Mouse Mammary Tumor Virus Carrying a Bacterial supF Gene Has Wild-Type Pathogenicity and Enables Rapid Isolation of Proviral Integration Sites

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Mouse mammary tumor virus (MMTV) is a milk-transmitted, replication-competent retrovirus that causes mammary adenocarcinomas in female mice with an extended latency. It can also cause a low incidence of mammary tumors in males and lymphomas (4, 16, 22). In addition, a variety of premalignant structures in the mammary gland can be induced by MMTV infection, which enables the study of the multistep neoplastic process (23). Moreover, MMTV-induced mammary tumors often begin as hormone-dependent neoplasms in that they grow and regress depending on the pregnancy status of the female, thus allowing the hormonal regulation of tumorigenesis (24). The presence of supF in the LTR should circumvent the screening process for proviral insertion sites, since only those lambda clones with supF-containing proviral-cellular junction fragments should be able to form plaques on a lawn of wild-type Escherichia coli (i.e., lacking supF). The resulting virus (MMTVsupF) induced mammary tumors at the expected rate in infected mice, deleted the appropriate T-cell population by virtue of its superantigen gene, and stably retained the supF gene after passage via the milk to female offspring. To test the selective function of the system, size-selected DNA containing two proviral-cellular junction fragments from an MMTV supF-induced mammary tumor was ligated into AgtWES.AB, packaged, and plated on a supF-deficient bacterial host for selection of supF-containing clones. All plaques tested contained the desired cloned fragments, thus demonstrating the utility of this modified provirus for the rapid cloning of MMTV insertion sites.

MATERIALS AND METHODS

MMTVsupF provirus construction. A 0.2-kb EcoRI fragment containing the bacterial SulII tyrosine suppressor tRNA gene and its bacterial promoter was removed from plasmid pin31suIII (18) (gift of Pat Brown) and cloned into a PCR-generated EcoRI site in the MMTV LTR. The four primers used for introducing the EcoRI site were 5′-TTAGTGACTGCTTA-3′ (primer 1), 5′-GGGAATTCTATTCATAATAACTCA-3′ (primer 2), 5′-TGGAATTCTTATTGGCCCA-3′ (primer 3), and 5′-GGGAATTCTATTCATAATAACTCA-3′ (primer 4). Primers 1 and 2 were used to amplify the region 5′ of the EcoRI site, and the primers 3 and 4 were used to amplify the region 3′ of the EcoRI site. Primers 2 and 3 contained the new EcoRI site. The resulting PCR products and the supF gene fragment were then used in the reconstruction a full-length MMTV provirus containing supF in the 3′ LTR (MMTVsupF) [Fig. 1]). No MMTV sequence was lost or duplicated during the cloning process. The MMTV plasmid used in this construction was pUVEH-N, which contains a clonable, full-length hybrid provirus [MMTV(C3H)hyb] consisting of the 5′ half of Mtv1 and the 3′ half of MMTV(C3H) (33). A total of 207 bp was added to MMTV in the process of inserting the supF gene, including the 4 bp added to create the EcoRI site. The supF sequence used here corresponds to bases 113 through 315 of plasmid prVX (GenBank accession no. X14353), which is the source of supF sequences used to produce pin31suIII (18).
RNA and DNA isolation and analysis. Total cellular RNA was isolated and analyzed by Northern blotting as previously described (33). High-molecular-weight DNAs were isolated from cells and tissues and analyzed by Southern blotting. As described elsewhere (33), we used a 1.2-kb HindIII fragment of the envelope region of MMTV to detect the MMTV genomic and envelope RNAs. The MMTV LTR probe was a 1.1-kb PstI-SalI fragment.

A vector and host bacteria. AgWES.AB (14) contains an amber mutation in its p gene, a gene required for lytic growth. The two Escherichia coli strains used in this study as hosts for AgWES.AB were LE392 and W3110. LE392 is a supF-containing amber-suppressing strain which permits the growth of all recombinant AgWES.AB phages; W3110 is a nonsuppressing host which allows plaque formation only by phages carrying a supF gene. SacI fragments in the 2 to 4 kb size range from a supF-induced mammary tumor were purified and ligated into SacI-digested and phosphatase-treated AgWES.AB arms. The ligase products were packaged (Gigapack Gold; Stratagene) and labeled with mouse anti-supF antibodies.

RESULTS AND DISCUSSION

MMTV supF construction. To facilitate the cloning of MMTV insertion sites from tumors of infected mice, we introduced a bacterial supF suppressor tRNA gene into the LTR of the MMTV(C3H)\textsuperscript{blyb} virus (33). This supF gene carries its own prokaryotic promoter and should not be expressed in mammalian cells. Since the supF tRNA suppresses amber stop codons by inserting a tyrosine, selection for DNA fragments containing the supF gene can be accomplished in lambda vectors that contain amber mutations in genes required for phage replication. In an attempt to avoid disrupting important MMTV transcriptional regulatory elements, we cloned supF into a PCR-generated restriction site placed immediately downstream of the MMTV superantigen gene (sup) stop codon at base -234 relative to the U3/R boundary (Fig. 1). This site is not within any known positive or negative regulatory elements or promoters of MMTV (2, 17, 29).

MMTV supF gene expression in cell culture. We initially tested the MMTVs supF gene in cell culture to determine if the new supF sequences present in the LTR would disrupt normal viral gene expression or virion production. Plasmids containing the MMTVs supF provirus and a neomycin resistance gene were cotransfected into rat XC cells and selected in G418, and the resulting clones were isolated. Northern blot analysis of total cellular RNAs from several clones showed that abundant MMTV gene expression or virion production. Plasmids containing the MMTVs supF gene were packaged into lambda phage vectors that contain amber mutations in genes required for phage replication. In an attempt to avoid disrupting important MMTV transcriptional regulatory elements, we cloned supF into a PCR-generated restriction site placed immediately downstream of the MMTV superantigen gene (sup) stop codon at base -234 relative to the U3/R boundary (Fig. 1). This site is not within any known positive or negative regulatory elements or promoters of MMTV (2, 17, 29).

MMTV supF is superantigen-positive and infectious in vivo. The sag gene of MMTV encodes a superantigen which stimulates a large subset of T cells when it is expressed on the surface infected B cells or other antigen-presenting cells (1). Activated T cells then stimulate the infected B cells to proliferate, thus producing an expanded reservoir of infected cells (8, 9). The specificity of the T-cell reaction is dictated by the interaction of the Sag protein with specific T-cell receptor \textsuperscript{b}virions (Fig. 2A), suggesting that proviral gene expression, RNA stability and processing, and virion production are not negatively affected by the presence of the supF gene.

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above or the MMTV(C3H)hyb producer cells; a third cohort (uninfected group) was injected with normal XC cells. Upon tumor development or at 1 year of age, whichever was earliest, analysis of V\textsubscript{b}14 T-cell populations of these mice showed that those injected with MMTV\textsuperscript{supF} producer cells experienced V\textsubscript{b}14 T-cell deletions to an extent similar to those injected with wild-type MMTV(C3H)hyb producer cells, suggesting that MMTV\textsuperscript{supF} sag gene expression is normal and sufficient for T-cell activation and deletion (Table 1).

To test whether the life cycle of MMTV\textsuperscript{supF} was affected by the presence of sup\textsuperscript{F}, we bred the injected mice and allowed them to infect their offspring naturally via the milk. We then examined whether virus particles could be detected in the milk of these female offspring by immunodot blot analysis of milk samples using anti-MMTV SU antibody. Offspring in both the MMTV\textsuperscript{supF} and MMTV(C3H)hyb groups produced similar amounts of SU antigen into their milk, suggesting that the mammary glands were successfully infected by MMTV\textsuperscript{supF} and were producing virus particles at levels similar to those for glands infected with the wild-type virus (Fig. 2B).

**Tumorigenesis by MMTV\textsuperscript{supF}**. Mammary tumors induced by MMTV occur as a result of somatic insertional mutagenesis, with a median latency of approximately 8 months of age in breeding females (32). We compared the tumor incidence of mice infected with MMTV\textsuperscript{supF} to the tumor incidence of mice infected with MMTV(C3H)hyb, using both the i.p. injection and natural milk routes of infection. We found that the median age of tumor formation in mice infected with either virus and by either route of infection was approximately 8 months of age as expected (Fig. 3). Together with the results above, these data suggest that MMTV\textsuperscript{supF} is as infectious and oncogenic as wild-type MMTV(C3H)hyb. The similarity in tumor formation rates also suggests that the presence of sup\textsuperscript{F} sequences in the proviral LTRs does not inhibit the activation of neighboring proto-oncogenes by integrated proviruses.

Insertion of additional sequences, such as sup\textsuperscript{F}, into viral genomes can result in deletion of the foreign sequences if they present a negative influence on viral replication (18). To determine whether sup\textsuperscript{F} was deleted from the MMTV\textsuperscript{supF} LTR, either in virus-injected animals or during passage from mother to offspring, we analyzed the DNAs of mammary glands and tumors derived from both generations of mice. In Southern blots of these DNAs, capable of detecting internal 3' LTR

### Table 1. V\textsubscript{b}14 T-cell deletion by MMTV\textsuperscript{supF*}

<table>
<thead>
<tr>
<th>Group</th>
<th>V\textsubscript{b}14 CD4\textsuperscript{+} T cells (mean ± SD)</th>
<th>V\textsubscript{b}14 CD3\textsuperscript{+} T cells (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>9.76 ± 1.63</td>
<td>6.01 ± 0.98</td>
</tr>
<tr>
<td>Infected with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMTV\textsuperscript{supF}</td>
<td>3.77 ± 1.22</td>
<td>2.06 ± 0.65</td>
</tr>
<tr>
<td>MMTV(C3H)hyb</td>
<td>3.76 ± 0.94</td>
<td>2.70 ± 0.73</td>
</tr>
</tbody>
</table>

* Lymph node T cells from four to six BALB/cJ mice per group were analyzed as described in Materials and Methods.
restriction fragments of both MMTVsupF and MMTV (C3H)wt, the larger (by ~0.2 kb) LTR fragment of MMTV-supF was detected in the infected mother’s mammary gland DNA as well as in her offspring’s mammary gland and tumor DNAs (Fig. 4). The lack of smaller, wild-type LTR fragments in the offspring’s DNAs suggests that deletions did not occur at a significant level during passage via the milk. The minimum number of reverse transcription events required for the infection of the parent mouse and her offspring is three: one for the parent, if the injected virus directly and efficiently infects the mammary gland, and two for the sequential infection of her daughter’s lymphocytes and mammary cells. The number of replication cycles is probably greater, however, due to viral spread among lymphocytes and mammary epithelial cells in both animals. The inability to observe supF deletions in MMTV proviruses in either the parent or her offspring confirms that deletions did not occur at a detectable level and that the supF gene was stable in the MMTV LTR through multiple rounds of infection. Moreover, this result suggests that if deletions do occur, the resulting virus does not have a significant replication advantage over supF-containing viruses.

The lower intensity of the 1.3-kb signals in lanes 5 and 6 of Fig. 4 compared to the 1.1-kb signal in lanes 1 suggested that MMTVsupF-induced tumors may contain fewer proviral insertions than those induced by wild-type MMTV. However, when a larger number of tumors were tested by Southern blotting, we did not detect a significant difference in average insertion number between the two viruses (MMTVsupF average = 3.6 proviruses per tumor in 13 tumors; MMTV(C3H)wt average = 3.4 proviruses per tumor in 10 tumors).

The presence of a foreign sequence in the MMTV LTR could potentially affect the insertional activation of cellular

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genes, which for MMTV occurs primarily by an enhancer insertion mechanism. To look for evidence of such an effect, we tested 20 MMTVsupF-induced tumors for transcriptional activation of \textit{Wnt1} and \textit{Fgf3}, the two genes most commonly activated by MMTV insertion mutations. Northern blotting of tumor RNAs showed that \textit{Wnt1} was activated in 11 tumors (55%), \textit{Fgf3} was activated in 12 tumors (60%), and among these, both genes were activated in seven tumors (35%). These frequencies agree well with those observed previously for MMTV(C3H)\textsuperscript{f}l, which was found to activate \textit{Wnt1}, \textit{Fgf3}, or both at frequencies of 31 to 59%, 72 to 88%, or 28 to 50%, respectively (12). These data indicate that the presence of supF in the MMTV LTR does not significantly affect theinsertional activation of these genes.

**Facilitated cloning of MMTVsupF integration sites.** To demonstrate that the supF gene in the LTR of our modified provirus could facilitate the cloning of proviral insertion sites, we attempted to clone two such sites from a single tumor of an MMTVsupF-infected mouse. Southern blot analysis of this tumor using an MMTV LTR probe shows that it harbors three newly integrated proviruses whose SacI-digested proviral-host junction fragments are approximately 2.8, 2.4, and 1.4 kb in size (Fig. 5A). SacI-digested tumor DNA in the 2- to 3-kb size range was isolated from an agarose gel and ligated to SacI arms of \\textit{Agt}WES:AB, and the packaged phage were titered on a nonselective supF\textsuperscript{+} E. coli host (LE392). Approximately 5 \times 10\textsuperscript{9} PFU was then plated on a supF-deficient host (W3110) for the selection of phage containing the supF gene. Fifty plaques resulted, four of which were analyzed by restriction enzyme digestion and Southern blotting. Two of the four were found to contain the 2.4-kb junction fragment, and two contained the 2.8-kb fragment (data not shown). Finally, the cellular portions of these fragments were isolated and used sequentially as probes on the DNA blot used above (from Fig. 5A) after removal of the previous probe. These cellular DNA probes hybridized to the 2.8- and 2.4-kb junction fragments (Fig. 5B and C, respectively) in the tumor samples, as expected, as well as the normal cellular fragment from the unmutated paired chromosome present in both the tumor and control samples. Thus, the inclusion of the supF gene in MMTV functioned as designed to facilitate the cloning of proviral integration sites.

The modified MMTV provirus described here should significantly speed the labor-intensive process of cloning proviral insertion sites in MMTV-induced neoplasias. It allows for direct selection of MMTVsupF proviral-cellular junction fragments, thus obviating the need to screen for junction fragments using radioabeled probes. Furthermore, since the supF gene is located in bothLTRs of integrated proviruses, either junction fragment may be cloned with equal facility. Many lambda vectors contain amber mutations suitable for use with MMTVsupF, including vectors in the Charon and \textit{Agt} series, EMBL3a, as well as more modern vectors such as \textit{lambda}ZAP (Stratagene), which, after selection, can be induced to undergo in vivo excision resulting in an insert-containing plasmid (31, 34). Finally, supF may also be used as a selectable marker in plasmids, thus presenting the possibility of selectively cloning MMTVsupF-containing junction fragments directly from tumor DNAs into plasmids.

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