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Mouse mammary tumor virus is used as a mutagen in transgenic mice that carry an activated *Wnt-1* gene, to produce additional events such as activation of a second oncogene by nearby insertion of proviral DNA. In about 55% of the mammary tumors having new proviral insertions, no known targets of MMTV mutations or known *Fgf* genes are affected, implicating the involvement of other proto-oncogenes. In order to identify one of the activated proto-oncogenes from this group of tumors, the cellular sequences flanking a new proviral insertion site from tumor 76 was cloned, the insert mapped, and repeat free fragments isolated. Southern blots of tumor DNAs probed with the cellular fragments show rearrangements due to MMTV proviral insertions within this locus in 12 of 85 tumors analyzed. These results indicate that this is a common insertion site for MMTV. Since northern analysis and exon trapping failed to identify an activated gene within the cloned region, the region downstream is being cloned and characterized. No known targets of MMTV mutations or members of the FGF family seem to be activated in these tumors. Therefore it is likely that we would find a novel or unexpected proto-oncogene activated in these tumors.

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II. INTRODUCTION

Breast cancer, like other cancers, results primarily from accumulation of genetic mutations. Many of the identified mutations associated with cancer result in the activation of proto-oncogenes or inactivation of tumor suppressor genes. In most cases, a single chromosomal aberration is insufficient to cause carcinogenesis but is rather the result of mutations in two or more genes. In order to understand the development and progression to cancer, it is imperative to identify not only the single mutations involved, but also synergistically acting groups of cancer related genes.

The role of retroviruses in viral-induced cancers has been well established in mice. For example, MMTV has proven to be a powerful tool for studying murine mammary tumorigenesis. MMTV is a B type retrovirus that is known to cause mammary adenocarcinomas in certain inbred strains of mice (e.g. C3H, BR6, GR) (1, 2). The tumor inducing property of MMTV is intrinsically related to an obligatory step in its life cycle, the insertion of a proviral copy of its genome into host cellular DNA. The integration is a mutagenic event for the host cells and as a consequence may lead to the transcriptional activation of closely linked proto-oncogenes by the mechanism of insertional mutagenesis (3). The activation of the proto-oncogene by MMTV contributes to the transformation of the cell and development of a tumor. A number of proto-oncogenes activated by MMTV in mammary tumors have been identified. They are *Wnt-1, Wnt-3, Wnt-10b, Fgf-3, Fgf-4,* and *Int-3.*

While the structure of these genes are known, less is known about their function. A common factor among the MMTV activated genes is that they all appear to play key roles in early embryonic development (2). To prove the oncogenic potential of *Wnt-1*, transgenic mice containing the *Wnt-1* gene under the control of an MMTV enhancer were generated. Both male and female transgenics developed mammary adenocarcinomas following a generalized mammary hyperplasia (21). The median latency of mammary tumor formation, in female mice was ~ 5 months. Males developed tumors less frequently and later in life. The generalized hyperplasia coupled with the long latency and the sporadic nature of the tumor formation suggest that *Wnt-1* contributes to but is not sufficient for mammary tumorigenesis in these mice. Activation of *Wnt-1* is probably an early event in the process of tumor formation. Therefore other events, presumably genetic, are necessary for tumor progression.

In an attempt to identify genes acting in synergy in the multistep process of murine mammary tumorigenesis, these*Wnt-1* transgenic mice were mutagenized by infection with MMTV (22, 23). The strategy was that since MMTV transcriptionally activates proto-oncogenes by insertion of its own DNA near them (2), one could possibly identify additional oncogenes that oncogenically cooperate with *Wnt-1* by tagging them with viral DNA. Activation of the cooperating oncogene would therefore confer a growth advantage and would presumably produce a tumor composed mainly of cells that are clonally derived from the cell bearing the proviral insertion. Implicit in this hypothesis was the expectation of a reduction in tumor latency. As predicted, in MMTV infected *Wnt-1* transgenics the median latency of tumor formation decreased from ~5 months to 2.5 months and the number of tumors per mouse increased (23). Southern blot data reveal that most of these tumors contained clonal tumor-specific proviruses in addition to the endogenous proviruses found in laboratory mice (23). The advantage of this approach over other mutagenesis procedures is that tumors arising due to proviral insertions contain proviruses physically linked to the activated proto-oncogenes forming a molecular tag which permits easy identification and cloning of the activated genes (2).

Analysis of the tumor DNAs derived from infected *Wnt-1* transgenic mice by Southern blotting showed that at least 80 of 128 tumors (59%) contained clonal MMTV-specific proviruses (23). These tumors were examined for the insertional activation of proto-oncogenes known to be activated by MMTV: *int-2/Fgf-3, hst/Fgf-4, int-3* and *Wnt-3* (2, 24). Approximately 45% of these tumors contained insertionally activated *int-2* and/or *hst* (23). These results show the cooperation of *int-2* and *hst* with *Wnt-1*, which strongly corroborates prior findings indicating the same cooperation (25,26). I (in collaboration with a Hem/Onc fellow Craig MacArthur) recently identified another member of the FGF family of growth factors that is insertionally activated by MMTV in 8 of 80 mammary tumors with clonal tumor-specific proviral insertions (27). This gene (*Fgf-8*) was cloned from one of the tumors that had a single tumor specific proviral insertion as described in the methods section. *Fgf-8* is transcriptionally activated in the tumors from a silent state(27). This is the third member of the FGF family to be activated in this system, indicating that *Fgfs* and *Wnts* are strong collaborators in inducing mammary tumors.

As we have already demonstrated, this infected *Wnt-1* transgenic system can be used to identify novel or/and unexpected oncogenes that are involved in mammary tumorigenesis , thereby demonstrating oncogenic cooperation with Wnt-1 and elucidating the multiple steps involved in murine mammary tumorigenesis. We still have ~ 55% of the mammary tumors from infected *Wnt-1* transgenic mice with new proviral insertions in which the known targets of MMTV mutations are not affected. I had proposed to identify an insertionally activated gene(s) in tumors 47 and 76.

My specific aims:

1. Isolation and identification of proto-oncogenes (novel and unexpected) insertionally activated by MMTV in tumors of infected *Wnt-1* transgenic mice.
   - Identification of proviral-cellular junction fragments.
   - Clone cellular sequences flanking the proviral insertion
   - Locate and isolate the activated gene in the locus using Northern blot and exon trap strategies.
   - Determine the expression pattern of the gene in normal tissues and in tumors.

2. Characterization of the gene and analysis of the oncogenic potential of the identified proto-oncogene.
   - Demonstrate the oncogenic potential of the isolated proto-oncogene in cell culture transfection assays.
   - Demonstrate the gene's oncogenic potential *in vivo* using transgenic mice.

3. Demonstration of the cooperativity of *Wnt-1* with the proto-oncogene that is activated by MMTV.
   - Demonstrate cooperativity by cotransfection of C57MG cells.
   - Obtain definite proof of cooperativity by generating bitransgenic mice.

BODY OF ANNUAL REPORT

Isolation and identification of proto-oncogenes insertionally activated by MMTV in tumors from infected Wnt-1 transgenic mice.

Identification of Proviral-cellular junction fragment to be cloned:

The group of tumors that were known to have proviral insertions but no known activated gene were analyzed for the presence of a discrete clonal proviral-cellular junction fragment that could be cloned. Initial screening of the tumor DNAs using Southern blot analysis of EcoRV and Sst-I digested DNA samples showed proviral insertions within an apparently common locus in two independent tumors (tumors 47 and 76), as indicated by the common size of the MMTV-specific restriction fragments. MMTV integration into the host genome alters the restriction map of the locus because of the insertion of proviral DNA. Each clonal provirus will thus have different sized junction fragments depending on the restriction sites at the site of integration. Therefore, if there are proviral insertions from independent tumors within the same locus approximately at the same site (within a few hundred base pairs), then the sizes of the junction fragments will be the same with several enzymes and can be detected by hybridizing to MMTV-specific probes on Southern blots.

To confirm the initial observation made from the EcoRV and Sst I data, I made Southern blots of DNAs from these two tumors (47 and 76), digested with four different enzymes and probed them with a probe from the 5' end of MMTV(probe;gag). Junction fragments of similar sizes hybridizing to the MMTV probe were seen in each enzyme digest of these two tumor DNAs, but not of control DNA. Since the presence of proviral insertions within a common locus in two independent tumors is indicative of selection a particular phenotype (activation of a proto-oncogene), I decided to clone a junction fragment from Tumor 76.

Construction and Screening of a Subgenomic library from Tumor 76.

Fifty μg of tumor 76 DNA was digested with Sst-I and electrophoresed on an agarose gel. The 5.5 kb Sst-I junction fragment was size selected from the gel and purified using glass beads(Geneclean). The fragment was then cloned into lambda Zap II and packaged as phage using the Gigapack II kit (Stratagene). A subgenomic library (λ Zap 76)of ~ $1x10^6$ pfu of recombinant phage was generated. The library was probed with an MMTV LTR probe and 12 putative positive clones were isolated. These clones were subjected to two additional rounds of screening, and ultimately four clones were selected.

The newly cloned region was removed from λ Zap II by the process of *in vivo* excision; this process created a subclone of the cloned fragment contained in Bluescript phagemid (SS5.5). Next the subclone was restriction mapped using several single and double enzyme digests, and Southern blots. The entire subclone was screened for repeat sequences by hybridizing a southern blot containing restriction digests of the plasmid to

mouse genomic DNA(50ng), and two repeat free cellular fragments were identified: PP1.2 and XS0.8 (fig-1).

Identification of a common insertion locus for MMTV:

In order to look for insertions within the same locus in other tumors, the entire tumor panel was screened by Southern blotting using the two repeat-free cellular fragments as probes. Eighty five tumor DNAs were restriction digested with Xho-I, and EcoRV and Southern blotted. The resulting Southern blots were probed first with PP1.2(5') and then with XS 0.8(3') to look for rearranged or shifted restriction fragments. Rearranged bands were seen in 12 of 85 tumors with proviral insertions indicating that this was a common insertion locus for MMTV. These blots were stripped and reprobed with MMTV specific probes gag(5') and env(3') to determine the site and transcription orientation of the proviral insertions. These results were further confirmed by Southern blots of these 12 tumor DNAs digested with an additional three enzymes. A map of the locus with the location and orientation of MMTV integration was thus generated.

Exon trapping and Northern analysis:

In order to look for transcribed regions within the cloned cellular fragment, exon trapping procedure was performed. The 5.5 kb Sst-I fragment was cloned into the exon trap vector pML53In(30) in both orientations. Cos-7 cells were then transiently transfected with both the plasmids and the control vector by the Lipofectamine method (BRL). After transient transfection, RNA was made from the cells and reverse transcription polymerase chain reaction (RT-PCR) was performed using specific primers (27,30). Analysis of the PCR products showed only the 100 bp insulin fragment in all the lanes indicating that the splicing occurred between the 2nd and 3rd insulin exons (Control) but no exon was trapped from the cloned region.

To confirm this observation, I also did northern analysis on RNAs from some of the tumors that had proviral insertions within this locus. Both PP1.2 and XS 0.8 were used as probes for this analysis. No specific transcript was seen hybridizing to either of the probes. This confirmed the fact that I did not have any coding region within my cloned cellular region. Since the cloned region is only 4.5 kb and it is known that MMTV can activate genes even 10-20 kb away from the insertion ( ), I decided to do chromosome walking in order to identify the activated gene. The orientation of the MMTV proviruses within this locus seems to indicate that the activated gene may be downstream of the cloned region(fig). There seems to be two clusters of MMTV insertions, one upstream and one downstream (according to the map). I decided to clone the region between the two clusters (fig).

Cloning of the region downstream of SS 5.5:

Southern blot analysis of tumor 66 shows a Xho-I restriction fragment of 15 kb that hybridizes to both XS0.8 and MMTV gag. This tumor also has a fragment of 17 kb that hybridizes to MMTV env. This fragment will allow me to clone the region from the downstream cluster of insertions to the next Xho-I site. Cloning both these fragments would therefore give me approximately 23 kb of cellular DNA, and I should be able to locate the gene activated by these proviral insertions within this region easily.

I size selected the fragments as previously described, cloned them into Lambda Dash II vectors (Stratagene), and made two subgenomic libraries; Library-1 and 2. Library-1 was titered and screened using the XS0.8 and MMTV gag. I isolated four putative positives from library one, subjected them to two additional rounds of screening and ultimately two positives were selected. Lambda DNAs from the positive clones were made and I am in the process of generating restriction maps of the inserts. Library-2 has been titered and is currently being screened.

Once I have a good restriction map of the cloned region, I will perform the Exon trapping procedures using overlapping fragments spanning the entire region. The trapped fragment will then be analyzed as described in the methods section of the proposal. This locus is apparently a frequent site for MMTV proviral insertions in tumor DNAs (more insertions than Fgf-8) indicating that there is a very high probability of an activated proto-oncogene in the vicinity.

CONCLUSIONS:

I have successfully identified and cloned a proviral-cellular junction fragment from a tumor from MMTV-infected Wnt-1 transgenic mice. Using cellular probes derived from this cloned region I have found insertions in 12 of 85 tumors within this locus. The probability of the occurrence of proviral insertions within the same 30 kb locus in two independent tumors is only 10^{-10}(30). This result indicates that this is a new common insertion locus for MMTV. These tumors have been previously analyzed for the activation of known targets of MMTV activations and members of the FGF family(27). Since this so far seems to be an unique locus, it is likely that we would find a novel or unexpected proto-oncogene activated in these tumors. I am currently in the process of cloning and characterizing the region downstream of SS 5.5. I will soon search for activated gene(s) in this region using exon trap and Northern blot techniques. Once I have identified the gene, I will characterize it and prove its oncogenic potential as described in my proposal and statement of work.

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(9) APPENDIX

A NEW COMMON INSERTION LOCUS FOR MMTV

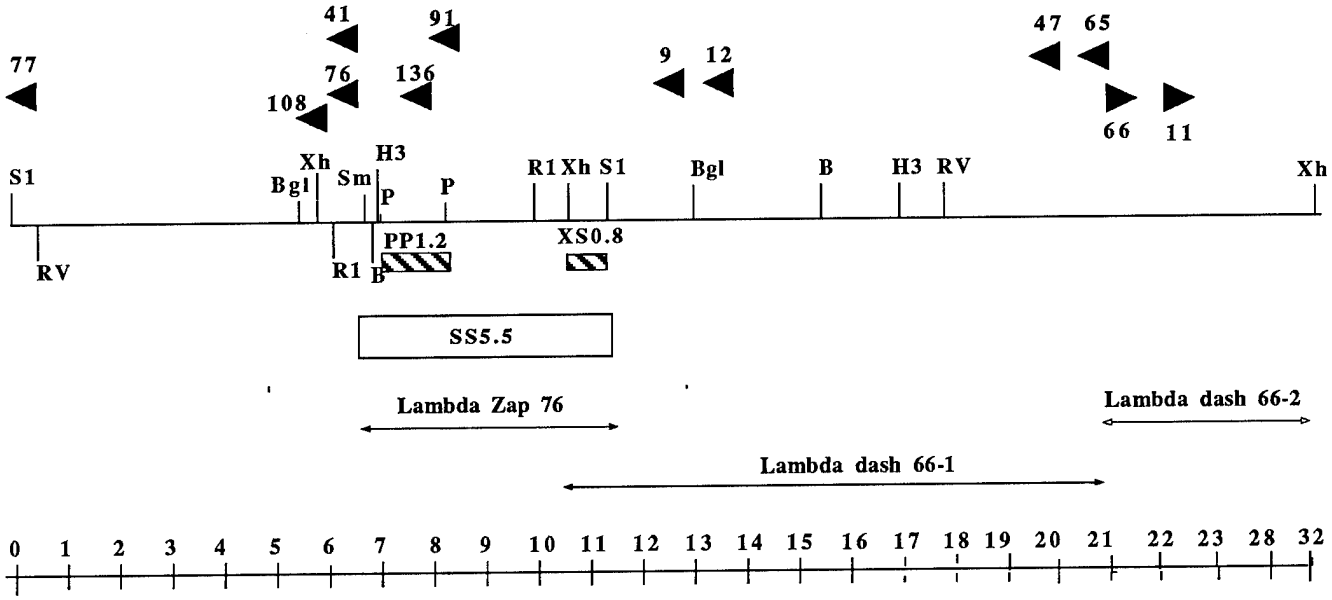

Figure-1: Map of the Tumor 76 locus . Arrowheads represent MMTV insertion sites and the orientation. R1represent EcoR1, Bgl: Bgl II, B: Bam HI, P: Pst I, H3: Hind III, RV: EcoRV, S1: Sst I, Sm: Sma I, Xh: Xho I. Filled in boxes represent repeat free probes. Lines with arrowheads represent the Lambda clones.