rat and mouse p9Ka mRNA was re-assessed using a 356 bp murine, p9Ka-cDNA probe (18A2, Jackson-Grusby *et al.*, 1987; kindly provided by Dr. D. Linzer, Northwestern University, Illinois). Duplicate Northern blots of rat and mouse total RNA were incubated with either the murine, p9Ka-cDNA probe (18A2) or the rat, p9Ka-cDNA probe (data not shown). The tissue distribution patterns of the rat and mouse p9Ka-mRNAs was the same as that already

described, irrespective of the cDNA probe used. Although the blot incubated with the murine, p9Ka-cDNA did not exhibit as marked a difference in rat and mouse expression as observed with the rat, p9Ka-cDNA, the levels of p9Ka mRNA in the rat were still generally greater than those in the mouse (with the exception of the spleen). This result confirms that expression of p9Ka mRNA in the rat is generally higher than that in mouse, but the question still remains as to how different these levels of expression are. To address the question of which p9Ka-cDNA probe is the more appropriate when comparing the two different p9Ka mRNAs, a direct comparison between the two probes used (i.e. between rat and murine p9Ka cDNA) was performed by DNA dot-blot analysis. Duplicate DNA dot-blots of dilutions of each type of probe were hybridised with either a rat, p9Ka-cDNA probe or a mouse, p9Ka-cDNA probe, and washed in the same way as in the Northern blot analysis. Analysis of autoradiographs of these blots revealed that the rat, p9Ka-cDNA probe hybridised more uniformly to both the two target cDNAs than did the murine, p9Ka-cDNA probe (data not shown). Thus, it would seem to follow that the analysis of mRNA levels with the rat, p9Ka-cDNA probe probably gives a more accurate result than that with the murine, p9Ka-cDNA probe, since the rat cDNA, when used as a probe, is able to detect both mouse and rat mRNAs at more equal efficiencies. However, it must be remembered that the properties of DNA-DNA hybrids may be different to those of DNA-RNA hybrids, due to differences in secondary structure.

The murine, p9Ka cDNA was also used to address the wider issue of the specific pattern of expression of p9Ka mRNA in different tissues of the mouse (since it proved to be more sensitive for mouse mRNA than the rat, p9Ka-cDNA probe). Despite the difference in the pattern of expression, and the much lower level of expression in the mouse (when compared to the rat), it was possible to detect mouse p9Ka mRNA in a wide range of tissues (data not shown). Expression of p9Ka mRNA was shown to occur in various mouse tissues including lung, mammary gland, spleen, kidney, heart, brain, uterus, stomach, ovary and thymus, and the pattern of expression observed was consistent with that seen previously (**Figure**

3.2.2).

3.2.3 Initial Analysis of Expression of Transgene mRNA

Since the range of tissues expressing p9Ka mRNA in the rat was so wide (**Figure 3.2.1**), it was decided to limit the range of tissues to be studied for expression of transgene mRNA. Lung was an obvious choice, since it was the highest expressing tissue, mammary gland was another choice, since it was a prime site of interest, and by contrast, brain was also analysed since it was a relatively low expressing tissue.

Initial analysis of mRNAs by Northern-blot of two or more of these tissues in the available transgenic mice (PK51, PK84, TPK9, TPK40 and TPK41) indicated that each line was expressing transgenic mRNA (data not shown). There was no major difference in the size of the p9Ka transgene mRNAs compared to the normal rat p9Ka mRNA (all major p9Ka mRNA bands were approximately 700 - 800 bases in length). There was some evidence of the tagged-p9Ka mRNA being larger (due to the presence of a 42 nucleotide tag-coding insert), but this was not always obvious. As with rat mRNA, minor high-molecular-weight bands believed to represent unspliced transcripts of p9Ka mRNA were present in some tissues.

The levels of p9Ka and tagged-p9Ka transgene mRNAs were so much greater than the level of murine p9Ka in non-transgenic mice, as assayed by hybridisation to a rat p9Ka cDNA probe, that this endogenous expression was barely detectable without long exposure times for these Northern blots. This preliminary analysis indicated that, in all transgenic mouse strains, the expression of the transgenes followed a similar pattern to that of the natural p9Ka gene as observed in the rat. The lung expressed p9Ka mRNA at the highest level of the tissues tested and brain expressed p9Ka mRNA at the lowest level, whilst the mammary gland expressed the p9Ka mRNA at an intermediate level. The transgenic mouse lines expressed different amounts of transgene mRNA; TPK41 expressed the most, followed by PK51, TPK40, TPK9 and finally PK84. A similar tissue distribution of expression

was seen for the endogenous mouse mRNA, but absolute levels were much lower .

3.2.4 Comparison of Expression of Transgene mRNA Between Individual Mouse Tissues and Between Transgenic Lines

Since there were large differences in levels of expression of p9Ka mRNA between the transgenic lines, it was important to determine the range of mRNA levels over which Northern blot hybridisations with p9Ka cDNA and with constitutive cDNAs (actin and G3PDH) were effective for the determination of relative levels of expression. A Northern blot containing a range of amounts of total RNA for each of two samples, Rama 29 and PK51 transgenic kidney, was produced. This blot was incubated consecutively with radioactively-labelled cDNAs for p9Ka, G3PDH and actin (**Figure 3.2.3**), and at least two autoradiographic exposures were performed for each hybridised blot. The autoradiographs were scanned and values of peak area (in arbitrary units) were taken as a measure of the level of mRNA (see Methods, 2.3.16). Graphs were then plotted of peak area against the amount of total RNA loaded on the gel (**Figure 3.2.4**). To account for possible loading inaccuracies, the peak area values of the different exposures were plotted against a single set of peak area values for each sample (**Figure 3.2.4**). The shapes of the graphs remained unaltered. From these graphs, the relationship between peak area and amount of RNA loaded was effectively linear for peak areas below 3×10^5, and above this threshold remained approximately linear, but with a decreased slope. Thus at the range of exposures produced in this experiment, the absolute measurement of peak area is proportional to the amount of mRNA loaded, but above a peak area of 3×10^5 the relationship was not the same as below this threshold. This effectively means that, although peak area can be taken as a direct measure of amount of mRNA present, care should be taken in interpreting blots on which peak areas exceed 3×10^5. The range of expression of transgenic mRNAs is so great that it is impossible to analyse accurately all tissues or all lines on the same blot without exceeding peak areas of 3×10^5 for some samples. However, since there is still a positive

relationship between peak area and amount of mRNA at higher peak areas, trends in expression can be shown over a wide range of mRNA levels.

Once Northern blot analysis was shown to be capable of measuring levels of p9Ka mRNA over a reasonable range, it was possible to extend the initial analysis to other tissues. The number of tissues analysed was extended from lung, mammary gland and brain, to include kidney, spleen (a lymphoid tissue) and heart (a muscle tissue); the latter three tissues express p9Ka mRNA at an intermediate level in the rat. Two sets of Northern blots were subsequently produced and probed for p9Ka, actin and G3PDH mRNAs. One set of blots consisted of two tissues of female mice from each transgenic mouse line, non-transgenic mouse and rat ("all line" blots, **Figures 3.2.5, 3.2.6 & 3.2.7**), and the other of all six selected tissues from mice of two transgenic mouse lines along with non-transgenic lung and brain ("all tissue" blots, **Figures 3.2.8, 3.2.9 & 3.2.10**). Each set of blots was hybridised with the requisite cDNAs, and all subsequent exposures to X-ray film performed at the same time, with the exception of the PK51 and TPK41 "all tissue" blot. In this case two short exposures replaced one longer one for the other "all tissue" blots since, from the initial analysis, these transgenic mice were known to express the highest levels of transgene mRNA.

Although the pattern of expression between transgenic mouse lines ("all line" blots, **Figures 3.2.5, 3.2.6 & 3.2.7**) or between tissues of individual mouse lines ("all tissue" blots, **Figures 3.2.8, 3.2.9 & 3.2.10**) is quite clear from the autoradiographs of blots probed for p9Ka mRNA, a true picture cannot be formed unless possible discrepancies due to unequal loading of the gels are ruled out. For this purpose peak areas of p9Ka mRNAs were corrected for possible variations in loading by comparison of peak areas of mRNA for each constitutive probe. The peak area values for p9Ka mRNA from any one autoradiograph were corrected using data from two autoradiographic exposures of blots probed for each constitutive mRNA, and an average of these four determinations was recorded. The use of two constitutive probes and two exposures of each effectively maximises variations in

correction due to the use of different peak areas for constitutive mRNAs (and this variation is indicated by the standard deviation of the mean). The average corrected peak area of any one p9Ka exposure, when plotted with an error bar representing the standard deviation of the mean is thus a good indicator of the level of p9Ka mRNA, providing the standard deviation is small. The same analysis was carried out individually for at least two separate exposures of each blot incubated with radioactively-labelled p9Ka cDNA.

Such an evaluation of p9Ka mRNA was performed for each blot, and graphs representing the variation in levels of transgene mRNA in any one tissue, between each transgenic mouse line are shown in **Figure 3.2.11**. Similarly, graphs representing the variation in levels of transgene mRNA between selected tissues in any one transgenic mouse line are shown in **Figure 3.2.12**. By taking comparable p9Ka exposures for each "all tissue" blot, an analysis of variation between lines for each tissue was performed. This analysis agreed well with that carried out from the "all line" blots. Similarly, analysis of the levels of mRNA in different tissues from the "all line" blots agreed well with that carried out from the "all tissue" blots.

The relative levels of transgene mRNA between the different transgenic mouse lines (**Figures 3.2.5, 3.2.6, 3.2.7 & 3.2.11**) conforms to a general pattern. In order of decreasing expression the pattern was TPK41, PK51, TPK40, TPK9, TPK45 and PK84. These levels of expression would seem to parallel broadly the copy number of the transgenes present in the various mouse lines. Thus, as mentioned previously, TPK41 has the highest copy number (approximately 66), followed by PK51 (approximately 17), TPK9 and TPK40 (both approximately 4), PK84 (approximately 2) and TPK45 (apparently only one integrated transgene).

In order to investigate further the copy-number dependence of mRNA expression for the p9Ka transgenes, a dot blot of total RNAs from lungs of transgenic mice, non-transgenic mouse and rat was produced and hybridised to a radioactively-labelled p9Ka cDNA. Several autoradiographic exposures of the resultant blots were analysed, in a similar manner to those of the Northern blots, and

a graph of the relationship between relative expression of p9Ka mRNA and transgene copy-number was produced (**Figure 3.2.13**). It is clear that the expression of transgene mRNA in the lung is dependent upon the number integrated copies of the transgnene.

The pattern of distribution of transgene mRNA in the different tissues of mice from the individual transgenic lines (**Figures 3.2.8, 3.2.9, 3.2.10 & 3.2.12**) is less clear due to the largely similar levels of transgene mRNA in mammary gland, spleen, heart and kidney. It is very clear, however, that, of those tissues tested, lung was always the highest expressing tissue and brain was the lowest. Hence, the general pattern was the same, and since no tissues that express p9Ka mRNA in any one line of transgenic mice failed to do so in the other lines, the results suggest that the distribution of transgene mRNA in the different tissues was approximately the same for all transgenic lines produced. Importantly, the distributions of transgene mRNAs in the different tissues were apparently the same for both the p9Ka and tagged-p9Ka transgenes, and the pattern of expression of the rat-derived p9Ka transgenes, in the various transgenic mouse lines, is more akin to that in the rat than that in the mouse (**Figures 3.2.2 & 3.2.12**).

It should be noted that although a male PK51 mouse was used in this particular "all tissue" analysis, other Northern blots that analysed the expression of p9Ka mRNA in the female PK51 mouse, or that compared directly PK51 male and female mice showed the same pattern of expression in different tissues irrespective of gender. The question of expression of transgene mRNA in male mice from other lines was addressed by analysis of Northern blot of RNA from lung and mammary fat pad (the latter was of particular interest since male mammary fat pad contains no glandular elements). Both these tissues expressed transgene mRNA, and in all transgenic mouse lines expression in the lung was greater than that in the mammary fat pad. Direct comparison of expression in male mammary fat pad and female mammary gland indicated that, despite obvious differences in the glandular nature of these tissues, they both expressed the p9Ka mRNA at similar levels relative to non-

transgenic controls (data not shown).

It was not possible to compare properly expression of p9Ka mRNA in the rat with that in the transgenic and normal mouse lines since it was not possible to correct for unequal loading between species (as discussed in section 3.2.2).

Whilst analysing the expression of p9Ka mRNA it was noted that the levels of the constitutive mRNAs in the different tissues were markedly different, as shown in the "all tissue" analysis (**Figures 3.2.8, 3.2.9 & 3.2.10**). Upon comparison of samples from the same tissue of different mice on the "all line" blots (**Figures 3.2.5, 3.2.6, 3.2.7**) and on the "all tissue" blots (**Figures 3.2.8, 3.2.9 & 3.2.10**) such differences seem not to be due to any loading anomalies. The inter-tissue variations of constitutive mRNAs does not effect the analysis presented here, since all corrections are limited to samples from the same tissue type and the two sets of Northern blots thus performed agreed well when compared.

3.2.5 Analysis of Expression of Transgene Protein by Western Blot

When this project was started, no antibody was available to study the expression of p9Ka protein and thus an epitope to a commercially available antibody was used to "tag" the p9Ka protein produced by the tagged-p9Ka transgene. However, as mentioned previously, our laboratory has produced an antibody to p9Ka, and it has been used to study the expression of p9Ka protein in the transgenic mice. The polyclonal nature of this antibody meant that it could also be used to detect tagged-p9Ka protein (although the relative affinity of the antibody to p9Ka and tagged-p9Ka is not known).

In order to show that the transgenes were expressed at the protein level, Western blots were performed on samples of total protein extracted from lung and mammary gland of transgenic and non-transgenic mice. Blots produced also included a sample of recombinant p9Ka (kindly donated by Fiona Gibbs, University of Liverpool) as a positive control since this material was used to produce the antibody. Immunochemical analysis (**Figure 3.2.14**) revealed that p9Ka protein

was expressed at greater levels in the PK51 and PK84 transgenic mice than in the non-transgenic control, with PK51 expressing at particularly high levels. Expression of tagged-p9Ka protein was detected in the tagged-p9Ka transgenic mouse lines, but whereas TPK41 expresses more transgene mRNA than PK51, the latter seems to express greater levels of transgene protein. This result could be accounted for by a difference in affinities of the antibody for the tagged and non-tagged proteins. However, since there was no pure tagged-p9Ka protein available, this difference could not be easily investigated. Despite this one anomaly, the levels of tagged-p9Ka protein seen in the various tagged-p9Ka transgenics confirmed the pattern of expression shown at the mRNA level. For all lines of mice, the expression of the transgenic protein in the mammary gland was less than in the lung, as might be predicted from the relative levels of transgenic mRNA in these tissues. The line of mice exhibiting the highest levels of p9Ka protein, PK51, was analysed further in terms of the distribution of p9Ka protein in different tissues (**Figure 3.2.14**). The pattern of expression of the p9Ka protein was the same as that described for p9Ka mRNA in all transgenic mouse lines and in the rat. Of the tissues analysed lung contained the highest level of p9Ka and brain contained the lowest level, with all other tissues expressing p9Ka protein at a level between these two.

The apparent molecular weight of the transgene p9Ka protein was shown to be the same as that for the recombinant p9Ka control, which in turn has been shown to be the same as native rat p9Ka (F. Gibbs, personal communication). The tagged-p9Ka protein had a larger molecular weight than the p9Ka protein, as one would expect from the inclusion of a 14-amino-acid insert. The respective molecular masses, derived from the putative translation products, were 10.4 kDa for p9Ka and 12.1 kDa for tagged-p9Ka.

Expression of p9Ka protein was not detected in the non-transgenic mouse, as might be expected, given the sensitivity of this technique and the low level of expression of p9Ka mRNA in mouse lung and mammary gland. However, it is possible that the polyclonal antibody raised to rat recombinant-p9Ka has a lower

affinity for the mouse protein. This is unlikely, given the similarity in the proteins (only four amino-acids difference), but the question cannot be addressed formally due to the lack of a good supply of pure mouse p9Ka protein.

The monoclonal antibody to the tag epitope is, of course, unable to detect non-tagged p9Ka and would not be expected to detect any protein in the non-transgenic mouse. This antibody can be used to look at expression of tagged-p9Ka, which is apparently not detected particularly well with the polyclonal antibody to p9Ka. However the use of the antibody to the tag was not without difficulty.

Any second antibody used to visualise the first antibody to the tagged-p9Ka might be expected to detect not only the mouse monoclonal to the tag epitope, but also any other mouse immunoglobulins in the sample. Such potentially serious problems can be overcome by the appropriate choice of second antibody (in this case a peroxidase-conjugated goat $F(ab')_2$ fragment raised against mouse IgG), the use of a low concentration of second antibody, and importantly, the difference in size between the tagged-p9Ka and the mouse immunoglobulins. When a control analysis in the absence of first (anti-tag) antibody was performed, the reaction of second antibody with immunoglobulins from the mouse protein samples was shown to be minimal at the concentration of antibody used (data not shown). When the antibody to the tag was used, it detected not only the control synthetic peptide (supplied with the antibody) but also the tagged p9Ka in the samples of tissues (**Figure 3.2.14**). This tagged p9Ka was the same size as that detected by the polyclonal antibody produced to p9Ka (data not shown). However, problems of additional staining were encountered even at quite low concentrations of the antibody to the tag (**Figure 3.2.14**). These problems consisted of not only (presumably) non-specific staining of additional proteins in the tissue-samples but also of considerable background staining of the PVDF membrane, in particular where the gel had been in contact with the membrane. Although it was still possible to identify the tagged-p9Ka band due to its characteristic size, these additional staining properties were undesirable. Additional blocking agents (Tween-20, bovine serum albumin and a higher

concentration of Marvel) proved to be only partially successful in combating this background staining (data not shown). The observed staining due to DAB was not the result of any peroxidase activity in the preparation of antibody to the tag, since the omission of the peroxidase-conjugated second antibody erradicated the staining seen (data not shown). Since the antibody to the tag was prepared from mouse ascites, it might well be that additional mouse immunoglobulins in the preparation were the prime cause of the background and non-specific protein staining observed. This observation was consistent with the staining of additional mouse proteins seen when using this preparation of antibody since these proteins were only detected, by anti-mouse IgG serum, when the blot was first incubated with the antibody to the tag.

In an attempt to avoid any problems due to use of an anti-mouse second antibody, the preparation of antibody to the tag was biotinylated and used together with an avidin-peroxidase conjugate to detect tagged-p9Ka on Western blots (data not shown). Essentially the problems encountered were similar to those already described (staining of additional proteins and a high background). It would therefore seem that the preparation of antibody to the tag purchased was rather impure and contained other immunoglobulins. These immunoglobulins could be biotinylated and lead to "non-specific" protein staining, or they could be detected with an anti-mouse IgG serum and again seen as "non-specific" protein staining. These additional immunoglobulins, the anti-tag monoclonal or other elements of the commercial antibody preparation were responsible for the relatively high background levels of staining observed.

Despite the high background it was possible to show that the expression of tagged-p9Ka protein paralleled the relative expression of p9Ka mRNA (**Figure 3.2.14**). This was true not only with respect to distribution of p9Ka in different tissue (as determined for the TPK41 line), but also for the variation in expression of tagged-p9Ka protein between the transgenic mouse lines (as shown by expression in the lung).

3.2.6 Analysis of Expression of Transgenic Proteins by Immunocytochemistry

In order to investigate the distribution of the protein products of the p9Ka transgene and tagged-p9Ka transgenes at the cellular level, immunocytochemical analysis was performed on a variety of tissues from transgenic and non-transgenic mice.

The polyclonal antibody to p9Ka was used predominantly to examine the distribution of p9Ka protein in non-transgenic and PK51 transgenic mice. This line was selected, from the available transgenic mouse lines, since it expressed the highest level of p9Ka protein, as determined by Western blot analysis with the same antibody to be used for immunocytochemistry. The immunocytochemical results for tissues from other lines confirmed the pattern of expression of the transgene protein shown by analysis using Western blot. These results confirmed the apparently lower levels of tagged-p9Ka protein than might be expected from the level of its mRNA. Again, whether this was due to different affinities of the antibody for the different transgenic proteins, or a genuine disparity between levels of expression of mRNA and protein was unclear.

When the p9Ka antibody was used at the relatively high levels needed to detect mouse p9Ka in non-transgenic mice (a 1 in 4 dilution of the stock solution), the immunocytochemical staining for rat p9Ka in the PK51 mouse tissues was strikingly high (**Figures 3.2.15 & 3.2.16**). In the highest staining tissues, the extent of staining for p9Ka was such that a study of the distribution of p9Ka at the cellular level was difficult, in that expression in two cell-types which were expressing p9Ka at high but different levels might not be distinguishable. Therefore selected tissues were also incubated with lower concentrations of p9Ka antibody (e.g. spleen **Figure 3.2.16**). Controls carried out with no primary antibody (**Figure 3.2.16**), or with primary antibody preincubated with recombinant p9Ka, indicated that the staining pattern was exclusively due to the presence of immunoreactive p9Ka. Cross-reactivity with other proteins including calcyclin, S-100 and MRP-8 (all members of S-100-related family of calcium-binding proteins) has been shown to be undetectable

upon immunochemical staining of Western blots (F. Gibbs, personal communication).

The cellular distribution of endogenous murine p9Ka when observed using the p9Ka antibody was not entirely clear, since it was difficult to detect significant levels of endogenous murine p9Ka in many cells. Whilst the mouse and rat versions of the p9Ka protein differ only very slightly (i.e. by four amino acids), it cannot be shown (due to the lack of a supply of the pure mouse protein) that the affinity of the antibody raised is the same for both proteins. Nevertheless, some immunocytochemical staining in mice was observed at the high level of antibody used, and this staining correlated reasonably well with the pattern of staining seen for PK51-transgenic-mouse tissues that were incubated with lower concentrations of antibody (**Figure 3.2. 15 & 3.2.16**).

The cellular distribution of rat and mouse p9Ka in various tissues using a high antibody concentration is given below. The distribution of p9Ka in some tissues (lung, mammary gland, spleen, uterus, brain, salivary gland) has been confirmed using a more dilute antibody.

Tissue	Observed Staining Pattern
Lung	**PK51** Strong uniform staining of all cellular constituents. (When a more dilute antibody was used, smooth muscle elements and macrophages stained more strongly than epithelium.) **non-transgenic** Only stratified squamous epithelium, macrophages and scattered vascular-associated cells were stained (the nucleus of the latter was especially strong.)
Mammary Gland	**PK51** Intense staining of the mammary gland epithelial and myoepithelial cells and strong staining of associated connective tissue, periductal stroma, adipose, vascular elements and occasional macrophages. **non-transgenic** Some periductal connective tissue was moderately

stained and macrophages showed strong staining.

Spleen **PK51** At high concentrations of antibody, uniform, intense staining was seen, which was slightly less intense in the white pulp. (At lower concentrations of antibody staining in white pulp was limited to a few cells and red pulp staining was moderate but more widespread.)

non-transgenic Moderate staining of cells in red pulp but only occasional cells were stained in the white pulp.

Heart **PK51** Moderate staining of cardiac muscle with stronger staining of of nuclei. Some strong staining of blood vessels.

non-transgenic Weak staining with the same pattern as for PK51.

Kidney **PK51** Ductal cells stained strongly, particularly in collecting ducts and distal (ascending) elements, endothelial cells and blood vessels stained intensely.

non-transgenic Epithelial cells of proximal convoluted tubules show pale staining of basal cytoplasm and more intense staining was seen in cells of the distal convoluted tubules and collecting tubules.

Brain **PK51** Cells of the outer granular layer were moderately stained together with tracts of white matter, other neural and glial cells showing no definite pattern were stained intensely.

non-transgenic Similar pattern to that of PK51 above, and only moderately weaker.

Lymph Node **PK51** Strong staining of all elements. (Less intense staining of the follicular areas when using a lower antibody concentration.)

non-transgenic Cells in diffuse cortical areas were stained as were occasional macrophages, follicular areas were unstained or only weakly stained.

Uterus **PK51** Intense and virtually uniform staining of epithelial, stromal, vascular and smooth muscle elements.

non-transgenic No epithelial staining, weak muscular staining but

	intense staining of rounded unidentified cells.
Liver	**PK51** Moderate staining of hepatocytes, in particular those around the central vein.
	non-transgenic Staining pattern as for PK51 above, but paler.
Stomach	**PK51** Stratified squamous epithelia of the fore-stomach was intensely stained; the epithelial cells of the gastric glands were moderately stained with oxyntic cells showing a peripheral band of more intense staining; the smooth muscle was moderately but uniformly stained.
	non-transgenic Stratified squamous epithelia of the fore-stomach showed a degree of staining apart from the basal layer of cells; smooth muscle staining was weak and patchy.
Salivary Gland	**PK51** Moderate staining of acinar and duct cells in serous gland.
	non-transgenic Acinar cells and intralobular ducts were unstained, but epithelial cells of interlobular ducts showed weak but definite staining.

In summary, the cellular distribution of staining for endogenous p9Ka in the non-transgenic mouse was generally the same as that for the PK51 transgenic mice, although at greatly reduced levels. The main exception was that staining in non-transgenic mice was generally greater in the lymphoid cells found within the tissues than in the tissues themselves and was particularly strong in the lymph node (**Figure 3.2.15**) and spleen (**Figure 3.2.16**), when compared to other non-transgenic mouse tissues. This was in keeping with the fact that the non-transgenic mice exhibited an unusually high expression of p9Ka mRNA in the spleen (**Figure 3.2.2**). Although it would seem that murine p9Ka was expressed predominantly in cells of lymphoid origin, expression can be seen in the same range of cell-types as for the transgene, but at a much lower level. The expression of p9Ka protein in the rat (as determined by immunocytochemistry with the p9Ka antibody by Fiona Gibbs) will be discussed later (Section 4), with particular attention to similarities with the expression of transgene p9Ka and expression of murine p9Ka.

3.2.7 Summary

The expression of p9Ka, of the p9Ka transgene and of the tagged-p9Ka transgene have been studied successfully in transgenic mice, both at the level of mRNA and protein. The tissue-specific patterns of expression of the mouse and rat homologues of p9Ka are seen to be different. The mouse gene is expressed at lower levels than that of the rat, and with a tissue and cellular pattern of expression which suggests that the bulk of the expression of p9Ka in mice occurs in cells of lymphoid origin. The rat gene, on the other hand, is expressed at higher levels and in a greater number of tissues (although the latter fact may be merely a reflection of our inability to detect p9Ka mRNA or protein in low expressing mouse tissues). Expression of transgenic mRNA has been shown to occur with the same general pattern in all lines of transgenic mice, and expression of the transgenic protein has been shown by immunochemical analysis of protein on Western blots. The level of expression of transgenic mRNA varies between transgenic mouse lines in approximate correlation with the transgene copy-number. Expression of the transgenic p9Ka and tagged-p9Ka mRNAs follow the pattern of expression of the rat gene from which these constructs were produced. Moreover, expression of these genes at the protein level follows that at the mRNA level. The pattern of cells expressing the p9Ka transgene protein can be shown to be similar to that of the endogenous rat gene, despite the wide differences in levels of expression in the different situations.

Figure 3.2.1

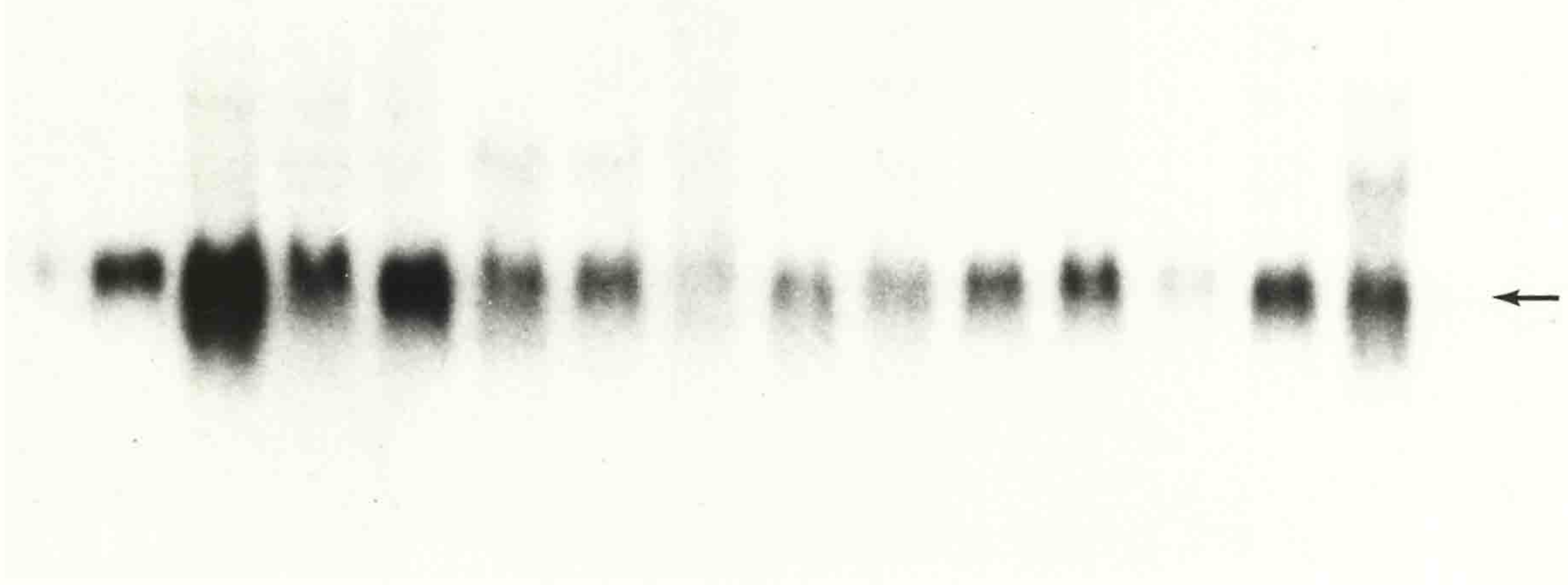
Expression of p9Ka mRNA in rat tissues. Northern blot analysis was performed as described in Methods, using 10 μg of total RNA from tissues of an adult, female, Ludwig-Wistar rat.

The following tissues were analysed: lung (Lu), mammary gland (MG), spleen (Sp), heart (H), kidney (Ki), brain (Br), Uterus (Ut), stomach (St), ovary (Ov), Thymus (Thy), salivary gland (SG), lymph node (LN) and large intestine (LI).

1 μg of Rama 29 total RNA (R29) was loaded as a positive control.

The size of the p9Ka mRNA (indicated by the arrow) was as previously reported (700-800 bases; Barraclough *et al.*, 1984b).

R29
1μg Lu MG Sp H Ki Br Ut St Ov Thy SG LN LI



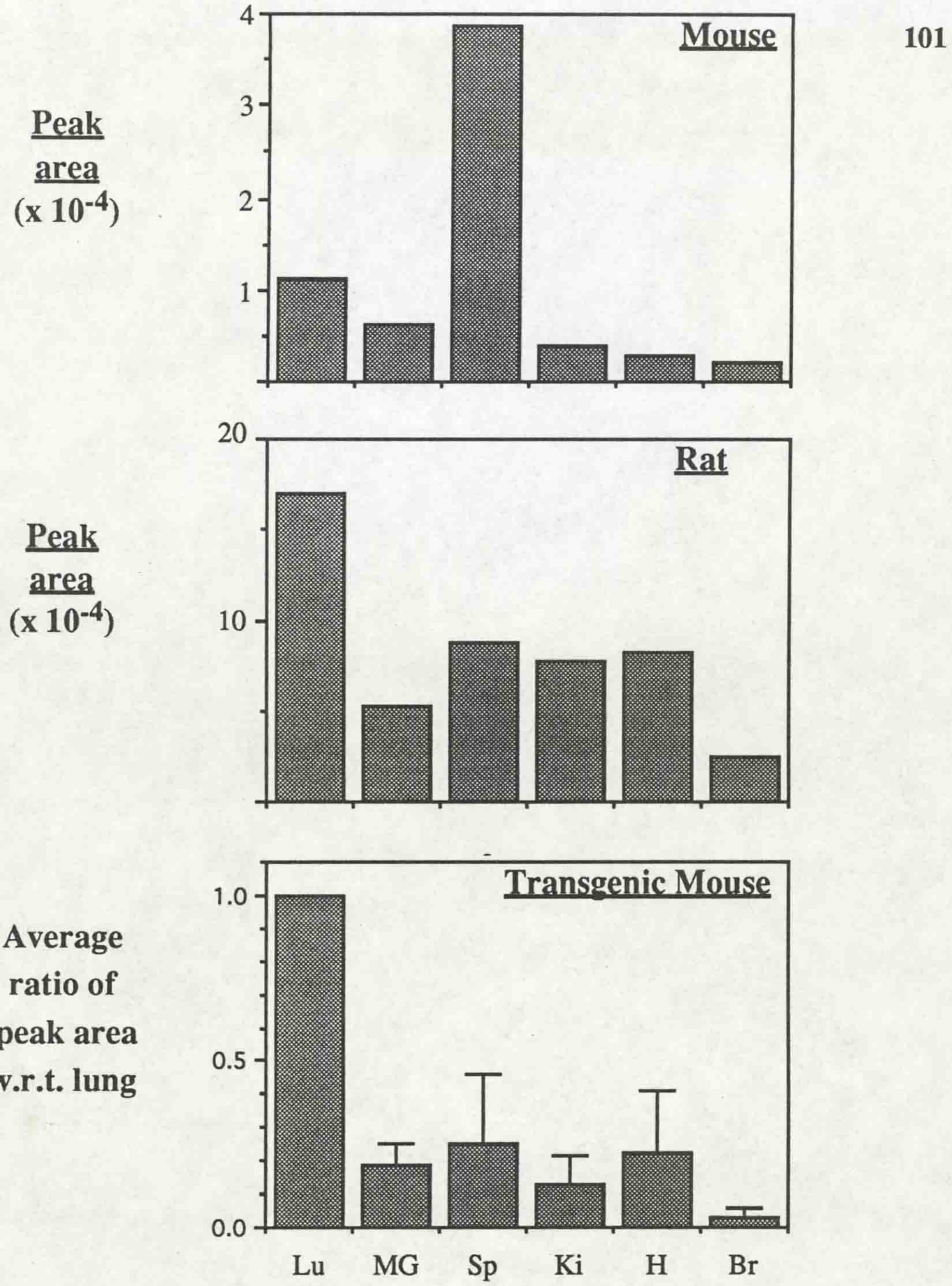


Figure 3.2.2

Comparison of the patterns of expression of p9Ka mRNA in mouse (top), rat (middle) and transgenic mouse (bottom), showing relative levels of p9Ka mRNA in lung (Lu), mammary gland (MG), spleen (Sp), kidney (Ki), heart (H) and brain (Br). For mouse and rat, peak areas of scanned data from Northern blots probed with rat p9Ka cDNA were taken from a single autoradiograph, but were not corrected for unequal loadings due to difficulties in interpreting data from blots hybridised with constitutive probes (see Results 3.2.2). N.B. Scale sizes are different.
For transgenic mice, the ratio of peak area with respect to that of lung was taken from corrected data for "all tissue" analysis (Figures 3.2.7, 3.2.8, 3.2.9 & 3.2.11); the means of the ratios for each tissue were calculated from data for all transgenic mouse lines and error bars represent the standard deviations of the means.

Figure 3.2.3

Analysis of hybridisation intensity as a function of RNA amount using all three probes utilised for Northern blot analysis of the expression of p9Ka mRNA (p9Ka, actin and G3PDH). Northern blot analysis was performed as described in Methods, using amounts of total RNA specified. The total RNA used was from either Rama 29 cells or from a preparation of kidney RNA from a male transgenic PK51 mouse. The size of the p9Ka mRNA was as previously reported (700-800 nucleotides; Barraclough *et al.*, 1984b), and the size of other mRNAs agreed with those obtained previously (approximately 2000 nucleotides for actin and 1800 nucleotides for G3PDH). The additional bands seen in PK51 kidney RNA probed with actin (i.e. just below the actual actin bands for the higher amounts of RNA) are due to the presence of a weak G3PDH signal; this is a remnant from the previous hybridisation (G3PDH hybridisation gave a particularly strong signal for RNA from kidney).

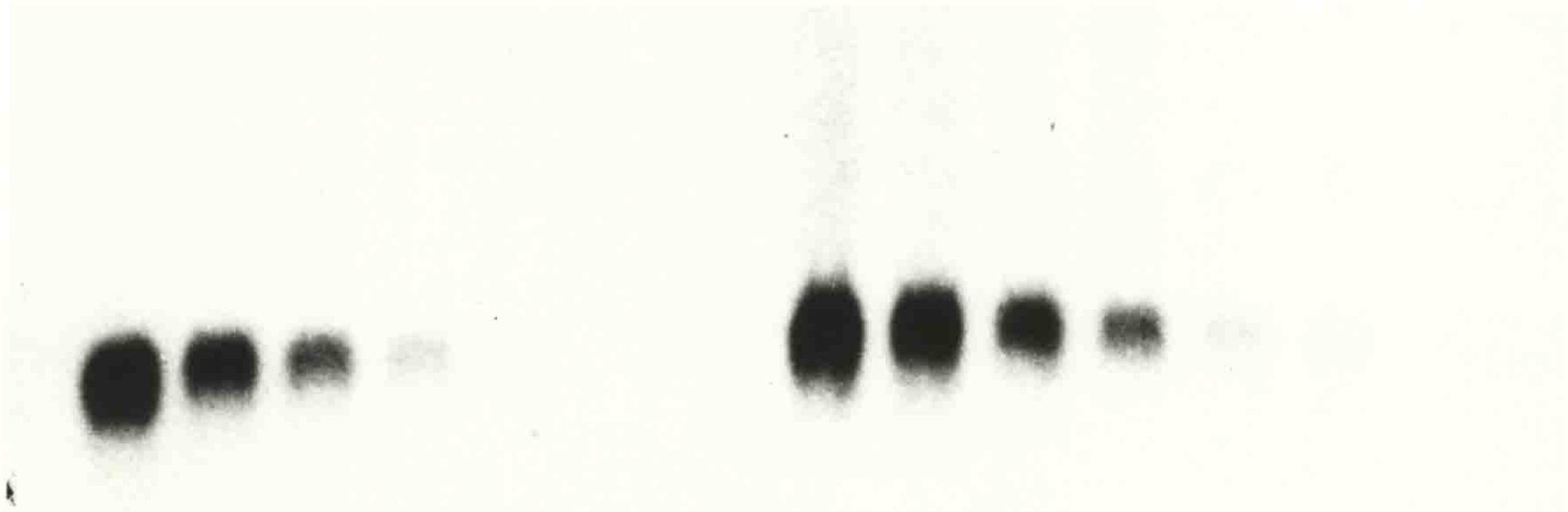
Rama29 total RNA (μg)

4.5 2 1 0.5 0.25 0.1

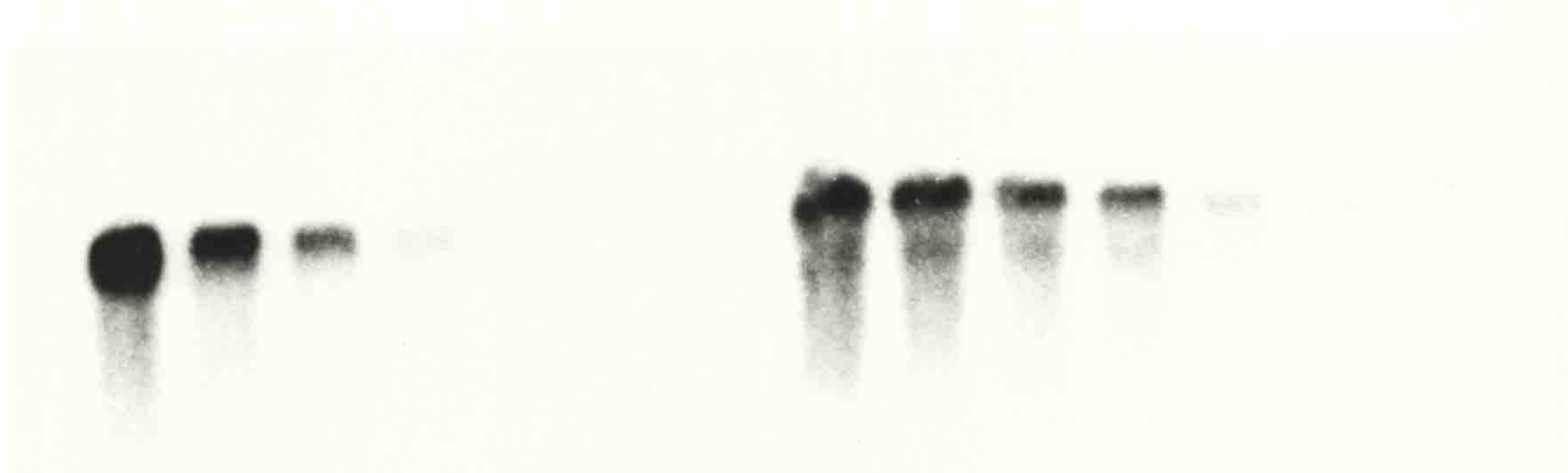
PK51.3 (+ve, male) Kidney total RNA (μg)

20 15 10 5 2 1 0.5

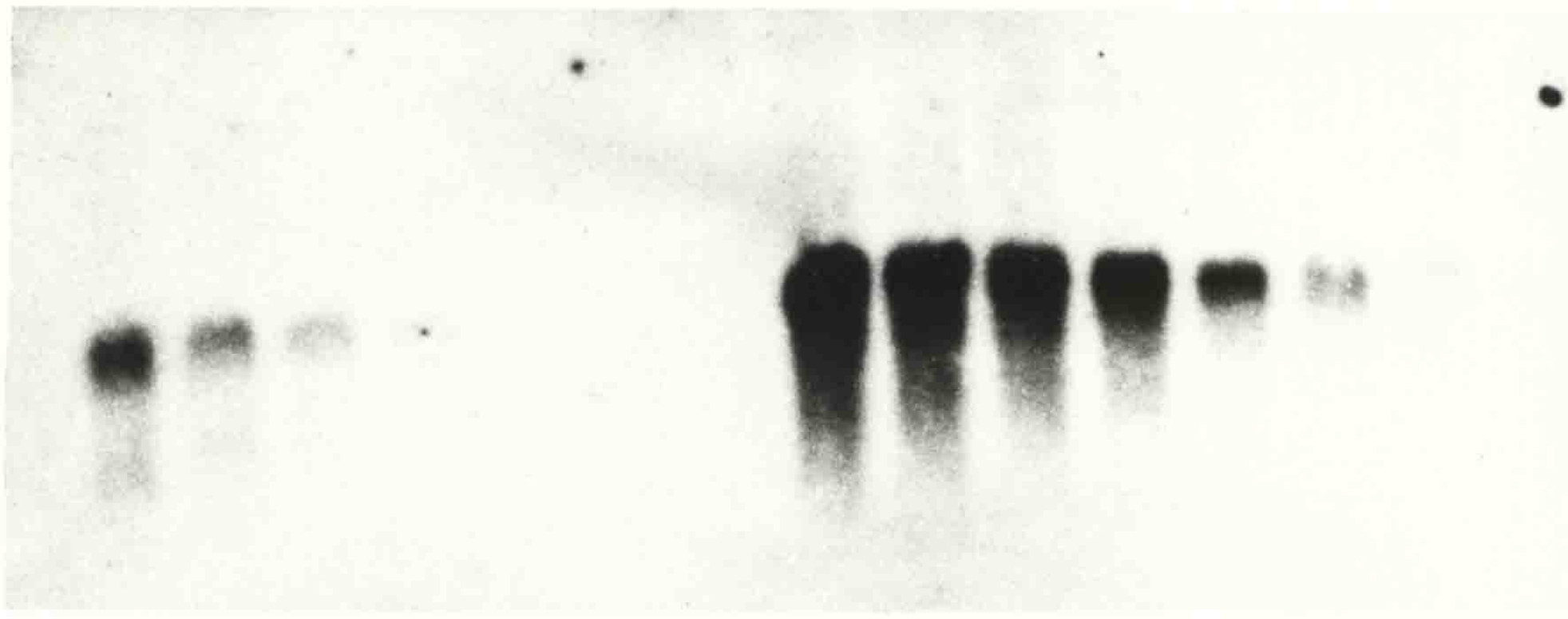
p9Ka



Actin



G3PDH



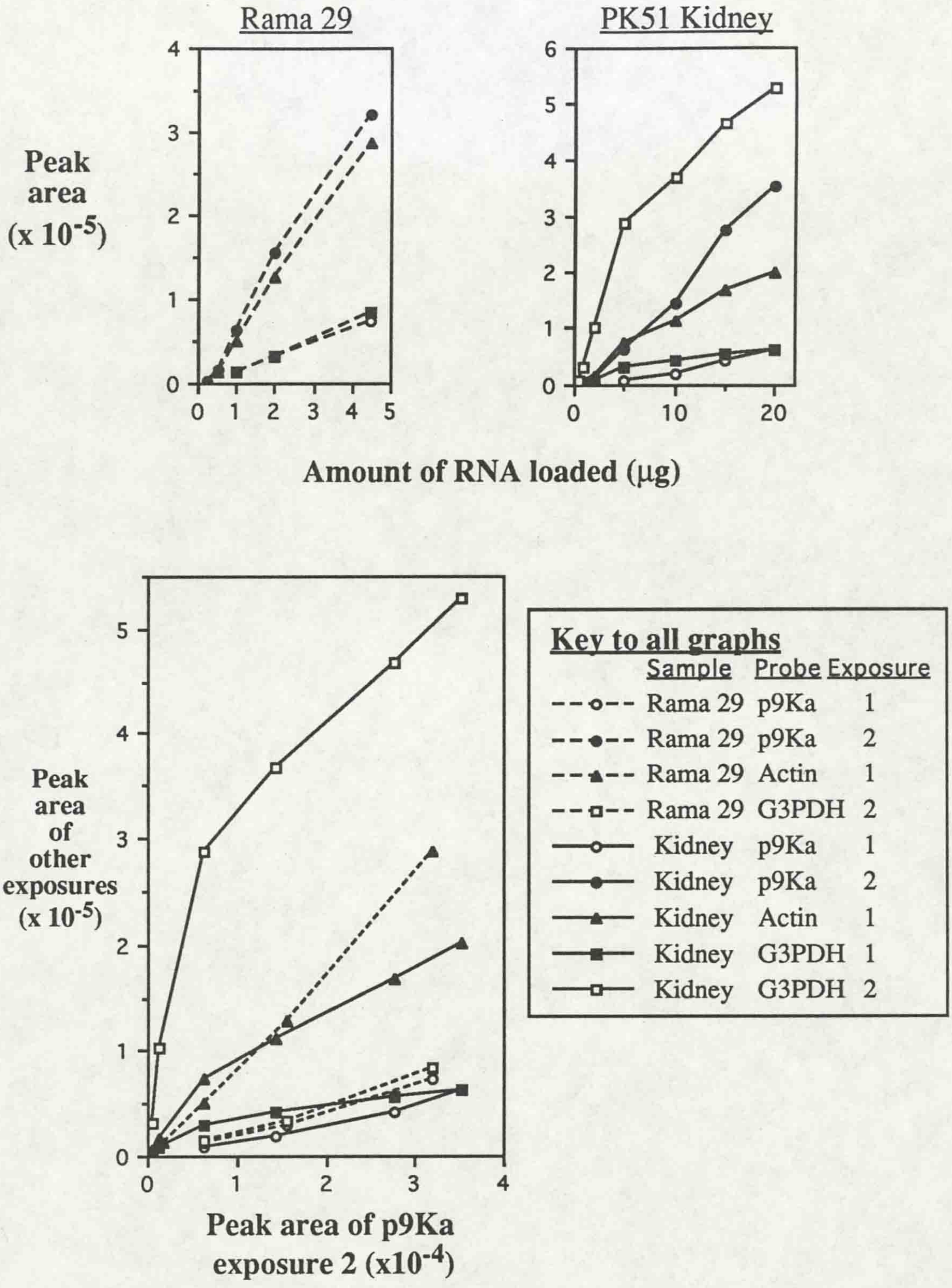


Figure 3.2.4

Graphical analysis of peak areas for probed blots of Rama 29 and PK51 kidney RNA for a range of dilutions of total RNA (for blots see Figure 3.2.2). Peak areas were determined as described in Methods and plotted against µg of total RNA loaded (top) or, to account for possible loading anomalies, against a single set of peak-area values (bottom).

Figure 3.2.5

"All Line" analysis of the expression of mRNA in lung and mammary gland. Northern blot analysis was performed as described in Methods, using 10 μg of the total RNA specified (unless stated otherwise). R29 = Rama 29 RNA; Rat = Ludwig-Wistar rat RNA; -ve = RNA from non-transgenic mouse; transgenic mouse identifiers are as given and all animals were adult females. The size of p9Ka mRNA was as previously reported (700-800 nucleotides) and the size of other mRNAs agreed with those obtained previously (approximately 2000 nucleotides for actin and 1800 nucleotides for G3PDH).

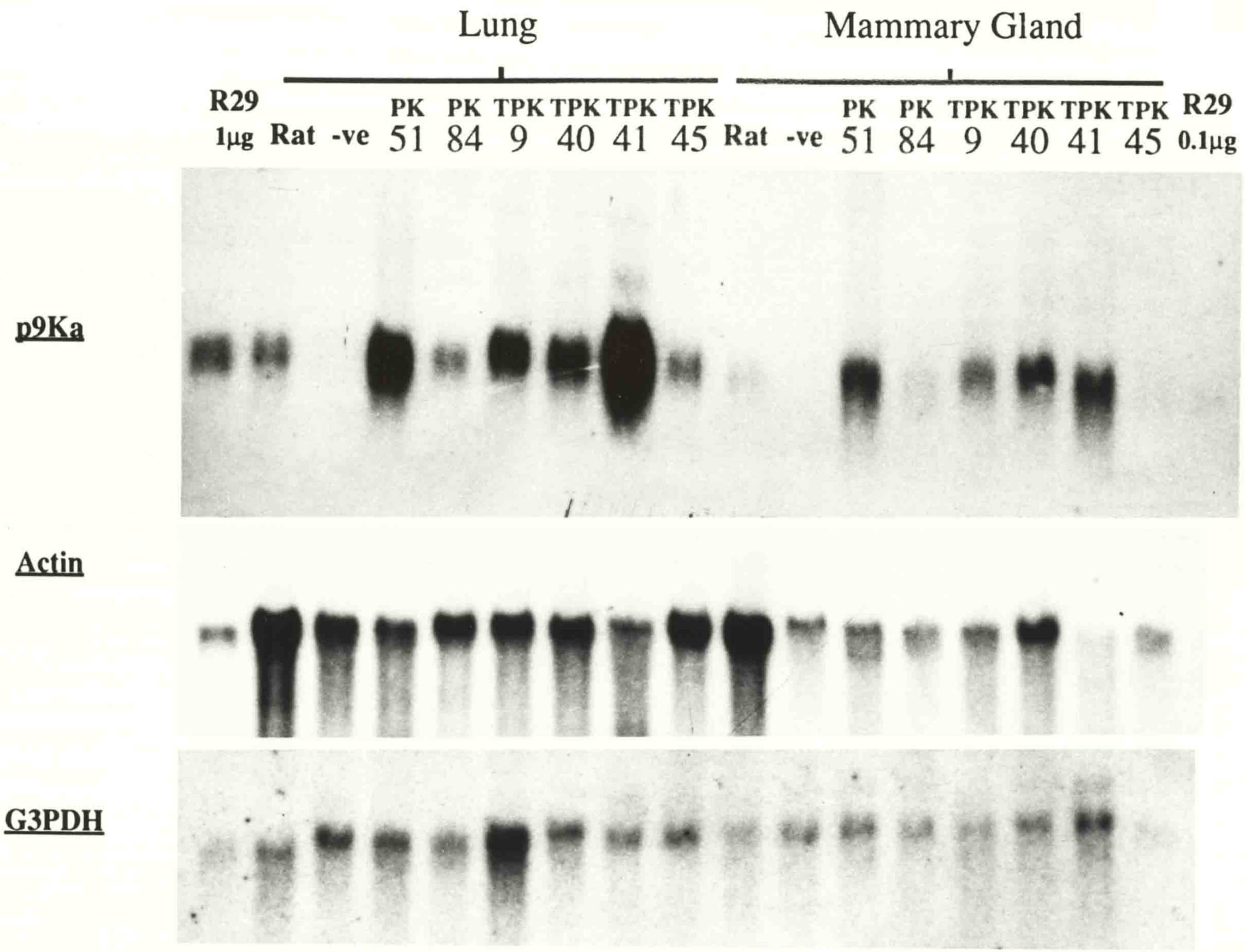

Lung
Mammary Gland
R29 1µg
Rat
-ve
PK 51
PK 84
TPK 9
TPK 40
TPK 41
TPK 45
Rat
-ve
PK 51
PK 84
TPK 9
TPK 40
TPK 41
TPK 45
R29 0.1µg
p9Ka
Actin
G3PDH

Figure 3.2.6

"All Line" analysis of the expression of mRNA in spleen and kidney. Northern blot analysis was performed as described in Methods, using 10 µg of total RNA specified (unless stated otherwise).

R29 = Rama 29 RNA; Rat = Ludwig-Wistar rat RNA; -ve = RNA from non-transgenic mouse; transgenic mouse identifiers are as given and all animals were adult females. The size of p9Ka mRNA was as previously reported (700-800 nucleotides) and the size of other mRNAs agreed with those obtained previously (approximately 2000 nucleotides for actin and 1800 nucleotides for G3PDH).

N.B. The TPK9 spleen and kidney RNA samples were loaded in the wrong lanes; i.e. for this line the lane marked as kidney is spleen, and vice-versa.

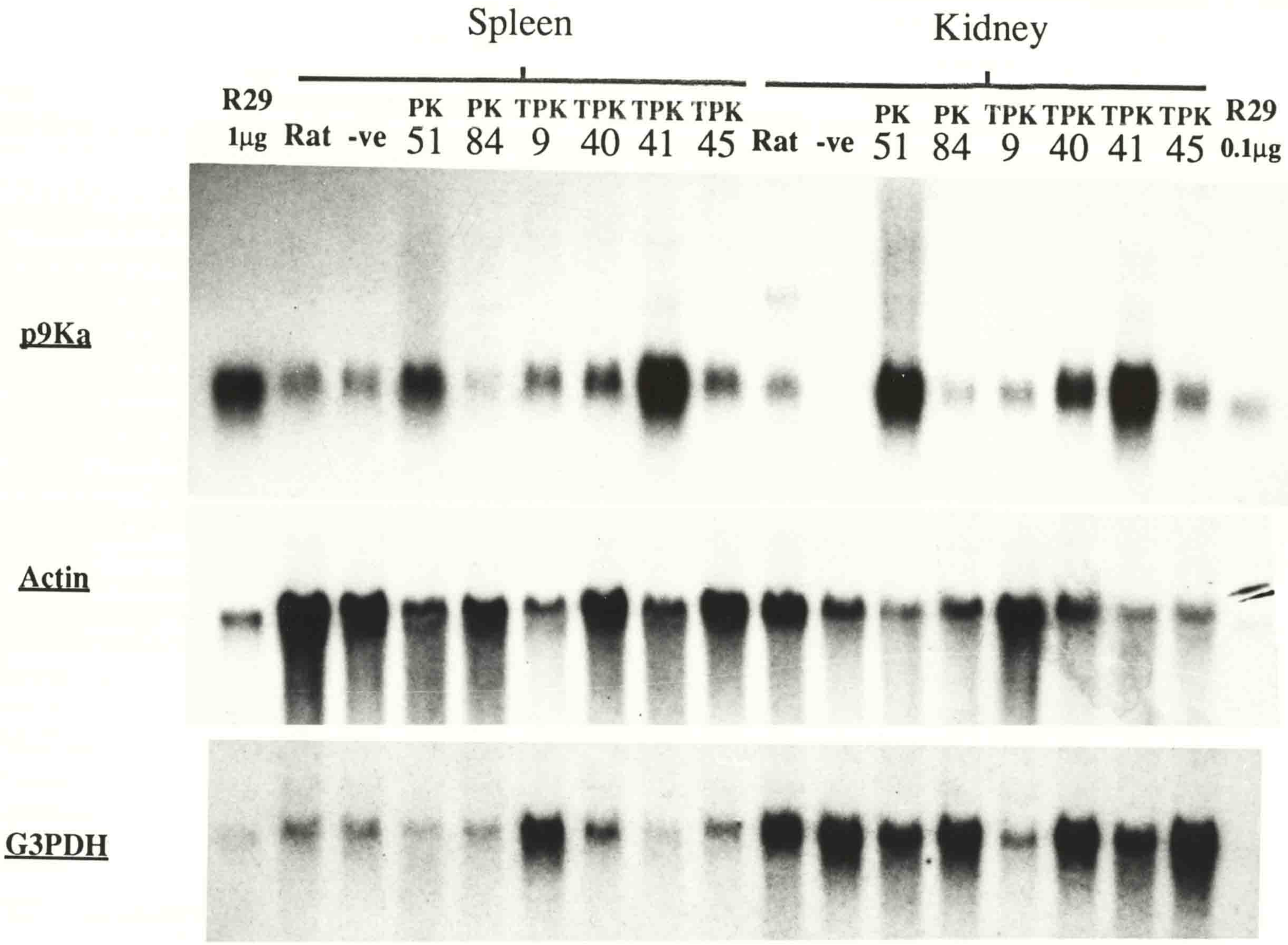

Spleen
Kidney
R29 1µg
Rat
-ve
PK 51
PK 84
TPK 9
TPK 40
TPK 41
TPK 45
Rat
-ve
PK 51
PK 84
TPK 9
TPK 40
TPK 41
TPK 45
R29 0.1µg
p9Ka
Actin
G3PDH

Figure 3.2.7

"All Line" analysis of the expression of mRNA in heart and brain. Northern blot analysis was performed as described in Methods, using 10 μg of the total RNA specified (unless stated otherwise).

R29 = Rama 29 RNA; Rat = Ludwig-Wistar rat RNA; -ve = RNA from non-transgenic mouse; transgenic mouse identifiers are as given and all animals were adult females. The size of p9Ka mRNA was as previously reported (700-800 nucleotides) and the size of other mRNAs agreed with those obtained previously (approximately 2000 nucleotides for actin and 1800 nucleotides for G3PDH).

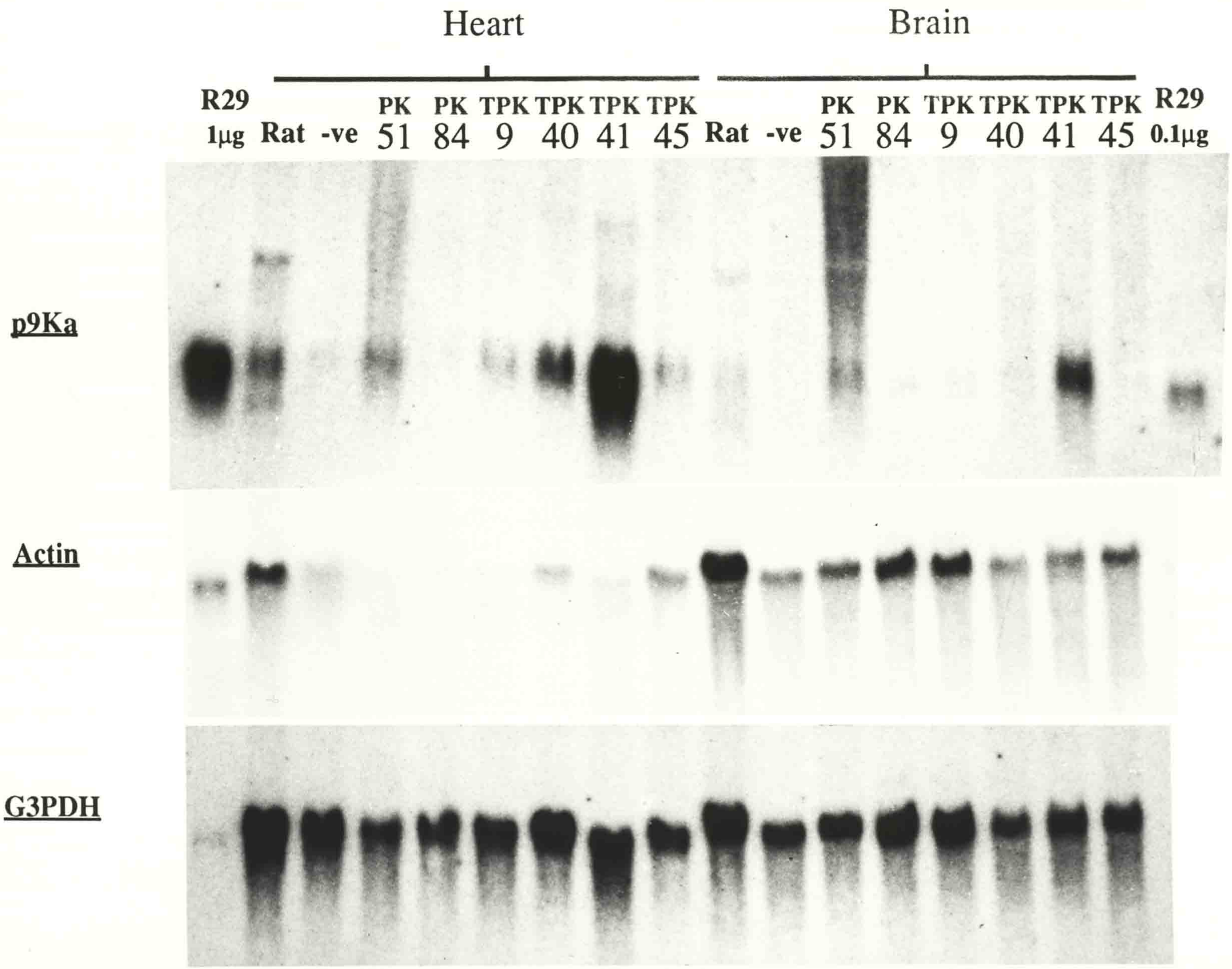

Heart
Brain
R29 1µg
Rat
-ve
PK 51
PK 84
TPK 9
TPK 40
TPK 41
TPK 45
Rat
-ve
PK 51
PK 84
TPK 9
TPK 40
TPK 41
TPK 45
R29 0.1µg
p9Ka
Actin
G3PDH

Figure 3.2.8

"All Tissue" analysis of mRNA expression in PK51 and TPK41 transgenic mice. Northern blot analysis was performed as described in Methods, using 10 μg of total RNA specified (unless stated otherwise).

R29 = Rama 29 RNA; -ve = RNA from non-transgenic mouse;

Lu = lung; MG = mammary gland; Sp = spleen; H = heart; Ki = kidney and Br = Brain.

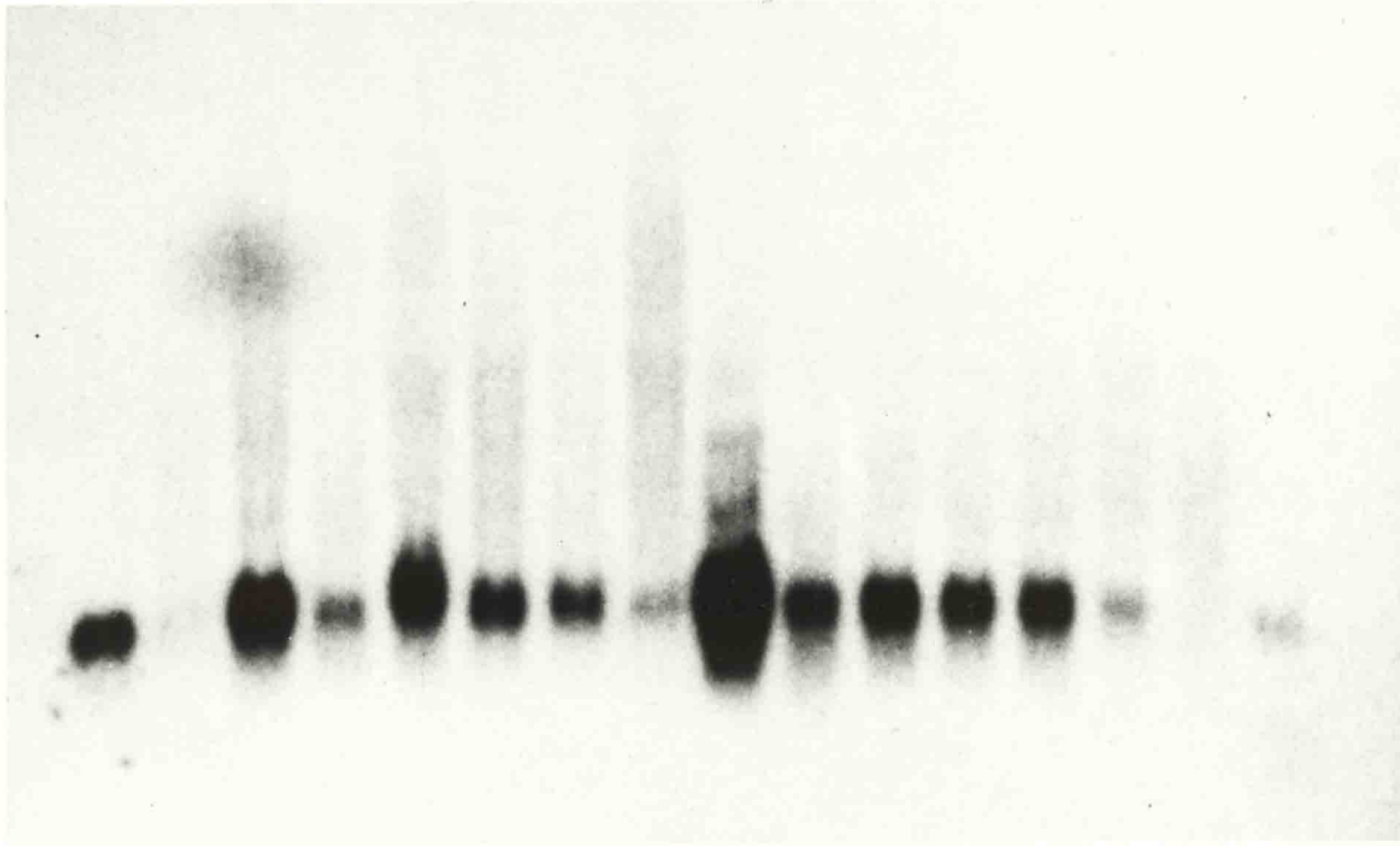
The p9Ka mRNA size was as previously reported (700-800 nucleotides) and the size of other mRNAs agreed with those obtained previously (approximately 2000 nucleotides for actin and 1800 nucleotides for G3PDH).

PK51 male

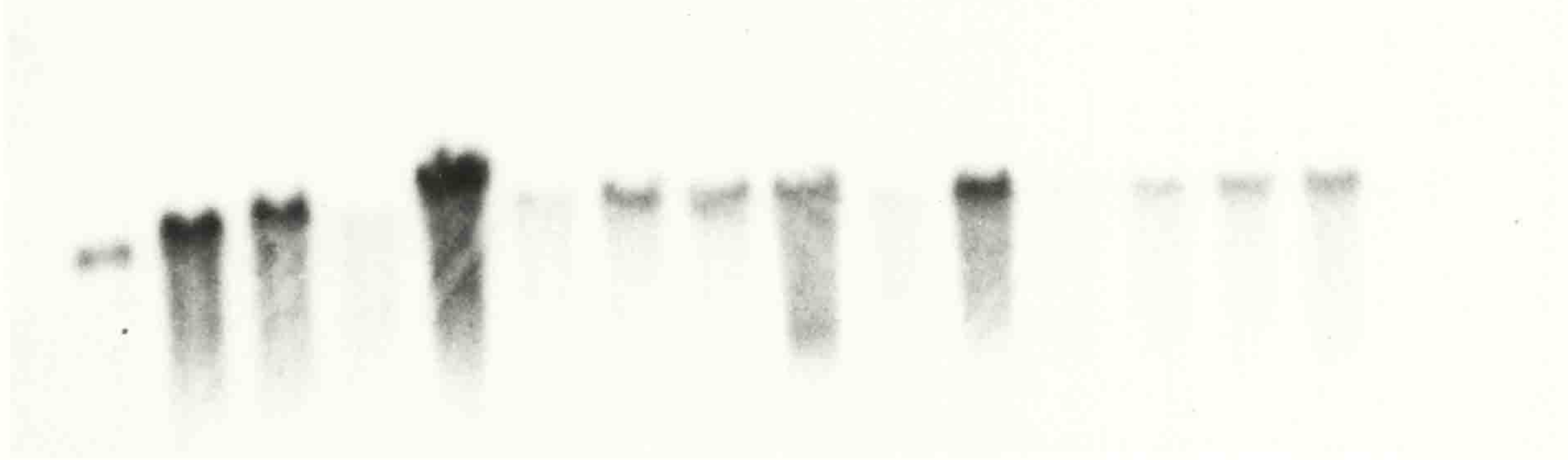
TPK41 female

R29 1μg | -ve Lu | Lu | MG | Sp | H | Ki | Br | Lu | MG | Sp | H | Ki | Br | -ve Br | R29 0.1μg

p9Ka



Actin



G3PDH

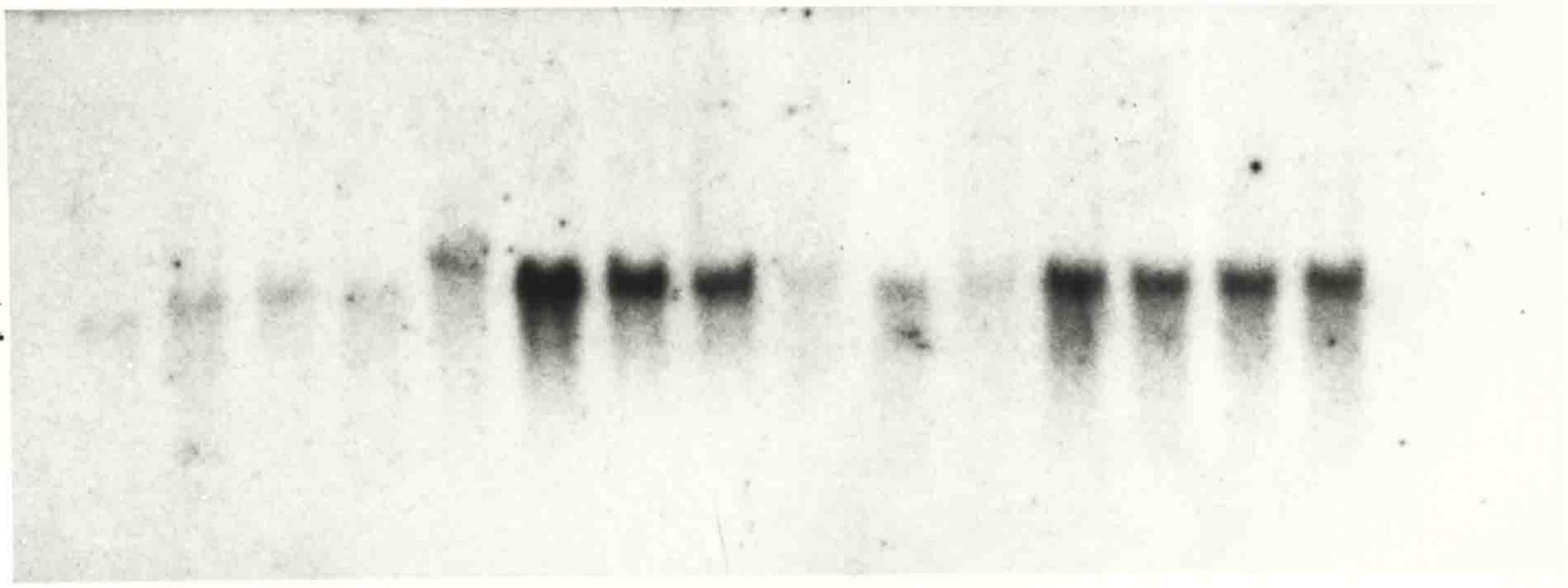


Figure 3.2.9

"All Tissue" analysis of mRNA expression in PK84 and TPK9 transgenic mice. Northern blot analysis was performed as described in Methods, using 10 μg of total RNA specified (unless stated otherwise).

R29 = Rama 29 RNA; -ve = RNA from non-transgenic mouse;

Lu = lung; MG = mammary gland; Sp = spleen; H = heart; Ki = kidney and Br = Brain.

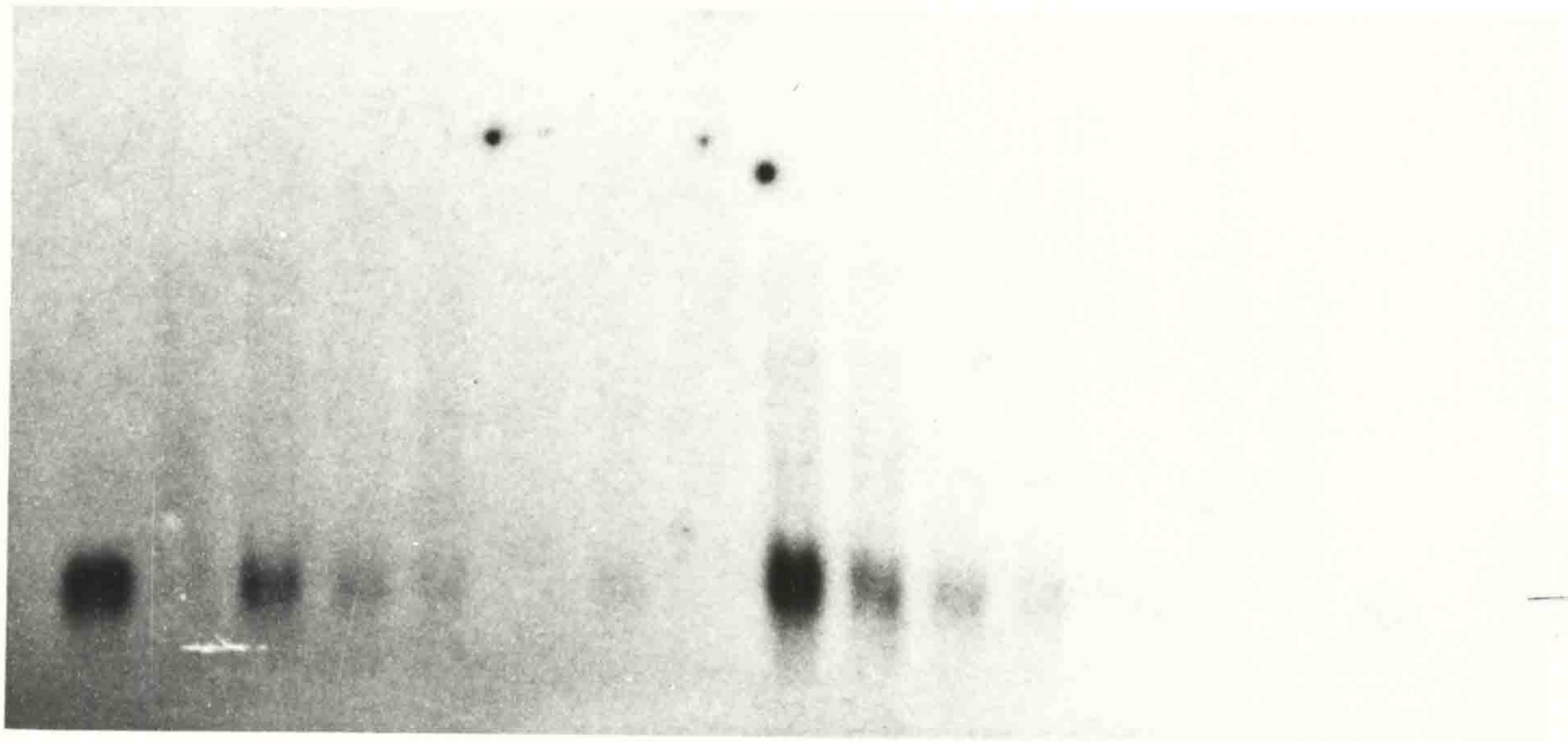
The p9Ka mRNA size was as previously reported (700-800 nucleotides) and the size of other mRNAs agreed with those obtained previously (approximately 2000 nucleotides for actin and 1800 nucleotides for G3PDH).

PK84 female

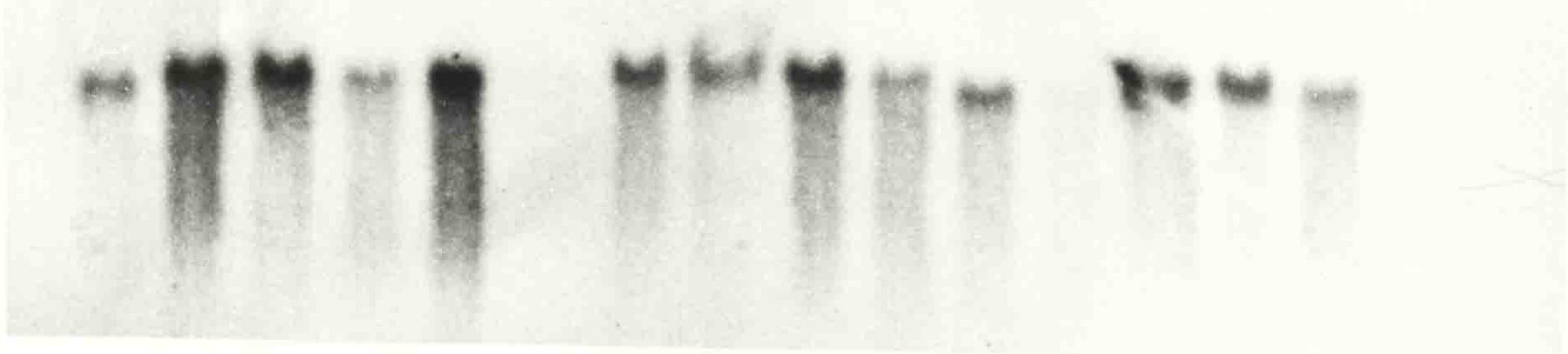
TPK9 female

R29 1µg | -ve Lu | Lu MG Sp H Ki Br | Lu MG Ki H Sp Br | -ve Br | R29 0.1µg

p9Ka



Actin



G3PDH

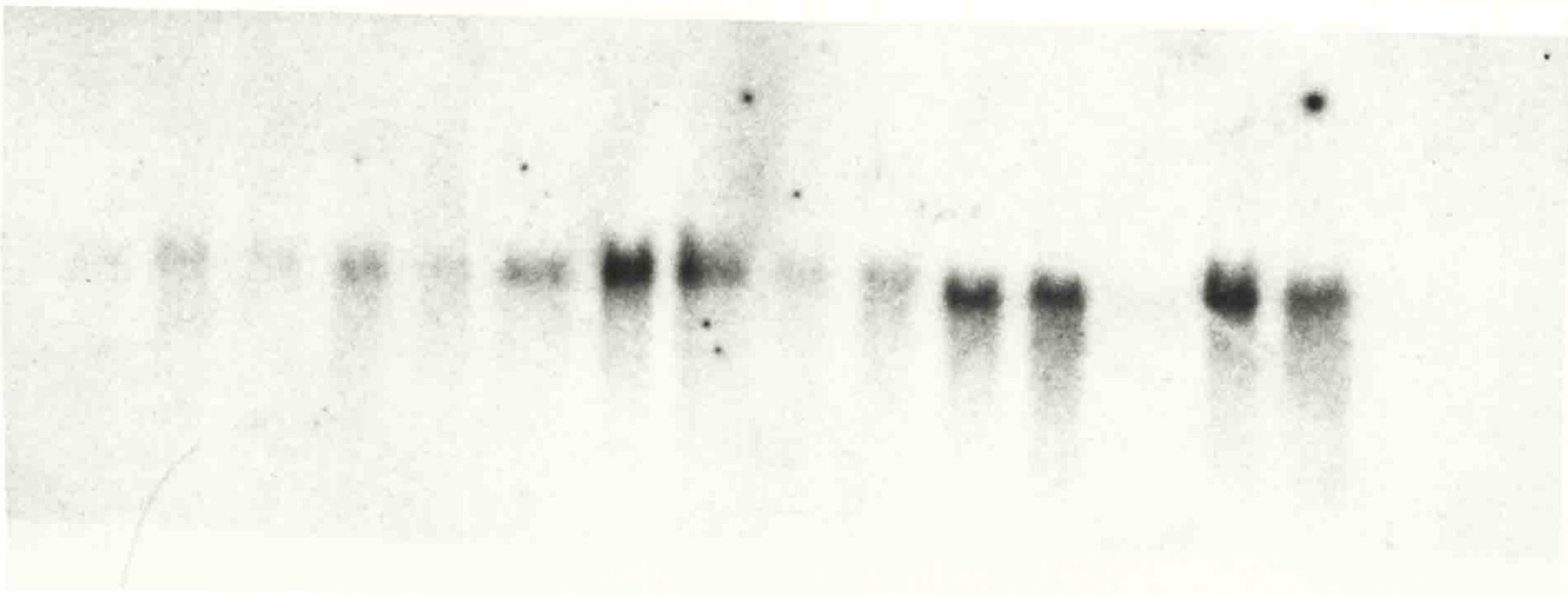


Figure 3.2.10

"All Tissue" analysis of mRNA expression in TPK40 and TPK45 transgenic mice. Northern blot analysis was performed as described in Methods, using 10 μg of total RNA specified (unless stated otherwise).

R29 = Rama 29 RNA; -ve = RNA from non-transgenic mouse;

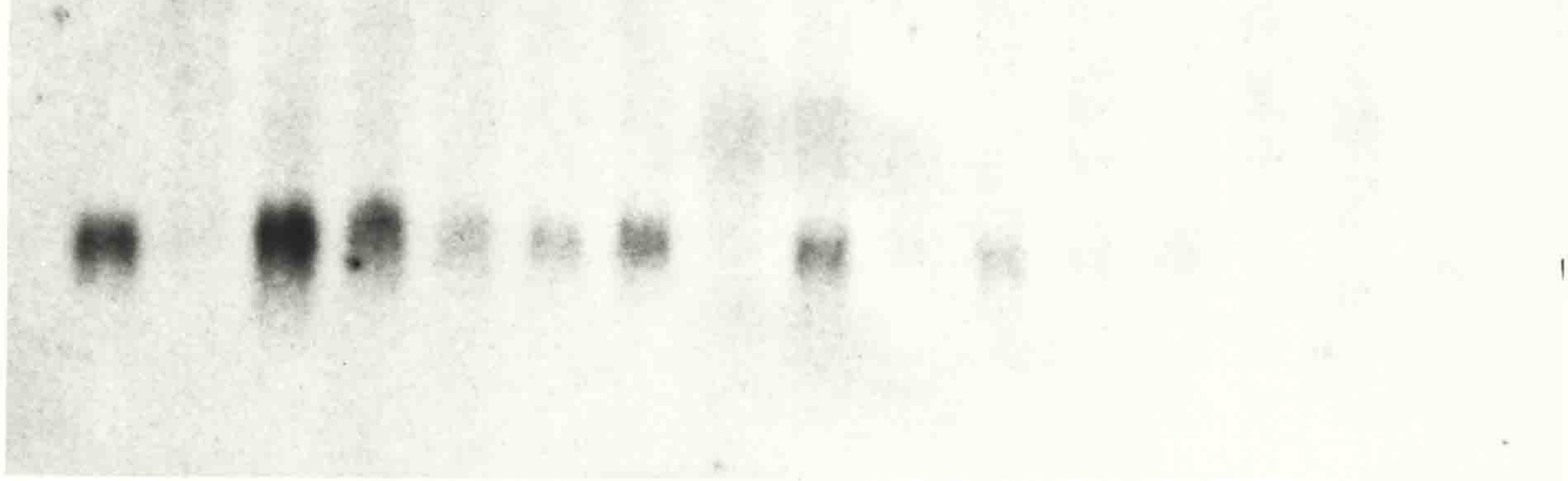
Lu = lung; MG = mammary gland; Sp = spleen; H = heart; Ki = kidney and Br = Brain.

The tagged-p9Ka mRNA size was as previously reported (750-850 nucleotides) and the size of other mRNAs agreed with those obtained previously (approximately 2000 nucleotides for actin and 1800 nucleotides for G3PDH).

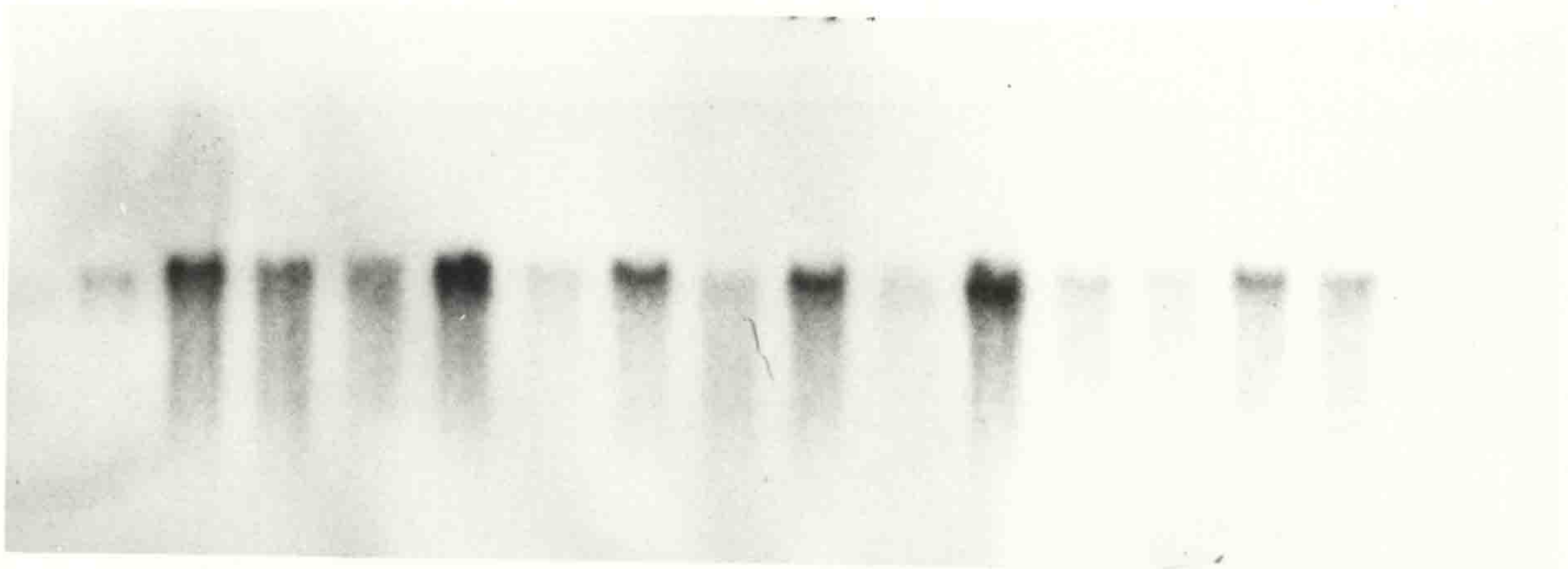
TPK40 female TPK45 female

R29 1μg | -ve Lu | Lu MG Sp H Ki Br | Lu MG Sp H Ki Br | -ve Br | R29 0.1μg

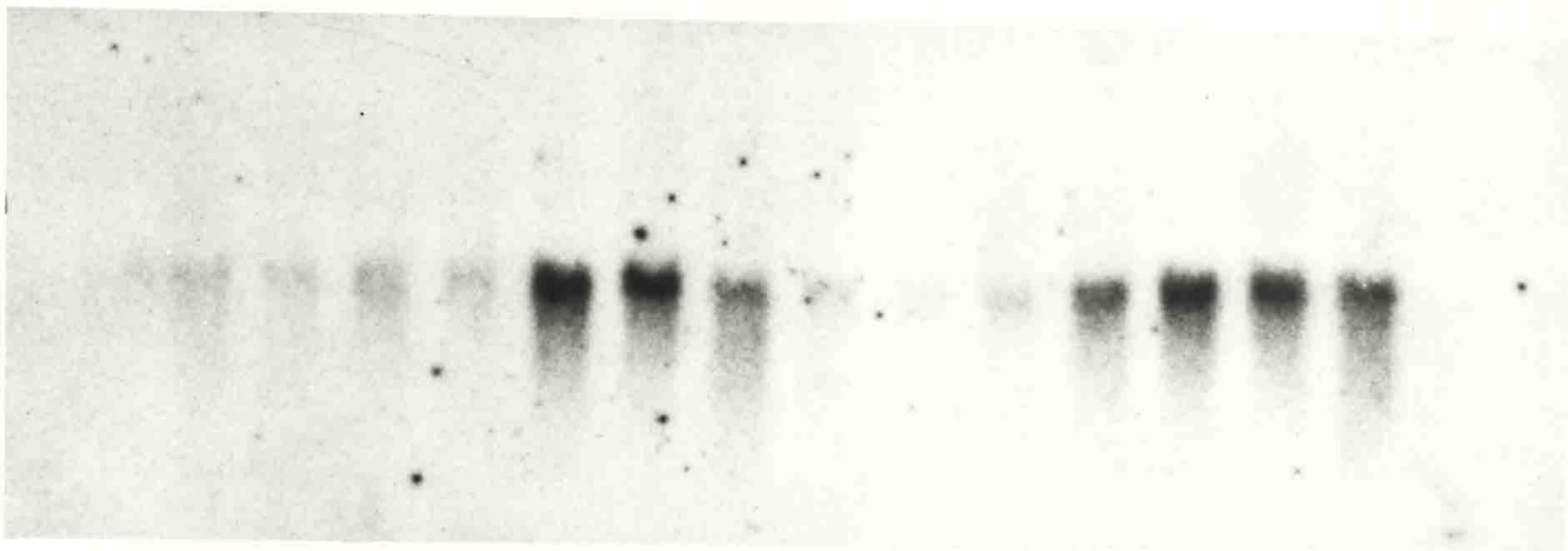
p9Ka



Actin



G3PDH



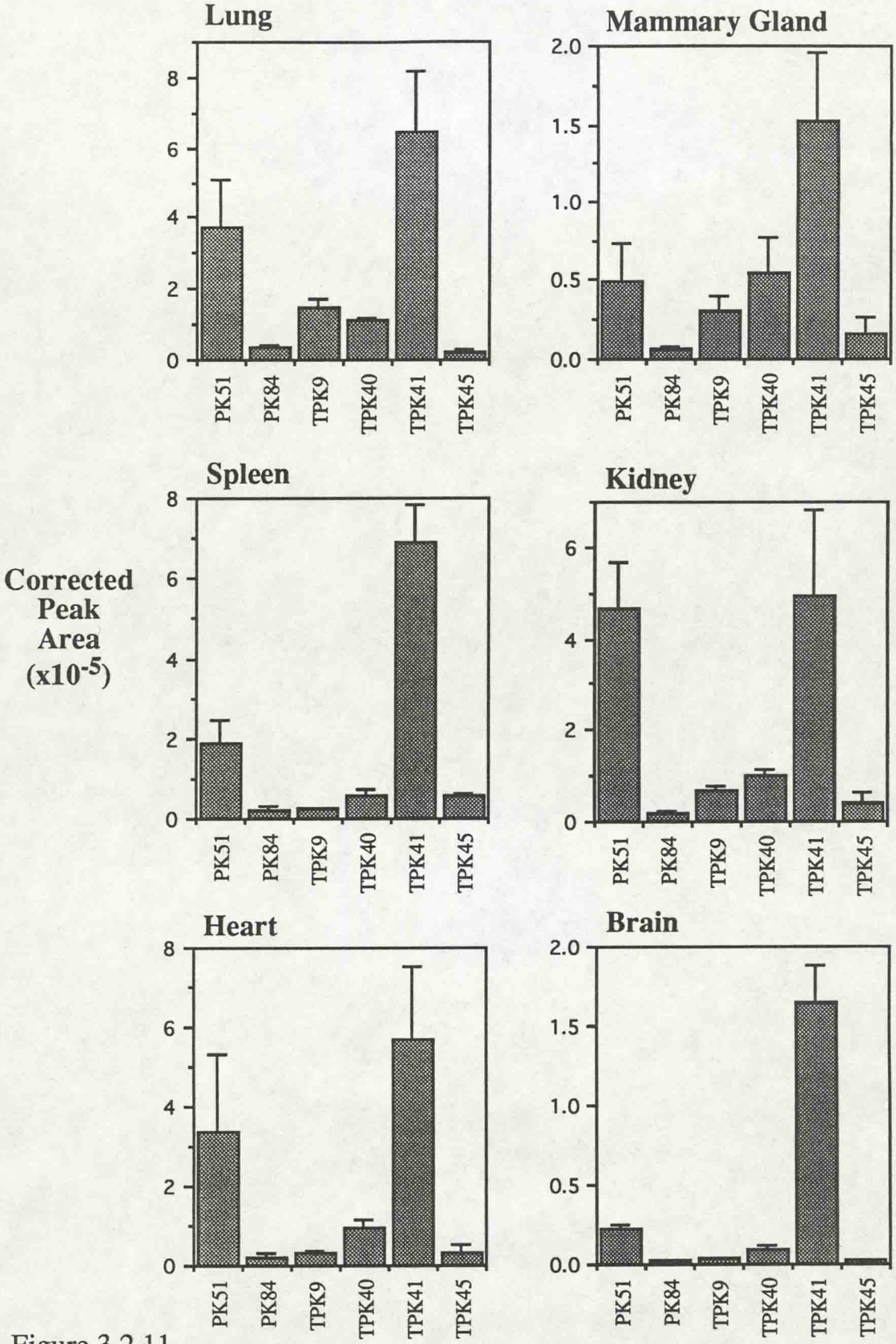


Figure 3.2.11

Bar charts comparing the expression of p9Ka mRNA between transgenic mouse lines for individual tissues. All values plotted are averaged corrected peak areas from "All Lines" northern blots (Figures 3.2.3, 3.2.4 & 3.2.5), and error bars represent the standard deviation of the mean for individual corrected peak areas.

N.B. Scale size varies.

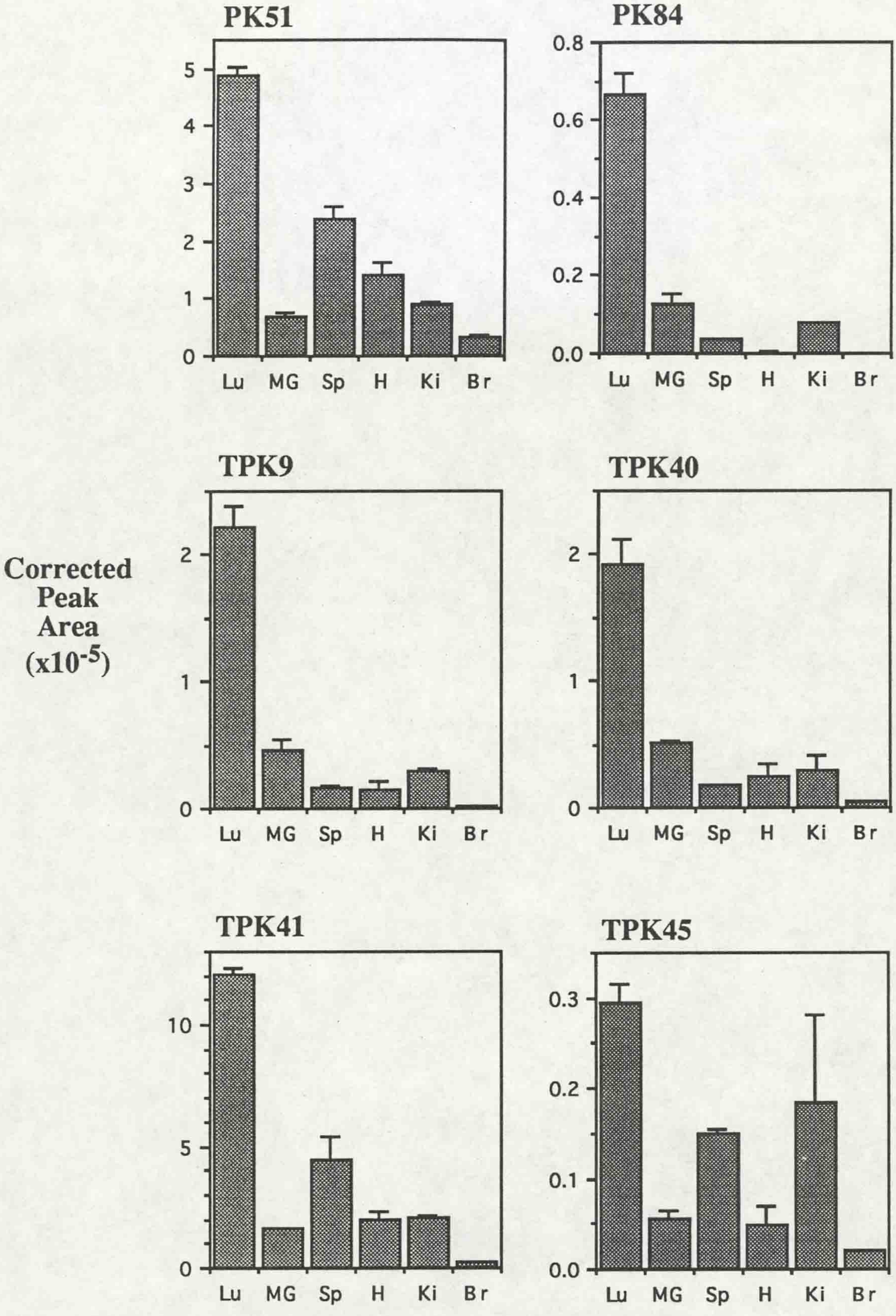


Figure 3.2.12

Bar charts comparing the expression of p9Ka mRNA between individual tissues for transgenic mouse lines . All values plotted are averaged corrected peak areas from "All Tissues" northern blots (Figure 3.2.6, 3.2.7 & 3.2.8), and error bars represent the standard deviation of the mean for individual corrected peak areas.

N.B. Scale size varies.

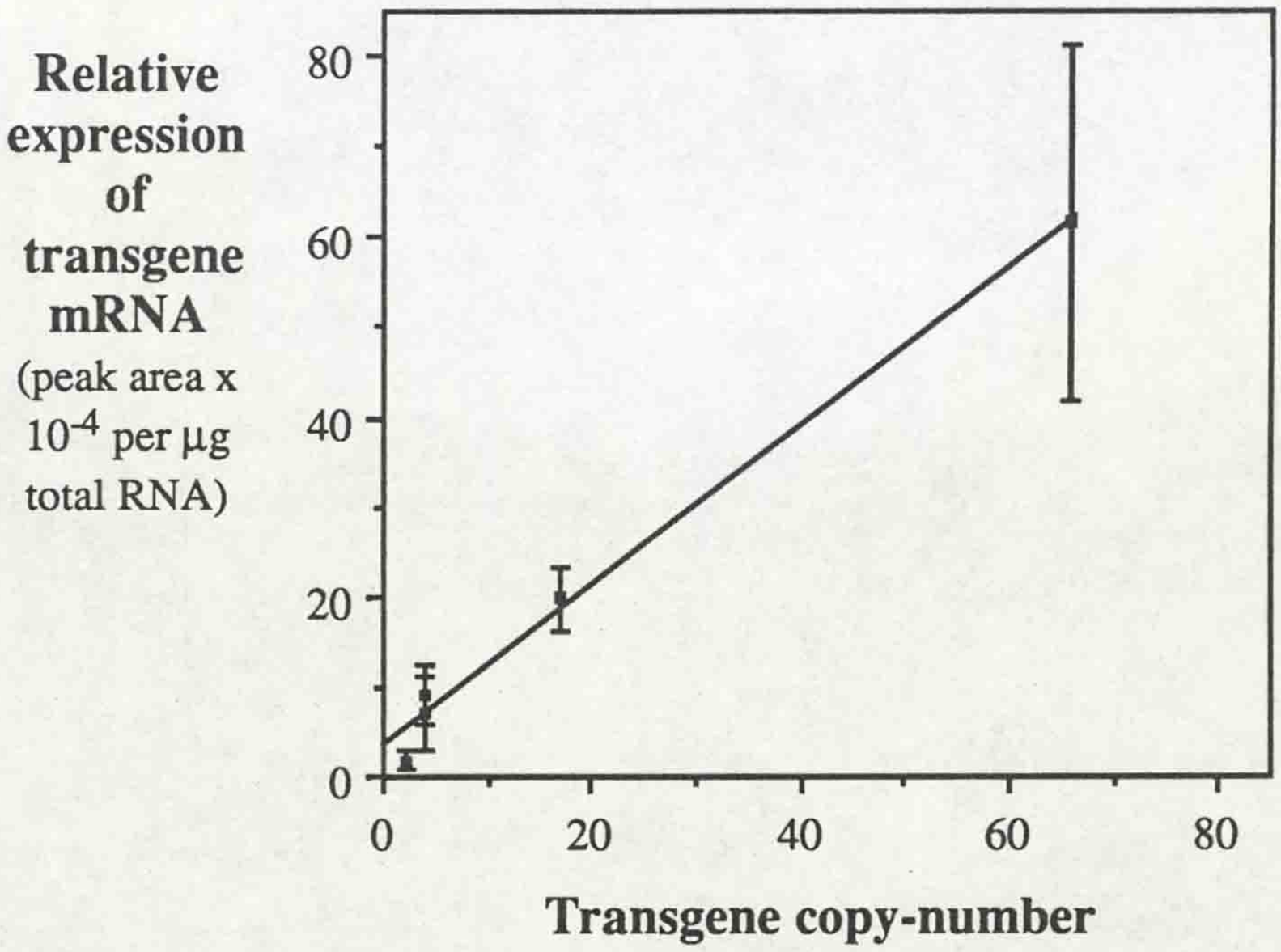


Figure 3.2.13

Copy-number dependence of the expression of p9Ka transgene mRNA.

Relative expression of transgene mRNA was calculated, from scanned exposures of a dot blot, as the peak area ($x10^{-4}$) per µg of total RNA loaded. Values from three separate exposures were corrected for time of exposure and an average value calculated (n=11) for each transgenic strain, the error bars represent the standard deviations of the means.

Figure 3.2.14

Western blot analysis of the expression of transgene protein using antisera to p9Ka (A &B) and anti-tag monoclonal antibody (C&D).

Transgenic mouse identifiers are given as before, and all lanes of gels prior to blotting contained 100 μg of total protein, extracted from the specified tissue. Size markers given are in kDa.

p9Ka = recombinant p9Ka protein (approximately 4 μg) as positive control.

tag = synthetic proteins containing epitope used as tag (Cambridge Research Biochemicals).

-ve = non-transgenic mouse.

Lu = Lung MG = Mammary gland Sp = Spleen

Ki = Kidney H = Heart Br = Brain

Antibody dilutions used

for A & B: 1 in 40 dilution of antisera to recombinant rat p9Ka
1 in 200 dilution of goat anti-rabbit IgG (peroxidase conjugate)

for C & D: 1 in 250 dilution of antibody to tag
1 in 500 dilution of goat [$F(ab')_2$ fragment] anti-mouse IgG

N.B.

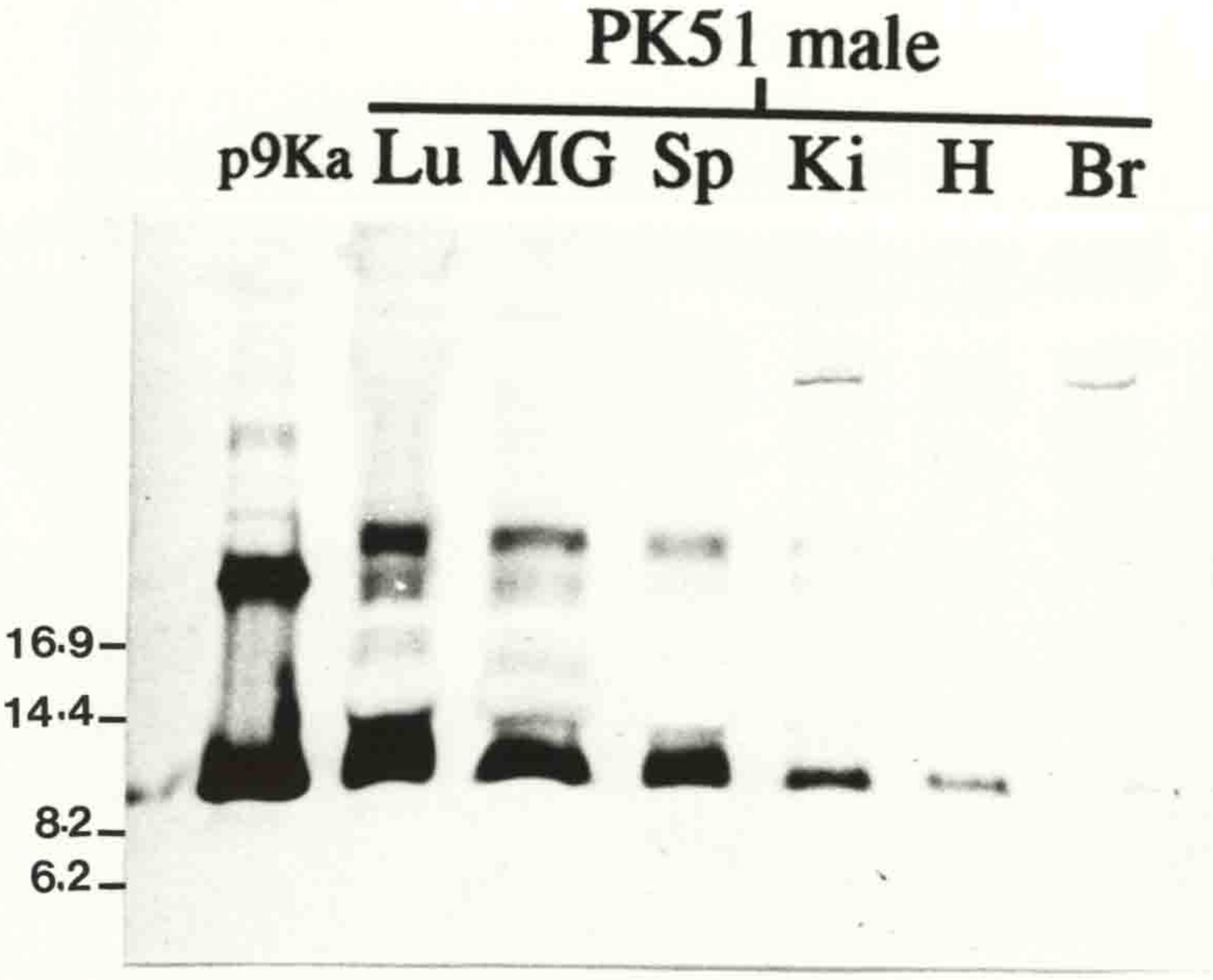
p9Ka is seen to run in both monomeric form (M) and dimeric form (D) on SDS-PAGE gels (as indicated in A).

In tissue extracts the migration of the p9Ka is somewhat retarded due to the interference of other proteins, when compared to the relatively pure recombinant p9Ka standard.

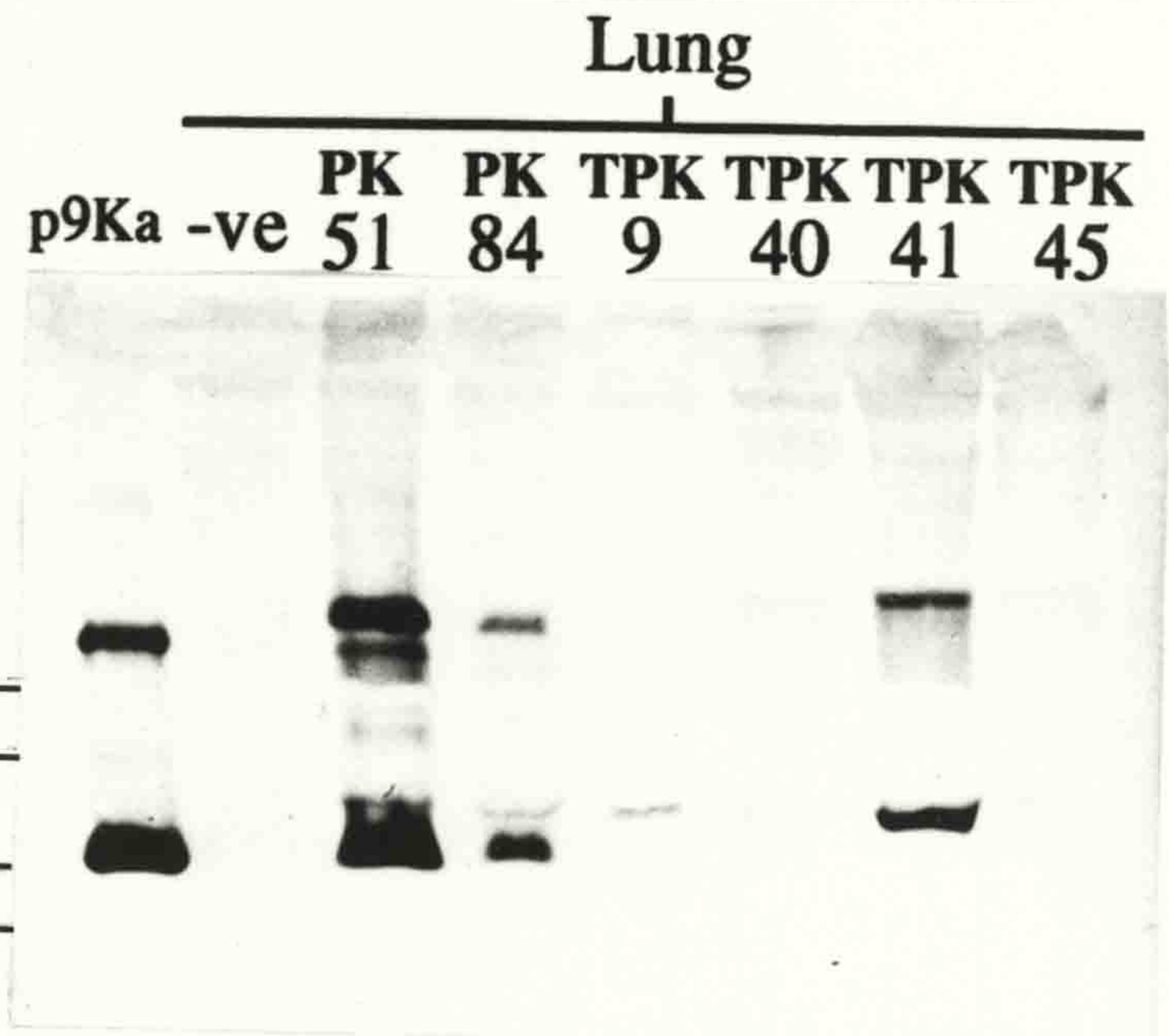
The tagged-p9Ka protein is seen to be larger than the recombinant p9Ka or the non-tagged transgene p9Ka, consistent with the insertion of the tag epitope (as seen in B).

In C and D the tagged-p9Ka band is indicated by the arrow.

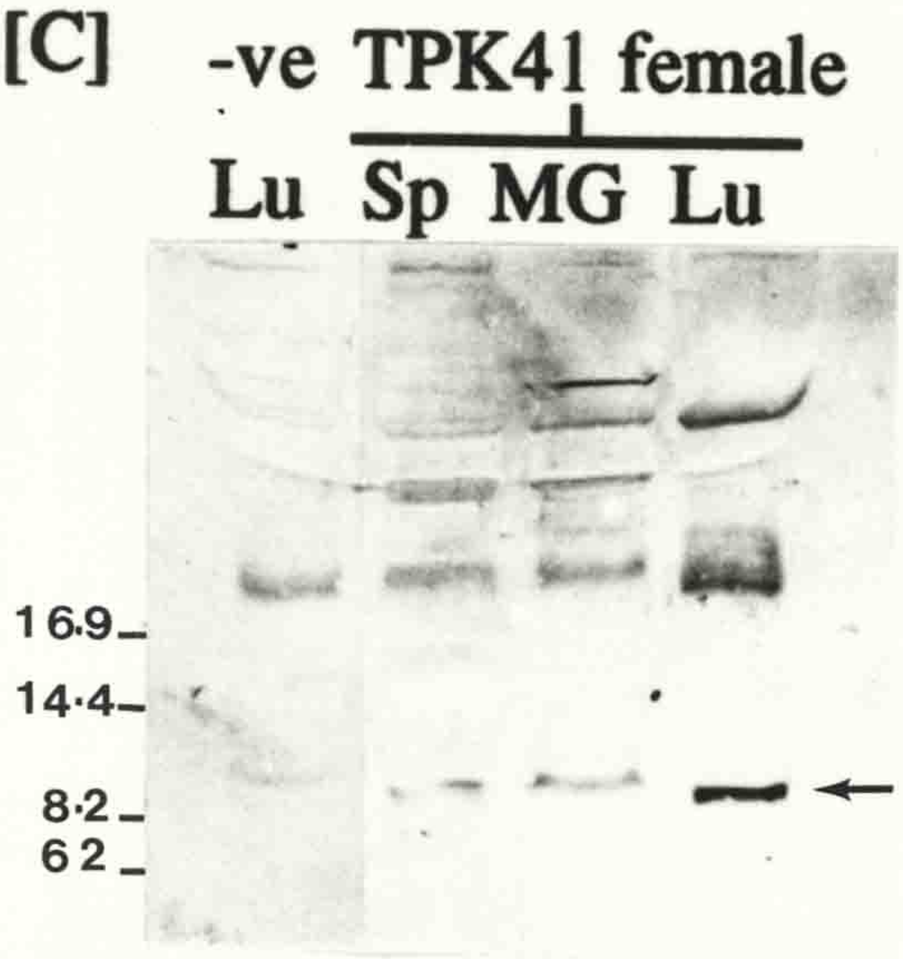
[A]


PK51 male
p9Ka
Lu
MG
Sp
Ki
H
Br
—D
—M
16·9
14·4
8·2
6.2

[B]


Lung
p9Ka
-ve
PK 51
PK 84
TPK 9
TPK 40
TPK 41
TPK 45
16.9
14.4
8.2
6.2

[C]


-ve
TPK41 female
Lu
Sp
MG
Lu
16·9
14·4
8·2
62

[D]

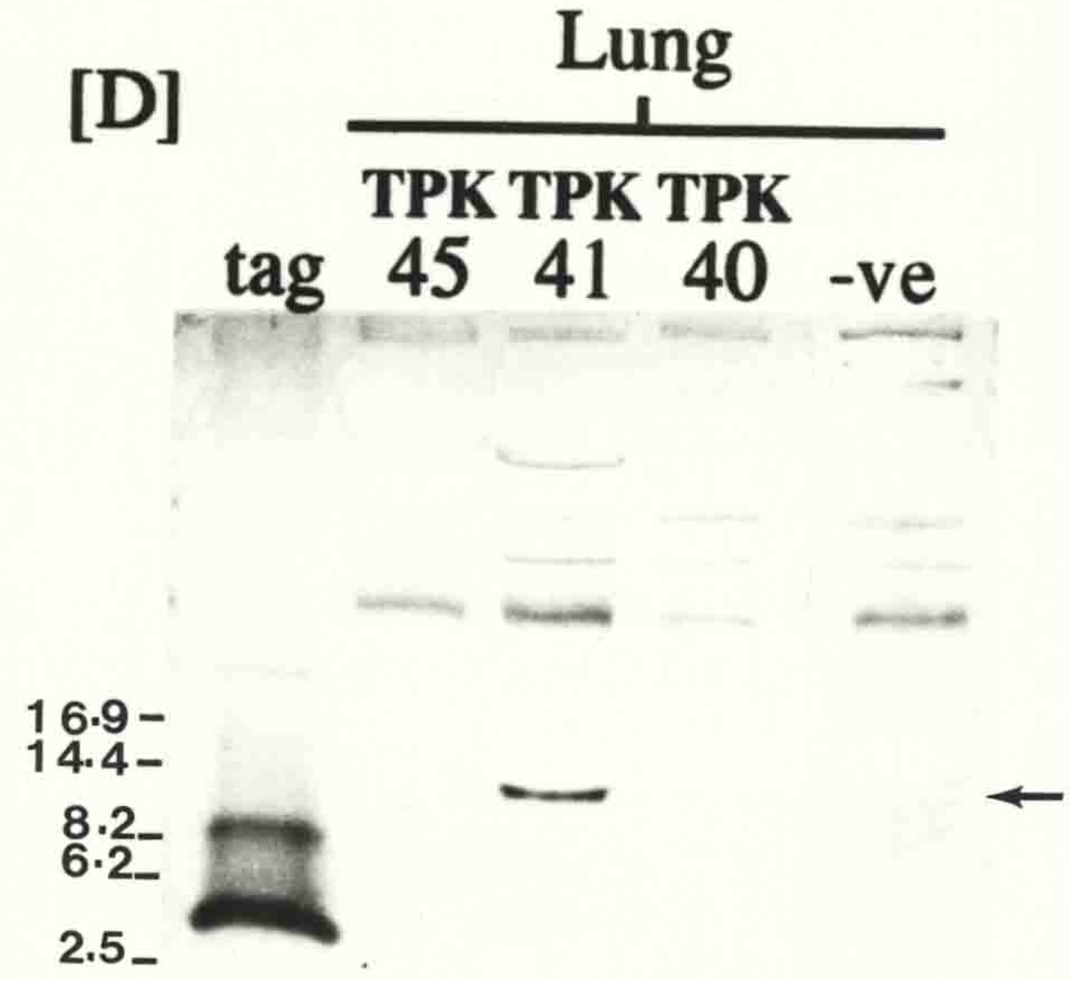

Lung
TPK
TPK
TPK
tag
45
41
40
-ve
16·9
14·4
8·2
6·2
2.5

Figure 3.2.15

Immunocytochemical analysis of the expression of p9Ka in PK51 transgenic (A, C, E, G) and non-transgenic (B, D, F, H) adult mouse tissues using a polyclonal antibody for p9Ka. A one in ten dilution of antibody to p9Ka was used for mammary gland (A, B) and lung (C, D), but a one in four dilution was used for the kidney (E, F), salivary gland and lymph node (G, H).

In G and H, the salivary gland is on the left of the photograph and a portion of an associated lymph node is shown on the right. Photographs were taken on a Reichart-Polyvar microscope, all photography was performed at the same magnification (x230) and the 50 μm bar shown relates to all photographs.

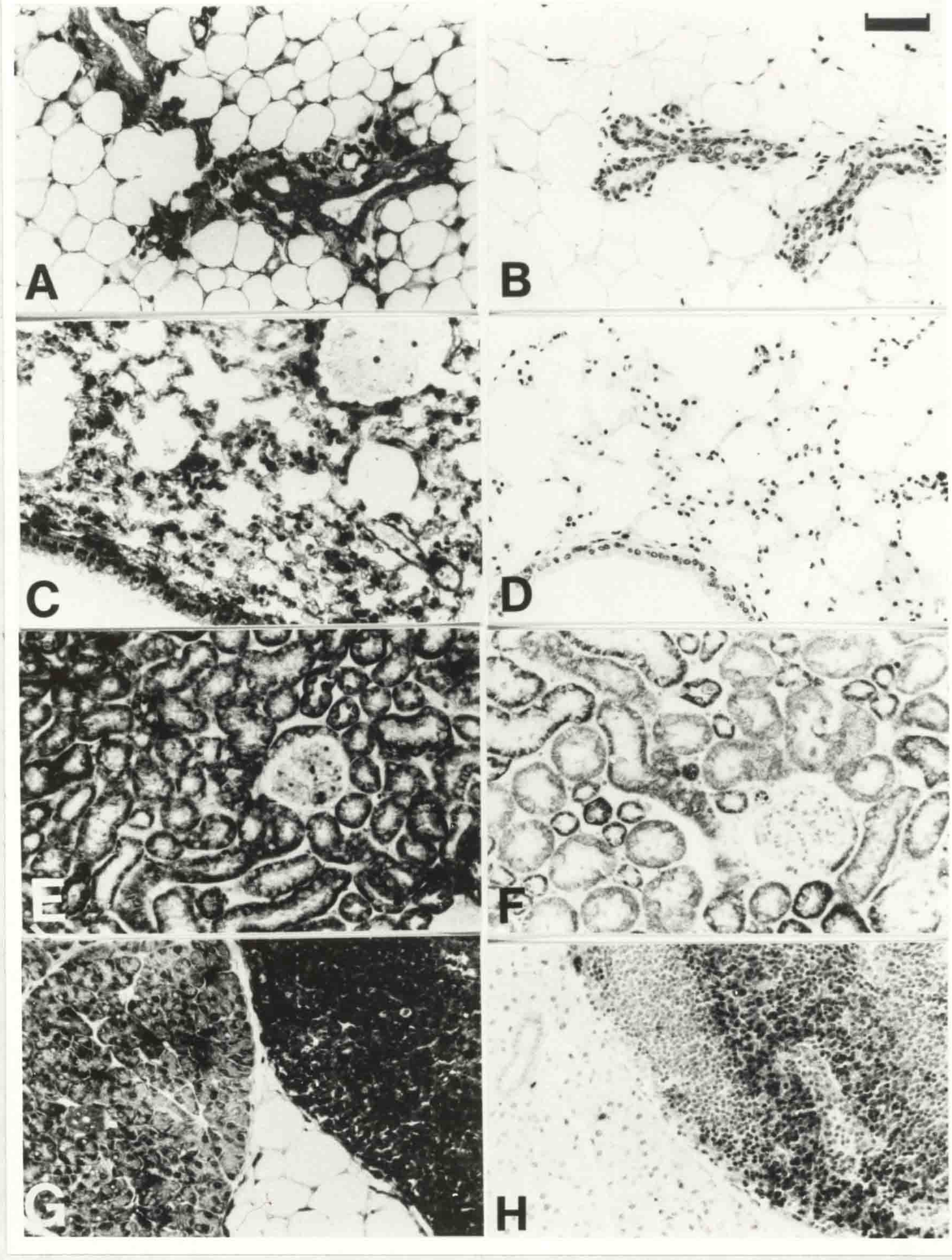
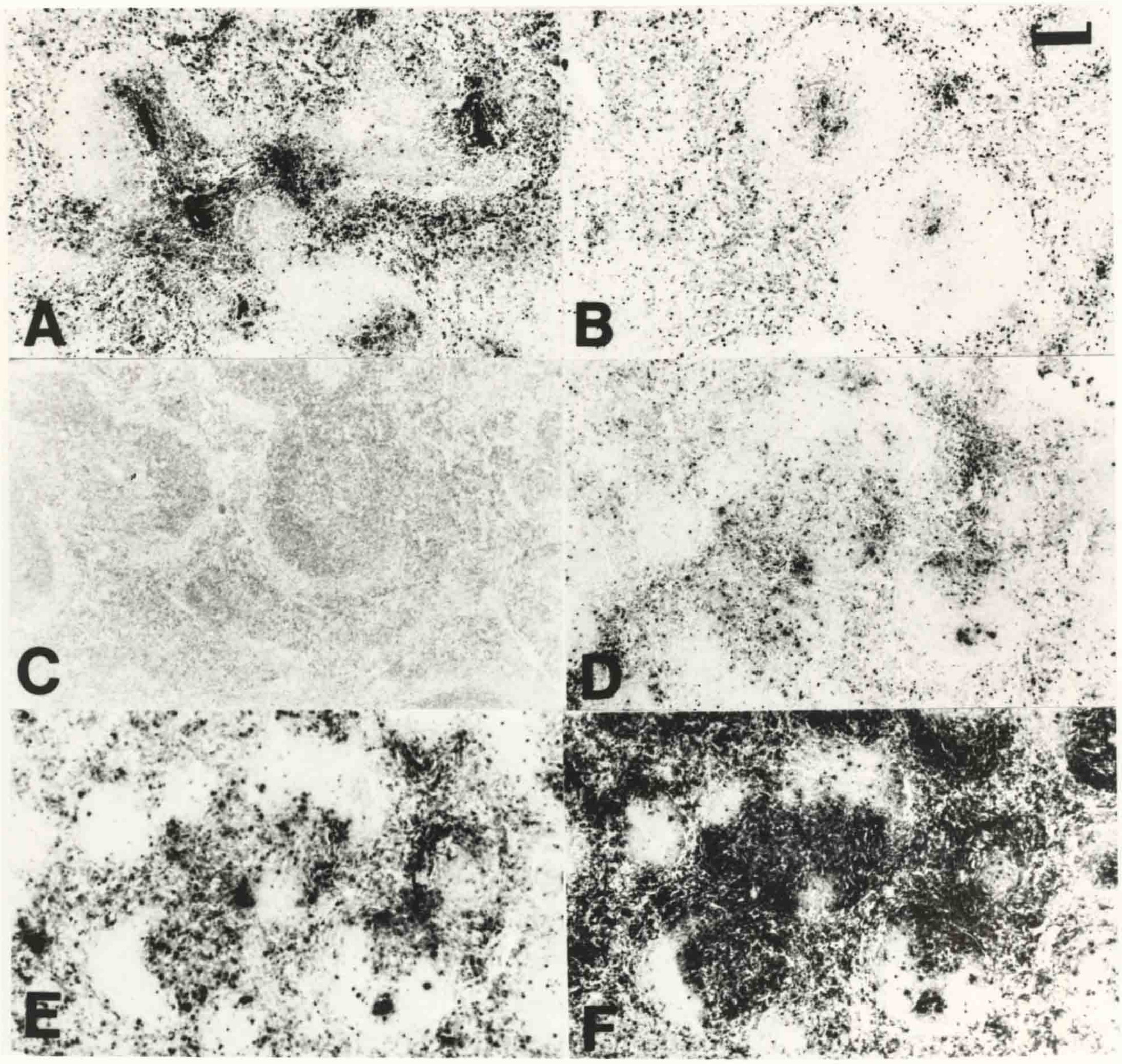

A
B
C
D
E
F
G
H

Figure 3.2.16

Immunocytochemical analysis of the expression of p9Ka in PK51 transgenic mouse, non-transgenic mouse and rat spleen.

Panel	Tissue	Antibody dilution
A	rat spleen	1 in 10
B	non-transgenic mouse spleen	1 in 10
C	PK51 transgenic mouse spleen	no primary antibody
D	PK51 transgenic mouse spleen	1 in 250
E	PK51 transgenic mouse spleen	1 in 50
F	PK51 transgenic mouse spleen	1 in 10

Immunocytochemistry was performed as described in the Methods (section 2.3.9) using a polyclonal antibody raised in rabbit to recombinant rat p9Ka (kindly provided by Fiona Gibbs, University of Liverpool). Photographs were taken on a Reichart-Polyvar microscope, all photography was performed at the same magnification (x57.5) and the (200 μm) bar shown in **A** relates to all photographs. Exposure times for all photographs was the same, so as to allow direct comparison of the staining pattern and intensity between panels.


A
B
C
D
E
F

3.3 Phenotype of Transgenic Mice

The production of mice transgenic for the p9Ka and tagged-p9Ka constructs, and demonstration that these transgenes are highly expressed relative to the endogenous p9Ka gene, warrants a study of the resultant mice produced for any difference in phenotype from their non-transgenic counterparts. For this study to be accomplished reasonable numbers of mice from each transgenic line have to be analysed, together with non-transgenic littermates which act as negative controls. These studies have to be carried out over as long a period as possible in order to allow for the late appearance of any new phenotypic traits, e.g. the appearance of neoplasia.

Transgenic mice and non-transgenic littermates were observed regularly for signs of illness or any traits which might be considered to constitute an altered phenotype. Since limited numbers of mice and limited resources were available, this study relied on the ability to maintain as many mice as possible and only limited numbers were used for analysis of expression. Where such analysis was necessary, full autopsies were usually performed and tissues were examined histologically. Such analysis is not without problems, the majority of which can be grouped as "sampling errors". That is to say putative phenotypes are observed which on longer term or more extensive (e.g. histological) analysis turn out to be false. Reasons for such misinterpretations include: comparison of mice of different ages; comparison of mice housed in different conditions (e.g. alone, in groups, in different animal-house environments); and, analysis based on small numbers of available mice at any one time. One possibility that always has to be considered is that an apparent phenotype can be due to an infectious agent rather than the transgene itself. A good example of the way in which a potential phenotypic effect can be linked initially to a particular mouse line, but which can, by thorough and unbiased analysis, be shown to be neither phenotypic nor (in all probability) linked, is given in Section 3.3.1 "Rheumatism in the PK51 Transgenic Mouse Line".

The other main analysis performed to try and determine a phenotype for the transgenic mouse lines is an autopsy, which includes post-mortem dissection, observation and histology. This analysis is performed on three groups of mice: mice which die unexpectedly from an unknown cause; mice which are sacrificed to save them from undue pain of an observed illness or injury; and, mice which, after they have been observed for a long time, display no gross phenotype, but are sacrificed in order to try and discern a phenotype upon autopsy. The numbers of mice subjected to long-term observation and the numbers of those which were subsequently subjected to post-mortem examination are given in **Table 3.3.1**. In general mice were observed for as long as possible before they were sacrificed for autopsy, but mice from longer-established lines (such as PK51) were often older when sacrificed and mice from later-established lines (such as TPK45) were often younger. **Table 3.3.2** shows the average age at autopsy for each transgenic mouse line, together with the range of ages over which autopsies were carried out.

Great care was taken to observe all the major organs upon autopsy for signs of abnormalities, and any tissue seen to differ from normal was subjected to histological examination. If several mice of the same line exhibited abnormalities in any one tissue, that tissue was examined histologically in other mice of the line, and also in non-transgenic controls and in selected mice of other lines.

3.3.1 Rheumatism in the PK51 Transgenic Mouse Line

When they were routinely examined, several mice were seen to be suffering from a swelling of one of their limbs. A total of eight mice were affected, five in the front, left limb and three in the rear, left limb, and all were PK51 females. Despite the fact that only a relatively small number of non-transgenic female littermates were available as controls, there were some mice of other lines in the same cage-rack and this phenotype seemed to be restricted to PK51 animals. Upon further investigation, the symptoms resembled "mouse rheumatism" and, whilst it would be an interesting phenotype, this disease is known to be caused primarily by infection. To check for

the presence of the causal agent, *Streptobacillus moniliformis* (a gram-negative bacillus), mice were sacrificed and a swab of the caseous exudate, which was located in the swollen limbs, sent for analysis to the Department of Veterinary Pathology at the University of Liverpool. The causal agent was isolated from these swabs and further examination of the animals confirmed some of the other symptoms of this disease, including enlargement and focal necrosis of the spleen; conjunctivitis; oedematous, ankylosed and arthritic limbs in which the joints show signs of disintegration with caseous exudation.

After the infectious agent had been identified, the probable source of infection was identified as wild rats, which are known carriers of the causal bacteria, but do not suffer any ill effects. Contact between a colony of wild rats, which were infesting the animal facilities, and the affected mice was most probably via urination by the wild rats, whilst feeding on the mouse-cage food supply. (It should be noted that the animal facilities in question have since been refurbished to a very high standard.)

Despite the fact that the disease suffered by the PK51 mice was due to *Streptobacillus moniliformis*, the causal agent of mouse rheumatism, some interesting questions were raised by this infection. Why were only females infected, why were only the left limbs affected, and, perhaps most interestingly, why were only PK51 mice were infected? The possibility of a link between increased expression of p9Ka and susceptibility to pathogens that have a causative role in rheumatism is intriguing. However, since this disease constitutes an unwelcome health risk, is communicable between mice and necessitates humane killing of the affected animals to relieve the painful symptoms, it was not considered wise to carry out any experiments to address the apparent susceptibility of the PK51 mice.

3.3.2 Putative Phenotype - Death

One phenotype which could arise in transgenic mice is a shorter life-span (early death) or increased death rate, relative to non-transgenic mice. This phenotype could

be due to a number of more-subtle phenotypes caused by the transgenic nature of the mice. Several mice did die unexpectedly with no defined cause, but the lack of knowledge about the cause of death was often due to widespread decomposition. The ages at death for the various mice, together with the proportion of premature deaths in the population under study, are shown in **Table 3.3.3**. This analysis excluded those mice culled for preparation of tissues or due to illness. The analysis also excluded those mice for which the cause of death was not disease (e.g. one case of death due to lack of water, resulting from a faulty bottle).

Whilst the range of ages of the mice at death were consistent between those transgenic lines in which death was seen, the proportion of mice that died (due to causes unknown) did vary. However, in only one transgenic line were there significantly more deaths than in the non-transgenic littermates. The homozygous TPK41 mice were quite clearly prone to death at a relatively young age. Although the ages at which death occurred in this group were not significantly different from premature deaths seen for other mice (**Table 3.3.3**), the proportion of the population that died prematurely was significantly different [$P < 0.01$, Fischer's exact test (Zar, 1984)]. For the TPK41 line, the number of homozygotes which died was significantly different from the number of hemizygous transgenics which died, and this result warranted individual examination of mortality. However, the premature death of these mice is probably related to the other phenotypic traits seen in the TPK41 line (see section 3.3.5 "Dwarfism in the TPK41 Transgenic Mouse Line").

In three cases, death was apparently related to neoplasia (Section 3.3.3).

3.3.3 Putative Phenotype - Neoplasia

Any increase in the incidence of neoplastic disease was monitored both by long-term observation and after autopsy. One of the difficulties in monitoring neoplasia is that tumours may not always be obvious on inspection of live mice, or they may have no obvious deleterious effects on an affected mouse. Even after

autopsy, any tumours present may not be obvious due to small size or their location. Tumour histopathology was confirmed independently by two other investigators (Prof. P.S. Rudland and Dr E.W. Parry).

When an assessment is made of any increase in tumorigenic rate, it is important to consider any sampling errors as mentioned previously, especially with regard to those which may occur in the control group of non-transgenic littermates. These considerations are particularly pertinent when investigating small increases in tumorigenicity in a population which is prone to age-related neoplasia. Such age-related neoplasia is heavily dependent on the strain of mouse used, and it is unfortunate that the genetic background of the transgenic lines produced is not one which has been well studied with regards to its neoplastic predisposition. Much of the comparison with regard to age-related tumour incidence is therefore taken from studies on a range of other strains of mice as summarised in Frith and Ward (1988) and Faccini *et al.* (1990), and age-matched controls were studied when possible. In the present study, many types of neoplasia were seen in a range of mice, most of whom were of a considerable age. The types of tumour were grouped and the data is shown in **Table 3.3.2**, together with information of the average age at autopsy for each line of mice. Further details on the type of tumour observed, and the age of the affected animals, are given below.

Lung tumours were seen, with an adenoma in one non-transgenic mouse, and adenocarcinomas in one TPK9 mouse, two PK51 mice and one TPK45 mouse. Such low levels of incidence of a tumour type which is relatively common in the better-studied mouse lines, cannot be considered to be of any significance. No significant difference in incidence from the non-transgenic mice or from each other, was seen for any line, using Fischer's exact test (Zar, 1984). Of all the tumours observed, the tumours of the lung were apparently the earliest arising and were found in mice of 17, 40, 70, 45 and 35 weeks old, respectively.

Lymphomas were seen in the PK84 line (a single, fatal case in the thymus), in the TPK9 line (a single case in the thymus) and in the PK51 line (two cases in the

Peyer's patches of the duodenum, both leading to malnutrition due to obstruction of the alimentary canal). One of the PK51 mice died from malnutrition associated with blockage of the gut, but the other was sacrificed before death could occur naturally. No significance could be attached to any data using Fischer's exact test. Once again (even without any incidence in the control group) the occurrance of these tumous must be considered as no more than sporadic. Nevertheless it is interesting to note that lymphoid tissue is a prime site for expression of p9Ka, even in the non-transgenic mouse.

Angiosarcoma was one type of neoplasia which had a serious, deleterious effect on the affected animals, and was therefore obvious from simple inspection. This malignancy occurred in two PK51 mice (a female at 69 weeks and a male at 76 weeks of age) and in various sites (mammary gland, spleen and bone in the female, and liver, spleen and lung in the male). The female mouse died due to haemorrhage associated with the angiosarcoma of the spleen, but the male was sacrificed before death could occur naturally. Multiple sites of disease are very common in this type of neoplasia, and there is some argument whether this is due to metastatic spread, or multiple lesions arising independently through some common pathway (Faccini *et al.*, 1990). The occurrence of this type of lesion in mice which over-express a gene which may be involved in metastasis is very interesting. Although it is not possible to state categorically that this type of malignancy is conferred to the PK51 line ($P = 0.09$, Fischer's exact test of the incidence in PK51 mice verses the incidence in all other lines), there were no other such cases in the control group of age-matched, non-transgenic mice, or in other transgenic lines. Since the PK51 mice express (non-tagged) p9Ka at levels considerably greater than any other mouse line studied, this is an interesting observation. However, this type of neoplasia has been reported to be common in old mice, and the two cases reported here occurred in mice of a considerable age (69 and 76 weeks). It is therefore possible that the greater, average age of the PK51 mouse line is responsible for the incidence of this neoplasia, but, nevertheless, this type of malignancy is worthy of further investigation.

Liver tumours were the most common type of neoplasia seen, and occurred mainly in old mice of the PK51 line (53, 82, 82 and 89 weeks old). These tumours were of different types (one poorly differentiated hepatocellular carcinoma, one trabecular hepatocellular carcinoma, one spindle-cell tumour and one basophilic adenoma with areas of necrosis and chlorangiosarcoma). A single case of a liver tumour was seen in the PK84 line (a vacuolated cell hyperplasia with poorly differentiated hepatocarcinoma). Although the incidence of liver tumours in the PK51 line of mice is not statistically significant when compared to the non-transgenic control mice (P=0.17, Fischer's exact test), its incidence is significantly higher than the incidence in all other mice (P=0.03, Fischer's exact test). This apparent statistical discrepancy is simply due to the greater significance that can be attached to data from a larger sample size (i.e. 117 non-PK51 mice as opposed to just 28 non-transgenic mice). The incidence of tumours in the liver is well known to increase with age in various mouse lines, and it is probable that the higher incidence in this line is primarily due to the greater age of these mice. Unfortunately, the liver tumours in the PK51 mice occurred predominantly in the oldest mice, for which no age-matched controls are at present available. Moreover, the fact that all tumours were of different pathology does not support a putative phenotype based on transgene-induced liver tumorigenesis in the PK51 line.

Sarcomas (other than those already mentioned) were found in a variety of mice: a TPK40 mouse (spindle-cell tumour of the skin), a TPK41 mouse (spindle-cell sarcoma in the mesentery), a PK84 mouse (leiomyosarcoma), a PK51 mouse (leiomyosarcoma) and a control mouse (a sarcoma of indistinct origin). Again no significance can be attached to the incidence of this type of tumour.

No *mammary tumours* were seen in any lines of transgenic mice, or in non-transgenic controls. This is of particular relevance given the proposed role for p9Ka in malignant progression of breast neoplasia.

In terms of the overall incidence of tumours in the various lines (**Table 3.3.2**), the PK51 line is apparently more tumorigenic and the incidence in the TPK9

line is also considerably higher than the other transgenic lines or the non-transgenic mice. No statistical significance can be attached to any incidence of tumours when compared to the non-transgenic mice (in all cases $P > 0.07$, Fischer's exact test). However, for the PK51 line only, compared to all non-PK51 mice, the overall incidence of tumours is significant ($P = 0.01$, Fischer's exact test). Again the PK51 data is statistically significant because it can be compared to data of a large sized sample. Despite the fact that the overall incidence of tumours is significantly greater than in the general population of other mice used in this study, it is hard to attach any real significance to the role of the p9Ka transgene in production of these tumours. Although the PK51 mice express the (non-tagged) p9Ka at a considerable level, the generally greater age attained by the PK51 mice is probably a more important fact in the ætiology of these tumours than the expression of p9Ka. The fact that most tumours were only visible after autopsy of aged mice, taken together with the known occurrence of neoplasia in mice of this age, strongly implies that the neoplasias that have arisen are age-related. If the transgenes were having any positive effect on the occurrence of tumours, greater number of tumours in younger mice, would be expected.

3.3.4 Putative Phenotypes - Non-neoplastic Lesions

Non-neoplastic (or in some cases pre-neoplastic) lesions can form the basis of a phenotype, especially those related to developmental abnormalities. It is important to consider age when analysing such results, since there are many examples of non-neoplastic lesions that occur more frequently in old age.

In the case of the transgenic lines, there were two clear phenotypic effects which fall into this category and they were: the dwarfism witnessed in the TPK41 mouse line (for this and all related TPK41 data, see section 3.3.5 "Dwarfism in the TPK41 Transgenic Mouse Line"); and the rheumatism seen in the PK51 mouse line (see section 3.3.1 "Rheumatism in the PK51 Transgenic Mouse Line"). Other lesions which were seen consisted of four main types: liver abnormalities including

focal necrosis, vacuolated cell foci, basophilic cell foci and eosinophilic cell foci; enlargement of the coagulating gland together with hyperplasia; ovarian cysts; and cystic endometrial hyperplasia of the uterus. All of these lesions were seen in all transgenic and non-transgenic lines of mice except TPK9 (N.B. small sample size). They largely occurred in old age since they were found upon autopsy of aged mice, and hence no evidence of phenotypic trait can therefore be offered with regard to these lesions.

3.3.5 Dwarfism in the TPK41 Transgenic Mouse Line

When adult, female littermates of the TPK41 line, which had been produced from two hemizygous parents (and therefore included hemizygous, homozygous and non-transgenic individuals, **Figure 3.1.12**) were observed, some mice were visibly smaller than others (**Figure 3.3.4**). The size of the animals was linked to whether they were non-transgenic (largest), hemizygous (smaller) or homozygous (smallest).

In order to make a quantitative analysis of the above phenomenon, the mice were weighed and their body weight taken as an indicator of size. Nose to tail measurement was not performed since this data would be affected adversely by the prior removal of the tail-tips, performed to prepare genomic DNA. Other invasive or post-mortem techniques (such as bone measurement) were also ruled out since it was not reasonable, at that time, to sacrifice the numbers of mice necessary to produce statistically relevant results. Adult mice (divided by sex) from all lines were weighed and their mean body weights determined (**Figure 3.3.4**). Since adult mice of different ages were being compared, the data was first analysed to check the dependance of body weight on age (**Figure 3.3.5**). In the range of ages used, it was found that there was no dependance of body weight on age, and the data was therefore valid for comparison of body weight between mouse strains. Only the hemizygous and homozygous female mice of the TPK41 line exhibited any significant difference in body weights from the mean for the other lines or non-

transgenic littermates. The hemizygotes were significantly lighter than the non-transgenic controls ($P = 0.0033$, Student's t-test, assuming homogeneous variances). The homozygotes were not only significantly lighter than the non-transgenic controls ($P < 0.0001$, Student's t-test, assuming homogeneous variances) but also significantly lighter than their hemizygous littermates ($P = 0.0056$, Student's t-test, assuming homogeneous variances).

Upon autopsy and histological examination of several TPK41 homozygous mice, a number of interesting abnormalities were noted. In two cases, abnormalities were seen in the kidneys of female mice; a homozygote possessed one small, atrophic kidney and a hemizygote possessed an atrophic and cystic kidney. Both of these cases seemed to be caused by developmental abnormalities, since no evidence of chronic or acute disease was seen. More generally, the smaller mice, particularly the homozygotes, as well as possessing smaller tissues, had a lower level of body fat when observed at autopsy. Some mice were almost totally devoid of body fat, either in their mammary fat pads or associated with their viscera, at an age when normal (non-transgenic) mice exhibited considerable amounts of fat. This lack of white adipose tissue was, at least in the homozygous females, paralleled by a similar paucity of brown adipose tissue. Histological analysis of the interscapular brown adipose tissue of the homozygous females showed signs of developmental retardation in keeping with the dwarf phenotype of these mice. Development of the mammary glands was also severely retarded in the homozygous females. In one particular case, the mammary gland of a twenty-two-week old TPK41 homozygous female resembled that of an eight-day old mouse. These abnormalities are apparently linked to the dwarfism found in this line of mice.

Since the pituitary is a prime site of interest in control of bodily growth through the production and release of growth-regulating hormones, several pituitaries from non-transgenic and TPK41 transgenic mice were examined histologically. Although the pituitaries of the transgenic (particularly the homozygous) mice appeared smaller than those from control (non-transgenic) mice, this result was in keeping with the

level of dwarfism seen in these mice, and no histological abnormalities were seen.

Both hemizygous and homozygous mice were fertile, but early litters from female TPK41 mice were not nursed, apparently due to lack of production of milk. This lack of production of milk is in keeping with the delayed development of the mammary gland seen in the homozygous females. Although hemizygous females did not exhibit the extreme developmental abnormality witnessed in (non-parous) homozygotes, there was also an apparent inability to nurse first litters. Multiparous hemizygotes were able to nurse their young, and this fact may be due to the additional developmental stimulation arising from multiple pregnancies. This data is purely observational and before any greater significance can be attached to these results, multiple pregnancies in several hemizygous and homozygous females will have to be induced under more tightly controlled conditions.

That the characteristics of the dwarf phenotype were found only in female mice of the TPK41 line is interesting, since it might relate to the transgene being X-linked or to a sex dependent action of the transgene expression. However, since the female progeny of hemizygous males were not all transgenic, and a male homozygote was identified, it is not possible that the transgene is on the X chromosome of the TPK41 mice, and both male and female TPK41 mice express similar levels of transgene. It should be emphasised that the dwarf phenotype was much more obvious in the homozygotes of which there was only one male example. Hence, a thorough study of any effects in male homozygotes was not possible, especially since this mouse died without a full autopsy being possible. With regard to body-weight, we might have expected to see a significantly lower mean body weight for TPK41 hemizygous males. That this was not the case, despite the fact that TPK41 mice had the lowest mean body-weight, can probably be explained by the greater variation in body weight of the male compared to the female mice (**Figure 3.3.5**). Indeed this is obvious when the male non-transgenic and transgenic mice of all lines are observed, since they can be seen to have a tendency toward obesity in old age. It is therefore not possible to categorically state that the dwarfism is seen solely in the females of this

strain.

To find out whether the observed dwarfism was due to the expression of the tagged-p9Ka transgene (the TPK41 line was the highest expressing tagged-p9Ka transgenic mouse line), or to an insertional event in the host genome caused by integration of the transgene, further studies were performed to determine the relative levels of expression of the transgene between hemizygous and homozygous TPK41 transgenic mice. The premise for these experiments was that, since the homozygous mice were significantly smaller than the hemizygous mice, which in turn were significantly smaller than their non-transgenic littermates, the expression of the tagged-p9Ka would have to be significantly different between homozygous and hemizygous, as well as between hemizygous and non-transgenic mice for these differences to be due to expression of the transgene.

Firstly, the level of tagged-p9Ka mRNA expression was assessed by Northern blot analysis of total RNA from the spleen, heart, kidney and brain of homozygous and hemizygous TPK41 female mice (**Figure 3.3.6**). It had already been shown that the hemizygous TPK41 mice typically express the tagged-p9Ka mRNA at levels some 100 fold or more than the non-transgenic mice express the murine p9Ka mRNA, as determined using the rat p9Ka cDNA probe. By this criterion, the homozygous mice did not express the p9Ka transgene at significantly greater levels than their hemizygous littermates. A second Northern blot was performed on total RNA from the spleen and kidneys of non-transgenic, TPK41 hemizygous and TPK41 homozygous mice. Whilst both hemizygous and homozygous mice expressed significant amounts of tagged-p9Ka mRNA, when compared to the non-transgenic mouse, the homozygous mouse did not express the transgenic mRNA at a significantly greater level than that of the hemizygote, and certainly not at twice the level, in any of the tissues tested. The pattern of expression of tagged-p9Ka protein, as determined from by Western blots of protein from lung and mammary gland, also showed that the tagged-p9Ka transgene was not significantly over-expressed in the homozygous TPK41 mouse when compared to the hemizygous TPK41 mouse. The

relevance of these studies of transgene expression to the phenotypes seen in hemizygous and homozygous TPK41 mice is discussed later (Chapter 4).

3.3.6 Summary

Long-term observation and post-mortem examination were neccessary to assess the phenotypic effects of transgene expression or integration. Expression of the p9Ka or tagged-p9Ka transgenes would seem to have little effect on neoplasia or any other gross phenotype in the mice studied. None of the lines could be shown to have any significant incidence of a particular neoplastic disease, and the greater general incidence of tumours in PK51 mice could be attributed to the greater age of these mice with respect to the control group. There were no lesions of pre-neoplastic nature which could be linked to a particular line or lines of transgenic mice, and again these lesions occurred in old mice. Whilst, the PK51 line was seen to suffer from a rheumatic condition, this was shown to be due to an infection by *Streptobacillus moniliformis*, the causal agent of mouse rheumatism. In the case of the TPK41 line, a phenotypic dwarfism has been shown in terms of the body-weight of female mice. Also various histological anomalies were seen and mortality was greater in homozygous TPK41 animals, irrespective of sex. The dwarfism was apparently more closely linked to the transgenic status of the animals than to the level of expression of the transgene.

Mouse Line	Mice available for study	Mice found dead	Mice culled for tissues	Mice culled for autopsy	Mice culled due to illness
PK51	65	11	5	52	8
PK84	42	6	2	25	0
TPK9	16	1	3	11	0
TPK40	29	0	6	24	1
TPK41	25	4	5	16	1
TPK45	31	0	2	13	2
Non-transgenic	56	6	4	28	1
Total	*258*	*28*	*27*	*169*	*13*

Table 3.3.1

Summary Table of the Fate of Transgenic Mice and Non-transgenic Littermates

Key

Mice available for study = All mice transferred to Liverpool animal facilities.

Mice found dead = Number of mice discovered dead during routine observation (not all mice were autopsied fully due to an advanced state of decomposition).

Mice culled for tissues = Mice sacrificed to provide source of RNA and protein for analysis.

Mice culled for autopsy = Mice sacrificed (> 40 weeks old) upon which a full post-mortem dissection was performed (this includes some mice culled for tissues).

Mice culled due to illness = Mice found in a state of ill-health or discomfort and subsequently sacrificed to remove the burden of pain.

N.B. All other transgenic and non-transgenic mice produced during the course of this project have been either kept as breeding stock or culled.

	Mouse Line							
	PK 51	**PK 84**	**TPK 9**	**TPK 40**	**TPK 41**	**TPK 45**	**-ve**	***all mice***
Tumour								
Lung	2	0	1	0	0	1	1	*5*
Lymphoma	2	1	1	0	0	0	0	*4*
Sarcoma	1	1	0	1	0	0	1	*4*
Liver	4	1	0	0	0	0	0	*5*
Angiosarcoma	2	0	0	0	0	0	0	*2*
Total tumours	*11*	*3*	*2*	*1*	*0*	*1*	*2*	***20***
Total mice	52	25	11	24	16	13	28	*169*
Incidence (%)	21	2	18	4	0	8	7	*12*
Average age at autopsy (weeks)	66	*55*	50	51	52	41	50	*55*
Range	35-89	17-65	17-70	16-79	25-79	25-48	35-81	*17-89*

Table 3.3.2

Incidence of neoplasia in transgenic and non-transgenic (-ve) mice, and incidence in the total population studied (*all mice*).

Tumours are defined by either the type of tumour (sarcoma, lymphoma or angiosarcoma) or the site (lung or liver). More details of the exact nature of the tumours are given in the text (section 3.3.5). Incidences, expressed as the percentage of mice in which neoplasia was observed, are given for the total number of tumours in any one line of mice.

To indicate the age of mice upon autopsy, the average age of the autopsied mice is given together with the range of ages over which mice were autopsied. The age at which any particular tumour occurred can be found in the text (section 3.3.5).

[A]

Mice	Ratio of premature deaths to number of mice studied	P value for significant difference from non-transgenic mice
non transgenic	6/51	not applicable
PK51	10/52	0.13
PK84	6/40	0.22
TPK9	1/13	0.38
TPK40	0/22	0.11
TPK41 (hemi)	0/14	0.22
TPK41 (homo)	4/5	0.0025*
TPK45	0/27	0.070

[B]

Mice	Age at Death (weeks)	Average	s.d.
non-transgenic	20, 25, 31, 36, 38, 40	31.7	7.9
PK51	22, 32, 42, 50, 54, 59, 62, 68, 69, 89	54.7	19.4
PK84	17, 24, 25, 29, 32, 52	29.8	12.0
TPK9	47	47	
TPK41 (homo)	16, 32, 47, 49	36.0	15.3

Table 3.3.3

Mortality of transgenic mice and non-transgenic littermates.

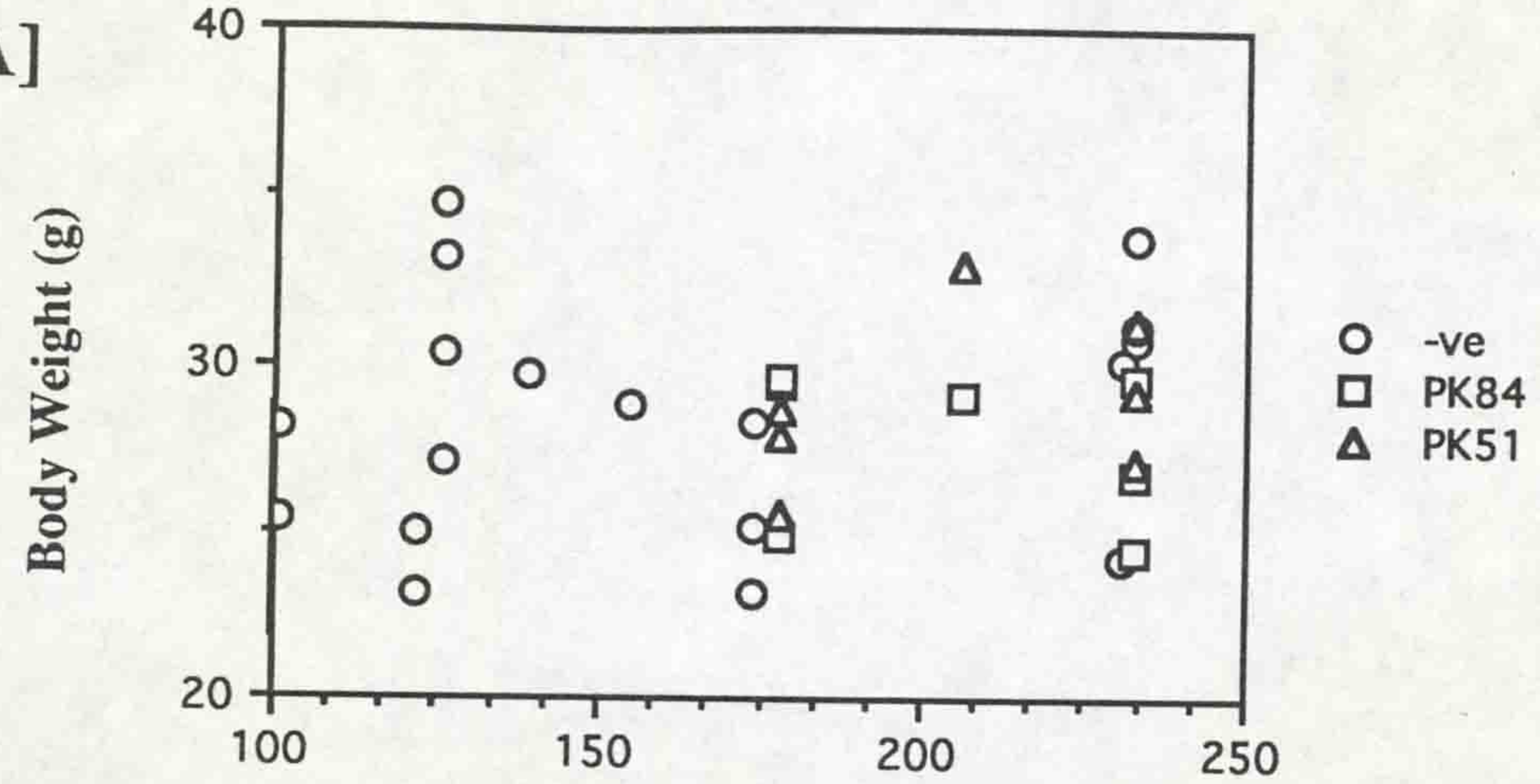
In [A], the number of mice found dead upon routine examination, and the numbers taken as "mice studied" do not include those mice who were sacrificed for tissues (at a relatively early age) or those sacrificed due to illness, but do include those culled for autopsy. P values were calculated, by comparison of each ratio with that for the non-transgenic mice, using Fischer's exact test. TPK41 mice were designated hemizygous (hemi) or homozygous (homo), since there is reasonable cause to expect these two groups of mice to be phenotypically distinct. The asterisk denotes the only value deemed to be significantly different from the normal (non-transgenic) control, i.e. the TPK41 homozygous mice ($P < 0.01$).

In [B] the ages at death are given to allow comparison. There was no significant difference in the average age at death or the range of ages at which the deaths occurred.

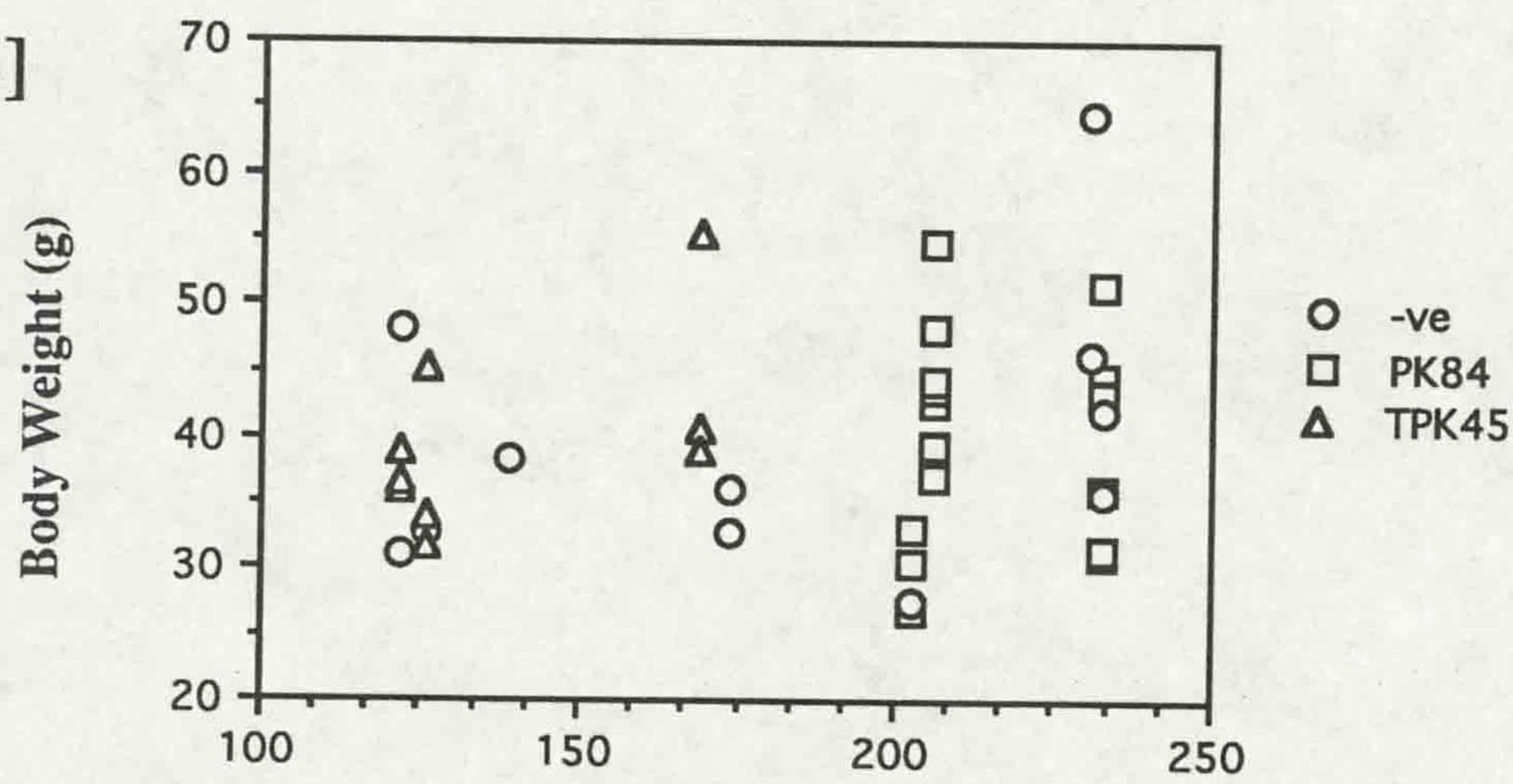
Figure 3.3.4

Photographic and graphical evidence of the small size of TPK41 hemizygous and homozygous mice. In the photograph, the normal mouse is pictured at the top, the TPK41 homozygous mouse is in the middle, and the TPK41 hemizygote is at the bottom (all mice are female littermates). The graphs show the mean weight of both male and female adult mice; the error bars represent the standard errors of the means. The mouse line is indicated on the x-axis; TPK41 hemizygotes are indicated by TPK41+ and homozygotes by TPK41+/+, all other lines are represented by hemizygous mice, non-transgenic mice are indicated as -ve (negative). An asterisk (*) indicates a significant difference from the non-transgenic mean (as determined by Student's t-test); a double asterisk (**) indicates both a significant difference from the non-transgenic mean and from the mean for the hemizygous TPK41 mice (as determined by Student's t-test).

[A]



[B]



[C]

Mice	Male	Female
non-transgenic	11	19
TPK41 hemizygotes	6	6
TPK41 homozygotes	0	5
PK51 hemizygotes	8	14
PK84 hemizygotes	17	11
TPK9 hemizygotes	5	2
TPK40 hemizygotes	5	12
TPK45 hemizygotes	9	15

Figure 3.3.5

Scatter diagrams showing lack of dependence of body weight on age for [A] adult female and [B] adult male mice. Only data from the largest three data groups are shown. N.B. The scale size for [B] is greater than that for [A].
[C] gives the numbers of mice used in analysis of body weight [i.e. for graphs in Figure 3.3.2 and statistics given in text (section 3.3.5)].

Figure 3.3.6

Analysis of the expression of mRNA in TPK41 hemizygous and homozygous transgenic mice. Northern blot analysis was performed as described in Methods, using 10 μg of the total RNA specified (unless stated otherwise).

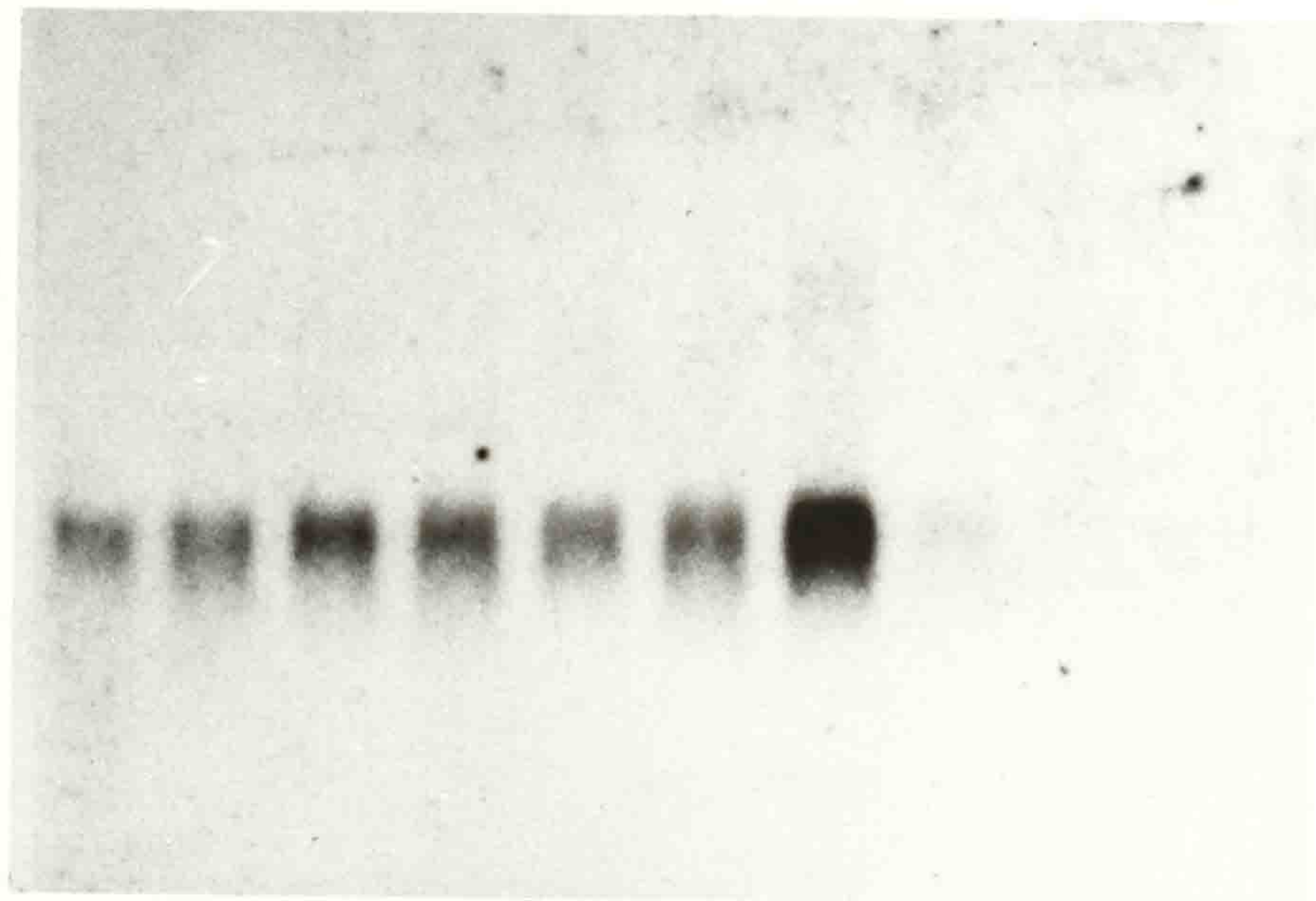
R29 = Rama 29 RNA + = hemizygous +/+ = homozygous

Sp = Spleen; H = Heart Ki = Kidney Br = Brain.

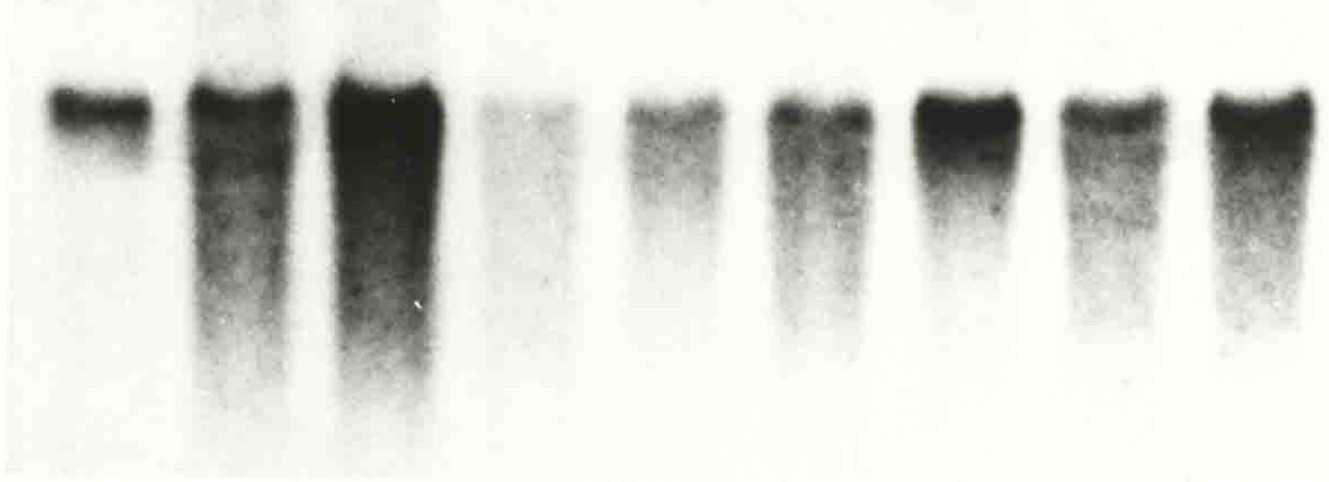
The size of tagged-p9Ka mRNA was as previously reported (750-850 nucleotides), and the size of other mRNAs agreed with those obtained previously (approximately 2000 bases for actin and 1800 bases for G3PDH).

R29 1μg	+ Sp	+/+ Sp	+ H	+/+ H	+ Ki	+/+ Ki	+ Br	+/+ Br	R29 0.1μg

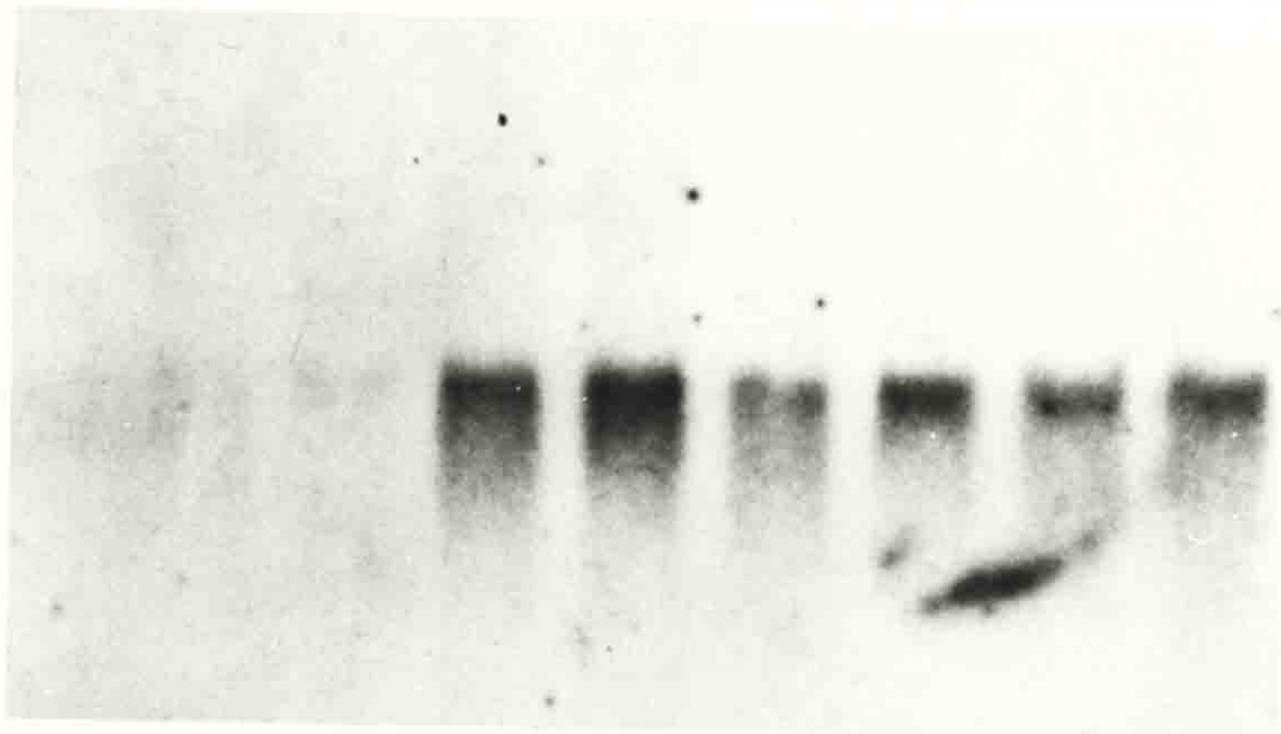
p9Ka



Actin



G3PDH



4. Discussion

Analysis of the Expression of p9Ka in Transgenic and Non-Transgenic Rodent Tissues

In order to assess properly the patterns of expression of p9Ka mRNA and protein shown in rat, in normal mouse and in transgenic mice it is first important to address a few technical aspects, some of which have already been discussed to some extent.

The hybridisation with cDNA probes for constitutive mRNAs (e.g. actin and G3PDH) in order to account for different amounts of total RNA loaded on Northern blots is common. Results produced here suggest that this procedure is not necessarily very good for comparing levels of RNA between tissues of different types, or between the same tissue in different species. All corrections for unequal loading of RNA on Northern blots were, therefore, made by comparison only of samples of the same tissue from a single species. Unfortunately, it was not possible to make similar allowances for the differences in the levels of hybridisation to constitutive mRNAs which were seen between rat and mouse, since it is not easy to determine whether such differences between species are due to genuine differences in expression of the relevant mRNAs, or to differences in hybridisation efficiency of the cDNA probes to the respective mRNAs. A similar problem is encountered for the cDNA probes used to investigate the expression of p9Ka mRNA, but differences in the ability of the individual probes to detect each p9Ka mRNA species are not enough to account for the differences in expression between rat and mouse or between transgenic and non-transgenic mice.

When data from Northern blots or Western blots of distribution in different tissues are examined, it is important to realise that because a certain tissue is seen to express only very low levels (or none at all), it does not necessarily mean that all cell-types within that tissue fail to express that mRNA or protein. Alternatively, it may be that even in a tissue expressing a relatively high level of the mRNA or protein of interest, that expression is limited to a small number of cells.

Immunocytochemistry is a potentially more sensitive technique than Northern or Western blots, since it can detect low levels of expression in a very small minority of cells within a tissue, despite the fact that Western blots apparently indicate no expression in this tissue. However, immunocytochemical analysis cannot be easily quantified for direct comparison of expression in different tissues. Immunocytochemical analysis is dependent upon the use of varying concentrations of antibody to detect different levels of protein, and although a relatively low concentration of antibody may indicate no expression, higher concentrations can show that expression does indeed occur. Since a single tissue-section contains a number of different cell types, which may express the protein at a wide range of different levels, the staining pattern can vary considerably depending on the concentration and sensitivity of the antibody used (e.g. expression of p9Ka in transgenic mouse spleen, **Figure 3.2.16**). It is therefore largely dependent upon the investigator to assimilate the different levels of information into a coherent pattern.

It is often stated in the literature that certain mRNAs or proteins are expressed only in certain tissues but not in others (e.g. expression of p9Ka in the mouse is reported to occur only in lymphoid tissues, Ebralidze *et al.*, 1987). Whilst this may well be true in some cases, the definition of expression is to a certain extent subjective, and based upon the sensitivity of the method used to detect it. Thus for murine p9Ka mRNA, we have shown that it is expressed in tissues that some groups state do not express it. This discrepancy arises primarily as a result of the use of a more sensitive methodology.

Bearing in mind the problems discussed above, a critical review of the patterns of expression of p9Ka performed to date is constructive. The expression of p9Ka mRNA in normal tissues of various species is interesting, since some differences have been reported. The rat p9Ka mRNA has been reported to be expressed in several tissue types including mammary gland, spleen, uterus and liver (Barraclough

and Rudland, 1991). The mouse p9Ka mRNA has been reported to be expressed only in "lymphoid organs" such as spleen, thymus and bone marrow, and in blood stream lymphocytes (Ebralidze *et al.*, 1987). Nevertheless, p9Ka mRNA has also been detected in mouse uterus and placenta (Jackson-Grusby *et al.*, 1987). Recently published studies, of differences in expression between human and mouse p9Ka mRNA (Engelkamp *et al.*, 1992), seem to indicate that the expression of human p9Ka mRNA is relatively wider and greater (strong expression in virtually all tissue studied including heart, skeletal muscle, stomach, kidney, lung and thymus) compared to the mouse (low expression in lung, kidney, stomach and thymus but none in heart and skeletal muscle). Some of these differences may be due to the use of a human cDNA clone for study of both the human and mouse RNAs, since differences in hybridisation levels for p9Ka and actin cDNA probes between different species have been reported here (section 3.2.2). Much of the earlier published work on the expression of p9Ka has been carried out at the mRNA level due to the fact that no suitable antibody was available. An antibody to bovine p9Ka (calvasculin) has recently been produced and p9Ka protein has been detected in bovine aorta but not in lung, brain, heart or testis (Watanabe *et al.*, 1992a). The detection of the expression of p9Ka in some tissues but not in others may be the result of different cellular expression patterns, but, as discussed above, the levels of sensitivity achieved also play a part in defining the tissues where expression was detected. To be even more critical, it must also be stated that the range of tissues analysed in each case is usually quite limited, and reflects not only the availability of tissues but also the particular interests of any one group of investigators.

The expression of rat p9Ka transgenes in mice provides an interesting way of investigating differences in the patterns of expression of p9Ka seen between these species. Together with further studies of the expression of p9Ka in normal rat and mouse tissues, the results from the transgenic mice can be used to validate any differences in the pattern of expression.

Initial discussion will be limited to expression of p9Ka in non-transgenic rat

and mouse tissues. The first point to note is that expression of p9Ka in the mouse was generally very low apart from in the so called lymphoid tissues. Nevertheless, there was expression in other tissues at both the mRNA and protein levels, in contrast to the lack of expression shown in the same tissues by other investigators (Goto *et al.*, 1988; Ebralidze *et al.*, 1989; Engelkamp *et al.*, 1992). To be more precise, mouse expression of p9Ka mRNA has been shown to occur in spleen, lung, mammary gland, heart, kidney, brain, uterus, stomach, ovary and thymus. Moreover p9Ka protein has been additionally detected by immunocytochemistry in small intestine, large intestine, salivary gland, lymph node and bladder. Whereas previous studies have not detected p9Ka in these tissues, this expression is apparently real, although notably generally lower than in the rat.

Turning to the rat, every tissue investigated at the mRNA level exhibited some degree of expression of p9Ka (**Figure 3.2.1**). In terms of a specific pattern of expression of p9Ka mRNA in different tissues of the rat, the main features were a high level of expression in the lung and a low level of expression in brain, other tissues expressed an intermediate level of p9Ka mRNA (**Figure 3.2.2**). These features were borne out in later experiments carried out in parallel with the analysis of transgenic mice (**Figures 3.2.5, 3.2.6 & 3.2.7**).

The levels of expression of both p9Ka mRNA and protein in the rat would appear to be relatively greater than in the mouse, and more akin to that seen in the human (Engelkamp *et al.*, 1992), both in terms of the levels detected and the range of tissues exhibiting expression. The different pattern of expression between rat and mouse is apparently due to a generally lower expression of p9Ka mRNA and protein across the whole range of tissues of mice but additional expression in the lymphoid organs. Since expression of p9Ka mRNA was seen in bloodstream lymphocytes in the mouse (Ebralidze *et al.*, 1987), it could be postulated that any expression of p9Ka mRNA shown on Northern blots of whole tissue-extracts of RNA may be due to these cells and merely reflect their levels within the tissue. However, it was clear

from immunocytochemical studies that expression of p9Ka in these tissues is not limited to circulating lymphocytes.

In terms of the mechanisms that control expression of p9Ka it is important to assess whether the pattern of expression of the rat p9Ka gene in the transgenic mice follows that of the rat or that of mouse. The pattern of expression of transgenic p9Ka mRNA in different tissues, as shown by Northern blots hybridised with radioactively-labelled p9Ka cDNA, is basically the same as that seen for the rat gene, and thus different from that of the endogenous mouse p9Ka gene (**Figure 3.2.2**). This pattern of expression of p9Ka mRNA was shown for both the p9Ka and tagged-p9Ka transgenes, in all the lines of transgenic mice produced (**Figures 3.2.2 & 3.2.12**). The distribution of transgene protein in different tissues largely paralleled the patterns of expression of the mRNAs for both the PK51 and TPK41 lines (**Figure 3.2.14**).

Immunocytochemical analysis of the cell types in which the expression of the p9Ka transgene (using PK51 transgenic mice) and expression of the endogenous mouse p9Ka are seen apparently indicate that the cellular patterns of expression were similar. Obviously expression of p9Ka protein in the PK51 mice was considerably greater than in non-transgenic mice, but when different concentrations of antibody were used (i.e. lower concentrations for transgenic tissues) the pattern could be shown to be very similar (**Figure 3.2.16**). The one exception was that, expression of p9Ka was apparently higher in lymphoid cells of the non-transgenic mouse, in relation to other cell-types, than in the transgenic mouse, and such a situation would account for the pattern of distribution of p9Ka mRNA in different tissues of the mouse.

Since expression of p9Ka is controlled, at least in part, at the transcriptional level (Barraclough *et al.*, 1984b; Barraclough and Rudland, 1991), and the rat expresses p9Ka mRNA at a level intermediate to that in PK51 and in non-transgenic mice (**Figures 3.2.5, 3.2.6 & 3.2.7**), we may expect that expression of p9Ka

protein in the rat would be intermediate to that in PK51 and in non-transgenic mice, and indeed this would seem to be the case. Immunocytochemical analysis of different tissues of the rat (Fiona Gibbs, personal communication and Ph.D. Thesis) reveals some similarities with the expression described in the mouse, but with some additional staining similar to that seen in the transgenic mouse. As for non-transgenic mice, the lymph nodes of the rat exhibit diffuse cortical staining, but no follicular staining, and the breast exhibits staining only in the periductal stroma and connective tissue. The rat spleen is seen to stain in a similar pattern to the mouse but at a slightly greater level (**Figure 3.2.16**). As with the results in the lymph node, the similarity in staining pattern and intensity at the same concentration of antibody in the spleens of rats and mice is probably due to the greater expression of p9Ka protein in these tissues relative to others in the mouse. Staining patterns similar to both transgenic and non-transgenic mice but at an intermediate level are seen in heart, kidney, liver, brain and salivary gland of rats. Greater and more diverse staining is seen in other rat tissues compared to that in the same tissue from mice, and the pattern is similar to the staining seen for the PK51 transgenics, but at lower levels (e.g. lung and uterus). Thus, the pattern of expression of the endogenous rat p9Ka gene and the (rat-derived) transgenes is similar both at the level of the tissue and at the level of the individual cell. Apart from the lymphoid cells, the pattern of expression of the transgene, or the endogenous expression of the rat gene, would also appear to be similar to that of the endogenous mouse p9Ka gene at the level of the cell.

In all lines of transgenic mice, the expression of p9Ka mRNA still follows a pattern of distribution that is the same as the pattern for the rat gene (**Figure 3.2.2 & 3.2.12**), and is dependent on the number of copies of the integrated transgene (copy-number dependent) (**Figure 3.2.13**). These results would seem to indicate that transcriptional control of the transgene is independent of the position of integration within the genome (position-independent). Position independence is a property associated with a small number of transgenes (β-globin, Grosveld *et al.*,

1987; CD2, Lang *et al.*, 1988; lysozyme, Bonifer *et al.*, 1990; *fps/fes*, Greer *et al.*, 1990; α-globin, Higgs *et al.*, 1990; and β-lactoglobulin Whitelaw *et al.*, 1992). Position-independent, copy-number dependent expression of transgenes would appear to be reliant on those transgenes possessing regulatory elements acting at the chromosomal level, e.g. the "locus control region" (LCR) of the β-globin gene (Orkin, 1987; Kollias and Grosveld, 1992). Several such regulatory regions (e.g. those of the α-globin, β-globin and CD2 genes) are apparently also involved in conferring selective expression in specific tissues (tissue-specific expression) and contain tissue-specific, DNaseI-hypersensitive sites (Sippel *et al.*, 1992). Further analysis of the regulatory elements of the p9Ka gene may help to identify a similar regulatory region. This regulatory region could be used when position-independent expression of a transgene is required in a wide range of tissues. The ability of the p9Ka transgene to act independently of its environment is quite important, if we are to consider the implications of differences in transcriptional control between rat and mouse genes.

The fact that the pattern of expression of the p9Ka mRNA from the rat gene does not conform to that of the mouse when expressed *in vivo* in the transgenic mice could be taken as an indication that the rat transgenes do not respond to some trans-acting regulatory factors involved in limiting transcription of murine p9Ka mRNA. Alternatively the transgene construct may be lacking the cis-acting control regions which are responsible for limiting the p9Ka mRNA level in mouse tissues. The fact that the endogenous rat gene is also relatively highly expressed indicates that, rather than being a property of the transgene construct, this tissue-specific mRNA expression and high level expression truly reflect that of the rat gene.

Interestingly, recent experiments on the murine p9Ka gene (*mts*1) seem to indicate that cis-acting regulatory elements immediately upstream of the first intron play little or no part in expression, but that loss of methylation of the DNA may be involved in transcriptional activation (Tulchinsky *et al.*, 1992). Further

characterisation of the gene revealed a number of elements within the first intron that may be involved in regulation of the expression of the p9Ka gene (Tulchinsky *et al.*, 1993).

A reasonable level of nucleotide sequence homology has been shown between the first intron of murine p9Ka and the CD3δ enhancer element, particularly in the core region of the enhancer (Tulchinsky *et al.*, 1993). Homologous core enhancer elements are believed to be responsible for lymphoid-specific expression of genes controlled by the viral enhancers of SL3 and RadLV (Georgopoulos *et al.*, 1988). It is interesting to note that the CD3δ enhancer elements can act to initiate the expression of certain genes specifically in lymphoid cells, and this result may explain the significantly higher expression of p9Ka in lymphoid cells-types of the mouse. However, the first intron of the rat p9Ka gene exhibits the same level of homology with the CD3δ enhancer core element. Since this enhancer element is apparently present in both rats and mice and is also present in the transgene construct, its presence may not explain the differences between expression of p9Ka from the mouse and rat p9Ka genes. However, only a minor difference in this putative enhancer region could be responsible for the differences seen in the level and pattern of p9Ka expression. The fact that this particular putative control element is present within the first intron of the rat gene, validates our use of genomic-based transgenes in the expression of rat p9Ka.

The use of *in vivo* footprinting of the p9Ka gene, in both high and low expressing mouse mammary epithelial cell lines, has revealed a 16 nucleotide region of the first intron which is protected from nuclease digestion only in the highly expressing cell-line (Tulchinsky *et al.*, 1993). The nucleotide sequence of this 16 bp protected element is identical in rat and mouse genes, and differences in the ability of trans-acting factors to bind to this region cannot, therefore, account for differences shown in expression of p9Ka in rats and mice. Hypermethylation may be involved in negative regulation of gene expression by this element, since hypermethylation has

been reported in the low-expressing cell-line (Tulchinsky *et al.*, 1992; 1993). Although the rat transgene is present in transgenic mice from the single-cell embryonic stage of the mouse, and undergoes the same changes in chromatin methylation during development as the rest of the mouse genome, it is unlikely to be integrated at the same chromosomal location as the mouse p9Ka gene and therefore may not be methylated in the same manner as the mouse gene. The differences in methylation of the mouse gene may be involved in transcriptional regulation. However, such differences in methylation of the DNA can only provide correlations and do not give formal proof of particular methylation patterns in the control of expression of specific genes. Thus, differences in methylation may well be simply an unrelated phenomenon in two divergent cell-lines. How the situation in these cell lines, originally derived from a carcinoma, relates to non-neoplastic cells *in vivo* is unclear. Similar studies in tissues from transgenic mice which harbour the rat gene may provide an important insight into regulation of expression of the p9Ka gene.

The experiments conducted to investigate cis-acting promotor and enhancer regions of the murine p9Ka gene (Tulchinsky *et al.*, 1992) have been performed using transfection of cells with plasmids which contain partial sequences of the p9Ka gene together with a reporter gene (chloroamphemicol acetyl transferase). Such transfections result in the transient expression of the gene of interest. As indicated in the Introduction, such studies are invaluable in identifying putative control elements, but they fail to take into account fully the effects of chromatin structure and DNA methylation on transcriptional activity of a gene. Both these effects on the DNA help to control expression of mammalian genes *in vivo*, and must not be ignored in any investigation of expression. Moreover, control of gene expression can also occur at the post-transcriptional level. The translational and post-translational control of the expression of genes rely primarily on the structure of the mRNA and the resultant protein, respectively. Both these structures are clearly different when a reporter gene is used. The use of significantly-abbreviated fragments of the p9Ka gene in these experiments, albeit necessary, may also preclude consideration of potential long-

range control elements, either in conjunction with, or independently from, the putative control elements under investigation. The use of a significantly greater portion of the p9Ka gene in the transgenic mouse studies presented here, and the lack of any reporter gene (except for the tag epitope), provide some potentially important benefits for the study of gene regulation.

The fact that expression of the rat p9Ka gene at the transcriptional level in transgenic mice follows the pattern of expression of the rat gene rather than that of the mouse does not negate the potential importance of any putative transcriptional control elements described for the mouse (Tulchinsky *et al.*, 1992; 1993). As more species are investigated for expression of p9Ka mRNA and more p9Ka genes are sequenced, more subtle differences in gene structure may emerge that can be demonstrated to contribute to inter-species variation in expression of this particular gene. The production of transgenic animals harbouring p9Ka genes from a different species provides an important tool for the investigation of such features.

Phenotype of the TPK41 Transgenic Mice

Direct evidence of a phenotype associated with the tagged-p9Ka transgene was seen in the TPK41 line (section 3.3.5). This phenotype could arise from an insertional mutation caused by integration of the transgene, or from expression of the transgene. The dwarf phenotype in female mice of this line was dependent on the transgenic status of the animal, but not on the level of expression of the transgene in the range of tissues studied. The increased rate of mortality that was also seen in the TPK41 homozygous mice (see section 3.3.2 and **Table 3.3.3**) may be linked to the dwarfism found in these same mice.

The dwarf phenotype of the TPK41 transgenic mice could be due to an insertional effect of transgene integration.

One logical explanation of the dwarf phenotype seen in the TPK41 line, and shown to be linked to the transgenic status of the mice, is that it is due to an

insertional effect of the transgene integrant. It is eminently plausible that such a large insertion (66 copies of a 10.3 k.b.p. transgene) could have such marked effects, and the more pronounced dwarfism seen in the homozygotes would be due to the insertion of the transgene into copies of the affected gene from both maternal and paternal genomes (Hogan *et al.*, 1986). Since the level and pattern of expression of the p9Ka transgenes are apparently independent of chromosomal location (section 3.2.4), it is possible that the locus of the integrated transgene can act independently of the surrounding DNA in terms of transcriptional activity. Hence, although one transgene integrant is apparently subject to transcriptional inactivation, this does not necessarily imply that the surrounding chromosomal DNA is in an inactive form. Thus, the insertional effect on the second copy of the affected gene may be evident in the phenotype of the homozygous animals due to a double mutational inactivation. Alternatively, if the inactivation of one of the transgene integrants affects surrounding chromosomal DNA, this could have the same effect on the affected gene as the transgene insertion. Insertional effects are not uncommon in transgenic mice (Hogan *et al.*, 1986), and there are several identified dwarf alleles which could act as sites for such an insertional effect (van Buul-Offers, 1983; Green, 1990).

At least seven allelic mutants for dwarfism have been characterised, all at distinct chromosomal loci (Green, 1990). Three of the dwarf mutations, achondroplasia (*cn*), brachymorphic (*bm*) and stubby (*stb*) (Lane and Dickie, 1968), produce disproportionate dwarfing, but no evidence of abnormal body proportions was seen in the TPK41 mice. Two other types of dwarf mice, Snell dwarf (*dw*) and Ames dwarf (*df*), are considerably smaller and less fertile than the TPK41 mice (Green, 1990). The hypothyroidism (*hyt*) dwarfism is thought to be due to lack of response of the mutant thyroid glands to thyroid-stimulating hormone (Beamer *et al.*, 1981). These dwarf mice are infertile, unlike the TPK41 mice, homozygotes of which have been used successfully in mating experiments. The pygmy (*pg*) mutation gives rise to mice which are noticeably smaller at birth and

only grow to one third of normal size (King, 1950), this phenotype is seen in both sexes and is rather more severe than that observed in the TPK41 mice. The *lit* (little) mutation is on chromosome 6 and produces mice whose adult body weight is about two-thirds that of controls, a condition which resembles the human condition of isolated growth hormone deficiency type I (Eicher and Beamer, 1976). Of all the dwarfisms identified, the TPK41 mice most resembles these *lit* mice in terms of body size and proportions. Although female *lit* mice are fully fertile, they fail to nurse their first litters. Some evidence of this trait was seen when homozygous TPK41 female mice were used in breeding, and the failure to produce milk was probably due to developmental retardation of the mammary gland in these mice. Together with the Ames dwarf and Snell dwarf mice, the *lit* mice exhibit defects in their levels of growth hormone and prolactin (Cheng *et al.*, 1983), but there are apparently no gross chromosomal deletions in the growth hormone gene (Phillips *et al.*, 1982).

The process of control of bodily growth in both mouse and man is extremely complex, and whilst some of the defects caused by mutations in dwarf mice have been identified, others, including the primary genetic events, are as yet unidentified. Of the identified defects in hormonal regulation of growth, such as low plasma levels of growth hormone, many are shared by two or more types of dwarf mice and the characterisation of a new dwarfism by these criteria is complicated by the possibility of several different mutational events giving the same hormonal profile. Given the complexity of control of bodily growth, an attempt to classify the insertional site of the TPK41 transgene integrant(s) based on biochemical analysis, could be both complicated and unrewarding. Two potentially more-productive lines of investigation are feasible for the further study of the genetic basis of the TPK41 dwarfism. In-situ hybridisation of the tagged-p9Ka transgene to chromosome spreads could be used in order to try and identify the site of insertion of the transgene (Hogan *et al.*, 1986) and to find out whether the transgene has integrated at, or near, a previously identified dwarf loci, or at a new one. It would certainly be worthwhile

to perform an allelic analysis, by mating the TPK41 mice with well characterised mouse lines, so as to identify whether one of the known dwarf alleles is involved in the dwarf phenotype of the TPK41 transgenic mice.

The dwarf phenotype of the TPK41 transgenic mice could be due to the expression of the tagged-p9Ka transgene.

Apparently, despite an effective doubling of the transgene copy-number in the homozygous mice (section 3.1.6), and the copy-number dependence of transgene expression in hemizygous mice transgenic for either of the p9Ka transgenes (section 3.2.4), there is no significant difference in expression of the transgene between hemizygous and homozygous transgenic mice in any tissue analysed (section 3.3.5). Given that the pattern of expression of the tagged-p9Ka transgene is consistent for all TPK41 mice analysed, it would seem unlikely that an enhanced expression in the homozygous TPK41 mice is responsible for their smaller size when compared to hemizygous TPK41 mice. Nevertheless, it is still possible that the homozygous mice do express the transgene at significantly greater levels in a tissue, or at a specific stage of development, that has not been studied here.

The possibility that the expression of tagged-p9Ka is involved in the dwarf phenotype raises interesting questions as to the role of p9Ka in growth and the mechanism by which the tagged-p9Ka can inhibit growth. That other lines of mice (e.g. TPK40 and TPK9) did express significant levels of tagged-p9Ka, but failed to exhibit any gross dwarfism may simply reflect the fact that the TPK41 line was the strain of mouse that expressed the most tagged-p9Ka. Similarly, the lack of any positive effects on bodily growth in the p9Ka transgenic lines may be due to the fact that expression of the p9Ka transgene failed to surmount a threshold for this phenotypic effect. Alternatively, the lack of a contrary effect in the p9Ka transgenic lines could reflect the fact that the insertion of the tag has an unexpected effect on the structure of the tagged-p9Ka.

It is interesting to speculate that the expression of the tagged-p9Ka may act as

as an antagonist of the normal function of p9Ka. It is reasonable to expect that insertion of the tag epitope into p9Ka acts to disrupt some function of the p9Ka protein. If, for example, this disruption prevents the tagged-p9Ka from activating its target protein but does not prevent its actual binding, then high expression of the tagged-p9Ka could maybe inhibit the function of p9Ka. This, or other mechanisms, may then lead to retarded growth in the TPK41 mice.

Although, arguably, the best-known transgenic mice which have been produced are those which exhibit increased growth due to expression of growth hormone (Palmiter *et al.*, 1982, 1983), there are strains of transgenic mice which exhibit dwarfism due to expression of certain transgenes, rather than due to insertional events. So-called dwarf transgenic mice (DTM) which express a mutant bovine growth hormone (Chen *et al.*, 1991) apparently exhibit dwarfism due to antagonism of the mutated bovine growth hormone with the endogenous mouse growth hormone. By analogy, the growth defects seen in the TPK41 mice could be due to endocrinological antagonism between tagged-p9Ka and endogenous mouse p9Ka.

Other transgenic mice have been produced, which exhibit inducible dwarfism (Borrelli *et al.*, 1989; Behringer *et al.*, 1988). The dwarfism in these mice was induced using the "thymidine-kinase obliteration" system under the control of a growth-hormone promotor (Borrelli *et al.*, 1989), or the diptheria-toxin gene fused to the growth-hormone promotor (Behringer *et al.*, 1988). In both cases effective genetic ablation of cell lineages expressing growth hormones was achieved (somatotropes and lactotropes were ablated to varying degrees in each system), and the resultant mice developed as dwarfs. These results indicate the importance of the somatotropes and lactotropes, presumably through their respective growth hormone and prolactin-producing activities, in normal growth. Such radical cellular defects were not seen in the TPK41 mice, which exhibited no gross histological abnormalities in the pituitary, other than their small size, which was in keeping with

the general anatomical observation of dwarfism. If we are to believe that a functional antagonism between tagged-p9Ka and normal p9Ka is involved in the dwarf phenotype seen in the TPK41 mice, we might expect to find some evidence for a role of p9Ka in cellular growth and some supportive evidence for antagonism between the two forms of p9Ka.

Several proteins of the S-100 family, including p9Ka, are associated with alterations of the growth of cultured cells (Hilt and Kligman, 1991). A role for murine p9Ka in growth-regulation and cell-cycle progression has been postulated from the increase in abundance of its mRNA after quiescent mouse fibroblasts have been stimulated by addition of serum (Jackson-Grusby *et al.*, 1987). A similar role has also been suggested for the closely-related calcyclin protein in human fibroblasts (Calbretta *et al.*, 1986; Ferrari *et al.*, 1987). Whereas the expression of calcyclin is induced during the G_0 to G_1 phase of the cell cycle, expression of p9Ka is induced during the S phase. These results suggest different roles for these two closely-related proteins (Hilt and Kligman, 1991). However, the appearance of these proteins has only been correlated with stimulated cell growth, and evidence for their direct role in control of cellular growth, or suggestions of how these proteins might act in this respect, have not been forthcoming. A better defined role in growth for such molecules has been put forward for an S-100-related protein that was copurified with the prolactin receptor (PRA; Murphy *et al.*, 1988). This S-100-related protein was not detected in a human breast cancer cell-line which, although it expressed the prolactin receptor and bound prolactin, did not respond to prolactin either biochemically or morphologically. It has been suggested that PRA may be responsible, in part, for the transduction of the signal from the prolactin receptor in a calcium-dependent manner (Hilt and Kligman, 1991). The correlation of expression of p9Ka (Goto *et al.*, 1988; Ebralidze *et al.*, 1989; De Vouge and Mukarjee, 1992) and other S-100-related proteins (Heizmann and Hunziker, 1990; Hilt and Kligman, 1991) with the transformed phenotypes of various cells could also be cited as evidence of a role for p9Ka in control of cell growth.

A possible role of p9Ka in the growth of cells is supported by studies of tumorigenesis and metastasis in which some growth-related effects were seen upon transfection and expression of the p9Ka gene and the tagged-p9Ka gene in cells *in vivo* (Davies, 1993; Davies *et al.*, 1993). The same constructs of the p9Ka gene that were used to produce transgenic mice were coupled to a drug-selectable gene and were transfected separately into a benign rat mammary epithelial cell-line. The resultant transfectants, which grew in medium containing the selective drug, were identified and isolated. These transfectants were grown in culture, and shown to be expressing enhanced levels of the transfected genes. They were then injected into the mammary fat pad of syngeneic female rats. Transfection of the benign tumour-forming cell-line with the p9Ka gene construct resulted in a reduction in the mean latent period for the appearance of tumours. This result implied that the tumours grew more rapidly than those produced by the non-transfected cell-line, and was interpreted as conferring a positive growth property on the transfected cells (Davies *et al.*, 1993). A more interesting and pertinent result with regard to the TPK41 dwarf phenotype comes from the transfection of the same cell-line with a tagged-p9Ka construct (Davies, 1993). Whereas this cell-line normally produced tumours in approximately 50% of the injected rats, in this instance no tumours were detected (Davies, 1993). The transfected cell line expressed high levels of tagged-p9Ka mRNA and protein prior to injection. These results suggested the possibility that this tagged-p9Ka suppressed the tumorigenic potential of the benign mammary cell line used and perhaps indicated an antagonistic role for the tagged-p9Ka in the formation of neoplasias. This possible tumour-suppressing effect could also be interpreted as a negative growth property when the tagged-p9Ka was expressed in this cell system. In this case the cells used were derived from pools of transfectants, and they thus contained a variety of separate transfectants with (presumably) different integration sites for the p9Ka constructs. Hence the site of integration of the tagged-p9Ka gene is not considered to be important in these studies. Whilst these experiments imply

some growth-related activity for p9Ka and tagged-p9Ka, the situation is not really comparable to that of growth in the whole animal. Not only is expression of p9Ka limited to one cell-type, but those cells, by virtue of their ability to grow indefinitely in tissue-culture and to produce benign tumours in syngeneic hosts, already possess different growth properties from their counterparts *in vivo*.

An alternative explanation for the loss of tumorigenicity in the cells transfected with the tagged-p9Ka gene is that the epitope used to tag the p9Ka was recognised as foreign by the immune system of the rats. This tag epitope (Evan *et al.*, 1985) was derived from an amino acid sequence of human c-*myc* protein which is not present in the rodent c-*myc* homologue. This fact, together with the high expression of the tagged-p9Ka, may be responsible for effective immune surveillance in what is otherwise a completely syngeneic system. The presence of a complete immune response in the rats would thus be responsible for the drastic inhibitory effects on the formation of tumours. Since no immune response is mounted against the expressed products of transgenes, or more specifically on expression of similar tag epitopes when expressed as part of a transgene (Munro and Pelham, 1987; Pelham, 1988), such an explanation cannot be applied to the dwarf mice.

It could be postulated that, rather than interfering with the p9Ka, the tag might somehow interact with some aspect of the function of endogenous c-*myc*. However, whilst the involvement of c-*myc* in cell growth makes this an attractive hypothesis, as mentioned previously, the mouse and rat c-*myc* proteins do not contain the epitope used as the tag, and this explanation would therefore seem unlikely.

Whilst the ideas of a role for p9Ka in growth control and functional antagonism at an intracellular location have already been broached, an explanation for the TPK41 dwarfism based on that shown for the DTM mice expressing a mutant growth hormone (Chen *et al.*, 1991) would depend on p9Ka having an endocrine action. The possibility of an extracellular role for p9Ka is supported by evidence of the local

trophic action by S-100β (see below), by the fact that the S-100-related proteins MRP-8 and MRP-14 are found in serum and that they may act as inflammatory cytokines in cutaneous lesions (Wilkinson *et al.*, 1988), and by the fact that p9Ka itself has been shown to be secreted from bovine smooth muscle cells (Watanabe *et al.*, 1992b). Given that S-100β is nearly identical to the neurite-extension factor from bovine brain (Kligman and Marshak, 1985), it is interesting to postulate that S-100 proteins may act as trophic factors in the brain. Other evidence for trophic effects of S-100β includes: enhanced survival and morphological differentiation of various neuronal populations by disulfide-linked, dimeric, exogenous S-100β (Kligman and Marshak, 1985; Winningham-Major *et al.*, 1989; Azmitia *et al.*, 1990; Van Eldik *et al.*, 1991); S-100β-induced stimulation of glial cell proliferation (Selinfreund *et al.*, 1991); increased astroglial proliferation in transgenic mice over-expressing S-100β (Yarowsky *et al.*, 1991); and increased survival of embryonic chick spinal motor-neurones following spinal transection or natural cell-death (Bhattacharyya *et al.*, 1992). These activities may be modulated via intracellular increases in cytoplasmic free calcium, as demonstrated in glial and neuronal cells that were stimulated with disulphide-linked, dimeric S-100β (Barger and Van Eldik, 1992). Despite this evidence of possible paracrine and autocrine activities for p9Ka-related proteins, there have been no reports of endocrine action for any member of the S-100-related family of small calcium-binding proteins, and most of the potential roles of these proteins are believed to be intracellular (Heizmann and Hunziker, 1990; Hilt and Kligman, 1991).

Future Work on the Dwarfism Seen in the TPK41 Transgenic Mice

Without further lengthy analysis, it may not be possible to determine exactly why the TPK41 mice exhibit dwarfism. Nevertheless, whether the dwarf phenotype is due to an insertional inactivation event or to the high level of expression of the tagged-p9Ka, it should prove interesting to show how such events lead to a retardation of growth in these mice. This one transgenic line, together with the other tagged-p9Ka transgenic mice and the p9Ka transgenics, will prove invaluable in

examining this potentially-novel form of dwarfism. If expression does prove to be important, the other tagged-p9Ka transgenics, which express their transgenes at a lower level, will provide a means to investigate the more subtle effects of tagged-p9Ka expression. Meanwhile, the p9Ka transgenic lines may be expected to exhibit contrary phenotypic effects. The PK51 line in particular, due to its abundant expression of p9Ka protein, could be crossed with the TPK41 line, in order to investigate the possible antagonistic actions of these two transgenes. If insertional mutagenesis is responsible for the dwarfism, it should prove possible, by means already outlined (*in-situ* chromosome hybridisation and allelic matings) to identify whether the integration site is within a known dwarf locus. If the locus has not already been identified, the transgene itself will act as a marker and could be used to clone flanking mouse genomic regions for comparison with known growth-related genes.

Towards an Animal Model of Breast Cancer Metastasis From Transgenic Mice Expressing p9Ka

The studies presented here, involving expression of p9Ka in transgenic mice, were performed with two broad objectives. The first, to understand better the differences in expression of rat and mouse p9Ka genes, has already been discussed extensively. The second, to investigate any role for expression of p9Ka in breast neoplasia and metastatic progression, was performed as expression of p9Ka at the mRNA (Dunnington *et al.*, 1984; Ebralidze *et al.*, 1989) and protein (Dunnington *et al.*, 1984) levels had been correlated with metastasis in two independent rodent models.

That there was no obvious incidence of neoplastic breast disease or dysplasia in transgenic mice containing additional copies of the p9Ka gene and having greatly enhanced expression of p9Ka in their tissues, would initially seem to dispel the notion that p9Ka is involved in the genesis of malignant breast tumours. However, it

is important that the nature of tumorigenesis and metastasis be considered, along with other recent experiments that have further implicated p9Ka in metastatic progression.

Tumorigenesis is widely considered to be a multi-step phenomenon involving mutation or over -expression of a range of proto-oncogenes and the loss of function of tumour suppressor genes (Weinberg, 1989; Bishop, 1991). That most transgenic models of mammary neoplasia rely on additional stochastic events to produce tumours (Andres *et al.*, 1982; Stewart *et al.*, 1984; Andres *et al.*, 1987; Sinn *et al.*, 1987; Schöenenberger *et al.*, 1988; Bouchard *et al.*, 1989; Iwamoto *et al.*, 1990; Jhappan *et al.*, 1992) would seem to support this multi-stage hypothesis for breast cancer. Further evidence of the need for multiple genetic events comes from the fact that even when two oncogenes act synergistically to produce tumours they are still stochastic (Sinn *et al.*, 1987; Andres *et al.*, 1988). Other transgenic models, utilising activated c-*erb*B2 and polyoma middle T oncogenes, produce polyclonal tumours with a short latency (Muller *et al.*, 1988; Guy *et al.*, 1992; Lucchini *et al.*, 1992). Whether this effect is due to the tyrosine kinase-associated activities of these oncogenes that lead to pleiotropic effects, or to an artefact is unclear at present. Nevertheless, even the nature of neoplasia in these transgenic mice does not preclude the assertion that multiple targets must be hit before a neoplastic phenotype is produced. Together with evidence from many other model systems, the involvement of multiple events in tumour formation is clear.

As discussed in the Introduction (section 1.3), the various models of mammary neoplasia so far produced have provided many important insights into oncogenesis in the mouse mammary gland and will provide a useful tool for testing novel therapeutic agents. However, the pertinence of some of these transgenic models to human breast cancer is questionable (section 1.3; Van de Vijver and Nusse, 1991), and the important question of metastatic spread of the mammary tumours produced has not been fully addressed (section 1.4). The importance of studying breast cancer metastasis lies in the fact that it is this aspect of mammary neoplasia which is

responsible for causing the majority of deaths (Harris *et al.*, 1992), and only an intact animal model can be used to investigate fully metastatic spread.

Not all neoplastic cells are necessarily metastatic and the acquired phenotype of the metastatic cell is considerably more complex that of the primary tumour (Fidler *et al.*, 1978; Fidler, 1991). In order to form metastatic tumours, cells must possess the ability to invade surrounding tissue, blood vessels and/or lymphatics; survive in the circulation; adhere to the vascular endothelium; extravasate out of the bloodstream or lymphatic vessel; and, grow in the secondary site (Fidler, 1991). Thus progression to the fully-malignant phenotype includes the subversion of many more cellular activities than does the formation of a primary tumour. However, it must be stressed that metastasis is a sequential series of events and, theoretically at least, cells of the final metastatic tumour need not necessarily still possess all the phenotypic traits necessary to produce that tumour (Fidler *et al.*, 1978).

Given the fact that in order for metastasis to occur there must firstly be primary tumour formation, and that even the formation of primary tumours relies on more than one tumorigenic event, it is not surprising that the expression of p9Ka cannot, by itself, lead to malignant breast cancer. Moreover, since there is now more evidence of the involvement of p9Ka in enhancing tumorigenesis and leading to metastatic progression (see below), it is interesting that it apparently does not contribute to the initiation of primary tumours, as demonstrated by the absence of any predisposition to mammary, or other, neoplasia in the transgenic mice.

Transfection of the p9Ka gene has recently been shown to lead to an increased tumour incidence, a shorter latent period before tumours are detected and an increased incidence of metastasis in a rat mammary model system (Davies *et al.*, 1993). This system relies on the ability of potentially metastatic DNA when transfected, together with a selectable marker (pSV2*neo*; Southern and Berg, 1982), into a benign rat mammary cell-line (Rama 37), to induce metastasis in syngeneic hosts (Jamieson *et al.*, 1990). This rat mammary model system (Dunnington *et al.*, 1983) overcomes problems of interpreting results from highly aneuploid, genetically

unstable cells, or cells with a pre-existing but low metastatic potential, when they are injected into non-syngeneic, immune-deficient rodents. It also mimics many aspects of human breast cancer (Rudland, 1987). The transfection of such cells with the same rat p9Ka gene as used in the production of transgenic mice, cloned into the pSV2*neo* vector, caused a significant increase in tumour formation (an average of 94% as opposed to controls of 46%), a decrease in tumour latent period (from 31 to 12 days), and a significant increase in the incidence of metastatic tumours (55% of animals with primary tumours compared to none in the controls). The transfected mammary epithelial cells expressed significantly more p9Ka mRNA and protein than the original untransfected cells or control cells transfected with pSV2*neo* alone. The enhanced metastatic potential of mammary epithelial cells expressing p9Ka is in keeping with that previously seen with spontaneously-arising malignant mammary epithelial cells in rat (Dunnington *et al.*, 1984) and metastatic sub-clones of a mouse mammary carcinoma (Ebralidze *et al.*, 1989).

Both the induction of metastasis in the rat model system (Davies *et al.*, 1993) and the correlation with metastatic potential in the mouse mammary system (Ebralidze *et al.*, 1989) rely on the use of cell-lines which possess an innate ability to form primary tumours. Despite the fact that they failed to form metastatic lesions upon introduction into their respective hosts, the cell-lines used in these experiments must, due to their immortalised and tumour-forming phenotypes, be considered to have progressed some way towards the metastatic phenotype, especially when compared to the cells in which expression is induced in the transgenic mice described here.

Although p9Ka would seem to be able to increase the pre-existing tumorigenicity of mammary cells (Davies *et al.*, 1983), and its enhanced expression has been reported in a number of cell-types upon transformation (Goto *et al.*, 1988; De Vouge and Mukarjee, 1992), it would appear that enhanced expression of p9Ka in a wide range of cell-types in transgenic mice does not produce any predisposition to transformation within the time scale examined. It would therefore appear that the

role of p9Ka in neoplasia is most likely in the latter stages of tumour development, and in particular in progression to the metastatic phenotype.

There is, however, one line of investigation which may support a requirement for expression of p9Ka in more fundamental neoplastic events. The same rat mammary model as described above was used to transfect the tagged-p9Ka construct (Davies, 1993). As discussed above in reference to the dwarf phenotype in the TPK41 mouse line, the transfection of the tagged-p9Ka gene apparently not only failed to induce more tumours as seen with the (non-tagged) p9Ka gene, but totally inhibited the formation of tumours upon introduction of the transfected cells into the syngeneic hosts. It is possible that the tag effectively acts as a "mutation" in the p9Ka, and expression of this mutant p9Ka may inhibit the normal function of the endogenous p9Ka. Although p9Ka is apparently not expressed in the epithelial cells used, there is reason to believe that expression of p9Ka protein is merely below detectable levels and some evidence of expression of p9Ka mRNA was seen. If competitive inhibition of p9Ka function with tagged-p9Ka does indeed lead to the reduced tumorigenicity observed, then p9Ka would seem to be necessary for primary tumorigenesis but not, as previously discussed, responsible for it. However, before any more significance can be attached to these results, it will be necessary to prove the potential antagonism between these two gene products. Another possible explanation for the lack of tumour formation in the rat mammary model system is that the human c-*myc* epitope used to tag the p9Ka is recognised as foreign by the (fully competent) immune system of the syngeneic host, and the tumour cells are cleared before a tumour can form. The tagged-p9Ka transgenic mice were never considered to be a feasible system for the basis of a transgenic mouse model of breast cancer metastasis, due to the probability of the tag insert somehow interfering with the function of p9Ka. Nevertheless, the possibility of antagonism between tagged and non-tagged p9ka would make it intriguing, once a tumorigenic and metastatic model has been produced, to use these mice to probe this possibility further, and hopefully

discern the role of p9Ka.

In order to produce a transgenic mouse model of breast cancer metastasis using mice transgenic for p9Ka, it is first necessary to produce primary breast neoplasia in these mice. Unlike other transgenic mouse models of carcinogenesis (Berns *et al.*, 1989; Bailleul *et al.*, 1990) which can be used to investigate "secondary events" involved in tumour formation, a model for metastasis relies on reproducible primary events. There are several methods by which primary neoplasia may be induced in p9Ka transgenic mice, and these will be discussed below.

Mice of a similar genetic background to those produced here have been shown to have a very low incidence of mammary tumours, but when such mice are treated with chemical carcinogens, such as 7,12-dimethylbenzanthracene (DMBA), the incidence of mammary tumours rises to 70% after nine months (Medina, 1982). Thus it may prove possible, by administration of chemical carcinogens, to induce primary tumours in transgenic and non-transgenic mice and to monitor any differences in metastatic potential. In epidermal carcinogenesis of the mouse *in vivo* using DMBA (Quintanilla *et al.*, 1991), the reproducibility of the presumed primary oncogenic event (A:T to T:A transversion at codon 61 of the H-*ras* gene) was remarkably high (>90%). The use of similarly selective carcinogens in p9Ka transgenic mice could facilitate the production of primary tumours from which metastasis could occur. However, the reproducibility of tumour incidence, and of the genetic events involved in the formation of tumours, induced by chemical carcinogens in the mammary gland is questionable. Whilst this method of induction of tumours may be productive in identifying primary events with which the action of p9Ka can synergise, if the mice are to be used to mimic human breast cancer the use of chemical agents may not be prudent.

If p9Ka transgenic mice were bred with mice with a genetic background that was susceptible to tumour induction by MMTV, primary tumours could be induced by infection with MMTV. Unlike murine leukaemia viruses which have been used in a similar way to detect synergising oncogenes in transgenic mice (Berns *et al.*,

1989), the insertional events which would be expected to give rise to the primary mammary tumours upon infection with MMTV are reasonably well characterised (Nusse, 1988; Van de Vijier and Nusse, 1991). Again, there are potentially multiple pathways that could give rise to the formation of tumours in this system, and this possibility could provide an opportunity to investigate which events are synergistic with over-expression of p9Ka. However, the relevance of MMTV-induced tumours to human breast cancer is controversial (Callahan, 1987; Van de Vijver and Nusse, 1991).

The ability to mate transgenic mice carrying different oncogenes provides a potentially more rewarding means of producing a mouse model of malignant breast cancer based on the expression of p9Ka. In this system the collaborating oncogene(s) are better defined, and could be chosen so as to mimic those with a proven link to human mammary neoplasia. The lack of tumorigenicity in the p9Ka transgenic mice may prove to be an advantage when such matings are performed, since it effectively minimises the background levels of neoplasia when comparing bitransgenic offspring with their singly transgenic littermates. It is hoped that by mating p9Ka transgenic mice with mice transgenic for other oncogenes, and exhibiting a predisposition to mammary neoplasia (as discussed in the Introduction, section 1.3), an enhanced level of neoplasia and a significant level of metastasis will be seen in the bitransgenic progeny. Work performed along the same lines (Sinn *et al.*, 1987; Andres *et al.*, 1988; Cardiff *et al.*, 1991) indicates that not all pairs of oncogenes are necessarily synergistic and that different oncogenes would seem to act by different routes to produce tumours of various pathologies. Thus, it will be interesting to investigate the metastatic progression of mammary tumours produced when different oncogenic mice are used in these experiments.

The possibility that increased expression of p9Ka may be involved in the metastatic progression of several other types of human tumours has been raised by analysis of the expression of p9Ka in a range of metastatic and non-metastatic tumour

cells (Ebralidze et al., 1990). Specimens obtained from metastatic tumour xenografts of four melanomas, three sarcomas and an adrenal carcinoma all exhibited enhanced levels of p9Ka mRNA expression when compared to non-metastatic tumours. Considering the wide range of tissues that express p9Ka in the transgenic mice produced here, it may prove possible to construct models of many types of malignant cancer by crossing p9Ka transgenic mice with different transgenic models of primary oncogenesis.

The production of two lines of transgenic mice exhibiting different levels of over-expression of p9Ka, should allow us to dissect the involvement of p9Ka *in vivo* in the progression of mammary, and other, tumours to a metastatic phenotype. By using these p9Ka transgenic mice to produce new mice bearing not only the p9Ka transgene, but other transgenes involved in the formation of primary tumours, it may prove possible to construct a model of metastatic breast cancer which can be used to develop novel therapeutic agents. The eventual aim of such studies will be the production of strategies to combat the metastatic spread of tumours, which is responsible for the majority of deaths from neoplastic disease.

Summary

The principle aims of producing mice transgenic for the rat p9Ka gene were to study differences in expression of the rat and mouse p9Ka genes and to investigate the possible role of p9Ka in malignant mammary neoplasia.

The patterns of expression of the rat and mouse p9Ka genes were different in that expression of p9ka in the mouse was generally lower than in the rat and was predominantly, but not exclusively, seen in cells of lymphoid origin. The rat-derived p9Ka transgenes were expressed in a copy-number dependent, position-independent manner and exhibited the same pattern of expression as the endogenous rat p9Ka gene. Further analysis of the transgenic mice produced here may identify the factors underlying the differences between expression of the rat and mouse p9Ka genes.

Whilst the dwarfism identified in the TPK41 transgenic mice was more closely correlated with the transgenic nature of the mice than with levels of transgene expression, it is possible to speculate that the dwarfism is due either to an insertional effect of transgene integration or as a result of the expression of the tagged-p9Ka. Further analysis of tagged-p9Ka transgenic mice could show whether the dwarfism is due to an insertional event at a potentially-novel dwarf loci, or to functional antagonism between tagged-p9Ka and endogenous mouse p9Ka proteins.

The lack of any overt effect of p9Ka over-expression on neoplasia in the PK51 and PK84 transgenic mice supports the idea that p9Ka expression may be involved only in the latter stages of malignant neoplasia. It will prove interesting to examine the effect of increased p9Ka expression on metastatic potential when primary tumours are induced in the mammary gland, or other tissues, of the p9Ka transgenic mice. It is hoped that the production and analysis of transgenic mice expressing elevated levels of p9Ka will further the understanding of metastasis and ultimately lead to an animal model in which to test novel therapies.

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