Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 4149–4153, July 1984
Genetics

Construction of mutants of Moloney murine leukemia virus by suppressor-linker insertional mutagenesis: Positions of viable insertion mutations

(virus replication/synthetic DNA/oligonucleotides)

LESLIE I. LOBEL AND STEPHEN P. GOFF

Department of Biochemistry and Institute for Cancer Research, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032

Communicated by Harold S. Ginsberg, March 20, 1984

ABSTRACT **A highly efficient method for the generation of insertion mutations is described. The procedure involves the use of a 220-base-pair (bp) *Eco*RI fragment bearing the *SuIII*$^+$ suppressor tRNA gene as an insertional mutagen. The plasmid DNA to be mutagenized is linearized by a variety of means, and the suppressor fragment is ligated into the site of cleavage. Successful insertion mutants can be readily detected in *Escherichia coli* carrying *lac*$^-$ amber mutations on MacConkey lactose plates; virtually 100% of the red colonies contain insertions of the fragment. Subsequent removal of the *SuIII*$^+$ gene and recyclization leaves a 12-bp insertion if the original cleavage was blunt-ended and a 9-bp insertion if the original cleavage generated 3-bp cohesive termini. This technique, as well as conventional linker mutagenesis with decamer and dodecamer linkers, was used to generate a large library of insertion mutations in cloned DNA copies of the genome of Moloney murine leukemia virus. A number of viable mutants were isolated bearing 9-, 10-, and 12-bp insertions in various domains of the genome. The map positions of the viable mutations suggest that the viral long terminal repeats and portions of the *gag* and *env* genes are quite insensitive to alteration. Although most of the mutations were stable for many passages, some of the mutants lost the inserted DNA; we presume that the insertion was somewhat deleterious in these mutants and that continued passage of the virus selected for overgrowth by a revertant.**

The *in vitro* mutagenesis of cloned genes has been one of the most powerful tools in the elucidation of the relationship between structure and function of DNA sequences. By introducing defined alterations in a sequence and correlating the changes with alterations in gene function, it has been possible to define such features as transcriptional promoters (1–4), RNA splicing signals (5), RNA polyadenylylation signals (6), ribosome binding sites (7), and protein domains needed for particular enzymatic activities (8, 9). Our power to carry out such analyses has been increased enormously as the techniques used to make the defined alterations have become increasingly sophisticated (10).

The use of these techniques is sometimes limited by our ability to choose likely target regions for mutagenesis. When there is no information defining which parts of a gene are essential for a particular function, then narrowly targeted *in vitro* mutagenesis is not helpful in the localization of that function. In such cases, it is necessary to generate a large library of mutants with defined alterations scattered throughout the cloned DNA and to screen these mutants for a desired phenotype. One of the best ways to construct such a library is by "linker insertion" mutagenesis (11): a circular target DNA is linearized with a nuclease, and a short synthetic oligonucleotide sequence containing a restriction enzyme recognition site is inserted at the position of cleavage in the target DNA. The resulting mutations can be readily mapped simply by determining the site of cleavage by the restriction enzyme that recognizes the inserted sequence.

We have constructed several libraries of mutants of Moloney murine leukemia virus (M-MuLV) by the insertion of *Eco*RI linkers at random positions in a cloned DNA copy of the viral genome. During the course of these studies, we found that the efficiency of the process was highly variable; linkers were added poorly to ends formed by some nucleases and very few linker insertions could be successfully formed. To circumvent this difficulty, we have developed an improved method by which rare insertion events can be readily detected, obviating the screening of large numbers of clones by electrophoresis to identify insertions. By this method, large libraries of mutants, free of wild-type parental DNA, can be efficiently constructed. In this report, we describe the method and its application to the genetic analysis of the genome of M-MuLV. We have screened libraries of mutants and, after transfection of NIH/3T3 cells, have recovered numerous clones that are still replication competent. The positions of the mutations in these clones define noncoding and coding regions of the viral genome that are dispensible for replication or that can tolerate insertions without deleterious effects on the virus.

MATERIALS AND METHODS

Bacteria. *Escherichia coli* strain HB101 (*rec A13*$^-$, *hsdR*$^-$, *hsdM*$^-$, *lacY1*, *supE44*) was used as the recipient cell for most DNA transformations (12). To detect the presence of the *SuIII*$^+$ suppressor gene, strain CC114 [*del(ara,leu)7697*, *lacZ*$^-$ *amY14*, *galU*, *galK*, *hsdR*$^-$, *hsdM*$^+$, *strA*^{r}, *rif*^{r}, *argE*$^-$ *am*, *srl*::Tn*10*, *recA1*] was used as recipient. This strain, constructed and generously provided by C. Manoil, gave high transformation efficiencies comparable to that of HB101.

Plasmid DNAs. Plasmids used in this work include p8.2 (13), a complete permuted copy of the M-MuLV genome inserted in the *Hin*dIII site of pBR322; pACYC177 (14), a vector containing no *Eco*RI sites; pMOV9 (15), containing an integrated proviral copy of the M-MuLV genome; pBR328 (16), a vector with a single *Pvu* II site; and piVX (17), a plasmid carrying the *SuIII*$^+$ gene.

Enzymatic Reactions. Synthetic oligomers (New England Biolabs) were phosphorylated with T4 polynucleotide kinase (Bethesda Research Laboratories) at a concentration of 1 μg per 10 μl in buffer (50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/10 mM dithiothreitol) containing 1 mM ATP (5 Ci/mmol; 1 Ci = 37 GBq) for 1 hr at 37°C. One-tenth of the preparation (0.1 μg) was ligated to DNA (1–5 μg) in the same

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: M-MuLV, Moloney murine leukemia virus; kb, kilobase(s); bp, base pair(s).

buffer overnight at 15°C before purification by agarose gel electrophoresis. Restriction enzymes were used according to the specifications of the manufacturer (New England Biolabs). Partial cleavages were carried out at 25°C for various times (1–60 min). Typically, 40 μg of DNA in 200 μl of the recommended buffer was digested with 1 unit of enzyme, and aliquots were removed for analysis. Optimal times for formation of full-length linear DNAs were usually 20–40 min. Cohesive ends of DNA fragments were filled in with the Klenow fragment (18) of DNA polymerase I (New England Biolabs) in buffer (50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/10 mM dithiothreitol) containing the four deoxyribonucleotides (1 mM each). Ends were blunted before addition of linkers by treatment with S1 nuclease (19) (Boehringer Mannheim) in buffer (300 mM NaCl/30 mM sodium acetate, pH 4.6/4 mM $ZnCl_2$) at 20°C for 20 min. Reactions with terminal transferase (20, 21) obtained from Bethesda Research Laboratories were carried out with ≈0.5 μg of DNA in 100 μl of buffer (200 mM potassium cacodylate, pH 7.2/1 mM mercaptoethanol/1 mM $CoCl_2$/50 μM dGTP or dCTP) for 1–4 min at 25°C. DNAs to be joined were mixed, heated to 70°C for 5 min, and then cooled to 40°C for 4 hr before transformation of bacteria.

DNA sequence analysis was as described (22).

Bacterial Transformations. Transformation of strain CC114 was carried out according to standard protocols, treating the cells with buffer (50 mM $CaCl_2$/10 mM Tris·HCl, pH 7.4) to induce competence. Growth on MacConkey plates was allowed for 24 hr for unambiguous identification of colonies bearing the suppressor gene. We have noticed that clones harboring the *SuIII*$^+$ gene in high copy number have an increased generation time and, therefore, become visible later as macroscopic colonies on the plates.

Animal Cell Transfections. Viral DNAs were introduced into NIH/3T3 cells by the DEAE dextran method (23) or by calcium phosphate coprecipitation (24, 25) with equivalent results. Permuted viral inserts were excised by cleavage with *Hin*dIII and polymerized with T4 DNA ligase before transfection; proviral clones were used without modification.

Analysis of Viral DNA. Preintegrative viral DNA was extracted from cells by the Hirt (26) procedure 24 hr after infection. The DNA was treated with a selected restriction enzyme, displayed by agarose gel electrophoresis, transferred to nitrocellulose (27), and detected by hybridization with a radioactive viral DNA probe.

Detection of Virus. Fully wild-type replication-competent virus was detected by the XC plaque assay (28); transmissibility was determined by reverse-transcriptase assay (29).

RESULTS

Conventional Linker Insertion Mutagenesis. To construct a library of mutants of M-MuLV, we chose to insert synthetic oligonucleotides containing an *Eco*RI restriction site, because the M-MuLV genome contains no site for cleavage by the *Eco*RI restriction enzyme, and because these linkers are readily available. Two plasmids encoding the genome of M-MuLV were constructed containing no *Eco*RI sites in other positions, including the vector. In one construct, the permuted copy of the viral genome in plasmid p8.2 (9, 13) was excised from the vector and transferred into the *Hin*dIII site of the vector pACYC177 (14) to form the plasmid termed pA8.2. In the other construct, the proviral insert of pMOV9 (15) was excised from its vector and transferred by G·C tailing (21) into the vector consisting of the 2.4-kilobase (kb) *Hae* II fragment of pACYC177. The resulting plasmid, termed pT11, was free of *Eco*RI sites and contained a minimum (5 kb) of nonviral sequences.

In our initial experiments, conventional linker insertions were made (Fig. 1). To mutagenize the clones, plasmid DNAs were linearized by treatment with restriction enzymes *Hae* III or *Alu* I. These enzymes produce blunt ends and can make cleavages at a large number of sites in the M-MuLV genome: a total of 64 and 36 sites, respectively, are present in the 8.8-kb provirus. After limited digestion, linkers were added with T4 DNA ligase, and the full-length linear molecules were isolated by agarose gel electrophoresis. The mixture was digested with *Eco*RI, circles were reformed by ligation, and clones were prepared from bacterial transformants. With this technique, our best experiments produced populations in which ≈50% of the DNAs were found to contain novel *Eco*RI sites. In some cases, however, only 10% contained an *Eco*RI site. Furthermore, independently isolated inserts at a given site sometimes generated different phenotypes, suggesting that sometimes insertions of different lengths had occurred.

The positions of a large number of these insertion mutations were determined by digestion with pairs of restriction enzymes (*Eco*RI and *Hin*dIII, or *Eco*RI and *Sal* I) followed by agarose gel electrophoresis. Once the approximate map position was determined from these digests, the exact site of insertion was mapped by measuring the distance from the linker to a nearby restriction site on acrylamide gels. When two *Hae* III or *Alu* I sites were close together, DNA sequence analysis was used to determine the insertion site. Mutations were assigned numbers according to the base position of the insertion starting at the left edge of the left long terminal repeat of a proviral copy of the M-MuLV genome. One collection of such insertions is shown in Fig. 2. Insertions are fairly uniformly distributed along the genome.

Suppressor-Linker Insertional Mutagenesis. To improve these methods, a novel linker was constructed (Fig. 3). A 220-base-pair (bp) fragment containing the *SuIII*$^+$ tyrosine tRNA gene (*SupF*) was excised from the piVX plasmid (17) and the 4-bp cohesive ends were filled in by treatment with the Klenow fragment of DNA polymerase I. Synthetic octamer linkers containing the cleavage site for *Pvu* II were added with T4 DNA ligase, restoring the complete *Eco*RI sites at each end of the fragment (Fig. 3). Subsequent cleavage with *Pvu* II formed a blunt-ended DNA fragment con-

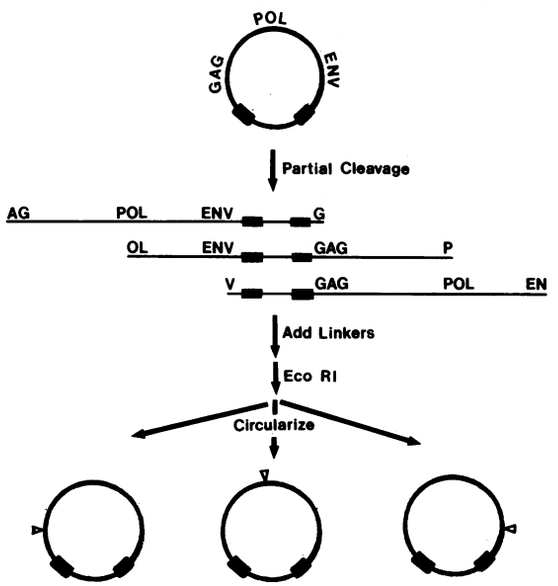

FIG. 1. Linker insertion mutagenesis. A circular plasmid containing a DNA copy of the genome of M-MuLV is cleaved by limited digestion with a selected restriction enzyme. The full-length linear DNAs are purified and ligated to *Eco*RI linkers. Finally, the DNAs are treated with *Eco*RI and recircularized by treatment with DNA ligase at low DNA concentrations. The result is a library of mutant DNAs each containing a single *Eco*RI linker insertion.

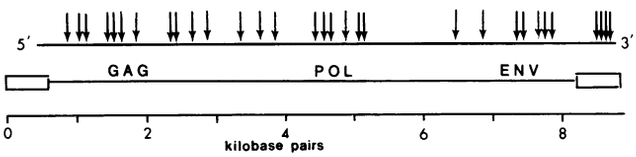

FIG. 2. The position of insertion of *Eco*RI linkers in a library of mutants of M-MuLV. Thirty-one insertion mutations into plasmid pA8.2 were mapped by cleavage with *Hin*dIII and *Eco*RI, and with *Sal* I and *Eco*RI. The approximate positions of the new *Eco*RI sites in the Moloney genome are indicated by the arrows.

taining the *SuIII*$^+$ gene, flanked by *Eco*RI recognition sequences 3 bp from each end. This molecule was amplified by cloning in the *Pvu* II site of the vector pBR328; large amounts of this reagent could be readily prepared as needed by excision of the fragment with *Pvu* II and by purification on an acrylamide gel.

Libraries of mutants were generated by ligating this blunt-ended fragment to a linearized target DNA and directly transforming *lac*$^-$ *am E. coli* (strain CC114) with the ligation mix. The cells were plated on MacConkey lactose plates containing ampicillin, and red (*Lac*$^+$) colonies were picked and pooled. One collection of such mutants was generated with pT11 DNA cleaved with *Sma* I (Table 1); only 30% of the colonies were red, but every red colony (60/60) contained an insertion mutant (data not shown). DNA was prepared from pooled red colonies, cleaved with *Eco*RI to remove the *SuIII*$^+$ gene, recircularized, and introduced into cells. Every white (*Lac*$^-$) colony contained DNA with a single simple insertion of the *Eco*RI site. Sequence analysis of one clone showed that 12 bp had been inserted as expected (data not shown). From this library, all five possible insertions at *Sma* I sites were recovered (Table 1).

A second library of mutants was generated by an extension of the above procedure. Plasmid pT11 DNA was linearized by partial digestion with *Sau*96I, which cleaves frequently (87 times in M-MuLV) and which leaves 3-bp cohesive ends. The linear molecules were blunted with S1 nuclease under mild conditions and ligated to the suppressor linker. As before, every red (*Lac*$^+$) colony contained an insertion mutant; in this case, the ability to detect these mutants by a plate assay was essential, because the fraction of the total colonies containing inserts was often low (5%–10%). After excision of the suppressor fragment from DNAs of pooled red colonies, clones were formed that contained the same 12-bp insertion as before, but with a loss of 3 bp to yield a net insertion of 9 bp. Mapping the position of the insertion in many of the clones showed a wide distribution of insertion sites as had been found for synthetic linker insertions. Those DNAs with inserts outside the proviral DNA were not analyzed further.

Isolation of Viable Insertion Mutants of M-MuLV. Members of the libraries generated as described above were tested for their ability to induce XC syncytia after DNA transfection of NIH/3T3 cells in culture (9). Viable mutants from 10-bp insertion libraries were detected, but at low frequencies; all such mutants were clustered in noncoding regions (Table 2). Thus, any frameshift mutations that mapped in the viral genes were apparently lethal. Viable mutants from 12-

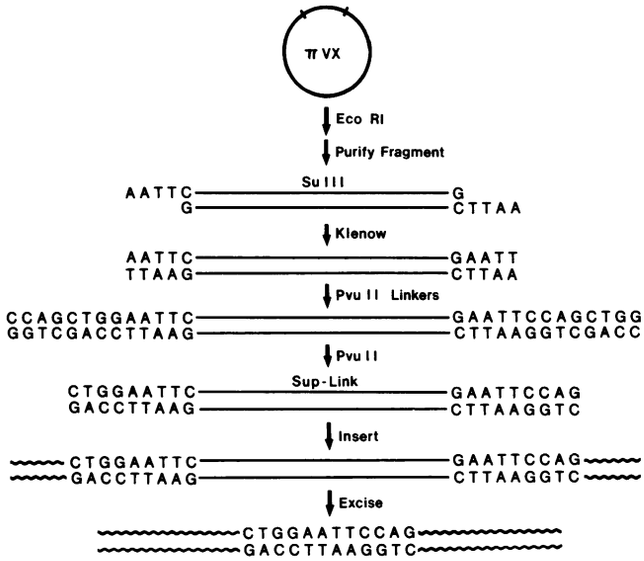

FIG. 3. Construction and use of the suppressor-linker (Sup-Link) DNA fragment. The 220-bp fragment from plasmid piVX bearing the *E. coli SuIII*$^+$ gene was ecised by *Eco*RI cleavage, and octamer linkers containing the recognition sequence for *Pvu* II were added. Cleavage with *Pvu* II yielded the "Sup-Link" DNA, a blunt-ended DNA containing the *SuIII*$^+$ gene flanked by *Eco*RI sites. Successful insertion of this DNA into a target plasmid (wavy lines) could be readily detected by the suppression of a *lac* amber gene resident in the bacterial host chromosome. Excision of the insert generates a 12-bp insertion (bottom line).

and 9-bp insertions were very common, appearing as 15% and 33% of the total mutants, respectively (Table 1). The insertions in these mutants were located at many positions in the viral genome (Table 2). Many were found in the U3 region of the long terminal repeat sequences, containing transcriptional promoter and enhancers. Other mutants were obtained with insertions in the region upstream of the *gag* gene, in gag proteins P12 and P10, in the 3′ part of the *pol* gene, and throughout the *env* gene. Thus, these regions are at least moderately flexible without serious effects on the replication of the virus. Conspicuously absent from the viable mutants were any insertions in gag proteins P15 and P30 and in the 5′ portion of the *pol* gene. These regions may be very sensitive to alterations and may be very critical for the growth of the virus (ref. 30; unpublished observations).

Several of the mutants in coding regions of the genome, although XC positive, reproducibly yielded fewer plaques per μg of applied DNA than the wild type (Table 2). Thus, these mutants may show quantitative defects in replication functions.

Stability of the Insertion Mutations. Those mutant viruses capable of inducing XC plaques were analyzed further to assess the stability of the alteration. Virus was recovered from the cell cultures after transfection and was used to infect fresh NIH/3T3 cells at high multiplicity. The preintegrative DNA intermediates were isolated (26) and analyzed by agarose gel electrophoresis followed by blot hybridization with a labeled virus-specific probe. The retention of the *Eco*RI site

Table 1. Libraries of insertion mutants

Library	Parent plasmid	Mutagen	Target site	Members, no.	Viable/tested	% viable
1	pA8.2	12-mer linker	*Alu* I	12	1/5	
2	pA8.2	10-mer linker	*Hae* III	142	3/12	
3	pT11	12-mer linker	*Hae* III	75	9/60	15
4	pT11	Sup-Link	*Sma* I	5	3/5	
5	pT11	Sup-Link	*Sau*96I	120	11/33	33

Sup-Link, suppressor-linker.

Table 2. Properties of selected mutants

Mutant	Site of insertion	Nucleotide position of insertion	Region affected	Mutagen	XC plaque formation*	Transmissibility†	*Eco*RI site maintained‡
*in*479-12	*Sma* I	479	R in 5′ LTR	Sup-Link	+	+	−
*in*845-12	*Hae* III	845	gPr80gag	12-mer	+	+	+
*in*1525-12	*Hae* III	1525	P12	12-mer	+	+	+
*in*1568-9	*Sau*96I	1568	P12	Sup-Link	+	+	ND
*in*1688-12	*Hae* III	1688	P12	12-mer	+	+	ND
*in*2504-12	*Hae* III	2504	P30/P10	12-mer	+/−	+	−
*in*2636-9	*Sau*96I	2636	P10	Sup-Link	+	+	+
*in*2645-9	*Sau*96I	2645	P10	Sup-Link	+	+	+
*dl*5395-447	*Sau*96I	5395	Pol	Sup-Link	−	+	−
*in*6199-12	*Sma* I	6199	Pol	Sup-Link	+	+	+
*in*6438-12	*Hae* III	6438	Env	12-mer	+	+	+/−
*in*7030-9	*Sau*96I	7030	Env	Sup-Link	+/−	+	+/−
*in*7407-9	*Sau*96I	7407	Env	Sup-Link	+	+	+
*in*7442-9	*Sau*96I	7442	Env	Sup-Link	+/−	+	+/−
*in*7601-12	*Hae* III	7601	Env	12-mer	+/−	+	ND
*in*8299-12	*Alu* I	8299	U3 in 3′ LTR	12-mer	+	+	+
*in*8397-10	*Hae* III	8397	U3 in 3′ LTR	10-mer	+	+	+
*in*8397-12	*Hae* III	8397	U3 in 3′ LTR	12-mer	+	+	+/−
*in*8442-10	*Hae* III	8442	U3 in 3′ LTR	10-mer	+	+	+
*in*8470-9	*Sau*96I	8470	U3 in 3′ LTR	Sup-Link	+	+	ND
*in*8472-12	*Hae* III	8472	U3 in 3′ LTR	12-mer	+	+	+
*in*8517-10	*Hae* III	8517	U3 in 3′ LTR	10-mer	+	+	+
*in*8530-9	*Sau*96I	8530	U3 in 3′ LTR	Sup-Link	+	+	−
*in*8743-12	*Sma* I	8743	R in 3′ LTR	Sup-Link	+	+	−
*in*1074-12	*Hae* III	1074	P15	12-mer	−	−	ND
*in*6527-12	*Sma* I	6527	Env	Sup-Link	−	−	ND
*in*6684-12	*Sma* I	6684	Env	Sup-Link	−	−	ND

Those insertion mutants found to be replication competent are listed. Three defective viruses whose insertions were mapped in detail are also shown. LTR, long terminal repeat; Sup-Link, suppressor-linker.

*+, Wild-type levels (≈1000 XC plaques per 100 ng); +/−, low levels (1–10 per 100 ng); −, no plaques.

†+, Release of particles after viral infection as judged by reverse transcriptase assay (29).

‡+, Complete sensitivity to *Eco*RI after 2 weeks of passage; +/−, partial resistance (10%–50%) after 2 weeks; −, complete loss of the site; ND, not done.

could be demonstrated by sensitivity of the DNA to *Eco*RI; loss of the mutation was indicated by the appearance of *Eco*RI-resistant DNA. The mutants that gave rise to a wild-type number of XC plaques proved to be quite stable: the DNAs were completely sensitive to *Eco*RI digestion and gave rise to DNA fragments of the appropriate sizes predicted by the location of the insertion (Fig. 4). After extended passage in culture, some of these mutants did reveal the appearance of low levels of *Eco*RI-resistant DNA (Fig. 4). Those mutants that consistently yielded few XC plaques showed the most rapid loss of the site. Two mutants with insertions in the R region of the long terminal repeat also showed rapid loss of the mutations; these mutants (*in*479-12, *in*8743-12) are exceptional because loss can occur by special means during retroviral replication. Analysis of these mutants will be described elsewhere (unpublished results).

DISCUSSION

The techniques of *in vitro* mutagenesis have manifold applications in the correlation of structure and function of genes. We have described a procedure for the generation of 9- and 12-bp insertion mutations in which a short DNA fragment carrying a selectable marker is used as the initial insertional mutagen. The method is an improvement over previous methods (11) for synthetic DNA linker insertions at blunt restriction sites in that fewer steps are involved and 100% of the resulting clones have acquired insertion mutations. Thus, the creation of large libraries of independent insertion mutants is an easy task. In the construction of insertions at sites that are cut to leave cohesive ends, these advantages become even more important. The efficiency of successful ligation of dodecamers to nuclease S1-blunted ends by the usual procedures is often low (on the order of 10%), and many colonies must be analyzed to recover a few mutants. The use of the suppressor-linker fragment allows the rapid detection of mutants by a simple plate test and obviates the

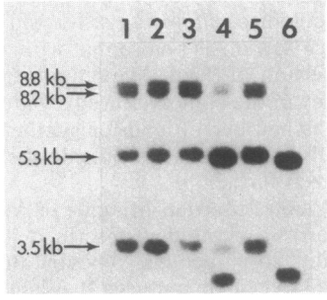

FIG. 4. Southern blot of viral DNAs synthesized by mutants of M-MuLV. Mutant viral DNAs were introduced into cells by transfection, and virus was allowed to spread through the cultures for a period of 2 weeks. Virus was then harvested and used to infect fresh NIH/3T3 cells at high multiplicity. The unintegrated viral DNAs were isolated, cleaved, and analyzed by gel electrophoresis and blot hybridization. Lanes 1, 3, and 5, DNAs were cleaved with *Hin*dIII. Circular molecules were cleaved once to yield 8.8- and 8.2-kb linear molecules; linear molecules were also cleaved once to yield 5.3- and 3.5-kb fragments. Lanes 2, 4, and 6, DNAs were cleaved with *Hin*dIII and *Eco*RI. New cleavages indicate the existence of an *Eco*RI site. Lanes 1 and 2, wild-type virus. A plasmid DNA bearing an *Eco*RI insertion outside the transcribed provirus was applied to cells. Lanes 3 and 4, mutant *in*8397-12. Lanes 5 and 6, mutant *in*8472-12.

screening of large numbers of clones by more arduous procedures. We have made the most extensive use of the procedure to make 12-bp insertions after removal of 3-bp cohesive ends, yielding a net 9-bp insertion. The same linker could also be used to make 15-bp insertions by joining to cohesive ends that are filled in with the Klenow fragment of DNA polymerase I. In our hands, however, the efficiency of this procedure was lower than that of joining to nuclease S1-blunted ends.

After the suppressor-linker is inserted and the suppressor gene is excised from a clone, an *Eco*RI site is generated at the point of insertion. This makes it easy to map the mutation and, in addition, can be used to detect the presence of the mutation after reintroduction of the DNA into the natural host. Further experiments (unpublished) have made use of the sites as genetic markers in studies of recombination between homologous regions of two different virus genomes.

We were able to assess the stability of the mutations during passage of the viruses by examining the persistence of the recognition site. Most insertions were quite stable and were retained over many generations. Occasionally, sites were lost during passage; the structure present after the loss is not yet known. Those sites lost from the central parts of the genome may be lost by point mutation, by deletion, or possibly by recombination with endogenous sequences; it is likely that the virus may not be restored completely to the wild type. It is interesting that the mutants that yielded fewer XC plaques after transfection often lost the site. We propose that these mutations are somewhat deleterious and that loss of the site results in selective growth advantage. Two mutants were exceptional (*in*479-12 and *in*8743-12); these DNAs yielded normal numbers of plaques but quickly lost the *Eco*RI site. Further studies have suggested that in these cases loss occurs during reverse transcription.

The creation of a unique *Eco*RI site in a DNA clone can serve as a point of entry for deletion mutagenesis in sequences otherwise devoid of infrequent cleavage sites and bearing only highly reiterated restriction sites. One could use the suppressor-linker to create deletions and simultaneously establish unique sites at the position of the deletion if the DNA is treated with an exonuclease before ligation to the suppressor-linker.

The general technique of insertion mutagenesis has enabled us to define several domains of the M-MuLV genome that are amenable to small alterations without deleterious effects on the virus. Using decamer linker inserts, we have been able to generate viable mutations in a variety of noncoding regions. No insertions that would result in frameshift mutations of the *gag*, *pol*, or *env* genes were found to leave the viral genome competent to replicate. Numerous viable mutations in viral genes were created with 9- and 12-bp insertions; these mutations define flexible regions of the viral proteins encoded on the viral genome. This procedure could be as readily applied to other cloned genes. During the course of analyzing the mutations in coding regions, we found that the frequency of appearance of viable mutants in a population was dependent on the size of the insertions. Specifically, 33% of the 9-bp insertions were viable, while only 15% of the 12-bp insertions were viable. Thus, a net insertion of three amino acids appears to be less disruptive to the gene products of M-MuLV than four amino acid insertions, at least in the limited number of cases tested. One explanation is that the 9-bp insertions might, by virtue of the difference in sequence, result in the insertion of less destabilizing amino acids than the 12-bp insertions. Clearly, each insertion can encode any of three different sets of amino acids depending on the translational reading frame through the insertion; it is not obvious by inspection alone that the 12-bp linker insertions would necessarily encode unusually disruptive amino acids relative to the amino acids inserted by the suppressor linker. It seems likely that, in general (and in any given gene), short insertions may generate a higher frequency of functionally active gene products.

No viable insertions have yet been mapped to the *gag* genes encoding P30 or P15, or to the region of the *pol* gene encoding reverse transcriptase. This result suggests that the products of these genes play critical roles in the life cycle of M-MuLV and that the proteins have strict structural requirements. The conclusion is supported by other genetic data (refs. 9 and 30; unpublished data).

We would like to thank Dr. Colin Manoil for the construction of strain CC114, Jan van Oostrum for generous gifts of T4 DNA ligase, and Margaret Grimes for excellent technical assistance. This work was supported by National Cancer Institute Grant 1 R01 CA 30488. Additional support was provided by awards from the Irma T. Hirschl Foundation and the Searle Scholars Program.

1. Dierks, P., Van Ooyen, A., Mantei, N. & Weissman, C. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 1411–1415.
2. Grosschedl, R. & Birnsteil, M. L. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7102–7106.
3. Benoist, C. & Chambon, P. (1981) *Nature (London)* **290,** 304–309.
4. McKnight, S. & Kingsbury, R. (1982) *Science* **217,** 316–325.
5. Montell, C. E., Fisher, E., Caruthers, M. & Berk, A. J. (1982) *Nature (London)* **295,** 380–384.
6. Fitzgerald, M. & Shenk, T. E. (1981) *Cell* **24,** 251–260.
7. Taniguchi, T. & Weissman, C. (1978) *J. Mol. Biol.* **118,** 533–565.
8. Crawford, S. & Goff, S. P. (1984) *J. Virol.* **49,** 909–917.
9. Schwartzberg, P., Colicelli, J. & Goff, S. P. (1983) *J. Virol.* **46,** 538–546.
10. Shortle, D., DiMaio, D. & Nathans, D. (1981) *Annu. Rev. Genet.* **15,** 265–294.
11. Heffron, F., So, M. & McCarthy, B. J. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 6012–6016.
12. Boyer, H. W. & Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41,** 459–469.
13. Shoemaker, C., Goff, S. P., Gilboa, E., Paskind, M., Mitra, S. W. & Baltimore, D. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3932–3936.
14. Chang, A. C. Y. & Cohen, S. N. (1978) *J. Bacteriol.* **134,** 1141–1156.
15. Chumakov, I., Stuhlmann, H., Harbers, K. & Jaenisch, R. (1982) *J. Virol.* **42,** 1088–1098.
16. Soberon, X., Covarrubias, L. & Bolivar, F. (1980) *Gene* **9,** 287–305.
17. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual.* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 353–362.
18. Jacobsen, H., Klenow, H. & Overgaard-Hansen, K. (1974) *Eur. J. Biochem.* **45,** 623–627.
19. Vogt, V. M. (1980) *Methods Enzymol.* **65,** 248–271.
20. Bollum, F. J. (1974) *Methods Enzymol.* **10,** 145–163.
21. Roychoudhury, R. (1981) in *Gene Amplification and Analysis*, eds. Chirikjian, J. G. & Papas, T. S. (Elsevier/North-Holland, New York), Vol. 2, pp. 41–83.
22. Maxam, A. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
23. Mertz, J. E. & Berg, P. (1974) *Virology* **62,** 112–124.
24. Graham, F. L. & van der Eb, A. J. (1973) *Virology* **52,** 456–467.
25. Graham, F. L. & van der Eb, A. J. (1973) *Virology* **54,** 536–539.
26. Hirt, B. (1967) *J. Mol. Biol.* **26,** 365–369.
27. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
28. Rowe, W. P., Pugh, W. E. & Hartley, J. W. (1970) *Virology* **42,** 1136–1139.
29. Goff, S. P., Traktman, P. & Baltimore, D. (1981) *J. Virol.* **38,** 239–248.
30. Schwartzberg, P., Colicelli, J. & Goff, S. P. (1984) *J. Virol.* **49,** 918–924.