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Immunology

Allelic inclusion in the pre-B-cell line 18-81

(pre-B-cell hybridoma/Southern blot analysis)

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ABSTRACT **In an Abelson-virus-transformed mouse lymphoid cell line with pre-B-cell characteristics, a few cells continuously produce heavy chains from both homologs. Each chain has a different variable region. These cells thereby exhibit allelic inclusion rather than allelic exclusion.**

The monospecificity of B lymphocytes necessitates allelic exclusion. Allelic exclusion describes the fact that at each of the loci encoding immunoglobulin (Ig), only one of the two alleles is functional in a B lymphocyte (1, 2). Pre-B cells do not necessarily have to observe allelic exclusion, because heavy (H) chain is present only in the cytoplasm (3) and is not yet displayed on the membrane to the antigenic universe.

We have previously shown that the 18-81 cell line is a pre-B-cell line; it contains cells synthesizing only H chain. No light (L) chain is produced (4), because the *L* loci are still in the embryonic configuration (5), as in pre-B-cell hybridomas (6). The cells of this line generally show allelic exclusion for the *H* locus. They can switch from μ to γ2b chain synthesis *in vitro* (5). Both μ and γ2b chains have the same variable region (V) and are encoded by the same chromosomal homolog. The switch is accompanied by loss of DNA sequences between the joining region (J_H) and γ2b constant region ($C_\gamma 2b$) gene segments including the C_μ gene segment (5). We have observed, however, that the subclones that we have classified to be γ2b producers usually contained $\approx$0.5% cells that also produce μ chain. These cells might use the usually silent C_μ allele for μ-chain production and therefore would exhibit allelic inclusion rather than exclusion.

MATERIALS AND METHODS

Subcloning. Cell line 18-81 was subcloned in soft agar or by limiting dilution (0.15 cell per well) with syngeneic or xenogeneic (Wistar rat) peritoneal exudate cells as feeder cells or by micromanipulation.

Immunofluorescence. The purification and fluorochrome conjugation of goat antibodies specific for mouse H chain isotypes and methods for immunofluorescent detection of intracellular Ig have been described (7).

Cytogenetic Analysis. Metaphase chromosomes were prepared and banded with Giemsa/trypsin stain by standard methods (8, 9).

Cell Fusions. Fusions between 18-81 subclones and the myeloma Ag8653 were carried out as described (4).

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Hybridoma cells were cultured in the presence of [^{35}S]methionine. Ig was immunoprecipitated from cell lysates, reduced, and analyzed on 10% NaDodSO$_4$/polyacrylamide gels as described (4)

Southern Blot Analysis. High molecular weight DNA was isolated (10) from the various cell lines, digested to completion with the different restriction enzymes (from Bethesda Research Laboratories and New England BioLabs), electrophoresed in 0.8% (or 1.5% for *Hin*dIII digests) agarose, and transferred to nitrocellulose filters (11). The filters were hybridized with the indicated probes that had been labeled with [^{32}P]dCTP by nick-translation. (For restriction enzyme cleavage maps and DNA probes, see ref. 5.) The plasmid containing J_1 and J_2 (pJ_0) was kindly provided by K. Marcu. The other plasmids were generously provided by F. Blattner.

RESULTS

Cells Producing μ and γ2b Chains Simultaneously. We succeeded in isolating a cell clone that produces both μ and γ2b chain (Fig. 1); in subclone A33-1 (and its subclone A33-11), 99% of the cells are μ-chain positive and 50% of these also produce γ2b, as shown by immunofluorescence. Long-lived messenger RNA for μ or γ2b can be ruled out as an explanation for double production in the clone A33-1 and its subclone A33-11, because we found double-producing cells for several generations: 40% of cells were positive for both μ and γ2b when the clone had expanded from one cell to 5×10^8 cells. These clones have the same karyotype as the mother clone 18-81, which has a diploid complement of chromosome 12 (4) that carries the *H* locus (12, 13). Therefore, fusion of a μ-chain-producing cell with a γ2b-chain-producing cell cannot account for the existence of the double producers. The *L* loci were in embryonic configuration (data not shown). When fused with the myeloma Ag8653, 77 out of 95 hybridomas of the A33-11 clone (95% μ and 5% γ2b at the time of fusion) stably produced both μ and γ2b chains (Fig. 2), 8 produced μ chain only, 7 produced γ chain only, and 3 produced no H chain.

The Double Producer Has Lost a C_μ Gene Segment That Was Previously Expressed. In the 18-81 cell line, the expressed C_μ gene segment is lost on switching to γ2b production. If this has happened in clone A33-1, then this double producer must use the usually silent allele (on the other homolog) for μ production.

Subclone A33-1 is derived from clone A33, a μ-chain producer. Clone A33 contains two copies of a C_μ gene segment, as revealed by Southern blot analysis. The enzyme *Bam*HI cuts within the C_μ segment, so that two bands [9.4 and 11.2 kilobases (kb)] are found in embryonic DNA when hybridized with a μ cDNA probe (Fig. 3*a*). As the enzyme also cuts between J_2 and J_3, any rearrangement to form an active transcriptional VDJ unit with J_3 or J_4 or any deletion 3′ to this restriction site will change the size of the 9.4-kb embryonic fragment. In clone A33, the two *J*-C_μ alleles are found on 5.1- and 3.5-kb restriction fragments. On switching to γ2b (clone A33-1) the 3.5-kb restriction fragment is lost. This band represents the active allele in the 18-81 cell line (5) and is also lost when other 18-81 cells switch to γ2b production. This is confirmed by the fact that there is no C_μ gene seg-

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Abbreviations: Ig, immunoglobulin; H and L, heavy and light chain, respectively, of Ig; V, J, and C, variable, joining, and constant regions, respectively, of Ig chain; kb, kilobase(s).

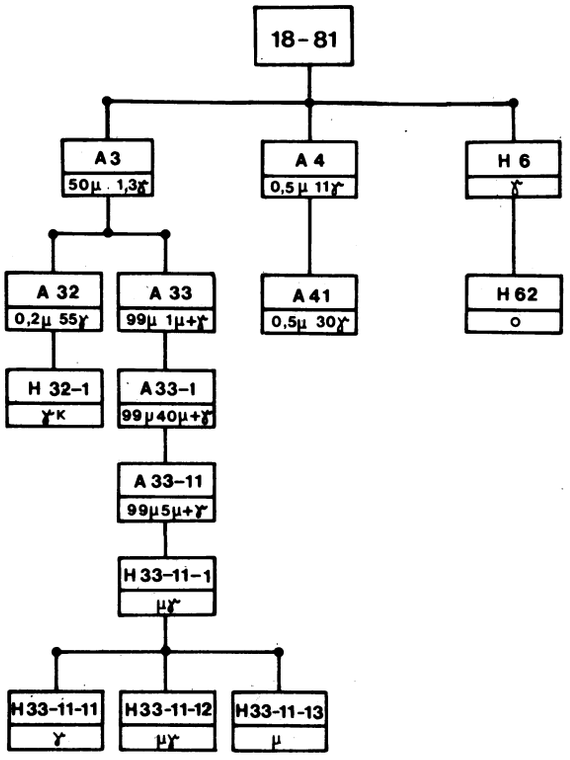

FIG. 1. Genealogy of selected subclones and hybridomas derived from the Abelson-virus-transformed line 18-81. Abelson-virus-transformed cells are designated "A," and their hybridomas are designated "H." The percentages of μ and γ2b cells in the "A" lines are listed below the subclone designation.

ment (Fig. 3*b*) in the γ chain only producing hybridoma H-33-11-11.

The Double Producer Exhibits Allelic Inclusion Rather than Exclusion. In the 18-81 cell line, only one *V* allele is usually used for μ and γ2b expression (5, 16). We have also identified this allele in the γ2b-producing hybridoma H6. This hybridoma has two *Eco*RI restriction fragments, 1.8 and 2.4 kb, that hybridize with J34 probe and do not come from the myeloma (Fig. 4, lane 2). Only the 2.4-kb band also hybridizes with a probe containing J_1 and J_2 (unpublished data). A subclone of H6, H62, no longer makes γ2b and has lost the 1.8-kb band (lane 3). Therefore, the 2.4-kb *Eco*RI fragment must represent the silent allele.

Restriction enzyme digestion of DNA of the double-producing hybridomas and Southern blot analysis with the relevant DNA probes showed that in these cells μ and γ2b chains are encoded by different homologs. The hybridoma H33-11-1 produces both μ and γ2b chains within a single cell. Subclones of this hybridoma were of three types: μ and γ2b double-producer H33-11-12, μ only producer H33-11-13, and γ2b only producer H33-11-11. Digestion of DNA from H33-11-12 with *Eco*RI and hybridization with J34 probe gave three bands, 6.4, 6.2, and 2.4 kb (Fig. 5*a*, lane 4). The 6.4-kb band is contributed by the myeloma Ag8653 (lane 1). In the $\gamma^+ \mu^-$ hybridoma H33-11-11 the 2.4-kb band is missing (lane 3), as is the only C_μ gene segment found in H33-11-12 (Fig. 3*b*). (Because of deletion of the *Eco*RI site 3′ to the J region, the size of the *Eco*RI fragment hybridizing with J34 probe in H33-11-11 is different from the analogous fragment in H6.) In the $\gamma^- \mu^+$ hybridoma H33-11-13 the 6.2-kb band is missing (Fig. 5*a*, lane 2). Therefore, this hybridoma must use the allele represented by the 2.4-kb *Eco*RI fragment for μ-chain production. (This allele is silent in the hybridoma H6.) That is, the H33-11-1 hybridomas use different VDJ arrangements for μ and γ2b, thus using both of the homologs of chromo-

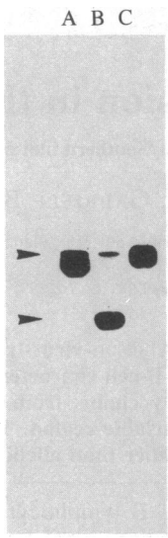

FIG. 2. $NaDodSO_4$ gel electrophoresis of immunoglobulin chains produced by hybridomas made with subclones of the 18-81 cell line. Hybridomas presented: H13, a μ-chain producer, cell lysate precipitated with anti-μ (lane A), and H33-11-2, which produces μ and γ2b chains simultaneously, cell lysates precipitated sequentially with anti-γ2b (lane B), then with anti-μ (lane C). The distances migrated by intracellular μ and γ2b chains from spleen-cell-derived hybridomas are indicated by the upper and lower arrows, respectively. The μ chain produced by H13 (lane A) is slightly smaller than normal. Similar size heterogeneity, apparently independent of glycosylation differences, has also been found in μ chains produced by hybridomas made with pre-B cells in fetal liver (3, 14). H33-11-2 produces a normal-sized μ chain. A protein with the same mobility as μ chain is shown in lanes A and B. We have previously described this protein and provided evidence that it is not μ chain (4). In recent studies this protein was found to bind to Ig H chains (15).

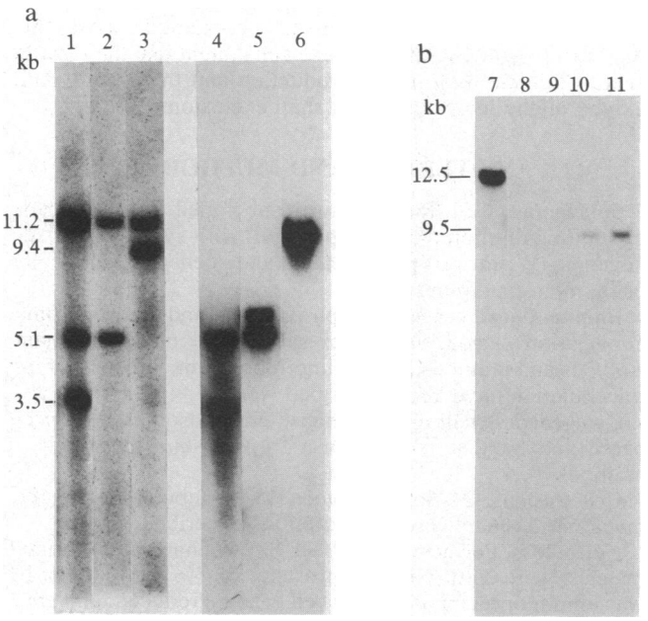

FIG. 3. Southern blot analysis of the double-producing clone A33-1 and its hybridomas, showing the loss of one active C_μ allele. (*a*) *Bam*HI digests hybridized with C_μ (lanes 1–3) or J34 (lanes 4–6) probe. (*b*) *Eco*RI digests hybridized with C_μ probe. For restriction enzyme cleavage map, refer to ref. 5. Lanes: 1 and 4, clone A33 (μ-chain producer); 2 and 5, subclone A33-1 (μ- and γ-chain producer); 3, 6, and 7, kidney; 8, myeloma Ag8653; 9, hybridoma H33-11-11 (γ-chain producer); 10, hybridoma H33-11-12 (μ- and γ-chain producer); 11, hybridoma H33-11-13 (μ-chain producer).

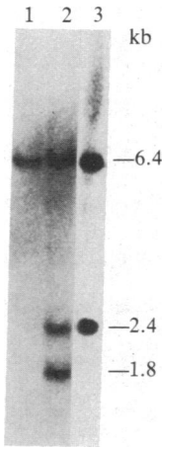

FIG. 4. Southern blot analysis for identification of the usually silent allele in the 18-81 cell line. *Eco*RI digests hybridized with the *Xba* I–*Eco*RI fragment of J34 probe (lanes 1 and 2) or with J34 probe (lane 3). Lanes: 1, myeloma Ag8653; 2, hybridoma H6 (γ-chain producer); 3, hybridoma H62 (produces no H chain).

some 12 that are present in 18-81. Fig. 5 shows an analysis with the enzyme *Xba* I, *Hin*dIII, and with *Bst*EII, all leading to the same conclusion. This conclusion is confirmed by our finding that (*i*) in the γ^+ μ^+ hybridoma H33-11-12, the gene for the γ2b chain uses the J_3 segment, whereas the gene for the μ chain uses the J_2 segment, as revealed by sequential hybridization with J12 and J34 probe (data not shown). DNA sequences of the rearranged alleles indicated that J_2 and J_3 are contained in the *V* alleles in 18-81 (16). (*ii*) The μ chain from the γ^+ μ^+ double producer migrates in $NaDodSO_4$/polyacrylamide gel electrophoresis differently from the μ chain that is usually expressed in 18-81. (*iii*) In some γ^+ μ^+ double producers that also express κ chain the L chain combines exclusively with the γ chain, suggesting that the V regions are different (unpublished results).

Frequency of Allelic Inclusion. Since we were able to clone out a μ and γ2b double-producing cell line, we interpret the frequency of γ2b cells also producing μ chain as the frequency of allelic inclusion (breakage of allelic exclusion). In cell clones A32 and A4 that have mainly γ2b cells and have deleted one C_μ allele, the frequency of μ-chain-producing cells is $\approx$0.5%, a frequency that was maintained on subcloning the γ2b cells. This fits well with the general frequency of double producers, which is also 0.5% of the γ2b-producing cells in a clone with predominantly μ cells. In cells that produce only μ, production of μ chains encoded by both homologs will not be immediately evident. However, we assume that the majority of cells in clone A33 represents such a situation, because all of the cells that have switched to γ2b production also produce μ chain; in the mother clone A3, only 0.5% of the γ2b cells also produce μ chain. Of 133 clones, only in clone A33 did the γ2b cells all contain μ chain.

DISCUSSION

Mechanism of Activation of H Chain Synthesis from the Silent Allele. It has been reported that in the 18-81 cell line the VDJ segment on the silent homolog is also rearranged in a functional way. This rearrangement results in a 2.4-kb *Eco*RI fragment that hybridizes with a J probe (16). As mentioned, we also find that the silent homolog is represented by the 2.4-kb fragment. H chain expression by the silent homolog is prevented by the amber termination codon TAG in the nucleotide sequence of its D segment (17). In this case, inhibition of translation might be overcome in several ways: by recombination between homologs, by reversion to a codon specifying an amino acid, or by reading through the stop codon.

To explain translation of the usually silent allele, a single recombination event between homologs would have to alter the VDJ segment, the new V_H, and at least part of the new D_H coming from the active allele; therefore, the size of restriction enzyme fragments hybridizing with the J probe would also be altered. We did not find any size difference when the usually silent allele was compared among the clones A3, A31, A32, A33, and A33-1, and their hybridomas, after digestion with the enzymes *Eco*RI, *Xba* I, *Hin*dIII, *Bst*EII, and hybridizing with a J probe (an example is shown

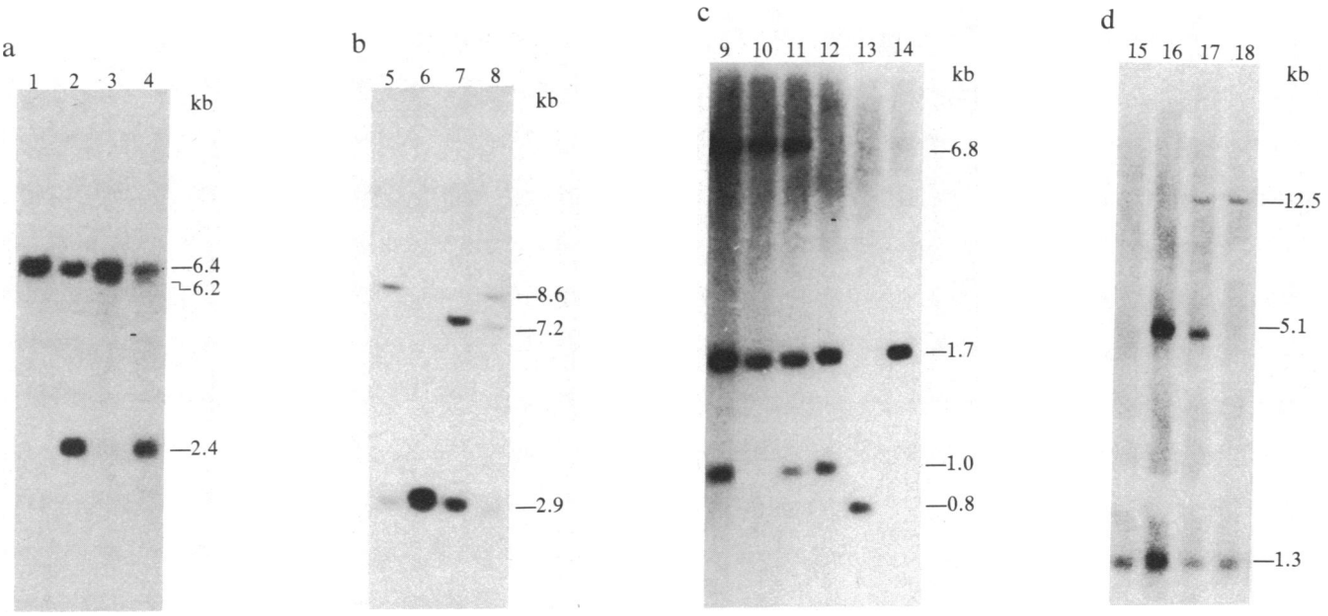

FIG. 5. Southern blot analysis of a double-producing hybridoma and its subclones, showing allelic inclusion. DNA was digested with (*a*) *Eco*RI, (*b*) *Xba* I, (*c*) *Hin*dIII, and (*d*) *Bst*EII, and hybridized with J3 (*c* and *d*) or J34 probe (*a* and *b*). Lanes: 1, 6, 14, and 15, myeloma Ag8653; 2, 5, 10, and 18, hybridoma H33-11-13 (μ-chain producer); 3, 7, 12, and 16, hybridoma H33-11-11 (γ-chain producer); 4, 8, 11, and 17, hybridoma H33-11-12 (μ- and γ-chain producer); 9, hybridoma H32-1 (γ- and κ-chain producer); 13, kidney.

in Fig. 5*c*). We know from their sizes that the restriction fragments cover at least part of the V_H and all of D_H of the usually silent allele. This excludes a single but not a double recombination event ("gene conversion").

The mechanism involved in the activation of the normally silent allele in 18-81 should be clarified by sequence determination of the D segment, which is currently being carried out.

Cell Biological Consideration. We have shown that allelic exclusion need not occur in pre-B cells. It has been postulated that allelically included cells have a growth disadvantage as compared to the allelically excluded cells, because of H-chain toxicity (18). This growth disadvantage could operate at the pre-B or at the B-cell level (18). We are currently investigating the growth behavior of the 18-81 subclone described here.

We also want to note that the preferential combination of a L chain with one of the H chains can also contribute to allelic exclusion of the H chain. By fusion of a double-producer clone with a nonproducer myeloma, we obtained some hybridomas that produce μ, γ2b, and κ simultaneously (unpublished data). In those cells, the κ chain is associated only with the γ2b chain, and only the complete IgG2b molecule is secreted.

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