Volume 10 Number 8 1982 Nucleic Acids Research

A generalized method of subcloning DNA fragments by restriction site reconstruction: application to sequencing the amino-terminal coding region of the transforming gene of Gazdar murine sarcoma virus

Daniel J.Donoghue and Tony Hunter

Tumor Virology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, CA 92138, USA

Received 11 February 1982; Revised and Accepted 5 April 1982

ABSTRACT

The technique of restriction site reconstruction was generalized so as to allow the subcloning of any DNA fragment and its subsequent reexcision with EcoRI, XbaI, XhoI or HindIII. After excision, the 3' terminus of each strand will be derived from the starting nucleic acid, permitting the use of such fragments as primers for nucleotide sequencing by primer extension methods. The technique was used to subclone a 56 base pair BstNI-DdeI fragment of Moloney murine sarcoma virus (Mo-MSV) as a unique HindIII-HindIII fragment. This fragment then served as a primer to sequence a portion of the RNA genome of Gazdar murine sarcoma virus (Gz-MSV). The nucleotide sequence which was obtained indicated that the transforming gene of Gz-MSV arose by at least two recombination events involving murine leukemia virus (MLV) and the cellular homologue c-*mos*. This analysis suggests that a virus indistinguishable from Mo-MSV was an intermediate in the formation of Gz-MSV.

INTRODUCTION

Techniques of RNA sequencing have been greatly facilitated by the use of specific DNA primer fragments which are extended by reverse transcriptase to generate a complementary cDNA copy. Currently, two variations of this technique are employed. In one method (1-4), a ^{32}P-labeled primer DNA fragment is extended to generate an end-labeled cDNA copy, which is then sequenced by Maxam-Gilbert techniques (5). In the second method, an unlabeled DNA primer fragment is extended by reverse transcriptase in the presence of α-^{32}P-deoxynucleoside triphosphates (dNTP's) plus 2',3'-dideoxynucleoside triphosphates (ddNTP's), generating a dideoxy-terminated sequencing ladder (6).

In some instances, application of these techniques has been impractical due to difficulty in obtaining sufficient quantities of appropriate DNA primer fragments. This report presents a generalized technique for subcloning restriction fragments to be used as primers for sequencing by primer extension techniques. This methodology was employed to sequence a portion of Gazdar

0305-1048/82/1008-2549$ 2.00/0

murine sarcoma virus (Gz-MSV), in an effort to understand its relationship to Moloney murine sarcoma virus (Mo-MSV).

The original Moloney MSV/MLV complex was isolated from a tumor in a Balb/c mouse injected with Moloney murine leukemia virus (Mo-MLV) (7), and consists of a mixture of related but uncloned viruses. Thus, Mo-MSV constitutes a family of replication-defective retroviruses that transform fibroblasts in vitro and induce rapidly progressing fibrosarcomas in vivo. All clonal isolates of Mo-MSV share a common viral transforming region, termed v-*mos*^{Mo}, located near the 3' terminus of the genome (8). As demonstrated by nucleotide sequence analysis (9,10), by in vitro translation of Mo-MSV virion RNA (11-13), and by expression in *E. coli* (14), v-*mos*^{Mo} encodes a protein of 41,000 daltons. Recent data indicate that this protein, designated p37^{mos} based on its apparent molecular weight, is synthesized in cells transformed by Mo-MSV and thus is the most likely candidate for the transforming protein (15).

Mo-MSV arose by recombination between the replication-competent Mo-MLV and the endogenous cellular homologue of *mos*, termed c-*mos* (see Fig. 1 for the relationship between Mo-MLV and Mo-MSV). The single copy of c-*mos* present in the Balb/c mouse genome has been cloned (16) and sequenced (10,17). Comparison of the nucleotide sequence of a clonal isolate of Mo-MSV, designated 124-MSV, with its parental sequences, i.e. Mo-MLV and c-*mos*, has located its two recombination points in the v-*mos*^{124} sequence. This comparison reveals that the 41,000 dalton protein encoded by v-*mos*^{124} is actually encoded by a chimeric sequence, consisting of the NH_2-terminal region of the Mo-MLV *env* gene fused to an open reading frame in *mos*. Thus, the first five codons of the 41,000 dalton v-*mos*^{124} reading frame, including the initiator ATG, are derived from the NH_2-terminus of the Mo-MLV *env* gene.

In an earlier study, the MLV-*mos* junctions in various isolates of Mo-MSV were studied by electron microscopic heteroduplex analysis (8). To the limit of resolution, all the isolates derived from the original Mo-MSV tumor, i.e. 124-MSV, HT1-MSV, NP-MSV, m1-MSV and m3-MSV, displayed a common 5'-proximal recombination point between the MLV-derived sequence and the v-*mos*^{Mo} sequence. However, Gz-MSV, which was reported to have been derived independently of Mo-MSV from a spontaneous tumor in a NZW/NZB F_1 mouse (18), differed significantly from the Moloney-derived isolates. Although the transforming gene of Gz-MSV (termed v-*mos*^{Gz}), was highly related to v-*mos*^{Mo}, electron microscopy revealed that v-*mos*^{Gz} was inserted about 1 kb downstream in the parental MLV *env* gene as compared with all the isolates of Mo-MSV (see Fig. 1). Given that the NH_2-terminus of the 41,000 dalton v-*mos*^{124} protein is donated by the *env*

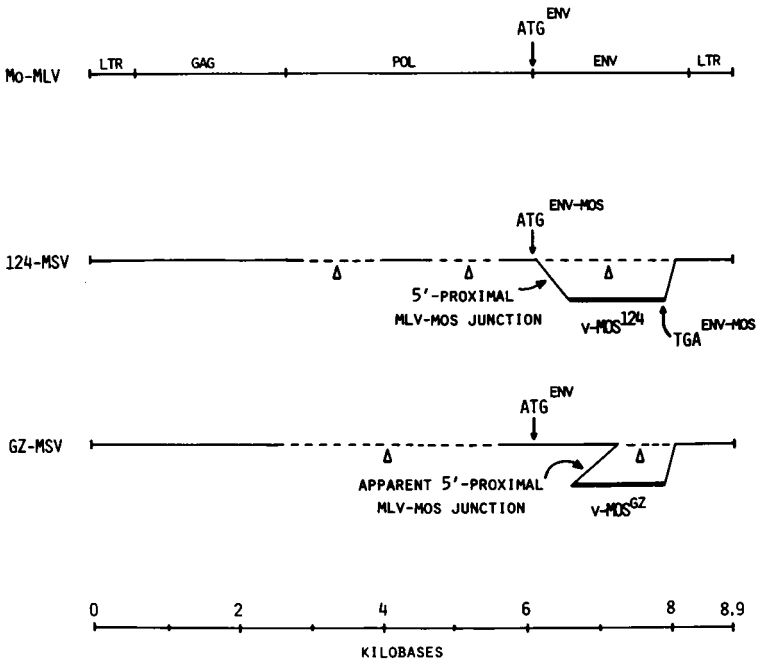

Figure 1. Genome structures of Mo-MLV, 124-MSV, and Gz-MSV. Deletions (Δ) which have occurred in 124-MSV and Gz-MSV are shown by dashed lines. The v-*mos* gene, which has been inserted into the *env* gene to generate 124-MSV and Gz-MSV, is shown by the bold line. Other small deletions or substitutions detected in 124-MSV by nucleotide sequencing (17) are not shown here. The structure of Gz-MSV was determined previously by electron microscopy (8), which detected an "apparent" 5'-proximal recombination point in Gz-MSV downstream of that seen in 124-MSV.

gene, one might therefore expect v-*mos*^{Gz} to encode a protein with a very different NH_2-terminus.

For this reason, it was of interest to determine the nucleotide sequence at the 5'-proximal MLV-*mos* junction of Gz-MSV. As this virus has not yet been molecularly cloned, this sequence could most easily be obtained by sequencing a cDNA copy of the Gz-MSV viral RNA. This was accomplished by preparing an appropriate fragment of DNA from a molecular clone of 124-MSV which would serve as a primer for reverse transcription in the presence of ddNTP's.

In choosing an appropriate DNA primer fragment, three considerations must be borne in mind: i) the primer must be long enough to form a stable hybrid with the template RNA, ii) the primer must be short enough to allow convenient resolution of the sequencing ladder, since each DNA chain in the sequencing

ladder will have the primer at its 5' terminus, and iii) if possible, the primer should hybridize near the region of interest, so that even relatively short cDNA molecules will extend into this region. Based on the nucleotide sequence of 124-MSV, the optimal primer was a BstNI-DdeI fragment located near the 5'-proximal MLV-*mos* junction. It was impractical, however, to obtain sufficient quantities of this fragment by ordinary techniques, due to the prevalence of sites for the enzymes BstNI and DdeI, and also the prohibitive cost of these enzymes. It was also impossible to subclone the desired fragment using oligonucleotide linkers, as this would have rendered the 3' terminus of the priming strand nonhomologous to the template RNA. Therefore, the desired primer fragment was subcloned using a technique whereby the BstNI-DdeI restriction fragment was transmuted to a HindIII-HindIII fragment in pBR322. This technique, although altering the sequence at the 5' terminus of the priming strand, left intact its 3' terminus.

This technique of subcloning by restriction site reconstruction was extended so as to be generally applicable. A vector was constructed which permits any known DNA terminus to be cloned into one of four unique restriction sites: XbaI, EcoRI, XhoI, or HindIII. This technique thus allows any restriction fragment to be rapidly subcloned, without the use of oligonucleotide linkers, in such a way that it can be readily excised as a unique restriction fragment.

MATERIALS AND METHODS

Plasmid constructions

The MSV-specific plasmid pDD0 has been described previously (9). The plasmid pGGB, containing the rat growth hormone gene (19), was obtained from Marcia Barinaga and Ron Evans (Salk Institute).

DNA fragments were recovered from agarose gel slices using the glass powder technique (20). 5'-overhangs of restriction sites were filled in by the Klenow fragment of *E. coli* DNA Polymerase I (Boehringer Mannheim) in a buffer of 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 1 mM dithiothreitol, with each dNTP present at 20 μM. When the terminus was to be ^{32}P-labeled during the fill-in reaction, 100 μCi of the desired α-^{32}P-dNTP was added, and the total concentration for this dNTP was lowered to 2 μM.

In order to identify pDD39, DNA molecules were ligated (21) as indicated in Figure 2, and transfected (22) into *E. coli* C600 $r_K^- m_K^-$ (23). Ampicillin-resistant colonies were screened with a ^{32}P-labeled MSV-specific probe, pre-

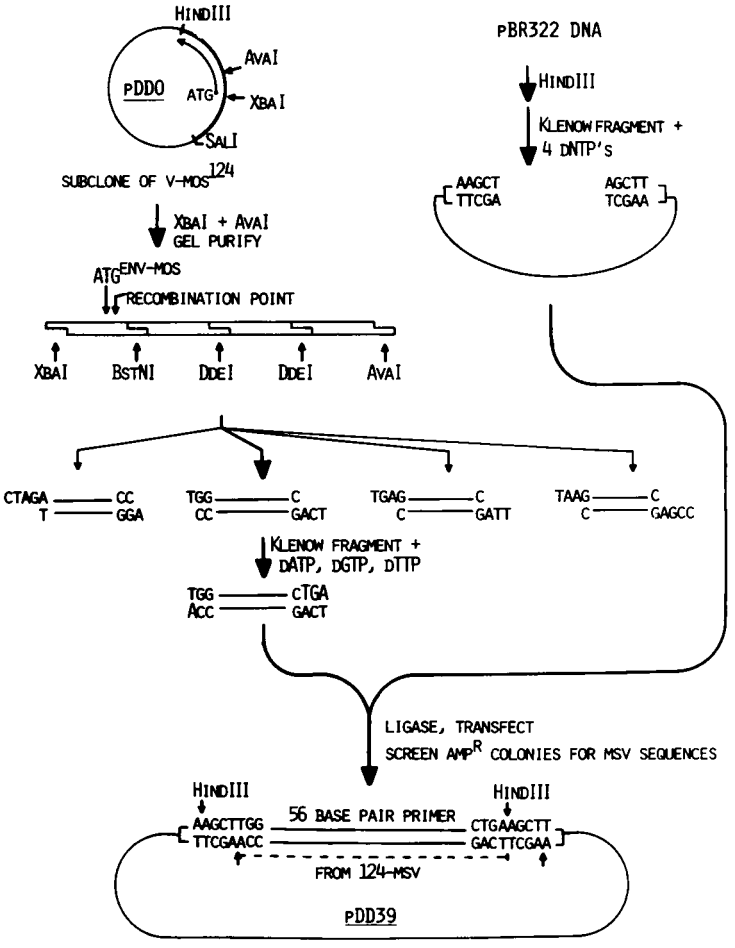

Figure 2. Construction of the primer clone pDD39. This figure shows how the desired primer fragment was subcloned by reconstructing a BstNI-DdeI fragment to a HindIII-HindIII fragment.

pared by nick translation (24) of gel-purified XbaI-HindIII MSV fragment from pDDO. As only 56 base pairs of homology would exist between the desired clone and the ^{32}P-labeled MSV-specific probe, the sensitivity of the hybridization screening was improved as follows. Ampicillin-resistant colonies were picked into broth and grown overnight. Using pasteur pipets, a drop of each culture was spotted onto a fresh plate, resulting after incubation in a grid of bacterial spots about 1 cm in diameter. These "spot colonies" were then transferred to nitocellulose filters and hybridized (25). Following overnight

hybridization at 60°C, the hybridization waterbath was slowly cooled to room temperature (about 6 h) in order to favor stable formation of short duplexes. The filters were then washed in 2X-SSC at 37°C.

DNA was prepared from positive colonies by the rapid boiling method of Holmes and Quigley (26). To examine plasmids for a HindIII-excisable insert, aliquots were digested with HindIII and simultaneously 3'-end-labeled by filling-in with Klenow fragment in the presence of dATP, dCTP, dGTP, and α-^{32}P-dTTP. The reaction products were electrophoresed on an 8% acrylamide gel and detected by autoradiography (Fig. 3).

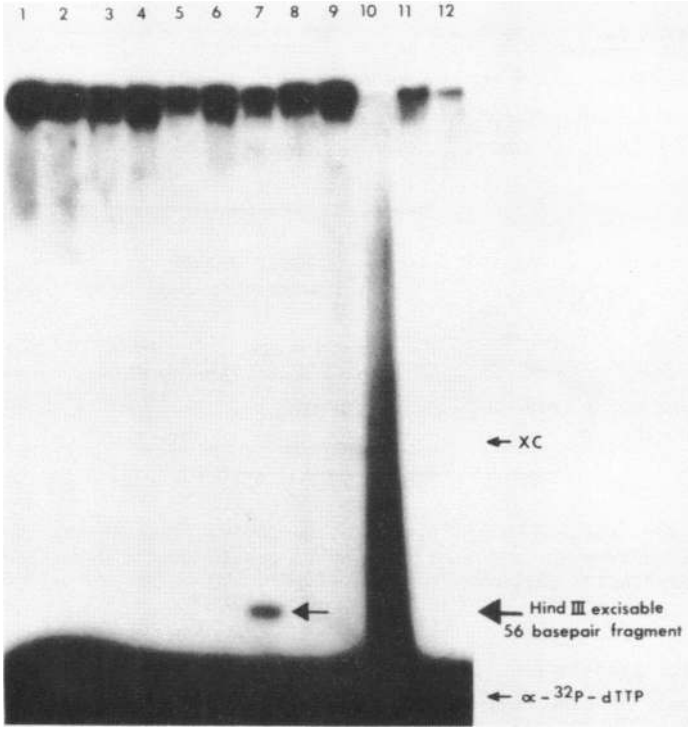

Figure 3. Screening ampicillin-resistant MSV-positive clones for a HindIII-excisable fragment. Aliquots of DNA minipreps were cleaved with HindIII and simultaneously ^{32}P-labeled with Klenow fragment plus appropriate dNTP's. The gel shown is an 8% nondenaturing acrylamide gel. XC indicates the position of the xylene cyanol dye. The unincorporated α-^{32}P-dTTP runs at the bromphenol blue dye front. The arrow in lane 7 marks the 56 base pair HindIII-HindIII fragment present in pDD39.

Conditions for sequencing by primer extension

About 20 µg of pDD39 DNA was cleaved with HindIII, and 5'-^{32}P-labeled using T4 polynucleotide kinase according to standard protocols (5). The ^{32}P-labeled 56 base pair primer fragment was then isolated by electrophoresis in an 8% nondenaturing acrylamide gel and recovered from the gel slice (5).

RNA was prepared from sucrose gradient-purified virions of Gz-MSV grown in RTG-1 cells (27). About 40 µg of total Gz-MSV virion RNA, which had been extracted with phenol but not otherwise purified, was dissolved in 80 µl of 62% formamide, 0.4 M NaCl, 20 mM Pipes pH 6.4, 2 mM EDTA. The precipitated ^{32}P-labeled primer fragment was then dissolved with the RNA, and the mixture heated to 85°C for 5 min to denature all nucleic acids. The mixture was then placed in a 65°C waterbath, which was allowed to slowly cool to room temperature during 6 h. The RNA-DNA hybrids were then precipitated from the hybridization mixture with ethanol.

Hybrids were redissolved in the buffer described by Bina-Stein et al. (6) containing 50 mM Tris pH 8.3, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and all four dNTP's at 20 µM each. This was split into four aliquots, each of which was supplemented with one of the four ddNTP's (P-L Biochemicals). The following ratios were arrived at empirically: ddATP/dATP, 1.3; ddCTP/dCTP, 0.4; ddGTP/dGTP, 0.3; ddTTP/dTTP, 0.9. Reverse transcription was carried out for 1 h at 40°C using 15 units of AMV reverse transcriptase, kindly provided by Dr. J. W. Beard (Life Science, St. Petersburg, Fl). Reaction products were precipitated with ethanol, resuspended in formamide-dye sample buffer for sequencing gels, and analyzed on an 8% acrylamide sequencing gel (5). Electrophoresis was carried out at 1000 V with the gel placed in a 45°C hot air oven to reduce curvature and elminate compression artifacts (9).

RESULTS

Preparation of a primer DNA fragment from 124-MSV

Due to the homology between v-*mos*^{124} and v-*mos*^{Gz}, it was anticipated that DNA fragments prepared from molecular clones of 124-MSV would serve as primers for sequencing the RNA of Gz-MSV. Starting with the plasmid pDD0, which contains v-*mos*^{124}, a small BstNI-DdeI fragment was subcloned as a unique HindIII-HindIII fragment in the plasmid pDD39. The steps in this procedure are outlined in Figure 2.

As a first step, a convenient XbaI-AvaI fragment (193 base pairs) was prepared from the plasmid pDD0. This fragment included the NH_2-terminal

region of the v-*mos*^{124} open reading frame, and also the 5'-proximal MLV-*mos* recombination point (see Fig. 2). After digestion with BstNI and DdeI, the XbaI-AvaI fragment yielded four small restriction fragments. This mixture was then treated with the Klenow fragment of DNA Polymerase I plus dATP, dGTP, and dTTP; this was expected to render flush-ended all 5'-overhangs containing only T, C, and A. Both the BstNI ($\overset{\downarrow}{CCTGG}$ / $GGA\overset{\uparrow}{C}C$) and DdeI ($\overset{\downarrow}{C}TGAG$ / $GACT\overset{\uparrow}{C}$) termini of the desired fragment were thereby expected to become flush-ended, and to display a 5'-terminal T residue. None of the other fragments would be completely filled-in at both ends.

In order to prepare an appropriate vector, pBR322 DNA was cleaved with HindIII ($\overset{\downarrow}{A}AGCTT$ / $TTCGA\overset{\uparrow}{A}$) and the 5'-overhangs were completely filled-in with Klenow fragment plus all four dNTP's. This was then blunt-end ligated to the mixture of 124-MSV fragments described above. After transfection, the resulting ampicillin-resistant clones were screened for the presence of MSV-specific sequences using a ^{32}P-labeled DNA probe (see Materials and Methods). DNA was then prepared from positive colonies by a rapid "miniprep" procedure (26). These samples were digested with HindIII and simultaneously ^{32}P-labeled by the addition of Klenow fragment, dATP, dCTP, dGTP and α-^{32}P-dTTP. The reaction products were then analyzed on an 8% nondenaturing acrylamide gel as shown in Figure 3.

One of the clones examined clearly yielded a unique HindIII-excisable fragment of 56 base pairs, and was designated pDD39. Nucleotide sequencing confirmed the presence of the MSV sequence indicated in Figure 2, bounded by two HindIII sites, as anticipated (data not shown). Note that after excision with HindIII, each strand possessed a 5'-overhang of 4 bases, none of which was derived from 124-MSV. The 3' terminus of each strand, however, was derived from 124-MSV. Hence, it was expected that the 3' terminus of the strand complementary to the MSV genomic (+) strand RNA would be available for priming after hybridization to RNA of Gz-MSV.

The other 11/12 clones examined in Figure 3 did not have the correct structure. These could have resulted from insertion of one of the other fragments present in the ligation mixture, each of which had one blunt end and one partially filled-in terminus. Alternatively, some of these clones might have resulted from insertion of the correct fragment, but with its termini incompletely filled-in.

Sequencing the 5'-proximal MLV-*mos* joint in Gz-MSV

To prepare the primer fragment, pDD39 DNA was digested with HindIII, treated with bacterial alkaline phosphatase, and 5'-end labeled with γ-^{32}P-ATP plus T4 polynucleotide kinase. The ^{32}P-labeled primer fragment of 56 base pairs was isolated by acrylamide gel electrophoresis and hybridized to Gz-MSV virion RNA. After hybridization, nucleic acids were precipitated with ethanol and, subsequently, reverse transcription was carried out in the presence of ddNTP's as described in Materials and Methods.

As shown in Figure 4, when the reverse transcriptase products were analyzed in an 8% acrylamide sequencing gel, an unambiguous sequence was obtained. This sequence, which is interpreted in detail in Figure 5 and schematically in Figure 6, revealed that Gz-MSV must be the product of at least two recom-

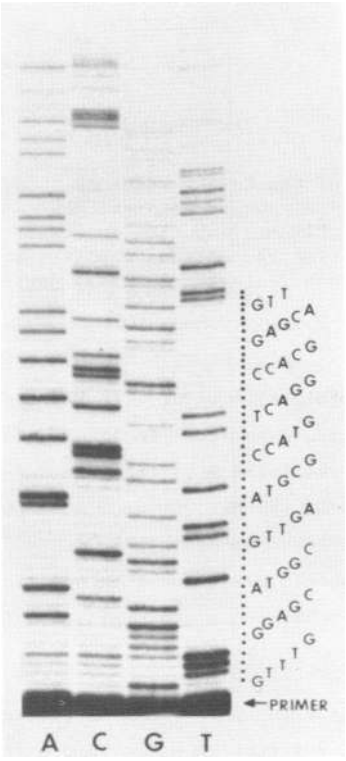

Figure 4. Sequence of the 5'-proximal MLV-*mos* junction of Gz-MSV. The sequence as read up the gel corresponds to the (-) strand of the Gz-MSV RNA. See Figure 5 for an interpretaion of this sequence. This gel is an 8% acrylamide sequencing gel.

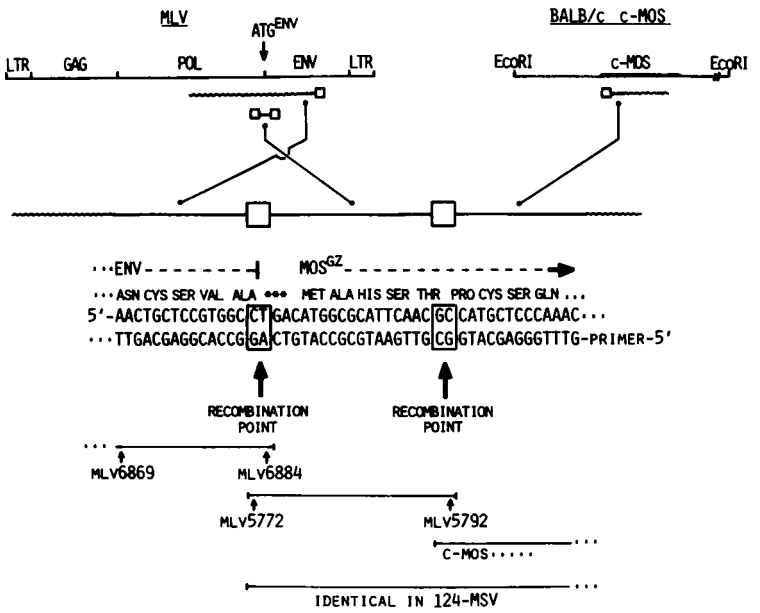

Figure 5. Interpretation of the Gz-MSV sequence. Comparison of the Gz-MSV sequence with the two parental sequences, i.e. MLV (29) and c-*mos* (10), shows the "apparent" 5'-proximal MLV-*mos* junction in Gz-MSV to be the product of two recombination events. The recombination points are shown by the boxes around the nucleotides, which indicate the small degree of homology between the recombining sequences.

bination events. One recombination event with MLV fused c-*mos* to the NH_2-terminus of the MLV *env* gene, exactly as in 124-MSV (9,10,28). Another recombination event, also involving MLV, then fused c-*mos* plus its MLV-derived NH_2-terminus into the body of the MLV *env* gene at a point about 1 kb downstream. The exact point of recombination was located by inspection of the MLV sequence of Shinnick et al. (29), and this position was in very good agreement with the earlier electron microscopic data (8).

A generalized method of subcloning

Table 1 summarizes the steps involved in subcloning DNA fragments by reconstructing the initial restriction sites into more convenient restriction sites. Essentially, a vector plus an insert are blunt-end ligated so as to reconstruct a 6 base pair restriction site, either XbaI, EcoRI, XhoI or HindIII. Five of the six base pairs in the site must be contributed by the vector DNA, after cleavage with the appropriate enzyme and filling-in by

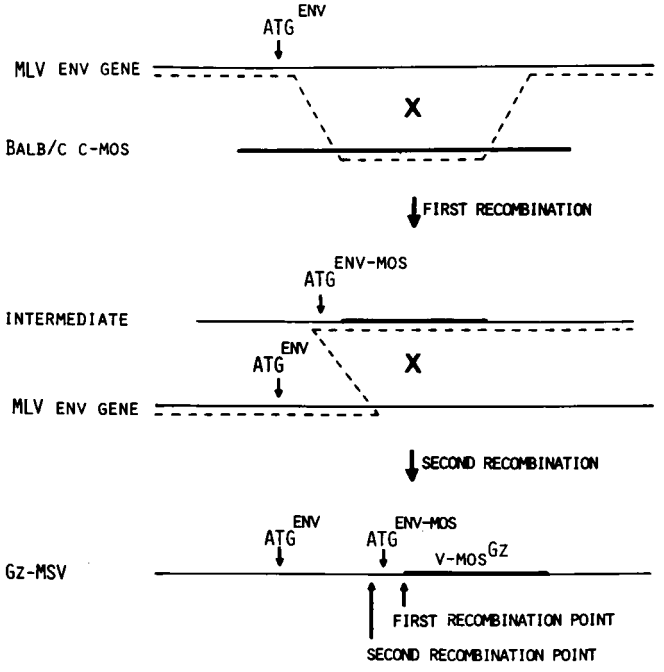

Figure 6. Suggested generation of Gz-MSV by two successive recombination events involving the cellular homologue c-*mos* and MLV.

Table 1. Acceptor sites in pDD54.

pDD52 acceptor site		after restriction	after filling in	will accept:
XbaI	∿∿TCTAGA∿∿ ∿∿AGATCT∿∿	∿∿T ∿∿AGATC	∿∿TCTAG ∿∿AGATC	A∿∿ T∿∿
EcoRI	∿∿GAATTC∿∿ ∿∿CTTAAG∿∿	∿∿G ∿∿CTTAA	∿∿GAATT ∿∿CTTAA	C∿∿ G∿∿
XhoI	∿∿CTCGAG∿∿ ∿∿GAGCTC∿∿	∿∿C ∿∿GAGCT	∿∿CTCGA ∿∿GAGCT	G∿∿ C∿∿
HindIII	∿∿AAGCTT∿∿ ∿∿TTCGAA∿∿	∿∿A ∿∿TTCGA	∿∿AAGCT ∿∿TTCGA	T∿∿ A∿∿

Klenow fragment plus dNTP's. The sixth base pair of the site must be donated by the terminal base pair of the insert.

The four restriction sites XbaI, EcoRI, XhoI and HindIII were chosen because they will accept, after restriction and filling-in, termini bearing a 5'-terminal A, C, G or T, respectively. No convenient plasmid vector contained all four sites, necessitating the construction of pDD52 as shown in Figure 7. The insertion of a BglII fragment from the rat growth hormone gene (19,30), containing sites for XbaI and XhoI, into the BamHI site of pBR322, resulted in a plasmid containing the four unique restriction sites.

Table 2 indicates the appropriate acceptor site in the vector so as to subclone DNA fragments produced by most commercially available restriction enzymes. For instance, to subclone a RsaI-DpnI fragment, Table 2 indicates that one would need only to ligate this fragment to the vector pDD52, which would have been cleaved with XbaI and HindIII and filled-in. The RsaI-DpnI fragment would thus be transmuted to an XbaI-HindIII fragment. In the more common instance, inserts with 5' or 3' overhangs would first be converted to blunt-ended molecules by filling-in or by S1-nuclease digestion, before blunt-end ligation with the appropriate site in the vector.

By consulting Table 2, any DNA fragment can be readily cloned into the vector, provided that the insert is flush-ended and that the vector is first cleaved and filled-in at the appropriate sites.

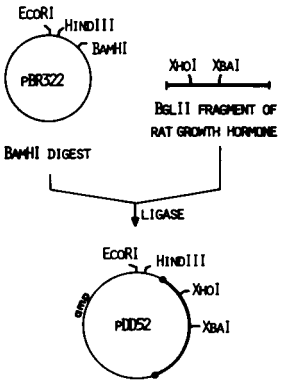

Figure 7. Construction of the vector pDD52. Any flush-ended DNA fragment can be cloned into some combination of the sites shown in pDD52, permitting its reexcision as a unique fragment.

Table 2. Restriction site termini for use with pDD52. Most commercially available enzymes are included in this table, except for those that have a partial degeneracy in their recognition sequences after filling-in, e.g. BstNI ($CC^{\downarrow}{}^{A}_{T}GG$), HinfI (G$\downarrow$ANTC), etc. For these enzyme sites, simply determine what will be the 5'-terminal base after the fragment is rendered flush-ended by filling-in, and consult Table 1 for the correct acceptor sites in the vector pDD52.

pDD52 acceptor site	will accept:	RESTRICTION SITES THAT CAN BE LIGATED TO ACCEPTOR SITE: enzymes leaving blunt ends	enzymes leaving 3' or 5' extensions, followed by nuclease treatment	enzymes leaving 5' extensions, followed by filling in
XbaI - filled in	5'-A∿∿ T∿∿	HpaI RsaI	AccI BclI TaqI XbaI	EcoRI HindIII HinfI
EcoRI - filled in	5'-C∿∿ G∿∿	AluI BalI FnuDII HaeIII PvuII StuI ThaI	AvaII NarI BamHI PvuI BstEII SacI CfoI SalI EcoRI Sau96I HgiAI SphI HhaI SstI HinfI XorII KpnI	EcoRII HpaII MspI NarI TaqI XbaI XmaI
XhoI - filled in	5'-G∿∿ C∿∿	MstI SmaI	AvaI PstI BstNI SacII DdeI SstII Fnu4HI XhoI HpaII XmaI MspI XmaIII MstII	AvaII Sau96I BamHI XmaIII BclI BglII BstEII MboI Sau3AI
HindIII - filled in	5'-T∿∿ A∿∿	DpnI	BglII HindIII	DdeI MstII SalI XhoI

DISCUSSION

Based on previous electron microscopic heteroduplex analysis, Gz-MSV appeared to possess the same v-*mos* sequence as found in 124-MSV, but inserted in a different (downstream) position within the MLV *env* gene (8). Nucleotide sequencing of v-mos^{124} (9,10,28) and characterization of its translation products (11-13) indicate that the open reading frame which encodes the *mos* gene product is actually a fused *env-mos* reading frame, for which the NH_2-

terminal portion is donated by the MLV *env* gene. Since v-*mos*^{Gz} is located in a different position in the *env* gene, one would expect Gz-MSV to encode a *mos* gene product with an altered NH_2-terminus, compared with the gene product of 124-MSV.

Contrary to this expectation, the MLV-*mos* junction in Gz-MSV was identical to that in 124-MSV, having been juxtaposed downstream in *env* by a second recombination event (see Fig. 5 & 6). This second recombination event generated a terminator codon in the *env* reading frame. Initiation at the first *env* gene ATG in Gz-MSV would thus be expected to yield a truncated *env* gene product. Consistent with this, in vitro translation of Gz-MSV virion RNA yields a 37,500 dalton product which can be immunoprecipitated by an antibody against the Mo-MLV *env* gene product (J. Papkoff, personal communication). Initiation at the second *env* gene ATG would be expected to yield the same "*env-mos*" fusion protein as does 124-MSV.

Although the COOH-terminal portion of the v-*mos*^{Gz} coding region has not been sequenced, two observations indicate that it is likely to be very similar to 124-MSV: i) By electron microscopy, the 3'-*mos*-MLV junction of Gz-MSV is indistinguishable from 124-MSV (8). ii) In vitro translation of Gz-MSV virion RNA yields a 37,000 dalton *mos* protein which comigrates with the 37,000 dalton in vitro translation product encoded by 124-MSV virion RNA, and which can be specifically immunoprecipitated by antiserum (12) directed against the 12 COOH-terminal residues of the 124-MSV *mos* protein (J. Papkoff, personal communication).

Since the 5'-proximal MLV-*mos* junction in Gz-MSV and 124-MSV were identical, it is impossible to decide if Gz-MSV represents an independent isolate, distinct from the Mo-MSV-derived isolates. The two simplest interpretations are as follow. First, either Gz-MSV *does* represent an independent isolate, but proper expression of the *env-mos* reading frame required selection of the same MLV-*mos* junction on two different occasions. According to the alternate hypothesis, Gz-MSV *does not* represent an independent isolate, but rather, resulted from infection of an NZW/NZB F_1 mouse with the Moloney MLV/MSV complex, which subsequently underwent additional rearrangement. Regardless, the conservation of the *env-mos* junction suggests the importance of the *env* gene NH_2-terminus in the activation of the *mos* gene.

The sequence of the MLV-*mos* junction in Gz-MSV was obtained by reverse transcription from a primer DNA fragment hybridized to Gz-MSV virion RNA. This primer fragment, initially a BstNI-DdeI fragment obtained from a clone of 124-MSV, was subcloned into a plasmid vector as a unique HindIII-HindIII

fragment. This permitted preparation of this primer fragment in large amounts. The plasmid containing the HindIII-excisable fragment should be useful in sequencing other MSV-specific RNA's, e.g. intracellular mRNA's, or virion RNA's of other isolates or mutants of MSV.

Using the techniques and vector described in this work, any restriction site terminus can be transmuted to either an XbaI, EcoRI, XhoI or HindIII site. This allows the potential conversion of any restriction fragment into a form such that it will be available as a uniquely-excisable restriction fragment in a plasmid. This technique also has the advantage that the 3' termini of both strands of the fragment, after reexcision, will be derived from the starting nucleic acid. This may be an important benefit, compared with the alternative method of subcloning with oligonucleotide linkers, if the eventual goal is the preparation of priming fragments for nucleotide sequencing. Fragments prepared by this technique would also be suitable for use in analysis of spliced mRNA's by the method of Berk and Sharp (31), where it is desired to use a 3'-end-labeled DNA fragment as the probe.

ACKNOWLEDGEMENTS

We thank Jon Cooper, Jackie Papkoff and Jack Rose for valuable criticism of the manuscript. D.J.D. gratefully acknowledges a fellowship from the Helen Hay Whitney Foundation. This work was supported by grants from the National Cancer Institute, CA-17096 and CA-28458.

REFERENCES

1. Ghosh,P.K., Reddy, V.B., Piatak,M., Lebowitz,P., and Weissman,S.M. (1980) Methods in Enzymol. 65, 580-595.
2. McReynolds,L., O'Malley,B.W., Nisbet,A.D., Fothergill,J.E., Givol,D., Fields,S., Robertson,M., and Brownlee,G.G. (1978) Nature 273,723-728.
3. Baralle,F.E. (1977) Cell 12,1085-1095.
4. Szostak,J.W., Stiles,J.I., Bahl,C.P., and Wu,R. (1977) Nature 265,61-63.
5. Maxam,A. and Gilbert,W. (1980) Methods in Enzymol. 65,499-560.
6. Bina-Stein,M., Thoren,M., Salzman,N., and Thompson,J.A. (1979) Proc. Natl. Acad. Sci. 76,731-735.
7. Moloney,J.B. (1966) Cancer Inst. Monogr. 22,139-142.
8. Donoghue,D.J., Sharp,P.A., and Weinberg,R.A. (1979) J. Virol. 32,1015-1027.
9. Donoghue,D.J. (1982) J. Virol., in press.
10. Van Beveren,C., Galleshaw,J.A., Jonas,V., Berns,A.J.M., Doolittle,R.F., Donoghue,D.J., and Verma,I.M. (1981) Nature 289,258-262.
11. Papkoff,J., Hunter,T., and Beemon,K. (1980) Virology 101,91-103.
12. Papkoff,J., Lai, M.H., Hunter,T., and Verma,I.M. (1981) Cell 27,109-119.
13. Cremer,K., Reddy,E.P., and Aaronson,S.A. (1981) J. Virol. 38,704-711.
14. Donoghue,D.J. and Hunter,T. (1982) Proc. Natl. Acad. Sci., 79,800-804.
15. Papkoff,J., Verma,I.M., and Hunter,T. (1982) Cell, in press.

16. Oskarsson,M., McClements,W.L., Blair,D.G., Maizel,J.V., and Vande Woude, G.F. (1980) Science 207,1222-1224.
17. Van Beveren, C., van Straaten,F., Galleshaw,J.A., and Verma,I.M. (1981) Cell 27,97-108.
18. Gazdar,A.F., Chopra,H.C., and Sarma,P.S. (1972) Int. J. Cancer 9,219-233.
19. Doehmer,J., Barinaga,M., Vale,W., Rosenfeld,M.G., Verma,I.M., and Evans, R.M. (1982) Proc. Natl. Acad. Sci., in press.
20. Vogelstein,B. and Gillespie,D. (1979) Proc. Natl. Acad. Sci. 76,615-619.
21. Ferretti,L. and Sgaramella,V. (1981) Nucl. Acids Res. 9,85-93.
22. Morrison,D.A. (1979) Methods in Enzymol. 68,326-331.
23. Thomas,M., Cameron,J.R., and Davis,R.W. (1974) Proc. Natl. Acad. Sci. 71, 4579-4583.
24. Rigby,P.W.J.,Dieckmann,M., Rhodes,C. and Berg,P. (1977) J. Mol. Biol. 113,237-247.
25. Grunstein,M. and Hogness,D.S. (1975) Proc. Natl. Acad. Sci. 72,3461-3465.
26. Holmes,D.S. and Quigley,M. (1981) Anal. Biochem. 114,193-197.
27. Pang,R.H.L., Phillips,L.A., and Haapala,D.K. (1977) J. Virol. 24,551-556.
28. Reddy,E.P., Smith,M.J., Canaani,E., Robbins,K.C., Tronick,S.R., Zain,S. and Aaronson,S.A. (1980) Proc. Natl. Acad. Sci. 72,4650-4654.
29. Shinnick,T.M., Lerner,R.A. and Sutcliffe,J.G. (1981) Nature 293,543-548.
30. Page,G.S., Smith,S. and Goodman,H.M. (1981) Nucl. Acids Res. 9,2087-2103.
31. Berk,A.J. and Sharp,P.A. (1977) Cell 12,721-732.