Replication-competent Moloney murine leukemia virus carrying a bacterial suppressor trNA gene: Selective cloning of proviral and flanking host sequences

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ABSTRACT A bacterial suppressor trNA gene was introduced into the long terminal repeat of the Moloney murine leukemia virus (Mo-MuLV) proviral genome to construct a retrovirus that allows easy cloning of the provirus with flanking host sequences. A replication competent virus, Mo-MuLV<sup>sup</sup> containing a trNA amber suppressor gene, was derived that replicates to high titers in tissue culture cells and stably transduces the bacterial gene. The recombinant virus can efficiently replicate in vivo when microinjected into midgestation embryos or when injected into newborn mice and it is shown that high levels of sequence, and it is not site specific. Furthermore, differential expression, of at least two distinct substrains of mice may be seen (12). This suggests that the same gene in different chromosomes may become activated differentially during development. Furthermore, retroviruses capable of inserting into many chromosomal regions are able to affect expression of cellular genes. This can result in the inactivation of adjacent genes by insertional mutagenesis (4–7) or in enhancement of transcription as seen in the activation of cellular oncogenes (8).

The general question of how a retrovirus selects its target site is far from being solved. Analyses of integration sites have not yet revealed any specificity on the level of the host DNA sequence, and it is possible that other and unknown levels of chromosomal organization influence the site of provirus insertion. Clearly, the elucidation of the mechanism of integration, of position dependent expression, and of insertional mutagenesis requires molecular cloning of the provirus together with flanking sequences. However, the recovery of proviral integration sites in the mouse is hampered by the extensive homology between the multiple murine endogenous and exogenous retroviruses, which complicates the screening in conventional cloning procedures. Recently, Mo-MuLV-based vector systems have been devised that make use of bacterial selectable markers and allow replication in vivo, thus allowing direct rescue of integrated viral copies in E. coli (9). In these systems the vector itself is replication defective and, thus, requires a replication-competent helper virus for its own propagation. This limits its use for in vivo experiments, where efficient virus replication and virus spread are needed to infect different animal tissues (10).

In this report we describe a replication competent Mo-MuLV vector that efficiently replicates in animals and can be directly selected in bacteria after packaging in phage λ. Furthermore, the use of proviral sequences as a bacterial vector assures high cloning efficiency and a high capacity to recover cellular sequences flanking the provirus.

MATERIALS AND METHODS

Plasmid Constructions, DNA Isolation, and Blotting. Restriction enzymes were purchased from Boehringer Mannheim, Bethesda Research Laboratories, and New England Biolabs and were used under recommended conditions. DNA fragments were purified on 0.5–2% agarose gels and Na-45 ion-exchange membranes (Schleicher & Schüll). Ligations were performed with T4 DNA ligase (Bethesda Research Laboratories) at DNA concentrations of 100–200 µg/ml. Linker reactions (linkers supplied by Boehringer Mannheim and P-L Biochemicals), transfection into E. coli HB 101 or DP-54, and plasmid growth were done by standard methods (11). Isolation of DNA from tissue culture cells and mouse organs. DNA blotting, and hybridization with [32P]DNA labeled by nick translation have been described (2, 12).

Transfection and Infection of Cells. Plasmids pMo<sup>sup</sup>-1, -2, -3, and -4 (see legend to Fig. 1) were digested with Cla I, and the proviral inserts were isolated and ligated. NIH 3T3 cells were transfected with 2 µg of ligated plasmid DNA and pSV2-gpt DNA (13) at a molar ratio of 1:3 and with 30 µg of carrier DNA as described (12). Two days after transfection the cells were trypsinated and plated into selective medium containing mycophenolic acid (13) and assayed for virus production by the XC plaque assay (12).

NIH 3T3 cells were infected with filtrated supernatant at a multiplicity of infection of 2–4. The embryonal carcinoma cell line PCC4 (14) was infected by cocultivation for 6 days with 100 µg/ml of carrier DNA and 3 µg/ml of mycophenolic acid (15) producing Mo-MuLV<sup>sup</sup>-1, which contains a trNA amber suppressor gene.

Selective Cloning of Integrated Mo-MuLV<sup>sup</sup>. Cloning in

Abbreviations: Mo-MuLV, Moloney murine leukemia virus; LTR, long terminal repeat; wt, wild type; kb, kilobase(s).
phage λ was done by described procedures (11, 16). Briefly, 5 µg of high molecular weight DNA of Mo-MuLVsup-1 and -3 infected NIH 3T3 cells was digested completely with EcoRI and ligated to purified phage λ Charon 4A arms at a molar ratio of 1:2. DNA was packaged in vitro, and the resulting phages were titrated on LE 392 (su'). Subsequently, suppressor-containing clones were selected on MC 1061 (su', a gift of H. Huang). Filter replicas of plaques were hybridized with nick-translated representative Moloney probe (2) or suppressor probe (pAN7, a gift of H. Huang).

Infection of Embryos and Newborn Mice. Embryos were microinjected at day 8.5 of gestation with 10^3 to 10^4 3T3 cells producing Mo-MuLVsup-1 as described (10, 17). Postnatal mice were injected intraperitoneally with 10^5 to 10^6 plaque-forming units of Mo-MuLVsup-1. Viremia was monitored by radioimmunoassay for p30 (18) when the animals were 4 to 6 wk old.

RESULTS

Construction of Mo-MuLVsup Plasmids and Test for Infectivity. To construct Mo-MuLV derivatives that retain the ability to replicate in their natural host cells, we introduced the selectable marker, a 200-base-pair (bp)-long synthetic tRNA gene, into the viral LTRs. This suppressor gene was excised from plasmid πvx (19) with EcoRI and ligated to BamHI or Xba I linkers, thereby removing the EcoRI sites. 3' long terminal repeat (LTR) subclones derived from the infectious provirus pMov-3 (16), extending from the Cla I site in env to the 3' end of the LTR, were used to insert the tRNA gene. The first two modified LTRs were derived by introduction of the suppressor tRNA gene, provided with Xba I ends, in either orientation into the Cla I site the 3' end of the enhancer sequences in the LTR (pLTRsup-4 in the same and pLTRsup-3 in the opposite transcriptional sense of the viral DNA). Other constructs were obtained by first converting the Sau3A site upstream of the enhancer with linkers into a BamHI site and then inserting the tRNA gene carrying terminal BamHI sites in either orientation into this site (pLTRsup-2 in the same and pLTRsup-1 in the opposite transcriptional sense) (Fig. 1). The next step was the substitution of the wild-type Mo-MuLV (Mo-MuLVwt) LTR with the four different modified LTRs in a plasmid containing a circularly permuted copy of an infectious Mo-MuLV provirus (see Fig. 1). The inserts of the resulting four circular permuted Mo-MuLVsup clones (pMoLVsup-1, pMoLVsup-2, pMoLVsup-3, pMoLVsup-4) were released by Cla I treatment, purified on agarose gels, and ligated in vitro to produce polymers (Fig. 1). In vitro ligated fragments were cotransfected with pSV2-gpt using NIH 3T3 cells as recipients. Mycophenolic acid-resistant cells were grown to confluency and tested for virus production by the XC plaque assay. All four constructs were shown to be infectious in this assay.

Production of Infectious Virus and Stability in Tissue Culture Cells. To test whether Mo-MuLVsup would be infectious for different cell types and whether the suppressor gene was stably transcribed in the Mo-MuLV genome throughout complete replication cycles of the virus, we infected NIH 3T3 and PCC4 cells. After several passages, high molecular DNA was isolated from the cells and subjected to Southern blot analysis. To distinguish between the LTRs of Mo-MuLVwt and Mo-MuLVsup, we performed a Kpn I digestion, which produces a 3' LTR fragment of 1.28 kilobases (kb) and 1.48 kb, respectively. Fig. 2 shows the presence of the 1.48-kb band in Mo-MuLVsup-1-infected 3T3 (Fig. 2A, lane b) and PCC4 (Fig. 2B, lane a) cells. An additional band, corresponding in size to the 1.28-kb wt LTR band (Fig. 2A, lane f) was seen in Mo-MuLVsup-2, -3, and -4-infected 3T3 cells.

As expected, the 1.48-kb Kpn I fragments reacted with the labeled suppressor probe, whereas the 1.28-kb fragments did not (Fig. 2A, lanes g-j; Fig. 2B, lane c), suggesting deletion of the suppressor gene. The Mo-MuLVsup-1 virus was stable in a subsequent round of virus infection, whereas the fraction of the deletion variant increased with each passage of the Mo-MuLVsup-3 virus stock (data not shown). This indicated that the deletion variant had a replication advantage over the suppressor-containing virus.

Mo-MuLVsup-1-infected 3T3 cells produced a titer of 10^8/ml of infectious virus, which is similar to the titer produced by cells chronically infected with Mo-MuLVwt. No virus production was observed in Mo-MuLVsup-1-infected PCC4 cells, indicating that the modified virus, like Mo-MuLVwt (15), cannot replicate in embryonal carcinoma cells.

Selective Cloning of Mo-MuLVsup Proviral Copies. To show that not only the suppressor sequence but also the desired function was retained in the viral genome, we performed two independent cloning experiments. From 3T3 cells infected with Mo-MuLVsup-1 or -3 (Fig. 2), DNA containing approximately five proviral copies per cell was cleaved with EcoRI, which does not cut Mo-MuLV proviral DNA. After ligation to phage λ Charon 4A arms carrying amber mutations and in vitro packaging into phage particles, sup' and su' E. coli cells were infected. Table 1 shows that, from a total of 10^8 to 10^9 phages plated (monitored on the su' host), 80 Mo-MuLVsup-1 and 120 Mo-MuLVsup-3 phages, respectively,
Fig. 2. Analysis of tissue culture cells infected with different Mo-MuLVsup constructs. (A) DNA from infected NIH 3T3 cells (15 µg each) was digested with Kpn I, run on a 1.5% agarose gel, and hybridized with a nick-translated pMU3 probe (a Rsa I–Rsa I clone including the U3 region of Mo-MuLVsuP, a gift of Ph. Soriano; lanes a–f) or with pAN7 (a plasmid carrying the suppressor gene, a gift of H. Huang; lanes g–j). DNA was from cells infected with Mo-MuLVsup-2 (lanes a and g), Mo-MuLVsup-1 (lanes b and h), Mo-MuLV1a (lanes c and i), Mo-MuLVsup–3 (lanes d and j); from uninfected 3T3 cells (lane e); and from Cl1-la, a cell line carrying Mo-MuLVsuP (ref. 12; lane f). (B) Same digest of DNA from PCC4 cells infected with Mo-MuLVsup-1 (lane a) and from uninfected cells (lane b) and probed with pMU3. The same DNA digest as in lane a but probed with pAN7 is shown in lane c. The diagram is a schematic representation of proviral 3'LTR restriction digests, and probes used for hybridization. Solid lines, viral DNA sequences; waved lines, host DNA sequences.

The animals grew under selective conditions on the su− host. These numbers indicate a high efficiency of recovery of the retroviral integration sites, considering the average size of clonable EcoRI fragments, the size of the murine genome, and the average number of proviral copies in the infected 3T3 cells. All of the selected plaques showed hybridization to a Mo-MuLV probe and to a suppressor probe (Table 1), indicating that the applied selection scheme is highly specific. Integrat ed complete Mo-MuLVsup genomes could be recovered since EcoRI, a noncutter for Mo-MuLV, was used for cloning.

Fig. 3 shows a Southern blot analysis of 10 phase clones that were digested with Sst I and hybridized with a representative Moloney probe. A 5.8-kb and a 2.6-kb fragment, which are characteristic for the Mo-MuLVsup proviral genome (Fig. 3, lane k), were seen in four clones, whereas the other six clones contained rearranged Mo-MuLV genomes. Two of the nonarranged clones were transferred

Table 1. Selective cloning of integrated Mo-MuLVsup proviral genomes

<table>
<thead>
<tr>
<th>DNA from NIH 3T3 cells infected with</th>
<th>Total no. of</th>
<th>Phases growing on su− host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo-MuLVsup–1</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>Mo-MuLVsup–3</td>
<td>1.8</td>
<td>120</td>
</tr>
</tbody>
</table>

DNA from NIH 3T3 cells infected with Mo-MuLVsup-1 or -3 was digested with EcoRI, ligated to phase λ Charon 4A arms, packaged in vitro, and titrated on LE 392 (su− host) (11). Whole packaging reactions were plated on MC 1061 (su−), and filter replicas of the plaques were hybridized with a nick-translated representative Mo-MuLV probe and with the suppressor gene containing plasmid pAN7.

Table 2. Induction of viremia in mice infected as embryos or newborns with Mo-MuLVsup–1

<table>
<thead>
<tr>
<th>Time of infection</th>
<th>Mouse strain</th>
<th>Viremic animals/total injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 8.5 of gestation</td>
<td>C57BL</td>
<td>7/8</td>
</tr>
<tr>
<td>Days 3–5 after birth</td>
<td>C57BL</td>
<td>7/9</td>
</tr>
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</table>

Embryos were injected at day 8.5 of gestation with Mo-MuLVsuP 1 virus-producing cells (17), and postnatal mice were infected intraperitoneally with virus. The animals were tested for viremia (18) when 4–6 wk old.
the observed instability of the recombinant virus is not specific for certain organs or the developmental stage at which the animals were infected. Its appearance and extent seem to be variable from animal to animal.

In summary, these results show that Mo-MuLVsup,1 closely resembles Mo-MuLVwt in its replication properties in vivo (10). All fetal tissues become infected efficiently, whereas virus replication is restricted to spleen and thymus when the mice are infected after birth. The suppressor tRNA gene is lost from the virus with a low frequency when replicating in animals but appears to be stable when the virus replicates in tissue culture cells (Fig. 2).

**DISCUSSION**

Molecular cloning of integrated Mo-MuLV as a means to study biological properties of the virus and characterize cellular integration sites has been a laborious procedure. Cross-reaction of available Mo-MuLV probes to endogenous viruses and the need for multiple rounds of recombinant phage purification have hampered the rapid and efficient recovery of clones.

We have circumvented these problems by introducing an amber suppressor tRNA gene as a selectable marker into the LTRs of Mo-MuLV. Four constructs were obtained having the tRNA gene incorporated in either orientation 5' or 3' of the enhancer sequences in the Moloney LTR. After transfection into NIH 3T3 cells, these constructs produced infectious virus. The proviral copies could be recovered from infected cells by molecular cloning in λ phages by using the tRNA gene as a selectable marker in E. coli. The selection system was very efficient, as demonstrated by the high number of clones obtained per genome equivalent, and was highly specific because all selected clones contained Mo-MuLV proviral sequences. Because of the size requirements for packaging in phage particles, between 1 and 11 kb of flanking sequences are recovered with each provirus. Thus, genomic flanking sequences can be cloned with higher efficiency as compared to plasmid-derived “shuttle” vectors constructed for this purpose (9). In our experiments using a bacterial strain with a high recombination frequency, a considerable fraction of the recombinant phages contained rearranged Mo-MuLVsup sequences. It is likely that rearrangements can be minimized by using a recombination-deficient su- host for the suppressor selection.

All four Mo-MuLVsup viruses were able to replicate in NIH 3T3 cells. However, only one construct, Mo-MuLVsup,1, was stable during three cycles of infection and produced a virus titer comparable to Mo-MuLVwt. The other Mo-MuLVsup viruses lost the suppressor gene at different frequencies.

When injected into midgestation embryos or newborn mice, Mo-MuLVsup,1 induced viremia efficiently. Analyses of the tissue distribution of the proviral sequences revealed that Mo-MuLVsup,1 is able to replicate in all fetal tissues when introduced at midgestation and in spleen and thymus when injected postnatally. Thus, Mo-MuLVsup retains the characteristic tissue tropism of Mo-MuLVwt (10). At present we do not know whether viremic animals develop neoplastic disease at later stages.

Although no instability of Mo-MuLVsup,1 was detected in tissue culture, the suppressor gene was deleted at a low frequency in animals. The apparently different stability of the four Mo-MuLVsup constructs seems to depend on the orientation and location of the suppressor gene in the LTR. Remarkably, the two least stable constructs, Mo-MuLVsup,2 and -4, contain the suppressor gene in the same transcriptional orientation as the virus [the noncoding (+)-strand is part of the viral RNA], suggesting a role of RNA secondary
structure in the deletion process. Another possible mechanism could involve homologous recombination across the linker sequences within the LTR. This explanation is supported by the finding that, within the limits of resolution, the deletion products are very similar in size to the Mo-MuLV™ genome. Once the deletion variant has formed, it appears to outgrow the Mo-MuLV™ virus due to a replication advantage.

The described Mo-MuLV™sup-1 virus represents a powerful tool for studying parameters in virus–host chromosome interactions. These interactions may include the recognition of a specific sequence, as in the case of a transposable element in Drosophila that codes for a protein with homology to reverse transcriptase (21). Moreover, a change of chromatin conformation after retrovirus insertion close to a hypersensitive site has been reported recently (22). To test the general significance of this finding, it will be necessary to clone a number of different integration sites in order to analyze their chromatin organization. Finally, this virus will be useful in studies on developmentally regulated, cell type-specific interactions of retroviruses in animals as well as in retroviral mutagenesis experiments (4, 6) by facilitating the cloning of the flanking sequences.

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