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Immunoglobulin heavy chain switch region recombination within a retroviral vector in murine pre-B cells

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We have employed a retroviral vector, ZN(S_μ/$S_{\gamma 2b}$)tk1, as a substrate for detecting the presence of immunoglobulin heavy chain constant region (C_H) gene switch (S) recombination activity in murine pre-B cells. ZN(S_μ/$S_{\gamma 2b}$)tk1 contains a neomycin (*neo*) resistance gene in addition to the herpes simplex virus thymidine kinase (Htk) gene which is positioned between murine S_μ and $S_{\gamma 2b}$ sequences. Stable acquisition of the ZN(S_μ/$S_{\gamma 2b}$)tk1 vector was selected in G-418 and switch region recombination within these proviruses was selected by resistance to the drug bromodeoxyuridine (BUdR). Fluctuation analyses of ZN(S_μ/$S_{\gamma 2b}$)tk1 infected 18-8tk$^-$ and 38B9tk$^-$ pre-B lines revealed Htk gene inactivations with apparent frequencies of 5×10^{-5} and 1×10^{-5} events/cell/generation, respectively, while G-418 resistant Ltk$^-$ fibroblasts lost the HTK phenotype at an apparent rate of 4×10^{-8}. Southern blot analysis demonstrated that switch recombination caused the deletion of the Htk gene in all pre-B clones examined while the loss of Htk in Ltk$^-$ clones was not mediated by S region recombination. In 21 out of 24 pre-B clones, the recombinations involved the tandemly repetitive portions of the S_μ and $S_{\gamma 2b}$ sequences. These results demonstrate that the C_H gene S region segments inserted into ZN(S_μ/$S_{\gamma 2b}$)tk1 are sufficient for B-cell-specific recombination/deletion within the S region tandem repeats.

Key words: C_H gene switching/pre-B cells/retroviral vector/S region recombination

Introduction

The committed expression of IgG, IgE and IgA antibodies by B lymphocytes is manifested by rearrangement of Ig heavy chain constant region (C_H) gene segments in a process termed C_H class switching (reviewed in Marcu, 1982; Shimizu and Honjo, 1984; Radbruch *et al.*, 1986a). These DNA rearrangements result in the deletion of the originally expressed C_H gene segment and the placement of a downstream C_H gene segment proximal to the functionally rearranged variable (V) gene segment. C_H switching occurs by recombination between switch (S) regions, located ~2 kb 5′ of every C_H gene segment except δ (reviewed in Marcu, 1982; Shimizu and Honjo, 1984; Radbruch *et al.*, 1986a). Switch region recombination has been examined in murine plasma cell tumors (Cory *et al.*, 1980; Rabbitts *et al.*, 1980; Coleclough *et al.*, 1980; Davis *et al.*, 1980; Sakano *et al.*, 1980; Dunnick *et al.*, 1980; Kataoka *et al.*, 1981; Obata *et al.*, 1981; Lang *et al.*, 1982; Nikaido *et al.*, 1982; Eckhardt *et al.*, 1982; Eckhardt and Birshtein, 1985; Tilley and Birshtein, 1985; Szurek *et al.*, 1985), hybridomas (Hurwitz *et al.*, 1980; Sablitzky *et al.*, 1982; Kipps and Herzenberg, 1986), B lymphomas (Stavnezer *et al.*, 1982; Stavnezer *et al.*, 1985; Stavnezer and Sirlin, 1986), normal B cell populations (Cebra and Hurwitz, 1982; Radbruch and Sablitzky, 1983; Radbruch *et al.*, 1986b); Epstein-Barr virus (EBV) transformed human B cells (Brown *et al.*, 1985; Webb *et al.*, 1985) and in Abelson murine leukemia virus (A-MuLV) transformed pre-B cells (Alt *et al.*, 1982a; Akira *et al.*, 1983; Burrows *et al.*, 1983; DePinho *et al.*, 1984; Yancopoulos *et al.*, 1986b). Several mechanisms for this recombination have been proposed: (i) loop out and loss of intervening C_H gene segments (Honjo and Kataoka, 1978), (ii) unequal homologous chromosome exchange (Sablitzky *et al.*, 1982; Kipps and Herzenberg, 1986) and (iii) sister chromatid exchange (Obata *et al.*, 1981; Tilley and Birshtein, 1985). However, the precise molecular requirements of this developmentally controlled phenomenon have largely remained obscure due to the limited number of lymphoid cell lines which possess only a low level of constitutive or inducible C_H switch potential.

Switch (S) regions are composed of tandemly repeated, short DNA sequences that vary in homology between different S segments (Davis *et al.*, 1980; Kataoka *et al.*, 1981; Nikaido *et al.*, 1981, 1982; Marcu *et al.*, 1982; Stanton and Marcu, 1982; Szurek *et al.*, 1985; Mowatt and Dunnick, 1986). The 5′ portion of the S_μ region contains the sequence YAGGTTG which is repeated in other S regions and is also found 5′ of a number of switch recombination sites in mouse myelomas (Marcu *et al.*, 1982). The 3′ portion of S_μ is comprised of a tandemly repeated block of two pentameric sequences (GAGCT and GGGGT) which are also generally repeated in other downstream S regions and are commonly found near S–S recombination sites (Nikaido *et al.*, 1981, 1982; Shimizu and Honjo, 1984). The four S_γ switch regions are comprised of homologous 49-mer repeat units (Kataoka *et al.*, 1981; Nikaido *et al.*, 1981, 1982; Stanton and Marcu, 1982; Szurek *et al.*, 1985; Moatt and Dunnick, 1986). The degree of S_μ homology to the four S_γ segments diminishes with the C_γ gene order such that S_μ is most homologous to $S_{\gamma 3}$ and least homologous to $S_{\gamma 2a}$ (Shimizu *et al.*, 1982; Stanton and Marcu, 1982). S_ϵ and S_α, the 3′ most S regions, display the highest S_μ homology (Nikaido *et al.*, 1982; Stanton and Marcu, 1982). A characteristic repeat unit has not been described for S_ϵ (except for the presence of GAGCT and GGGGT sequences) (Nikaido *et al.*, 1982), while S_α consists of 80-mer repeats (Davis *et al.*, 1980; Obata *et al.*, 1981). Precise conserved sequences, which could serve as recognition sites for switch recombination, have not been observed near all S region recombination sites, making this type of Ig gene recombination very distinct from V_L and V_H gene formation (Marcu *et al.*, 1982; Shimizu and Honjo, 1984). The absolute requirements for DNA sequences flanking S region repeats remain unknown.

The existence of novel repeat units in different S regions and the variations in their overall sequence homologies originally led to the suggestion that isotype switching may involve C_H class specific recombinases (Davis *et al.*, 1980). This idea has gained

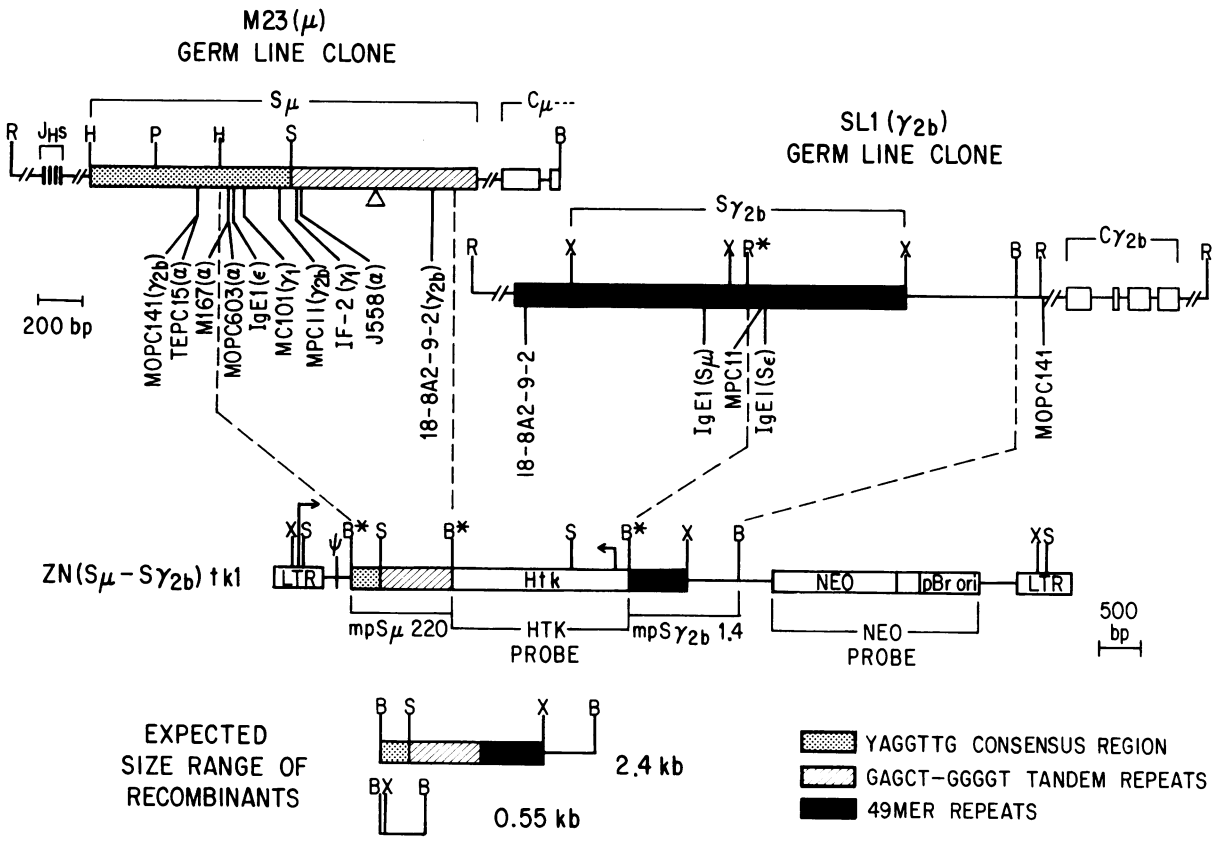

Fig. 1. Derivation and construction of ZN(S_μ/$S_{\gamma 2b}$)tk1. The two Charon 4A germline genomic clones (M23 and SL1) (Marcu *et al.*, 1980; Lang *et al.*, 1982) that provided the respective S_μ and $S_{\gamma 2b}$ switch region segments are shown at the top. The general sequence composition of the regions are shown by the various shaded boxes. Switch sites used by various mouse myelomas are indicated under the genomic clone maps (Nikaido *et al.*, 1981; Marcu *et al.*, 1982; DePinho *et al.*, 1984). An internal deletion in S_μ occurred during phage propagation (Marcu *et al.*, 1980) and is shown as an open triangle. The map of the ZN(S_μ/$S_{\gamma 2b}$)tk1 construct shows the packaged virus genome and the origin of the chosen switch region segments. The probes used are denoted by the brackets under the maps. The predicted maps of recombinants that utilize the repetitive regions are shown along with their predicted sizes. Enzyme abbrevations are: B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; R*, *Eco*RI site added by cloning procedure; S, *Sst*I; X, *Xba*I.

support from the documented propensity of A-MuLV transformed pre-B cell lines to switch from μ to γ_{2b} (Alt *et al.*, 1982a; Akira *et al.*, 1983; Burrows *et al.*, 1983; DePinho *et al.*, 1984; Yancopoulos *et al.*, 1986b), the preferential C_H isotype switching in a murine B lymphoma (Stavnezer *et al.*, 1982, 1985), the restricted pattern of C_H gene recombination in some myeloma tissue culture variants (Eckhardt *et al.*, 1982; Eckhardt and Birshtein, 1985; Tilley and Birshtein, 1985) and the selective expression of γ_3 or γ_1 isotypes in normal splenic B cells which have been stimulated with LPS or LPS/BSF-1, respectively (Radbruch and Sablitzky, 1983; Radbruch *et al.*, 1986b). However, it has also been proposed that these apparent instances of directed C_H switching may be predetermined by factors which preferentially enhance the accessibility of different C_H gene segments to a general S region recombinase (Stavnezer *et al.*, 1984; Stavnezer-Nordgren and Sirlin, 1986; Alt *et al.*, 1986; Yancoupoulos *et al.*, 1986b). It is interesting to note in this context that the assembly of Ig V and C gene segments may also be associated with their unique transcriptional competence or accessibility in B lymphoid cells (Perry *et al.*, 1981; Yancopoulos and Alt, 1985, 1986; Yancopoulos *et al.*, 1986a; Alt *et al.*, 1986; Blackwell *et al.*, 1986).

To investigate directly the molecular requirements and regulation of C_H class switching, we have developed a selectable retroviral vector as an *in vivo* molecular substrate for switch recombination. Here, we describe the use of such a viral vector for assaying S_μ/$S_{\gamma 2b}$ switch recombinase activity in murine pre-B cell lines.

Results

Properties of the ZN(S_μ/$S_{\gamma 2b}$)tk1 retrovirus vector

The derivation of the ZN(S_μ/$S_{\gamma 2b}$)tk1 retorvirus vector is displayed in Figure 1. We chose to design a retroviral vector for monitoring the presence of switch-recombinase activities in B cells for a number of reasons. As a retrovirus, it can be introduced easily, with high efficiency into B cells which are normally refractory to most DNA transfection techniques. More importantly, retroviruses integrate in a single copy per cell (Varmus and Swanstrom, 1984) thereby allowing recombinations to be scored as unique events. Moreover, since retroviruses have a defined molecular mechanism of integration, constructs are integrated without perturbation of the sequences residing between the LTRs (Varmus and Swanstrom, 1984). The two independent markers (*neo* and Htk) allow for both the maintenance and the selection of proviruses which have undergone switch region mediated Htk gene deletion. The proximity of the LTR's promoter and enhancer sequences to the Htk gene and the two S regions would ensure that this portion of the vector is accessible for S region mediated recombination. Therefore, the intact Htk gene would be transcriptionally active in all *neo* expressing G-418 resistant transformants thereby obviating the necessity for prior

Table I. Fluctuation frequency analysis of the G-418/BUdR selections to score for loss of the HTK phenotype.

Cell lines containing $ZN(_{\mu}/S_{\gamma 2b})tk1$	Loss of HTK phenotype/cell/generation[a]	Apparent rate of HTK loss relative to 18-8[b]
18-8tk$^-$	5×10^{-5}	1
38B9tk$^-$	1×10^{-5}	0.2
Ltk$^-$	4×10^{-8}	8×10^{-4}

[a]Frequency values were derived by graphical solution of the fluctuation equation $r = aN_t\ln(aN_tC)$ (Luria and Delbruck, 1943) where r = observed number of G-418/BUdR resistant cells, N_t = number of cells in the G-418 resistant population prior to BUdR selection, C = number of independently derived populations and a = mutation rate. The equation was solved for aN_t for a range of r values with the appropriate C value for each cell line. The solutions were then graphically displayed and aN_t values were extrapolated for each observed r value.
[b]Frequency of HTK loss in 18-8tk$^-$ may be underestimated due to the diminished viability of γ_{2b} positive cells (see Results and Discussion).

selection in HAT media (Blackwell *et al.*, 1986). Fusion of the S_{μ} and $S_{\gamma 2b}$ sequences would result in Htk gene deletion which is selected, in a thymidine kinase deficient cell line, by resistance to bromodeoxyuridine (BUdR). The novel sizes of the retroviral S region restriction fragments allows them to be readily distinguished from the corresponding endogenous S regions. Recombinants are easily identified by *Bam*HI digestion which releases the S_{μ}, HTK and $S_{\gamma 2b}$ sequences of $ZN(S_{\mu}/S_{\gamma 2b})tk1$ on different-sized fragments. The S_{μ} and the $S_{\gamma 2b}$ switch regions possess a limited amount of sequence homology (Nikaido *et al.*, 1982; Stanton and Marcu, 1982) which should minimize the background of general homologous recombination.

Frequencies of HTK phenotype loss in 18-8tk$^-$, 38B9tk$^-$ and Ltk$^-$ cells

Two A-MuLV transformed murine pre-B cell lines (18-8tk$^-$ and 38B9tk$^-$) and a fibroblast line (Ltk$^-$), which lack functional thymidine kinase genes, were infected with $ZN(S_{\mu}/S_{\gamma 2b})tk1$ virus and then selected for resistance to G-418. The 18-8 line has been documented to spontaneously switch from C_{μ} and $C_{\gamma 2b}$ expression by S region mediated deletion (Burrows *et al.*, 1983; DePinho *et al.*, 1984; Yancopoulos *et al.*, 1986b). The 38B9 line has DJ rearrangements on both heavy chain alleles (Alt *et al.*, 1984) and has been shown to recombine D−J gene segments of both IgH and T cell receptor genes in plasmid vectors at a high frequency (Blackwell and Alt, 1984; Yancopoulos *et al.*, 1986a; Blackwell *et al.*, 1986). The Ltk$^-$ line serves as a non-lymphoid control which possesses an inert, endogenous IgH locus.

The frequencies of HTK phenotype loss were determined by selection in medium supplemented with G-418 and BUdR (Table I) (see Materials and methods for details). 18-8tk cells were the most efficient in eliminating the HTK phenotype while the 38B9tk$^-$ and Ltk$^-$ lines lost HTK function with frequencies that were 5- and 1250-fold lower, respectively, than 18-8tk$^-$.

Htk gene deletions in G-418/BUdR resistant 18-8tk$^-$ lines

To determine the mechanisms of HTK phenotype loss, G-418/BUdR resistant clones from the frequency analysis and limiting dilution cloning were expanded and genomic DNAs were prepared and submitted to Southern blot analysis with NEO, HTK and S region probes. As expected, each of these clones contained a *neo* gene but none retained an Htk gene (Figure 2A). All of the G-418/BUdR resistant 18-8tk$^-$ clones displayed a novel $S_{\gamma 2b}$ hybridizing *Bam*HI fragment (Figure 2B). The identical bands were also positive with an S_{μ} specific probe (Figure 2B). Therefore, the novel $S_{\mu}/S_{\gamma 2b}$ containing *Bam*HI fragment in each independent clone was generated by recombination between the S_{μ} and $S_{\gamma 2b}$ sequences which deleted the Htk gene and its bordering 5′ and 3′ *Bam*HI sites. The potential size range of the S region recombination products resulting in complete Htk gene deletion is shown in Figure 1. Recombination between the 3′ end of S_{μ} and the 5′ most portion of $S_{\gamma 2b}$ would produce the largest expected fragment size of 2.4 kb while fusion of the 5′ most portion of S_{μ} with the 3′ most repeat of $S_{\gamma 2b}$ would produce the smallest expected fragment size of 0.55 kb. Fourteen independent 18-8tk$^-$ clones were analyzed and the structures of all the recombined S regions are displayed in Figure 3. S region recombination products ranged in size from 0.80 to 2.0 kb with a median size of 1.3 kb with no two clones having the same size.

DNA samples were digested with other enzymes to localize the recombination sites within the S region sequences and restriction maps of these recombinants are presented in Figure 3. The 5′ 265-bp *Bam*HI−*Sst*I portion of the S_{μ} segment in the vector does not contain tandem $(GAGCT)_nGGGGT$ repeats (Sakano *et al.*, 1980) but harbors YAGGTTG consensus motifs (Marcu *et al.*, 1982). The 3′ 890-bp *Sst*I−*Bam*HI portion of the S_{μ} segment is a continuous tandem repeat of $(GAGCT)_nGGGGT$ sequences (Sakano *et al.*, 1980; Nikaido *et al.*, 1981; Marcu *et al.*, 1982). The $S_{\gamma 2b}$ insert can also be divided into two parts. The 5′ 670-bp *Bam*HI−*Xba*I portion contains a high density of 49-mer repeats while these sequences are not prevalent in the 3′ adjacent 580-bp *Xba*I−*Bam*HI segment (Kataoka *et al.*, 1981). *Sst*I cuts in both LTRs and once within the S_{μ} segment (see above and Figure 1) if the latter site is not deleted by S region mediated recombination. The deletion of the S_{μ} *Sst*I site would result in a 0.7-kb addition to the size of the rearranged *Sst*I fragment that hybridizes with the NEO probe (Figure 1). The presence of the $S_{\gamma 2b}$ *Xba*I sites is assayed in a similar way. *Xba*I cuts in both LTRs and once within the $S_{\gamma 2b}$ segment. If the $S_{\gamma 2b}$ *Xba*I site was deleted, then the size of the *neo*-containing *Xba*I fragment within the original vector (3.9 kb) would be increased by the combined length of the rearranged $S_{\mu}/S_{\gamma 2b}$ *Bam*HI fragment (Figure 2A) and the vector sequences 5′ of this rearranged fragment. Only two clones out of fourteen contained a recombination site within the *Bam*HI−*Sst*I 5′ S_{μ} segment (see maps of clones NB3 and NB32 in Figure 3), while none recombined 3′ of the *Xba*I site within the non-repetitive portion of the $S_{\gamma 2b}$ segment. The recombinant events in all of the remaining clones occurred within the repetitive portions of the S_{μ} and $S_{\gamma 2b}$ segments (Figure 3). Therefore, the loss of the HTK phenotype in these G-418/BUdR resistant 18-8tk$^-$ lines is entirely due to switch region mediated recombination.

Htk gene deletions in G-418/BUdR resistant 38B9tk$^-$ lines

The 38B9tk$^-$ cell line was infected and selected in parallel with the 18-8tk$^-$ cells. Southern hybridizations of *Bam*HI restricted genomic DNAs from G-418/BUdR resistant 38B9tk$^-$ clones revealed co-hybridizing fragments with S_{μ} and $S_{\gamma 2b}$ specific probes. None of the 38B9tk$^-$ clones retained Htk sequences (data not shown). The S region recombination products ranged in size from 0.60 to 2.3 kb in 10 independent clones (Figure 3) and Southern results on six representative lines are shown in Figure 4. Restriction with *Xba*I and *Sst*I confirmed that S region tandem repeats were involved in all of these recombinations with one exception (Figure 3). Therefore, the retroviral S region recombinations in 38B9tk$^-$ and 18-8tk$^-$ cells are analogous.

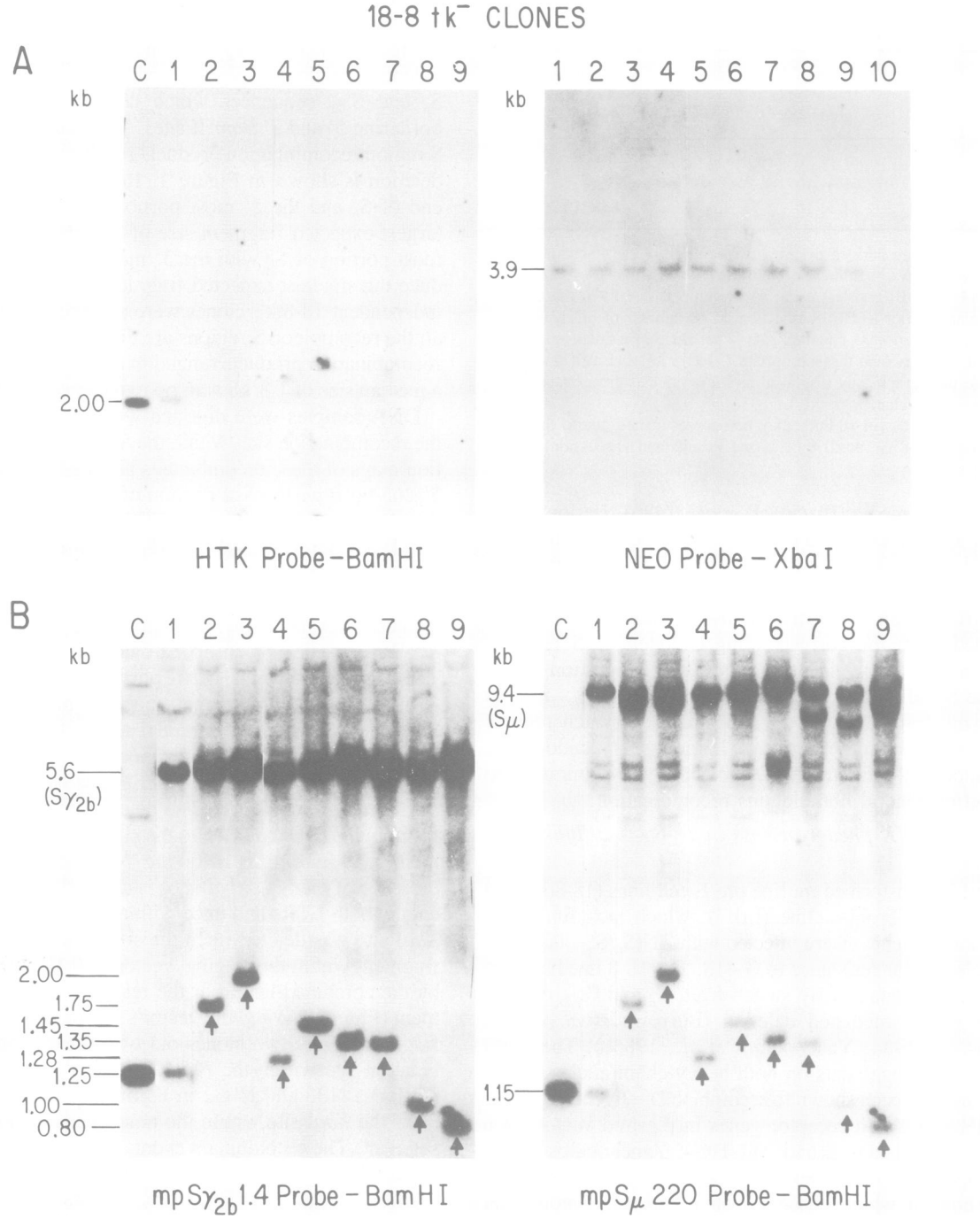

Fig. 2. Southern blot analysis of DNAs from ZN(S_μ/$S_{\gamma 2b}$)tk1 containing 18-8tk$^-$ G-418/BUdR resistant clones. **Panel A**: Southern blots of *Bam*HI and *Xba*I digested genomic DNAs hybridized to HTK or NEO probes, respectively. **Panel B**: *Bam*HI digestions hybridized to $S_{\gamma 2b}$ and S_μ probes, respectively. **Lane C**: pZN(S_μ/$S_{\gamma 2b}$)tk1 vector control; **lane 1**: ZN(S_μ/$S_{\gamma 2b}$)tk1 in 18-8tk$^-$ cells before G-418/BUdR selection; **lanes 2–9**: G-418/BUdR resistant clones NB39, NB38, NB30, NB26, NB5, NB1, NB3 and NB2, respectively. **Lane 10** in **panel A** is the uninfected, parental 18-8tk$^-$ line. Bands co-hybridizing with both probes are denoted by arrows. The endogenous S sequences are present above the construct bands.

Switch region mediated HTK gene deletions do not occur in Ltk$^-$ cells

The status of the Htk sequences in G-418/BUdR resistant clones of ZN(S_μ/$S_{\gamma 2b}$)tk1 infected Ltk$^-$ cells was analyzed by *Bam*HI digestion. Ten independent lines contained intact Htk genes or internal Htk gene rearrangements and results with five representative clones are shown in Figure 5. The smaller Htk hybridizing bands in Ltk$^-$ clones NB4 and NB7 in Figure 5 do not involve rearrangement with adjacent S sequences because rehybridization with S region probes revealed that both S_μ and $S_{\gamma 2b}$ segments remain in their original context (data not shown). We conclude that the Htk gene is generally inactivated in Ltk$^-$ murine fibroblasts by non-deletional mechanisms even in the presence of adjacent S region sequences.

18-8tk$^-$ clones contain deletions in their endogenous S_μ regions

The 18-8tk$^-$ cell line partially deletes its S_μ region possibly as a prelude to the actual switching event (Alt *et al.*, 1982a; DePinho *et al.*, 1984). The contexts of the endogenous S_μ regions of the

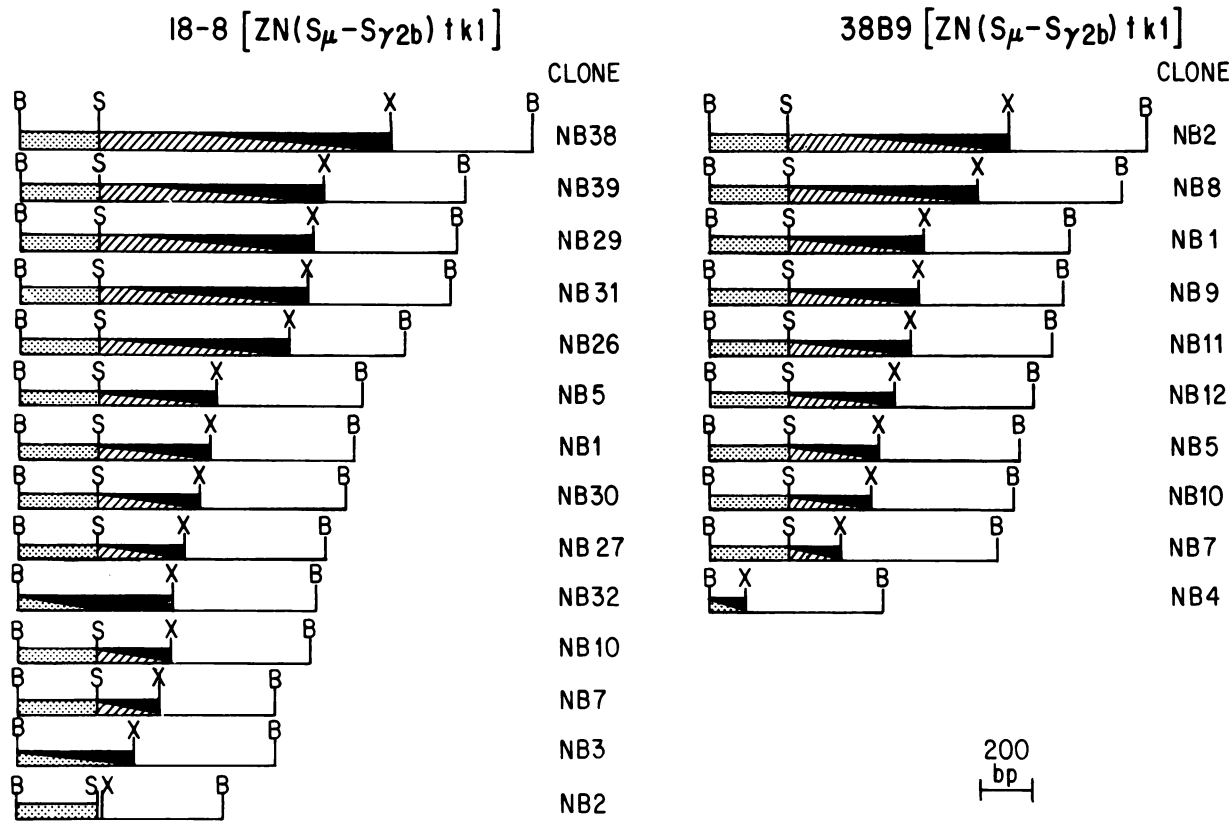

Fig. 3. Southern blot maps of the 18-8tk$^-$ and 38B9tk$^-$ ZN(S_μ/$S_{\gamma 2b}$)tk1 containing clones. Genomic DNAs were mapped by hybridization of *Bam*HI, *Sst*I and *Xba*I digestions to NEO and S region probes. Recombined inserts are displayed with a diagonal line showing the approximate area where the recombinations have taken place.

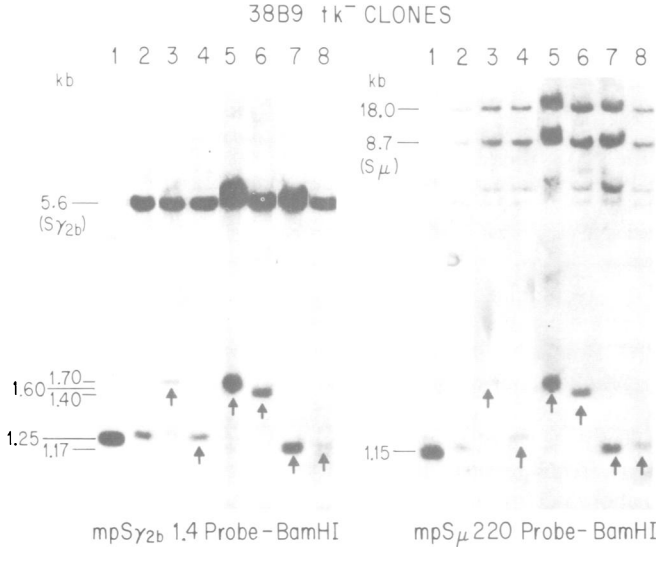

Fig. 4. Southern blot analysis of *Bam*HI-restricted DNAs from ZN(S_μ/$S_{\gamma 2b}$)tk1 containing 38B9tk$^-$ G-418/BudR resistant clones using $S_{\gamma 2b}$ and S_μ probes. **Lane 1**: $_p$ZN(S_μ/$S_{\gamma 2b}$)tk1 vector; **lane 2**: 38B9tk$^-$ cells containing ZN(S_μ/$S_{\gamma 2b}$)tk1 before G-418/BUdR selection; **lanes 3–8**: clones NB2, NB12, NB8, NB9, NB10, NB5, respectively. Bands cohybridizing with both probes are denoted by arrows. Endogenous sequences are denoted above the construct band.

original 18-8tk$^-$ line and the G-418/BUdR resistant clones were compared by probing Southern blots of *Xba*I digested DNAs with a 5′ S_μ probe (5′ S_μXP) (Figure 6) which is not present in the construct. Deletions within the 5.8-kb *Xba*I S_μ fragment of the 18-8tk$^-$ line generated rearranged bands of 2.1–5.0 kb (Figure 6). Twelve out of 14 clones each contain a unique endogenous S_μ deletion while the remaining two clones do not have a rearranged band that can be distinguished from the original 18-8tk$^-$ S_μ band. Southern data for eight representative 18-8tk$^-$ clones are shown in Figure 6. These results were corroborated by hybridizing *Bam*HI digested DNAs with a DNA probe containing J_H segments 3 and 4 (data not shown) and an S_μ probe (Figure 2b). However, there was no evidence of an endogenous C_μ to $C_{\gamma 2b}$ switch when the same *Bam*HI blot was rehybridized with an $S_{\gamma 2b}$ probe (Figure 2B). Similar results were obtained by *Xba*I digestion (data not shown). The 38B9tk$^-$ and Ltk$^-$ cell lines did not show any rearrangement of their endogenous S_μ and $S_{\gamma 2b}$ regions (Figure 4 and data not shown) in this analysis although on occasion S_μ deletions have been observed in some 38B9 subclones (F.Alt, unpublished data).

Expression of cytoplasmic μ and γ_{2b} heavy chains

Immunofluorescence staining with FITC and TRITC conjugated anti μ and anti γ isotype specific antibodies was performed on the original 38B9tk$^-$ and 18-8tk$^-$ lines and 26 G-418/BUdR resistant 18-8tk$^-$ clones. The 38B9tk$^-$ line was $\mu^-\gamma^-$ while the 18-8tk$^-$ line contained 80% $\mu^+\gamma^-$ and 20% $\mu^-\gamma^+$ cells. All 26 G-418/BUdR resistant 18-8tk$^-$ clones also contained a small number of γ^+ cells which did not co-stain with anti μ. For six of the clones, a battery of fluorescent antibodies specific for all the γ subclasses were individually used to confirm that the γ staining clones were γ_{2b}. These compositions range from 0.1 to 20% γ_{2b}^+ and are likely to represent cells that have switched from

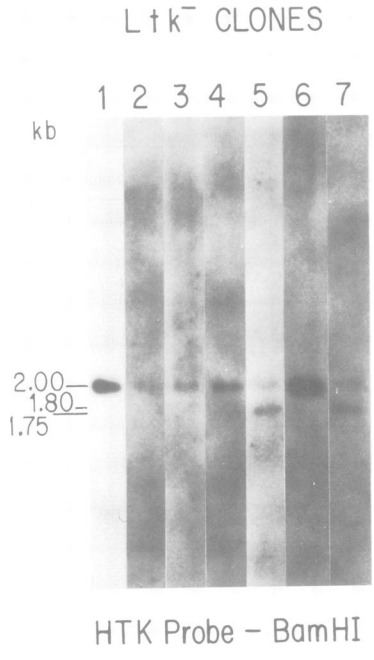

Fig. 5. Southern blot analysis of *Bam*HI-digested DNAs from $ZN(S_\mu/S_{\gamma 2b})tk1$ containing Ltk^- G-418/BUdR resistant clones hybridized to an HTK probe. **Lane 1**: $_pZN(S_\mu/S_{\gamma 2b})tk1$ vector, **lane 2**: Ltk^- cells containing $ZN(S_\mu/S_{\gamma 2b})tk1$ before G-418/BUdR selection; **lanes 3–7:** G-418/BUdR resistant clones NB3, NB5, NB4, NB10 and NB7, respectively.

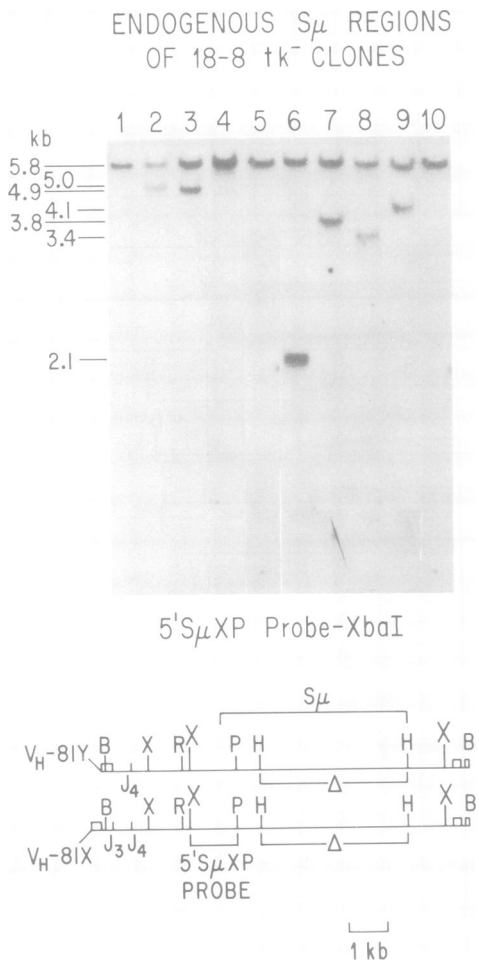

Fig. 6. Southern blot analysis of *Xba*I-digested DNAs from 18-8tk^- clones harboring $ZN(S_\mu/S_{\gamma 2b})tk1$ probed with a 5′ S_μ probe (5′ S_μXP) to assess the status of the endogenous S_μ regions. **Lane 1**: 18-8tk^- cells containing $ZN(S_\mu/S_{\gamma 2b})tk1$ before G-418/BUdR selection; **lanes 2–9**: clones NB39, NB38, NB30, NB26, NB7, NB3 and NB2, respectively; **lane 10**: 18-8tk^- parental line. Restriction maps of the 81Y and 81X VDJ rearrangements in the 18-8 line (Alt *et al.*, 1982a,b) and the approximate location of the S_μ deletions are shown below the blot. The allele which has undergone an S_μ deletion in each of the independent clones has not been determined.

μ to γ_{2b} expression subsequent to cloning. No 100% γ_{2b} staining clones were found which explains our inability to detect an endogenous μ to γ_{2b} switch in Southern blotting experiments. The inability of the 18-8tk^- line to accumulate more than 20% μ to γ_{2b} switch cells implies that the γ_{2b}^+ cells are less viable (Burrows *et al.*, 1983).

Discussion

We have shown that IgC_H S region sequences in a retoviral vector can mediate DNA recombinations in two murine pre-B cell lines but not in a fibroblast line. Limited portions of S_μ and $S_{\gamma 2b}$ sequences were sufficient as switch recombinase substrates excluding the absolute requirements of other flanking sequences as well as the C_H gene segments. Recombination sites were only observed within S_μ and $S_{\gamma 2b}$ and not in flanking DNA or in vector sequences. The absence of 'switch-like' recombination within this S region substrate vector in Ltk^- fibroblasts demonstrates that the expressed provirus's open chromatin environment is insufficient to cause S region mediated rearrangements by general homologous recombination in non-lymphoid cells. The frequency of S region mediated recombination in both pre-B cells lines was 50- to 200-fold higher than the expected frequency of $10^{-6}-10^{-8}$ events/cell/generation (Varmus *et al.*, 1981; Jenkins *et al.*, 1981; Liskay and Stachelek, 1983; Subramani and Rubnitz, 1985; Stringer *et al.*, 1985; Brenner *et al.*, 1986) for strictly homologous recombination between chromosomally integrated sequences. This difference in recombination frequency is even more striking considering that the S_μ and $S_{\gamma 2b}$ segments are not exceptionally homologous (Shimizu *et al.*, 1982; Stanton and Marcu, 1982; Nikaido *et al.*, 1982). This indicates that the general homologous recombination system is not involved; is specifically modified in B cells to recognize switch repeat homologies; or is superactive in B cells. The lack of this recombination/deletion event in Ltk^- cells, which have been used in other recombination studies (Liskey and Stachelek, 1983; Brenner *et al.*, 1986), lends support to the argument that a general cellular recombination system is not directly responsible for these S region rearrangements.

The presence of unique endogenous S rearrangements in the G-418/BUdR resistant 18-8tk^- clones suggests that these recombinations occurred in a co-ordinated fashion with the retroviral S sequence deletions. If this was not the case, we would expect to observe a heterogenous pattern of endogenous S_μ rearrangements in these clonal isolates unless all the S_μ deletions occurred before the vector rearrangements. It is important to note that Southern blot analysis of a G-418 resistant, non-BUdR selected 18-8tk^- population showed no evidence of S_μ deletion with either *Bam*HI (Figure 2, panel B, lane 1) or *Xba*I (Figure 6, lane 1). The appearance of γ_{2b} expressing cells in all the G-418/BUdR resistant 18-8tk^- clones demonstrates that these cells have undergone C_H switching after cloning possibly when the $S_{\gamma 2b}$ region becomes accessible to the switch-recombinase. However, in all cases examined the γ_{2b}^+ cells never represent

more than 20% of each clone; this observation is most likely explained by the reduced viability of the γ_{2b}^+ cells (Burrows *et al.*, 1983). It follows that the observed rate of S region deletions within the retroviral vector is underestimated in the 18-8tk$^-$ clones due to the preferential loss of γ_{2b} switched cells from the original population.

S region mediated rearrangements were 5-fold less frequent in 38B9tk$^-$ compared with 18-8tk$^-$ pre-B cells. This observation is most likely explained by different levels of switch-recombinase activity in these two pre-B lines. It follows that the lack of endogenous S_μ deletions and γ_{2b} switching in the G-418/BUdR resistant 38B9tk$^-$ line cannot be due to the absence of switch-recombinase activity in these cells. The absence of a functional VDJ rearrangement in 38B9tk$^-$ suggests that it is less differentiated than 18-8tk$^-$ (Alt *et al.*, 1984) although there is evidence with another A-MuLV pre-B line that switching can occur before VDJ rearrangement is completed (Akira *et al.*, 1983). It is also conceivable that the environment of the 38B9tk$^-$ endogenous S_μ sequences may not be accessible to this switch-recombinase activity. The absence of sterile γ_{2b} transcripts in the 38B9 line suggest that its γ_{2b} gene would also not be accessible to switch-recombinase machinery (Yancopoulos *et al.*, 1986b).

The repetitive portions of the S region substrate were used in these pre-B cell specific recombinations. The absence of recombination in the 3′ half of the $S_{\gamma 2b}$ segment, which lacks the charactristic 49-mer $S_{\gamma 2b}$ repeats demonstrates the importance of these motifs for the switch-recombination process in pre-B cell lines. The construct utilizes the highly repetitive $(GAGCT)_nGGGGT$ portion of the S_μ region as opposed to the adjacent 5′ YAGGTTG region for most of these recombination events. This observation suggests that the S_μ YAGGTTG region is not an obligatory requirement for S region recombination in pre-B cells. However, these results do not exclude the involvement of YAGGTTG-like sequences in switch-recombination since this region's length in the S_μ segment within the viral vector is only 24% of its normal size in the mouse genome. It is conceivable that the presence of Sμ YAGGTTG-like sequences near C_H switch-recombination sites in murine plasma cells tumors resulted from secondary deletions which removed all the remaining S_μ tandem repeats (Szurek *et al.*, 1985). The loss of S_μ tandem repeats could prevent additional switch events thereby stabilizing isotype expression in terminally differentiated B cells.

Materials and methods

Cell lines, retroviral infections and marker selections

18-8tk$^-$ and 38B9tk$^-$ pre-B lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μM mercaptoethanol and Ltk$^-$ cells were grown in DME with 10% calf serum. Recombinant retroviruses were produced by the ψ2 helper-free packaging cell line (Mann *et al.*, 1983) as previously described (Cepko *et al.*, 1984) except for Ltk$^-$ which required the amphotypic capsid provided by PA12 (Miller *et al.*, 1985). Retroviral infections were carried out in DME supplemented with 20% calf serum and 8 μg/ml polybrene (Sigma) for 2−3 h with either 2×10^6 cells for the pre-B lines or a 30% confluent plate for Ltk$^-$ (Cepko *et al.*, 1984). After 48 h, media were supplemented with G-418 at 2 mg/ml for pre-B cells and 400 μg/ml for Ltk$^-$. After G-418 resistant clones were obtained, thymidine kinase negative cells were selected by growth in media with BUdR (10^{-4} M and 10^{-2} M for pre-B and Ltk$^-$ cells, respectively) in conjunction wtih G-418 to ensure the retention of a functional neomycin resistance gene.

Construction of the $_pZN(S_\mu/S_{\gamma 2b})$tk1 retroviral vector

A 1.15-kb *Hin*dIII fragment, containing a portion of the BALB/c S_μ region (Marcu *et al.*, 1980) (see Figure 1), was inserted into the unique *Bam*HI site of the $_p$ZipNeoSV(X)1 retroviral vector which was kindly provided by C.Cepko and R.Mulligan (Cepko *et al.*, 1984) by employing *Bam*HI linkers. A 2.0-kb *Pvu*II fragment, containing the herpes simplex virus thymidine kinase (Htk) gene and its promoter, was converted into a *Bam*HI fragment and inserted 3′ of the above S_μ segment by partial *Bam*HI cleavage. A retroviral construct with an Htk gene inserted in opposite trancriptional orientation relative to the LTR promoter was selected for the next step to avoid the possibility of promoter interference (Emerman and Temin, 1984a,b) which could reduce the amount of full length retrovirus transcripts and thereby diminish the virus titer produced by transfected ψ2 and PA12 cells. Finally, a 1.25-kb portion of $S_{\gamma 2b}$ sequences derived from a BALB/c germ line $S_{\gamma 2b}$ subclone (Lang *et al.*, 1982) (Figure 1) was inserted downstream of the S_μ segment after the Htk gene by employing *Bam*HI linkers and a second cycle of partial *Bam*HI digestion to generate $_pZN(S_\mu/S_{\gamma 2b})$tk1.

Frequency analysis for loss of HTK phenotypes

Cells containing a *neo* expressing virus were selected with G-418 and separate mixtures of 25−100 G-418 resistant clones were expanded to a population size of 2×10^7 cells and then maintained in non-selective medium for ~1 month. In separate experiments, seven 18-8tk$^-$ and thirteen 38B9tk$^-$ independent populations of G-418 resistant cells were prepared in this manner. For each population, dilutions of known numbers of cells were plated into 96-well microtiter plates containing G-418 and BudR. After incubation for 7−14 days, the numbers of positive wells were counted at each dilution. Plates with no more than 37% positive wells were considered to be clonal and the number of wells were multiplied by the dilution factor to yield the number of G-418/BUdR resistant cells in the population. Cloning efficiencies of the 18-8tk$^-$ and 38B9tk$^-$ lines were 100%. The numbers of resistant cells were analyzed by fluctuation test (Luria and Delbruck, 1943) in order to determine the frequency of loss of the HTK phenotype (see Table I legend). The analogous frequency analysis for infected Ltk$^-$ cells was performed by maintaining each of six independently derived populations containing 5×10^6 G-418 resistant cells for ~1 month in non-selective medium. Cells (5×10^6) from each of these six populations were subsequently plated into a 150-mm dish in media supplemented with G-418 and BUdR. Resistant colonies were counted after 10−20 days in selective media.

Molecular analysis

Southern blotting was carried out by standard techniques (Southern, 1975; Marcu *et al.*, 1983) using nylon membranes (Schleicher and Schuell). Probes were either subcloned into M13 mp12 and labeled with hybridization probe primer (BRL) or restriction fragments were purified by agarose gel electrophoresis and nick translated as described (Rigby *et al.*, 1977; Hu and Messing, 1982).

Immunofluorescence

The expression of endogenous μ and γ_{2b} heavy chains was examined in 38B9tk$^-$ and 18-8tk$^-$ cells by immunofluorescence. Cells were fixed on polylysine-coated microscope slides and reacted with either fluoresceine (FITC) conjugated goat anti-mouse antibody or tetramethyl rhodamine (TRITC) conjugated goat anti-mouse μ or γ_{2b} antibody (Southern Biotechnology) as described (Stavnezer *et al.*, 1985). The antibody-stained cells were examined under a Lietz fluorescence microscope.

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