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Genetics

Targeted gene disruption of the endogenous c-*abl* locus by homologous recombination with DNA encoding a selectable fusion protein

(electroporation/embryonic stem cells/neomycin resistance)

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ABSTRACT **We have introduced a substitution mutation into the c-*abl* locus of murine embryonic stem cells by homologous recombination between exogenously added DNA and the endogenous gene. Model constructs were initially generated that consisted of a promoterless selectable neomycin resistance marker inserted into the v-*abl* gene of the complete Abelson murine leukemia virus genome, designed to be expressed either as a fusion protein or by translational restart. Tests of these viral genomes for transmission of v-*abl* and *neo* markers showed more stable coexpression in a protein fusion construct. The *neo* fusion was subcloned from this v-*abl* construct into a promoterless c-*abl* fragment, and the resulting DNA was used to transform embryonic stem cells. Direct screening of genomic DNAs showed that a high proportion of drug-resistant clones arose from homologous recombination into the endogenous c-*abl* locus.**

Abelson murine leukemia virus (Ab-MuLV) is a replication-defective retrovirus that transforms fibroblast lines and lymphoid precursors in culture and induces a rapidly progressive lymphosarcoma of B-cell origin in mice (1–3). The Ab-MuLV genome contains a single open reading frame, formed by the fusion of the 5′ portion of the viral *gag* gene to exons from the body of a mouse gene termed c-*abl* (4, 5). The viral gene product, pp160$^{gag\text{-}abl}$ (6, 7), is a potent tyrosine-specific protein kinase with sequence similarity to the *src* family of kinases (8, 9). The high-level kinase activity is essential for the transforming function of the virus (10–12). A large C-terminal region of the protein is dispensable for the transformation of fibroblast lines, though alterations in this area can affect transformation of lymphocytes and induction of disease in animals (13, 14). This domain is also responsible for the cytotoxic effects of the v-*abl* protein in certain cell types (15).

The normal c-*abl* gene spans >200 kilobases (kb) (16) on mouse chromosome 2 (17). The gene is expressed in a broad spectrum of cell types to form at least two distinct mRNAs, termed types I and IV, in approximately equal abundance (18, 19). Each mRNA contains a distinctive first exon that is spliced onto the remaining common exons that constitute the body of the gene (20, 21). A third mRNA, truncated in the 3′ untranslated region, has been found at high levels in post-meiotic spermatids (22, 23).

The role of the c-*abl* protein kinase in the physiology of the cell, or in development of the animal, remains uncertain. The protein, like many other tyrosine kinase enzymes, may act in signal transduction, but because it contains no extracellular domain it must act in concert with other membrane proteins, and so the identification of relevant ligands is difficult. It seems likely that a genetic approach—the analysis of mutations in the c-*abl* gene in mice—could be helpful in understanding its function.

One method to generate targeted mutations in the mammalian genome is to select for homologous recombination between cloned DNAs and the endogenous chromosomal locus in embryonic stem (ES) cells and then to use these cells to pass the mutation into the germ line (24). Work from a number of investigators has demonstrated that DNA introduced into mammalian cells by available methods integrates by random, illegitimate recombination ≈100–100,000 times more frequently than by homologous recombination (25–29). Thus, attempts to target DNA must include some strategy for the selection or detection of these rare homologous events. Several procedures have been devised, including large-scale screening (25); selection for activation of promoterless genes (30–33); "positive-negative" selection (34); and screening by polymerase chain reaction (33, 35–37). A number of genes have been disrupted in mammalian cells in culture by these methods (for review, see ref. 38).

In this paper, we report the use of homologous recombination to introduce a substitution mutation into the c-*abl* locus of mouse ES cells. Detection of homologous integrants was facilitated by the activation of a promoterless selectable marker embedded in the gene sequences of interest. In earlier work using similar procedures, homologous recombination was studied between exogenous DNA and target DNA sequences that had been previously integrated into the mammalian genome (30–32) or that are counterselectable (33). We demonstrate here that this selection mechanism is also useful for the detection of homologous integration into nonselectable endogenous chromosomal loci.

EXPERIMENTAL PROCEDURES

Cloned DNAs. Plasmids p16-1, p12-27, and p12-28, linker insertion mutants of v-*abl* plasmid pTabl, were as described (11). p16-1 contains a 16-base-pair (bp) insertion including an *Eco*RI site at position 4186 of v-*abl*. pXVX, a derivative of pRSVneo containing multiple cloning sites in front of the second amino acid of the *neo* gene and a *Xho* I site after the poly(A) signal, was a generous gift of G. Gaitanaris (Columbia University). Bluescript KS(−) was obtained from Stratagene, pIBI31 was from IBI, and pSV2neo (39) was a gift of P. Berg (Stanford University).

Plasmid Constructions. Cloning manipulations were carried out by standard methods (40).

v-abl-neo *protein fusion constructs.* Provirus pVXS, containing *neo* sequences fused at the *Xho* I site of v-*abl*, was constructed by inserting the 2-kb *Xho* I fragment of the *neo*

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Abbreviations: ES, embryonic stem; Ab-MuLV, Abelson murine leukemia virus; ^{r}, resistant.

plasmid pXVX into a fragment of the Abelson provirus produced by digestion of p16-1 with *Xho* I plus *Sal* I. Provirus pV16-1XR was constructed by first subcloning the 1-kb *Xho* I–*Sma* I *neo*-containing fragment of pXVX into pIBI-31 and then subcloning the 1-kb *Xho* I–*Eco*RI *neo*-containing fragment from this intermediate into provirus p16-1 digested with *Xho* I plus *Eco*RI.

v-abl-neo *translational restart constructs*. Provirus pV16-1RR was constructed by first inserting an *Eco*RI site in front of the AUG of SV2neo by site-directed mutagenesis of the *Bgl* II–*Sma* I fragment of pSV2neo in M13mp18 and then subcloning the 1-kb *Eco*RI fragment thus generated into p16-1 at the *Eco*RI site. Provirus pV16-1BR was constructed by ligating 10-nucleotide *Eco*RI linkers (New England Biolabs) onto the termini of the *Bgl* II–*Sma* I fragment of pSV2neo and inserting that fragment into the *Eco*RI site of p16-1. Proviruses pV12-27BR and pV12-28BR were constructed by inserting the same 1-kb *neo*-containing *Eco*RI fragment from pV16-1BR into the *Eco*RI sites of p12-27 and p12-28, respectively.

c-abl *constructs*. Plasmids pAbXb1 and pAbXb2 were constructed by subcloning, respectively, the 7.5-kb and the upstream 5-kb *Xba* I fragments from phage λab12 (5) into Bluescript KS(−). The c-*abl-neo* plasmid pAbXR1 was constructed by using the *Xho* I–*Sal* I piece containing the *neo* insert from pV16-1XR to replace the corresponding fragment of pAbXb1.

Cells. CCE ES cells were maintained on mitomycin-treated STO feeder layers as described (41). Cells used for electroporations were at passage 9–12. NIH 3T3 and STO fibroblasts were maintained in Dulbecco's modified Eagle medium plus 10% calf serum (HyClone).

Introduction of DNA into ES Cells. Transformation of NIH 3T3 cells with viral DNAs was by the DEAE-dextran method (42). CCE cells were transformed (43) in an electroporator (Anderson Electronics, Boston) under the following conditions. Cells were plated out at 3×10^6 cells per 6-cm dish, fed on the following 2 days, and trypsinized 2 hr after the second feeding. Trypsinized cells were washed once with phosphate-buffered saline (PBS) and resuspended in PBS at a concentration of 4×10^7 per ml. One-half milliliter of the cell suspension was mixed with 10–20 μg of plasmid pAbXR1 DNA digested with *Xba* I and electroporated at a variety of voltages and capacitances to determine the best electroporation conditions. Optimal conditions were obtained at 200 V and 1000 μF. Cells (5×10^6) were plated onto 10-cm dishes containing G418-resistant ($G418^r$) feeder layers. Selections were carried out in either of two ways. In some experiments cells were fed fresh medium the next day and refed with medium containing G418 at 400 μg/ml 1.5 days after electroporation. In other experiments cells were refed at 1 and 2 days after electroporation and then passed onto fresh $G418^r$ feeder layers in medium containing G418. Cells were refed with medium containing G418 every 2 days. Colonies were picked into 24-well cloning trays 9–12 days after plating into selective medium, and the G418 concentration in the medium was lowered to 100 μg/ml. Cells from the 24-well trays were plated onto two 6-cm dishes in nonselective medium. Aliquots of these cells were frozen and used to make high molecular weight DNA. After screening, selected clones were thawed, grown for two passages, and refrozen.

Probes for Southern and Northern Analyses. Probe EX was prepared by isolating the ≈160-bp *Eco*0109–*Xba* I fragment of plasmid pAbXb2 after electrophoresis in low-melting agarose (Seaplaque); the DNA was labeled by extension of hexanucleotide primers (Pharmacia) with Klenow DNA polymerase in the presence of [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) and the three other unlabeled triphosphates (44). After use, blots were stripped of probe by washing twice in 0.5 M NaOH/1.5 M NaCl for 10 min at room temperature. Stripped blots were prehybridized and rehybridized with nick-translated pSV2neo probe.

RESULTS

Experimental Strategy for Disruption of the c-*abl* Locus. To enrich for cells that incorporated DNA by homologous recombination, we designed constructs in which a promoterless selectable marker, the *neo* gene, was flanked on both sides by regions of homology to the c-*abl* target gene. After most nonhomologous insertions, the *neo* gene would remain transcriptionally silent, leaving the cell sensitive to the drug G418. A homologous insertion event, mediated by two crossovers in the flanking DNA, would activate the *neo* gene and render the cell G418 resistant (Fig. 1). The *neo* gene would be expressed by translation of a bifunctional hybrid mRNA, transcribed from the promoter of the endogenous c-*abl* gene. In all cases we positioned the *neo* sequences downstream of the protein kinase domain of the *abl* gene, so as to retain kinase activity but eliminate the C-terminal one-third of the protein.

Construction of v-*abl-neo* Fusions: Tests of Model Gene Fusions. To ensure that the planned promoterless *neo* constructs would indeed confer resistance to the drug G418 when homologous recombination occurred, we first inserted the *neo* gene in v-*abl*—i.e., in Ab-MuLV—and tested the constructs for biological activity. We generated v-*abl-neo* constructs that would express the *neo* gene sequences in either of two ways. In some constructs, the *neo* gene was designed to be expressed by a "ribosomal restart," or translational

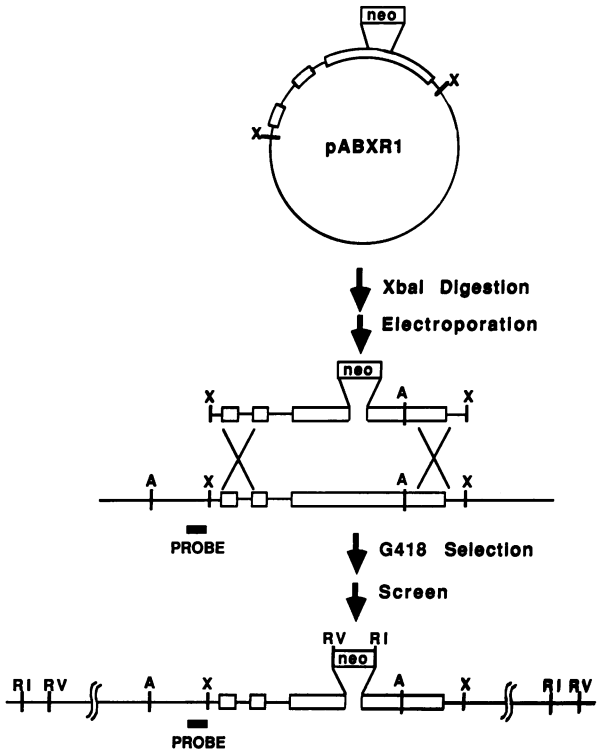

FIG. 1. Scheme for the replacement of the normal c-*abl* gene with a c-*abl-neo* fusion by homologous recombination. A plasmid DNA containing the *neo* gene embedded in c-*abl* sequences, but devoid of signals for transcription and translation, is linearized by digestion with *Xba* I and introduced into cells by electroporation. A double crossover in the flanking c-*abl* sequences replaces the normal gene with the fusion and activates expression of the *neo* gene. After digestion of DNA from drug-resistant clones, hybridization with the flanking probe EX detects DNA fragments of additional sizes from the mutant allele as well as fragments of the normal size from the unaltered allele. A, *Apa*L1; X, *Xba* I; RI, *Eco*RI; RV, *Eco*RV.

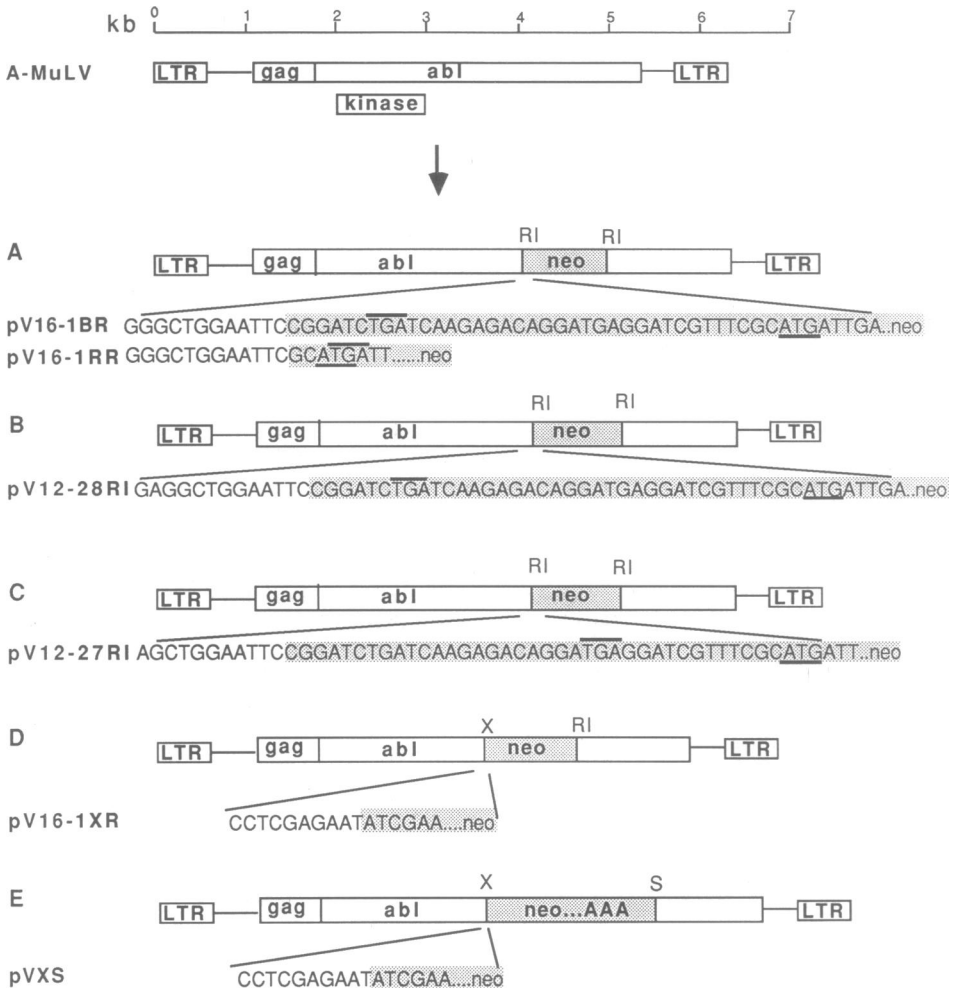

FIG. 2. Structures of v-*abl-neo* constructs and nucleotide sequences at the *abl-neo* boundaries. LTR, long terminal repeat. Top line: structure of the wild-type Ab-MuLV genome; the position of the tyrosine-specific protein kinase domain is indicated by the box. (*A–E*) Structure of v-*abl-neo* genomes. The *neo* sequences are shaded, and restriction sites flanking the *neo* sequences are indicated. The sequences at the *abl-neo* boundary for each of the constructs are shown in the expanded region underneath. (*A–C*) Translational restart constructs. The introduced terminator codons are overlined and the AUG initiator codons for *neo* expression are underlined. (*D* and *E*) Protein fusion constructs. In *E*, the *neo* fragment includes the poly(A) addition sequence derived from pSV2neo (40). RI, *Eco*RI; X, *Xba* I; S, *Sal* I.

reinitiation (Fig. 2 *A–C*). In these constructs, a terminator codon was inserted in the v-*abl* sequence, effectively truncating the C terminus of the protein product; a complete copy of the *neo* gene, including an AUG initiation codon, was positioned immediately downstream of the terminator. The *neo* sequences would then be translated to yield a separate protein, after premature termination of translation of the v-*abl* gene. In other constructs, the *neo* sequences were simply inserted in frame into the coding region of the v-*abl* gene, substituting the *neo* gene for a portion of the v-*abl* sequences (Fig. 2 *D* and *E*). Such a construct would encode a *gag-abl-neo* fusion protein. Since all the constructs introduced alterations in the v-*abl* coding region downstream of the tyrosine kinase domain, we expected them simultaneously to transform fibroblasts and confer G418 resistance.

The v-*abl-neo* constructs were introduced into NIH 3T3 fibroblasts along with DNA of the helper virus Moloney murine leukemia virus. Virus was allowed to spread through the culture for 3 days, and the cells were plated to assay either for focus formation or for G418 resistance. All constructs were able to produce foci and G418^r colonies. However, only some of the transformed foci from the translational restart constructs were able to grow in G418, and the majority of the colonies initially selected for drug resistance were not morphologically transformed. Examination of the viral DNA from the recipient cells revealed that the integrated proviruses were generally rearranged (data not shown). In contrast, the constructs encoding v-*abl-neo* protein fusions were able to express both markers stably. Foci of morphologically transformed cells derived from the protein fusion viruses grew in G418, and the majority of the colonies selected initially in G418 appeared morphologically transformed. The proviral DNA integrated in these cells was unrearranged as judged by Southern analysis (data not shown). We therefore concluded that the fusion virus was indeed able to express both functions and was stably transmitted through rounds of virus replication.

The v-*abl-neo* constructs were expressed at high levels from the potent transcriptional promoter contained in the retroviral long terminal repeat. To ensure that this high level of transcription was not required for the expression of drug resistance, we made an additional construct in which the fusion was placed downstream of the relatively weak promoter of the herpes simplex virus thymidine kinase gene. Transformation of cells with this construct demonstrated that even weak transcription was sufficient to provide drug resistance (data not shown).

Homologous Recombination of c-*abl-neo* Fusions into the Endogenous c-*abl* Gene of ES Cells. The region of v-*abl* containing the *abl-neo* fusion was excised from the Ab-MuLV genome of plasmid pV16-1XR (Fig. 2*D*) and used to replace the corresponding region of the c-*abl* gene in a cloned 7.5-kb *Xba* I fragment of the c-*abl* genomic sequence, form-

Table 1. DNA fragment sizes altered by insertion of *neo* sequences by homologous recombination

Enzyme	Fragment size, kb: Before insertion	After insertion
*Apa*L1	6.5	6.5, 7.1
Xba I	7.5	7.5, 8.1
*Eco*RI	≈32	≈32, 23, 9.5
*Eco*RV	≈40	≈40, 30, 10

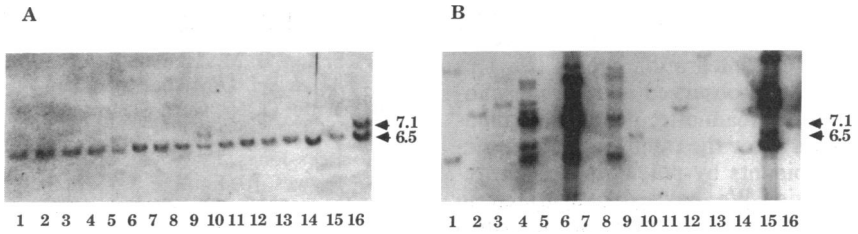

FIG. 3. Southern blot analysis of genomic DNA from individual G418^r cell lines. DNA preparations from various clones were digested with *Apa*L1 and analyzed by Southern blotting. Lanes 1–15, clones 1–15 from experiment 3 (see Table 2); lanes 16, clone 2b1 from experiment 1. (*A*) Blot was hybridized with the Abelson-specific EX probe. The bands derived from the parental c-*abl* allele (6.5 kb) and from the rearranged allele (7.1 kb) are indicated by arrows. (*B*) The filter was stripped and rehybridized with a pSV2neo probe.

ing the plasmid pABXR1 (ref. 5; see Fig. 1). This c-*abl* fragment contains c-*abl* exons 10, 11, and 12 but lacks any transcriptional promoter or translational start site. When this DNA was introduced into fibroblasts, at least 75-fold fewer G418^r colonies per μg were obtained than with the corresponding viral construct.

We carried out all further experiments in the CCE ES cell line, which has been extensively characterized and has been shown to maintain a euploid number of chromosomes in culture (41). Furthermore, this cell line has been shown to provide extensive contribution to many developmental lineages of chimeric animals, a crucial feature for further studies. The plasmid containing the c-*abl-neo* fusion was digested with *Xba* I, the DNAs were introduced into CCE ES cells by electroporation, and the cells were plated into medium containing G418 (see *Experimental Procedures*).

To detect homologous recombination events, we prepared genomic DNAs from individual G418^r clones and analyzed the DNA by Southern blotting after digestion with *Apa*LI. The filters were hybridized with a labeled probe (EX) homologous to a region of c-*abl* outside the introduced DNA fragment. Integration of the exogenous *neo* DNA fragment by homologous recombination should convert one copy of the 6.5-kb *Apa*LI wild-type fragment to a new 7.1-kb fragment (Fig. 1; Table 1). Examination of DNAs from many clones revealed the presence of this alteration in several cases (for examples, see Fig. 3*A*, lanes 5, 9, and 16). The submolar ratio of rearranged to wild-type bands in some lanes results from contamination of the ES cells by feeder cells containing only the wild-type alleles. In four separate experiments, we identified a total of seven independent (single-copy) homologous integration events out of 239 colonies screened, giving a frequency of 1 in 34 G418^r clones (Table 2). In these seven cases, reprobing the blots with a probe containing *neo* sequences showed hybridization only to the 7.1-kb rearranged band (Fig. 3*B*).

The genomic DNA of five of the clones was examined further by digesting with several other restriction enzymes, blotting, and hybridizing with a larger v-*abl* probe. The expected fragment sizes, based on the known structure of the c-*abl* gene, are listed in Table 1. In four of the five clones, the new restriction fragments were as expected for a simple replacement of the c-*abl* region by the added DNA via a double crossover (Fig. 4). In one case, however, integration was accompanied by a rearrangement at the 3′ end of the region (Fig. 4, lanes 6). Similar rearrangements have been observed by other investigators (refs. 31, 33; G. Gaitanaris, M. Gottesman, D. Grieco, S. Cocozza, and E. Avvedimento, personal communication).

Table 2. G418^r colonies carrying a mutation at the c-*abl* locus

Exp.	Colonies screened, no.	Single-insert homologous recombinants, no.	Multiple inserts at c-*abl* locus, no.
1	43	1	2
2	44	0	1
3	32	2	0
4	120	4	6
Total	239	7	9

The frequency of single-copy homologous events = 1 in 34; the frequency of all integration events at c-*abl* = 1 in 15.

We also obtained nine clones that contained alterations at the c-*abl* locus that did not conform to the pattern expected from a single substitution. Reprobing these samples with *neo*-specific sequences indicated that multiple, probably tandem, copies of the added DNA integrated at the c-*abl* locus (Table 2). Perhaps a low level of expression of drug resistance produced from the fusion construct enriched for the recovery of clones containing multiple inserts. If we include these clones, the proportion of the drug-resistant clones that have undergone recombination at the c-*abl* locus is 1 in 15. Although these clones contain mutations at the c-*abl* locus, the complex structure of the inserted DNA dictated against further use of these lines in genetic analyses of the c-*abl* mutation.

DISCUSSION

The use of promoterless selectable markers as a means of selecting for rare homologous recombinants has been described by several investigators to target mutations to defined regions of the genome of tissue culture cells (30, 31, 33). To date, most of these mutations have been targeted to sequences, including simian virus 40 and polyoma viral DNA, that were previously introduced into the genome. Our work adds to the growing list of genes that have been targeted in the mammalian genome and demonstrates that this method is a useful form of enrichment for rare homologous recombinants into endogenous, nonselectable loci. In the case of c-*abl*, we found that 1 in 34 drug-resistant clones had undergone a

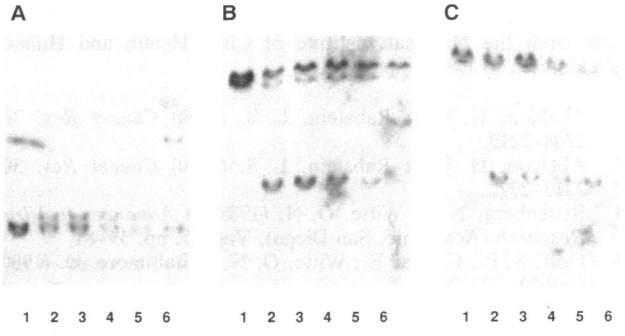

FIG. 4. Southern blot analysis of genomic DNAs from five drug-resistant cell lines. DNAs were digested with *Xba* I (*A*), *Eco*RI (*B*), and *Eco*RV (*C*) and analyzed by Southern blotting; hybridization was with the nick-translated pAb3sub3 probe (4). Lanes: 1, CCE control line; 2, clone 2b1; 3, clone 34a3; 4, clone 53b6; 5, clone 55b1; 6, clone 56a8.

homologous recombination event. If we assume an ≈100-fold enrichment using this technique, the ratio of unselected homologous to nonhomologous integration events is ≈1 in 3400, comparable to frequencies observed at other chromosomal loci (refs. 30, 31, 45; L. Jeannotte, personal communication). These frequencies allow the rapid and easy detection of homologous recombinants by polymerase chain reaction or Southern analysis. We have demonstrated in subsequent experiments that the c-*abl* mutation described here can be transmitted through the germ line of chimeric mice obtained with the cell clones generated in this study (46).

Promoterless selectable markers embedded in the gene sequence of interest can be designed to be activated by that gene's promoter in a number of different ways. The selectable marker can be placed at or near the beginning of the coding region of a gene and translated directly from its own AUG codon (30, 45). Alternatively, placement of a stop codon in the gene followed closely by the AUG of the selectable marker can allow expression of the selectable marker by ribosomal restart (30, 33, 47, 48). Although this technique has been successfully applied by other investigators, we were not able to obtain active, stable constructs with insertions in the C-terminal portion of v-*abl*. When we selected for expression of drug resistance using these constructs, we observed rearrangements of the retroviral genome. Perhaps the location of the ribosomal restart region near the 3′ end of the mRNA or the context in which the restart sequence was embedded did not provide a high enough rate of ribosomal reinitiation to efficiently express the *neo* gene. We have utilized a third approach, in which we fused the *abl* sequences to the second codon of the *neo* gene, creating a c-*abl-neo* fusion. This construct was based on a similar one successfully used to target a mutation to the thyroglobulin gene (G. Gaitanaris, M. Gottesman, D. Grieco, S. Cocozza, and E. Avvedimento, personal communication). We believe that the activity of this gene fusion relies on the fact that the *neo* sequences begin very near to the normal N terminus.

The activation of a selectable marker is a powerful method for the isolation of the desired clones of cells, but it is likely to be applicable only to transcriptionally active target genes. Other methods, such as the positive-negative selection (34) and the use of polymerase chain reaction for screening (33, 35–37) may permit targeting of any gene sequence for homologous recombination, whether or not it is expressed. The use of ES cells, and the subsequent use of these cells to generate chimeric mice, will ultimately permit the analysis of mutations in any gene in the context of the whole organism.

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