MOLECULAR AND CELLULAR BIOLOGY, Jan. 1989, p. 100–108
0270-7306/89/010100-09$02.00/0

Vol. 9, No. 1

Construction and Properties of Replication-Competent Murine Retroviral Vectors Encoding Methotrexate Resistance

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Received 12 July 1988/Accepted 13 October 1988

A series of replication-competent Moloney murine leukemia virus vectors was constructed in which each vector contained a mutant dihydrofolate reductase (DHFR) cDNA insert in the U3 region of the viral long terminal repeat. Two of the resulting viruses, MLV (murine leukemia virus) DHFR*-5 and MLV DHFR*-7, were able to stably transfer methotrexate resistance to infected fibroblast cells upon multiple rounds of virus replication and in the absence of drug selection. Cell lines producing recombinant virus with high titers were established, which indicated that the insert did not grossly interfere with viral replication functions. These vectors should be useful for introducing and expressing foreign genes in vivo in tissues and whole animals in which virus spread is needed for efficient infection.

Most retroviral vectors described to date are replication defective and are propagated through the use of wild-type virus or helper-virus-free packaging cell lines which provide all of the functions in *trans* necessary for encapsidation of a recombinant genome. In the latter case, the virus generated is able to efficiently infect cells and stably integrate the viral genome yet is unable to spread to adjacent uninfected cells (20). Although for most applications the use of such helper-free recombinant virus is adequate and, in fact, desirable, one can envisage situations in which the ability of a recombinant virus to spread from cell to cell might be advantageous. In particular, such vectors might provide a powerful means of directly introducing foreign DNA sequences into somatic cells in vivo.

Most of the replication-competent vectors constructed so far have been derived from the avian Rous sarcoma virus. In most cases, the foreign DNA sequences were inserted in place of the coding region for *src*, which is dispensable for virus replication (7, 11, 15). Other vectors, based on the replication-competent avian spleen necrosis virus, have been constructed and enable the introduction of foreign sequences downstream from the coding sequences for *env* (8). A third series of vectors, derived from Moloney murine leukemia virus (Mo-MLV) has been described in which a bacterial suppressor tRNA gene was inserted into the U3 region of the viral long terminal repeat (LTR). These M-MuLV^{sup} vectors replicate at high efficiency and permit the facile recovery of provirus genomes and associated flanking host sequences (17, 24).

To determine whether replication-competent Mo-MLV-based vectors could for the expression of protein-coding sequences could also be generated, we constructed a number of recombinant retroviral genomes in which a mutant murine dihydrofolate reductase (DHFR) cDNA (27) was introduced into the viral LTR sequences. Here, we describe both the transmission characteristics of such genomes as well as their ability to express the DHFR* gene and confer methotrexate resistance on infected cells.

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MATERIALS AND METHODS

Construction of plasmids and replication-competent MLV (murine leukemia virus) DHFR* vectors. pSV2-based expression vectors for DHFR* were constructed by inserting a *Hin*dIII-linkered, 660-base-pair (bp) cDNA fragment of a mutant DHFR* from the *Fnu*4HI site to the *Bgl*II site (27) into the *Hin*dIII-*Bgl*II sites of pSV2 DHFR (29). pSV2 DHFR* vectors containing the human β-globin poly(A) site were obtained by inserting a 225-bp *Eco*RI-*Aha*III fragment from the human β-globin gene into the *Xho*I-linkered *Hpa*I-*Bam*HI sites or the *Xho*I-linkered *Bgl*II-*Bam*HI sites of pSV2 DHFR*.

3′ LTR intermediate constructions were obtained by inserting *Bam*HI-linkered fragments containing the DHFR* cDNA alone or with simian virus 40 (SV40) promoter and intervening sequences and/or the human β-globin poly(A) site from the pSV2 DHFR* vectors into the *Bam*HI site of pLTR subclones (24) at position 7911 (26) in either orientation. Replication-competent MLV DHFR* vectors were reconstructed by ligating a 8.5-kilobase (kb) *Eco*RI-*Cla*I fragment from pMov9, containing the 5′ end of Mo-MLV, to a *Cla*I-*Pst*I fragment from the pLTR DHFR* subclones and a 3.6-kb *Pst*I-*Eco*RI fragment of pBR322. All plasmid constructions were performed by standard procedures (18).

Cell culture, DNA transfection, and virus infection. NIH 3T3 and ψ2 cells (19) were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum. DNA transfections were performed by the method of Graham and van der Eb as modified by Parker and Stark (21). Virus harvest from transiently transfected ψ2 cells and virus infection were performed as described by Cepko et al. (3). Transfected ψ2 cells and virus-infected NIH 3T3 cells were grown for 2 days to allow expression of transformed phenotype and then split 1:10 into Dulbecco modified Eagle medium containing 10% dialyzed calf serum and 0.25 μM methotrexate (Lederle Laboratories, Pearl River, N.Y.).

For passage of virus and titration of methotrexate resistance, NIH 3T3 cells were plated at a density of 5×10^4 cells per well in 24-well petri dishes; for the XC assay, density was 0.5×10^4 cells per well. Virus supernatant was filtered through 0.45-μm-pore-size membrane filters (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) and serially diluted from 10^{-1} to 10^{-5}; 0.2 ml of each dilution was

used for infection. After 2 days, cells from one well were transferred into methotrexate selection medium; cells from a parallel well were passaged three times without methotrexate selection to allow virus spread. For analysis of infectious virus titer, the cells were subjected to an XC plaque assay (25) 5 to 6 days after virus infection. NIH 3T3 populations infected with the highest virus dilution and still producing infectious, methotrexate-resistant virus were used for further virus passage or single-cell cloning by endpoint dilution, without or with methotrexate selection.

Southern hybridization and analysis. Agarose gel electrophoresis and Southern hybridization were performed by standard procedures (18), using Zetabind membranes (AMF/Cuno) for DNA transfer. A 660-bp DHFR* cDNA fragment or the pMu3 plasmid (24) was nick translated by standard procedures (18).

RNA preparation and Northern (RNA) hybridization analysis. Total cellular RNA was prepared by the method of Chirgwin et al. (4) as described by Cone et al. (5). Samples (5 μg) of RNA were electrophoresed on formaldehyde-agarose gels as described elsewhere (5). Gels were transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.), and the filters were hybridized by using a nick-translated 660-bp DHFR* cDNA fragment or a 1.38-kb *Hpa*I fragment of pMoV9, containing the *env* region of Mo-MLV, as a probe.

S1 nuclease analysis. S1 nuclease analysis was performed essentially as described by Hentschel et al. (10), using 1 μg of total cellular RNA and 5 μg of yeast tRNA as carrier for each reaction. Either a ^{32}P-5′-end-labeled 840-bp *Ssp*I-*Bam*HI fragment or a ^{32}P-5′-end-labeled 1,300-bp *Ssp*I-*Sca*I fragment, each from a pLTR subclone containing an SV40 promoter-DHFR* cDNA insert, was used as a probe. Hybridization was performed with an RNA concentration of 0.5 μg/μl at 52°C for 18 h. The hybrids were digested with 30 U of S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 1 h at 37°C. Protected DNA fragments were analyzed by electrophoresis through 7% polyacrylamide-urea gels.

Analysis of DHFR protein. Protein was extracted from approximately 5×10^7 cells by threefold freezing and thawing of the cell pellets in 200 μl of 50 mM potassium phosphate, pH 7.5, and centrifugation at 100,000 × *g* at 4°C for 1 h in a TLA-100 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The supernatants (S-100) were stored in liquid N_2. The amount of protein was determined by using the protein assay of Bio-Rad Laboratories, Richmond, Calif.

Endogenous DHFR protein was separated from the mutant DHFR* by isoelectric focusing, using thin-layer 8% urea-polyacrylamide gels with carrier ampholytes (5% [vol/vol] 3/10 Ampholite, 2% [vol/vol] 8/10.5 Ampholite; Pharmacia Fine Chemicals, Piscataway, N.J.) in a horizontal electrophoresis cell (Bio-Rad). Samples (10 μl) of S-100 supernatants were applied with filter paper strips (Schleicher & Schuell), and isoelectric focusing standards with a pI range of 4.6 to 9.6 (Bio-Rad) were run in parallel as markers. Electrophoresis was at 10 W (constant power) for 45 min and 950 V (constant voltage) for 1.5 h, using 0.1 N NaOH as the cathode buffer and 0.1 N H_3PO_4 as the anode buffer. The isoelectric point is 8.0 to 8.5 for the endogenous DHFR enzyme and is more basic for the mutant enzyme because of the substitution of an arginine for a leucine residue (9, 27).

Protein was blotted onto nitrocellulose filters (Schleicher & Schuell) by the method of Burnette (2). The filters were incubated with rabbit anti-DHFR antiserum (a gift from S. Srimatkandada and J. Bertino) and horseradish peroxidase-conjugated anti-rabbit antiserum (Organon Teknika, Malvern, Pa.). The blots were developed for 30 to 60 s in 1 part chloronaphthol (3 mg/ml in methanol) to 5 parts 50 mM Tris–200 mM NaCl (pH 7.4)–3.4 μl of 3% H_2O_2 per ml.

RESULTS

Construction of replication-competent MLV DHFR* vectors. To facilitate the analysis of replication-competent recombinant genomes, we chose to introduce a 660-bp murine DHFR* cDNA that encodes a methotrexate-resistant enzyme (27). We and others have previously shown that by using the appropriate eucaryotic transcriptional signals, the gene encoding this enzyme can be used as a dominant-acting selectable marker in mammalian cells (27; H. Stuhlmann and R. C. Mulligan, unpublished results). Because insertion of exogenous DNA sequences into most regions of the Mo-MLV proviral genome would necessarily disrupt essential viral gene expression, we chose to introduce the DHFR* cDNA and associated sequences into the viral LTR at position 7911 (26), since several groups have previously shown that insertions of a bacterial suppressor tRNA gene at that location do not affect virus transmission and replication (17, 24). The sequences introduced into the Mo-MLV genome (Fig. 1) included the DHFR* cDNA sequence alone as well as chimeric transcriptional units containing the cDNA linked to SV40 transcriptional sequences, intervening sequences, and/or the human β-globin poly(A) site. Each of these latter constructs was shown to yield methotrexate-resistant colonies after transformation of cells with both pSV2-based vectors and the 3′ LTR intermediate constructions (data not shown). To facilitate the final retroviral constructions, each segment was first introduced into a plasmid containing only the 3′ viral LTR in both transcriptional orientations; subsequently, the chimeric LTR was transferred to a plasmid containing an intact Mo-MLV proviral genome (see Materials and Methods). Although the resulting construction contained the foreign sequences only in the 3′ LTR, transmission of the viral genome would be expected to result in proviruses containing the sequences in both LTRs (30). All constructions (with the exception of pMLV DHFR*-8, carrying a 660-bp DHFR* insert in the opposite-sense orientation in the 3′ LTR) gave rise to methotrexate-resistant colonies upon transfection into NIH 3T3 cells (Fig. 1).

Production of infectious virus and stability of MLV-DHFR* virus upon replication in tissue culture. To determine whether the recombinant proviruses could give rise to infectious virus capable of faithfully transmitting the inserted sequences, each construct was introduced into ψ2 cells by DNA transfection. Virus was harvested from the supernatant 1 day posttransfection as described previously (3) and used to infect NIH 3T3 cells. This procedure for isolating virus was used because virus harvest from transiently transfected ψ2 cells should minimize the possibility of generating wild-type virus resulting from recombination between the LTRs during DNA transfection. After 2 days in nonselective medium, the 3T3 cells were transferred to medium supplemented with dialyzed calf serum and 0.25 μM methotrexate in order to assess expression of the mutant DHFR* cDNA. In addition, ψ2 cells transfected with the recombinant proviruses were directly selected for methotrexate resistance (Table 1). Although all constructs (with the exception of pMLV DHFR*-8) gave rise to methotrexate-resistant colonies upon transfection of ψ2 cells, only three constructs

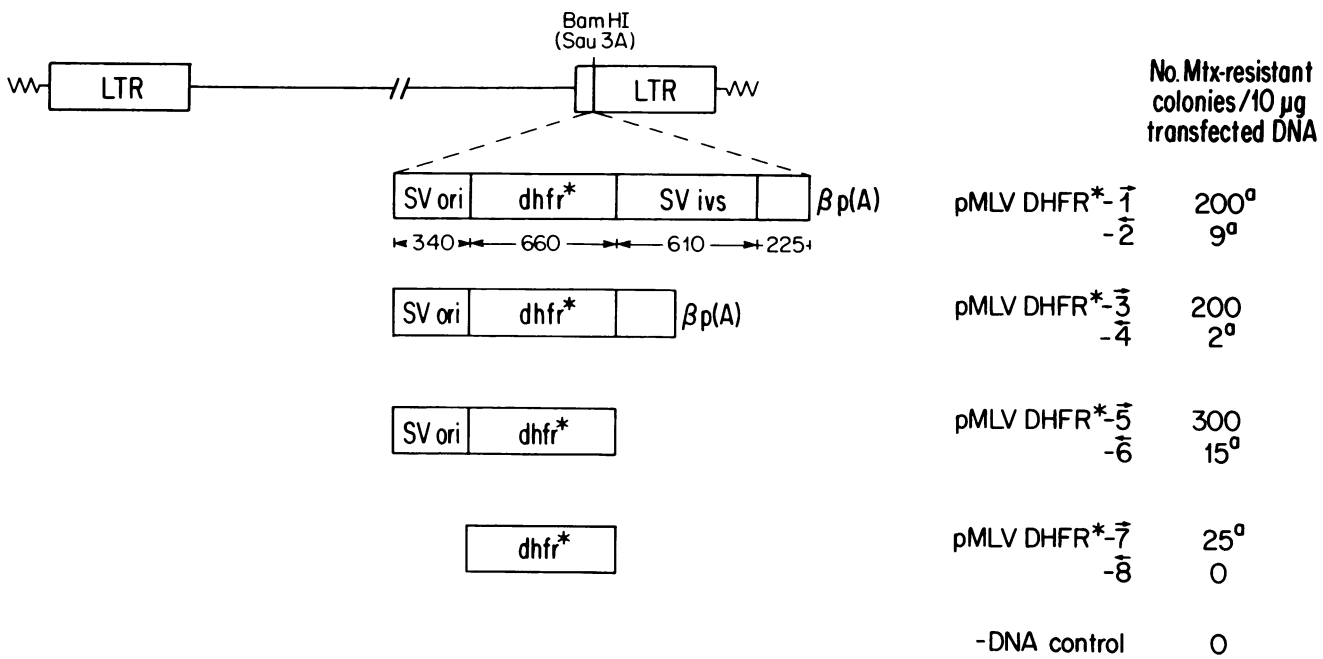


FIG. 1. Structure and infectivity of recombinant MLV DHFR* genomes with inserts in the U3 region of the viral 3' LTR. Numbers for fragments represent length in base pairs. The right-hand column shows average numbers of methotrexate (Mtx)-resistant NIH 3T3 colonies per plate from two independent transfections for each construct. Arrows above the constructs indicate orientations of the inserts with respect to that of the LTR. [a], Faint methotrexate-resistant colonies.

(pMLV DHFR*-3, -5, and -7), all containing the insert in sense orientation with respect to the LTR, were able to transmit infectious, methotrexate-resistant virus to 3T3 cells. No virus was produced from recombinant vectors carrying the insert in the opposite sense orientation in the LTR. The reasons for the lower transfection efficiencies in ψ2 cells (in comparison with 3T3 cells) are not clear.

We next examined the stability of infectious virus upon multiple rounds of replication in NIH 3T3 cells. For this study, six single methotrexate-resistant 3T3 colonies derived from the original transient harvest infection were expanded. Virus produced by these clones was then used to infect 3T3 cells at extremely low multiplicity of infection (see Table 1 footnote). After spread of the virus through the culture, the cycle of infection at low multiplicity and virus spread was repeated two more times without drug selection. After each passage, the supernatant of infected 3T3 populations was analyzed for methotrexate-resistant virus as well as for replication-competent virus by an XC plaque assay (25). MLV DHFR*-3 virus containing a 1.2-kb insert with SV40 promoter, DHFR* cDNA, and the β-globin poly(A) site was unstable upon passage in 3T3 cells, and no infectious, methotrexate-resistant virus could be detected after the first virus passage (Table 1). In contrast, some methotrexate-resistant 3T3 clones infected with transiently produced MLV DHFR*-5 virus gave rise to stable methotrexate-resistant virus in three rounds of virus infection, and in all cases MLV DHFR*-7 virus, containing only the DHFR* cDNA insert,

TABLE 1. Passage of methotrexate resistance phenotype and stability of replicating MLV DHFR* viruses[a]

Transfecting DNA	No. of resistant ψ2 colonies/ 10 μg of transfected DNA	No. of resistant 3T3 clones after transient harvest infection	Fraction of clones stably transmitting infectious resistant virus	Virus titer from 3T3 clones after 3rd passage	
				No. of resistant colonies/ml	No. of XC plaques/ml
pMLV DHFR*-1	4	0			
pMLV DHFR*-2	3	0			
pMLV DHFR*-3	9	8	0/6		
pMLV DHFR*-4	2	0			
pMLV DHFR*-5	13	15	2/6	1×10^4–5×10^5	1×10^6–2×10^7
pMLV DHFR*-6	6	0			
pMLV DHFR*-7	4	11	6/6	1×10^4–5×10^4	2×10^5–2×10^6
pMLV DHFR*-8	0	0			
Control	0	0			

[a] Recombinant viral DNA was transfected in ψ2 cells. Virus supernatants from transiently transfected cells were used to infect NIH 3T3 cells. Presented are average numbers of methotrexate-resistant colonies per plate from three independent experiments. The fraction of NIH 3T3 clones stably transmitting infectious, methotrexate-resistant virus was determined by threefold repeated infection of 3T3 cells with serial virus dilutions in the absence of methotrexate selection and spread throughout the cell population. Parallel plates were tested for methotrexate-resistant virus and XC-positive virus (see Materials and Methods). After the third virus passage, single-cell clones were isolated by endpoint dilution in the absence of methotrexate, and the supernatants were analyzed for methotrexate-resistant virus and XC titer. Shown are the average titers from six clones each.

transmitted infectious virus with a stable methotrexate resistance phenotype.

DNA was isolated from 3T3 populations at each virus passage and analyzed for the presence of provirus copies by *Kpn*I restriction enzyme digestion and subsequent gel transfer hybridization (Fig. 2A). With a 660-bp DHFR* cDNA fragment used as a probe, the expected size for the internal *Kpn*I fragment containing the 3′ LTR is 2.28 kb for MLV DHFR*-5 and 1.94 kb for MLV DHFR*-7 (Fig. 2B and 2A, lanes 9 and 17, respectively). MLV DHFR*-7-infected cell populations showed the expected 1.94-kb fragment (Fig. 2A, lanes 11 and 12). In contrast, distinct patterns of deletions in the 2.28-kb *Kpn*I fragment were detected upon passage of MLV DHFR*-5 (Fig. 2A, lanes 2 and 3), although the methotrexate resistance phenotype was retained, as shown above. The same pattern of *Kpn*I fragments was obtained when pMu3, spanning the U3 region of the Mo-MLV LTR (24), was used as a probe (data not shown). These results indicate that a 660-bp DHFR* insert in the U3 region of the wild-type Mo-MLV LTR is stable and gives rise to replication-competent virus (MLV DHFR*-7). In contrast, in the case of MLV DHFR*-5, which also contained the SV40 promoter sequences, the virus became unstable upon replication and underwent discrete deletions, some of which gave rise to replication-competent virus expressing the mutant DHFR*. These findings were confirmed by using *Pvu*II restriction enzyme digests for the analysis of DNA (data not shown).

To determine whether virus passaged nonselectively as described above would give rise to methotrexate resistance, single cell clones isolated after the third virus passage of MLV DHFR*-5 and of MLV DHFR*-7 were used as a source of virus. Virus supernatant was titered from six clones each for methotrexate resistance and XC plaques. High virus titers were obtained for MLV DHFR*-5 as well as for MLV DHFR*-7 (Table 1). Furthermore, the virus titers demonstrated single-hit kinetics (data not shown). For each clone, the XC titer characteristically ranged from 20- to 50-fold higher than the respective methotrexate resistance titer (see Table 1). Restriction enzyme analysis with *Kpn*I revealed that DNA from clones derived from infection with both viruses carried multiple integrated proviruses, in some cases between 10 and 15 MLV DHFR* copies per cell (Fig. 2A, lanes 4 to 8 and 13 to 16). The majority of provirus copies in MLV DHFR*-7-infected clones showed the expected 1.94-kb *Kpn*I fragment for the 3′ LTR internal fragment. This result was confirmed by *Pvu*II digests of the same DNA (data not shown). In contrast, *Kpn*I and *Pvu*II digestion of DNA from MLV DHFR*-5-infected clones revealed a small deletion of 50 to 100 bp in the 3′ internal fragments (Fig. 2A, lanes 4 to 8, and data not shown). More detailed mapping by restriction enzyme analysis (not shown) and S1 mapping (see below) localized the deletion to the SV40 promoter sequences present in the 1-kb insert of pMLV DHFR*-5.

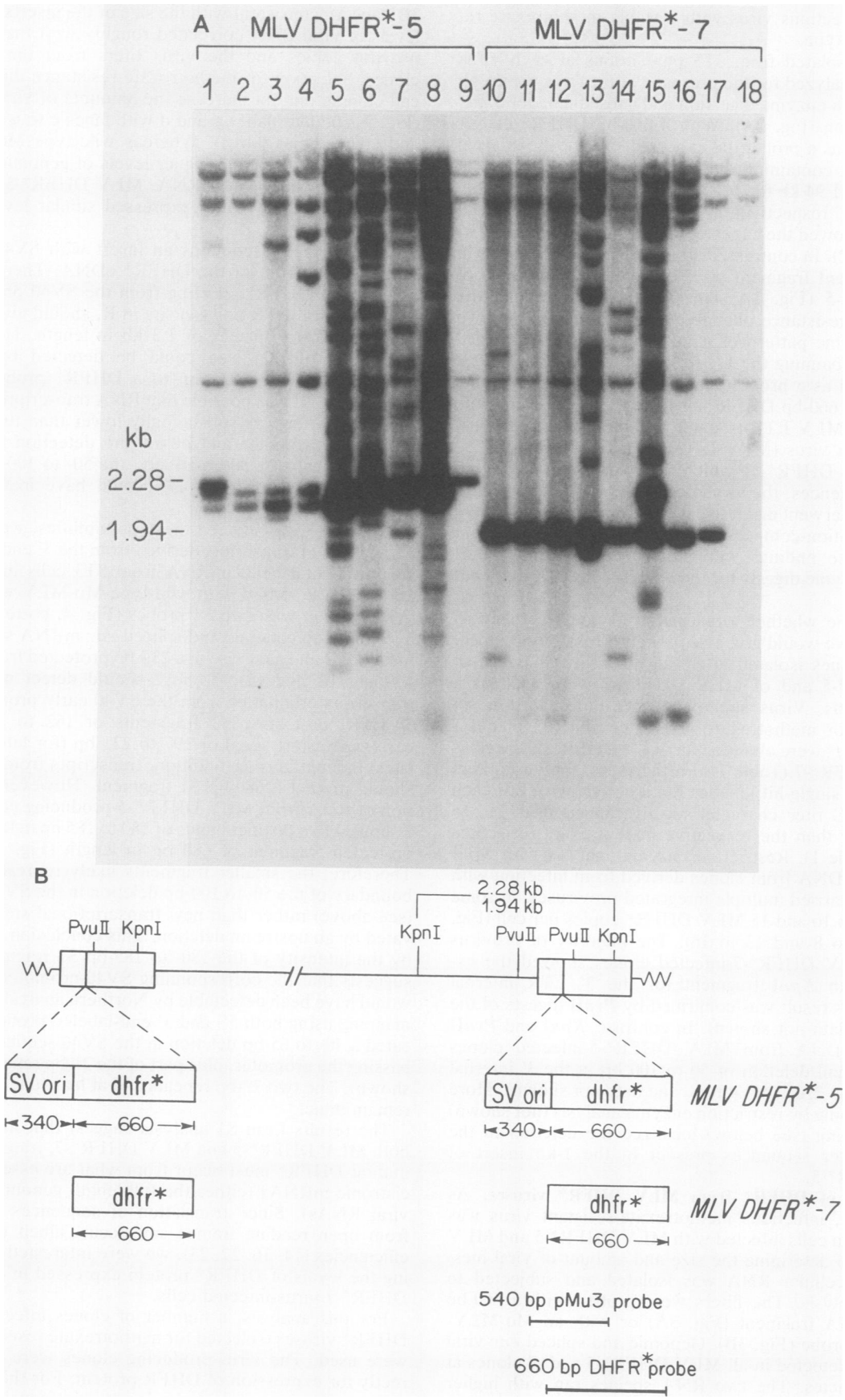
Expression of DHFR* from MLV DHFR* viruses. As shown above, infectious methotrexate-resistant virus was produced from cells infected with MLV DHFR*-5 and MLV DHFR*-7. To determine the size and amount of viral messages, total cellular RNA was isolated and subjected to Northern analysis. The filters were probed with a 660-bp DHFR* cDNA fragment (Fig. 3A) or with an Mo-MLV-specific *env* probe (Fig. 3B). Genomic and spliced *env* viral RNAs were detected in all MLV DHFR*-5 and -7 clones at high abundancies. The two RNA species ran with higher molecular weights than did wild-type Mo-MLV RNA (Fig. 3B, lane a), consistent with the size of the inserts in U3. The levels of viral RNA correlated roughly with the number of proviral copies and the virus titers from the respective clones. Selection for methotrexate resistance during single-cell cloning did not increase the amounts of viral messages (Fig. 3, compare lanes c and d with lanes e to g and lanes h and i with lanes j to l). Whereas wild-type Mo-MLV expressed about threefold-higher levels of genomic size compared with spliced *env* mRNA, MLV DHFR*-5- and MLV DHFR*-7-infected clones expressed similar levels of both RNA species.

MLV DHFR*-5 contains an insert with SV40 promoter sequences in front of the DHFR* cDNA. Therefore, transcription of DHFR*, starting from the SV40 promoter and using the viral LTR poly(A) site in R, should give rise to an additional RNA species of 1.3 kb in length. However, no transcripts of that size could be detected by Northern analysis after hybridization to a DHFR* probe (Fig. 3A, lanes c to g). It was possible that RNA transcription from the SV40 promoter was substantially lower than that from the viral LTR promoter and below the detection limit of the Northern analysis. Alternatively, the 50- to 100-bp deletion in the SV40 region (Fig. 2A) could have inactivated the promoter function.

To distinguish between these possibilities, we performed S1 analysis of transcripts derived from the 3′ end of the viral genome. Total cellular RNA from 3T3 cells infected with MLV DHFR*-5 and with wild-type Mo-MLV was used for hybridization with two S1 probes (Fig. 4, bottom). Probe 1 hybridized to genomic and spliced *env* mRNA starting from the 5′ LTR and gave rise to a 237-bp protected fragment (Fig. 4, lanes b1 and c1). Probe 2 should detect nonretroviral transcripts originating from the SV40 early promoter in the 3′ LTR, protecting S1 fragments of 162 to 170 bp (for early-early start sites) or 197 to 221 bp (for late-early start sites) in length (1). In addition, transcripts from the 5′ LTR should protect a 680-bp S1 fragment. However, hybridization of RNA from MLV DHFR*-5-producing cells to probe 2 showed two distinct bands of 180 to 185 bp in length and no protected fragment of 680 bp in length (Fig. 4, lane b2). Therefore, the smaller fragments likely represented the 3′ boundary of the 50- to 100-bp deletion in the SV40 promoter (see above) rather than new transcriptional start sites activated by an upstream deletion. This conclusion is supported by the intensity of the 180- to 185-bp S1 fragments, which suggests that the corresponding SV40-promoted transcripts would have been detectable by Northern analysis. Recent S1 mapping using both 5′- and 3′-end-labeled probes has delineated a 40- to 65-bp deletion in the SV40 sequences encompassing the promoter, plus part of the 21-bp repeats (data not shown). The two 72-bp repeats and at least one 21-bp repeat remain intact.

The results from S1 analysis suggest that in the case of both MLV DHFR*-5 and MLV DHFR*-7, translation of the mutant DHFR* must occur from what are essentially polycistronic mRNAs (either the full-length genomic or spliced viral RNAs). Since translation of sequences downstream from open reading frames can occur, albeit often at low efficiencies (14, 16, 22, 23), we were interested in determining the levels of DHFR* protein expressed in DHFR*-5 or DHFR*-7 virus-infected cells.

For this analysis, a number of clones infected with the DHFR* viruses (selected for methotrexate resistance or not) were used. The virus-producing clones were analyzed directly for expression of DHFR protein. For this procedure, cell extracts were run on an isoelectric focusing gel under


A
MLV DHFR*-5
MLV DHFR*-7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
kb
2.28-
1.94-
B
2.28 kb
1.94 kb
PvuII KpnI
KpnI
PvuII PvuII KpnI
SV ori
dhfr*
340
660
MLV DHFR*-5
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660 bp DHFR* probe

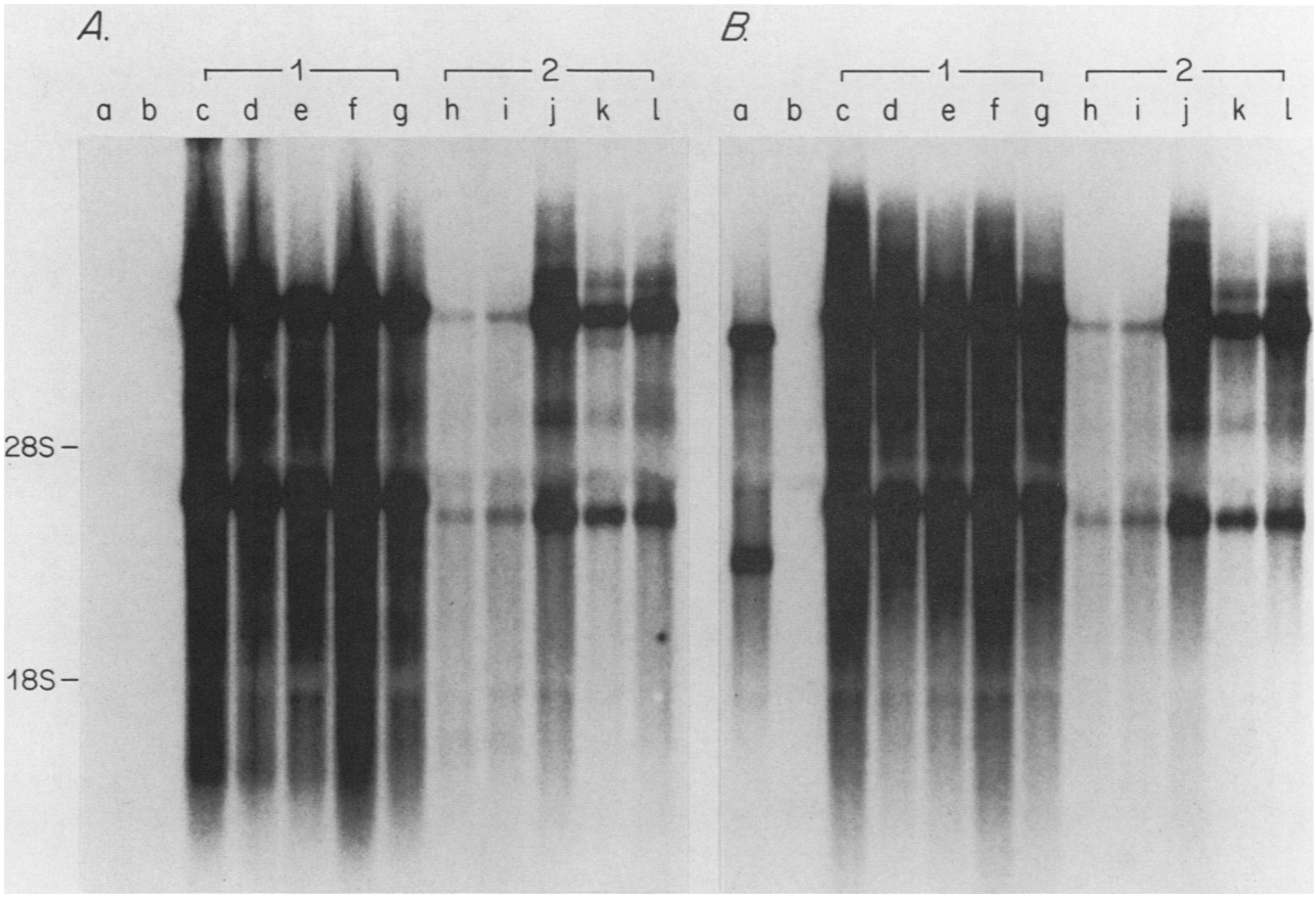

FIG. 3. Expression of DHFR* and viral transcripts in infected NIH 3T3 cells. Total cellular RNA (5 μg) was prepared and subjected to Northern hybridization analysis. Lanes: a, wild-type Mo-MLV-producing clone; b, uninfected 3T3 control; 1, clones infected with MLV DHFR*-5 virus after the third virus passage (c and d, selected in 10 μM methotrexate; e to g, no methotrexate selection); 2, clones infected with MLV DHFR*-7 virus after the third virus passage (h and i, selected in 0.3 μM methotrexate; j to l, no methotrexate selection). The filters were hybridized with 10^7 cpm of ^{32}P-labeled probe of a 660-bp DHFR* fragment (A) or of a 1.38-kb *Hpa*I fragment of pMov9 encompassing Mo-MLV *env* (B). Size markers on the left indicate positions of rRNA.

conditions that allow separation of the endogeneous DHFR from the introduced mutant DHFR* (see Materials and Methods). The proteins were detected by Western blotting (immunoblotting), using polyclonal rabbit α DHFR antibodies and horseradish peroxidase-conjugated α rabbit antibodies. Clones producing MLV DHFR*-5 virus produced about 10-fold-higher levels of the mutant DHFR* than of the endogenous protein even without selection for methotrexate resistance (Fig. 5, lanes d and e). In contrast, protein expression was low in MLV DHFR*-7-infected cells when no drug selection was applied and was increased at least 10-fold by methotrexate selection (Fig. 5, lanes a and b). Expression of the endogenous DHFR also appeared to be increased in the presence of methotrexate.

DISCUSSION

We describe here the generation of replication-competent Mo-MLV-derived vectors suitable for mammalian gene transfer studies. These vectors contain a mutant DHFR* cDNA (27) as a dominant selectable marker inserted into the U3 region of the viral LTR, upstream from viral promoter and enhancer. We show that two of the resulting viruses, MLV DHFR*-5 and MLV DHFR*-7, could stably transmit

FIG. 2. (A) Southern blot hybridization analysis of NIH 3T3 cells infected with recombinant MLV DHFR* viruses. Genomic DNA (10 μg), prepared from NIH 3T3 clones or populations, was digested to completion with *Kpn*I. The filter was hybridized with 2×10^7 cpm of a ^{32}P-labeled 660-bp DHFR* fragment. Lanes: 1, 3T3 clone 5 from transient harvest infection with MLV DHFR*-5, methotrexate selected; 2 and 3, second and third virus passages from clone 5, no methotrexate selection; 4 to 8, 3T3 clones A to E after the third virus passage, no methotrexate selection; 9, uninfected 3T3 DNA mixed with 15 pg of pMLV DHFR*-5; 10, 3T3 clone 1 from transient harvest infection with MLV DHFR*-7, methotrexate selected; 11 and 12, first and third virus passages from clone 1, no methotrexate selection; 13 to 16, 3T3 clones A to D after the third virus passage, no methotrexate selection; 17, uninfected 3T3 DNA mixed with 15 pg of pMLV DHFR*-7; 18, uninfected 3T3 control. Bands in lane 18 represent hybridization of the DHFR* probe with endogenous DHFR fragments. Markers indicate positions of the internal *Kpn*I fragment in pMLV DHFR*-5 (2.28 kb) and pMLV DHFR*-7 (1.94 kb). (B) Schematic diagram of integrated proviral DNA derived from MLV DHFR*-5 and MLV DHFR*-7 infection, showing the restriction enzymes and probes used for Southern hybridization analysis. ——, Proviral sequences; ∿∿, host DNA sequences.

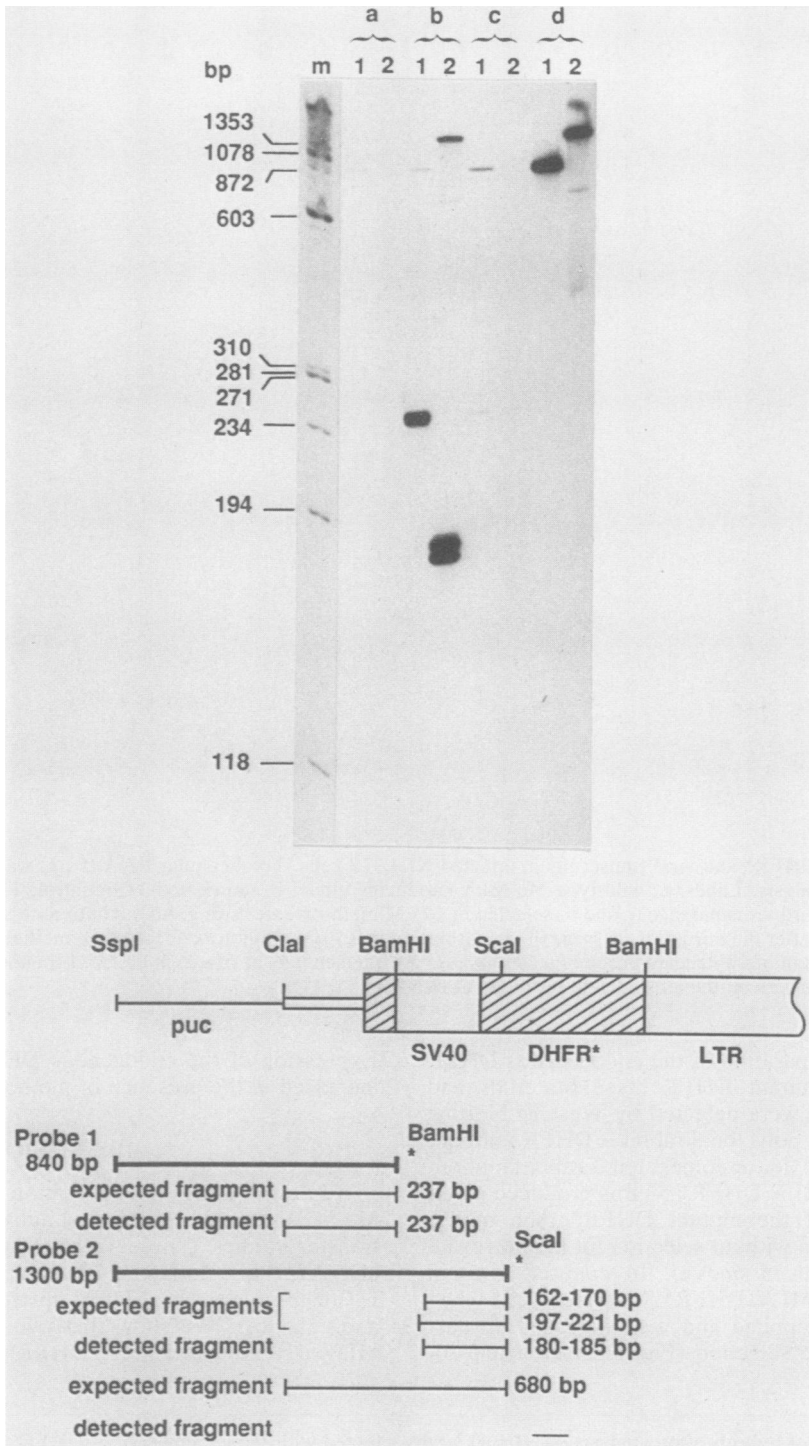

FIG. 4. S1 hybridization analysis of Mo-MLV- and DHFR*-specific transcripts. Total cellular RNA (1 μg) mixed with 5 μg of yeast tRNA was hybridized with 5×10^3 cpm of a ^{32}P-5′-end-labeled 840-bp *Ssp*I-*Bam*HI fragment (probe 1) or 1,300-bp *Ssp*I-*Sca*I fragment (probe 2). The protected S1 fragments were separated on a 7% polyacrylamide-urea gel. Lanes: m, ^{32}P-labeled ϕX marker, cut with *Hae*III (sizes of the ϕX *Hae*III fragments are indicated on the left); a, uninfected NIH 3T3 cells; b, 3T3 clone infected with MLV DHFR*-5 virus after the third virus passage; c, wild-type Mo-MLV-producing 3T3 clone; d, probes 1 and 2 alone. Structures and sizes of pLTR DHFR*-5 DNA, the DNA probes, and the expected and detected fragments that are protected by the S1 probes are indicated at the bottom. The probe used in each assay is shown above each track.

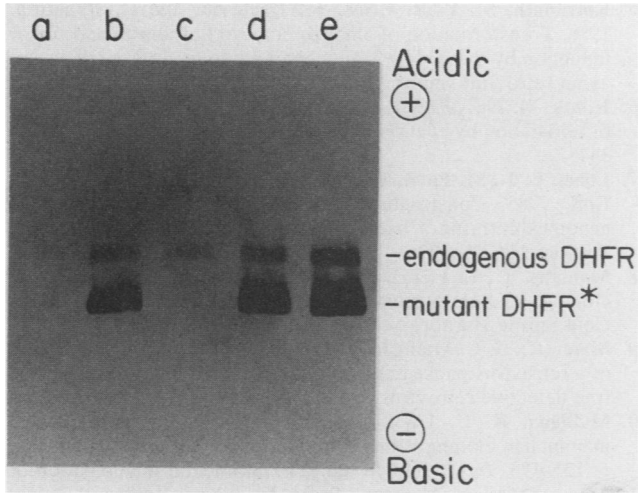

FIG. 5. Expression of DHFR protein in MLV DHFR*-infected cells. Total cellular extract (30 to 40 μg of protein per lane) was electrophoresed on an 8% urea-polyacrylamide isoelectric focusing gel and transferred to a nitrocellulose filter. The filter was incubated with rabbit anti-DHFR antiserum and horseradish peroxidase-conjugated anti-rabbit antiserum. DHFR-specific protein was detected by immunostaining as described in Materials and Methods. Lanes: a, 3T3 clone C after the third virus passage of MLV DHFR*-7, no methotrexate selection; b, 3T3 clone after the third virus passage of MLV DHFR*-7, selected in 0.3 μM methotrexate; c, 3T3 control; d, 3T3 clone E after the third virus passage of MLV DHFR*-5, no methotrexate selection; e, 3T3 clone after the third virus passage of MLV DHFR*-5, selected in 10 μM methotrexate. Positions of the endogenous and mutant proteins and of the cathode (−) and anode (+) during isoelectric focusing are indicated.

the marker gene when propagated in vitro under nonselective conditions. Cell lines that produced titers as high as those produced by wild-type Mo-MLV were established, which indicates that the inserts do not interfere with the replication function of the virus. However, viruses with inserts carrying regulatory sequences [SV40 promoter, SV40 intervening sequence, and the β-globin poly(A) site] in addition to the DHFR* cDNA were less stable and either generated no infectious virus or produced deleted, replication-competent variants, which outgrew the recombinant virus upon replication in NIH 3T3 cells. We cannot distinguish whether this instability results from a packaging size limit of the viral RNA, as has recently been suggested (8; S. Goff, personal communication), or from interference of nonviral regulatory sequences in close vicinity to viral promoter and enhancer with vital functions. The discrete deletions occurring in the SV40 promoter sequences upon early passage of MLV DHFR*-5 virus indicate that the latter possibility may hold true, at least for this particular construct.

Although MLV DHFR*-5 and -7 efficiently conferred methotrexate resistance on infected cells, the titers were consistently lower than measurements of virus titer obtained by using the XC plaque assay. Although this result could reflect the existence of a mixed population of viruses, experiments involving the use of endpoint-diluted virus and S1 analysis suggest that this is unlikely. One possible explanation for the discrepancy in titers is that although every inserted recombinant provirus yields enough expression of the viral proteins to give rise to an XC plaque, only a fraction of integrants express enough DHFR* to confer resistance to methotrexate.

The majority of single-cell clones isolated after low multiplicity of infection in the absence of methotrexate selection contained multiple (15 to 20) provirus copies, compared with the usual 1 to 5 copies per cell obtained with wild-type Mo-MLV (6). A possible explanation for this result is that the DHFR* insert interferes slightly with expression of the *env* gene, leaving the cells susceptible to superinfection until the level of *env* gp70 expression blocks Mo-MLV receptors on the cell surface.

Our data show that replication-competent, Mo-MLV-derived vectors carrying inserts of at least 1 kb in length can be generated and that they produce high titers of infectious virus and are stable upon multiple rounds of virus replication in vitro. Furthermore, insertion of a mutant DHFR* cDNA allows efficient selection of infected fibroblast cells in vitro for the dominant marker gene. Interestingly, the transcriptional analysis of both MLV DHFR*-5- and MLV DHFR*-7-infected cells suggests that the mutant DHFR* protein is translated from viral messages in which the cDNA sequences are located close to the 3′ end of the RNA. Several examples of the translation of two or more proteins from a single transcriptional unit have been demonstrated recently, and a termination-initiation mechanism has been proposed to account for translational reinitiation at an internal AUG (14, 16, 22, 23). Often, a decrease in translational efficiency of the downstream sequences in a polycistronic message was found (14, 16, 23). Although we have not directly compared the efficiency of translation of polycistronic viral mRNAs with that of other DHFR-containing RNAs, the data suggest that significant amounts of DHFR* protein are produced (3 to 10 times higher than endogenous levels of DHFR protein). Clearly, it will be important to determine whether such vectors can be useful for transmitting and expressing all cDNAs.

The availability of replication-competent vectors such as those described here raises the possibility of transferring genes into somatic cells in vivo at high efficiency. One of our major interests is to use the vectors to introduce and express foreign genes in somatic tissues of developing mouse embryos. It has been shown that wild-type Mo-MLV can replicate unrestricted in most somatic tissues when introduced at the postimplantation stage between days 8 and 10 of gestation (12, 13). However, replication-defective vectors carrying a dominant selectable marker, either in combination with a helper virus or alone, resulted in insertion of the marker gene in only a small fraction of cells from different organs, which indicates the need for virus spread (28; H. Stuhlmann, R. Jaenisch, and R. C. Mulligan, unpublished results). Thus, the vectors described here would greatly increase the possibility of manipulating the embryo at early stages of development. In addition, we are interested in the possibility of using these viruses to transduce other somatic tissues, perhaps in adult animals.

ACKNOWLEDGMENTS

We thank Jim Wilson and Paul Matsudaira for helpful advice with isoelectric focusing and S. Srimatkandada and J. Bertino for a gift of DHFR antiserum.

This work was supported by Public Health Service grants CA38497, HL37569, and HD00635 from the National Institutes of Health to R.C.M. H.S. was a fellow of the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. **Baty, D., H. A. Barrera-Saldena, R. D. Everett, M. Vigneron, and P. Chambon.** 1984. Mutational dissection of the 21 bp repeat

region of the SV40 early promoter reveals that it contains overlapping elements of the early-early and late-early promoters. Nucleic Acids Res. **12**:915–932.

2. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
3. **Cepko, C. L., B. E. Roberts, and R. C. Mulligan.** 1984. Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell **37**:1053–1062.
4. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
5. **Cone, R. D., A. Weber-Benarous, D. Baorto, and R. C. Mulligan.** 1987. Regulated expression of a complete human β-globin gene encoded by a transmissible retrovirus vector. Mol. Cell. Biol. **7**: 887–897.
6. **Fan, H., R. Jaenisch, and P. MacIsaac.** 1978. Low-multiplicity infection of Moloney murine leukemia virus in mouse cells: effect on number of viral DNA copies and virus production in producer cells. J. Virol. **28**:802–809.
7. **Foster, D. A., and H. Hanafusa.** 1983. *fps* gene without *gag* gene sequences transforms cells in culture and induces tumors in chickens. J. Virol. **48**:744–751.
8. **Gelinas, C., and H. M. Temin.** 1986. Nondefective spleen necrosis virus-derived vectors define the upper size limit for packaging reticuloendotheliosis viruses. Proc. Natl. Acad. Sci. USA **83**:9211–9215.
9. **Haber, D. A., S. M. Beverley, M. L. Kiely, and R. T. Schimke.** 1981. Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. J. Biol. Chem. **256**:9501–9510.
10. **Hentschel, C. C., J. C. Jrminger, P. Bucher, and M. Birnstiel.** 1980. Sea urchin histone mRNA termini are located in gene regions downstream of putative regulatory sequences. Nature (London) **285**:147–151.
11. **Hughes, S. H., J. J. Greenhouse, C. J. Petropoulos, and P. Sutrave.** 1987. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J. Virol. **61**: 3004–3012.
12. **Jaenisch, R.** 1980. Retroviruses and embryogenesis: microinjection of Moloney leukemia virus into midgestation mouse embryos. Cell **19**:181–188.
13. **Jähner, D., H. Stuhlmann, C. L. Stewart, K. Harbers, J. Lohler, I. Simon, and R. Jaenisch.** 1982. *De novo* methylation and expression of retroviral genomes during mouse embryogenesis. Nature (London) **298**:623–628.
14. **Kaufman, R. J., P. Murtha, and M. V. Davies.** 1987. Translational efficiency of polycistronic mRNAs and their utilization to express heterologous genes in mammalian cells. EMBO J. **6**: 187–193.
15. **Kornbluth, S., F. R. Cross, M. Harbison, and H. Hanafusa.** 1986. Transformation of chicken embryo fibroblasts and tumor induction by the middle T antigen of polyomavirus carried in an avian retroviral vector. Mol. Cell. Biol. **6**:1545–1551.
16. **Kozak, M.** 1987. Effects of intercistronic length on the efficiency of reinitiation by eucaryotic ribosomes. Mol. Cell. Biol. **7**:3438–3445.
17. **Lobel, L. I., M. Patel, W. King, M. C. Nguyen-Huu, and S. P. Goff.** 1985. Construction and recovery of viable retroviral genomes carrying a bacterial suppressor transfer RNA gene. Science **228**:329–332.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
20. **Mulligan, R. C.** 1983. Construction of highly transmissible mammalian cloning vehicles derived from murine retroviruses, p. 155–173. *In* M. Inouye (ed.), Experimental manipulation of gene expression. Academic Press, Inc., New York.
21. **Parker, B. A., and G. R. Stark.** 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. J. Virol. **31**:360–369.
22. **Peabody, D. S., and P. Berg.** 1986. Termination-reinitiation occurs in the translation of mammalian cell mRNAs. Mol. Cell. Biol. **6**:2695–2703.
23. **Peabody, D. S., S. Subramani, and P. Berg.** 1986. Effect of upstream reading frames on translation efficiency in simian virus 40 recombinants. Mol. Cell. Biol. **6**:2704–2711.
24. **Reik, W., H. Weiher, and R. Jaenisch.** 1985. Replication-competent Moloney murine leukemia virus carrying a bacterial suppressor tRNA gene: selective cloning of proviral and flanking host sequences. Proc. Natl. Acad. Sci. USA **82**:1141–1145.
25. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42**:1136–1139.
26. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukemia virus. Nature (London) **293**:543–548.
27. **Simonsen, C. C., and A. D. Levinson.** 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA **80**:2495–2499.
28. **Stuhlmann, H., R. Cone, R. C. Mulligan, and R. Jaenisch.** 1984. Introduction of a selectable gene into different animal tissue by a retrovirus recombinant vector. Proc. Natl. Acad. Sci. USA **81**:7151–7155.
29. **Subramani, S., R. Mulligan, and P. Berg.** 1981. Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid in simian virus 40 vectors. Mol. Cell. Biol. **1**:854–864.
30. **Varmus, H., and R. Swanstrom.** 1982. Replication of retroviruses, p. 369–512. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.