MOLECULAR AND CELLULAR BIOLOGY, Apr. 1990, p. 1697–1704
0270-7306/90/041697-08$02.00/0

Vol. 10, No. 4

A Novel Fluorescence-Based System for Assaying and Separating Live Cells according to VDJ Recombinase Activity

GEORGE D. YANCOPOULOS,[1†] GARRY P. NOLAN,[2] ROBERTA POLLOCK,[1‡] SUSAN PROCKOP,[1] SUZANNE C. LI,[1] LEONARD A. HERZENBERG,[2] AND FREDERICK W. ALT[1*]

The Howard Hughes Medical Institute and Departments of Biochemistry and Microbiology, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, New York 10032,[1] and Department of Genetics, Stanford Medical Center, Stanford, California 94305[2]

Received 5 October 1989/Accepted 27 December 1989

We describe two retroviral vector-based recombination substrate systems designed to assay for lymphoid VDJ recombinase activity in cultured cells. Both substrates incorporate a constitutive dominant marker gene (the simian virus promoter-driven *neo* gene) to allow selection of cells that stably integrate the substrate. Both substrates also include a second marker gene that becomes transcriptionally active only when inverted by a site-specific recombination event between flanking immunoglobulin variable-region gene segments. The first vector, similar in structure to previous retrovirus-based recombination substrates, utilizes the bacterial guanine-xanthine phosphoribosyltransferase gene (*gpt*) as its activatable marker; detection of inversion (VDJ recombinase activity) involves drug selection and Southern blotting analyses. We have used this vector to make a more extensive and quantitative survey of VDJ recombinase activity in B-lineage cell lines than has previously been performed with stable substrates, and we have compared our results with those of other studies that use transient recombination substrates. In the second vector, the activatable gene is the bacterial β-galactosidase gene (*lacZ*). Detection for inversional activation of this gene is achieved by a fluorogenic assay, termed FACS-Gal, that detects β-galactosidase activity in viable cells. The latter assay has the unique advantage of rapidly detecting cells that undergo recombination and also allows viable sorting of cells on the basis of the presence or absence of VDJ recombinase activity. We have used the *lacZ* vector to rapidly quantitate VDJ recombinase activity in B-lineage cell lines and compared the results with those obtained with the *gpt* vector. We have also used the *lacZ* vector to isolate variant pre-B-cell lines with low and high levels of VDJ recombinase activity.

The ability of the immune system to respond to an unlimited array of antigens depends on somatic assembly of genes encoding the antigen-binding receptors expressed by B and T cells (reviewed in references 12 and 20). The approximately 100 N-terminal amino acids of the polypeptide chains that comprise the antigen receptors are termed variable regions because they vary among chains of the same class. Variable regions of complementary polypeptide chains combine to form the antigen-binding pocket of the receptor. During somatic lymphocyte development, the genes encoding these variable regions are assembled from component gene segments. A variable region gene is assembled from either two (V and J) or three (V, D, and J) germ line segments. The genetic locus used to encode the variable region of a given polypeptide chain usually has multiple copies of each of the V, D (if present), and J segments. Combinatorial assortment of these different segments, coupled with further diversity that can be created at the junctions of segments joined together, allows for the assembly of an almost infinite number of different variable-region genes. All germ line variable-region gene segments are flanked by recombination recognition sequences (or signal sequences) that mediate the joining event (7, 18). Joining of two segments appears to initiate with recognition of the signal sequences and site-specific double-stranded breaks which separate the coding sequences from the signal sequences (1). Subsequently and in discrete joining steps, the two coding regions are brought together imprecisely while the signal sequences are joined back-to-back. The relative orientation of the segments to be joined determines whether the join will delete or invert the sequences between the two joined segments (see below).

Although neither the enzymes involved in VDJ recombination nor the genes encoding these enzymes have yet been isolated, several strategies have been used to assay for VDJ recombinase activity. These strategies have involved introduction of exogenous DNA constructs (termed recombination substrates because they contain variable-region gene segments that act as substrates for the VDJ recombinase) into cell lines that have VDJ recombinase activity. Recombination substrates can either be permanently integrated into chromosomal DNA to yield stable cell lines bearing the substrates (3, 13) or be propagated extrachromosomally for transient assays (9). Subsequently, the introduced DNA is analyzed to see whether it has undergone VDJ recombination. These assays have revealed that B and T cells express a common VDJ recombinase that can act on all classes of antigen receptor variable regions (21) and have suggested that the activity of the recombinase is targeted in a tissue- and stage-specific manner to particular genetic loci, depending on the accessibility of the given locus (4, 21). Limited surveys of stably introduced substrates, together with more extensive surveys using transiently introduced substrates, suggested that VDJ recombinase activity is limited to cell lines representing the early stages of the B, T, and macro-

* Corresponding author.
† Present address: Regeneron Pharmaceuticals, Inc., Tarrytown, NY 10591.
‡ Present address: Department of Biology, Occidental College, Los Angeles, CA 90041.

phage lineages (4, 5, 14, 19). Within the B-cell lineage, pre-B-cell lines (which do not express antigen receptors on their surface) generally expressed VDJ recombinase activity, whereas B-cell lines (which express surface antigen receptors) and terminally differentiated plasma cell lines (which secrete antibodies) did not.

The analyses of stably and transiently introduced recombination substrates have their respective advantages. Transient assays allow more rapid analysis of many cell lines and have been proposed to be more amenable to quantitative comparisons of recombination rates between cell lines (14). Stable substrate studies allow for the analysis and manipulation of the chromatin structure of the substrate, can allow for long-term studies of substrate recombination after treatment of perturbation of the cells, and may allow detection (because of more powerful selection techniques) of lower levels of recombinase activity (4, 19, 21). Here we make an extensive and semiquantitative survey of VDJ recombinase activity during B-cell development, using a conventional stably introduced recombination substrate. We then further manipulate this conventional substrate to develop a new stable recombination substrate system that can combine, and enhance, the advantages of both stable and transient recombination substrates. This system takes advantage of a new fluorescence-based assay for β-galactosidase activity (16), called FACS (fluorescence-activated cell sorting)-Gal, and allows for the rapid analysis of recombination within a stably introduced recombination substrate as well as for the separation of live cells on the basis of recombination activity.

MATERIALS AND METHODS

Recombination substrate constructions and analysis of rearrangements by Southern blotting. Constructions were made into the pDOL retroviral vector, using restriction fragments (with modified ends) containing kappa light-chain V or J segments, the *gpt* gene, and the *lacZ* gene as described in the legends to Fig. 1 and 2. Preparation of genomic DNA from cell lines containing the constructs, restriction enzyme digestion of genomic DNA, Southern blotting, preparation of radiolabeled probes, and hybridization were performed as previously described (21). Diagnostic digestions and probes that distinguish between the rearranged and unrearranged forms of the recombination substrates on Southern blots are described in the legends to Fig. 1 and 2.

Introduction of substrates into cell lines. The pV-gpt-J-neo and pV-lacZ-J-neo constructions were transiently transfected into PA317 cells; virus was harvested after 48 h and used to infect Ψ-2 cells to make permanent producer cell lines. These transfections, infections, and selections were performed as described by Korman et al. (11). The producer lines were cocultured for 2 days with the B-lineage cell lines in Dulbecco modified Eagle medium supplemented with 10% inactivated fetal calf serum, 50 μM β-mercaptoethanol, and 5 μg of Polybrene per ml, at which time both the Ψ-2 and lymphocyte cells were near confluence; the lymphocytes were then carefully removed by aspiration and selected in G418 (2.5 mg/ml). The *gpt* selection was performed by using mycophenolic acid (MPA) at 0.5 μg/ml.

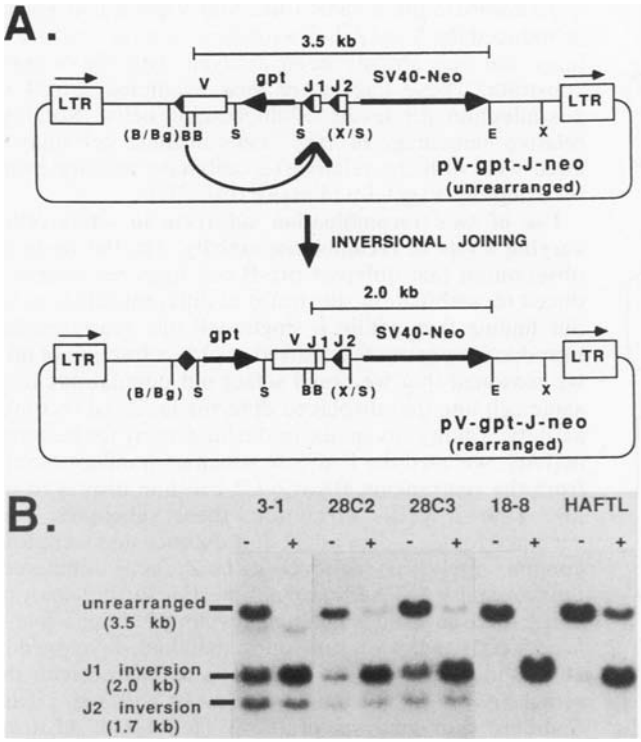
Fluorescence-based assay for *lacZ* activity. Fluorescein di-β-D-galactopyranoside (FDG) staining for β-galactosidase activity, fluorescence-activated cell analysis, and FACS were performed as previously described (16) except that the reaction was inhibited at 1 h by using phenylethylthiogalactoside at 1 mM to allow better quantitative comparison between samples (17).

Transient recombination assays. Recombination assays using transiently introduced substrates were performed by using the substrates and methods described by Lieber et al. (14).

RESULTS

Recombination substrate design. A recombination substrate designated V-gpt-J-neo, similar to previous retrovirus-based substrates (5, 19), was constructed within the pDOL retroviral vector and contained Vκ and Jκ segments as well as two drug-selectable marker genes (*gpt* and *neo*) (Fig. 1A). This substrate allows for constitutive expression of the *neo*-selectable marker gene (encoding resistance to G418) situated outside of the recombining V and J sequences but allows for expression of the internal *gpt*-selectable marker gene (encoding resistance to MPA) only after inversional joining between the flanking V and J segments. This vector was introduced into the Ψ-2 retroviral packaging cell line, resulting in a producer line that was used to transfer V-gpt-J-neo into a series of cell lines representing various stages of the B-cell lineage. Within the first week after coculture of each of the B-lineage cell lines with the producer cell line, populations of the B-lineage cell lines resistant to G418 rapidly emerged, indicating that polyclonal infection of the B-cell lines had occurred. Placing these G418-resistant cell lines into MPA-containing medium resulted in diverse responses. In all cases, MPA-resistant cells never grew out from cell lines representing B-cell stages more mature than the pre-B-cell stage. However, MPA-resistant cells grew out from all tested pre-B-cell lines containing the V-gpt-J-neo substrate, although at very different frequencies. Some pre-B-cell lines (i.e., 3-1 and 40E1) were not noticeably affected by MPA exposure, whereas other lines were mostly killed by MPA exposure. In the most extreme example, the pre-B-cell line 70Z only occasionally yielded MPA-resistant cells after the placement of 5×10^6 cells in MPA-containing medium, and these cells only appeared 3 to 4 weeks after exposure to MPA, suggesting that only very rare 70Z cells had rearranged the V-gpt-J-neo substrate.

The verify that MPA resistance in pre-B-cell lines resulted from conventional joining between the V and J segments, and to attempt to understand the differing responses of pre-B-cell lines to MPA treatment, genomic DNA was prepared from the cell lines 2 to 4 weeks after infection as well as after growth in MPA-containing medium. The DNA was subjected to Southern blot analysis, using restriction digests and a probe that conveniently distinguished between unrearranged and rearranged versions of the V-gpt-J-neo substrate (for details, see legend to Fig. 1B). The substrate DNA in all samples from MPA-resistant populations had undergone conventional V-J joining (representative data shown in Fig. 1B). However, analysis of samples before placement in MPA revealed different relative levels of the substrate in the unrearranged versus the rearranged form (Fig. 1B). Notably, cell lines (e.g., 3-1) that rapidly grew in MPA contained high levels of the rearranged form of the substrate even before selection in MPA, whereas cell lines (e.g., 18-8 and 70Z) that yielded MPA-resistant cells at low levels had undetectable levels of rearranged substrate before selection in MPA (Fig. 1B and C). Thus, we conclude that both ability to grow in MPA and the relative amounts of unrearranged and rearranged substrate before selection in



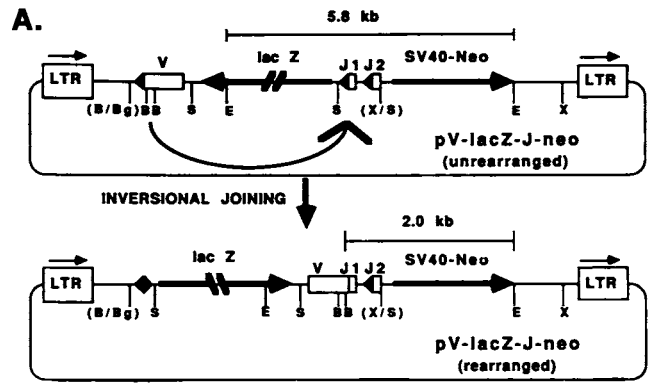
C.

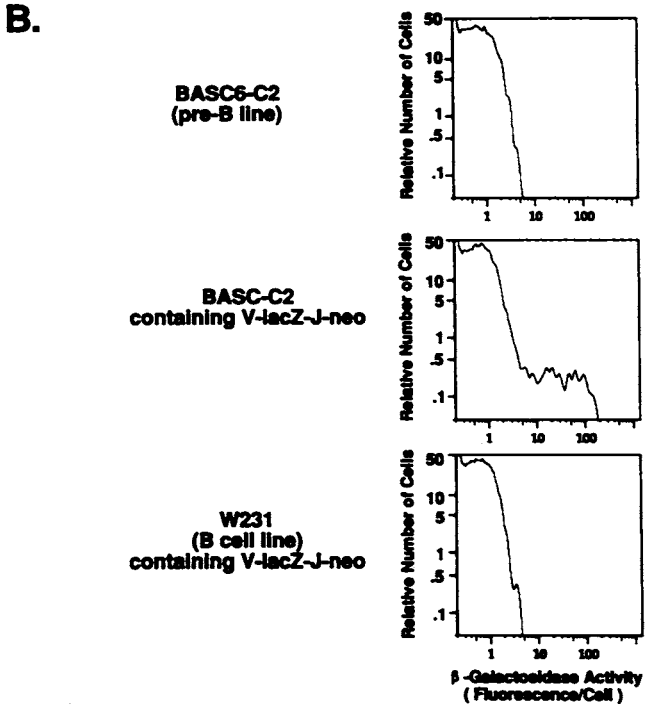
Pre-B Cell Lines:	RECOMBINASE ACTIVITY
HAFTL-1	++
BASC6-C2	++
40E1	+++
22D2	++
22D10	+++
28C2	++
28C3	++
28C5	+
300-19	++
18-8	+
3-1	+++
70Z	-/+
B Cell Lines:	
W231	-
W279	-
BCL1	-
Plasma Cell Lines:	
104E	-

FIG. 1. (A) Structures of unrearranged and rearranged V-gpt-J-neo within retroviral vectors. Three DNA segments (the 600-base-pair [bp] *Bgl*II-*Sph*I fragment containing Vκ21C [13] in which the *Sph*I site had been changed to a *Sal*I site; the 660-bp *Bgl*II-*Dra*I fragment spanning the bacterial *gpt* gene [17] in which both sites were changed to *Sal*I sites; and the 450-bp *Hin*dIII-*Ava*II fragment containing the Jκ1 and Jκ2 segments [15] in which the *Hin*dIII site was changed to *Sal*I and the *Ava*II site was changed to *Xho*I) were cloned in the order and orientations indicated into the pDOL retroviral vector (11), which had been linearized with *Bam*HI and *Sal*I; the cloning strategy destroys these *Bam*HI and *Sal*I sites. Because of the orientation of the V and J segments, normal joining results in the inversion of the internal *gpt* gene, allowing it to be expressed from the upstream LTR promoter. Diagnostic *Bam*HI-*Eco*RI fragments that hybridize to a probe for the *neo* gene and can distinguish the unrearranged (3.5-kilobase-pair [kb]) and rearranged (2.0-kb) forms of the substrate are indicated. Map is drawn to scale between the 5′ *Bam*HI-site and the 3′ *Xho*I site. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; S, *Sal*I; X, *Xho*I. (B) Rearrangement of V-gpt-J-neo in pre-B-cell lines. Genomic DNA isolated from pre-B-cell lines containing V-gpt-J-neo was digested with *Bam*HI and *Eco*RI, fractionated on 1% agarose gels, transferred to nitrocellulose, and probed with a radiolabeled *neo* probe. Diagnostic sized bands distinguish rearranged and unrearranged forms of the substrate. Genomic DNA was prepared either before (−) or after (+) selection for *gpt*-expressing cells with MPA. (C) VDJ recombinase activity in B-lineage cell lines as assayed with V-gpt-J-neo. −, No growth of MPA-resistant cells; +, rare MPA-resistant cells, with no evidence of rearrangement of the substrate (by Southern blot analysis as depicted in panel B) before selection with MPA; ++, evidence of rearrangement (by Southern blotting) before selection with MPA; +++, substrate was mostly in rearranged form when analyzed before selection in MPA. 70Z is given a −/+ rating since MPA-resistant cells only occasionally grew out after the placement of 5 million cells in MPA-containing medium. The progenitor B-cell lines HAFTL-1 and BASC6-C2 were the kind gifts of J. H. Pierce and W. F. Davidson (10). The Abelson murine leukemia virus-transformed pre-B-cell lines derived from fetal liver (40E1, 22D2, 22D10, 28C2, 28C3, and 28C5) and adult marrow (300-19, 18-8, and 3-1) have previously been described (2). The methylnitrosourea-induced pre-B lymphocyte line 70Z/3 and the surface immunoglobulin-positive B-cell lines WEHI231 (W231), WEHI279 (W279), and BCL1 were obtained from the American Type Culture Collection.

MPA reflect the inherent recombinase activity of cell lines containing the V-gpt-J-neo recombination substrate, allowing a crude quantitation of relative VDJ recombinase activity between different cell lines (summarized in Fig. 1C). Although recombinase activity was limited to lines representing the pre-B-cell stage of B-cell development, these cell lines expressed widely differing levels of recombinase activity.

A new fluorescence-based assay for recombinase activity. As demonstrated above, stably introduced recombination substrates based on drug resistance marker genes can be used to assay for relative VDJ recombinase activity in cell lines. However, this approach is tedious (requiring long-term drug selection, Southern blot analysis, or both), does not allow for precise quantitation of recombinase activity, and cannot be used to separate cells on the basis of the presence or absence of recombinase activity. To expand the utility of this system, we have modified our conventional inversion-based recombination substrate to take advantage of a recently developed fluorescence-based assay for β-galactosidase activity in live cells (16). Mammalian cells expressing the bacterial β-galactosidase gene (*lacZ*) can cleave the β-galactoside analog FDG to yield fluorescein, which can be detected in viable cells by using FACS. The *gpt*-selectable marker gene in V-gpt-J-neo was replaced by the *lacZ* gene (for details, see legend to Fig. 2A). Only inversional V-to-J joining within the introduced V-lacZ-J-neo substrate should yield cells that fluoresce after FDG substrate loading. To test the substrate and the fluorescence assay, a Ψ-2 producer cell line expressing V-lacZ-J-neo was used to infect a pre-B-cell (BASC6-C2) and B-cell (W231) line with V-lacZ-J-neo. Only the pre-B-cell line containing V-lacZ-J-neo developed a subpopulation (0.65%) of fluorescing (termed $lacZ^+$) cells (Fig. 2B and C).





C.

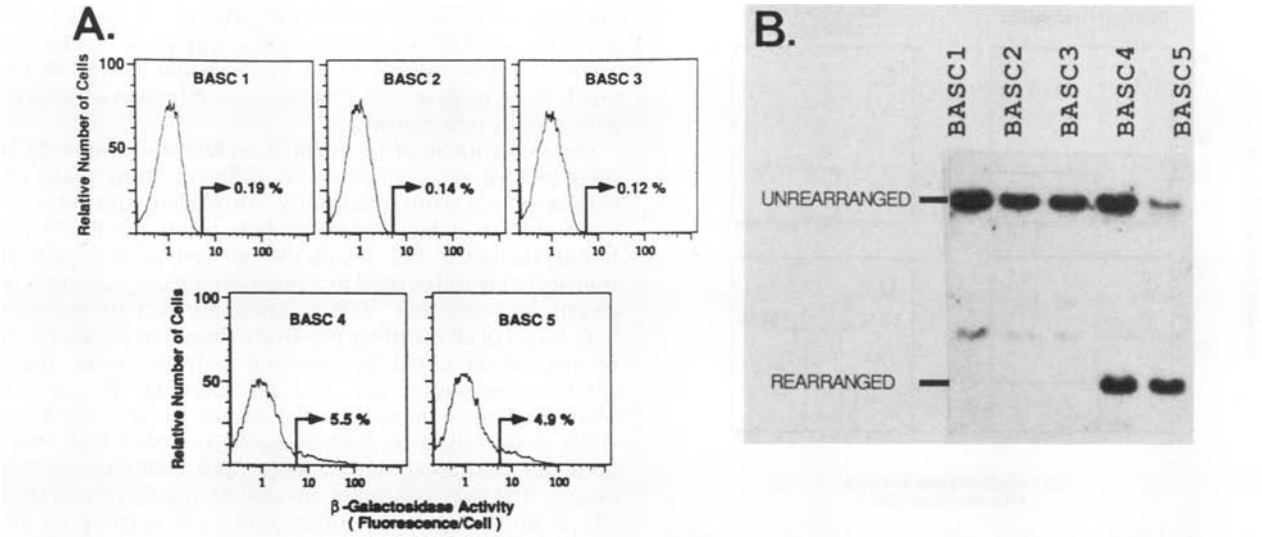
Cell Line	V-lacZ-J-neo assay	V-gpt-J-neo assay
40E1	2.0%	+++
28C3	1.8%	++
BASC6-C2	0.65%	++
HAFTL-1	0.50%	++
18-8	0.15%	+
28C3	0.09%	+
70Z/3	0.0%	-/+
W231	0.0%	-

FIG. 2. (A) Structures of unrearranged and rearranged forms of V-lacZ-J-neo within retroviral vectors. *Sal*I digestion removed the *Escherichia coli gpt* gene from V-gpt-J-neo; the *gpt* gene was replaced by the *Hin*dIII-*Dra*I fragment containing the *lacZ* gene from pCH110 (8), in which both the *Hin*dIII and *Dra*I sites were changed to *Sal*I sites. Because of the orientation of the V and J segments, normal joining results in the inversion of the internal *lacZ* gene, allowing it to be expressed from the upstream LTR promoter. Diagnostic *Eco*RI-*Eco*RI and *Bam*HI-*Eco*RI fragments that hybridize to a probe for the *neo* gene and can distinguish the unrearranged (5.8-kb) and rearranged (2.0-kb) forms of the substrate are indicated. Except for the interrupted *lacZ* gene, map is drawn to scale between the 5′ *Bam*HI site and the 3′ *Xho*I site. Restriction sites: B, *Bam*HckI; Bg, *Bgl*II; E, *Eco*RI; S, *Sal*I; X, *Xho*I. (B) Demonstration that only pre-B-cell lines harboring V-lacZ-J-neo develop a subpopulation of fluorescing cells. Data are presented as logarithmic histograms; *x* axis is scaled fluorescence per cell, and *y* axis indicates relative number of cells (note that scales are logarithmic). All cells were loaded with FDG as described in Materials and Methods. The parental W231 line into which the V-lacZ-J-neo substrate was introduced had a FACS plot identical to that of the population containing the substrate and is not depicted. (C) Correlation of V-lacZ-J-neo and V-gpt-J-neo recombinase assays. Percentage of $lacZ^+$ cells, presented for the *lacZ* assay, was calculated by using FACS-Gal-stained V-gpt-J-neo-infected cells as negative controls and the statistical analysis regimens of FACS-DESK (16). V-gpt-J-neo assay data are reproduced from Fig. 1C; the cell lines are described in the legend to Fig. 1C.

To compare the V-lacZ-J-neo and V-gpt-J-neo assays, we introduced the V-lacZ-J-neo substrate into several pre-B-cell lines that had already been assayed with the V-gpt-J-neo substrate. These lines were then examined 2 to 4 weeks postinfection for levels of fluorescing cells. Notably, the relative percentage of $lacZ^+$ cells in these cell lines correlated well with the relative recombinase activity estimated by using the V-gpt-J-neo assay (Fig. 2C).

Use of *lacZ* recombination substrate to select cells with varying levels of recombinase activity. On the basis of our observation that different pre-B-cell lines rearranged introduced recombination substrates at different rates, as well as our finding that within a single cell line rearrangement of introduced constructs occurred in only a fraction of all cells, we reasoned that we could select out populations from the same cell line that displayed differing levels of recombinase activity. Taking advantage of the *lacZ* assay for recombinase activity, we used the FACS to subclone nonfluorescing cells from the rearranging BASC6-C2 cell line displayed in Fig. 2B. After 2 weeks in culture, these subclones were rescreened for *lacZ*-dependent fluorescence and were found to contain widely varying levels of $lacZ^+$ cells compared with the parental BASC6-C2 population. For further analysis, we chose three subclones that had developed about 5-fold-fewer $lacZ^+$ cells and two subclones that had developed about 10-fold-higher levels of $lacZ^+$ cells compared with the parental BASC6-C2 line (compare Fig. 3A with Fig. 2B and C). Southern blot analysis of the V-lacZ-J-neo substrate revealed that subclones with very low levels of $lacZ^+$ cells also lacked detectable levels of the rearranged form of the substrate, whereas subclones with many $lacZ^+$ cells displayed notable levels of the rearranged form of the substrate (Fig. 3B). The Southern analysis was performed several weeks after the fluorogenic assay, partially explaining the imprecise correlation between percentage of $lacZ^+$ cells and percentage of the substrate in rearranged form in the BASC4 and BASC5 subclones (but see below).

These findings demonstrated that the *lacZ* fluorescence assay provided a quick and efficient method for isolation of cells that rearranged their introduced V-lacZ-J-neo substrate to varying degrees. To verify that differences in the substrate



C.

Cell Line	Experiment 1 CAM^r/AMP^r(%)	Experiment 2 CAM^r/AMP^r(%)	Experiment 3 CAM^r/AMP^r(%)	Experiment 4 CAM^r/AMP^r(%)
Parent	2/1100 (0.18)	3/560 (0.54)	8/3400 (0.24)	7/1800 (0.39)
BASC2	4/650 (0.62)	4/330 (1.2)	2/2400 (0.08)	4/935 (0.43)
BASC3	0/400 (0.0)	0/400 (0.0)	0/400 (0.0)	6/3100 (0.19)
BASC4	0/750 (0.0)	0/690 (0.0)	7/6200 (0.11)	6/1000 (0.60)
BASC5	9/1200 (0.75)	1/730 (0.14)	45/5400(0.83)	7/4500 (0.16)

FIG. 3. (A) Demonstration that subclones of BASC6-C2 harboring V-lacZ-J-neo display differing subpopulations of fluorescing cells. Data are presented as in Fig. 2B and C. Subclones were derived as described in the text. (B) Southern blot showing that the percentage of *lacZ*$^+$ cells in subclones correlates with rearrangement of the V-lacZ-J-neo substrate. Restriction digestion with *Bam*HI and *Eco*RI conveniently distinguishes DNA fragments (identified with a *neo* probe) representing unrearranged and rearranged forms of V-lacZ-J-neo within cell lines (see Fig. 2A). A faint band between the indicated rearranged and nonrearranged bands was seen in some experiments and did not correspond to a recombinase-mediated rearrangement of the substrate. (C) Results of transient recombination substrate assays of BASC6-C2 subclones described above; BASC1 was not tested. Values for recombination rates are as previously described (14), with CAM^r/AMP^r representing the fraction of rearranged-to-unrearranged plasmids. Experiments 1 and 2 were performed within 2 weeks of the fluorogenic assay, whereas experiments 3 and 4 were performed several months later, after freezing and thawing of the subclones.

rearrangement frequency within these subclones truly reflected differences in VDJ recombinase activity and did not simply reflect differences in the particular chromosomal structure or location of the recombination substrate in individual subclones, we assayed the subclones for recombinase activity with a second assay that involved the introduction of transient recombination substrates (9). Transient assays performed shortly after the fluoregenic assay (i.e., experiments 1 and 2, Fig. 3C) indicated that subclones with higher *lacZ*-dependent fluorescence showed recombination activity higher than that of the parental line, whereas subclones with lower *lacZ*-dependent fluorescence displayed no detectable recombinase activity by this assay. The unstable nature of the variant recombinase phenotypes became obvious with continued propagation of these subclones, as recombinase levels assayed by the transient assay eventually randomized (experiments 3 and 4, Fig. 3C).

Current limitations of the *lacZ* recombination assay. We can readily enrich for cells in which the V-lacZ-J-neo construct has rearranged and become transcriptionally activated, even from a starting cell population containing as few as 0.15% *lacZ*$^+$ cells (Fig. 4). Although we have found that unrearranged *lacZ*-negative cells can clearly contaminate the *lacZ*$^+$ enrichment, contributing to the significant *lacZ*-negative cell population seen in the reanalysis, we also found that cells harboring a rearranged *lacZ* substrate that all initially expressed the *lacZ* gene (as defined by fluorescence) gradually yielded a population of nonfluorescing cells. Cells initially sorted as *lacZ*$^+$ (Fig. 4A and C) rapidly developed significant *lacZ*-negative populations (Fig. 4B and D). Southern blot analysis revealed that these populations contained only the rearranged form of the V-lacZ-J-neo substrate, although a significant percentage of the population (more than 50%) no longer expressed *lacZ* (data not shown); furthermore, individual *lacZ*-negative subclones generated from the *lacZ*-negative population also had only the rearranged form of the substrate (data not shown). This apparent decrease in expression of a rearranged *lacZ* gene seems related to the proposed epigenetic down regulation of long terminal repeat (LTR) promoters seen in other systems (16, 22). Although the correlation between recombinase activities determined with the fluorogenic assay and the other two

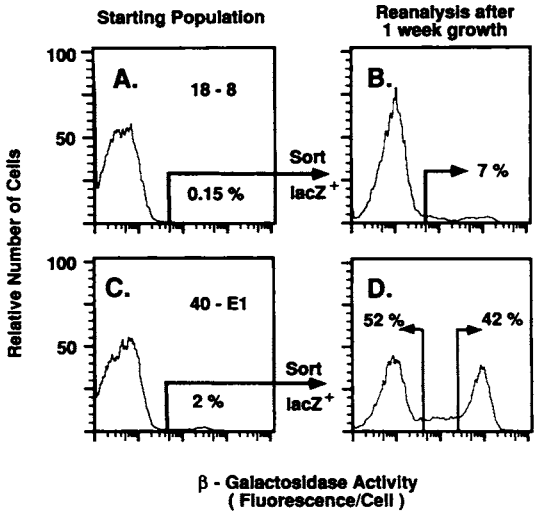


FIG. 4. Enrichment for cells expressing activated, rearranged V-lacZ-J-neo. A total of 10,000 $lacZ^+$ cells were sorted under sterile conditions for the V-lacZ-J-neo-containing cell lines 18-8 (A) and 40E1 (C). After growth in culture for 1 week, cells were reanalyzed by FACS-Gal (B and D). Data are presented as in Fig. 2B and C. Cell lines are described in the legend to Fig. 1C.

recombination assays (Fig. 2C and 3A and C) validates the utility of the fluorogenic assay for comparing relative recombinase activities between cell lines, the derivation of *lacZ*-negative cells from $lacZ^+$ cells currently makes it difficult to use the assay to precisely determine recombination rates in cell lines (see Discussion).

DISCUSSION

Survey of B-lineage cell lines by using stably introduced recombination substrates. The results of our survey using V-gpt-J-neo (Fig. 1C), although more extensive than earlier studies using stable recombination substrates (4, 5, 19), generally agree with previous findings that within the B-cell lineage, recombinase activity is limited to cell lines representing the pre-B stage. Furthermore, we were able to use V-gpt-J-neo to compare differing levels of VDJ recombinase activity among pre-B-cell lines. One potential problem with comparing recombination levels by using stably introduced substrates is that the recombination rate of a substrate within a given cell may depend on its particular chromosomal location. Our stable assays obviate this problem by examining recombination within polyclonally infected cell lines, consisting of cells harboring substrates in many different locations. The polyclonal nature of our infected cell lines, our ability to readily see substrate rearrangement before drug selection, and the correlation between the extent of this rearrangement and ability to grow in MPA-containing medium has allowed us to perform a semiquantitative comparison of VDJ recombinase activity in different pre-B-cell lines by using our stable V-gpt-J-neo substrate. Comparison of the V-gpt-J-neo assay, our novel fluorescence-based assay, and a transient substrate system on many of the same cell lines (Fig. 2C and 3A and C) confirms the utility of all the substrate systems for comparing relative recombinase activities in cell lines. The fluorescence-based assay has the additional advantages that it is rapid and easy to perform, can assay for recombination on a cell-by-cell basis in viable cells, and can be used to quickly obtain subclones with differing levels of recombinase activity. If some of the problems with LTR promoter down regulation can be overcome, the fluorescence-based assay should also allow for a much more precise and dynamic determination of recombination rates (see below).

Our comparison of recombination levels in B-lineage cell lines yielded results somewhat different from those of a similar survey using transiently introduced substrates. We were able to detect recombination in all pre-B-cell lines tested, including 70Z, which did not test positive with the transient substrates used in a previous survey (14). This line clearly had very low VDJ recombinase activity compared with levels of all the other pre-B-cell lines that we tested, and recombination could be detected only by using the V-gpt-J-neo substrate, not V-lacZ-J-neo (Fig. 1C and 2C). These findings indicate that the sensitivity of a stable substrate assay based on drug selection is higher than that of both our fluorescent-based assay and transient substrate assays. The previous study also found that there was generally a down regulation of recombinase activity as cells progressed through successive stages of pre-B-cell differentiation (14). With respect to normal physiology, it is not obvious why recombination activity would decrease during pre-B-cell differentiation; in fact, on the basis of the analysis of a similar number of cell lines, we did not find such a correlation (Fig. 1C). For example, the "pro-B" cell line BASC6-C2, which had the highest recombination activity (by at least an order of magnitude) as previously measured by the transient assay (14), did not have a particularly high activity by either of our stable substrate assays or when we measured its recombination rate by using the same transient assay that had previously been used (Fig. 1C, 2C, and 3C). Similarly, among lines representing later (cytoplasmic μ-chain positive) stages of pre-B-cell differentiation (e.g., 18-8 and 3-1), we saw wide variations in recombinase activity, with no consistent differences compared with lines representing earlier stages. Our ability to isolate subclones of the same cell line with widely differing recombinase levels suggests that different versions of the same cell line may actually contain different levels of recombinase activity, explaining some of the discrepancies between our rates and those previously obtained by using the transient assay. This possibility is also consistent with our earlier finding that levels of at least one apparent component of VDJ recombinase (terminal deoxytransferase) vary within cells of a given cell line (21).

Our survey for recombinase activity in B-lineage cell lines allows us to compare the specificity of this activity with the expression of a series of genes specific to different stages of B-cell development. There are a large number of genes constitutively expressed in all pre-B-cell lines examined that are turned off at the pre-B-to-B-cell junction (G. D. Yancopoulos, E. M. Oltz, M. Morrow, K. Kaplan, S. Prockop, and F. W. Alt, manuscript in preparation), as appears to be the case for recombinase activity. For example, the nuclear oncogene N-*myc* is generally expressed in all pre-B cells but is expressed at much lower levels in 70Z (23), which we demonstrate also displays much lower recombinase activity. These associations suggest the coordinate regulation of certain genes and recombinase activity and may eventually implicate some of these genes in the regulation or expression of recombinase activity.

Potential of the *lacZ* recombination substrate. We have demonstrated the validity of the *lacZ* recombination substrate as a marker of recombinase activity. Furthermore, we have used this assay to isolate cellular subclones expressing

varying levels of VDJ recombinase activity from a single recombinase-positive cell line; recombinase levels in these variants were apparently not stable after propagation in culture. On the basis of the frequency at which we obtained such variants, we presume that similar variants could have been isolated in a more laborious fashion by screening conventionally isolated subclones in transient recombination assays. However, the *lacZ* assay provides a much more efficient alternative for the rapid and repetitive screening and isolation of recombinase variants regardless of their frequency. Strategies based on such an assay would be much more practical than previously described assays for the isolation of cells stably expressing very low or very high levels of VDJ recombinase activity. Stable recombinase-negative variants could theoretically be isolated by repetitively sorting and screening for unrearranged subclones of one of the unstable recombinase-negative variants. V-lacZ-J-neo contains replication origins that allow for the transient replication of the substrate in mammalian cells (Fig. 1; 11); strategies designed to isolate (via FACS) cells displaying high levels of recombinase could thus be performed on cells transiently transfected with many copies of the *lacZ* inversion substrate. These applications of the *lacZ* inversion substrate may prove useful in the isolation and characterization of VDJ recombinase activity as well as in the isolation of regulatory variants of VDJ recombinase activity. Furthermore, mutations within a recombinase gene(s), if isolated, could be rapidly assayed for effects on recombinase activity in stable cell lines containing but not normally rearranging the *lacZ* recombination substrate.

Our attempts to use the *lacZ* recombination assay for long-term population studies of recombinase regulation have been hindered by the gradual down regulation of the LTR promoter included in the original substrate (16, 22). Genes driven by an immunoglobulin heavy-chain enhancer-promoter combination are expressed stably and at uniformly high levels both in pre-B-cell lines and in transgenic animals (e.g., reference 6). *lacZ* recombination substrates using these apparently stable transcriptional regulatory sequences may allow a very precise determination of actual recombination rates (i.e., a measure of substrates rearranged per cell per generation), which is not possible by any other recombination assay system. Such substrates should permit dynamic population studies of recombinase activity in pre-B-cell lines, in pre-B-cell lines treated with a variety of agents that modulate gene expression in lymphocytes (interleukins, lipopolysaccharide, etc.), and even in rare precursor lymphocytes isolated from transgenic mice harboring the substrates. Similarly, *lacZ* recombination substrates using appropriate transcriptional regulatory sequences that are generally expressed in transgenic animals could provide a rapid and sensitive assay for nonlymphocytic cells that might normally express recombinase activity in vivo.

ACKNOWLEDGMENTS

We thank A. Korman and R. Mulligan for providing the pDOL vector and for helpful advice. We also thank J. Hesse and M. Lieber for providing transient recombination substrates.

This work was supported by the Howard Hughes Medical Institute and Public Health Service grants AI-20047 and CA-40427 (to F.A.) and CA-42509 and HD-01287 (to L.A.H.) from the National Institutes of Health. G.D.Y. was supported by a Lucille P. Markey fellowship, R.P. was supported by a special fellowship of the Leukemia Society of America, and S.L. is a fellow of the Arthritis Foundation.

LITERATURE CITED

1. **Alt, F. W., and D. Baltimore.** 1982. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc. Natl. Acad. Sci. USA **79**:4118–4122.
2. **Alt, F. W., G. D. Yancopoulos, T. K. Blackwell, C. Wood, E. Thomas, M. Boss, R. Coffman, N. Rosenberg, S. Tonegawa, and D. Baltimore.** 1984. Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J. **3**:1209–1219.
3. **Blackwell, T. K., and F. W. Alt.** 1984. Site-specific recombination between immunoglobulin D and JH segments that were introduced into the genome of a murine pre-B cell line. Cell **37**:105–112.
4. **Blackwell, T. K., M. W. Moore, G. D. Yancopoulos, H. Suh, S. Lutzker, E. Selsing, and F. W. Alt.** 1986. Recombination between immunoglobulin variable region gene segments is enhanced by transcription. Nature (London) **324**:585–589.
5. **Desiderio, S. V., and K. R. Wolff.** 1988. Rearrangement of exogenous immunoglobulin VH and DJH gene segments after retroviral transduction into immature lymphoid cell lines. J. Exp. Med. **167**:372–389.
6. **Dildrop, R., A. Ma, K. Zimmerman, E. Hsu, A. Tesfaye, R. DePinho, and F. W. Alt.** 1989. IgH enhancer-mediated deregulation of N-myc gene expression in transgenic mice: generation of lymphoid neoplasias that lack c-myc expression. EMBO J. **8**:1121–1128.
7. **Early, P., H. Huang, M. Davis, K. Calame, and L. Hood.** 1980. An immunoglobulin heavy chain variable region is generated from three segments of DNA: VH, D, and JH. Cell **19**:981–992.
8. **Hall, C. V., P. E. Jacob, G. M. Ringold, and F. Lee.** 1983. Expression and regulation of E. coli lacZ gene fusions in mammalian cells. J. Mol. Appl. Genet. **2**:101–109.
9. **Hesse, J. E., M. R. Lieber, M. Gellert, and K. Mizuuchi.** 1987. Extrachromosomal DNA substrates in pre-B cells undergo inversion or deletion at immunoglobulin V-(D)-J joining signals. Cell **49**:775–783.
10. **Holmes, K. L., J. H. Pierce, W. F. Davidson, and H. C. Morse.** 1986. Murine hematopoietic cells with pre-B or pre-B/myeloid characteristics are generated by in vitro transformation with retroviruses containing fes, ras, abl, and src oncogenes. J. Exp. Med. **164**:443–457.
11. **Korman, A. J., J. D. Frantz, J. L. Strominger, and R. C. Mulligan.** 1987. Expression of human class II major histocompatibility complex antigens retrovirus. Proc. Natl. Acad. Sci. USA **84**:2150–2154.
12. **Kronenberg, M., G. Sui, L. Hood, and N. Shastri.** 1986. The molecular genetics of the T-cell antigen recognition. Annu. Rev. Immunol. **4**:529–591.
13. **Lewis, S., A. Gifford, and D. Baltimore.** 1981. Joining of V to J segments in a retroviral vector introduced into lymphoid cells. Nature (London) **308**:425–428.
14. **Lieber, M. R., J. E. Hesse, K. Mizuuchi, and M. Gellert.** 1987. Developmental stages specificity of the lymphoid V(D)J recombination activity. Genes Dev. **1**:751–761.
15. **Max, E. E., J. V. Maizel, and P. Leder.** 1981. The nucleotide sequence of a 5.5 kilobase DNA segment containing the mouse kappa immunoglobulin J and C region genes. J. Biol. Chem. **256**:5116–5120.
16. **Nolan, G. P., S. Fiering, J. F. Nicolas, and L. A. Herzenberg.** 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on beta-gal activity after transduction of E. coli lacZ. Proc. Natl. Acad. Sci. USA **85**:2603–2607.
17. **Pratt, D., and S. Subramani.** 1983. Nucleotide sequence of the E. coli xanthine-guanine phosphoribosyl transferase gene. Nucleic Acids Res. **11**:8817–8829.
18. **Sakano, H., R. Maki, Y. Kurosawa, W. Roeder, and S. Tonegawa.** 1980. Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy-chain genes. Nature (London) **286**:676–683.
19. **Schatz, D. G., and D. Baltimore.** 1988. Stable expression of immunoglobulin gene V(D)J recombinase activity by transfer into 3T3 fibroblasts. Cell **53**:107–115.

20. **Tonegawa, S.** 1983. Somatic generation of antibody diversity. Nature (London) **302**:575–581.
21. **Yancopoulos, G. D., T. K. Blackwell, H. Suh, L. Hood, and F. W. Alt.** 1986. Introduced T cell receptor variable region gene segments recombine in pre-B cells: evidence that B and T cells use a common recombinase. Cell **44**:251–259.
22. **Xu, L., J. K. Yee, J. A. Wolff, and T. Friedmann.** 1989. Factors affecting long-term stability of Molony murine leukemia virus-based vectors. Virology **171**:331–341.
23. **Zimmerman, K. A., G. D. Yancopoulos, R. C. Collum, R. K. Smith, N. E. Kohl, K. A. Denis, M. M. Nau, O. N. Witte, D. Toran-Allerand, C. E. Gee, J. D. Minna, and F. W. Alt.** 1986. Differential expression of *myc* family genes during murine development. Nature (London) **319**:780–783.