Replication of enhancer-deficient amphotropic murine leukemia virus in human cells

Frank U. Reuss†, Bianca Berdel, Martin Ploss, and Ricarda Heber

Deutsches Krebsforschungszentrum, Angewandte Tumorvirologie F0400, 69120 Heidelberg, Germany

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Amphotropic murine leukemia virus (MLV) replicates in cells from various mammalian species, including humans, and is a potential contaminant in MLV vector preparations for human gene transfer studies. The generation of replication-competent virus is considered less likely with vectors that delete the viral transcription elements. This conclusion is based on data obtained in rodents, where MLV replication depends on the expression of viral genes under the control of 75-bp enhancer elements in the long terminal repeat. We demonstrate here that in some human cells replication of amphotropic MLV is possible in the absence of these enhancer elements. Replication of the enhancer-deficient virus MLV-(MOA)ΔE is observed in selected human sarcoma and B lymphoma lines and proceeds at a lower rate than that of the intact virus. No insertion of a foreign promoter or enhancer into the long terminal repeat was detected. Our data suggest the presence of a secondary enhancer element within the MLV provirus that can in selected human cells mediate virus transcription and replication in the absence of the 75-bp U3 enhancers.

Amphotropic murine leukemia virus (MLV) originally was isolated from feral mice and found to replicate in cells from various mammalian species including humans (1). The *env* gene of this isolate (4070A) determines the host range of the virus (2) and was subsequently used for transduction of primate cells with the newly developed MLV vectors (3, 4). Most retroviral vectors in use for experimental and clinical gene therapy have been derived from the Moloney strain of MLV (Mo-MLV) (5). Human gene transfer studies frequently use MLV-based vectors with amphotropic host range to introduce and express marker or therapeutic genes (5). Recombinant, replication-competent amphotropic MLV is a potential contaminant and safety risk in these vector preparations (6, 7). However, only very limited information is available about the replication and gene expression of amphotropic MLV in human cells (8).

For both replication-competent MLV and most MLV-based, replication-deficient vectors, gene expression requires transcription from the MLV long terminal repeat (LTR). Mo-MLV transcription and gene expression is observed in a wide range of cell types. Mo-MLV transcription in murine cells is determined by a promoter and two copies of a 75-bp transcriptional enhancer element in the U3 region of the viral LTR (9). Cellular transcription factors were characterized that interact with the 75-bp repeats of Mo-MLV (10). LTRs with a deletion of both enhancers show a drastically reduced promoter activity ($\leq$0.1%) in reporter gene assays (11). As a consequence, Mo-MLV mutants that lack both 75-bp enhancer elements are replication-deficient in murine 3T3 fibroblasts (11). It is widely assumed that MLV transcription and gene expression in human cells is regulated by the same mechanisms as in rodent cells. Reduced transcription of self-inactivating MLV vectors, which lack U3 promoter and enhancers, suggest a similar function of these elements in human cells (12). However, detailed studies of LTR-controlled transcription in human cells are restricted to the Jurkat T cell line (13). None of the studies address the transcription mechanisms that regulate the replication of amphotropic MLV in human cells. We have previously analyzed replication of infectious, amphotropic MLV in human mammary epithelial cells (F.U.R., R.H. & B.B., unpublished work). In this study we have extended these experiments to transcriptional regulation and replication in human sarcoma and lymphoma lines.

Materials and Methods

Recombinant Viral Constructs, Cell Lines, and Transfection Procedure. Plasmid pMLV-(MOA) contains an MLV provirus with amphotropic host range cloned into *Nhe*I and *Eco*RI sites of the plasmid pGEM-3 (Promega). The provirus MLV-(MOA) was assembled from a *Xba*I/*Spe*I fragment from pHIT110 (14) containing a Moloney-murine sarcoma virus (MSV) 5′ LTR with the U3 region replaced by the cytomegalovirus (CMV) immediate early promoter/enhancer unit, a *Spe*I/*Sal*I fragment from p63–2 (15) with *gag* and the 5′ part of the *pol* gene, a *Sal*I/*Nhe*I fragment with the 3′ portion of the *pol* gene and the amphotropic *env* gene from pAMS (2), and a PCR-generated *Nhe*I/*Eco*RI fragment with the MLV 3′ LTR of p63–2. In plasmid pMLV-(MOA)ΔE the 3′ LTR of pMLV-(MOA) was replaced with the 3′ LTR from pMLV/CRBΔMo (15). We have used the murine fibroblast line NIH/3T3, the human fibrosarcoma line HT-1080 (ATCC CCL121), the human breast carcinoma-derived lines MCF-7 and MDA-MB-435S (supplied by Deutsches Krebsforschungszentrum Tumor Bank, Heidelberg), human B lymphoma lines Ramos and MHH-PREB-1 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), and the human kidney line 293. HT-1080, MCF-7, MDA-MB-435S, 293 and NIH/3T3 cells were cultured in DMEM supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Ramos and MHH-PREB-1 cells were grown in RPMI medium containing the same supplements. For transfection between $1 \times$ and 2×10^5 cells were plated in a 25-cm^2 culture flask and grown for 24 h before transfection. Recombinant viral plasmids were introduced into cells by using Lipofectamine (GIBCO/BRL) and Optimem medium (GIBCO/BRL). After exposure of cells to the Lipofectamine/DNA mixture for 4 h at 37°C the medium was removed and fresh medium was added. When the cultures reached confluency the medium was harvested and the cells were diluted into fresh medium. MLV replication was monitored with a RNA-dependent DNA-polymerase (RT) assay.

Virus Production, Infection of Cells, and RT Assay. Virus was produced in 293 cells transfected with 2 μg of the proviral expression plasmids. Target cells (2×10^5) for infection were plated in a 25-cm^2 culture flask and grown for 24 h. Virus-containing medium was filtered through a 0.45-μ Millex filter unit (Millipore), and serial dilutions of this medium were tested for RT activity. RT activities were determined from the linear range of

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Abbreviations: MLV, murine leukemia virus; Mo-MLV, Moloney strain of MLV; LTR, long terminal repeat; MSV, murine sarcoma virus; CMV, cytomegalovirus; RCMV, replication CMV; RT, RNA-dependent DNA polymerase.

†To whom reprint requests should be addressed. E-mail: f.reuss@dkfz.de.

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the dilution curve. For infection the medium was replaced by 2 ml of fresh medium, and virus-containing medium equivalent to 10^5 cpm of RT activity was added. After 4 h of incubation the medium was removed and replaced by fresh medium. Cell culture supernatants were harvested, and cellular debris was removed by centrifugation at 5,400 rpm (5,000 × *g*) for 10 min at 4°C in a Heraeus Megafuge1.0R (Rotor BS4402/A) and stored at −70°C. Virions from 1-ml aliquots were pelleted by centrifugation at 75,000 rpm for 10 min at 4°C with a TLA-100.3 rotor in a TL-100 ultracentrifuge (Beckman Instruments). The pellet was resuspended in 20 μl RT buffer [50 mM Tris·HCl, pH 8.3/125 mM NaCl/20 mM DTT/0.58 mM $MnCl_2$/0.05% Nonidet P-40/10 μM desoxyribosylthymine 5′-triphosphate (TTP)/0.1 mCi/ml α-[^{35}S]TTP or α-[^{33}P]TTP (NEN)/10 μg/ml poly(rA)/5 μg/ml oligo($dT_{12–18}$) and incubated for 60 min at 37°C. The reaction was terminated by addition 200 μl stop solution (15 mM NaCl/100 μg/ml BSA/0.1% tetrasodiumpyrophosphate) and 25 μl of 60% trichloroacetic acid (TCA), kept on ice for 15 min, and filtered through HAWP02500 membranes (Millipore). Filters were successively washed with 6% TCA and 70% ethanol and dried at 80°C for 30 min. The TTP incorporation was determined in a 1450 MicroBeta Trilux liquid scintillation counter (Wallac Oy, Turku, Finland) for 1 min. Conditioned medium from Abelson MLV-infected 2 M3/M cells was used as positive control (16).

Extraction of Viral RNA, RT-PCR Analysis, and DNA Sequence Determination. For isolation of viral RNA cell culture supernatants were cleared and virions were pelleted by ultracentrifugation as described above. The virus-containing pellet was resuspended in 1 ml of buffer (0.15 M NaCl/20 mM Tris·HCl, pH 7.5/5 mM EDTA/100 μg yeast RNA/ml). After addition of pronase and SDS to a final concentration of 250 μg/ml and 1%, respectively, the sample was incubated at 37°C for 30 min, extracted with phenol, and precipitated with ethanol. The RNA was reverse-transcribed by using Superscript MLV reverse transcriptase (Life Science, Arlington Heights, IL/BRL). The cDNA was amplified by using recombinant *Pwo*-DNA polymerase (Peqlab, Erlangen, Germany) and primers FS1 (5′-TGG CAA GCT AGC TTA AGT-3′) and FS3 (5′-AAG CTT GCG GCC GCT GCA ACT GCA AGA GGG TT-3′) for the Mo-MLV R region or HR2 (AAG CTT GCG GCC GCT GCA AAC AGC AAG AGG CTT) for the Mo-MSV R region. The PCR products were separated on a 5% nondenaturing polyacrylamide gel and silver stained as described (17).

Isolation of Genomic DNA, Southern Blot Analysis, and Provirus Mapping. Cells were lysed in lysis buffer (50 mM Tris·HCl, pH 8.0/10 mM EDTA/0.5% SDS), and incubated in the presence of 20 μg/ml RNase A at 37°C for 1 h. The lysate was extracted with phenol and chloroform/isoamylalcohol (24:1), ethanol-precipitated, and resuspended in Tris/EDTA. For Southern blot analysis, 12 μg DNA was digested with a restriction enzyme, run on a 0.8% agarose gel, and transferred to a Hybond-N (Amersham Pharmacia) membrane. Radiolabeled probes were generated from isolated DNA fragments by random hexamer oligonucleotide-primed synthesis with Klenow DNA polymerase in the presence of [^{32}P]dCTP. Probe 1 corresponds to a 1.8-kb *Hin*dIII fragment from position 452 in the 4070A integrase coding region (18) to position 1120 in the 4070A *env* gene (19). Probe 2 represents a *Bam*HI/*Cla*I fragment from positions 1219 to 1901 within the *env* gene (19). The CMV promoter probe was isolated from plasmid pHIT110 as a 513-bp *Xba*I/*Sac*I fragment (positions 1–513) (14). Hybridization was performed as described (20). For provirus amplification we used the Expand long template PCR kit (Roche Diagnostics) with primers FX1 (ACA GAA CCC GGG TAC CCG TAT TCC CAA T) and FX2 (ACT AGC CCC GGG CGA CGC AGT CTA TCG) representing positions 28–49 and 13–33 of the MSV R region.

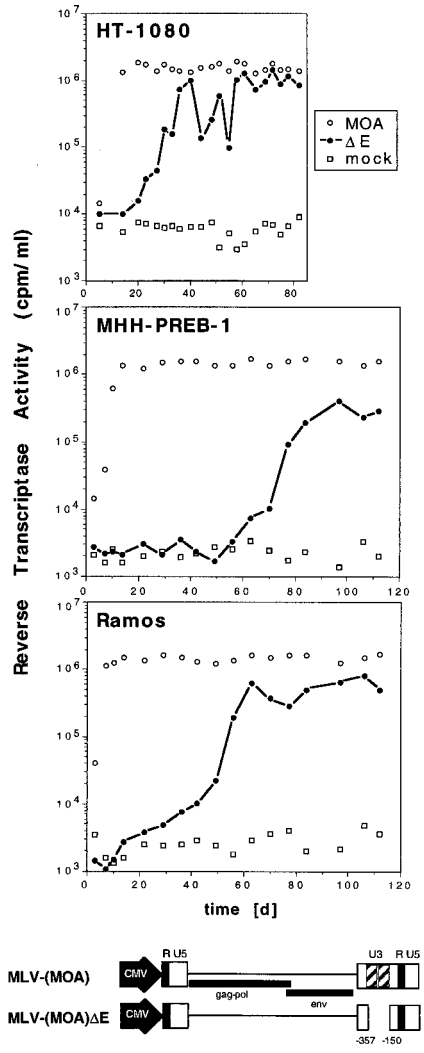

Fig. 1. Replication of amphotropic MLV in human sarcoma and lymphomas. Cell lines infected by either MLV-(MOA) (○) or MLV-(MOA)ΔE (●) or mock-infected (□) were passaged for the indicated time periods, and the RT activity in the supernatants was determined. One representative experiment is shown for each cell line.

Results

Replication of Enhancer-Deficient Amphotropic MLV in Human Cells. Information about the replication of amphotropic MLV in human cells is very limited. In murine cells replication of MLV is determined by transcriptional enhancer elements within the viral LTR (11). To characterize the effect of these enhancers on virus transcription and replication in human cells we have previously generated the amphotropic host range variant MLV-(MOA) of Mo-MLV (F.U.R., R.H. & B.B., unpublished work) (Fig. 1). The corresponding enhancer-deficient mutant MLV-(MOA)ΔE is characterized by a deletion that extends from position −357 to −150 in the U3 region and removes both enhancer copies at positions −341 to −257 and −266 to −182 (11). The initial transcription of viral RNAs in both MLV-(MOA) and MLV-(MOA)ΔE is under the control of the CMV immediate early promoter to exclude recombination between wild-type and mutant U3 regions. We have previously analyzed

MICROBIOLOGY

replication of MLV-(MOA) in human breast carcinoma cell lines (F.U.R., R.H. & B.B., unpublished work) and found that these cells were nonpermissive for MLV-(MOA)ΔE. However, in two of these lines a replication-competent virus MLV-(RCMV) was generated through spontaneous insertion of the CMV promoter into the MLV U3 region.

Here we extend our earlier studies on replication of the amphotropic MLV-(MOA) and the enhancer-deficient MLV-(MOA)ΔE to human sarcoma and lymphoma lines. Infectious virus was produced either by direct transfection of proviral expression plasmids into the target cells (for sarcomas) or separately by transfection of 293 human kidney cells followed by infection with cell-free virus (for lymphomas). After infection of target cells either the wild-type or mutant U3 region is found in both LTRs of the provirus, and additional rounds of virus replication require transcription of these proviruses. Replication-competent virus spreads in the culture until all susceptible cells are infected and superinfection resistance is established. Cells were maintained for a period of up to 113 days to allow virus spread. When cultures reached confluence, cells were diluted into fresh medium. Medium from confluent cultures was used to determine virus-associated RT activity as a measure of virus replication.

In these experiments, we observed that three of the cell lines were permissive for virus arising from both the intact virus MLV-(MOA) and the enhancer-deficient mutant MLV-(MOA)ΔE (Fig. 1). In the human fibrosarcoma line HT-1080 RT activity above mock control level was detectable after 5 days for MLV-(MOA) and after 20 days for MLV-(MOA)ΔE. Direct infection of HT-1080 cells yields identical results (see Fig. 6). Similarly, in the B lymphoma lines Ramos and MHH-PREB-1 virus replication was detectable after 3 days for MLV-(MOA) and after 22 and 63 days for MLV-(MOA)ΔE (Fig. 1). Replication of MLV-(MOA)ΔE in all three lines occurred at a rate that was slower then that of the intact virus MLV-(MOA). A delay in replication, as previously observed for the MLV-(RCMV) virus (F.U.R., R.H. & B.B., unpublished work), was observed in MHH-PREB-1 cells, but not in HT-1080 and Ramos. The RT activity in the medium of cells infected with the intact virus reached levels that were at least 2 orders of magnitude higher than in the corresponding mock control samples. RT values above 2×10^6 cpm/ml were not resolved in this experiment. Maximal RT values for the enhancer-deficient MLV were lower than for the intact virus. As determined in serial dilution experiments, expression of the enhancer-deficient virus in HT-1080, Ramos, and MHH-PREB-1 cells reached 20%, 7%, and 30% of the respective wild-type levels. FACS analysis of infected MHH-PREB-1 cells indicates that 30–40% of the population expresses the viral gp70 antigen for both virus types. Levels of MLV-(MOA)ΔE expression are reduced (data not shown).

We previously have found that the defect in gene expression and replication in the enhancer-deficient virus mutant MLV-(MOA)ΔE can be restored by spontaneous integration of a CMV promoter into the viral LTR to generate MLV-(RCMV). To determine the structure of MLV proviruses in HT-1080, Ramos, and MHH-PREB-1 cells, we isolated the genomic DNA of uninfected, MLV-(MOA)-infected, and MLV-(MOA)ΔE-infected cells after 82 days (HT-1080) and 112 days (Ramos, MHH-PREB-1) in culture and characterized the proviruses in a Southern blot experiment (Fig. 2). DNA isolated from HT-1080 cells was directly compared with DNA from MLV-(RCMV)-infected MCF-7 and ZR-75–1 cells and the proviral plasmids. To confirm the presence of MLV in infected lines, *Hin*dIII-digested DNA was hybridized to MLV probe 1, representing the *pol* and *env* genes of the virus (Fig. 2*A*). We found that for the individual cell lines, the cultures infected with the intact MLV-(MOA) and the enhancer-deficient MLV-(MOA)ΔE contained comparable numbers of proviruses. The number of proviral copies per

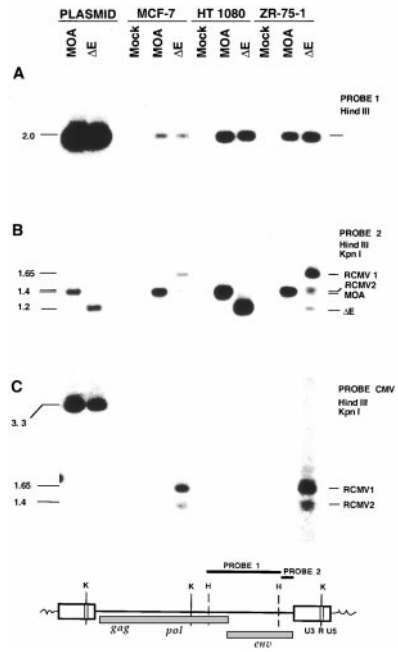

Fig. 2. Southern blot analysis of infected HT-1080 cells. Proviral expression plasmids (Plasmid), pMLV-(MOA) (MOA), and pMLV-(MOA)ΔE (ΔE) were compared with DNA from mock-infected (Mock), MLV-(MOA)-infected (MOA), or MLV-(MOA)ΔE-infected (ΔE) MCF-7, HT-1080, and ZR-75–1 cells. DNA was either *Hin*dIII-digested and analyzed with probe 1 (*A*), or *Hin*dIII and *Kpn*I-digested and tested with either probe 2 (*B*) or a CMV promoter probe (*C*). A provirus restriction map is shown below the blots.

microgram DNA was lower in MCF-7 than in the two other lines. The size of the *Hin*dIII fragment (2.0 kb) was identical for MLV-(MOA), MLV-(MOA)ΔE, and the proviral plasmids, excluding major rearrangements in this part of the provirus. No MLV was detected in the parental lines.

Compensatory changes in the MLV-(MOA)ΔE viral LTRs such as insertion or duplication are likely to increase the size of the U3-R region. To determine the size of U3-R in proviral 3′ LTRs, the genomic DNAs described above were digested with *Hin*dIII and *Kpn*I, hybridized to MLV probe 2 representing the 3′ portion of the *env* gene, and compared with the proviral expression plasmids (Fig. 2*B*). The parental proviral plasmids pMLV-(MOA) and pMLV-(MOA)ΔE yielded 1.4-kb and 1.2-kb fragments, respectively. The size difference reflects the 207-bp deletion that removes the two 75-bp enhancer elements from the LTR in MLV-(MOA)ΔE. All three cell lines infected with the intact MLV-(MOA) contained a 1.4-kb fragment characteristic for the intact virus. In HT-1080 cells infected with MLV-(MOA)ΔE the proviral U3 region is identical in size to the U3 region of the corresponding plasmid clone. U3 regions with the size of the intact MLV-(MOA) or MLV-(RCMV) were not detected in HT-1080 cells. As a control, MCF-7 and ZR-75–1 cells infected with either MLV-(MOA) or the CMV-recombinant MLV-(RCMV) including the previously characterized subpopulations RCMV1 and RCMV2 are shown on the same blot. In these cells only a minor proportion of the proviruses maintained the structure of the parental MLV-(MOA)ΔE provirus.

To directly test for potential CMV promoter integrations in the U3 region and in other parts of the provirus, we used a CMV promoter probe for the hybridization to *Hin*dIII/*Kpn*I-digested DNA (Fig. 2*C*). The proviral expression plasmids pMLV-(MOA) and pMLV-(MOA)ΔE digested with the same enzymes served as control and generated a 3.3-kb CMV promoter-

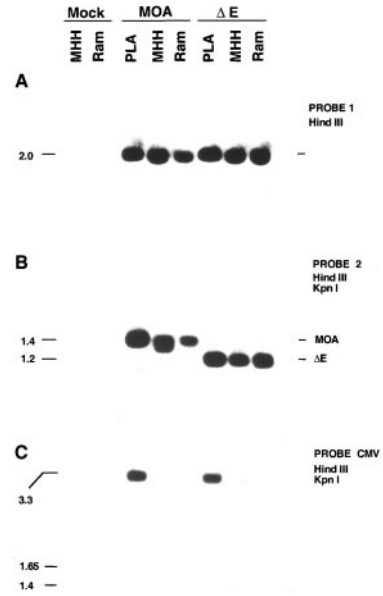

Fig. 3. Southern blot analysis of infected lymphoma cells. DNA from mock-infected (Mock), MLV-(MOA)-infected (MOA), or MLV-(MOA)ΔE-infected (ΔE) MHH-PREB-1 (MHH) and Ramos cells (Ram) was compared with the corresponding proviral expression plasmids (PLA). DNA was either *Hin*dIII-digested and analyzed with probe 1 (*A*), or *Hin*dIII- and *Kpn*I-digested and tested with either probe 2 (*B*) or a CMV promoter probe (*C*). For provirus restriction maps and probes see Fig. 2.

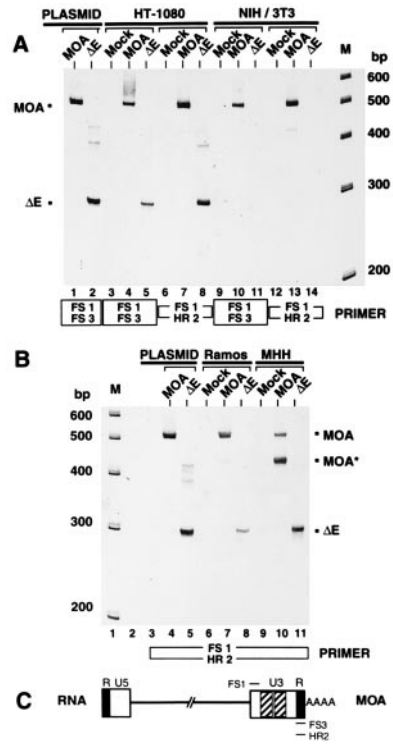

Fig. 4. RT-PCR analysis of viral RNA. Virus RNA isolated from HT-1080 and NIH/3T3 cells (*A*) or Ramos and MHH-PREB-1 cells (MHH) (*B*) that are either mock- (Mock), MLV-(MOA)- (MOA), or MLV-(MOA)ΔE-infected (ΔE) was used for RT-PCR amplification of the U3-R region (see *C*) with primer sets FS1/FS3 and FS1/HR2. Products were separated on a polyacrylamide gel and silver-stained.

positive fragment. None of the tested cellular DNAs contained detectable amounts of the proviral plasmids used for transfection, excluding amplification of the plasmid inside the cell. CMV promoter DNA was detected in MCF-7 and ZR-75–1 cells transfected with the enhancer-deficient provirus plasmid pMLV-(MOA)ΔE but not in mock- or pMLV-(MOA) transfected cells. As reported before (F.U.R., R.H. & B.B., unpublished work), the CMV promoter was present in 1.65-kb and 1.4-kb fragments in both cell lines. No CMV promoter containing fragment was detected in HT-1080 cells. Southern blot analysis of MLV proviruses in Ramos and MHH-PREB-1 human lymphomas produced identical results (Fig. 3). In addition, in MLV-(MOA)-infected MHH-PREB1, but not in Ramos cells, a 1.3-kb U3 fragment was found (Fig. 3*B*) that was as abundant as the 1.4-kb intact U3 region and may represent either a spontaneous deletion variant or a restriction site polymorphism. These data demonstrate that replication of the amphotropic virus MLV-(MOA) in HT-1080 sarcoma, Ramos, and MHH-PREB-1 lymphoma cells is possible in the absence of the 75-bp MLV enhancer elements. The replication of enhancer-deficient MLV-(MOA)ΔE in these cells does not depend on the insertion of a CMV promoter or other transcription elements into the viral U3 region.

Primary Structure of the MLV U3 Region Isolated from Viral RNA and Restriction Analysis of Amplified Proviruses. The replication of enhancer-deficient virus in the absence of major rearrangements in the U3 region raises the question whether point mutations may complement this defect. To precisely determine the structure of the U3 region in MLV-(MOA)ΔE viruses replicating in HT-1080, Ramos, and MHH-PREB-1 cells, we isolated virus RNA from the culture supernatants of these cells. The cDNA generated from these RNAs was used in a PCR to amplify the viral U3-R region. Because of slightly divergent R regions at the 5′ and 3′ terminus of the initial viral transcript—the R/U5 region is from Mo-MSV and the U3/R region from Mo-MLV (see *Materials and Methods*)—we used either primer FS3 (for the MLV R region) or HR2 (for the MSV R region) in combination with the U3 primer FS1 (Fig. 4*C*). The corresponding region from the proviral expression plasmids was amplified as control. The PCR products were resolved on a polyacrylamide gel to detect even small size differences between the U3 regions from the transfected plasmid and the resulting virus (Fig. 4). The DNA was visualized by silver staining.

We found that virus from MLV-(MOA)-infected HT-1080 cells contained a 492-bp U3-R fragment characteristic for the intact MLV-(MOA) (Fig. 4*A*, lanes 4 and 7). In virions from MLV-(MOA)ΔE-infected HT-1080 cells (Fig. 4*A*, lanes 5 and 8) the size of the U3 region was identical to the enhancer-deficient plasmid control. The control cell line NIH/3T3 is permissive for MLV-(MOA), but nonpermissive for MLV-(MOA)ΔE (11) (F.U.R., R.H. & B.B., unpublished work). This result is confirmed by the presence of virions with an intact U3 region in the medium of MLV-(MOA)-infected cells, and the lack of viral RNA in the medium of MLV-(MOA)ΔE-infected NIH/3T3 cells (Fig. 4*A*, lanes 9–14). No viral RNA was detected in mock-infected cultures. As in HT-1080 cells, enhancer-deficient virus grown in the lymphoma lines Ramos and MHH-PREB-1 maintained the original enhancer deletion (Fig. 4*B*, lanes 5, 8, and 11). In support of our Southern blot data in Fig. 3*B*, we detected a virus population (MOA*) with a U3 region that is reduced in size by ≈70 bp in MLV-(MOA)-infected MHH-PREB-1, but not in Ramos cells. The PCR products obtained were cloned into a plasmid vector, and at least two independent plasmids representing each product were selected for sequence analysis. For HT-1080-derived virus we found that the MLV enhancers are absent from the U3 region of MLV-(MOA)ΔE (Fig. 7, which is published as supporting information on the PNAS web site, www.pnas.org). Individual clones differed in sequence from the

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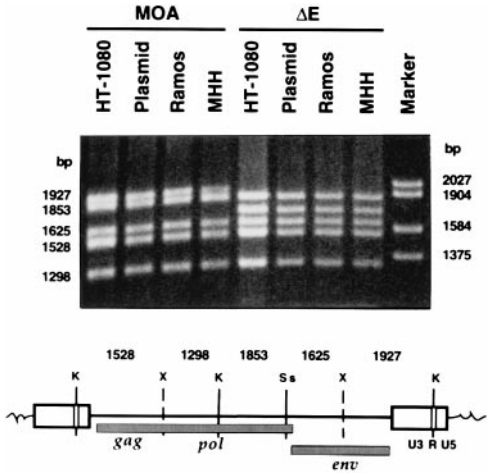

Fig. 5. Restriction analysis of PCR-amplified amphotropic MLV provirus. Genome-size provirus fragments from R to R region were PCR-amplified, digested with *Kpn*I (K), *Xho*I (X), and *Ssp*I (Ss) and separated on a 0.8% agarose gel. Template DNA was genomic DNA from HT-1080, Ramos, and MHH-PREB-1 cells (MHH) infected with either MLV-(MOA) or MLV-(MOA)ΔE (MOA, ΔE). The corresponding viral expression plasmids (PLASMID) were used as control templates. A provirus restriction map is shown below the gel.

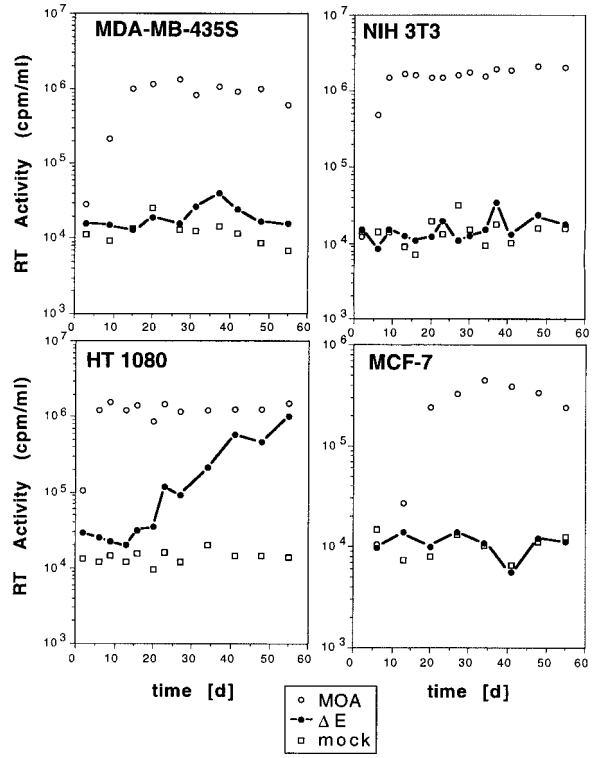

Fig. 6. Infection studies with intact and enhancer-deficient amphotropic MLV. MLV-(MOA)- or MLV-(MOA)ΔE-containing media from infected HT-1080 cells were harvested and filtered, and the RT activities were determined. Virus-containing medium equivalent to 10^5 cpm of RT activity were used for infection of MDA-MB-435S, NIH 3T3, HT-1080, and MCF-7 cells. Cells were passaged for 60 days, and RT activity was determined.

intact virus by point mutations, but none of these mutations was consistently present in all of the clones. The same is true for MLV-(MOA)ΔE virus produced from lymphoma cells (Fig. 8, which is published as supporting information). A cytosin residue at the site of enhancer deletion (position 68) is present in the original (MOA)ΔE provirus. The MLV population (MOA)* in MHH-PREB-1 cells was identified as a spontaneous deletion mutant that has lost one copy of the 75-bp U3 enhancer element. These data demonstrate that the replication of MLV-(MOA)ΔE does not require compensatory mutations within the U3 region.

To identify potential insertions and rearrangements in MLV regions other than the U3 region we used a long-range PCR assay to amplify genome-sized parts of provirus from the 5′ R region to the 3′ R region. Restriction analysis was performed such that the amplified provirus was cut into five fragments between 2.0 and 1.3 kb that were analyzed for length polymorphisms and differences in abundance relative to the corresponding amplified provirus clone (Fig. 5). We found for both intact and enhancer-deficient MLV in HT-1080 cells decreased amounts of the 1,625-bp *env* gene fragment and increased amounts of the 1,528-bp fragment. This may result from a restriction polymorphism within the *env* gene in part of the virus population. These changes, however, are not related to the enhancer defect, but represent an adaptation to replication in HT-1080 cells. The reduced amount of the 1,927-bp *env*/U3-fragment in MHH-PREB-1 cells infected by the intact virus with increased amounts of the 1,853-bp fragment confirms the sequence data about the MOA* single enhancer population in these cells. No changes were detected for MLV-(MOA)ΔE proviruses in Ramos and MHH-PREB-1 lymphoma cells.

Transmission and Cell Type Specificity of Enhancer-Deficient MLV. To determine the biological characteristics of the enhancerless virus MLV-(MOA)ΔE we compared the replication competence and rate of MLV(MOA) and MLV-(MOA)ΔE in selected cell lines. MLV-(MOA)- or (MOA)ΔE-containing medium from chronically infected HT-1080 cells was used for infection. Equivalent amounts of virus, as calculated from the RT activity in the medium samples, were used to infect NIH/3T3, MDA-MB-435S, HT-1080, MCF-7, Ramos, and MHH-PREB-1 cells. The infected cells were maintained for a period of 55 days, and RT activity in the medium was determined as a measure of virus replication (Fig. 6). MLV-(MOA) replicated in all of the cell lines tested. By contrast, we found that MLV-(MOA)ΔE replicated in HT-1080 cells, but not in NIH/3T3, MDA-MB-435S, and MCF-7 cells. The replication kinetic of this virus in HT-1080 cells was very similar to the initial culture (Fig. 1): Virus was detectable 16 days after infection with a 14-day delay relative to MLV-(MOA). The replication rate was reduced relative to the intact virus and similar in both experiments. These data suggest that no adaptive genetic changes in the genome of MLV-(MOA)ΔE are required to compensate for the missing 75-bp MLV enhancers. For (MOA)ΔE virus harvested from HT-1080 cells replication also was observed in Ramos and MHH-PREB-1 cells (data not shown). We conclude that MLV-(MOA)ΔE is an infectious virus that can be transmitted via cell-free media. Replication of MLV-(MOA)ΔE is restricted to specific cell lines.

Discussion

Amphotropic MLV is a potential contaminant and risk factor in preparations of MLV-based vectors for human gene transfer. Such replication-competent viruses can principally be formed at all stages of vector production by recombination between vector and packaging genes. Exposure of immuno-suppressed rhesus monkeys to replication-competent amphotropic virus produced from transplanted bone marrow stem cells was associated with T cell lymphomas in three of 10 animals (7). Acknowledging these potential hazards, the Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration recently has released guidance on testing procedures for such viruses (21). The recommendations are aimed at the detection of a standard, intact replicating virus. Variant amphotropic MLV with differ-

ent biological properties currently is not considered. We have previously characterized spontaneous, replication-competent MLV recombinants that harbor the CMV immediate early promoter (F.U.R., R.H. & B.B., unpublished work). We demonstrate here that amphotropic MLV that lacks the transcription enhancer elements in the U3 region can replicate in certain human fibroblast- and B lymphocyte-derived cells. The biological properties of this virus differ from the standard, intact amphotropic MLV. The replication of enhancerless virus is particularly important, because "self-inactivating" MLV vectors that delete the viral promoter and/or enhancer elements during transduction (12) are considered relatively safe with respect to the generation of replication-competent virus. This assumption is largely based on the fact that replication of Mo-MLV in the absence of the two 75-bp enhancer elements has not been observed in murine cells (11). However, Akv MLV replication in murine cells is possible for virus with partial deletions in the U3 enhancer region (22). In this case, the remaining enhancer portion may be responsible for the residual virus replication. Two options must be considered to explain the replication of enhancerless amphotropic MLV in human cells: cis-acting sequences either within the provirus or adjacent to the site of provirus integration may compensate for the enhancer defect.

It is our current hypothesis that a secondary enhancer is present within the provirus that can, either in its original sequence or after subtle mutations, recruit nonubiquitous transcription factors and stimulate transcription from the MLV promoter. Because enhancer elements can exert their effects from large distances, such a secondary enhancer may be present in either the remaining part of the U3 region, the R region, U5 region, or the internal coding part of the provirus. For mouse mammary tumor virus we have previously described a B lymphocyte-specific enhancer element within the *pol* gene (23), but in MLV no such elements within the coding region are known. Our data also may be explained by a differential, enhancerlike activity of either the "distal" elements, such as the C/EBP-binding site (9) or the negative regulatory ELP-binding element (24) of the U3 region.

Gene expression and replication of Mo-MLV is severely compromised in murine embryonal carcinoma cells by the lack of MLV enhancer activity (15). However, this defect can be overcome in a very small fraction of cells where the provirus is inserted in the vicinity of active cellular enhancer elements (25–28). Enhancer-deficient amphotropic MLV may use such stimulation of viral transcription by nearby cellular enhancers to sustain a replication at a reduced rate. However, two arguments speak against this model: (*i*) Assuming similar numbers of active genes in different cells this model does not explain the inability of the virus to replicate in otherwise permissive cell types. In addition, NIH/3T3 cells that have been successfully used for the identification of cellular transcription elements in a complementation assay with enhancer-deficient MLV vectors (28) do not support replication of the enhancer-deficient virus. (*ii*) The cellular enhancer stimulation model predicts that fewer than 1 in 10^3 proviruses is expressed. This prediction implies that cells that contain inactive proviruses cannot establish superinfection resistance and are targets for further infections. Cells infected with the enhancer-deficient MLV would contain a drastically increased number of proviruses when compared with the same cell line infected with the intact MLV-(MOA). Our experimental data demonstrate identical numbers of proviruses in cells infected by either the intact or enhancer-deficient viruses and strongly contradict this model.

With the demonstration that replication of enhancerless amphotropic MLV is possible in certain human cells the question arises whether such a virus poses a risk for patients that receive MLV vector-mediated gene transfer. To address this problem we are currently testing whether the same phenomenon is observed in primary human cells in cell culture. Direct pathogenicity studies would require infection of rhesus monkeys as *in vivo* model (7). We suggest that routine tests to detect replication-competent MLV in human cells should be designed to include enhancer-deficient MLVs. The currently recommended procedure (21) consists of virus amplification in a permissive cell line such as *Mus dunni* fibroblasts followed by detection in an indicator cell line such as PG-4 S^+L^-. We have not yet determined whether *M. dunni* cells are permissive for MLV-(MOA)ΔE and whether the virus is detectable in the PG-4 assay. The use of HT-1080 fibrosarcoma cells in the virus amplification step for a period of at least 40 days would strongly enrich both intact and enhancer-deficient MLV for subsequent detection.

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