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Genetics

Retroviral insertional mutagenesis of a target allele in a heterozygous murine cell line

(proviral insertion/β_2-microglobulin gene/viral rescue/Abelson murine leukemia virus/Moloney murine leukemia virus)

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ABSTRACT **A clonal murine cell line that is heterozygous at the β_2-microglobulin locus (*B2m*) was obtained by Abelson murine leukemia virus (Ab-MuLV) transformation of liver cells from (C57BL/6 × BALB/c) F_1 fetuses. To obtain proviral insertional mutants, we superinfected a subclone of these cells, which does not express the env surface protein of the Moloney leukemia virus (Mo-MuLV, the helper virus that was used to transmit the defective Ab-MuLV genome during transformation), with Mo-MuLV. Mutant clones that fail to express the C57BL/6 allele of *B2m* ($B2m^b$) were then immunoselected by using a monoclonal antibody that specifically recognizes the $B2m^b$ gene product and not that of the $B2m^a$ allele. Of 22 independent clones obtained, one contains a proviral insertion that is near or in the first exon of the $B2m^b$ gene. Surprisingly, the insertion is of the Ab-MuLV genome and not of replication-competent Mo-MuLV. This indicates that superinfection with Mo-MuLV "rescued" the defective Ab-MuLV genome, which then inserted into the $B2m^b$ gene. We conclude that when an allele-specific selection procedure exists, proviral insertion is a potential method for obtaining mutations in heterozygous mammalian cells. This approach may thereby provide a method for molecular cloning of such selectable genes, using a retroviral hybridization probe.**

Insertional mutagenesis by defined genetic elements is an important tool for identification and molecular cloning of genes from prokaryotic and eukaryotic organisms. Bacteriophage mu (1), yeast *Ty* elements (2), and *copia* (3) and *P* elements (4) of *Drosophila* all provide examples of insertions into genetic loci that have either occurred spontaneously or have been used actively as mutagenic agents. In mammalian systems, retroviral genomes appear to integrate randomly into the cellular host genome (5). A few examples of proviral insertions into specific cellular genes are known (6–11). These were obtained either by infection of cells in tissue culture or by infection of germ-line cells in intact animals.

Generally, retroviral infection results in only a small number of integrated proviruses in the infected cell. This property facilitates molecular cloning of loci that contain such insertions. However, mutations caused by retroviral insertion are likely to be recessive; that is, they occur in only one copy of a gene in the diploid cell, leaving the other copy functional. Thus, mutagenesis of most genes by use of retroviruses is prohibitively difficult due to the low probability of simultaneous mutation of multiple gene copies.

Retroviral infection of cells that are heterozygous at a given locus, followed by selection for mutations at a specific target allele of that locus, offers a potential means for surmounting this problem. We tested this hypothesis by infecting cells that are heterozygous at the β_2-microglobulin (β_2m) locus (*B2m*). β_2m, the light chain associated with H-2 class I cell-surface molecules, is encoded by a single-copy gene that bears a genetic polymorphism in C57BL/6 and related strains of mice that is not present in most other strains, including BALB/c (12). Cell lines derived from (C57BL/6 × BALB/c) F_1 mice codominantly express both allelic forms of light chain on the cell surface. By immunoselection using a monoclonal antibody that recognizes only the product of the C57BL/6 allele, we have been able to isolate mutants that no longer express the C57BL/6 form of β_2m (unpublished results). Therefore, we attempted to obtain retroviral insertional mutations by infecting a *B2m*-heterozygous cell line with Moloney murine leukemia virus (Mo-MuLV) prior to immunoselection.

MATERIALS AND METHODS

Development of an Appropriate Cell Line. $B2m^b$ mutants were derived from the clonal cell line 439.4, which was generated by transforming fetal (17–19 days of gestation) liver cells from a (C57BL/6 × BALB/c) F_1 mouse with the P160 strain of Abelson murine leukemia virus (Ab-MuLV P160), as described (14). The genotype of this cell line at the *B2m* locus is $B2m^a/B2m^b$. Because the multiplicity of infection used to generate clonal Ab-MuLV-transformed cell lines is <1, it was expected that many of the isolates would be negative for gp70 production. For these experiments, five clones, 439.1–439.5, were screened for the presence of gp70 by using a live-cell fluorescence test and an anti-gp70 monoclonal antibody. All five clones were negative in this test. To confirm the absence of Mo-MuLV-encoded proteins, all five clones were metabolically labeled with [^{35}S]methionine, and cell lysates were immunoprecipitated with an anti-Mo-MuLV antiserum; neither gag nor env viral proteins were detected (data not shown). These five clones then were tested for susceptibility to Mo-MuLV superinfection and all were found to be susceptible to the virus. After these analyses, the five clones were tested for optimal cytotoxicity with an anti-β_2m^b monoclonal antibody and rabbit complement (described below); the clone 439.4 was chosen and subcloned and is now referred to as 439.4.2. This subclone was retested for Mo-MuLV susceptibility and found to retain this property of the 439.4 parent clone. Subclone 439.4.2, which was used in this study, is maintained as a suspension culture in RPMI 1640 medium supplemented to contain 10% fetal calf serum and 50 μM 2-mercaptoethanol.

Viral Infection. Twenty independent cultures of 439.4.2 cells seeded at 100 cells per dish were grown to 10^5 cells each; this ensures the isolation of independent mutations at $B2m^b$. These were infected with 3×10^6 plaque-forming units of Mo-MuLV clone 2 (15), in the presence of Polybrene at 8 μg/ml. Cells were cultured for ≈7 days (to allow maximal

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Abbreviations: β_2m, β_2-microglobulin; *B2m*, gene encoding β_2m; Mo-MuLV, Moloney murine leukemia virus; Ab-MuLV, Abelson murine leukemia virus; kb, kilobase(s).

spread of virus, integration of the viral genome, and potential disruption of the $B2m^b$ gene) before immunoselection was performed.

Immunoselection. Complement-mediated lysis of cells expressing $B2m^b$ was performed on 22 separate cultures of Mo-MuLV infected 439.4.2 cells and on 2 uninfected cultures. The procedure for immunoselection has been described (13). Briefly, 5×10^5 cells were treated with a 1:100 dilution of the anti-β_2m^b monoclonal antibody S19.8 (16) in the presence of rabbit complement for 3 hr at 37°C. After this incubation, cells were cloned in soft agar. Clones producing macroscopic colonies after 7–10 days were isolated, grown in mass culture, and further characterized. None of the surviving clones express $B2m^b$.

The mutants obtained by immunoselection after Mo-MuLV superinfection can be divided into four classes. Nine of the 22 mutants contain a deletion starting near at least one repeat element within the first intron of $B2m^b$ and extending to a common point at least 8 kilobases (kb) upstream of $B2m^b$. Another 9 of the 22 appear to contain no $B2m^b$ gene and two copies of the $B2m^a$ gene. The mutants within each set are apparently identical to one another and to either of those from the two uninfected cultures and thus probably represent the background of $B2m^b$ mutations for this cell line (unpublished results). Three of the 22 mutants contain altered $B2m^b$ genes in addition to the normal $B2m^a$ gene but are not similar to the mutants described above. The mutation in $B2m^b$ in these three mutants is not of an intact Ab-MuLV proviral insertion, and the basis of their mutation is not yet known. The one mutant of the fourth type, referred to as mutant X, is described in this paper.

Southern Blot Analysis. Genomic DNA was extracted from cells by using procedures previously described (17) and then was cleaved with restriction endonucleases and electrophoresed in agarose gels. The DNA was denatured *in situ* and blotted onto nitrocellulose paper according to Southern (18).

Hybridization Probes for *B2m* and Ab-MuLV. The C57BL/6 *B2m* gene was obtained as an *Xho* I fragment cloned in the plasmid vector pKC7 (from J. Seidman, Harvard Medical School, Boston). We subcloned the *Xho* I–*Hin*dIII fragment in pKC7; this probe contains the first exon of *B2m* and sequences flanking both sides of it (Fig. 1*a*). The Ab-MuLV probe, originally designated pAbl subclone 3, is an internal *Bgl* II fragment of the Ab-MuLV P120 genome, and has been cloned in pBR322 (23). This probe, which contains sequences homologous only to c-*abl* and not to Mo-MuLV (Fig. 1*c*), was obtained from S. Goff (Columbia University). Both probes were radiolabeled with [^{32}P]dCTP by nick-translation and were hybridized to Southern blots as described (17).

RESULTS

Experimental Design. Mutants in the $B2m^b$ gene were generated by infecting 439.4.2 cells, which are heterozygous at the *B2m* locus, with Mo-MuLV. Cells that failed to express $B2m^b$ were selected by a single round of immunoselection using an anti-β_2m^b monoclonal antibody and rabbit complement-mediated cell lysis. Mutant clones then were screened by the Southern blot procedure to determine whether proviral insertions into the $B2m^b$ gene had occurred.

Two criteria were most important in choosing the right cell line for these experiments: (*i*) that it be heterozygous for $B2m^b$ and (*ii*) that it could be infected with Mo-MuLV. An Ab-MuLV-transformed cell line was chosen because this and other virus-transformed cell lines had been used successfully for immunoselection against cells expressing β_2m and H-2 class I antigens (13); in our hands, the chromosome number and structure remain relatively intact. Since Mo-MuLV cannot superinfect cells that express viral envelope glycoprotein gp70, we used a clone of cells, which does not express gp70, for subsequent infection with Mo-MuLV.

Immunoselection was performed with the anti-β_2m^b monoclonal antibody S19.8. This antibody recognizes the allotypic determinant Lym-11, which is found only on the C57BL/6 form of β_2m and not the BALB/c form (16); a single round of selection is sufficient to obtain cells that do not express β_2m^b.

Serological analysis of the clones surviving the selection was performed by microcytotoxicity. All surviving clones failed to express the product of the $B2m^b$ locus (the Lym-11 antigenic determinant) but continued to express all the H-2 class I antigens (H-2K^b, H-2D^b, H-2D^d, and H-2L^d) characteristic of a (C57BL/6 × BALB/c) F_1 heterozygous cell line. This implies that $B2m^b$ mutant clones also continued to express the $B2m^a$ allele of *B2m*, since normal expression of H-2 class I antigens requires β_2m expression (24, 25).

Southern Blot Analysis of an Immunoselected $B2m^b$ Mutant. The $B2m^a$ and $B2m^b$ allelic polymorphism is defined by a single base substitution at the codon for amino acid 85. $B2m^a$ codes for an aspartic acid at this position, whereas $B2m^b$ has an alanine codon there. This substitution creates a *Bgl* I restriction site in $B2m^b$ that is not present in $B2m^a$ (19). Therefore, the *B2m* first-exon probe will hybridize to two fragments in *Bgl* I-digested DNA from $B2m^a/B2m^b$ heterozygous cell lines, a 9.9-kb fragment from the $B2m^a$ allele and an 8.7-kb fragment from $B2m^b$ (Fig. 1*a*). The results of

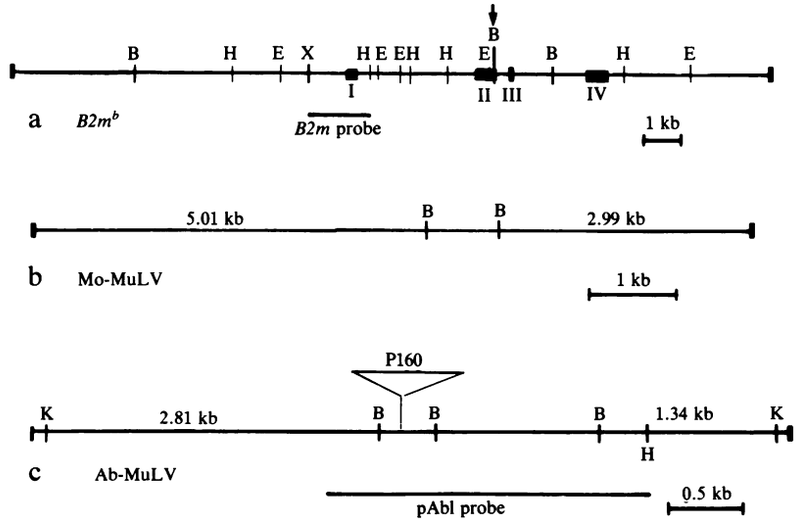

FIG. 1. (*a*) Molecular map of the $B2m^b$ gene, showing the cleavage sites of several restriction enzymes and the four exons (I–IV) (19). The *Bgl* I restriction site (arrow) represents the restriction polymorphism not found in $B2m^a$. The first exon probe is indicated here. (*b*) *Bgl* I restriction map of a Mo-MuLV proviral genome showing the sizes of junction fragments (20). (*c*) Molecular map of an Ab-MuLV proviral genome, showing the sizes of *Bgl* I junction fragments, the pAbl probe, and the insertion that distinguishes the Ab-MuLV P160 genome from that of Ab-MuLV P120 (21, 22). Restriction sites: B, *Bgl* I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; X, *Xho* I.

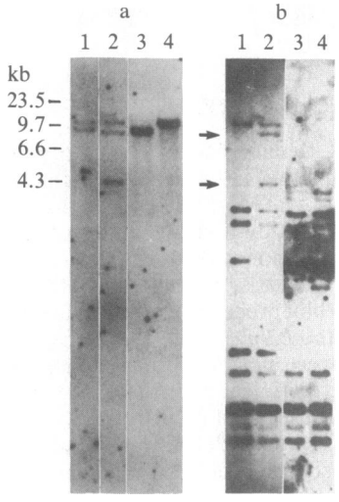

FIG. 2. Southern blot analysis of 439.4.2 (lanes 1), mutant X (lanes 2), C57BL/6 (lanes 3), and BALB/c (lanes 4) DNAs digested with *Bgl* I and probed with the *B2m* first-exon probe (*a*) or with pAbl (*b*). Arrows indicate mutant X bands, at 8.4 kb and 4.5 kb, hybridizing to both probes. Markers at left indicate positions of bands from *Hin*dIII-digested λ DNA.

hybridizing this probe to Southern blot of *Bgl* I-digested genomic DNA from the cell line 439.4.2, a $B2m^b$ mutant, and the two parental types of mouse are shown in Fig. 2*a*. As expected, 439.4.2 (wild type) contains both $B2m^a$ and $B2m^b$ *Bgl* I fragments, and each parental type (C57BL/6 and BALB/c) contains only one. The blot of DNA from mutant X shows three bands. The 9.9-kb band corresponds to the $B2m^a$ allele, but the 8.4 kb and 4.5 kb bands are found only in the mutant X DNA and suggest that this mutation may be an insertion of sequences containing one or more *Bgl* I restriction sites within the region hybridizing to the first-exon probe.

If the mutation were due to a single proviral insertion, then the *Bgl* I restriction fragments from the two ends of the linear provirus would contribute the extra 4.2 kb of DNA. Such an insertion is not consistent with the Mo-MuLV genome, which contains *Bgl* I junction fragments (those linked to cellular DNA in a typical proviral insertion) that total 8.0 kb (Fig. 1*b*). However, insertion in this region of $B2m^b$ is consistent with the total length of *Bgl* I junction fragments of the Ab-MuLV genome, which is 4.2 kb (Fig. 1*c*). We thus considered whether superinfection with MoLV had rescued an Ab-MuLV proviral genome, which then inserted in the $B2m^b$ gene, and tested this hypothesis by using pAbl to probe Southern blots of mutant X and 439.4.2 DNA.

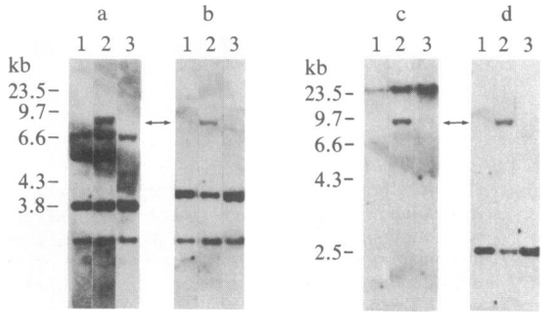

FIG. 3. Southern blot analysis of 439.4.2 (lanes 1), mutant X (lanes 2), and C57BL/6 (lanes 3), DNAs digested with *Hin*dIII (*a* and *b*) or *Eco*RI (*c* and *d*) and probed with pAbl (*a* and *c*) or the *B2m* first-exon probe (*b* and *d*). Arrows indicate the band present only in mutant X DNA.

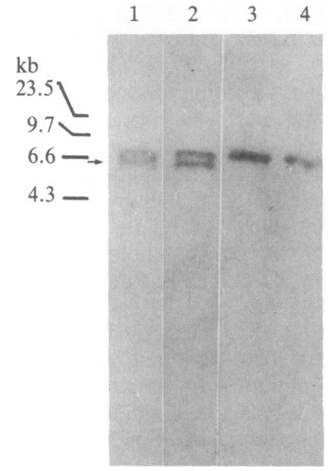

FIG. 4. Southern blot analysis of 439.4.2 (lane 1), mutant X (lane 2), C57BL/6 (lane 3), and BALB/c (lane 4) DNAs digested with *Kpn* I and probed with pAbl. Both mutant X and wild-type DNA contain two fragments that hybridize with the probe. The larger fragment, present in all four lanes, represents cross-hybridization of pAbl with the cellular gene c-*abl*. The band at ≈6.0 kb (arrow) is the almost full-length Ab-MuLV proviral genome and is present in only wild-type 439.4.2 and mutant X DNAs. Because mutant X contains no other bands, the Ab-MuLV insertion at $B2m^b$ must be of an intact provirus.

Fig. 2*b* shows a Southern blot of *Bgl* I-digested genomic DNA probed with pAbl. The bands present in parental C57BL/6 and BALB/c DNA extracted from mouse liver represent cross-hybridization of pAbl with the cellular gene c-*abl*. The bands in 439.4.2 (wild type) DNA and absent from the liver DNA represent pAbl hybridization to junction fragments and internal fragments of the Ab-MuLV proviral genome that initially transformed the cell line. Mutant X contains two additional bands detected by pAbl. These bands comigrate with those detected with the *B2m* probe. These observations are consistent with insertion of an Ab-MuLV provirus into the $B2m^b$ gene. To further test this hypothesis, we performed Southern blots using two other restriction enzymes, *Hin*dIII and *Eco*RI (Fig. 3). Both showed results similar to those for the *Bgl* I analysis; that is, there is a band in each, unique to mutant X, that is detected by both pAbl and *B2m* probes.

The restriction endonuclease *Kpn* I cleaves once in each long terminal repeat sequence in the Ab-MuLV genome and does not cleave internally (Fig. 1*c*). Therefore, hybridization of pAbl to *Kpn* I-digested DNA from cells containing intact Ab-MuLV proviruses should show a band corresponding to an almost full-size Ab-MuLV genome. Fig. 4 shows a Southern blot of *Kpn* I-digested DNA probed with pAbl. The lanes containing C57BL/6 and BALB/c DNA each show a band of ≈7.2 kb, representing cross-hybridization of pAbl with the cellular gene c-*abl*. Subclone 439.4.2 (wild-type) DNA has an additional band at ≈6.0 kb corresponding to the Ab-MuLV provirus. Mutant X DNA contains the same bands but no additional bands, again indicating that the insertion into $B2m^b$ is most likely a full-length Ab-MuLV P160 genome.

DISCUSSION

We have isolated, by immunoselection of a $B2m^a/B2m^b$ heterozygous cell line, mutants that no longer express $B2m^b$. Twenty-two of these mutants were obtained after infection of the 439.4.2 parental cell line with Mo-MuLV and are briefly described in *Materials and Methods*. The mutant described here, mutant X, was shown by Southern blot analysis to contain two anomalous $B2m^b$ *Bgl* I restriction fragments in

addition to the $B2m^a$ fragment. This mutant does not contain a Mo-MuLV genome inserted into $B2m^b$. Furthermore, the fragments unique to mutant X comigrate with those on a duplicate blot probed with pAbl. To confirm that this mutant contained an intact insertion of the Ab-MuLV provirus, we performed similar Southern blot experiments using two other restriction enzymes, *Eco*RI and *Hin*dIII (Fig. 3). Again, the unique $B2m^b$ band in mutant X comigrated with the novel band found in this mutant by hybridization with pAbl, whereas there were no such bands in the wild-type cell line 439.4.2. Hybridization of the pAbl probe to *Kpn* I-digested mutant DNA showed no bands other than the ones representing c-*abl* and the full-length Ab-MuLV provirus, which further implies that the insertion into $B2m^b$ was that of an intact, full-length Ab-MuLV provirus.

We can determine the orientation and approximate position of the Ab-MuLV insertion into $B2m^b$ based on the sizes of the *Bgl* I and *Hin*dIII restriction fragments. Fig. 5 shows that the integration site is very close to or in the first exon of $B2m^b$.

Fine-structure mapping by cloning and sequencing the mutant gene is, of course, necessary to determine the precise location of the Ab-MuLV insertion; that is, whether it is within the first exon or inserted in noncoding, possibly regulatory sequences. Such information will be necessary for determination of the mechanism by which insertion of Ab-MuLV blocks $B2m^b$ expression.

From the observations reported here, we can offer a theory of how the Ab-MuLV provirus, and not Mo-MuLV, inserted into $B2m^b$. Immunoselection of the Ab-MuLV-transformed cell line was performed 1 week after infection of the cells. It is possible that Mo-MuLV superinfected many, but not all, of the cells and "rescued" the defective Ab-MuLV genome from one of these. Therefore, during the week of culture, the supernatant may have contained infectious Ab-MuLV virus. Previously uninfected cells may then have been infected with Ab-MuLV, using Mo-MuLV as a helper virus in a second round of infection. Evidence supporting this is that mutant X expresses both *env*- and *gag*-encoded Mo-MuLV proteins, indicating that it was superinfected with Mo-MuLV. Furthermore, mutant X cells contain an additional Mo-MuLV proviral genome (data not shown).

Our observations argue that selection for mutants generated by proviral insertion into a gene in diploid cells requires the gene to be in a hemizygous or heterozygous state; this increases the probability of obtaining insertional mutants. In our hands, it has not been possible to obtain mutants of any kind in homozygous loci of cell-surface antigens without multiple rounds of selection and chemical mutagenesis (26). It is likely that proviral insertional mutants in diploid cells will only be observed when the disrupted gene is either present in one copy per diploid genome or requires complete expression of both copies for the normal phenotype.

At least three other proviral insertions into known genes in cell lines have been reported. Wolf and Rotter (9) demonstrated that in an Ab-MuLV-transformed cell line, a Mo-MuLV-like provirus is inserted into the cellular gene encoding p53 tumor antigen. Although the p53 gene is normally present in two copies in diploid cells, it appears that there is no intact copy of the p53 gene in this particular cell line. It was not clear in this study (9) whether both copies contained the proviral insertion or whether one contained the insertion and the other contained a different mutation rendering the gene structurally abnormal. Since it seems unlikely that both copies contained identical independent proviral insertions, it is possible that the chromosome containing the proviral insertion in the p53 gene was duplicated by mitotic nondisjunction after loss of the normal chromosome. Alternatively, this cell line may be hemizygous at the p53-encoding locus. Kuff *et al.* (10) reported the insertion of intracisternal A-particle (IAP) genes, which are retroviral-like elements, into two κ immunoglobulin-light-chain genes. These were cloned from two hybridoma cell lines defective in κ light chain production. Immunoglobulin-producing cells have been shown to undergo allelic exclusion at light chain-producing loci and are thus effectively hemizygous for them. Varmus *et al.* (27) described a system in which a single copy of Rous sarcoma virus, integrated into a rat cell line, was mutagenized by superinfection of the cell line with Mo-MuLV. Two independent, intact Mo-MuLV insertional mutants that blocked expression of the *src* gene and the transformed phenotype were obtained. We interpret these results as well as our own to indicate that mutagenesis by retroviruses in tissue culture cells generally requires the target allele to be in a hemizygous or heterozygous state.

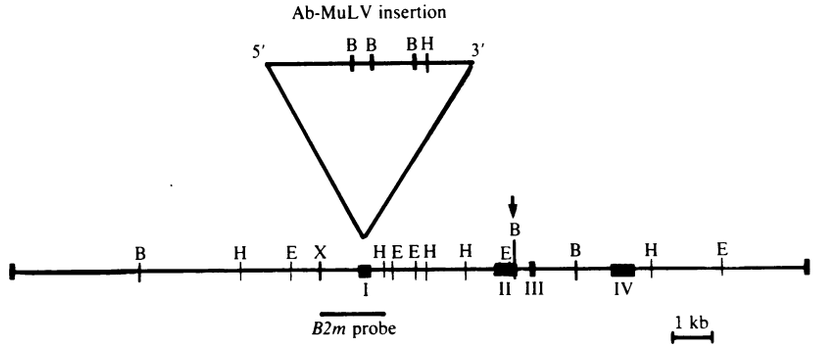

FIG. 5. Orientation of the Ab-MuLV proviral insertion in $B2m^b$ (see Fig. 1). The precise location of the insertion is not known; by Southern blot analysis, it is estimated to be within 0.5 kb of the first exon of $B2m^b$. We approximated the position of Ab-MuLV in the $B2m^b$ gene by interpretation of the *Bgl* I Southern blot data (Fig. 2). Since Ab-MuLV is inserted into the region that corresponds to the *B2m* first-exon probe, the particular orientation must agree with the minimum sizes of the regions flanking this region bounded by the *Bgl* I restriction sites. These are 4.7 kb (5' side) and 3.3 kb (3' side). Other Southern blots, using a *B2m* 3' probe (data not shown), revealed that the 4.5-kb *Bgl* I fragment in mutant X corresponds to DNA on the 3' side of the first exon. The Ab-MuLV provirus cannot be inserted in the 3'→5' transcriptional orientation because the 2.81-kb *Bgl* I Ab-MuLV junction fragment plus the minimum size of the 3' flanking sequence (3.3 kb) is larger than the observed 4.5-kb band. If the proviral orientation were in the 5'→3' orientation, the observed *Bgl* I bands for mutant X would be in accord with this model. Subtracting the sizes of the *Bgl* I Ab-MuLV junction fragments from the sizes of *Bgl* I bands unique to mutant X, we place the proviral insertion very near the first exon of $B2m^b$, which contains the 5' untranslated sequence, signal peptide coding sequence, and the first two amino acid codons of *B2m* (19). This approximate position is confirmed by the *Hin*dIII Southern blot data (Fig. 3 *a* and *b*), which shows a novel fragment that is the sum of the size of the 5' *Hin*dIII fragment of the Ab-MuLV genome (5.4 kb, Fig. 1*c*) plus the distance from the first exon of *B2m* to the next 5' *Hin*dIII site (3.8 kb, Fig. 1*a*), or ≈9.2 kb. Restriction sites shown: B, *Bgl* I; H, *Hin*dIII; X, *Xho* I.

Retroviral insertions into genetic loci have also been observed in intact mice. Jenkins *et al.* (6) reported that the dilute-color-coat mutation was the result of an ecotropic Mo-MuLV proviral insertion at the *dilute* locus; excision of the provirus resulted in reversion of the mutant to normal coat color. Since the new provirus was present in all tissues of strains carrying *dilute*, it seemed that the spontaneous original insertion occurred in germ-line, haploid cells. Hence, the dilute phenotype is only observed when mice are made homozygous for *dilute*. Jaenisch *et al.* (11) inserted a Mo-MuLV genome into a mouse embryo and later observed that this mouse and its progeny contained a Mo-MuLV proviral genome inserted into the $\alpha 1$ procollagen gene. This observation was made because the mutation is lethal when progeny are made homozygous for the Mo-MuLV insertion. From the cases of proviral insertions reported so far, it appears that such mutations are usually recessive. With intact mice, this therefore requires breeding to obtain animals homozygous for the proviral insertional mutation before the mutant phenotype can be observed.

In our study, selectable insertional mutagenesis of a single-copy mammalian gene was achieved by using retroviral superinfection followed by immunoselection against the $B2m^b$ gene in a $B2m^a/B2m^b$-heterozygous murine cell line. It is probable that Mo-MuLV infection rescued the defective Ab-MuLV genome, which then mutagenized $B2m^b$. Although immunoselection of a polymorphic cell-surface antigen has many advantages over other selection schemes, it is conceivable that insertional mutagenesis of heterozygous cell lines is feasible for many selectable cellular genes, provided that proviral insertion is random (a hypothesis that is consistent with known data but difficult to prove rigorously). Such mutations should then be useful for the molecular cloning of the genes of interest.

Note Added in Proof. We have analyzed the other 21 mutants in greater detail and found that 2 of these contain insertions of Mo-MuLV provirus in the $B2m^b$ gene. One insertion is in or near the first coding sequence of the gene, and the other in the first intron. The orientation of both these proviruses is such that their transcription would be in the reverse direction of that of the *B2m* gene. Therefore, of the 22 independent mutants, 3 contain proviral insertion in the target gene. This proportion (about 14%) appears to be similar to that reported by Varmus *et al.* (27) and King *et al.* (28). A final point worth noting is that most of the 21 mutants contain only one copy of Mo-MuLV. This low copy number of the insertion element should facilitate molecular cloning of the target gene.

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