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Genetics

Looping out and deletion mechanism for the immunoglobulin heavy-chain class switch

(inversion/recombination between homologs/unequal sister chromatid exchange/pre-B-cell line)

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ABSTRACT **In the mouse pre-B-cell line 18-81, cells can switch production *in vitro* from immunoglobulin μ chain to γ2b chain. The gene encoding the γ2b chain is created by a rearrangement of the μ gene. This rearrangement always takes place within a homolog. In cells with a γ2b gene, most of the time the gene segment encoding the constant region of the μ chain is deleted, but often the rearrangement leads to cells that produce no immunoglobulin, and all DNA sequences are retained. The latter result is due to an inversion. Inversions exclude the unequal sister chromatid exchange model of the heavy-chain class switch. Looping out is an intermediate step in the process of generating an inversion. Our findings demonstrate that the switch rearrangement occurs by looping out and deletion.**

When injected with an antigen, higher vertebrates can respond by producing antibodies that are immunoglobulins of various classes. These classes differ in the heavy (H) chain that combines with the light chain to form the complete immunoglobulin molecule. In the mouse, the early immune response is dominated by IgM, which contains the H chain μ, and the later immune response is dominated by IgG3, IgG1, IgG2b, IgG2a, IgA, and (rarely) IgE, which contain the H chains γ3, γ1, γ2b, γ2a, α, and ε, respectively. At the genetic level, the gene encoding the μ chain consists of a variable (V)-region gene segment, which has been somatically assembled from one each of the V_H, diversity (D), and joining (J_H) segments, and the nearby C_μ gene segment, which encodes the constant (C) region of the μ chain. Upon H-chain switching, which occurs within a clone derived from a committed lymphocyte (1, 2), the V region remains the same, but the C region changes. C_μ is replaced by $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$, C_ε, or C_α, which are the C-region gene segments of the respective chains and are closely linked to C_μ in that order (reviewed in refs. 3 and 4).

This rearrangement event generally results in deletion of the DNA sequences between a site within the intron 5′ to C_μ and a site within the intron 5′ to the particular C-region gene segment that is to be expressed. The sites of rearrangement (breakpoints) often fall within regions of repetitive sequences, the switch (S) regions, but sometimes they do not (reviewed in ref. 5). *A priori*, there are three types of genetic events that could cause the deletion: (i) recombination within a chromatid, (ii) unequal sister chromatid exchange, and (iii) unequal recombination between homologs. Both unequal sister chromatid exchange and unequal recombination between homologs would yield, along with cells having deletions of DNA sequences including C_μ, cells that contain three copies of C_μ. We previously tested this prediction in the Abelson virus-transformed pre-B-cell line 18-81, in which switching from the μ to the γ2b chain continually occurs (6, 7). We found no cell with three C_μ segments in its genome and concluded that the switch rearrangement takes place within a chromatid (8).

In the mouse pre-B-cell line 18-81, clones can be selected that contain two functional μ alleles at the H-chain locus (9). From those, clones can be selected that have spontaneously switched from the μ gene to a γ2b gene on one allele (i.e., one $C_{\gamma 2b}$ allele is in a rearranged configuration), whereas the other $C_{\gamma 2b}$ allele remains in the germ-line configuration. Because the two $C_{\gamma 2b}$ alleles can be distinguished by Southern blot analysis, the analysis of a second switch from μ to γ2b, on the other allele, is simplified. We report here a molecular genetic analysis of such cells.

MATERIALS AND METHODS

Cell Culture and Subcloning. Cells were grown in RPMI medium as previously described (13).

Southern Blot Analysis. Southern blot analysis was done as described (9). The μ cDNA probes were provided by F. Blattner, the γ2b cDNA probe was provided by P. Tucker, and the 5′-$S_{\gamma 2b}$ probe and J12 probe were from K. Marcu. Sites of switch rearrangement were determined by restriction enzyme mapping with *Sac* I, *Bcl* I, *Bam*HI, *Eco*RI, and *Hin*dIII.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Cells were cultured in RPMI medium containing [^{35}S]methionine (100 μCi/ml; specific activity of 800 Ci/mmol; 1 Ci = 37 GBq; Amersham). Immunoglobulin was immunoprecipitated from cell lysates, reduced, and analyzed on 10% NaDodSO$_4$/polyacrylamide gels as described (6).

DNA Sequencing. DNA from clone 56 was isolated and digested with the restriction enzymes *Sst* I and *Hin*dIII. A 1.4-kilobase (kb) piece hybridizing with the μ cDNA probe was directly inserted into phage M13mp18 and sequenced as described by Sanger *et al.*(10)

RESULTS AND DISCUSSION

Genealogy of 18-81 Clones. The genealogy of the clones studied is given in Fig. 1. We started with A3, a subclone of 18-81 (7). Both alleles at the H-chain locus have correctly assembled genes for μ-chain production. Allele *V3* utilizes the J_{H3} gene segment; *V2* utilizes J_{H2}. In the A3 subclone, *V3* directs the synthesis of full-size μ chain, but *V2* does not, because its V-region gene segment contains a termination codon (11). In all other clones named in Fig. 1, *V3* encodes the γ2b chain, and the C_μ gene segment has been deleted. From these switch variants, we selected mutant clones in which the termination codon in the V-region gene segment of the *V2* allele had reverted to a sense codon (12). These revertants produced full-size μ chain encoded by *V2*. From the revertants, or subclones thereof, we selected clones that did not produce μ chain. Many of the clones so derived are

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Abbreviations: H, heavy; V, variable; D, diversity; J, joining; C, constant; S, switch.

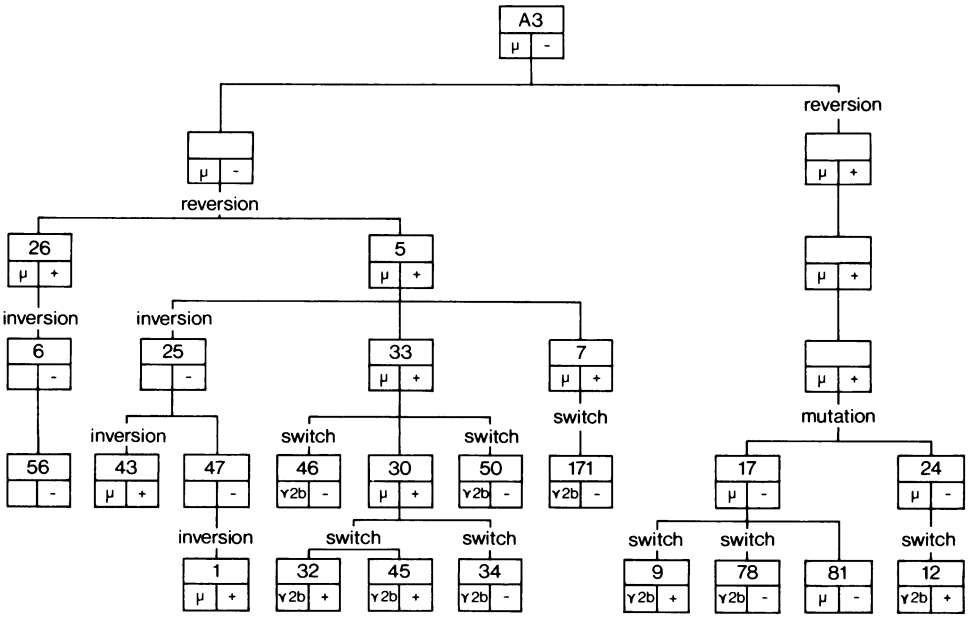

FIG. 1. Genealogy of the 18-81 subclones described in this report. The left box under each clone designation denotes the gene present in the *V2* allele; the right box indicates production of full-size H chain. Switch and inversion denote independent rearrangement events in which sequences 5′ to $C_{\gamma 2b}$ participate. Reversion indicates a reversion of the termination codon in the V-region gene segment. Mutation indicates introduction of a termination codon in the second exon of C_{μ}.

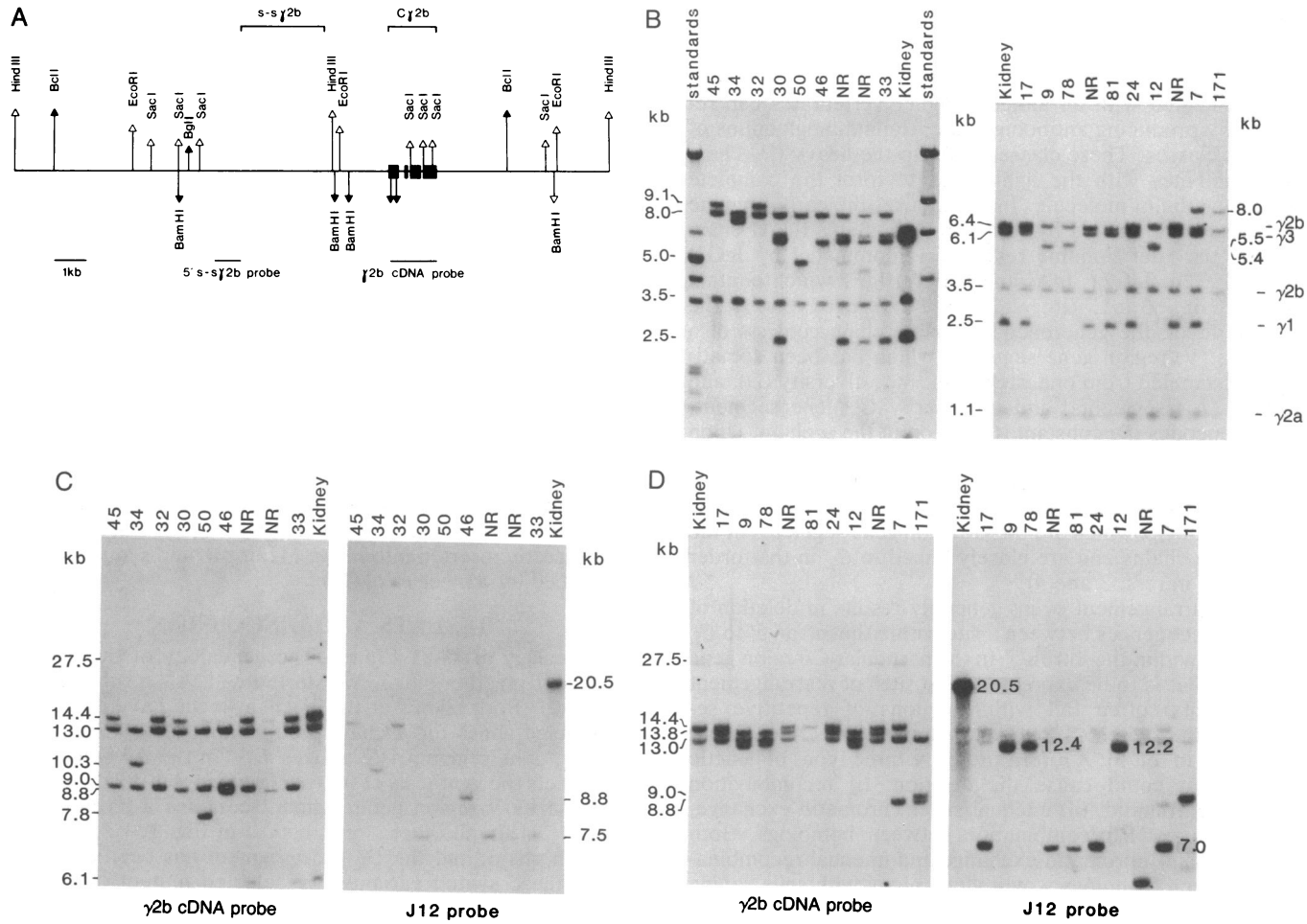

FIG. 2. (*A*) Restriction map of the γ2b locus and probes used for Southern blot analysis. The location of the sites is taken from references 14–16, except for the *Bcl* I site, which was mapped in this study. The 5′-$S_{\gamma 2b}$ probe (5′S-Sγ2b) is a 0.8-kb *Eco*RI–*Bgl* I fragment (17, 18); the γ2b cDNA probe is a *Kpn* I–*Bam*HI fragment (19). Filled rectangles indicate the exons of $C_{\gamma 2b}$. (*B*) Southern blot analysis of *Sac* I-digested DNA from various A3 subclones. Hybridization is with the γ2b cDNA probe. Kidney DNA represents the embryonic configuration. NR, DNA from clones not relevant to this study. Assignment of the bands for the various immunoglobulin subclasses is from ref. 20. Standards are fragments of the phage λ digested by *Eco*RI and *Hin*dIII (*Left*) or by *Hin*dIII only (*Right*). (*C* and *D*) Southern blot analysis of *Bcl* I-digested DNA from various A3 subclones. Hybridization is with the γ2b cDNA probe (*Left*) and the J12 probe containing J_{H1} and J_{H2} (21) (*Right*).

normal switch variants with a γ2b gene on the *V2* allele. Although the *V3* allele also directs γ2b synthesis, *V3*-encoded γ2b can be distinguished from γ2b encoded by the *V2* allele because the *V2*-encoded γ2b is more highly expressed and migrates differently in electrophoretic gels. But often, clones failed to produce μ chain for other reasons.

As will be demonstrated below, clones 56 and 25 each contain an inversion involving the $S_{\gamma 2b}$ region. In clones 46, 50, 171, 34, and 78, the *V2* allele contains a γ2b gene in which both the V-region and $C_{\gamma 2b}$ gene segments are of correct size; we do not yet know why these clones fail to produce a γ2b chain.

Clones 17 and 24 fail to produce full-size μ chain because the μ gene contains a termination codon within the second exon of C_μ (13). In clones 9 and 12, recombining the V-region gene segment with the $C_{\gamma 2b}$ gene segment allows expression of full-size γ2b chain encoded by *V2*.

In Fig. 1, independent rearrangement events are denoted switch or inversion. Independence of events is obvious for the clones that have different mother clones containing the μ gene (e.g., clones 50 and 34). When the breakpoints differ, we also infer that the γ2b genes were probably derived from independent rearrangement events (e.g., in clones 46 and 50). It is possible that these clones might have a common mother cell that diversified after the switch to a γ2b gene, in which case our assumption of independence would be wrong for 3 of the 12 cases studied. Because their breakpoints are identical, clones 32 and 45 are thought to be derived from a common mother cell that had switched to γ2b.

The Switch Rearrangement Occurs Within a Homolog. In clones 26, 5, 17, and 24, the *V3* allele contains a functional γ2b gene; hence, the $C_{\gamma 2b}$ gene segments should be in a rearranged configuration. In Southern blot analysis of DNA in embryonic configuration digested with *Sac* I, five bands hybridized with a γ2b cDNA probe (Fig. 2*A* and *B*). The 6.4-kb and 3.5-kb fragments contain part of the $C_{\gamma 2b}$ gene segment. The γ2b cDNA probe also hybridized with the sequences of $C_{\gamma 3}$, located on the 6.1-kb band, with sequences of $C_{\gamma 1}$, located on the 2.5-kb band, and with sequences of $C_{\gamma 2a}$, located on the 1.1-kb band. In clones 33 and 7 (and clones 26 and 5, not shown), the additional 8.0-kb band represents the rearranged $C_{\gamma 2b}$ gene segment on the *V3* allele. In clones 17 and 24, the switch rearrangement occurred 5′ to the *Sac* I and *Bgl* I sites (Fig. 2*A*); this explains why the fragments containing the $C_{\gamma 2b}$ sequences are in the germ-line configuration (Fig. 2*B*).

When the μ gene of the *V2* allele undergoes switch rearrangement to generate a γ2b gene, either the $C_{\gamma 2b}$ gene segment of the *V2* allele, in the germ-line configuration, or the $C_{\gamma 2b}$ gene segment of the previously rearranged *V3* allele could, in principle, be used. Fig. 2*B* shows that in clones 45, 32, 50, 46, and 171, the 8.0-kb fragment of the previously rearranged $C_{\gamma 2b}$ gene segment remains unchanged and that the 6.4-kb germ-line band is lost to yield new bands of various sizes. Therefore, we conclude that the switch rearrangement occurs within a homolog. This is confirmed by the fact that in clones 45, 32, 50, 46, 9, 78, 12, and 171, there is not a copy of the $C_{\gamma 3}$ and $C_{\gamma 1}$ gene segments (Fig. 2*B*) and also not a copy of the C_μ and C_δ gene segments (not shown). Southern blots of DNA digested with *Bcl* I and probed with γ2b cDNA lead us to the same conclusion. In Fig. 2*C* and *D*, kidney DNA of embryonic configuration shows two strong bands of 14.4 kb and 13.0 kb. The 14.4-kb fragment contains $C_{\gamma 2b}$, and the 13.0-kb fragment contains $C_{\gamma 2a}$. The weaker bands at 27.5 kb and 6.1 kb contain $C_{\gamma 3}$ and/or $C_{\gamma 1}$. The 9.0-kb band of clone 33 represents the γ2b gene of the *V3* allele; it remains unchanged when the *V2* allele switches to γ2b. The same is found in clones 17 and 24 for the 13.8-kb band, which represents the γ2b gene of the *V3* allele; it is unchanged in subclones 9, 78, and 12. When we rehybridized the Southern blots with the J12 probe, containing J_{H1} and J_{H2}, the bands containing the $C_{\gamma 2b}$ gene segment of the *V2* allele were stained (Fig. 2*C* and *D*), demonstrating that the

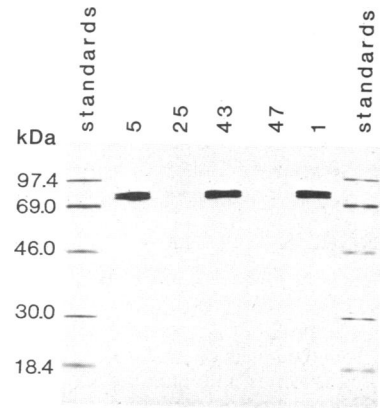

FIG. 3. Fluorograph obtained after $NaDodSO_4$/polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled μ chains produced by various cell clones.

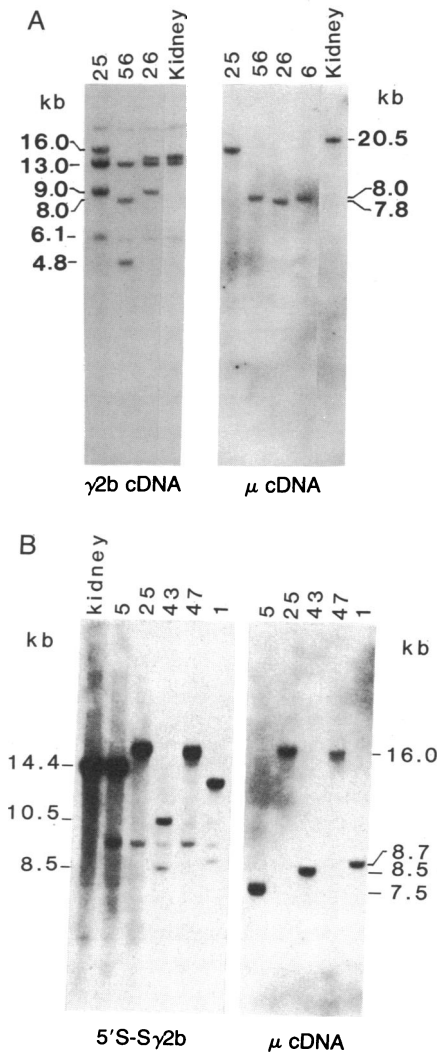

FIG. 4. Southern blot analysis of *Bcl* I-digested DNA from various A3 subclones. Hybridization is with γ2b cDNA and μ cDNA probes (*A*) or 5′-$S_{\gamma 2b}$ (5′S-Sγ2b) and μ cDNA probes (*B*). The μ cDNA probe was prepared from the *Pst* I-digested plasmid pMKμ-1 made by M. Knapp.

V-region gene segment has been brought into close proximity to the $C_{\gamma 2b}$ gene segment of the *V2* allele.

It could be argued that the *V3* allele had already used up some of the S-region sequences at the first switching event and that therefore the *V2* allele would be prone to use its own pristine $S_{\gamma 2b}$ region. This is not the case for the γ2b-producing subclones of clones 17 and 24, because in these clones the switch rearrangement on the *V3* allele took place upstream of the $S_{\gamma 2b}$ region 5′ to the *Bgl* I site.

Inversions. From the μ-producing clones 5 and 26, we obtained subclones 25 (Fig. 3) and 56, both of which failed to produce μ chain yet did not contain a complete γ2b gene on the *V2* allele. In Southern blot analysis of *Bcl* I-digested DNA, an 8.0-kb fragment of clone 56 hybridized to both μ cDNA and γ2b cDNA (Fig. 4*A*); a 16.0-kb fragment of clone 25 hybridized, in addition, to the 5′-$S_{\gamma 2b}$ probe (Fig. 4*B*). From this we inferred that an inversion had occurred in clones 25 and 56. Mapping with other restriction enzymes (Fig. 5*A* and *B*) and sequencing around one of the two breakpoints (Fig. 5*C*) confirmed this inference. Fig. 5*D* shows the breakpoints for clones 25 and 56 within the μ gene. It was verified by Southern blot analysis that the $C_{\gamma 1}$, $C_{\gamma 2a}$, and $C_{\gamma 3}$ as well as C_{μ} and $C_{\gamma 2b}$ gene segments are present in the inversion clones.

From clone 25 and its subclone 47, we isolated the μ-producing clones 43 and 1 (Fig. 3). The *V2* allele of these clones has undergone another inversion (Fig. 4*B*) to reform a complete μ gene.

Clones 25 and 56 were selected for their loss of μ-chain production. Because rearrangement at the *V2* allele involved the γ2b locus in both cases, we believe that this recombination event is a result of the enzymatic activity of the usual switch recombinase that generates predominantly γ2b genes in the cell line 18-81 (6). Inversions exclude both unequal recombination between homologs and the unequal sister chromatid exchange model of the H-chain class switch, leaving as an explanation recombination within a chromatid by looping out and deletion.

The Looping Out and Deletion Model. The loop in the DNA presumably forms by attachment of a component of the switch recombinase system to an unknown site that it recognizes somewhere in the H-chain locus. After diffusion of the DNA strand, we arrive at the state diagrammed in Fig. 6. The enzyme cuts the DNA at two places, which leaves four ends. These ends may be rejoined in three different ways. One way restores the original configuration. Another way leads to a deletion; the deleted sequences form a circle, which is either lost or replicates as an episome. The third way leads to an inversion with no sequences lost. The DNA arrangement of all our switch variants can be explained by the looping out and deletion model.

It has been argued that the repetitive sequences of the S regions facilitate homologous recombination (22) or focus the putative switch recombinase (23) or both. Although there is an intriguing pseudo-mirror symmetry at the inversion breakpoint we sequenced (pseudo because the 5′ → 3′

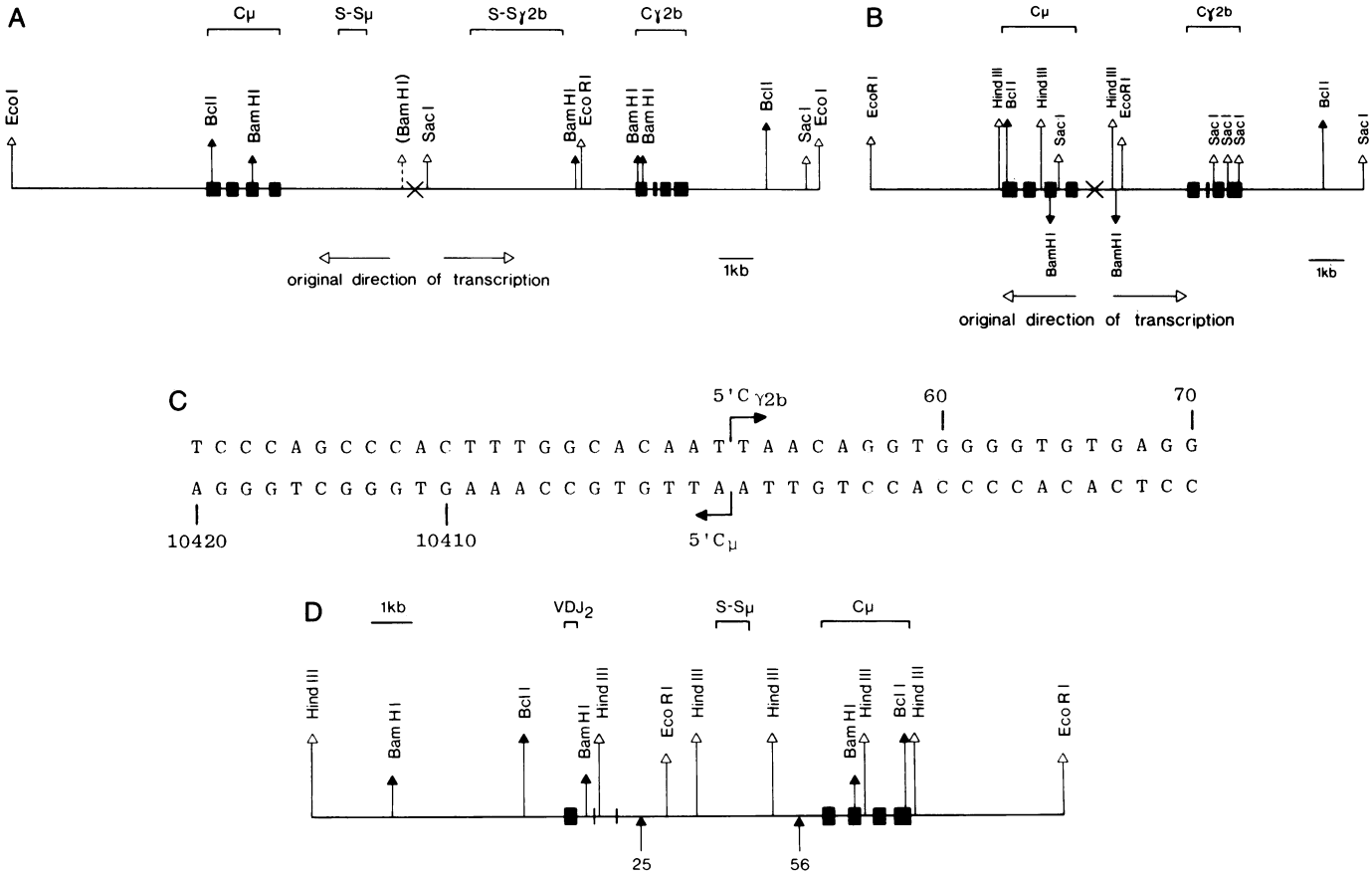

FIG. 5. Restriction map of the region around one of the two breakpoints (X) resulting from the inversion in clone 25 (*A*) and clone 56 (*B*). For clarity, some restriction sites are omitted. In *A* the *Bam*HI site indicated in parentheses, which is present at the γ2b locus in embryonic configuration, is deleted. EcoI, *Eco*RI. (*C*) Partial sequence of the 1.4-kb *Sac* I–*Hin*dIII DNA fragment from clone 56 containing the breakpoint (diagrammed in *B*). The numbering of base pairs 10,410 and 10,420 in the sequence 5′ to C_{μ} is according to P. Tucker (personal communication); the numbering of base pairs 60 and 70 in the sequence 5′ to $C_{\gamma 2b}$ is according to GenBank (MUSIGDJC126) (16). (*D*) Restriction map of the μ locus on the *V2* allele. There is a 3.2-kb deletion in the major intron between J_H and C_{μ}, which leaves 1.5 kb for the S_{μ} (S-Sμ) region. The numbers 25 and 56 denote the breakpoints for clones 25 and 56, respectively. The filled rectangles indicate exons.

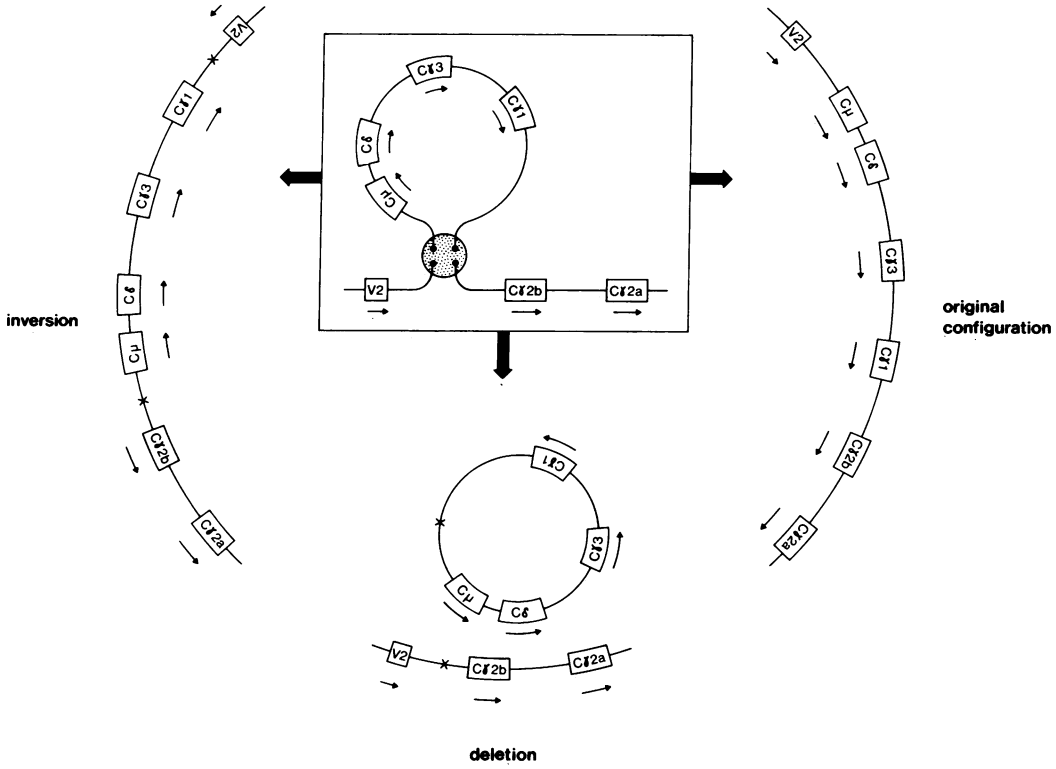

FIG. 6. Schematic representation of the H-chain locus showing looping out and deletion and the resulting recombination products. Thin arrows underneath the exons show the original direction of transcription. In the center, the putative switch recombinase, represented by the hatched circle, is bound to the four loose DNA ends that have been produced previously. These four ends can be combined in three different ways to yield the three results shown at the periphery.

direction is not reflected), there is no homology between the sequences that have recombined.

The γ2b Preference of 18-81. An intriguing aspect of H-chain class switching in the 18-81 cell line is the fact that the cells consistently switch to the γ2b gene (6, 7). Using Southern blot analysis, we found that there is only one copy of the Abelson virus present in our line, and this copy is not integrated at the H-chain locus (unpublished observations). Because the switch leads to a γ2b gene on *both* homologs, the integration site of the virus cannot be directly responsible for the switch to the γ2b gene. Therefore, we think that there may be a recombinase that promotes switching to a particular H-chain C-region gene segment (23). If this is due to the virus, then the Abelson virus phosphokinase would have to modify the putative switch recombinase in such a way that it is specific for the switch to γ2b.

If a loop, like that shown in Fig. 6, forms between the $S_{\gamma 2b}$ region and sites other than those 5′ to C_μ, then the μ gene would be retained while the $C_{\gamma 2b}$ segment might be lost. In fact, we have found three clones with this genotype, in addition to several clones that have rearranged their γ2b locus in the absence of C_μ loss (unpublished observation).

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