JOURNAL OF VIROLOGY, Jan. 1984, p. 214–222
0022-538X/84/010214-09$02.00/0

Vol. 49, No. 1

Two Base Changes Restore Infectivity to a Noninfectious Molecular Clone of Moloney Murine Leukemia Virus (pMLV-1)

A. DUSTY MILLER AND INDER M. VERMA*

Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California 92138

Received 25 July 1983/Accepted 27 September 1983

The complete nucleotide sequence of a molecular clone of Moloney murine leukemia virus (pMLV-1) has previously been reported (Shinnick et al., Nature [London] **293**:543–548, 1981). However, pMLV-1 does not generate infectious virus after transfection into cells (Berns et al., J. Virol. **36**:254–263, 1980). The lesion in pMLV-1 has been localized by determining the biological activity of recombinants containing DNA from an infectious clone of Moloney murine leukemia virus (pMLV-48) and pMLV-1. Replacement of a 1.0-kilobase pair region which spans the *gag-pol* junction of pMLV-1 with the corresponding DNA fragment from the infectious clone restores its infectivity. Nucleotide sequence analysis of this fragment obtained from the infectious clone (pMLV-48) and pMLV-1 reveals two single base pair changes, one in the $p30^{gag}$ gene and the other in the 5′ end of the *pol* gene. The mutation in the *pol* gene does not affect the production of infectious virus but renders them XC negative, whereas the mutation in the *gag* gene appears to be lethal. The complete nucleotide sequence of an infectious clone of Moloney murine leukemia virus can now be deduced.

Moloney murine leukemia virus (Mo-MLV) is a replication-competent C-type retrovirus which, upon inoculation into newborn mice, gives rise to leukemias of mostly T cell origin (8). In cells infected with Mo-MLV, the viral genome is converted into double-stranded DNA form, some of which integrates into host chromosomal DNA as a provirus (29). Unintegrated viral DNA and proviral DNA have been molecularly cloned and shown to be biologically infectious (1, 10, 23). The complete nucleotide sequence of a molecular clone of unintegrated Mo-MLV DNA, referred to as pMLV-1, has been determined (22). The Mo-MLV genome contains 8,332 nucleotides which encode *gag*, *pol*, and *env* proteins (2, 22). pMLV-1, however, is not biologically active. The defect(s) has been localized in the 5′ two-thirds of the viral genome, which includes the *gag* gene and part of the *pol* gene (2).

The *pol* protein of Mo-MLV is derived from a single polyprotein precursor which also contains *gag*-related amino acid sequences. The precise mechanism of synthesis of the *gag-pol* polyprotein is not understood, but the general consensus is that it results from translational readthrough of the stop codon at the end of the *gag* gene (4, 22). In vitro translation experiments involving yeast suppressor tRNAs which enhance the synthesis of *gag-pol* polyprotein also support this notion (15, 16). Sequence analysis of pMLV-1 indicates that a *gag-pol* polyprotein can be synthesized by this mechanism (22). Additionally, restriction endonuclease analysis revealed that there are no major alterations in the 5′ sequence of pMLV-1 as compared with corresponding sequences of infectious molecules (1, 2). Thus, there are no apparent reasons for the lack of infectivity of pMLV-1.

To define the lesion(s) in pMLV-1, we took an approach which involved replacing portions of pMLV-1 with corresponding DNA fragments obtained from an infectious clone, pMLV-48. The resulting chimeric plasmids were analyzed for their ability to produce infectious virus after transfection into cells. The results show that a 1.0-kilobase-pair DNA fragment from pMLV-48, which spans the *gag-pol* junction, restores infectivity to pMLV-1. Furthermore, sequence analysis of this fragment from the infectious clone and from pMLV-1 reveals two base pair changes.

* Corresponding author.

MATERIALS AND METHODS

DNA transfection. All mammalian cells used in this study were maintained in Dulbecco/Vogt modified Eagle medium supplemented with 10% fetal bovine serum (Sterile Systems, Logan, Utah). NIH 3T3 cells lacking thymidine kinase (TK) activity (TK^-) (28) were plated at 5×10^5 to 1×10^6 cells per 5-cm dish 1 day before transfection. The cells were transfected with DNA, using the calcium phosphate precipitation technique of Graham and van der Eb (7) as modified by Corsaro and Pearson (3). Test DNA was either gradient-purified plasmid DNA or unpurified DNA ligation products. Test DNA plus 10 μg of carrier DNA (from NIH 3T3 TK^- cells) in 0.4 ml of transfection buffer was applied to cells after the culture medium was replaced with 4 ml of fresh medium.

Virus infection. Recipient NIH 3T3 TK^- cells were plated at 5×10^5 to 1×10^6 cells per 5-cm dish 1 day before virus infection. Immediately before infection, the culture medium was replaced with 4 ml of fresh medium containing 4 μg of polybrene per ml. Culture medium containing virus was centrifuged at 5,000 × *g* for 5 min to remove cells and filtered through 0.45-μm filters (Gelman Sciences, Inc., Ann Arbor, Mich.), and samples were added to the recipient cells.

XC plaque assay. The number of cells producing virus after DNA transfection or after viral infection was quantitated by using the XC plaque assay (12, 20). At 16 h after DNA transfection or viral infection of NIH 3T3 TK^- cells, the cells were split 1:10 into 5-cm dishes. Three days later, the cells were killed by UV irradiation and overlaid with 10^6 XC cells. Two days after XC cell overlay, the cells were stained with Coomassie blue (1 g/liter in 40% MeOH–10% acetic acid), and plaques were counted. The NIH 3T3 TK^- cell line was chosen as the virus recipient in this assay because of the large, well-defined XC plaques produced after a 3-day incubation.

Recombinant DNA constructs. DNA fragments generated by restriction endonuclease digestion of plasmid DNA were separated by agarose gel electrophoresis and purified from

the agarose by adsorption to glass powder (27). For linear constructs requiring ligation of two DNA fragments, one of the fragments was treated with bacterial alkaline phosphatase (BAP) before ligation to reduce unintended ligation products. For linear constructs requiring ligation of three fragments, the two terminal fragments were treated with BAP before ligation. In both cases, restriction endonuclease sites at the termini of the constructs were chosen to minimize interference with ligation of internal restriction endonuclease-generated DNA termini. Ligations were performed by using T4 DNA ligase, and the unpurified products were used directly to transfect cells.

Plasmid constructs were made in pBR322. In two or three fragment ligations, the plasmid fragment carrying the drug marker(s) was treated with BAP before ligation. Ligated DNA was used directly to transform *Escherichia coli* DH-1 (9). Bacterial colonies were screened for the correct drug resistance and restriction enzyme pattern. Plasmid DNA used for transfection was prepared by detergent lysis of chloramphenicol-treated bacteria followed by cesium chloride-ethidium bromide ultracentrifugation (17).

DNA sequencing. DNA sequencing was performed by the chemical degradation method (14). DNA fragments were labeled with ^{32}P at both ends and were either cut with another restriction endonuclease or subjected to strand separation to give DNA fragments labeled at a unique end. Corresponding DNA fragments from pMLV-48 and pMLV-1 were electrophoresed next to each other on sequencing gels to simplify detection of differences between the clones and to minimize the chance of an extra base or base deletion being undetected.

Reverse transcriptase assay. Reverse transcriptase in cell culture medium was measured as described previously (26). One unit of activity is that amount of reverse transcriptase which catalyzes incorporation of 1 pmol of [^{3}H]dGMP in 1 h (1 pmol = 10^3 cpm). The detection limit is ca. 0.1 unit per ml of culture medium.

Immunoprecipitation of viral proteins. Cells were seeded at 10^6 cells per 35-mm dish 1 day before labeling. For labeling, cells were preincubated in Dulbecco/Vogt modified Eagle medium minus methionine (without serum) for 30 min and then were incubated for 1 h in the same medium plus 200 μCi of [^{35}S]methionine (New England Nuclear Corp.; >800 Ci/mmol). The cells were washed, dissolved in RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, and 10 mM Tris [pH 7.4]), and viral proteins were immunoprecipitated as previously described (21) by using goat antisera raised against disrupted Rauscher murine leukemia virus or the virion proteins p30 and gp70 (antibodies were obtained through the National Cancer Institute). Immunoprecipitates were electrophoresed in 10% acrylamide gels in the presence of sodium dodecyl sulfate (13), and labeled proteins were detected by autoradiography of vacuum-dried gels.

RESULTS

Construction and infectivity of recombinants. The structures of the two parental clones, pMLV-1 and pMLV-48, from which all of the recombinants were derived, are shown in Fig. 1. pMLV-1, which was cloned from an unitegrated circular DNA, is permuted with respect to the viral genome (2). As previously shown, the portion of pMLV-1 which is 3′ of the *Hin*dIII site is not responsible for the lack of infectivity of pMLV-1, since ligation to the 5′ portion of pMLV-48 (Fig. 1A) generated infectious virions. The converse construct (Fig. 1B) containing the 5′ portion of pMLV-1 and the 3′ portion of pMLV-48 was not infectious. Control ligations with the *Hin*dIII site showed that pMLV-48 (Fig. 1C) was infectious and that the nonpermuted form of pMLV-1 (Fig. 1D) was noninfectious, as measured by the XC plaque assay. Thus, the region 5′ of the *Hin*dIII site in pMLV-1 is responsible for its lack of infectivity.

To localize more precisely the sequences in pMLV-1 which rendered it noninfectious, we performed experiments similar to those described above, using the unique *Xho*I and *Sal*I endonuclease sites in Mo-MLV. Portions of pMLV-1 were exchanged with DNA fragments obtained from infectious pMLV-48. Constructs E and F in Fig. 1 localized the defect(s) in pMLV-1 to the region 5′ of the *Sal*I site. Constructs G and H in Fig. 1 further localized the lesion(s) in pMLV-1 to the region 3′ of the *Xho*I site, although the number of plaques produced by construct H was abnormally low. Thus, the defect(s) in pMLV-1 is located between the *Xho*I (nucleotide 1,560 from the 5′ cap of the viral genome [22]) and the *Sal*I (nucleotide 3,705) sites.

To confirm and extend the results obtained by using mixtures of ligated DNA molecules, we molecularly cloned the appropriate DNA constructs in pBR322 (Fig. 2). Compared with the ligated DNA mixtures, these recombinant plasmids gave much higher numbers of XC plaques per microgram of DNA due to their homogeneity. Construct I, the nonpermuted form of MLV-1 (see also construct D in Fig. 1), remained noninfectious. Construct J is similar to construct H in Fig. 1 and gave a high ratio of XC plaques per microgram of DNA. This result confirms that pMLV-1 is not defective 5′ of the *Xho*I site. Recombinant clones K and L, which have internal portions of the infectious clone pMLV-48 substituted into pMLV-1 (construct I), produced infectious virions. We conclude from construct L that the lesion(s) affecting the infectivity of pMLV-1 lies in the *Xho*I to *Sst*I region (nucleotides 1,560 to 2,554). In addition, since construct M (Fig. 2) did not produce XC plaques, we conclude that at least part of the lesion in pMLV-1 is located between the *Bgl*II (nucleotide 1,906) and *Sst*I (nucleotide 2,554) sites.

As a further check on the infectivity of construct L as compared with construct I or pMLV-48, cells transfected with these three DNAs were passaged for 2 weeks to allow for virus spread. At the end of this period, virus titers in the supernatant media were determined by XC plaque assay. The virus titer in supernatant from cells transfected with construct L (8×10^6 per ml) was not significantly different from that of cells transfected with pMLV-48 (10^7 per ml). No virus was detected in supernatant from cells transfected with construct I, the linear form of pMLV-1.

Nucleotide sequence analysis of *Xho*I to *Sst*I fragment. The replacement of the *Xho*I-*Sst*I fragment of pMLV-1 with the corresponding fragment obtained from pMLV-48 renders the plasmid infectious. Nucleotide sequence analysis of pMLV-1 reveals no apparent reason for the lack of infectivity of constructs containing the *Xho*I-*Sst*I fragment from pMLV-1. Furthermore, based on restriction endonuclease analysis, no major sequence alterations in this fragment were anticipated. Hence, we determined the nucleotide sequence of the *Xho*I-*Sst*I fragment obtained from pMLV-48 and compared it with the nucleotide sequence of the corresponding fragment obtained from pMLV-1. The sequences of the two fragments differed by only two bases (Fig. 3). Our sequence for pMLV-1 in this region agrees exactly with the previously published sequence (22). The first base difference at position 1,849 is a transition (TAG*A*GAG in pMLV-48 to TAG*G*GAG in

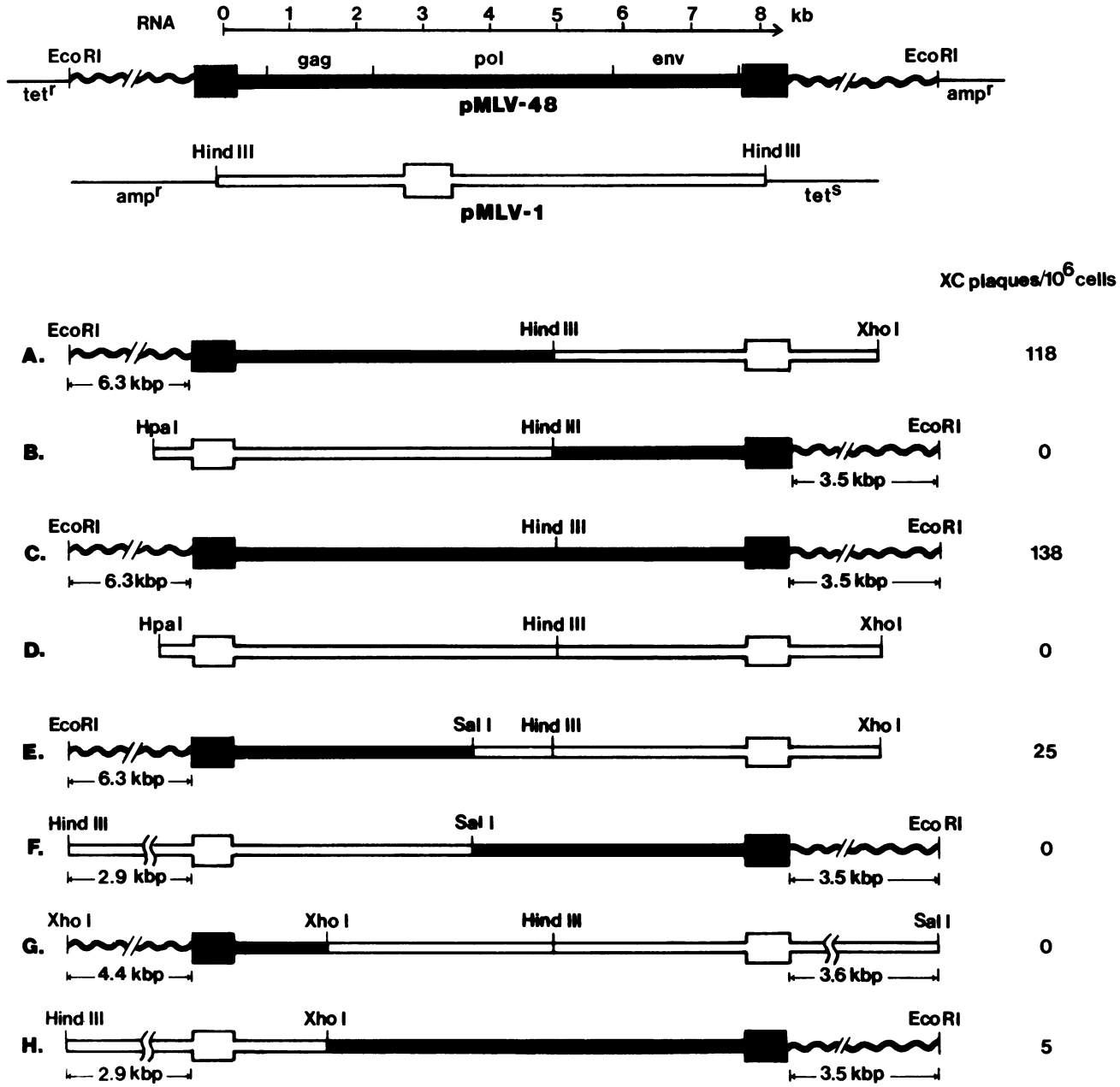

FIG. 1. In vitro DNA constructs. pMLV-48 and pMLV-1, from which these recombinant molecules were derived, are shown at the top of the figure. The 20-kilobase-pair (kbp) *Eco*RI insert of a molecular clone of Mo-MLV in Charon 4A (λ.48, reference 1) was subcloned in the *Eco*RI site of pBR322 to make pMLV-48. To make pMLV-1, the unintegrated circular form of Mo-MLV DNA was cleaved at the unique *Hin*dIII site and molecularly cloned in Charon 21A. The 8.35-kbp *Hin*dIII insert was then subcloned into the *Hin*dIII site of pBR322. For construction of the following recombinant molecules, plasmid DNAs were cleaved with appropriate restriction endonucleases and fractionated by electrophoresis on agarose gels; the DNA fragments were purified from the gel by adsorption to glass powder, and appropriate fragments were ligated. Construct A: pMLV-48 was digested with *Eco*RI and *Hin*dIII, and pMLV-1 was digested with *Hin*dIII and *Xho*I. An 8-μg portion of the 12-kbp *Eco*RI-*Hin*dIII pMLV-48 5′ fragment was mixed with 4 μg of BAP-treated 4.9-kbp *Hin*dIII-*Xho*I pMLV-1 3′ fragment and incubated with 10 U of T4 DNA ligase at 14°C for 16 h. Portions of the ligated DNA were analyzed on agarose gel to confirm that ligation had occurred. The ligation products were used directly for transfection. A similar procedure was followed for all other constructs. Construct B: pMLV-1 was cleaved with *Hin*dIII and *Hpa*I, and pMLV-48 was cleaved with *Hin*dIII and *Eco*RI. The *Hpa*I-*Hin*dIII 5′ fragment of pMLV-1 was ligated to the BAP-treated *Hin*dIII-*Eco*RI 3′ fragment of pMLV-48. Construct C: The *Eco*RI-*Hin*dIII 5′ portion of pMLV-48 was ligated to the BAP-treated *Hin*dIII-*Eco*RI 3′ portion of pMLV-48. Construct D: pMLV-1 was cleaved with *Hin*dIII and *Xho*I to isolate the 3′ portion, and with *Hin*dIII and *Hpa*I to isolate the 5′ portion of the molecule. The 5′ portion was ligated to the BAP-treated 3′ fragment. Construct E: The *Eco*RI-*Sal*I fragment from pMLV-48 was mixed with the *Sal*I-*Hin*dIII and *Hin*dIII to *Xho*I fragments of pMLV-1 in a three-way ligation reaction. A three-way ligation was required because of the permuted form of pMLV-1. The low number of XC plaques compared with constructs A and C most likely reflects the fact that in a three-way ligation, the number of desired products is lower than in a two-way ligation. Construct F: The *Hin*dIII-*Sal*I 5′ fragment of pMLV-1 and the *Sal*I-*Eco*RI fragment of pMLV-48 were ligated. Construct G: Three-way ligation between the *Xho*I 5′ fragment from pMLV-48 and the *Xho*I-*Hin*dIII and *Hin*dIII-*Sal*I fragments of pMLV-1. Construct H: 5′ sequences in a *Hin*dIII-*Xho*I fragment of pMLV-1 were ligated to the *Xho*I-*Eco*RI 3′ fragment of pMLV-48. The number of plaques produced from this construct was abnormally low, but the infectivity of this viral construct was later confirmed (Fig. 2J). Only the restriction endonuclease sites used in the constructions are shown. Open lines: pMLV-1; closed lines: pMLV-48; wavy lines: flanking cellular sequences of pMLV-48; open and closed boxes: long terminal repeats.

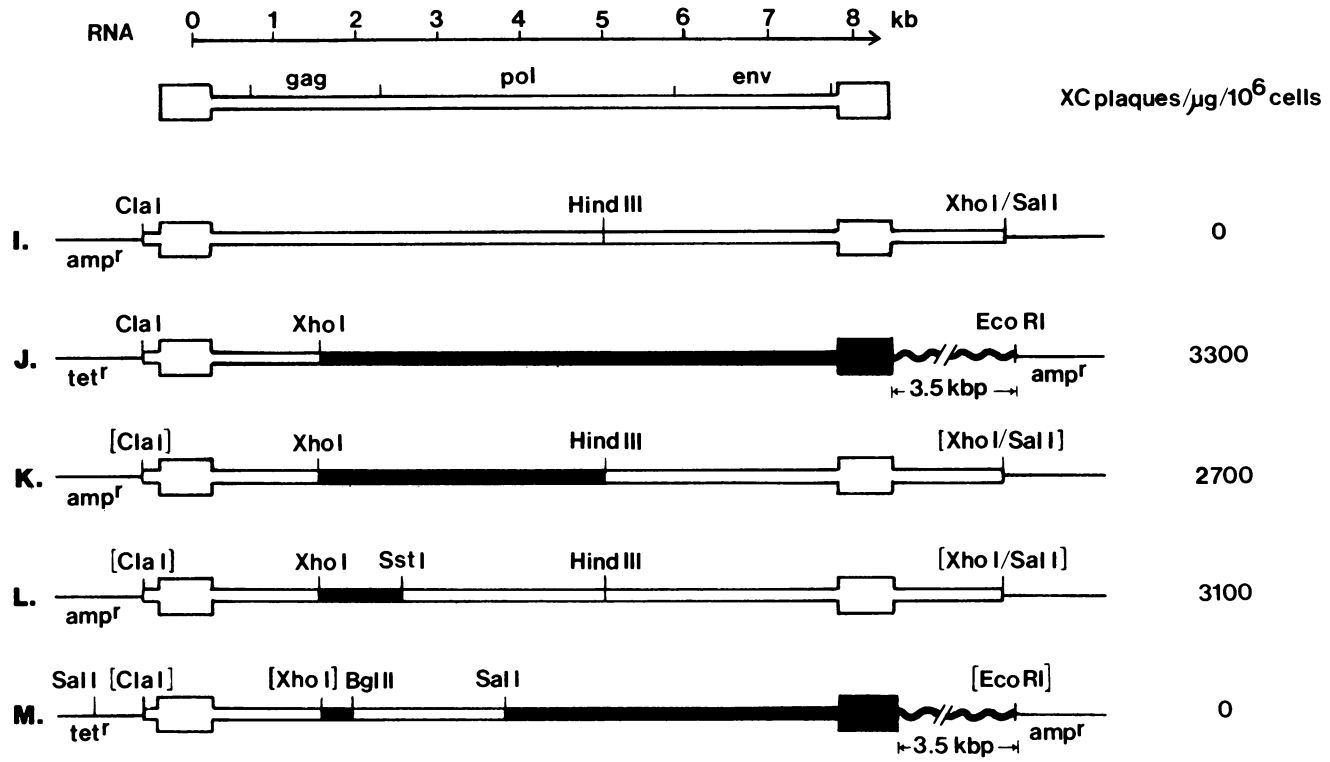

FIG. 2. Construction of recombinant plasmids. DNA fragments isolated from pMLV-1, pMLV-48, and pBR322 were combined to make these recombinant plasmids. Restriction endonuclease sites used for construction of recombinants are shown. The restriction endonuclease sites with brackets are included for clarity. The drug resistance of the recombinant plasmids and the number of XC plaques per microgram of plasmid DNA per 10^6 cells are shown. Construct I: Since pMLV-1 is permuted with respect to viral genome, a nonpermuted clone was constructed. The *Cla*I-*Hin*dIII fragment and the *Hin*dIII-*Xho*I fragment of pMLV-1 were mixed with the BAP-treated *Sal*I-*Cla*I pBR322 fragment of 3.7 kilobase pairs (kbp) (containing the amp^r gene) and ligated. Construct J: The *Cal*I-*Xho*I 5′ fragment of pMLV-1, the *Xho*I-*Eco*RI 3′ fragment of pMLV-48, and the BAP-treated *Cla*I-*Eco*RI fragment of pBR322 (containing the amp^r gene) were ligated. The resulting construct was also partially tet^r. Construct K: Construct I was cleaved with *Xho*I and *Hin*dIII, and the larger of the two resulting fragments was isolated and treated with BAP. Construct J was cleaved with *Xho*I and *Hin*dIII, and the 3.3-kbp internal *Hin*dIII-*Xho*I fragment derived from pMLV-48 was isolated. These two fragments were ligated and used to transform bacteria. Construct L: Construct I was cleaved with *Xho*I and *Hin*dIII, and the larger of the two resulting fragments was isolated and treated with BAP. Construct I was also cleaved with *Hin*dIII, *Sst*I, and *Xho*I, and the internal 2.3-kbp *Sst*I-*Hin*dIII fragment was isolated. Construct J was cleaved with *Hin*dIII, *Sst*I, and *Xho*I, and the internal 1.0-kbp *Xho*I-*Sst*I fragment was isolated. These three fragments were ligated to yield construct L. Construct M: Construct J was cleaved with *Sal*I and *Xho*I, and the largest of the three resulting fragments, containing 3′ pMLV-48-derived sequences and pBR322 sequences (including the amp^r gene), was isolated and treated with BAP. Construct J was also cleaved with *Sal*I and *Bgl*II, and the 3.1-kbp *Sal*I-*Bgl*II fragment, containing 5′ viral and pBR322 sequences, was isolated. pMLV-1 was cleaved with *Sal*I and *Bgl*II, and the internal 1.8-kbp *Bgl*II-*Sal*I fragment was isolated. These three fragments were ligated. The manipulations described for this construct were necessary because of the multiple *Bgl*II sites in Mo-MLV. Transformed bacteria were selected in ampicillin, although construct M also codes for partial tetracycline resistance. All of the ligation mixtures described above were used directly to transform DH-1 bacteria. All recombinant plasmids were cleaved with appropriate enzymes to verify the nature of the constructs.

pMLV-1) that changes the glutamic acid in pMLV-48 to glycine in pMLV-1. This amino acid change occurs in the p30^{gag} protein. The second change, a transversion (GGA*G*CCC in pMLV-48 to GGA*C*CCC in pMLV-1) occurs at position 2,255, altering a predicted glutamic acid residue in pMLV-48 to aspartic acid in pMLV-1. The base change at nucleotide 2,255 is in the region between the end of the *gag* coding region and the NH_2 terminus of the reverse transcriptase coding region (S. Oroszlan, personal communication).

Intracellular viral protein analysis. Nucleotide sequence analysis reveals two base changes which affect the infectivity of pMLV-1, neither of which affects the reading frames of the viral proteins. To test whether constructs I and M synthesize viral proteins, we performed the following experiment. Each plasmid containing a viral construct was cotrans-

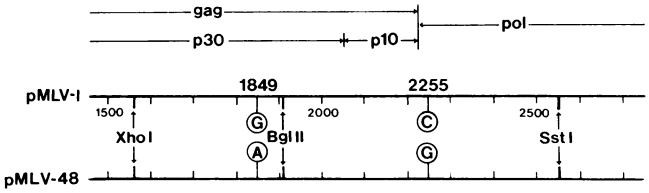

FIG. 3. Nucleotide alterations. Homologous regions of DNA from pMLV-1 and pMLV-48 were sequenced, including the *Xho*I to *Sst*I region responsible for the difference in infectivity of the two clones of Mo-MLV. The nucleotide numbering system is that used by Shinnick et al. (22). Base differences are indicated by circled letters, and the nucleotide numbers are shown. Landmark restriction endonuclease sites are indicated. Mo-MLV protein coding regions are shown at the top of the figure.

fected into NIH 3T3 TK$^-$ cells with a plasmid containing the herpesvirus TK gene. The plasmid containing the construct was in 10-fold excess. Colonies having TK activity (TK$^+$) were selected in HAT medium (30 μM hypoxanthine, 1 μM amethopterin, 20 μM thymidine), and multiple colonies were pooled. Such a cotransfection procedure ensures that a relatively high percentage of the cells contain viral construct DNA (30). In similar studies involving two selectable markers, we have previously found that approximately 10% of the cells selected for expression of one selectable marker also express the other selectable marker transfected in 10-fold excess (data not shown). Cells from the pooled TK$^+$ colonies were analyzed for viral proteins by immunoprecipitation with antisera to whole virus, p30^{gag} and gp70^{env}. Figure 4 shows the results obtained with constructs I, L, and M. Cells transfected with construct I appeared to synthesize small amounts of viral Pr65^{gag} and gPr80^{env} proteins (panels B and D) compared with infectious virus (construct L, panel F). This difference in protein levels is expected because infectious virus can spread and infect every cell in the population, whereas the noninfectious virus should be present in only ca. 10% of the cells. Pr180$^{gag\text{-}pol}$ was not detected in these cells. Instead, a 90,000-dalton protein (shown by an asterisk in panel D) was precipitated with sera directed against either whole virus or p30^{gag}. In contrast, cells transfected with construct M synthesized amounts of Pr65^{gag}, Pr180$^{gag\text{-}pol}$, and gPr80^{env} viral proteins comparable to infectious virus (Fig. 4, panels E and F). This result suggests that construct M is infectious, even though it was not detected in the XC assay. Hence, we tested for infectious virions in the supernatant from cotransfected cells. Supernatant medium (1.0 ml) from cotransfected cells was used to infect fresh NIH 3T3 TK$^-$ cells, which were passaged for 1 week, followed by immunoprecipitation of labeled proteins. No viral proteins could be detected in cells infected with supernatant obtained from either control cells or cells transfected with construct I. However, in cells infected with supernatant from cells cotransfected with either XC-positive construct L or XC-negative construct M, similar levels of viral proteins could be detected (Fig. 5, lanes 3 and 4). It thus appears that no infectious virus is released from cells transfected with pMLV-1 (construct I), whereas infectious virus is released from cells transfected with construct L or M. However, the virus released from cells transfected with construct M, which has only a single base change in the *pol* gene, is XC negative. We conclude that the inability of construct I (pMLV-1) to generate virus is due to the mutation in the *gag* gene at position 1,849.

The supernatant media from cells cotransfected with TK and construct L or M were analyzed for reverse transcriptase activity as another measure of virus production. Supernatant from cells cotransfected with construct L had nearly threefold higher activity (23 units) compared with supernatant from cells cotransfected with construct M (8 units), which had undergone a single base pair change in the *pol* gene. No reverse transcriptase activity could be detected in supernatant from control untransfected cells or cells cotransfected with TK and construct I (pMLV-1, Fig. 2). These results support the conclusions that constructs L and M are infectious and that construct I (pMLV-1) is noninfectious.

Structural analysis of the region containing the *pol* gene mutation. The mutation in the *pol* region does not alter infectivity, but it affects the ability of the virus to form XC plaques and probably affects the titer of virus produced by cells, as reverse transcriptase production from cells transfected with construct M is reduced in comparison with cells transfected with construct L. We noticed that the single base change in the *pol* region (position 2,255) had a profound effect on the mobility of single-stranded DNA containing this alteration in nondenaturing polyacrylamide gels. For example, *Bgl*II-*Sst*I DNA fragments obtained from pMLV-1 and pMLV-48 and labeled with ^{32}P at the *Bgl*II terminus on the 5′ DNA strand (Fig. 4C, row 1) were denatured with dimethyl sulfoxide, and the resulting single-stranded molecules were

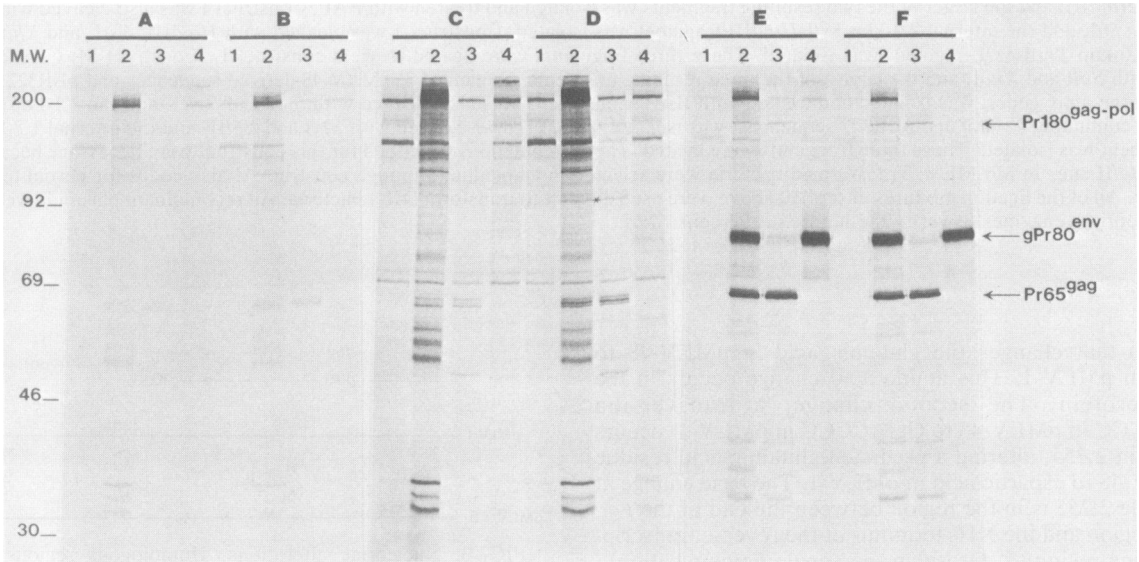

FIG. 4. Immunoprecipitation of viral proteins in cotransfected cells. Autoradiographs of immunoprecipitated ^{35}S-labeled viral proteins from NIH 3T3 TK$^-$ cells cotransfected with TK and various constructs are shown. Immunoprecipitation was carried out by using normal goat sera (lane 1) or antisera to whole virus (lane 2), p30^{gag} (lane 3), and gp70^{env} (lane 4). (A and C) Cells transfected with the TK gene only. (B and D) Cells cotransfected with TK and construct I. (E) Cells cotransfected with construct M. (F) Cells transfected with construct L. Panels A, B, E, and F resulted from equal exposure times, and panels C and D resulted from a 10-fold-longer exposure. The asterisk in panel D indicates the position of the putative 90,000-dalton *gag-pol* protein.

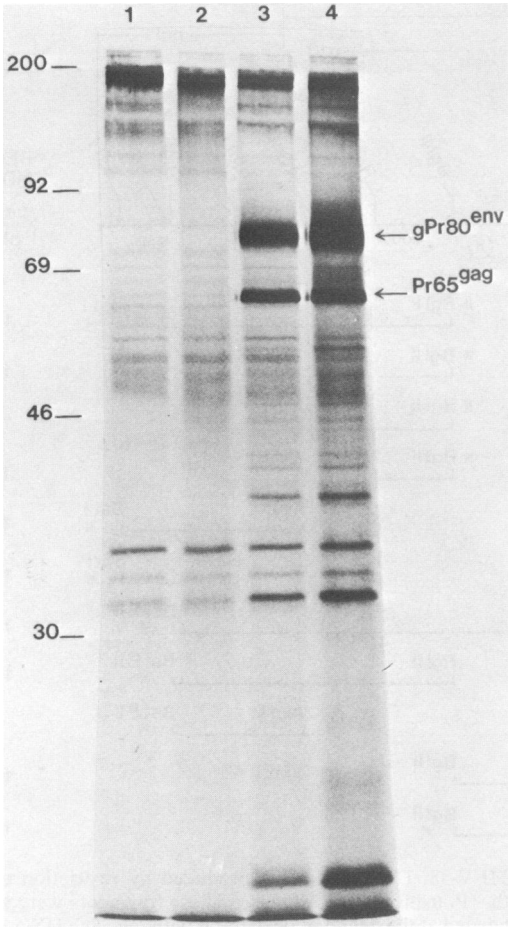

FIG. 5. Immunoprecipitation of viral proteins in infected cells. Autoradiographs of immunoprecipitated ^{35}S-labeled proteins are shown. Immunoprecipitation was performed by using whole virus antisera. Lane 1: Cells exposed to media from TK-transfected cells. Lane 2: Cells exposed to media from cells cotransfected with TK and construct I. Lane 3: Cells exposed to media from cells cotransfected with TK and construct M. Lane 4: Cells exposed to media from cells cotransfected with TK and construct L.

subjected to electrophoresis. Because dimethyl sulfoxide does not enter the gel, folding of the molecules can occur once the single-stranded molecules enter the gel. Only the 5′-*Bgl*II-*Sst*I-3′ DNA strand is detected on the autoradiograph of the gel because only the 5′ end of this strand is labeled. The labeled pMLV-48 single-stranded molecule moved 1.8 times faster than the corresponding pMLV-1 molecule in a nondenaturing gel run at room temperature (20°C) (Fig. 4A, lanes 1 and 3). A mixture of the pMLV-1 and pMLV-48 *Bgl*II-*Sal*I fragments was also denatured and electrophoresed to show that some unknown variable was not affecting the electrophoretic mobilities of the single strands (lane 2). When electrophoresis was carried out at relatively high temperatures (60 to 70°C), a difference in mobility could no longer be discerned (Fig. 6B). These results indicate that secondary structure, which is minimized at high temperature, is responsible for the mobility difference observed at low temperature. In comparisons of other single-stranded fragments from pMLV-1 and pMLV-48, differences in mobility are observed only for fragments containing the base changes at position 2,255 (Fig. 6C, rows 1, 2, 5, 7, 8, 9), not in those that do not contain the altered base (Fig. 6C, rows 3, 4, 6). For comparison, single-stranded fragments containing the base pair change at base 1,849 displayed no difference in mobility (Fig. 6C, rows 10, 11), showing that a single base does not always result in a change in mobility. Thus, the single base change at position 2,255 may influence the secondary structure of the viral mRNA (see below).

DISCUSSION

Two base pair changes restore infectivity. Infection by murine leukemia viruses leads to stable integration of viral DNA into host chromosomal DNA. The proviral DNA is transcribed by RNA polymerase II, and both 35S genome length and 24S subgenomic mRNA have been detected in infected cells (4). Formation of infectious virions requires the presence of *gag*, *pol*, and *env* proteins as well as intact 35S genome length viral RNA. We have reported the molecular cloning of unintegrated circular forms of Mo-MLV DNA (2). Unfortunately, the molecular clone pMLV-1 was not biologically infectious, and the lesion was localized in the 5′ two-thirds of the molecule (2). The restriction mapping and nucleotide sequence analysis of pMLV-1 did not reveal any clues as to why it should be noninfectious (2, 22). To localize the lesion, we used a strategy which involved substituting pMLV-1 DNA fragments with corresponding fragments obtained from an infectious clone (pMLV-48). Transfection by such recombinants showed that when the *Xho*I-*Sst*I fragment of pMLV-1 is substituted with the *Xho*I-*Sst*I fragment of pMLV-48, infectious virus particles are released. Thus, the lesion in pMLV-1 can be localized between nucleotide 1,560 and nucleotide 2,554 from the 5′ end of the genome.

The *Xho*I-*Sst*I fragment spans the *gag-pol* junction. Nucleotide sequence analysis of this fragment obtained from pMLV-1 and from infectious pMLV-48 revealed two base changes. One of the base changes occurs at position 1,849 which is in the p30^{gag} gene, whereas the other change occurs at position 2,255, which is in the *pol* gene. The mutation at position 2,255 in pMLV-1 generates an additional site for restriction enzyme *Ava*II. Analysis of corresponding fragments from pMLV-1 and pMLV-48 confirms this observation, lending further support to the nucleotide sequence analysis.

***gag* gene mutation.** The mutation in the *gag* gene of pMLV-1 at position 1,849 results in a glycine residue in place of the glutamic acid residue in pMLV-48. It is worth noting that nucleotide sequences of two strains of Moloney murine sarcoma virus, in which the p30^{gag} gene is similar to that in Mo-MLV, the corresponding nucleotide is that of pMLV-48 (19, 25). Thus, the change at position 1,849 does not merely reflect polymorphism but probably is a mutational event. Our results show that pMLV-1 (construct I), which contains the *gag* and *pol* mutations, is noninfectious by a variety of criteria. On the other hand, construct M, which contains the single base pair alterations in the *pol* region, generates infectious virus after transfection into cells. In addition, proteins synthesized from construct M are similar in amount and molecular weight to proteins synthesized by the infectious, XC-positive construct L. Therefore, we conclude that the proper base at position 1,849 in the *gag* gene is crucial for infectivity. Viral protein analysis suggests that loss of infectivity in construct I (pMLV-1) may be related to the synthesis of altered *gag*-related proteins; in particular, a new

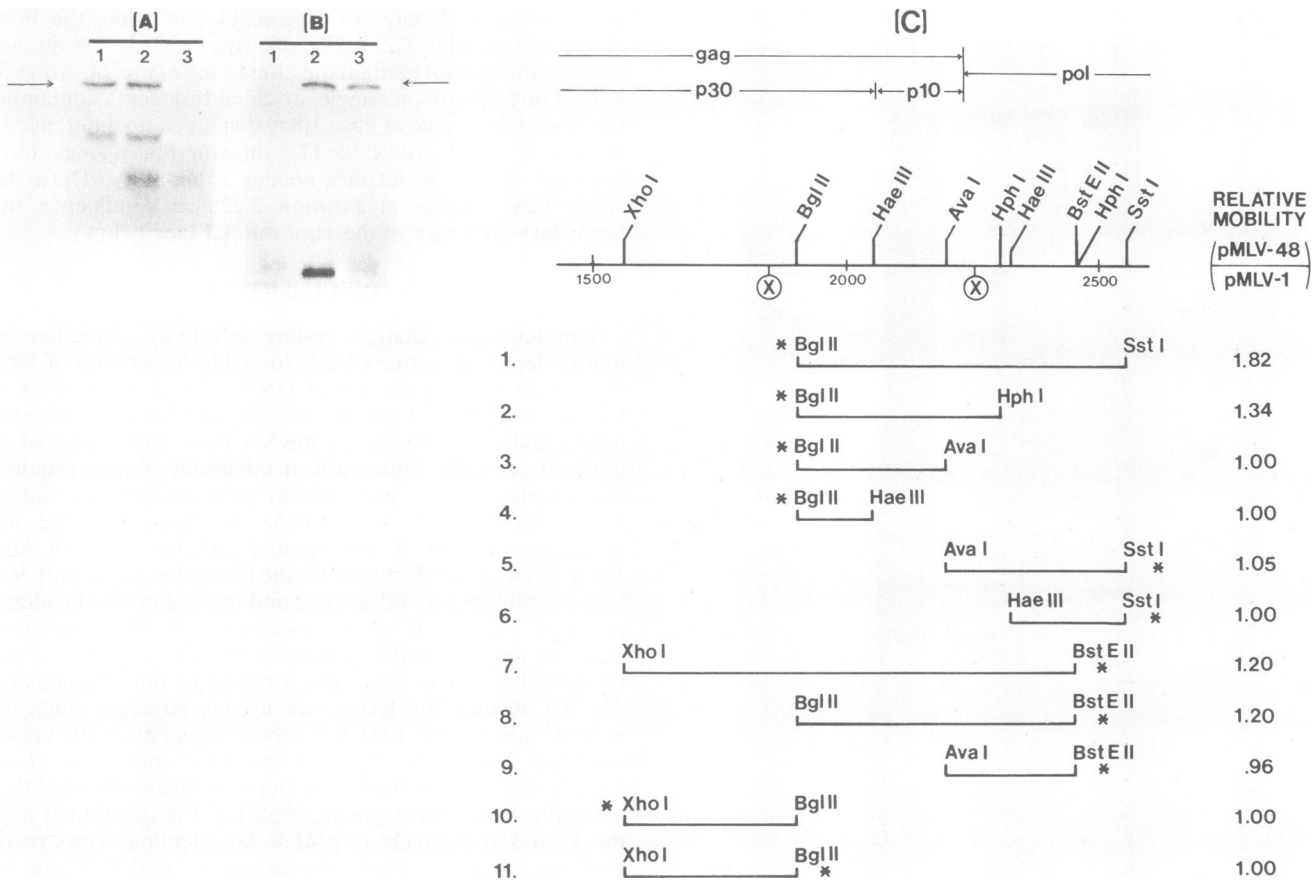

FIG. 6. Single-stranded DNA mobility differences between pMLV-1 and pMLV-48. DNA fragments produced by restriction enzyme cleavage of plasmid DNA were treated with BAP and labeled at their 5′ ends with ^{32}P, using T4 DNA kinase. These fragments were cleaved with additional restriction enzymes to generate double-stranded DNA molecules labeled at the 5′ end of one strand with ^{32}P. The DNA strands were separated by boiling in 10 μl of water plus 20 μl of dimethyl sulfoxide for 2 min, and 10 μl of dye solution (0.04% [wt/vol] bromophenol blue in 2 mM EDTA) was added. The samples were immediately loaded onto a 10% polyacrylamide gel (62.5:1, polyacrylamide to bisacrylamide) and electrophoresed with a Tris-borate-EDTA buffer (6 g of Tris base, 0.5 g of EDTA disodium salt, and 3 g of boric acid per liter). The labeled DNA molecules were detected by autoradiography. (A) Electrophoresis at room temperature (20°C). (B) Electrophoresis at high temperature (60 to 70°C). In both panels A and B, the DNA in lane 1 was derived from pMLV-1, the DNA in lane 2 was derived from pMLV-1 and pMLV-48, and the DNA in lane 3 was derived from pMLV-48. The migration distance of the bromophenol blue dye was the same for the autoradiographed gels shown in both panels. The arrows mark the top of the gels. (C) Relative mobilities of different end-labeled single-stranded DNAs are shown. Asterisks indicate the labeled (5′-^{32}P) termini.

90,000-dalton protein is present in cells cotransfected with construct I which is not present in control cells or in cells cotransfected with infectious virus (Fig. 4). It is not clear how the single amino acid change in $p30^{gag}$ renders pMLV-1 noninfectious. It is worth noting that human c-ras^{H} gene can be activated to transform NIH 3T3 cells by virtue of a single base change at position 12 from the N-terminal end (18, 24).

Despite an extensive knowledge of the structure and serology of the $p30^{gag}$ protein, little is known about its role in the life cycle of retroviruses. Oligonucleotide mapping shows that the specificity for N/B tropism of the virus is determined by the $p30^{gag}$ protein. N/B tropism appears to be due to the inability of the restricted virus to form supercoiled viral DNA in the nonpermissive cells (5, 6, 11). Thus, $p30^{gag}$ may play a crucial role in viral DNA replication or integration into chromosomal DNA. Alternatively, $p30^{gag}$ may play a role in virion assembly and subsequent maturation. The single base alteration in pMLV-1 in the $p30^{gag}$ gene, which renders pMLV-1 noninfectious, may be useful in elucidating the role of $p30^{gag}$ in the life cycle of retroviruses.

***pol* gene mutation.** The mutation in the *pol* gene at position 2,255 appears to be located at a region which is 5′ of the N terminal of the Mo-MLV polymerase (S. Oroszlan, personal communication). No viral protein has yet been assigned to this region. The single base change alters the predicted glutamic acid in pMLV-48 to aspartic acid in pMLV-1. The base pair at position 2,255 of pMLV-48 is the same as in clone $pMLV_{int}$-1 (C. Van Beveren, personal communication) which also restores infectivity to pMLV-1 (2). Again, two strains of Moloney murine sarcoma virus contain the base present in pMLV-48 at this position (19, 25). Thus, the base present in pMLV-1 probably represents a mutation. Cotransfection studies indicate that all viral proteins can be synthesized by construct M, which contains the *pol* mutation. Infectious virus is released by cells transfected with construct M as assayed by reverse transcriptase activity and by infection of fresh NIH 3T3 cells. The mutation at position 2,255, however, renders the infectious virus XC negative. The mechanism of formation of XC plaques by murine leukemia viruses is not fully understood, but the *env* protein

has been implicated in the fusion of XC cells (31). It is interesting that construct M, with a lesion in the *pol* gene, is unable to form typical XC syncytia. The anomalous electrophoretic mobility of the single-stranded DNA fragments containing the base change at position 2,255 indicates that the RNA transcripts of pMLV-1 may have altered secondary structures. Computer analysis of this region for hairpin loops reveals that the largest loops have altered structure at the termini of the loops due to the base change of G to C, including the hairpin loop noted by Shinnick et al. at the *gag-pol* junction (22). The altered secondary structure may affect virion RNA packaging and thus the amount of virus released by the cells, which would account for the reduced reverse transcriptase activity in supernatant from cells transfected with construct M.

Fine structural analysis of biological macromolecules has contributed much to our understanding of the structure and expression of genes. Knowledge of the nucleotide sequence of Mo-MLV is essential to an understanding of the role of various viral genes in virus replication. The availability of molecularly cloned pMLV-1 allowed the determination of its complete nucleotide sequence. Unfortunately, pMLV-1 is not biologically active, and thus its utility to construct recombinants delineating various regions of the viral genome is limited. It is therefore highly desirable to obtain the nucleotide sequence of a biologically active Mo-MLV DNA. Instead of determining the complete sequence of a biologically active molecular clone of Mo-MLV, we decided to determine the sequence of the DNA fragment of infectious pMLV-48 which restored infectivity of pMLV-1. Reconstruction of the nucleotide sequence of pMLV-1 and the DNA fragment from the infectious clone represents the nucleotide sequence of an infectious Mo-MLV (construct L).

In summary, we have shown that biological infectivity of pMLV-1 can be restored by replacing its *Xho*I-*Sst*I fragment with a corresponding fragment obtained from an infectious clone. There are only two base pair changes in this fragment, one of which is the $p30^{gag}$ gene and the other in the *pol* gene. pMLV-1 does not produce infectious virus. Constructs containing only the base pair change in the *pol* gene are able to generate infectious viruses, but these do not form XC plaques. We conclude that the mutation in the *gag* gene renders pMLV-1 totally noninfectious, whereas the mutation in the *pol* gene generates an infectious virus which is unable to make XC plaques. Future analysis of these base pair changes by making constructs which have only the change in the *gag* gene will further elucidate the mechanism of replication of Mo-MLV.

ACKNOWLEDGMENTS

We thank Hung Fan and Lee Bacheler for providing us with a lambda clone of Mo-MLV (λ.48). We thank Charles Van Beveren and Tom Curran for their many helpful discussions, Lisa Zokas for her technical assistance, and Sandy Haight for her illustrations.

A.D.M. is a fellow of the Leukemia Society of America. This work was supported by grants from the American Cancer Society and the National Institutes of Health (to I.M.V.).

LITERATURE CITED

1. **Bacheler, L., and H. Fan.** 1981. Isolation of recombinant DNA clones carrying complete integrated proviruses of Moloney murine leukemia virus. J. Virol. **37**:181–190.
2. **Berns, A. J. M., M. H. T. Lai, R. A. Bosselman, M. A. McKennett, L. T. Bacheler, H. Fan, E. C. R. Maandag, H. van der Putten, and I. M. Verma.** 1980. Molecular cloning of unintegrated and a portion of integrated Moloney murine leukemia viral DNA in bacteriophage lambda. J. Virol. **36**:254–263.
3. **Corsaro, C. M., and M. L. Pearson.** 1981. Enhancing the efficiency of DNA-mediated gene transfer in mammalian cells. Somatic Cell Genet. **7**:603–616.
4. **Dickson, C., R. Eisenman, H. Fan, E. Hunter, and N. Teich.** 1982. Protein biosynthesis and assembly, p. 513–648. *In* R. A. Weiss, N. Teich, H. E. Varmus, and J. M. Coffin (ed.), Molecular biology of tumor viruses, part III. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Duttagupta, S., and R. Soeiro.** 1981. Host restriction of Friend leukemia virus: gag proteins of host range variants. Proc. Natl. Acad. Sci. U.S.A. **78**:2320–2324.
6. **Faller, D., and N. Hopkins.** 1978. T1 oligonucleotide maps of N-, B-, and B → NB-tropic murine leukemia viruses derived from BALB/c. J. Virol. **26**:143–152.
7. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
8. **Gross, L.** 1970. Oncogenic viruses, 2nd ed. Pergamon Press, New York.
9. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
10. **Harbers, K., A. Schieke, H. Stuhlman, D. Jahner, and R. Jaenisch.** 1981. DNA methylation and gene expression: endogenous retroviral genome becomes infectious after molecular cloning. Proc. Natl. Acad. Sci. U.S.A. **78**:7609–7613.
11. **Jolicoeur, P.** 1979. The Fv-1 gene of the mouse and its control of murine leukemia virus replication. Curr. Top. Microbiol. Immunol. **86**:67–112.
12. **Klement, V., W. P. Rowe, J. W. Hartley, and W. E. Pugh.** 1969. Mixed culture cytopathogenicity: a new test for growth of murine leukemia virus in tissue culture. Proc. Natl. Acad. Sci. U.S.A. **63**:753–758.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
15. **Murphy, E. C., Jr., N. Wills, and R. B. Arlinghaus.** 1980. Suppression of murine retrovirus polypeptide termination: effect of amber suppressor tRNA on the cell-free translation of Rauscher murine leukemia virus, Moloney murine leukemia virus, and Moloney murine sarcoma virus 124 RNA. J. Virol. **34**:464–473.
16. **Philipson, L., P. Andersson, U. Olshevsky, R. Weinberg, D. Baltimore, and R. Gesteland.** 1978. Translation of MuLV and MSV RNAs in nuclease-treated reticulocyte extracts, enhancement of the gag-pol polypeptide with yeast suppressor tRNA. Cell **13**:189–199.
17. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye-buoyant-density method for detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **57**:1514–1521.
18. **Reddy, E. P., R. K. Reynolds, E. Santos, and M. Barbacid.** 1982. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma onc-gene. Nature (London) **300**:149–152.
19. **Reddy, E. P., M. J. Smith, and S. A. Aaronson.** 1981. Complete nucleotide sequence and organization of the Moloney murine sarcoma virus genome. Science **214**:445–450.
20. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42**:1136–1139.
21. **Sefton, B. M., K. Beemon, and T. Hunter.** 1978. Comparison of the expression of the *src* gene of Rous sarcoma virus in vitro and in vivo. J. Virol. **28**:957–971.
22. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukaemia virus. Nature (London) **293**:543–548.
23. **Shoemaker, C., S. Goff, E. Gilboa, M. Paskind, S. W. Mitra, and D. Baltimore.** 1980. Structure of a cloned circular Moloney murine virus DNA molecule containing an inverted sequence:

implication for retrovirus integrations. Proc. Natl. Acad. Sci. U.S.A. **77**:3932–3936.

24. **Tabin, C. J., S. M. Bradley, C. I. Bargmann, R. A. Weinberg, A. G. Papageorge, E. M. Scolnick, R. Dhar, D. R. Lowy, and E. H. Chang.** 1982. Mechanism of activation of a human oncogene. Nature (London) **300**:143–149.
25. **Van Beveren, C., F. van Straaten, J. A. Galleshaw, and I. M. Verma.** 1981. Nucleotide sequence of the genome of a murine sarcoma virus. Cell **27**:97–108.
26. **Verma, I. M., and D. Baltimore.** 1973. Purification of the RNA-directed DNA polymerase from avian myeloblastosis virus, and its assay with polynucleotide templates. Methods Enzymol. **29**:125–131.
27. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. U.S.A. **76**:615–619.
28. **Wei, C.-M., M. Gibson, P. G. Spear, and E. M. Scolnick.** 1981. Construction and isolation of a transmissible retrovirus containing the *src* gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. J. Virol. **39**:935–944.
29. **Weinberg, R. W.** 1977. Structure of the intermediates leading to the integrated provirus. Biochim. Biophys. Acta Rev. Cancer **473**:39–55.
30. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell **16**:777–785.
31. **Zarling, D. A., and I. Keshet.** 1979. Fusion activity of virions of murine leukemia virus. Virology **95**:185–196.