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Presently, the most common way to analyze gene function in a particular cell type *in vivo* is to generate a new transgenic line for each gene under study - a costly and time consuming endeavor. Here we describe an approach which utilizes mice expressing a retroviral receptor transgene (the Rous sarcoma virus receptor) to target infection of retroviral vectors *in vivo*. This allows directed infection, and thus directed gene expression, of cells expressing the viral receptor and provides a rapid and efficient method to test the mammary tumorigenic potential of genes in an **animal model**. An important difference between this approach and testing gene function in transgenic mice is that infection, and thus gene expression, can be **temporally controlled** allowing assessment of differences in oncogenic potential at different stages of mammary gland development. Finally, **multiple oncogenes** can be introduced by co-infection, allowing questions of **synergy** to be addressed. The work reported here documents our laboratory's efforts to develop murine leukemia virus-based vectors and optimize conditions for use of these vectors in this system.

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FOREWORD

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INTRODUCTION

The purpose of this project was to continue our laboratory's development a novel system for rapidly introducing genes into mammary tissue in an animal model for analysis of gene function in vivo. An important feature of the proposed scheme which distinguishes it from standard transgenic systems is that it allows temporal control of gene expression. This project relies on the ability of a receptor for a retrovirus (Rous sarcoma virus) expressed as a transgene in mammary cells to direct infection of these cells *in vivo*, thus allowing delivery of genes carried by retroviral vectors directly to the mammary cells for rapid assessment of the oncogenic potential of these genes. The goal of this IDEA grant was to prove that this targeted delivery system can work in the mammary gland. Additionally, we proposed to optimize protocols for production of viruses to use with the transgenic mice and for introduction of genes into mammary epithelial cells in vivo.

In the time since this proposal was written the use of tva transgenic mice has shown great utility for directed infection in vivo. Recent published reports demonstrate that glial cells in the brain are specifically infected by RSV vectors if these cells express the RSV receptor, Tva [1]. Indeed, the ability to direct infection of glial cells allowed development of a unique animal model for glioma [2, 3]. In another, unpublished report, genes can be delivered specifically to megakaryocytes expressing Tva (A. Leavitt personal communication). Again this provides a unique animal model for studying the effect of genes upon platelet development. Finally, as is discussed below, Dr. Yi Li in Harold Varmus' lab has recently produced mice that express Tva in the mammary epithelium and has in preliminary experiments demonstrated introduction of marker genes into the mammary epithelia (Yi Li and H. Varmus personal communication). Together with the published results [4] from Steve Hughes lab using tva expressed under the control of a muscle-specific promoter, these data from several labs clearly demonstrates the utility of tva for directed infection of cells in vivo. Indeed, because of the number of labs now using this system a one day meeting on in vivo Tva-directed infection and expression has been held annually at NIH for the past two years.

The Specific Aims of this research project were:

AIM 1) Characterize transgene expression in mice carrying an MMTV LTR/tva transgene.

AIM 2) Produce high titer MLV(RSV) pseudotyped viruses carrying histochemical markers.
AIM 3) Assay infection of mammary epithelial cells with these viruses and optimize the infection strategy.

BODY

AIM 1 Characterization of the MMTV-tva transgenic mice

When this grant was written we had recently established two mouse lines that carried a transgene consisting of the Mouse mammary tumor virus long terminal repeat (MMTV-LTR) driving expression of a cDNA copy of the quail gene encoding the subgroup A Rous sarcoma virus receptor, tva. Unfortunately, because of infections with mouse hepatitis virus (MHV) and pinworms these lines were both lost before a complete characterization could be performed.

Initially, we proposed to re-establish the MMTV LTR-tva transgenic lines in a new barrier facility at the University of Pennsylvania . However, discussions with Dr. Harold Varmus' laboratory at the National Cancer Institute prompted us to alter our plans. The Varmus lab had independently established several mouse lines that express a tva transgene under control of the MMTV LTR or other mammary-specific promoters such as the whey acidic protein (WAP) promoter. Because establishment of these lines was well underway at NCI, and in order to avoid duplicating their efforts, we have instead focused our efforts on developing new vectors that will greatly enhance our ability to introduce genes into mammary epithelia as detailed in AIM 2 of our original proposal and described below.

Characterization of the Tva transgenic mice has been performed in the Varmus lab (Yi Li personal communication). Most importantly, efficient infection of mammary epithelial cells by RSV vectors expressing marker genes (alkaline phosphatase and betagalactosidase) was demonstrated by injection of virus or cells expressing virus directly into the gland. This analysis completely supports our initial observation that we could obtain significant infection of our MMTV-tva transgenic mice by MLV(EnvA) betagalactosidase expressing vectors (P Bates and C. Rokos unpublished observation). We are now working with the Varmus lab to utilize the newly developed transgenic mice expressing tva in the mammary epithelium for our studies using MLV(EnvA) pseudotypes to introduce genes into the mammary gland.

AIM 2. Development of MLV(EnvA) vectors.

To date, avian vectors developed by Steve Hughes at NCI, based on the replication-competent Rous sarcoma virus, have been the only viruses used to introduce genes into transgenic mice expressing tva. The main advantage of the RSV-based vectors (so called RCAS vectors) is the very high titer of virus produced by avian cells infected with this virus. These high titers are achieved because RCAS is a replication-competent vector. Although RSV-based vectors are replication competent in avian cells, RCAS cannot replicate in murine cells due to blocks in RNA transcript splicing and virion assembly. Additionally, because RSV is an exogenous virus in mice, RCAS cannot recombine with or be helped by endogenous murine retroviruses thereby preventing spread of the RCAS vector beyond the initial targeted tissue. Despite these obvious strengths, a major limitation of the RCAS vectors is the size insert which they can accommodate. These are gene replacement vectors in which the introduced gene replaces the 2 kb src gene of RSV, thus the total insert size is limited to approximately 2kb. This is smaller than many cDNA's which one would like to study in the mammary gland and importantly makes it impossible to produce vectors which carry a marker gene to follow infection as well as a genes whose function is under analysis.

Because of the limitations of the RSV vector system, we proposed to develop a transient system to produce MLV viruses carrying the RSV EnvA protein which would allow directed infection of these viruses into cells expressing tva. One advantage of this system over replication-competent RSV vectors is that the simpler, defective MLV vector backbone accommodates much larger inserts (up to 7kb). Importantly, because the MLV system is widely used by numerous labs around the world to introduce genes into cells in culture, many of the genes one would wish to study in relation to mammary gland development or tumorigenesis have already been produced in MLV vectors.

Another major limitation of the replication-competent RSV vectors is that they encode the structural proteins of this virus. Therefore, when these vectors are introduced into adult mice they are likely to elicit an immune response, potentially resulting in clearance of infected cells carrying the transduced gene. In contrast, the defective MLV vectors do not encode any structural genes and the only exogenous genes one has to worry about are the genes under analysis. It is presently unclear whether the concerns about immune

responses to RCAS vectors are warranted, however given recent attempts to introduce genes in other replication-competent vectors (such as adenoviruses) it seems highly likely this will be the case [5, 6].

To fully take advantage of the transient MLV system we needed to extensively modify the original vector described for use in this system (pHIT110). The original vector was quite large (9.1kb) and contained a G418 resistance gene which we decided to remove to avoid potential immune recognition of infected cells. The plasmid we constructed, pHIT110 poly, is much smaller (5.1kb) and unlike pHIT110, it is derived entirely from known sequences. pHIT110 poly contains a strong cytomengalovirus immediate early enhancer-promoter fused to the R and unique 5' (U5) region of MLV, an extended MLV packaging signal for efficient incorporation of the genomic RNA into virions, a polylinker region with ten unique sites for introducing genes of interest, and the complete 3' MSV LTR for efficient expression of the transduced gene in the infected cells. We have inserted a nuclear localized β-gal gene into pHIT110 poly and produced MLV(VSV-G) pseudotypes. Using this modified vector titers averaging 1 X 10^6 IU/ml were routinely obtained. Thus the re-engineered MLV vector is functional and will allow construction of marker- or onco-gene encoding viruses.

Another feature required for vectors injected into the mammary gland is high titer. This requirement stems from the fact that very small volumes can be injected (maximum of 50μl). To achieve the highest titers possible we developed and optimized a protocol to concentrate the MLV(EnvA) viruses. Toward this end, we experimented with two procedures to produce high titer stocks, ultracentrifugation and ultrafiltration. We find that a high level of concentration can be achieved for EnvA pseudotyped viruses by ultracentrifugation while in our hands ultrafiltration results in a loss of infectivity. It appears that the most critical parameter for successful concentration by ultracentrifugation is slow resuspension of the pelleted virions (overnight at 4° without vortexing works best). Thus, the protocol routinely employed to concentrate MLV(EnvA) viruses is to harvest media from transiently transfected 293T cells 36 hours after the DNA is added. The media is clarified by low speed centrifugation (3600 rpm for 10 minutes) and then virions are concentrated by centrifugation at 80K X g for 15 minutes. The viral pellet is resuspended in Tris buffered saline overnight at 4°. Using two sequential concentration steps we have achieved a greater than 200-fold increase in MLV(EnvA) β-gal titers as assayed on cultured cells (average final titer

$2X10^8$IU/ml on quail QT6 cells). A viral stock of this titer allows sufficient virus in 25µl to inject of 5 X 10^6 IU of cell-free virus per gland.

Mammary glands taken from mice during lactation or immediately post-weaning appear to contain significant levels of endogenous β-gal activity which makes detection of infected cells using a β-gal marker gene. To avoid problems detecting a β-gal marker gene in this setting, we constructed vectors that carry markers other than β-gal for analysis of *in vivo* infection. One such vector is an MLV genome encoding green fluorescent protein (GFP) or variants of GFP that have been optimized for expression in eukaryotic cells or that have shifted excitation and emission spectra compared to wild type GFP. To avoid potential problems of in vivo immune responses to other proteins encoded by the MLV vectors, we are using the minimal viral vector described above that encodes GFP (pHIT-GFP) but no selectable marker gene. In addition, we have obtained a vector encoding a human alkaline phosphatase from Dusty Miller (pLNCAP). This vector encodes G418 resistance as well as alkaline phosphatase. While the pLNCAP vector will be used in preliminary infection studies optimizing infection parameters (see below), we are currently moving the AP gene into pHIT110 poly to produce pHIT-AP which should avoid immune response problems mentioned above and from our experience should result in higher titer MLV(EnvA) viruses.

The oncogene wnt-1 (initially called int-1) was initially identified during . Subsequently a family of genes related to int-1 and the drosophila analog wingless were discovered. This family of genes (Wnt genes) are important mediators involved in morphogenesis. To analyze the function of Wnt genes other than wnt-1 in mammary tumorigenesis and development we have obtained a collection of seven murine wnt genes from Jan Kitajewski (Columbia University). The encoded wnt proteins contain a C-terminal HA epitope tag that will allow the introduced proteins to be differentiated from endogenous murine wnt's. These genes are cloned into the MLV vector LNCX and have been used to generate MLV(EnvA) pseudotypes by transient transfection of 293T cells. Titters ranging from $3X10^5$ to $8X10^5$ G418 resistant colonies per ml have been obtained. We are collaborating with Dr. Yi Li of the Varmus lab to assess the ability of these wnt genes to induce mammary tumors in MMTV-tva and WAP-tva transgenic mice.

One of the long term goals of this project is to identify novel genes which have the capability to induce in mammary tumorigenesis. Toward this end, we have developed MLV-

based vectors that carry cDNA libraries from a variety of sources. We obtained MLV vector-based cDNA libraries from transformed murine early hematopoetic cells lines from Toshio Kitamura (University of Tokyo) [7]. In addition, we produced retroviral-based libraries from transformed avian (QT6) and human (HeLa) cell lines. We are in the process of making retroviral-based libraries from a murine mammary epithelial cell line, C57 MG. These libraries have been produced either in the original vector described by Kitamura which lacks a selectable marker or more recently in a bicistronic vector in which the cDNA is inserted upstream of an internal ribosome entry site (IRES) and GFP marker gene. These libraries will be used to identify genes that can participate in mammary tumorigenesis as described below.

AIM 3 *in vivo* infection of mammary epithelial cells

As discussed above, work on in vivo infection has been hampered by the loss of the two MMTV-tva transgenic lines. To begin optimizing the parameters for infection of mammary epithelial cells before the MMTV-tva mouse lines were re-produced, we attempted to utilize MLV vectors carrying envelope proteins that are known to direct infection of a wide variety of cells in culture. This work is in preparation for studies on directed infection with MLV(EnvA) viruses when the MMTV-tva transgenic animals become available. Toward this end, we have begun producing MLV viruses pseudotyped with either vesicular stomatitis virus glycoprotein (VSV-G) or the Ebola Zaire strain glycoprotein (EboGP). Both these envelope proteins mediate infection of a wide variety of cell types in numerous species, thus it is likely that they will allow infection of mammary epithelial cells. We chose to use these envelopes instead of MLV ecotropic envelope (which will also mediate infection of murine cells) because of the stability of VSV-G and EboGP. In both cases viral stocks can be concentrated by centrifugation as described above to produce the titers required for in vivo administration of viruses. This is not the case for the MLV ecotropic envelope protein which is more fragile and does not allow concentration. Currently we routinely obtain titers over 10^6 per milliliter for the Ebola pseudotyped viruses and up to 10^8 per milliliter for the VSV-G enveloped viruses.

MLV(EboGP) and MLV(VSV-G) viruses carrying pMX-GFP or pLNCAP are currently being prepared and concentrated. MLV-based vectors require mitotically-active cells to allow integration and expression of the viral genome. Thus we will need to assess infection

under conditions where mammary cells are dividing. Unlike most organs, the mammary gland undergoes extensive growth and development after birth in the juvenile and adult animal. Beginning at approximately 3 weeks of age and continuing through 9 weeks, there is extensive proliferation of ductal cells in the mammary gland. Another period of mammary cell proliferation occurs during pregnancy in preparation for lactation. To target cells during this phase of growth, adult (6 month) female mice will be mated, then 14 days after vaginal plugs are noted they will be injected with virus stocks. The two phases of mammary cell proliferation are not equivalent, so we may target different cells by using these two time frames for infection. MLV(VSV-G) or MLV(EboGP) vectors will be injected directly into the mammary gland of female mice at the stages of puberty or pregnancy discussed above to determine conditions during which mammary epithelial cells are good targets for retroviral infection in vivo. The optimal conditions determined by these studies will then be employed in future studies with MLV(EnvA) vectors and MMTV-tva mice.

CONCLUSIONS

Our work analyzing infection in vivo has been delayed by the loss of two transgenic lines expressing tva in the mammary epithelium. However, these lines have now been re-developed and this work can now proceed. While the mice we being re-developed, we concentrated our efforts on producing vectors based on the murine leukemia virus which could be used with the tva transgenic mice. These replication-defective viruses utilize the MLV core components and MLV vectors but are enveloped with RSV EnvA proteins. One limitation for use of traditional MLV vectors in vivo was the relatively low titers that could be obtained with viruses when the MLV envelope proteins were employed. Here we demonstrate that MLV(EnvA) pseudotyped virus can be efficiently produced and concentrated to high titer. Indeed the titers we routinely obtain with the protocols developed under this grant allow sufficient inoculum for use in vivo (up to $2X10^5$ per µl concentrated MLV(EnvA) virus).

In addition to optimizing the protocols of psuedotype production we spent a good deal of time producing MLV vectors for use in vivo. These include vectors expressing the marker genes alkaline phosphatase and GFP. More significantly, we have produced MLV vectors carrying cDNA libraries from transformed (QT6) and normal (CEF) cells. A library from

transformed murine mammary cells is under construction. Introduction of these libraries into mammary cells in vivo and scoring for tumorigenesis will allow identification of cDNA's capable of affecting mammary tumorigenesis. In addition, one can readily design complementing experiments in which these libraries are co-infected with a vector carrying a gene known to be involved in tumorigenesis but insufficient for full transformation.

In summary, during the relatively short period of this IDEA grant we have produced and developed pseudotyped MLV vectors that will extend significantly the utility of the tva-directed in vivo targeting system. Thus we are now poised to use these vectors to analyze the role(s) of specific genes in mammary development and/or tumorigenesis.

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