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The objective of this study was to characterize events in the uniformly selective association of H-*ras* gene activation, but not K-*ras* gene activation, in the rat mammary carcinogenesis model. Three primary objectives were investigated. First, the relative tumorigenic potency of activated H-*ras* and K-*ras*-2B was investigated. The activated H-*ras* gene was found to be approximately 10-fold more potent at inducing mammary tumors than the activated K-*ras* gene. Yet, the K-*ras* oncogene was still effective at mammary carcinoma induction. Second, the relative percentage of mammary transplants with mutations in H-*ras* and K-*ras* was investigated. This objective will be completed soon, but, as yet, no data is available. The third objective was to investigate the role of H-*ras* regulatory elements in rat mammary tumorigenesis using transgenic rats harboring a H-*ras* gene (HrHr transgenics) or K-*ras* gene (HrKr transgenics) controlled by H-*ras* gene regulatory elements. Mammary tumor development was decreased by 50% in the HrHr transgenics following NMU administration, whereas no effect on mammary tumor multiplicity was observed in HrKr transgenics. Mutation of the normal H-*ras* gene was 49% to 58% in tumors from both transgenic and non-transgenic rats. No mutations were found in the transgene of these tumors.

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Table of Contents

	Page
Introduction	1
Body	5
Conclusions	14
References	18
Appendix 1	21
Appendix 2	23
Appendix 3	25
Appendix 4	27
Appendix 5	29
Appendix 6	31
Appendix 7	33
Appendix 8	35
Appendix 9	37
Appendix 10	39

Introduction

Breast cancer development involves multiple poorly understood steps (25). Currently, several genes that may participate in breast cancer development are under investigation. The *ras* family of genes has been implicated in many different cancers and has also been observed to be overexpressed in human breast cancer (6,10,13,16). Interestingly, many cancers are associated with a specific member of the *ras* gene family (i.e. H-*ras*, K-*ras*, or N-*ras*)(12). For example, in humans, activation of the H-*ras* gene is found in bladder cancer (21), K-*ras* gene activation is found in colon (2,9) and pancreatic cancers (4), and N-*ras* gene activation is found in hematopoietic cancers (3). Therefore, *ras* genes play a central role in many different cancers. The goal of this research was to further characterize the role of the *ras* genes in mammary carcinogenesis.

The rat mammary carcinogenesis model is commonly used for investigations in breast cancer (11). Previous studies have found that the H-*ras* gene is frequently mutated in mammary tumors that result following the administration of chemical carcinogens (e.g. nitrosomethylurea or dimethylbenzanthracene) to rats (19,24). This high frequency of activation implicates *ras* as an initiation lesion in mammary carcinogenesis (24). Previous reports have found that the direct introduction of an activated Harvey *ras* gene into the mammary gland results in mammary carcinoma formation (22). In addition, carcinoma development was observed following the introduction of the wild-type Harvey *ras* gene into the mammary gland (20). This may be more analogous to the overexpression of Ras as found in human breast cancer (6,10,13,16). Yet, the steps and mechanisms that lead to the aberrant function of *ras* genes in cancer are poorly understood. The purpose of this work was to further define the steps important to selective *ras* activation in the rat mammary carcinogenesis model. This was accomplished by comparing the carcinogenicity of two

closely related *ras* family members using the rat mammary carcinogenesis model. That is, by comparing the carcinogenicity of H-*ras*, which is associated with mammary tumor development, to the K-*ras*, which has not been found to play a role in rat mammary carcinogenesis.

Although the H-Ras and K-Ras proteins are highly homologous, regions of variation in the two proteins are present, especially in the final 20 amino acids of the carboxyl terminus (1). To study the tumorigenic potential of *ras* genes, retroviral vectors were used to introduce either wild-type or activated H-*ras* or K-*ras* genes to the mammary parenchyma via the central lacteal. Previous studies have shown that the tumorigenic potential of the retrovirally expressed oncogene is proportional to the virus titer that is instilled into the mammary gland (23). Therefore, the potency of the H-*ras* and K-*ras* genes to form mammary carcinomas can be compared. Resultant tumors can then be histologically and molecularly characterized.

Another step that may contribute to the tissue selective association of an oncogene is the mutability of the genes at the DNA level. Previous studies have reported differences in DNA methylation patterns between the H-*ras* and K-*ras* genes in rat mammary tissue following nitrosomethylurea exposure (15). Both genes showed alterations in methylation patterns, although the H-*ras* gene was more sensitive to these effects in the mammary gland. In this report, a clonogenic transplantation assay (7) was used to investigate mutation induction by a carcinogen with a target gene. DNA was extracted from transplant growths that resulted from the introduction of mammary clonogens into the intrascapular fat pad of hormonally primed recipient rats. A variation of the PCR-based method developed by Cha *et al* (5) was used to determine whether DNA from the growths harbored a mutated H-*ras* gene. Thus, the relative percentage of H-*ras* gene mutations in the mammary gland after carcinogen exposure can be determined. This

information also provides an estimate of the relative penetrance of the H-*ras* gene for tumor formation versus mutation frequency.

Gene expression regulation largely defines the activity of a gene within a tissue type. Therefore, control of gene expression may be a major contributor to the selective association of an oncogene with a particular tumor type. Previous studies have shown that the K-*ras* gene is expressed at lower levels than the H-*ras* gene in rat mammary tissue (15). Yet, due to the many cell types and the different developmental stages of a particular cell type in the mammary gland, it cannot be concluded that the overall gene expression levels for the gland reflect expression levels for the breast cells important in tumor development.

To investigate the role of H-*ras* gene regulatory elements in the tissue-specific association of H-*ras* gene activation in rat mammary carcinogenesis, a transgenic animal model was developed that uses H-*ras* gene regulatory elements to drive expression of the rat K-*ras* gene. To validate this model, it was first necessary to develop a transgenic rat line that contains a H-*ras* transgene under the same regulatory elements as those used to drive expression of the K-*ras* transgene (i.e. H-*ras* gene regulatory elements). For each group, animals were characterized for expression of the transgene, transfer of the transgene through the germline in a Mendelian fashion, and determination of any anomalies that might arise from animals harboring the transgene, such as disease susceptibility, increased cancer incidence, and changes in lifespan. Acceptable transgenic lines were exposed to carcinogen and resultant tumors were assayed for mutation of the transgene and the endogenous H-*ras* gene.

This proposal addresses a fundamental question of cancer biology that has direct value to breast cancer. The results from these studies will help in understanding factors important in

controlling the association of oncogenes with a specific tumor type. It is hoped that this information will also provide insight for the development of organ specific prevention and therapeutic strategies.

Body

The three major objectives of this study were designed to further characterize events important in the selective activation of the H-*ras* gene as found in rat mammary carcinomas following exposure to the chemical carcinogen nitrosomethylurea (NMU). This selectivity was investigated by comparing the H-*ras* gene to the K-*ras* gene, which is closely related but not activated, in rat mammary carcinogenesis. The first set of experiments determined whether or not the *ras* gene products induce rat mammary tumors. The second objective was to determine the relative mutation frequency of the H-*ras* and K-*ras* genes in rat mammary transplants following carcinogen exposure. The final set of experiments investigated the role of H-*ras* gene regulation in chemically induced rat mammary carcinogenesis.

The first set of experiments determined whether H-*ras* and K-*ras*, in their wild-type and activated forms, could induce rat mammary carcinomas. This was done using methods developed in our laboratory using replication-defective retroviral vectors to deliver the *ras* genes directly to the mammary parenchyma through the 12 teats of sexually mature female Wistar Furth rats (22). This methodology stemmed from experiments performed to ablate the mammary parenchyma by injecting ethanol into the central mammary duct, thus allowing the cleared fat pad to be used for mammary cell transplantation studies (Jane Yasukawa-Barnes, personal communication). For effective infusion, the female rat must be sufficiently mature to allow recognition of distinct teats (i.e. rats must be approximately 40 days of age or older). The teat is clipped and the remaining orifice is cannulated using a syringe with a blunt-ended 27 gauge needle. Ten to 20 μl of viral stock are infused in a pigmented solution that is used to allow the determination of injection efficiency. After injection, the teat is held shut with forceps for several seconds to reduce reflux.

Replication-defective retroviruses were used to deliver expression vectors to the mammary parenchyma. The retroviral vectors used in this study are illustrated in Appendix 1. The activating mutation present in the *ras* gene of the JRHrasA and JRKrasA constructs is representative of the G to A transition mutation found in the second basepair of codon 12 (G35 to A) of *ras* genes following NMU exposure. Mammary carcinoma development was assessed for 18 weeks after virus infusion. In our initial study, using viruses at titers ranging from $3x10^6$ to $6x10^6$ CFU/ml, infusion of the activated form of both the H-*ras* (JRras and JRHrasA) and the K-*ras* (JRKrasA and JRKrasV) genes resulted in efficient carcinoma formation (Appendices 2 and 3). Histological analysis of tumors resulting from infusion of both vectors found they were mammary carcinomas (data not shown). The molecular biological procedures used in these studies were standard molecular biological techniques (17). Polymerase chain reaction (PCR) analysis was used to confirm the presence of viral DNA in the resultant tumors by amplifying the neomycin resistance gene, which is unique to the retroviral vectors. All tumors evaluated were found to carry vector DNA (data not shown). Although the viral titer was comparable for these vectors, the tumor multiplicity was greater for vectors expressing the H-*ras* gene (i.e. JRras) than for the K-*ras* gene (i.e. JRKrasG, JRKrasA, or JRKrasV - Appendix 3).

Tumors also resulted from infusion of vectors expressing wild-type H-*ras* (JRHrasG - Appendix 2), but not wild-type K-*ras* (JRKrasG - Appendix 3). On histological analysis of the wild-type H-*ras* generated tumors, regions of keratinization were observed (20). This has not previously been observed in tumors resulting from infusion of activated H-*ras*. Tumors resulting from the infusion of the wild-type H-*ras* gene were also small, slower growing, had a longer latency and slightly lower multiplicity compared to mammary tumors resulting from the infusion of the

activated form of the H-*ras* gene (Appendix 2). DNA sequencing from 3 tumors of the retrovirally expressed Harvey *ras* gene (i.e. from JRHrasG) found no mutations present from codons 12 through 61 (data not shown). Due to the small tumor size and low multiplicity of these tumors, not all of the necessary evaluations could be completed from the initial study, therefore, another study will be carried out to characterize carcinogenesis with wild-type H-*ras* gene from the JRHrasG vector.

A second study using the activated forms of H-*ras* (JRHrasA) and K-*ras*-2B (JRKrasA) expressing vectors was performed using equivalent viral titers ($7x10^6$ and $8x10^6$ CFU/ml, respectively). Again, the H-*ras* gene led to tumor development with greater multiplicity and shorter latency than by the K-*ras* expressing vector (Appendix 4). By comparing the level of tumorigenesis of the two vectors after infusion, the H-*ras* oncogene was found to be 5- to 10-fold more potent than the comparably activated K-*ras* gene product at inducing rat mammary carcinomas. A final study was carried out using the activated forms of H-*ras* and K-*ras*-2B vectors at $6x10^6$ and $3x10^7$ CFU/ml, respectively. Although the K-*ras* expressing vector was infused at a 5-fold higher titer, the H-*ras* expressing vector still led to double the number of tumors by 18 weeks after infusion (Appendix 5). All mammary tumors resulting from these studies were evaluated for histopathology and found to be mammary carcinomas (data not shown).

The second objective was to determine the percentage of H-*ras* and K-*ras* genes activated in the target cells believed responsible for mammary tumor development (i.e. mammary clonogens). This technique uses a mammary transplantation assay developed in our lab (7). Donor rats were treated with 50mg/kg NMU. Twenty-four hours later, abdominal and inguinal mammary glands were removed and de-aggregated in collagenase for 2 to 3 hours. Mammary tissue was then washed

to remove the remaining fat, digested briefly (~ 5 min) in 0.1% trypsin, and single-cells were obtained after filtering through a 53μ mesh. Cells were counted using a hemacytometer and resuspended in brain homogenate to a concentration of 10^6 cells per 60 μl. Sixty μl of mammary cell suspension was then injected using a 23μ needle into 3 independent sites of the intrascapular fat pad of hormonally-primed (i.e. rats treated with mammotropic pituitary tumor) recipient 50 day old female Wistar-Furths rats. Mammary growths were allowed to develop in the fat pad for 1 month, at which time they were removed and stored at -80°C until DNA extraction. This DNA was analyzed for G35 to A transition mutations in the H-*ras* or K-*ras* gene (as observed in NMU initiated tumors) using the mismatch amplification mutation assay (MAMA) developed by Cha *et al* (5).

Our initial studies indicated that there was potentially a problem with efficient clonogen growth when large numbers of cells are delivered to a single fat pad location. Therefore, cells expressing a unique marker (cells infected with a retroviral expression vector as described in the first objective) were instilled in the fat pad at varying numbers. The presence of the marker in resultant fat pad growths was determined using PCR to amplify sequences unique to vector DNA. Marker DNA was detected in all growth resulting from the instillation of up to 1000 clonogenic cells (data not shown). Therefore, 1,000 clonogenic cells (i.e. 10^6 total mammary cells) were infused per each of 3 instrascapular fat pad locations per a recipient animal.

Ninety-three transplant sites were isolated from control (non-NMU-exposed) growths and 108 transplant sites were isolated from NMU-exposed donor growths. These transplants were prepared for DNA extraction and stored at -80°C. Currently, DNA is being extracted from these samples with care taken not to contaminate the preparations with previously generated PCR

products. PCR product contamination is a common problem in PCR-based evaluations with the sensitivity required for this analysis. Following DNA extraction, these samples were tested for the percentage of G35 to A activating mutations in the *ras* genes of unexposed and NMU-exposed transplants.

This objective was delayed by several mishaps. Initially, the mammotropic tumor that was maintained in this laboratory was lost in a liquid nitrogen tank that dried up. Several months were required to regenerate a mammotropic tumor adequate for these studies. Also, the assay originally used to screen for mutations in the *ras* genes was not sufficiently sensitive. Therefore, a second assay has been developed (i.e. the MAMA assay) that provides the required sensitivity to screen for G35 to A Harvey *ras* gene mutations. Currently, a preliminary estimate of 1 in 10^5 mammary cells have been found harboring G35 to A mutations in the Harvey *ras* gene (data not shown). However, we also believe that the MAMA assay needs to be validated before this estimate is acceptable.

The final objective was to evaluate the role of H-*ras* gene regulation in the selective association of H-*ras* gene activation in rat mammary carcinogenesis. To do this, a transgenic rat model was developed that expresses the rat K-*ras* gene under the regulatory elements of the H-*ras* gene. To validate this model, it was first necessary to carry out a control study with transgenic animals that carry the H-*ras* gene under the same regulatory elements used to drive the K-*ras* transgene. Five founder animals carrying the H-*ras* transgene construct (designated HrHr) were produced. The HrHr transgene construct is illustrated in Appendix 6. The HrHr transgene was transferred in all animals in the expected Mendelian fashion. HrHr transgenic rats did not develop any notable pathologies, they did not have an increased incidence of cancers, and lived a normal lifespan compared to their non-transgenic littermates (data not shown).

The first HrHr founder animal (designated R8) was found to harbor 4 to 6 copies of the HrHr transgene as determined by Southern analysis. The original analysis of the R8 transgenic line found 15 to 20 copies of the transgene. Further evaluation shows that fewer copies of the transgene are actually present (i.e. ~5 copies). The transgene was expressed at the same level as the wild-type gene in mammary, skeletal muscle, lung, liver, kidney, and colon tissues (i.e. as determined by competative PCR) in R8 transgenic rats (data not shown). R8 male and female transgenic rats were mated in an attempt to generate a rat line homozygous for the HrHr transgene. To date, no homozygous animals have been identified based on PCR-product band intensities (data not shown). Therefore, transgenic males were mated with female Sprague-Dawley rats from the Harlan-Sprague Dawley colony (Madison, WI) to generate litters with heterozygous transgenic animals and non-transgenic littermates suitable for carcinogenesis studies.

Three transgenic founder animals carrying the K-*ras* gene driven by H-*ras* gene regulatory elements have been produced (designated HrKr). These animals transfer the HrKr transgene in the expected Mendelian inheritance fashion. To date, no **abnormalities** due to the presence of the transgene have been observed in these animals. The first HrKr strain produced (designated 4321) was found to carry approximately 7 copies of the transgene. Western analysis of R8, 4321, and control animals show a doubling of total Ras expression in the colon, mammary gland, and liver (Appendix 7). The R8 and 4321 transgenic lines were used for NMU-induced rat mammary carcinogenesis studies.

In the first transgenic rat mammary carcinogenesis study, 34 female R8 HrHr transgenic rats and 37 non-transgenic littermates were treated with 50mg/kg NMU at 42 to 50 days of age. Tumor development was assessed weekly for 18 weeks following NMU exposure and tumors reaching 10

mm in diameter were removed. Tumor samples were prepared for histological analysis and samples were stored at -80°C until DNA extractions were performed. Allele-specific oligonucleotide hybridization was used for the original evaluation of H-*ras* mutations in carcinomas that developed in non-transgenic animals (25). PCR product was transferred to a nylon blotting membrane and selective hybridization was carried out using a probe to screen for a G35 to A mutations in the H-*ras* gene. In addition, another PCR-based method was developed that recognizes the generation of a unique restriction site in PCR-products from amplification of DNA containing the G35 to A transition mutation in the *ras* genes (14). This method was used to screen for G35 to A mutations in both the endogenous H-*ras* genes and the transgenes of mammary tumors that developed in the HrHr and HrKr transgenic rats.

For statistical analysis of carcinoma development, Kaplan-Meier survival curve estimates were determined for each group. The time without tumor was determined for each group and used to compare the number of expected and observed events using a log-rank test. Pairwise comparisons were made using a log-rank test. If global comparisons at 3/4 or at the end of a study were significant, then a one-way analysis of variance for global comparison of each group was performed, followed by least significant difference pairwise comparison.

The latency of carcinoma development was significantly greater for HrHr transgenic animals compared to non-transgenic littermates ($P<0.05$ - Appendix 8A). Also, at 18 weeks, the multiplicity of tumor development in the HrHr transgenics was 50% that of non-transgenic controls (Appendix 8B). Examination of endogenous H-*ras* gene activation in tumors from non-transgenic animals showed that 55% possessed G35 to A mutations. The frequency of endogenous H-*ras* gene

mutation in the tumors derived from HrHr transgenics was found to be 58%. Only 1 G35 to A transition mutation was found in the HrHr transgene in any of the tumors screened (Appendix 10).

In a study analogous to that performed with the HrHr transgenics, a mammary carcinogenesis study was performed with the 4321 HrKr transgenic animal strain since this strain is similar to the R8 transgenics in transgene copy number and mammary gland Ras expression (Appendix 7). The HrKr transgene construct is illustrated in Appendix 6. Tumor latency following NMU exposure was not significantly different in 4321 transgenics and non-transgenic littermates (Appendix 9A). At 18 weeks after NMU-administration 4321 transgenics developed 87% as many tumors per rat as non-transgenic littermates which was not found to be significantly different (Appendix 9B). Fifty percent of the tumors that developed in non-transgenic littermates were found to have a G35 to A transition mutations in the endogenous H-*ras* gene. Comparably, 49% of tumors that arose in transgenics rats were found with G35 to A mutations in the endogenous H-*ras* gene. No mutations were found in the transgene of any of the mammary tumors screened (Appendix 10).

This concludes the results currently available for these studies. Several related studies are in progress. These include further characterization of the transgenic rat lines obtained during this study. To validate the results obtained with the first set of HrHr and HrKr transgenic rats, a second carcinogenesis study with the R8 and 4321 transgenic lines is in progress. Also, to determine whether these results are line specific, other HrHr and HrKr transgenic lines produced are being prepared for carcinogenesis studies. Also, a retroviral vector study to further investigate carcinogenesis by wild-type H-*ras* (JRHrasG) will be carried out.

In summary, the data provided in these studies further describe molecular events that contribute to the multistage process of mammary carcinogenesis. The most substantial finding was that Harvey *ras* is more potent than the closely related Kirsten *ras* in mammary carcinogenesis. Previous *in vitro* studies have shown the potential for differential activities of the *ras* family members, though this is the first study to directly illustrate the differential oncogenic potencies of Harvey *ras* and Kirsten *ras in vivo*. These results provide a basis for further investigations in the molecular mechanisms of carcinogenesis and the development of therapies directed at specific *ras* proteins for cancer treatment.

Conclusions

The first objective of this proposal was to determine the mammary carcinogenic potential of the K-*ras* gene. It has been observed that an activated H-*ras* gene can induce tumor development in the rat mammary carcinogenesis model (22). Yet, the carcinogenic potential of the K-ras gene in rat mammary carcinogenesis is not known. Therefore, retroviral vectors expressing activated forms of the K-*ras* gene product were constructed. Vectors expressing the activated H-*ras* gene were used as a positive controls for mammary carcinogenesis and, as expected, mammary tumors developed efficiently from infusion of the retroviral vector expressing the H-*ras* gene. Mammary tumors also developed from infusion of the vectors expressing the activated K-*ras* genes, but with lower efficiency than the H-*ras* gene expression vector. Therefore, although the activated K-*ras* gene is capable of leading to mammary carcinoma development, it is less potent than the activated H-*ras* gene in mammary carcinogenesis.

To better estimate the potency of the activated K-*ras* gene compared to activated H-*ras,* two additional studies were conducted. In the first study, vectors expressing either H-*ras* and K-*ras*-2B with a G35 to A transition mutation were used at comparable viral titers. Mammary tumors induced by the activated H-*ras* vector had a shorter latency and greater multiplicity than tumors induced by the comparable K-*ras* vector. Furthermore, when the activated K-*ras* virus was infused at a 5-fold higher titer than the activated H-*ras* vector, carcinoma development from infusion of the H-*ras* vector still resulted in twice as many mammary carcinomas at 18 weeks. Thus, the G35 to A activated form of the H-*ras* gene is at least 5 times more potent than the comparably activated form of the K-*ras*-2B gene in mammary carcinogenesis. Yet, the activated K-*ras* gene was still effective at inducing mammary carcinomas. Thus, the absense of K-*ras* activation in mammary

carcinogenesis is not due to the inability of the K-*ras* gene product to lead to mammary tumor development.

The second objective was to determine the percentage of H-*ras* and K-*ras* gene mutations induced by nitrosomethylurea in mammary clonogenic transplants. Our initial study established that 1000 clonogens could efficiently be transplanted to a fat pad site. Several complicating factors have hindered the completion of this objective. To date, mammary fat pad growths have been collected and DNA extraction have been carried out. Analysis for the percentage of transplants with H-*ras* and K-*ras* gene activation was recently performed, but these results must be validated before conclusions can be drawn. Therefore, this data is preliminary and not included in this report.

The final objective of this proposal was to evaluate the role of H-*ras* gene regulation in rat mammary carcinogenesis. Five founder animals have successfully been produced that express the normal H-*ras* coding region by H-*ras* gene regulatory elements (designated HrHr transgenics). The first founder group (designated R8) met all the criterion necessary for use in carcinogenesis studies. Also, 3 founder animals were produced that carry a K-*ras* transgene under the control of H-*ras* gene regulatory elements (designated HrKr transgenics). One HrKr strain (4321) expressing the transgene was chosen for carcinogenesis studies. Both transgenic strains chosen for carcinogenesis studies harbor 5 to 7 transgene copies. Expression of the transgene in the R8 (HrHr transgenic) and 4321 (HrKr) lines results in approximately a doubling of Ras protein expression.

Mammary carcinoma development in R8 transgenics was half that found in the non-transgenic control group. (This result contrasted our original hypothesis; that multiple copies of the H-*ras* gene would result in an increased number of mammary tumors following carcinogen exposure due to an increased number of initiation targets.) However, expression of the K-*ras* gene

controlled by H-*ras* gene regulatory elements in the 4321 line did not significantly alter the latency or multiplicity of mammary carcinoma development following NMU exposure when compared to non-transgenic littermates. These results further support the observation that H-*ras* and K-*ras* actions in the mammary gland differ at least on the basis of magnitude of activity. Of significance is the mammary tumor suppressive action of H-*ras* gene expression. In conjunction with the results from the first objective, these results support a more dominant role for Harvey *ras*, than Kirsten *ras*, in rat mammary gland biology.

On evaluation of *ras* gene activation in the resultant mammary tumors, it was observed that the endogenous H-*ras* gene was mutated as previously reported (24) in mammary tumors from control animals. For example, 55% of the control littermate tumors screened from the HrHr study and 50% in the HrKr study had a G35 to A transition mutation in the H-*ras* gene. Also, comparable frequencies of activation were observed in tumors derived from transgenic animals (i.e. 58% in HrHr transgenic mammary tumors and 49% in HrKr derived mammary tumors). Surprisingly, the *ras* transgene was rarely found mutated in tumors from either the R8 or 4321 transgenic lines. This, again, was in contrast to our running hypothesis; that expression of the H-*ras* transgene in the R8 strain was acting to suppress tumor formation in cells that would otherwise develop with an activated form of the H-*ras* gene. This hypothesis was supported by investigations showing that expression of the normal H-*ras* gene can suppress the tumorigenic phenotype of a mutated H-*ras* gene (8). Furthermore, the relative level of H-*ras* gene activation (i.e. ~ 50%) in mammary tumors that developed in non-transgenic rats was within reason for attenuation of tumor frequency if the H-*ras* transgene was suppressing tumors that would otherwise develop due to H-*ras* activation. Since this hypothesis was not supported by our latest data, two other possibilities are now being

investigated that integrate roles in which *ras* genes are known to participate - proliferation and differentiation.

The H-*ras* expressing transgene did not function as anticipated to increase the number of tumors developed from an increased number of *ras* gene targets. Thus, our goal of understanding how the H-*ras* gene regulatory elements contribute to H-*ras* selective mutation in the rat mammary carcinogenesis model was not realized in the current studies. However, of interest is the observation that deregulated expression of a wild-type H-*ras* gene from a retroviral vector led to carcinoma development, whereas overexpression of H-*ras* in the mammary gland, but with regulation by the normal H-*ras* gene control elements, in the HrHr transgenics did not result in mammary tumor development. Thus, regulation of H-*ras* expression may be an important contributor to the action of H-*ras* in mammary tumorigenesis.

The studies presented here clearly show differences between H-*ras*- and K-*ras*-induced carcinogenesis that should prove valuable in understanding the molecular and cellular events that lead to breast cancer. In particular, expression of mutated H-*ras* is more effective at mammary tumor development than the analogous mutant form of K-*ras*. Additional studies in understanding why these two highly homologous proteins differ in efficiency of tumor development are necessary. These studies should assist in the development of modalities for breast cancer prevention, diagnosis, and treatment.

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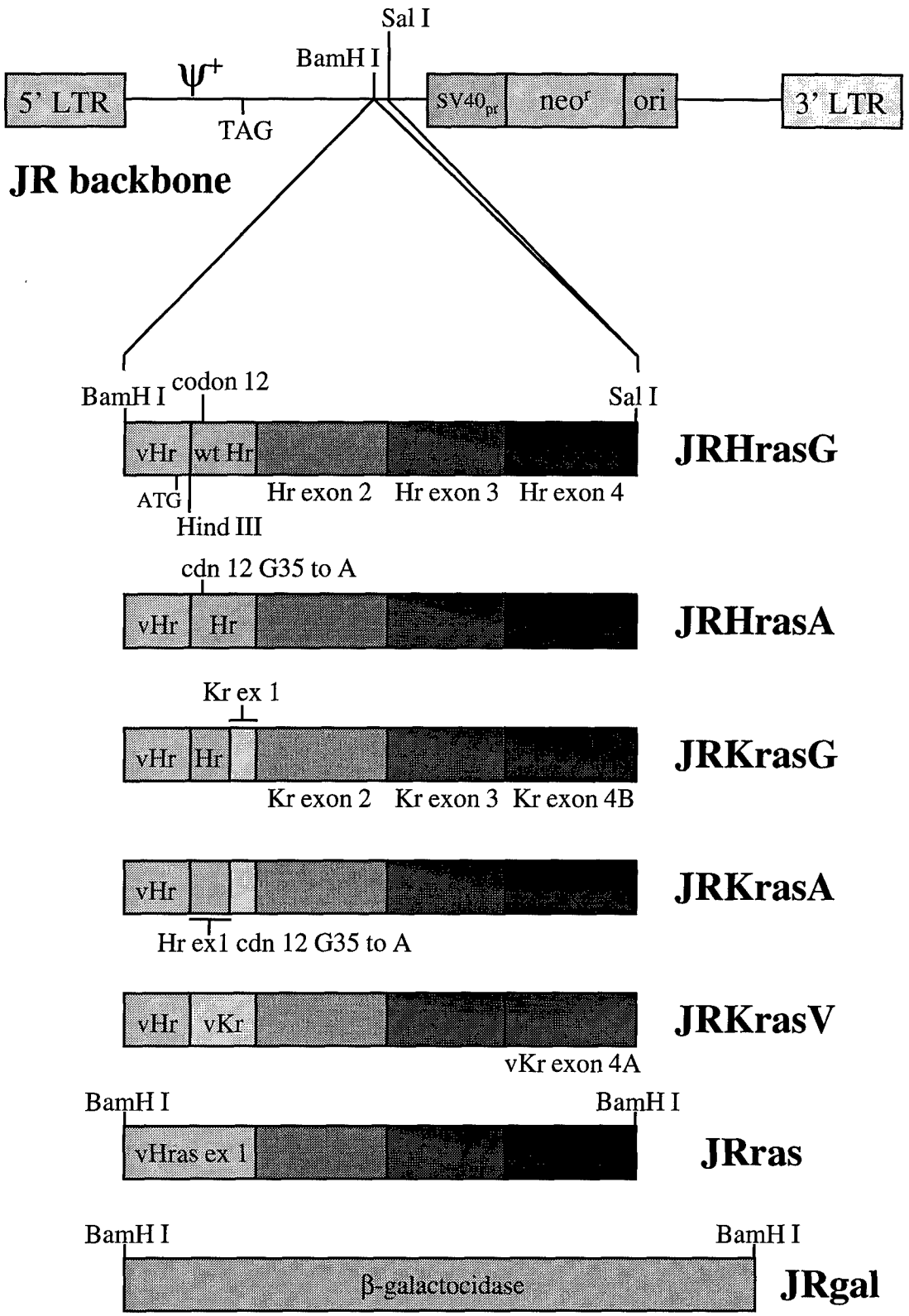
APPENDIX 1

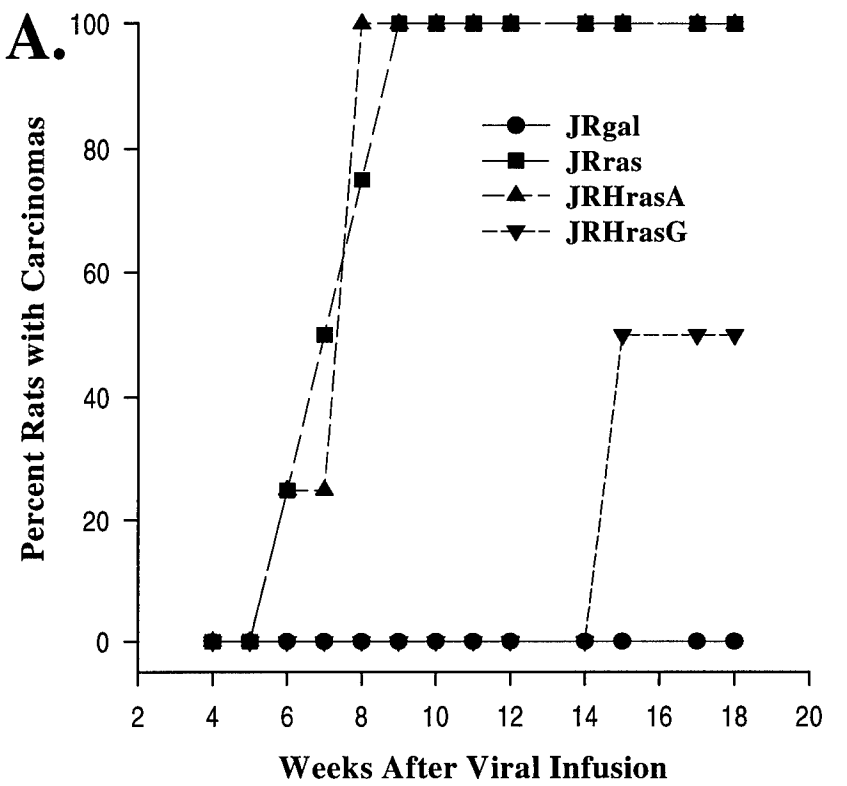
Retroviral vector constructs. The pJR backbone was used for construction of all *ras* expression vectors. *ras* genes were incorporated into the vector by ligation into the BamH I site and Sal I site of pJR. JRHrasG contains the wild-type Harvey *ras* gene; JRHrasA contains a G to A activating mutation in the second basepair of codon 12 of the Harvey *ras* gene (G35 to A); JRKrasG contains the first 28 codons of the wild-type Harvey *ras* gene followed by the coding region for the Kirsten *ras* 2B gene; JRKrasA contains the first 28 codons of the G35 to A mutated form of the Harvey *ras* gene followed by the coding region for the Kirsten *ras* 2B gene; JRKrasV contains the first 28 codons of the G35 to A activated form of the Harvey *ras* gene followed by the viral Kirsten *ras* gene (which contains exon 4A). JRras and JRgal were previously described[1] and used in these studies as positive and negative infusion controls, respectively.

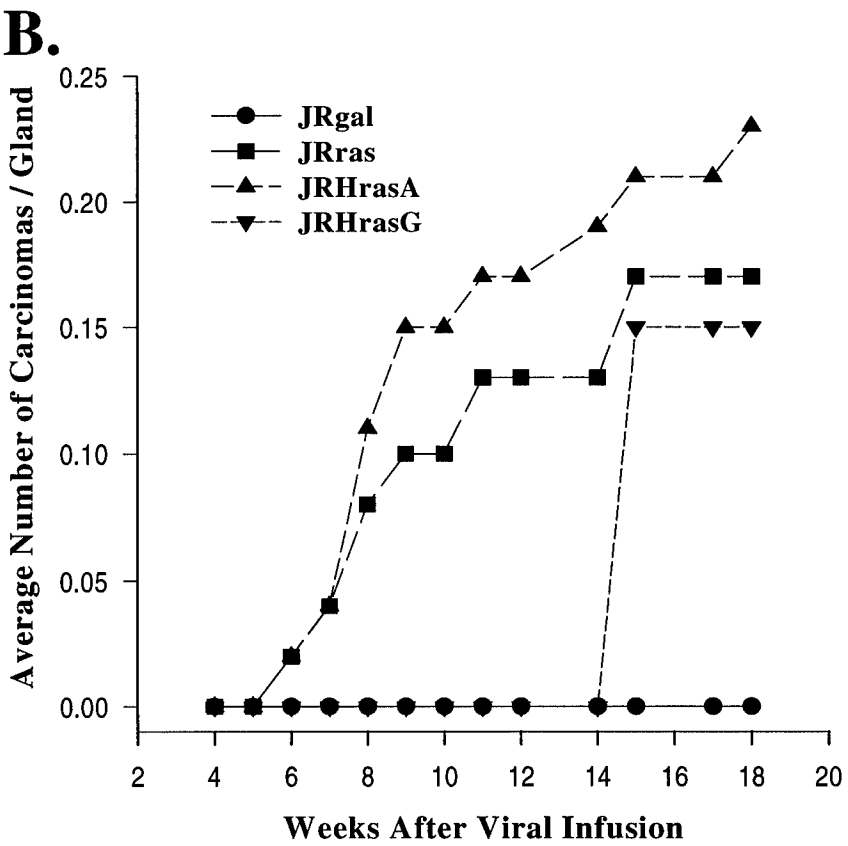
[1] Wang, B., Kennan, W.S., Yasukawa-Barnes, J., Linstrom, M.J., and Gould, M.N. Carcinoma induction following direct *in situ* transfer of v-Ha-*ras* into rat mammary epithelial cells using replication-defective retrovirus vectors. Cancer Res. 51: 2642-2648, 1991.


Sal I
BamH I
ψ+
5' LTR
TAG
SV40pr
neor
ori
3' LTR
JR backbone
BamH I
codon 12
Sal I
vHr
wt Hr
ATG
Hind III
Hr exon 2
Hr exon 3
Hr exon 4
JRHrasG
cdn 12 G35 to A
vHr
Hr
JRHrasA
Kr ex 1
vHr
Hr
Kr exon 2
Kr exon 3
Kr exon 4B
JRKrasG
vHr
Hr ex1 cdn 12 G35 to A
JRKrasA
vHr
vKr
vKr exon 4A
JRKrasV
BamH I
BamH I
vHras ex 1
JRras
BamH I
BamH I
β-galactocidase
JRgal

APPENDIX 2

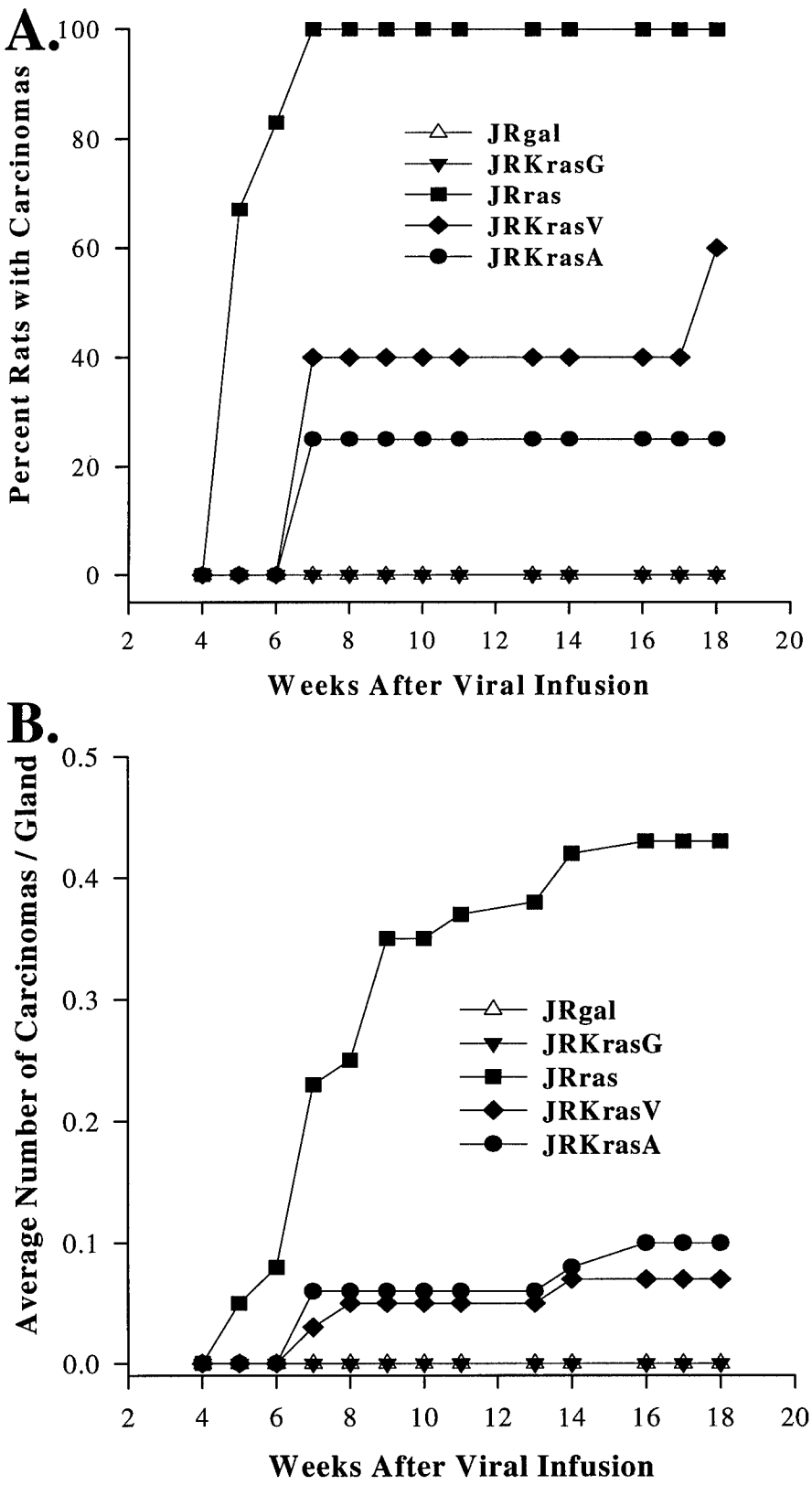
Latency and multiplicity analysis of mammary carcinoma development after infusion of retroviral vectors expressing wild-type and activated forms of the Harvey *ras* gene. A. Latency analysis of control vector expressing the β-galactosidase gene (JRgal) infused at 6.6 $x10^6$ CFU/ml (n=60 glands), the activated viral Harvey *ras* gene (JRras) infused at 5.5 x 10^6 CFU/ml (n=48 glands), the G35 to A mutant form of the Harvey *ras* gene (JRHrasA) infused at 4.3 x 10^6 CFU/ml, and the wild-type form of the Harvey *ras* gene (JRHrasG) infused at 2.7 x 10^6 CFU/ml (n=34). Latency for carcinomas resulting from infusions of JRgal was found to be significantly different than for JRras, JRHrasA, and JRHras G ($P<0.05$) B. Multiplicity analysis of mammary carcinoma development for JRgal, JRras, JRHrasA, and JRHrasG. At 18 weeks after viral infusion of mammary carcinoma multiplicity resulting from JRgal infusion was significantly different than for JRras, JRHrasA, and JRHrasG infusions ($P<0.05$).


A.
Percent Rats with Carcinomas
100
80
60
40
20
0
JRgal
JRras
JRHrasA
JRHrasG
2
4
6
8
10
12
14
16
18
20
Weeks After Viral Infusion


B.
Average Number of Carcinomas / Gland
0.25
0.20
0.15
0.10
0.05
0.00
JRgal
JRras
JRHrasA
JRHrasG
2
4
6
8
10
12
14
16
18
20
Weeks After Viral Infusion

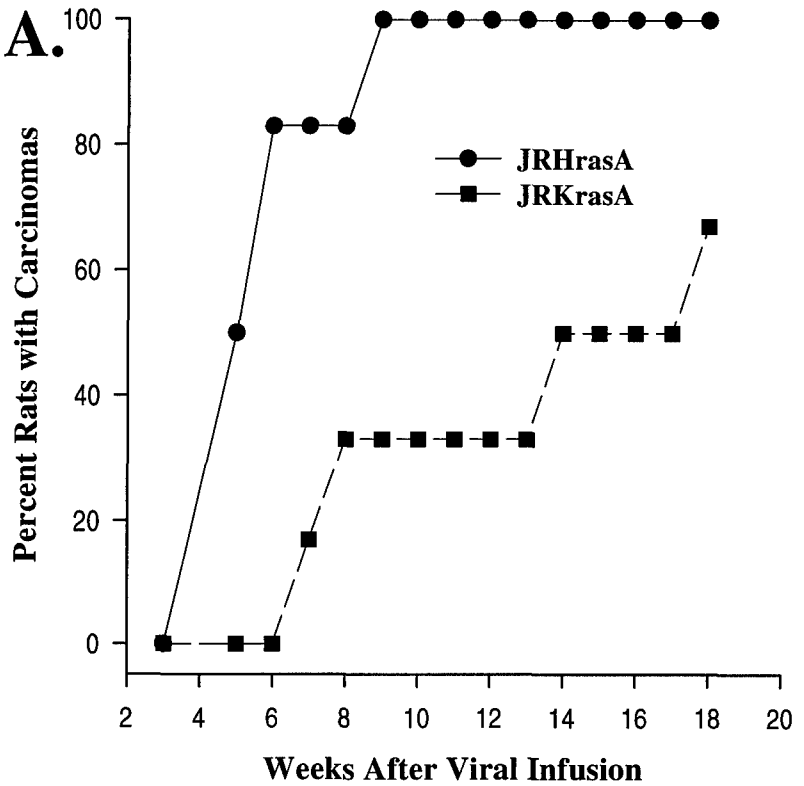
APPENDIX 3

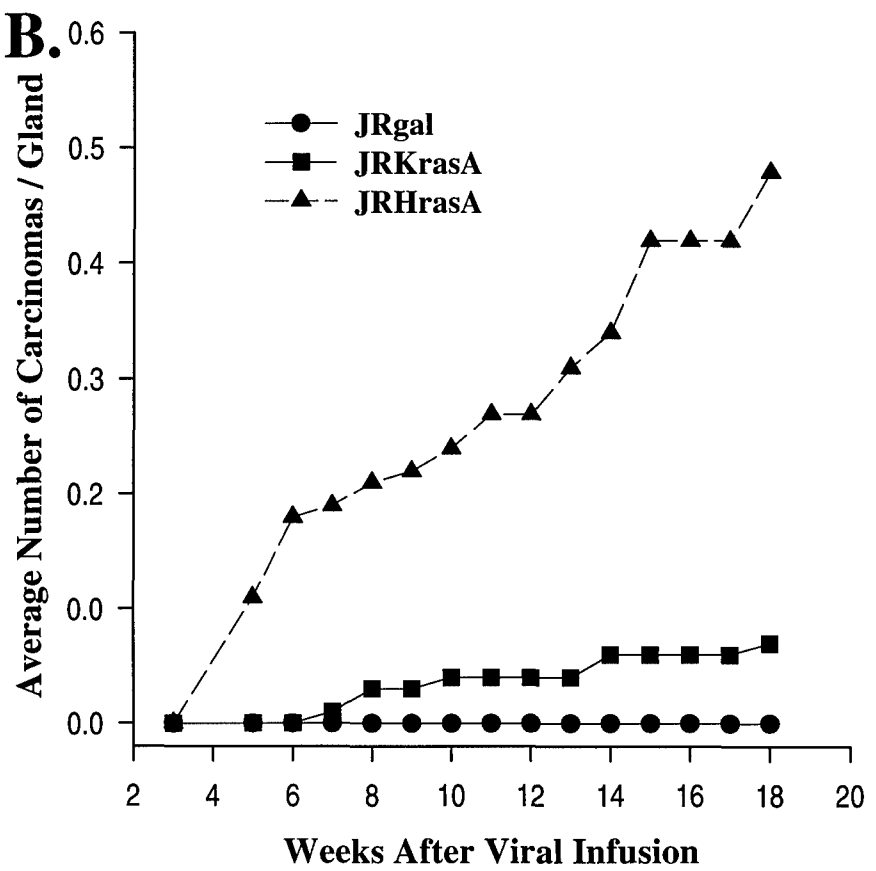
Latency and multiplicity analysis of mammary carcinoma development after infusion of retroviral vectors expressing the activated viral Harvey *ras* gene and vectors expressing wild-type or activated forms of the Kirsten *ras* genes A. Latency analysis of control vector expressing the β-galactosidase gene (JRgal) infused at 6.6 $x10^6$ CFU/ml (n=60 glands), the activated viral Harvey *ras* gene (JRras) infused at 5.5 x 10^6 CFU/ml (n=48 glands), the activated viral form of the Kirsten *ras* gene (JRKrasV) infused at 4.8 x 10^6 CFU/ml (n=60), the G35 to A mutant form of the Kirsten *ras* 2B gene (JRKrasA) infused at 3.3 x 10^6 CFU/ml, and the wild-type form of the Kirsten *ras* 2B gene (JRKrasG) infused at 3.1 x 10^6 CFU/ml (n=59). B. Multiplicity analysis of mammary carcinoma development for JRgal, JRras, JRKrasV, JRKrasA, and JRKrasG. Mammary carcinoma multiplicity was significantly different for tumors induced by JRras compared to JRKrasA and JRKrasV 18 weeks after viral infusion ($P<0.05$).


A.
Percent Rats with Carcinomas
100
80
60
40
20
0
JRgal
JRKrasG
JRras
JRKrasV
JRKrasA
2
4
6
8
10
12
14
16
18
20
Weeks After Viral Infusion
B.
Average Number of Carcinomas / Gland
0.5
0.4
0.3
0.2
0.1
0.0
JRgal
JRKrasG
JRras
JRKrasV
JRKrasA
2
4
6
8
10
12
14
16
18
20
Weeks After Viral Infusion

APPENDIX 4

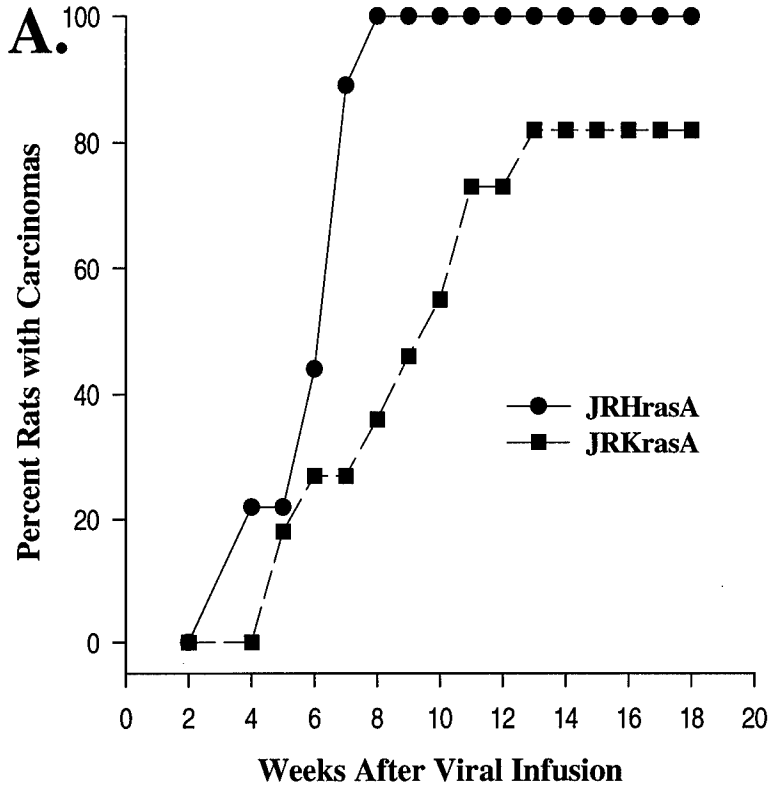
Latency and multiplicity plots for mammary carcinoma development after infusion of equivalent titers of G35 to A activated forms of either the Harvey *ras* (JRHrasA) or Kirsten *ras* (JRKrasA) retroviral expression vectors. A. Latency plot of mammary carcinoma development with JRgal infused at 3 x 10^6 CFU/ml (n=72 glands), JRHrasA infused at 6.6 x 10^6 CFU/ml (n=67 glands), and JRKrasA infused at 8.0 x 10^6 CFU/ml (n=71 glands). B. Multiplicity plot of mammary carcinoma development after infusion of JRHrasA and JRKrasA. Multiplicity was significantly different for JRHrasA-induced and JRKrasA-induced mammary carcinomas at 18 weeks following viral infusion ($P<0.05$).

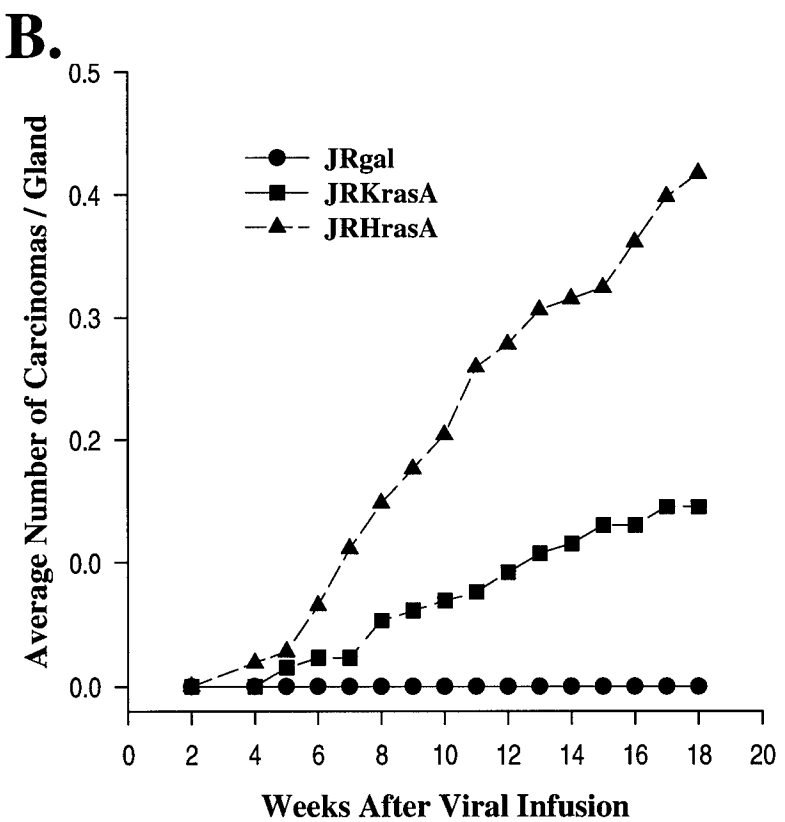

A.
Percent Rats with Carcinomas
100
80
60
40
20
0
JRHrasA
JRKrasA
2
4
6
8
10
12
14
16
18
20
Weeks After Viral Infusion


B.
Average Number of Carcinomas / Gland
0.6
0.5
0.4
0.3
0.2
0.0
0.0
JRgal
JRKrasA
JRHrasA
2
4
6
8
10
12
14
16
18
20
Weeks After Viral Infusion

APPENDIX 5

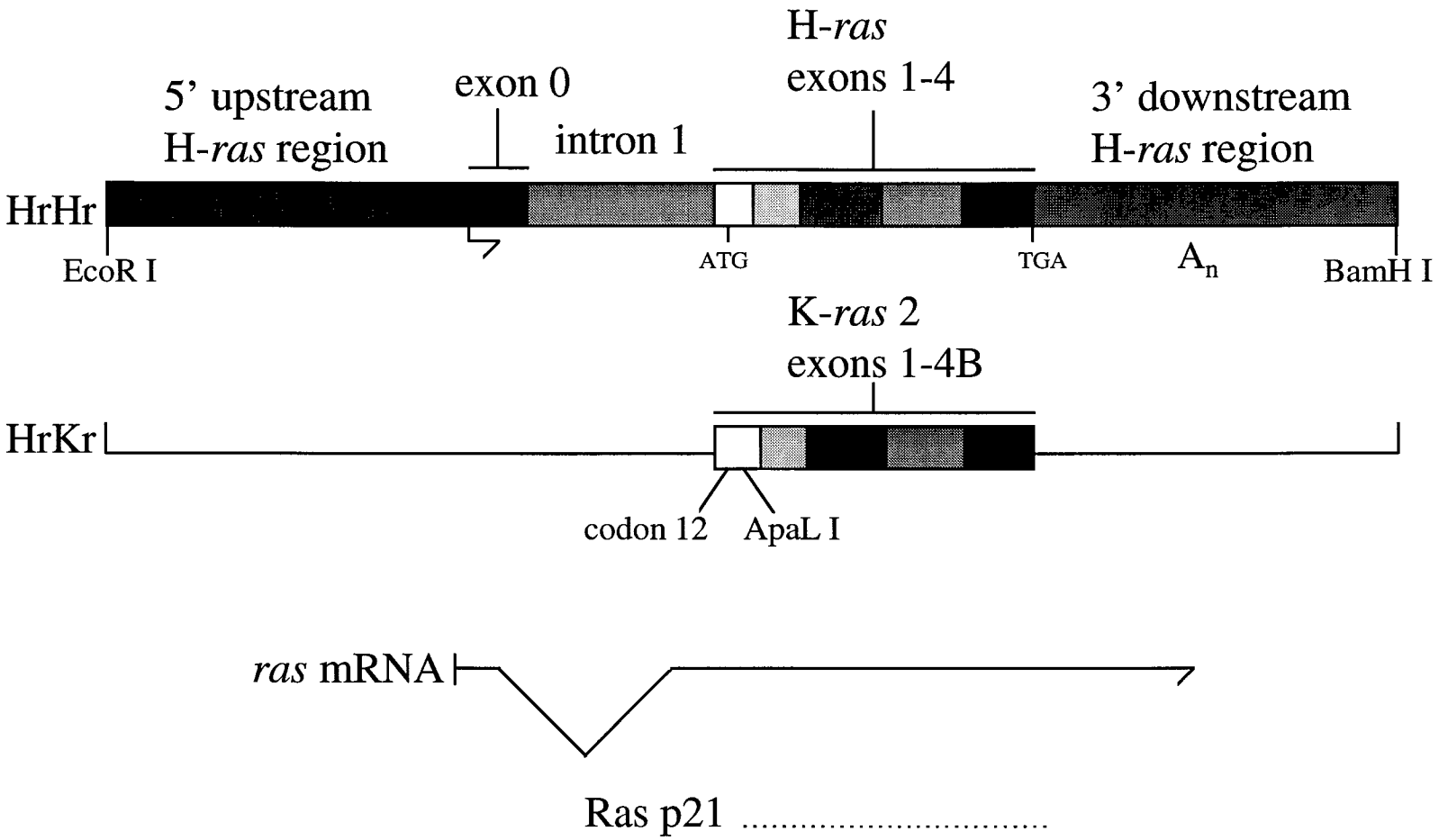
Latency and multiplicity plots for mammary carcinoma development after infusion of 5-fold higher titers of the activated Kirsten *ras* vector than the activated Harvey *ras* vector. A. Latency plot of mammary carcinoma development with JRgal infused at 1.8 x 10^6 CFU/ml (n=72 glands), JRHrasA infused at 6.0 x 10^6 CFU/ml (n=108 glands), and JRKrasA infused at 30 x 10^6 CFU/ml (n=131 glands). B. Multiplicity plot of mammary carcinoma development after infusion of JRHrasA and JRKrasA. Multiplicity of mammary carcinoma development induced by JRHrasA and JRKrasA was significantly different at 18 weeks ($P<0.05$).


A.
Percent Rats with Carcinomas
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
Weeks After Viral Infusion
JRHrasA
JRKrasA


B.
Average Number of Carcinomas / Gland
0.5
0.4
0.3
0.2
0.0
0.0
0
2
4
6
8
10
12
14
16
18
20
Weeks After Viral Infusion
JRgal
JRKrasA
JRHrasA

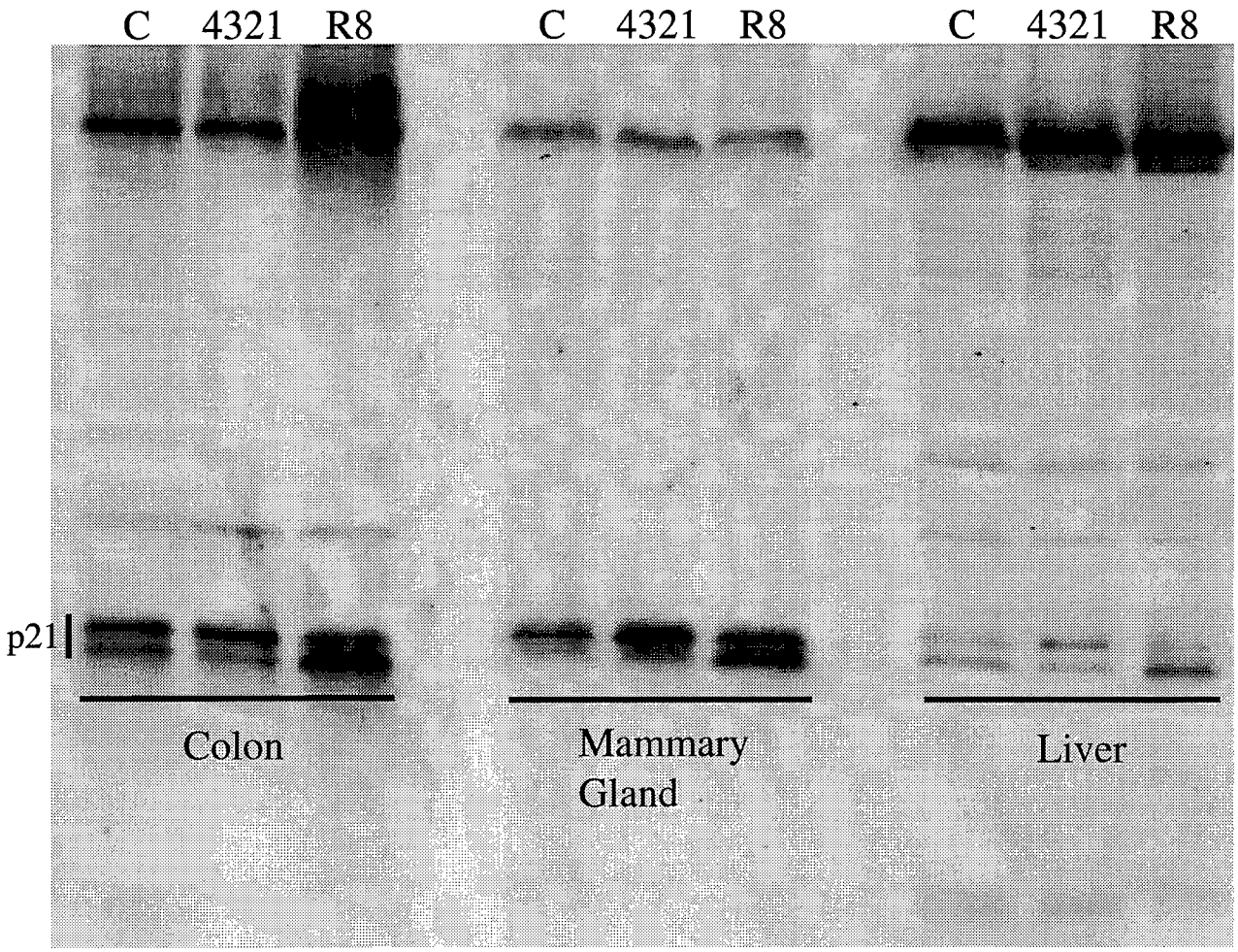
APPENDIX 6

HrHr and HrKr transgene constructs. Rat Harvey *ras* gene regulatory elements controlling the expression of the rat Harvey *ras* gene (transgene HrHr) or the rat Kirsten *ras* 2B gene (HrKr). The upstream regulatory elements of the transgene constructs including the rat Harvey *ras* gene are comprised of approximately 3,000 bp of the 5'upstream region of the Harvey *ras* gene, the first untranslated exon, and the first intron of the Harvey *ras* gene. The 3' untranslated region of the transgene construct includes approximately 2,900 bp of the rat Harvey *ras* gene downstream of the termination codon (TGA). The four coding exons of the HrHr transgene are shown. The first coding exon of the Harvey *ras* gene includes an ApaL I restriction site encompassing codons 17, 18, and 19; introduction of the ApaL I does not change the amino acid coding, but allows for distinction between the transgene generated message and the endogenous Harvey *ras* gene message. The four contiguous exons encompassing the HrKr coding regions are shown. The first exon contains the wild-type coding region of the Harvey *ras* gene, including the diagnostic ApaL I site. The fourth exon in the Kirsten *ras* transgene includes exon 4B. The message produced from the HrHr and HrKr constructs includes the first intron between the first untranslated exon (i.e exon 0) and the first translated exon and is terminated at the Harvey *ras* gene polyadenylation signal (A_n). The HrHr and HrKr generated *ras* mRNA leads to the production of Ras p21 indistinguishable from the endogenous Ras p21. Constructs are not to scale.


H-*ras*
exons 1-4
5' upstream
H-*ras* region
exon 0
intron 1
3' downstream
H-*ras* region
HrHr
EcoR I
ATG
TGA
A_n
BamH I
K-*ras* 2
exons 1-4B
HrKr
codon 12
ApaL I
ras mRNA
Ras p21 .....

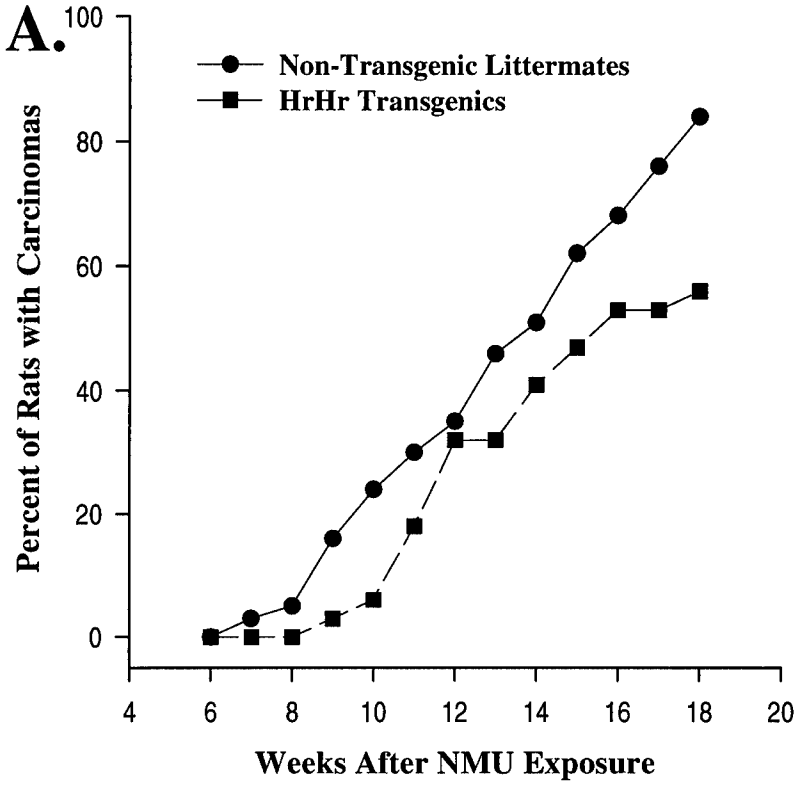
APPENDIX 7

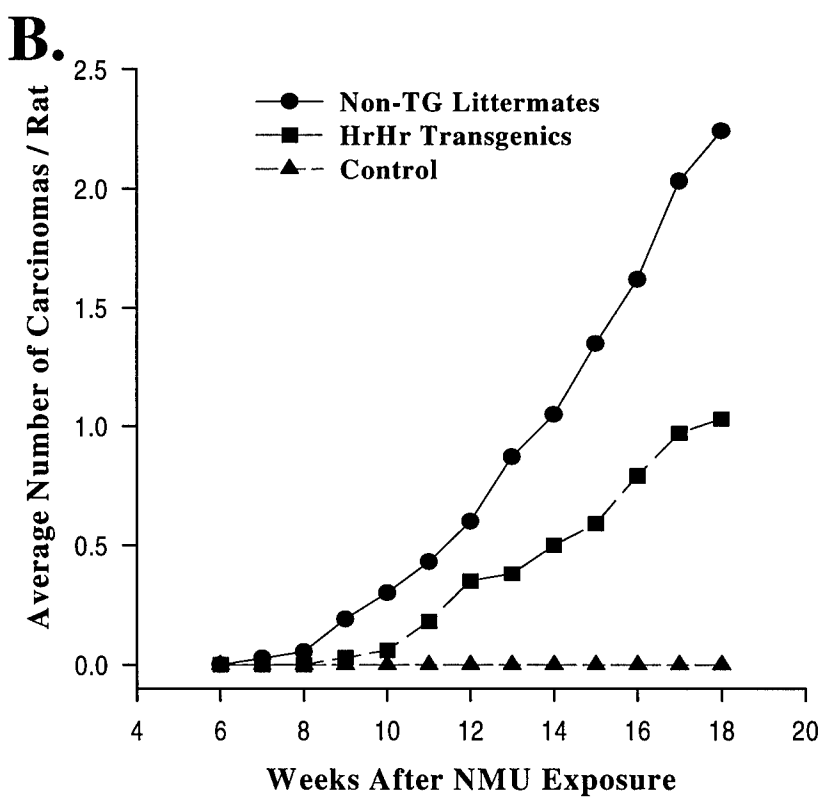
Expression of total Ras protein in colon, mammary gland, and liver tissues of non-transgenic (C), R8 HrHr transgenics, and 4321 HrKr transgenics at 3 months of age as determined by Western analysis. Mammary gland total Ras protein expression was found to be approximately 2-fold greater for R8 and 4321 transgenics than non-transgenic littermates.


C
4321
R8
C
4321
R8
C
4321
R8
p21
Colon
Mammary Gland
Liver

APPENDIX 8

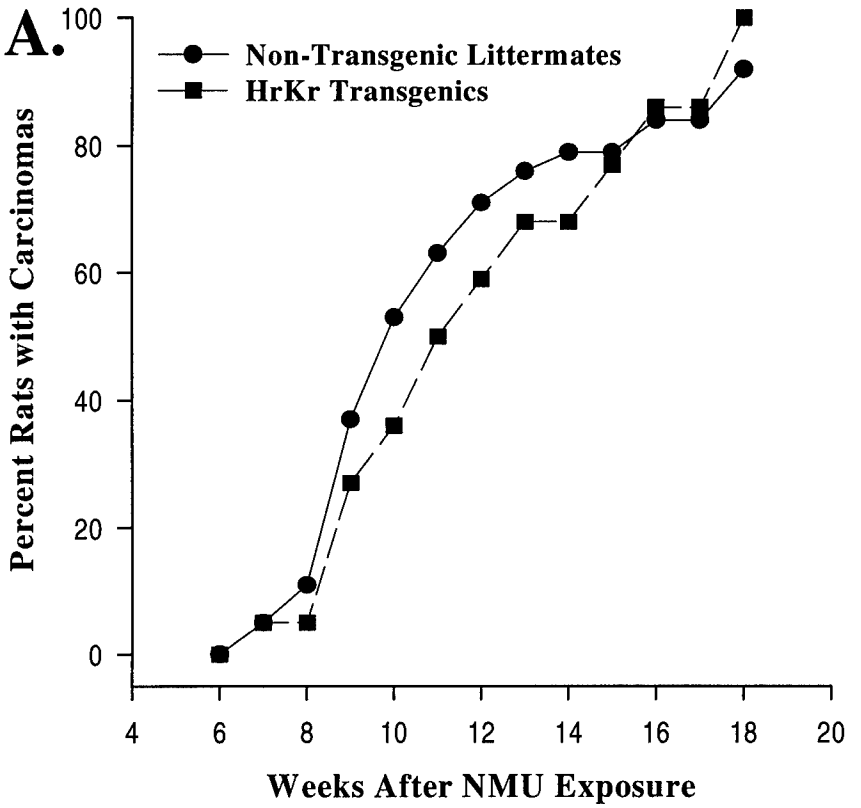
Latency (A.) and multiplicity (B.) analysis of NMU-induced mammary tumor development in R8 HrHr transgenic rats and non-transgenic littermates. Both latency and multiplicity of mammary tumors were significantly different between HrHr transgenics and non-transgenic R8 littermates ($P \leq 0.05$).

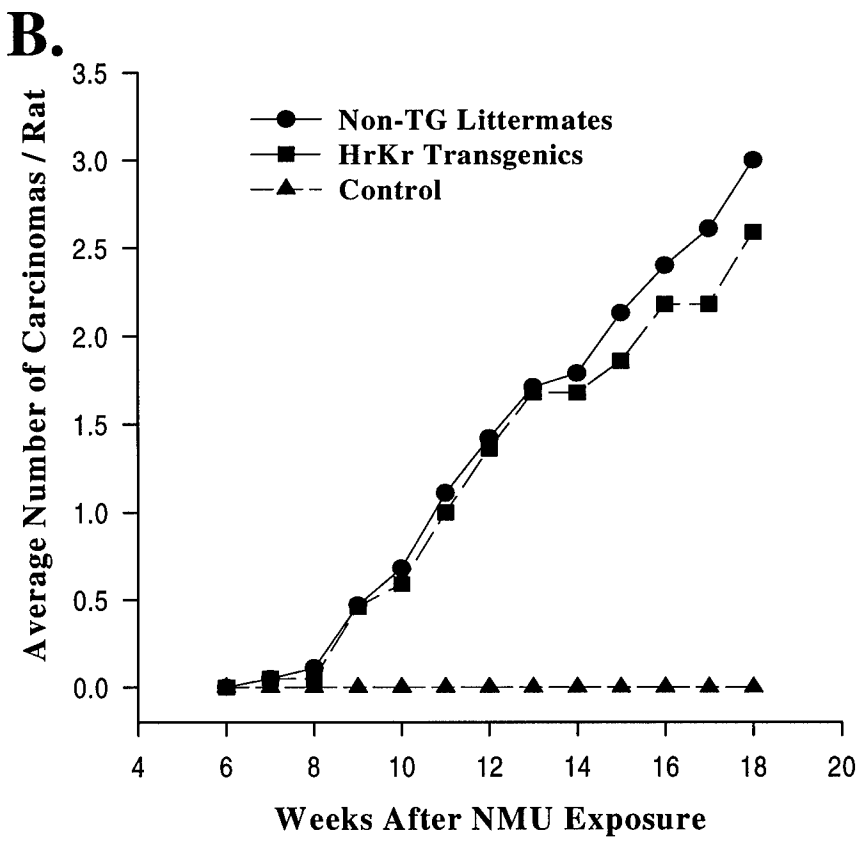

A.
Non-Transgenic Littermates
HrHr Transgenics
Percent of Rats with Carcinomas
0
20
40
60
80
100
4
6
8
10
12
14
16
18
20
Weeks After NMU Exposure


B.
Non-TG Littermates
HrHr Transgenics
Control
Average Number of Carcinomas / Rat
0.0
0.5
1.0
1.5
2.0
2.5
4
6
8
10
12
14
16
18
20
Weeks After NMU Exposure

APPENDIX 9

Latency (A.) and multiplicity (B.) analysis of NMU-induced mammary tumor development in 4321 HrKr transgenic rats and non-transgenic littermates. Latency and multiplicity were not significantly different for HrKr transgenic and 4321 non-transgenic littermate mammary tumor development.


A.
Non-Transgenic Littermates
HrKr Transgenics
Percent Rats with Carcinomas
0
20
40
60
80
100
4
6
8
10
12
14
16
18
20
Weeks After NMU Exposure


B.
Non-TG Littermates
HrKr Transgenics
Control
Average Number of Carcinomas / Rat
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4
6
8
10
12
14
16
18
20
Weeks After NMU Exposure

APPENDIX 10

Determination of G35 to A mutations in the endogenous Harvey *ras* gene from non-transgenic littermates or the endogenous Harvey *ras* gene and the HrHr or HrKr transgene of transgenic rats from NMU-induced mammary carcinomas

Animal Line	Number of tumors screened	# of tumors with mutated Endog. H-*ras*[a]	# of tumors with mutated transgene[a]
HrHr Non-TG Littermates	50	27/50	-
HrHr Transgenics	50	29/50	1/50
HrKr Non-TG Littermates	40	20/40	-
HrKr Transgenics	39	19/39	0/39

[a] Determined using the designed diagnostic restriction fragment length polymorphism assay (Kumar & Dunn, 1989).

1. A Bibliography of all publications, presentations, and meeting abstracts supported by the award:

Thompson, T.A. and Gould, M.N. A Comparison of mammary carcinoma induction by retrovirally transferred H- and K-*ras*. Proc. Annual Meeting Am. Assoc. Cancer Res. 35:A894, 1994.

Thompson, T.A. and Gould, M.N. Reduced mammary tumorigenesis in transgenic rats carrying multiple copies of the H-*ras* gene. Proc. Annual Meeting Am. Assoc. Cancer Res. 37: A3653, 1996.

Thompson, T.A. , Haag, J.D., and Gould, M.N. Age-dependent frequency of *ras* activation in NMU-induced rat mammary carcinogenesis. Proc. Annual Meeting Am. Assoc. Cancer Res. 37:A3961,1996.

"Comparison of Harvey-*ras* and Kirsten-*ras* activity in Rat Mammary Carcinogenesis". Environmental Toxicology Center Thesis Seminar, presented October 24, 1996 at the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI.

2. A list of personnel receiving pay from the above mentioned: Todd A. Thompson

3. Graduate degrees resulting from the award: The degree of Doctor of Philosophy (Environmental Toxicology) was awarded to Todd A. Thompson on April 29, 1997 due in a large measure to the work that he performed while being supported by the above mentioned grant.