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Genetics

Allelic inclusion in a pre-B-cell line that generates immunoglobulin heavy chain genes *in vitro*

(rates of gene assembly/allelic exclusion)

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ABSTRACT **In a pre-B-cell line that rearranges its heavy chain gene segments *in vitro*, we found that the rate of productive rearrangement on one allele was not influenced by the presence of heavy chain protein encoded by the other allele. This shows that allelic exclusion of heavy chain genes is not regulated at the genetic level.**

Immunoglobulin heavy (H) and light (L) chain genes are assembled from pools of different gene segments [called variable (V_H), diversity (D_H), and joining (J_H) for the H chain gene] in such a way that only one gene can be generated per allele. Because lymphocytes are diploid cells, this assembly process could create two genes at the H chain locus and six genes for the L chain isotypes κ and λ. Allelic and isotypic exclusion reduce these numbers to one functional gene for the H chain and one for the L chain, thereby ensuring the monospecificity of B lymphocytes. To explain allelic exclusion at the Ig H chain locus, several hypotheses have been proposed. Somatic segregation has been disproved (1), leaving three other hypotheses to be considered. The first states that the presence of H chain protein inhibits further rearrangements of the H chain gene segments to create a complete gene (2); in this model, regulation occurs at the genetic level. A second hypothesis considers allelic exclusion the statistical consequence of a low frequency of rearrangements that can encode functional H chain protein (3); in this model there is no regulation. A third hypothesis suggests that L chain is not able to saturate all H chains when H chains are produced from two alleles and that the toxicity of free H chains will eliminate these functionally diploid cells from the population of B lymphocytes (4); in this model allelic exclusion is achieved at the cellular level. The last two hypotheses are not mutually exclusive. A pre-B cell, which represents an early differentiation stage, produces H chain without L chain, and the H chain is confined to the cytoplasm. Because the specificity of the H chain is not yet displayed to the antigenic universe, allelic exclusion need not apply at this stage. We have tested these hypotheses about allelic exclusion in a pre-B-cell line that generates H chain genes *in vitro*.

MATERIALS AND METHODS

Compartmentalization Test. Single cells were distributed into microtiter plates at limiting dilution (0.15 cell per well) without feeder cells. The medium was supplemented with hybridoma growth factor (5) to support the growth of single cells. When the clone had reached the size of 10^5 cells, the cells were harvested and assayed for cytoplasmic μ H chain by immunofluorescence.

Southern Blot Analysis. Southern blot analysis was done as described (6).

Immunologic Blot Analysis. Proteins of 10^7 cells were precipitated with rabbit antiserum to μ and κ chains. The precipitate was dissolved and subjected to $NaDodSO_4$/PAGE (7). The proteins were then transferred onto a nitrocellulose filter in 25 mM Tris base, pH 8.3/192 mM glycine/20% (vol/vol) methanol and run at 60 V for 5 hr in a Bio-Rad Trans-Blot cell at room temperature. The filter was incubated with a solution of 3% (wt/vol) gelatin and ^{125}I-labeled antibody to mouse IgM.

$NaDodSO_4$/PAGE. Cells were cultured in the presence of [^{35}S]methionine (100 μCi/ml; specific activity 800 Ci/mmol; 1 Ci = 37 GBq; Amersham). Ig was immunoprecipitated from cell lysates, reduced, and analyzed on 10% $NaDodSO_4$/polyacrylamide gels as described (7).

RNA Blot Analysis. Cells of all lines were lysed in 0.5% Nonidet P-40, and the nuclei were removed by centrifugation at 2100 × *g* for 10 min at 5°C. The supernatant was digested with proteinase K, extracted three times with phenol/chloroform (1:1), and ethanol precipitated. The precipitate was dissolved in Tris EDTA (10 mM Tris, pH 8.0/1 mM EDTA) buffer and separated on a 1% agarose electrophoretic gel. RNA denatured with formaldehyde was transferred onto a nitrocellulose filter and probed with a ^{32}P nick-translated μ cDNA probe.

RESULTS AND DISCUSSION

The K cell line is a pre-B-cell line derived from Abelson-virus-transformed bone marrow cells of a BALB/c mouse (8). We found that most cells of this line produce neither L nor H chain, as determined by $NaDodSO_4$/PAGE of immunoprecipitated material and by immunofluorescence using goat antisera specific for the various Ig chains. However, μ chain is produced in 0.5% of the cells, which suggests that complete μ chain genes are being created in this line *in vitro*. Because the K cell line may have been in culture for a long time and consequently quite heterogeneous, we subcloned it to obtain subclone K.40 for further study.

DNA Arrangements at the H Locus. We determined the arrangement of gene segments at the two H chain alleles, arbitrarily designated A and B, by Southern blot analysis. When DNA digested with restriction enzymes *Bam*HI or *Eco*RI was hybridized with a radioactive probe containing the

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Abbreviations: H, heavy; L, light; V, variable; D, diversity; J, joining; C_μ, gene segment encoding the constant region of the μ heavy chain.

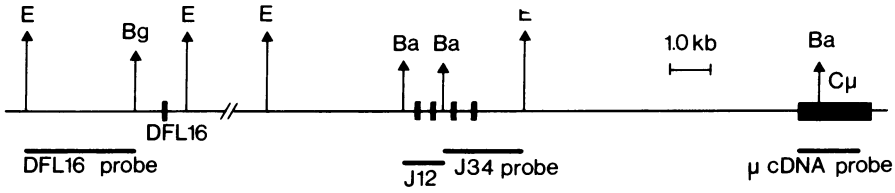

FIG. 1. Restriction map of part of the embryonic H chain allele and probes used for Southern and RNA blot analyses. The J12, J34, and μ cDNA probes were provided by F. Blattner, and the 5′ DFL16 and 5′ DSP2 probes were provided by F. Alt (9). The 5′ DSP2 probe is not shown here. E, *Eco*RI; Bg, *Bgl* II; Ba, *Bam*HI. Thick cross bars indicate the fragments to which respective probes hybridize—J_{H1}, J_{H2}, J_{H3}, and J_{H4} are located in the middle of the diagram.

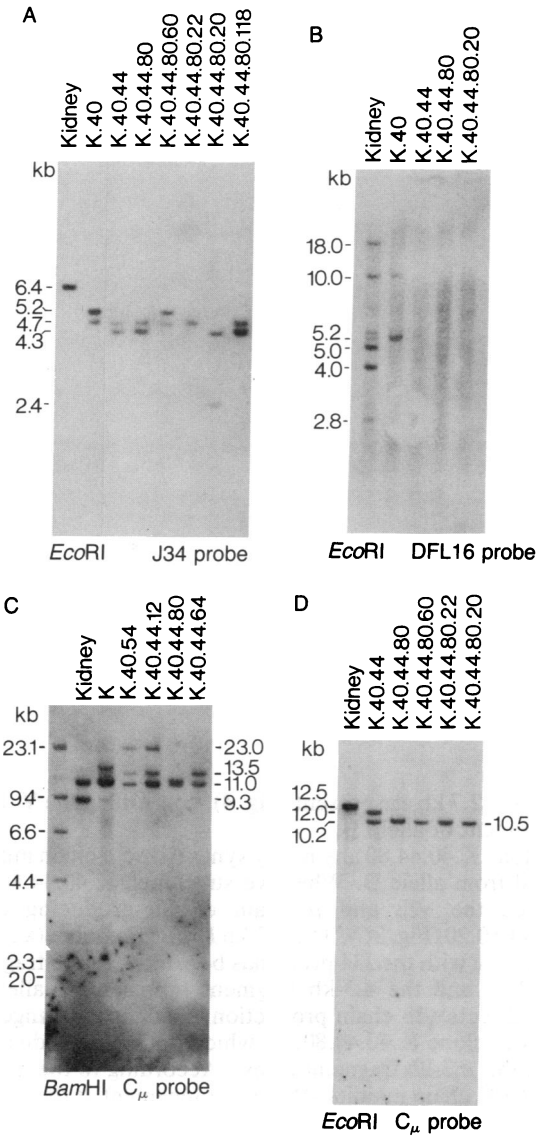

FIG. 2. Southern blot analysis of the DNA arrangement at the H locus in various K cell line subclones: (*A*) *Eco*RI digest and hybridization with the J34 probe; (*B*) *Eco*RI digest and hybridization with DFL16 probe; (*C*) *Bam*HI digest and hybridization with μ cDNA probe; K, original heterogeneous clone; (*D*) *Eco*RI digest and hybridization with μ cDNA probe. Clones K.40.44.64, K.40.44.80.22, and K.40.44.80.60 do not produce any H chain; clones K.40.54 and K.40.44.12 produce μ chain only; clone K.40.44.80.118 produces γ2b chain only. On both H chain alleles in the clone K.40, (different) embryonic sequences have been deleted in the major intron between J and C_μ (the constant region of the μ heavy chain).

J_{H1} and J_{H2} segments (Fig. 1), no bands were seen (data not shown). However, hybridization of *Eco*RI-digested DNA with a probe containing J_{H3} and J_{H4} (Fig. 1, J34) resulted in two bands of 5.2 kilobases (kb) (allele A) and 4.7 kb (allele B) (Fig. 2*A*). The embryonic fragment containing J_{H3} and J_{H4} has a length of 6.4 kb; therefore, both alleles A and B are not in the embryonic configuration. The 5.2-kb fragment also hybridized to a probe that contains part of the 5′-flanking region of the D region gene segment of FL16 type (Fig. 2*B*). This probe detects the most 5′ D segment, which in embryonic DNA is located on a 4.0-kb *Eco*RI fragment (Fig. 1), as well as a 10.0-kb fragment that is further 5′, but is not known to contain any D segment (10), and various other fragments that contain D segments. Because a D–J joining event does not alter sequences 5′ to D, but a V to D–J joining event deletes them, the 10.0-kb fragment should always be present in a D–J configuration but absent in a V–D–J configuration. Thus, allele A joined the most 5′ D_{FL16} segment to either J_{H3} or J_{H4}; because of the fragment size and its hybridization to the J34 probe, which is stronger than that of the 4.7-kb band (allele B), the D segment is probably joined to J_{H3}. Allele B did not hybridize with the 5′ D probe, and there was no embryonic 4.0-kb piece left; therefore, allele B does not have the D–J configuration. A probe covering part of the 5′ flanking region of a D segment of SP2 type hybridized to the same fragments as the DFL16 probe, although with different strengths. Because no H chain is made in most cells of the K.40 clone (see below), the B allele presumably represents a (nonproductive) $V–D–J^-$ rearrangement. Both alleles have constant region μ gene segments (C_μ). DNA digested with *Bam*HI and hybridized with a μ cDNA probe showed two nonembryonic bands in subclones K.40.54 and K.40.44.12 (Fig. 2*C*) that also hybridized with the J34 probe (data not shown).

Subclones That Have Rearranged Their Gene Segments. The rearrangement of alleles A and B was confirmed by the following subcloning experiments that are summarized in Table 1. The subclone K.40 is similar to the mother line in that most cells do not produce μ chain, but the cells do produce Dμ protein (11) (Fig. 3*A*). However, 7% of these cells produce μ chain, as determined by immunofluorescence and seen on $NaDodSO_4$/PAGE (Fig. 3*A*). Therefore, a productive V to D–J joining event must have occurred in at least one cell of this clone. In subclone K.40.44, most cells produce μ chain, as demonstrated by $NaDodSO_4$/PAGE (Fig. 3 *A* and *B*) and immunofluorescence, whereas Dμ protein is no longer pro-

Table 1. Rearrangements of nonproductive H alleles to productive ones in the pre-B-cell line K

Successive subclones	DNA arrangement at the *H* locus		Ig chain production	
	Allele A	Allele B	Allele A	Allele B
K.40	*D–J*, C_μ	*V–D–J*$^-$, C_μ	*Dμ*	None
K.40.44	*V–D–J*$^+$, C_μ	*V–D–J*$^-$, C_μ	*μ*	None
K.40.44.80	*V–D–J*$^+$, $C_{\gamma 2b}$	*V–D–J*$^-$, C_μ	γ2b	None
K.40.44.80.20	*V–D–J*$^+$, $C_{\gamma 2b}$	*V–D–J*$^+$, C_μ	γ2b	*μ*

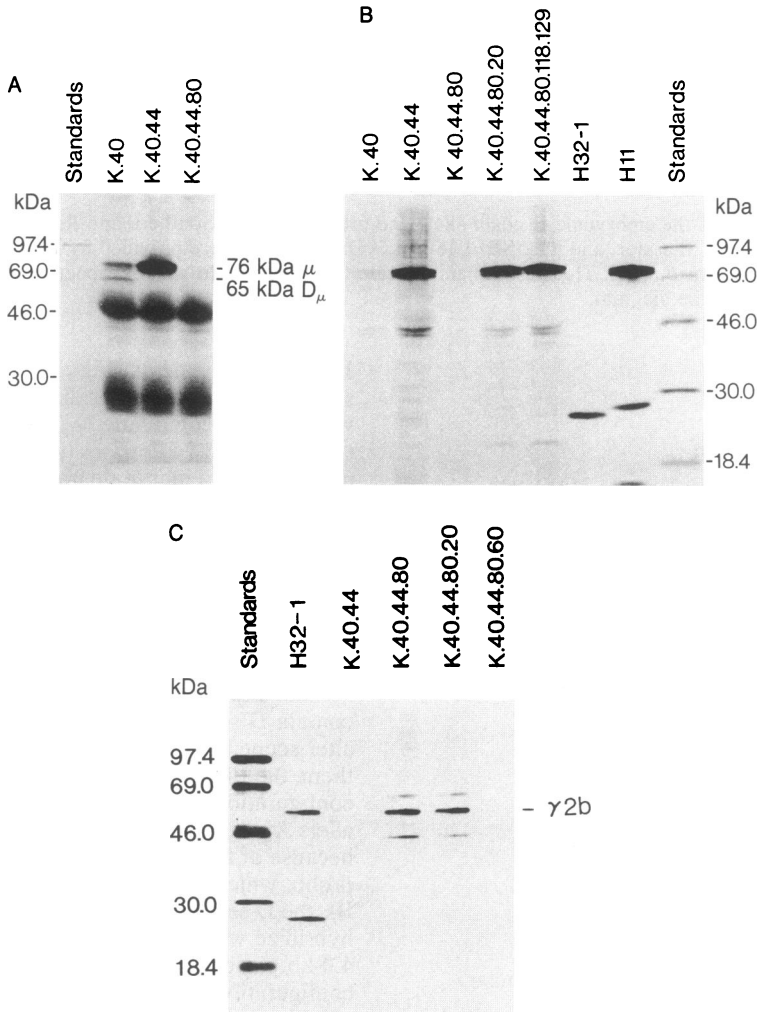

FIG. 3. (*A*) Immunologic blot analysis of proteins of three K subclones. In addition to mouse Dμ and μ chain protein, rabbit γ and L chain are detected by the radioactively labeled antibody. The Dμ protein at M_r of 65,000 is somewhat larger than originally described (11), as is μ chain at M_r of 76,000. (*B*) NaDodSO$_4$/PAGE of μ chains produced by various K clones. H32-1 is a γ2b- and κ-producing hybridoma; H11, a μ- and κ-producing hybridoma. (*C*) NaDodSO$_4$/PAGE of γ2b chains. K.40.44.80.60 is a clone that has lost γ2b chain production, as is also evident from immunofluorescence. Standards are indicated at the left.

duced. The 5.2-kb fragment representing allele A has moved to a band at 4.3 kb, and the 4.7-kb fragment (allele B) is unchanged (Fig. 2*A*). This 4.3-kb band was not detected by the 5′ DFL16 probe (Fig. 2*B*), nor did any other fragment hybridize with this probe. These observations confirm that allele A has a (productive) V–D–J$^+$ rearrangement in clone K.40.44 and that allele B retains the V–D–J$^-$ arrangement from the mother clone K.40. We repeated this analysis with clone K.62, which produces no μ chain. Subclone K.62.1 does produce μ chain, which is derived from the A allele.

When we subcloned K.40.44, we obtained clone K.40.44.-80, which had lost μ chain (Fig. 3 *A* and *B*) by switching to γ2b chain production as determined by immunofluorescence and NaDodSO$_4$/PAGE (Fig. 3*C*). In K.40.44.80 the 4.3-kb (allele A) and 4.7-kb (allele B) *Eco*RI fragments hybridizing with the J34 probe are the same as in the μ-producing mother clone K.40.44, but the C_μ on allele A is absent (Fig. 2 *C* and *D*). The fragment retaining its C_μ gene segment and representing the B allele has also deleted some DNA. This deletion must have occurred 3′ to the *Eco*RI site within the major intron between J and C_μ, because the *Eco*RI fragment hybridizing with the C_μ probe is separated from the fragment hybridizing with the J34 probe, which is unchanged. Allele B still does not direct μ chain synthesis, although μ mRNAs of 2.4 kb and 2.7 kb are present (Fig. 4), confirming the V–D–J$^-$ arrangement of allele B.

In clone K.40.44.80 any newly synthesized μ chain must be derived from allele B. When we subcloned K.40.44.80, we obtained the γ2b and μ chain double-producing clone K.40.44.80.20 (Fig. 3*C*). The 4.7-kb *Eco*RI fragment (allele B) hybridizing with the J34 probe has become a 2.4-kb fragment (Fig. 2*A*), and the 4.3-kb fragment representing allele A, which directs γ2b chain production, remains unchanged. In the sister clone K.40.44.80.22, which does not produce γ2b chain, this 4.3-kb fragment is lost. According to the hypothesis that H chain inhibits DNA rearrangement at the H locus and thus confers allelic exclusion, no additional H chain should be made in a pre-B cell in the presence of a functional H chain. The existence of H chain double producers in the K cell line seems to contradict this. Other instances of V_H replacement in a V–D–J$^-$ allele have been described recently (13–15).

Rates of Productive Rearrangements. To quantify our results, we used a compartmentalization test (16, 17) to measure the rates of creating productive rearrangements in cells of clone K.40, which lacks H chain, and in cells of clone K.40.44.80, which contains H chain. For each determination over 100 single cells were grown to clones of about 10^5 cells, and productive rearrangements were detected by testing for

μ chain production by immunofluorescence. Clones that arose from cells that produced no μ chains were analyzed as follows. The average number of events leading to a productive rearrangement per well, m, is given by

$$e^{-m} = N_0/N,$$

where N is the number of wells in an experiment and N_0 is the number of wells in which no productive rearrangement has occurred. The number of productive rearrangements per cell per generation, M, is then given by

$$M = m/2C,$$

where C is the number of cells per well. To account for variation in clone sizes, we determined the maximum-likelihood estimate of rate $\hat{M}$ using a computer program (17). Rates were approximately 1×10^{-5} and 2×10^{-6} productive rearrangements per cell generation for clones K.40 and K.40.44.80, respectively (Table 2). Because clone K.40 can generate a productive H chain gene on two alleles, whereas clone K.40.44.80 contains only one remaining allele for this purpose, the 5-fold difference in rates can decrease to a 2.5-fold difference per allele—a difference that hardly can be taken as an indication that H chain protein inhibits further rearrangement at the H locus.

According to our results allelic exclusion of H chain genes is not regulated at the genetic level. The mechanism of allelic exclusion of L-chain genes may be analogous, or it may differ completely; these experiments deal only with the H locus. It could be reasoned that no genetic regulation occurs *in vitro*, because the *in vitro* rate of rearrangement in the K cell line is lower than the rate *in vivo*. Unfortunately, a *rate* of rearrangement has not been measured *in vivo* or *in vitro*. On the other hand, it is obvious that a cell line rearranging its D–J

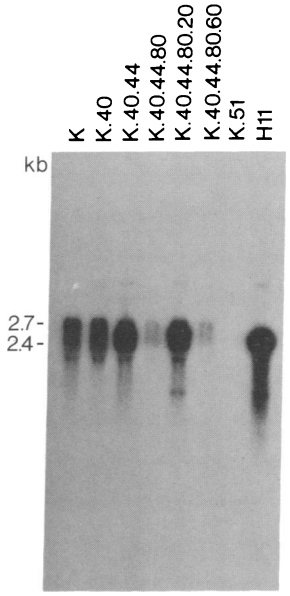

FIG. 4. RNA blot analysis of μ mRNA in K subclones. Clone K.51 has 0.5% cells that produce γ2b, no μ-producing cells, and no C_μ gene segments. Hybridoma H11 produces μ and κ chains. The small amount of μ mRNA in clone K.40.44.80 is derived from the B allele; it is the predicted size but is not translated. Less μ mRNA is derived from the unproductive B allele in clone K.40.44.80 than from the productive A allele in clone K.40.44—presumably owing to the decreased stability of untranslated μ mRNA (ref. 12; H.-M.J. and M.W., unpublished data). There is more μ mRNA in clone K.40, because μ mRNA from allele A of the 7% μ-positive cells is added to μ mRNA from allele B.

Table 2. Rate measurements of productive rearrangements in K subclones

	K.40	K.40.44.80
Total wells	115	105
Positive wells, no.	90	31
Negative wells, no.	25	74
Cells per well, $\overline{no.}$	1.08×10^5	1.08×10^5
Cells per positive well, $\overline{no.}$	1.11×10^5	1.13×10^5
Cells per negative well, $\overline{no.}$	0.95×10^5	1.06×10^5
Maximum-likelihood estimate of rate $\hat{M}$ per cell generation	7.5×10^{-6}	1.6×10^{-6}
$\hat{M}$, corrected for possible cell loss during centrifugation on microscopic slides	1.2×10^{-5}	2.2×10^{-6}

configuration cannot be very active in doing so, otherwise no D–J configuration would be left. It is also possible that μ chain inhibits further rearrangement and that γ2b chain does not, but this assumption requires that γ2b-producing pre-B cells be unphysiological, which is debatable (18). If the model of allelic exclusion of H chain genes states that μ chain closes off accessibility of recombination enzymes to the other allele, then one would have to add as a corollary that access can be regained, because clone K.40.44 does produce μ chain, and a subclone is a double producer.

The replacement of a nonproductive rearrangement with a productive one is probably mediated by the same recognition sequences and thus by the same enzyme(s) as the V to D–J joining (14). It is interesting to note that the rate for V to D–J joining and the rate for replacement of a V–D–J$^-$ arrangement with a productive one do not differ markedly. A low frequency of productive rearrangements (3) could be complemented by V_H segment replacement to create a higher number of pre-B cells with functional H chains. Even if V to D–J joinings were inhibited by a functional H chain, V_H segment replacement should sometimes create two functional H genes within a cell. This cell, we suggest, is later eliminated from the population because of growth disadvantage (4, 19).

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