LYMPHOID VDJ RECOMBINASE ACTIVITY: DEVELOPMENT OF A NOVEL FLUORESCENCE-BASED ASSAY SYSTEM

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Summary

We describe two retroviral vector-based recombination substrate systems designed to assay for lymphoid VDJ recombinase activity in cultured cells. Both incorporate a constitutive dominant marker gene (the simian virus promoter driven neo gene) to allow selection of cells that stably integrate the substrate. A second marker gene is included in the substrates that can undergo transcriptional activation when inverted by a site-specific recombination event between flanking immunoglobulin variable region gene segments. In the first vector, the activatible gene is the bacterial guanine-xanthine phosphoribosyl transferase gene (gpt); detection of inversion (VDJ recombinase activity) involves drug selection and Southern blotting analyses. We have used this vector to assay VDJ recombinase activity in a panel of cell lines representing various stages of the B cell differentiation pathway and compared the results to those of other studies that employ transient recombination substrates. In the second vector, the activatible gene is the bacterial beta-galactosidase gene (lacZ). Detection for inversional activation of this gene is achieved by a fluorogenic assay that detects beta-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 based on the presence or absence of VDJ recombinase activity. We discuss the potential applications of this vector for obtaining variant cell lines expressing differing levels of recombinase activity and for assaying VDJ recombinase activity in cells of transgenic mice.

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Introduction

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 by Tonegawa, 1983; Kronenberg et al., 1986). 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) germline 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) 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 germline variable region gene segments are flanked by "recombination recognition sequences" (or "signal sequences") that mediate the joining event (Early et al., 1980; Sakano et al., 1980). 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 (Alt and Baltimore, 1982). Subsequently and in discrete joining steps, the two coding regions are brought together with potential addition and/or deletion of nucleotides while the signal sequences generally are joined without loss of nucleotides. 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 enzyme(s) 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 substrate variable region gene segments) 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 (Lewis et al., 1981; Blackwell and Alt, 1984) or be propagated extra-chromosomally for transient assays (Hesse et al., 1987). Subsequently, the introduced DNA is analyzed for 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, and have suggested that the activity of the recombinase is targeted in a tissue- and stage-specific manner to par-

ticular genetic loci depending on the "accessibility" of the given locus (Blackwell et al., 1986; Yancopoulos et al., 1986). Limited surveys of stably introduced substrates, together with more extensive surveys using transiently introduced substrates, demonstrated that VDJ recombinase activity is limited to cell lines representing the early stages of B, T and macrophage lineages (Blackwell et al., 1986; Lieber et al., 1987; Schatz and Baltimore, 1988). Within the B cell lineage, precursor B (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 antigen-receptors) did not.

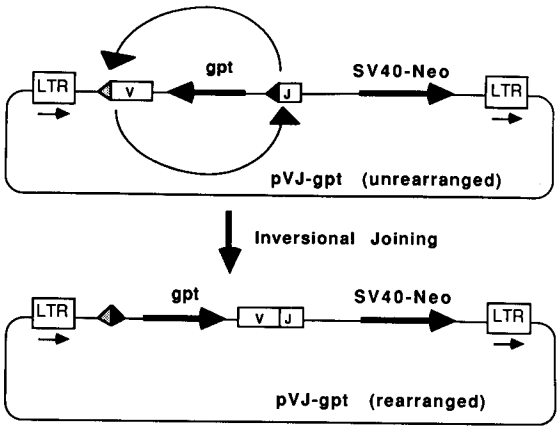
The analysis of stably or transiently introduced recombination substrates each has its advantage. Transient assays allow more rapid analysis of many cell lines and have been suggested to be more amenable to quantitative comparisons of recombination rate between cell lines (Lieber et al., 1987). Stable substrate studies allow for the analysis and manipulation of the chromatin structure surrounding the substrate, allow for long-term studies of substrate recombination following treatment or perturbation of the cells, and may allow detection (due to more powerful selection techniques) of lower levels of recombinase activity (Blackwell et al., 1986; Yancopoulos et al., 1986; Schatz and Baltimore, 1988). Here we make an extensive survey of VDJ recombinase activity during B cell development utilizing a conventional stably introduced recombination substrate. We also describe the development of 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 beta-galactosidase activity (Nolan et al., 1988) and allows for rapid analysis of recombination activity as well as the separation of live cells based on recombination activity.

Results

Recombination substrate design.

A recombination substrate (V-gpt-J-neo) containing V and J segments as well as two drug selectable marker genes (gpt and neo) was constructed within the pDOL retroviral vector (see Fig. 1A for details). The 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 the expression of the internal gpt selectable marker gene (encoding resistance to mycophenolic acid [MPA]) only subsequent to inversional joining between the flanking V and J segments. This vector was introduced into the Psi2 retroviral packaging cell line, resulting in a producer line that

A.



B.

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

FIG. 1. A: Structure of unrearranged and rearranged V-gpt-J-neo retroviral vector. Three DNA segments [the Bgl2/Sph1 fragment containing Vkappa21C (Lewis et al., 1981) in which the Sph1 site had been changed to a Sal1 site; the Bgl2/Dra1 fragment spanning gpt (Pratt and Subramani, 1983) in which both sites were changed to Sal1 sites; the Hind3/Ava2 fragment containing the Jkappa1 and Jkappa2 segments (Max et al., 1981) in which Hind3 was changed to Sal1 and Ava2 to Xho1] were cloned in the order and orientations indicated into the pDOL retroviral vector (Korman et al., 1987) which had been linearized with BamH1 and Sal1. Due to 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 long terminal repeat promoter. B: VDJ recombinase activity in B-lineage cell lines as assayed with V-gpt-J-neo. "-" indicates no growth of MPA-resistant cells. "+" indicates rare MPA-resistant cells, with no evidence of rearrangement of the substrate (by Southern blotting) prior to selection with MPA. "++" indicates evidence of rearrangement (by Southern blotting) prior to selection with MPA. "+++" indicates substrate was mostly in rearranged form when analyzed prior to selection in MPA.

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 following co-culture of each of the B-lineage cell lines with the producer cell line, populations of the B-lineage cell lines resistant to G418 emerged, indicating that polyclonal infection of the B cell lines had occurred. Placing these G418-resistant cell lines into MPA-containing media 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 were not noticeably affected by MPA exposure (i.e. cell lines 3-1 and 40E1), 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 following the placement of 5X106 cells in MPA-containing media, and these cells only appeared 3-4 weeks after exposure to MPA, suggesting that only very rare 70Z cells had rearranged the V-gpt-J-neo substrate.

To 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, DNA was prepared from the cell lines 2-4 weeks after infection as well as subsequent to growth in MPA-containing media. 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. The substrate DNA in all DNA samples from MPA-resistant populations had undergone conventional V-J joining (data not shown). However, analysis of samples prior to placement in MPA revealed different relative levels of the substrate in the unrearranged versus the rearranged form (not shown). Notably, cell lines that rapidly grew in MPA contained high levels of the rearranged form of the substrate even prior to selection in MPA, whereas cell lines that yielded MPA-resistant cells at low levels had undetectable levels of rearranged substrate prior to selection in MPA. Thus, we conclude that both ability to growth in MPA and the relative amounts of unrearranged to rearranged substrate prior to selection in MPA reflect the inherent recombinase activity of cell lines containing the V-gpt-J-neo recombination substrate (summarized in Fig. 1B). Furthermore, although recombinase activity is limited to lines representing the pre-B cell stage of B cell development, these cell lines express widely differing levels of recombinase activity.

A new 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 selection and/or Southern blot analysis), does not allow for precise quantitation of recombinase activity, and cannot be used to separate cells based on presence or absence of recombinase activity. To expand the utility of the system, we have modified the inversion-based recombination substrate to take advantage of a recently developed fluorescence-based assay for beta-galactosidase activity in live cells (Nolan et al., 1988). Mammalian cells expressing the bacterial beta-galactosidase gene (lacZ) can cleave the beta-galactoside analog fluorescein di-beta-D-galactopyranoside (FDG) to yield fluorescein, which can be detected in viable cells using the fluorescence-activated cell sorter (FACS).

The gpt selectable marker gene in V-gpt-J-neo was replaced by the lacZ gene (for details see Fig. 2A). Only inversional V to J joining within the introduced V-lacZ-J-neo substrate should yield cells which fluoresce after FDG substrate loading. To test the substrate and the fluorescence assay, a Psi2 producer cell line expressing V-lacZ-J-neo was used to infect a pre-B and B cell line with V-lacZ-J-neo. Only the pre-B cell line containing V-lacZ-J-neo developed a sub-population of fluorescing cells (Fig. 2B).

Use of lacZ recombination substrate to select cells with low recombinase activity.

Based on 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 non-fluorescing cells from the rearranging pre-B cell population displayed in Fig. 2B. These subclones were then re-screened for lacZ-dependent fluorescence. Some of the subclones remained completely negative for lacZ-dependent fluorescence, whereas other subclones had developed notable populations of fluorescing cells (Fig. 3A). Southern blot analysis of the rearrangement status of the V-lacZ-J-neo substrate in these subclones revealed that subclones lacking any lacZ-dependent fluorescence also lacked detectable levels of the rearranged form of the substrate, whereas subclones displaying lacZ-dependent fluorescence had detectable levels of the rearranged form of the substrate (Fig. 3B). These findings suggested that the lacZ substrate fluorescence assay provided a quick and efficient method for the isolation of non-rearranging cells within a rearranging cell line. However, isolation of non-rearranging cells was not necessarily equivalent to the isolation of cells lacking VDJ recombinase activity. It was also possible that rearrangement did not occur within individual cells because the particular chromosomal structure or location of the recombination substrate in these cells differed from that in

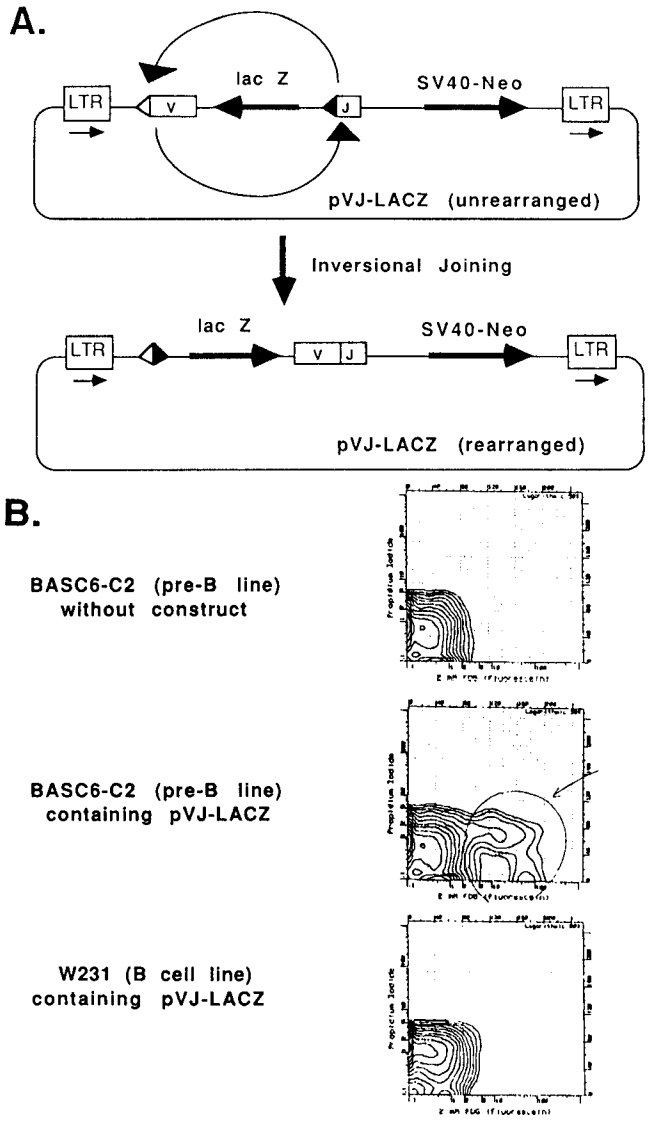


FIG. 2. A: Structure of unrearranged and rearranged forms of V-lacZ-J-neo retroviral vector. Sal1 digestion removed the gpt gene from V-gpt-J-neo; the gpt gene was replaced by the Hind3/Dra1 fragment containing the lacZ gene from pCH110 (Hall et al., 1983), in which both the Hind3 and Dra1 sites were changed to Sal1 sites. B: Only pre-B cell lines harboring V-lacZ-J-neo develop a sub-population of lacZ-expressing cells (which display increased fluorescence). Data is presented as 5% probability contour maps; fluorescence is indicated on the X-axis, and propidium iodide (which controls for non-specific fluorescence by dead cells) on Y-axis. The fluorescence-based lacZ assay was performed as described by Nolan et al. (1988).

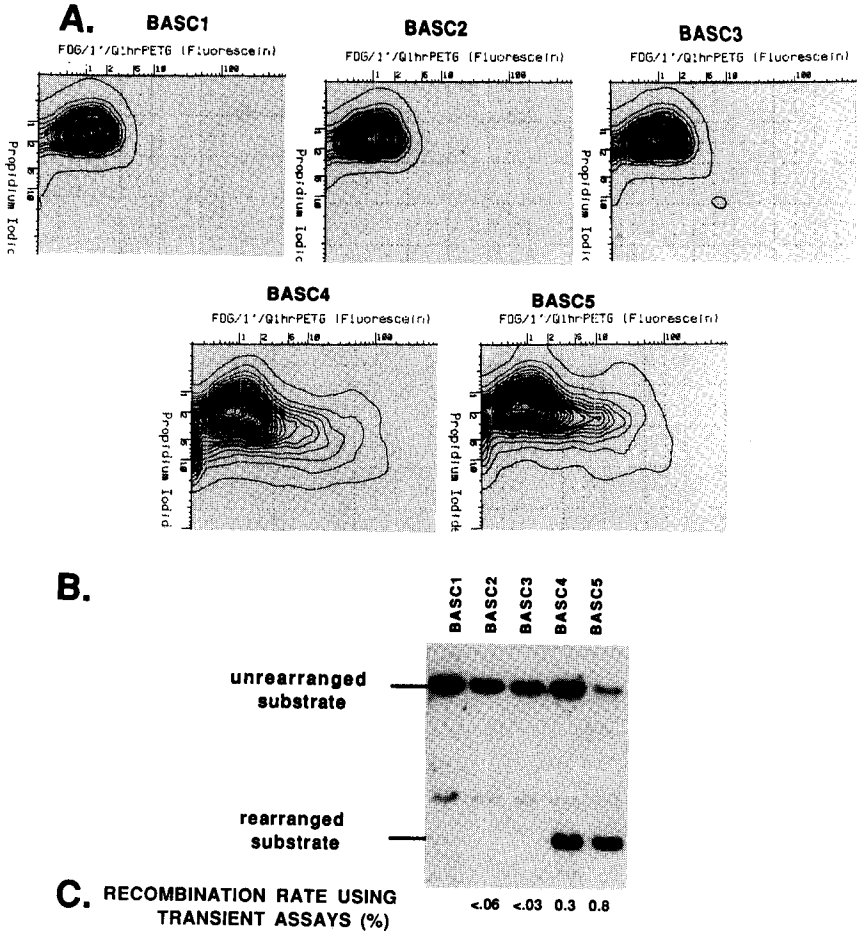


FIG. 3. A: Subclones of BASC6-C2 (BASC1 to BASC5) harboring V-lacZ-J-neo display differing levels of lacZ activity as assayed by fluorescence. Subclones derived as described in the text; fluorescence data presented as in Fig. 2B. **B:** Southern blotting reveals BASC6-C2 subclones lacking fluorescing sub-populations have not rearranged their V-lacZ-J-neo substrate. Restriction digestion with BamH1 and EcoR1 distinguishes neo-hybridizing DNA fragments that represent unrearranged and rearranged forms of V-lacZ-J-neo within cell lines. **C:** Results of transient recombination substrate assays of BASC-C2 subclones described in A and B. The transient recombination assay and substrates were those described by Hesse et al. (1987). No recombination seen with BASC2 and BASC3 subclones; BASC1 was not tested.

other cells of the same cell line which did rearrange their substrate.
To distinguish between these possibilities, we assayed the rearranging and non-rearranging subclones for recombinase activity by a second assay that utilized transient recombination substrates (Hesse et al., 1987). Significantly, the transient assay demonstrated that rearranging subclones displayed recombination activity similar to, or somewhat higher than, the parental line, whereas the non-rearranging subclones displayed no detectable recombinase activity by this assay (Fig. 3C).

Current limitations of the lacZ recombination assay.

The successful application of the lacZ substrate assay to isolate cells expressing low levels of recombinase activity suggested that the assay could be useful for understanding several features of recombinase regulation. We intended to use the assay to precisely determine recombination rates in cell lines, and also to follow changes in the recombination rate in pre-B cell lines treated with various agents (i.e. interleukins, lipopolysaccharide etc.). In the course of these experiments we encountered two significant problems. First, different pre-B cell lines fluoresced at different levels following rearrangement of the lacZ substrate (not shown). More importantly, cells harboring a rearranged lacZ substrate that all initially expressed the lacZ gene (as defined by fluorescence) gradually yielded a population of non-fluorescing cells (not shown). This down-regulation of the lacZ gene did not involve any secondary rearrangements of the substrate (not shown), and seemed related to the proposed epigenetic down-regulation of LTR promoters seen in other systems (Nolan et al., 1988).

Discussion

Survey of B-lineage cell lines using stably-introduced recombination substrates.

Our data agree with previous findings that within the B cell lineage, recombinase activity is generally limited to cell lines representing the pre-B stage. However, our survey of recombination in B-lineage cell lines yielded somewhat different results than 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 (Lieber et al., 1987). Although this line clearly had very low VDJ recombinase activity by our assay compared to other pre-B cell lines tested, our ability to detect it suggests that the sensitivity of stable substrate assays may be higher than that of transient substrate assays. The previous study concluded that there was a systematic variation in recombinase activity in early B cell ontogeny (Lieber et al., 1987). With respect to normal physiology, it is not obvious why

recombination activity would decrease during pre-B cell differentiation; in fact, based on the analysis of a similar number of cell lines, we did not find such a correlation. For example, the "pro-B" cell line BASC6-C2 that had the highest recombination activity (by at least an order of magnitude) as measured by the transient assay (Lieber et al., 1987) did not have a particularly high activity by our assay, whereas the "pro-GMB" cell line HAFTL-1 had a very low activity in the transient assay but an activity comparable to BASC6-C2 in our assay (Fig. 1B). Similarly, based on the transient assay, it was concluded that recombination activity decreased substantially in lines representing later stages of pre-B cell differentiation (Lieber et al., 1987), whereas we saw wide variation among such lines with no consistent differences compared to lines representing earlier stages. It may be that transient substrate uptake, nuclear localization and stability varies between cell lines, contributing to differences in the observed recombination rate that do not necessarily reflect differences in the inherent recombinase activities of different cell lines. One potential problem with assaying recombination rate using stably-introduced substrates is that the recombination rate of a substrate may depend on its particular chromosomal location. Our stable assay obviates this problem by examining recombination within a polyclonally infected cell line consisting of cells harboring substrates in many different locations.

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 which are turned off at the pre-B to B cell junction (Yancopoulos, Oltz, Kaplan, Prockop, and Alt, in preparation), as appears to be the case for recombinase activity. Furthermore, there are at least two genes (N-myc and PB.99) which are generally expressed in all pre-B cells, but are expressed at much lower levels in 70Z, which 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 of recombinase activity.

Potential of the lacZ recombination substrate.

We have demonstrated the utility of the lacZ recombination substrate as a marker of recombinase activity. Furthermore, we have used this assay to isolate cellular subclones not expressing VDJ recombinase activity from recombinogenic cell lines. Similar strategies should, in theory, allow for the isolation of cells expressing high levels of VDJ recombinase activity. V-lacZ-J-neo contains replication origins that allow for the transient replication of the substrate in mammalian cells (Fig. 1 and data not

shown); strategies designed to isolate (via FACS) cells displaying high levels of recombinase could thus be performed on transiently transfected cells. These applications of the lacZ assay may prove useful in the isolation and characterization of VDJ recombinase activity.

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 (see Nolan et al., 1988). Genes driven by an immunoglobulin heavy chain enhancer/promoter combination are expressed uniformly high both in pre-B cell lines and in transgenic animals (e.g. Dildrop et al., 1989). The use of lacZ recombination substrates utilizing these transcriptional regulatory sequences should allow dynamic population studies of recombinase activity both in pre-B cell lines and in developing lymphocyte subsets in vivo.

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