Virus-transformed Pre-B Cells Show Ordered Activation but Not Inactivation of Immunoglobulin Gene Rearrangement and Transcription

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Summary

Virus-transformed pre-B cells undergo ordered immunoglobulin (Ig) gene rearrangements during culture. We devised a series of highly sensitive polymerase chain reaction assays for Ig gene rearrangement and unrearranged Ig gene segment transcription to study both the possible relationship between these processes in cultured pre-B cells and the role played by heavy (H) chain (μ) protein in regulating gene rearrangement. Our analysis of pre-B cell cultures representing various stages of maturity revealed that transcription of each germline Ig locus precedes or is coincident with its rearrangement. Cell lines containing one functional rearranged H chain allele, however, continue to transcribe and to rearrange the allelic, unrearranged H chain locus. These cell lines appear to initiate but not terminate rearrangement events and therefore provide information about the requirements for activating rearrangement but not about allelic exclusion mechanisms.

The mature cells of the body arise by differentiation from immature precursors through many stages that together form a lineage. Generally, these stages are difficult to define because pure cell populations representing individual stages cannot be prepared. In the B lymphocyte lineage, virally transformed tumor cells of immature phenotype have provided extremely useful models of individual developmental stages. These models have limitations, however. Being tumor cells, they might not represent normal cells with fidelity. Furthermore, they often display a mixed phenotype because individual cells appear to slowly progress through the lineage, whereas a stem line of relatively fixed phenotype persists.

Characterization of these pre-B cell transformants has been extensive (1, 2). Ig gene rearrangements can be used as markers for successive developmental stages since the various H and L chain gene rearrangements are ordered during B cell development (2, 3). The most immature are lines derived by Palacios et al. (4), which have germline H and L chain loci but can be induced to differentiate under special conditions. LyD9 and BaF3, which display the B220 pan-B cell surface marker, are representatives. Pre-B cells that are mainly germline at the H chain loci but carry out the earliest stages of H chain gene rearrangement (D to J joining) are typified by HAFTL cell lines (5), fetal liver cells transformed by Harvey leukemia virus. Abelson virus–transformed fetal liver and bone marrow pre-B cells are somewhat more mature. These include early lineage cells such as 300-19 (6), which carry out predominantly H chain V to DJ rearrangements, and more mature cell lines like PD31, which have functional H chain gene rearrangements and rearrange the κ L chain locus in culture (7).

These pre-B cell analogues have been characterized largely by cumbersome subcloning experiments that are limited to examining the majority phenotypes. Also, H chain–related events have been studied more extensively than L chain–related events. These analyses suggested that only H chain–producing cell lines could rearrange κ L chain genes and that H chain–producing cells inactivate further H chain gene rearrangement and unrearranged gene transcription (2, 8).

We recently developed a semi-quantitative PCR assay for gene rearrangement and expression (9) that allows bulk cell populations to be assayed for many different events. Using this methodology, we showed that H chain protein was not necessary for κ gene rearrangement and that increased levels of germline κ gene transcription correlated with greater frequency of κ gene rearrangement (9). Here, we provide an extensive characterization of transformed pre-B cells that more completely defines their properties. We find that these cells effectively initiate but rarely terminate the events of B cell development and therefore provide good models for studying the requirements for rearrangement, but not necessarily for allelic exclusion (10, 11), an event thought to involve the regulated termination of rearrangement (3). We show that for both H and L chain genes, rearrangement is correlated to transcription of variable and constant region gene segments, and that a functional H chain gene rearrangement does not prevent further unrearranged H chain gene transcription or rearrangement in virally transformed pre-B cell lines.

Materials and Methods

Cell Lines and Culture. LyD9 (4) was provided by Dr. Ron Palacios (Basel, Switzerland), HAFTL (5) by Dr. Jacklyn Pierce (National Institutes of Health), 300-19 (6) by Dr. David Weaver (Dana-Farber Cancer Center, Boston, MA), and 223-18 (2) by Dr. Naomi Rosenberg (Tufts University, Boston, MA). Cell lines M8,

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M9, and M10 were generated by Abelson virus transformation of murine bone marrow (12) and kindly provided by Marjorie Oettinger (Whitehead Institute). K40F.1 and K40F1.2 were generated by Alan Shapiro and Dr. Tony DeFranco (University of California, San Francisco). K40F.1 was obtained by limiting dilution cloning of the Abelson virus–transformed cell line K40. K40F.2 is a H chain–producing derivative of K40F.1 obtained by sib-selection. With the exception of LyD9, all cells were maintained in RPMI supplemented with 10% inactivated FCS, 50 μM β-mercaptoethanol, penicillin, and streptomycin. LyD9 was grown in Iscove's media supplemented with 5% FCS, 10% WEHI-3-conditioned medium, 50 μM β-mercaptoethanol, penicillin, and streptomycin. Limiting dilution cloning was done in 96-well microtiter plates. Only clones from plates containing <30 positive wells were used.

Preparation of Nucleic Acids. Total RNA was prepared using the guanidinium method exactly as described (13). DNA was prepared for PCR by lysing 10^6 cells in 200 μl PCR lysis buffer (10 mM Tris, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 60 μg/ml proteinase K), incubating them at 55°C for 1 h, then inactivating the protease by heating to 95°C for 10 min. This DNA, at a concentration of 5,000 genomes/μl, was used directly for PCR.

DNA PCR Assays. 50-μl PCR reactions contained 2 μl template (10,000 genomes), 10 mM Tris, pH 8.4, at 20°C, 50 mM KCl, 2.0 mM $MgCl_2$, 100 μg/ml BSA (Bethesda Research Laboratories, Bethesda, MD), 100 ng of each primer (25 mers, see below), and 1 U Taq polymerase (Amplitaq; Cetus Corp., Emeryville, CA). 30 cycles of amplification were performed, using a DNA thermal cycler (Cetus Corp.), consisting of 1 min at 94°C, 1 min at 60°C, and 1.75 min at 72°C, followed by a single 10-min period at 72°C. One-fifth of each reaction was analyzed on a 1.4% agarose gel in tris-borate buffer. The gel was blot-transferred to a nylon membrane (Zetabind; American Bioanalytical, Natick, MA) and probed with ^{32}P-labeled DNA from the appropriate Ig constant region (see below).

The sequences of the various primers used in these experiments are shown below. The J primers are unique sequences 25 nucleotides in length. Germline alleles were detected using a primer (Mu0) 322 nucleotides 5′ to J_H1. The D_H R and L primers are mixtures of oligonucleotides degenerate at two and three positions, respectively, which are homologous to all of the members of the Dfl16 and Dsp2 D gene families (9 of the 10 known murine D minigenes). D to J rearrangements were detected as amplified fragments of ~1,033, ~716, or ~333 nucleotides depending on whether J_H1, J_H2, or J_H3 was rearranged. The assay for V to DJ rearrangement uses a mixture of three different degenerate (at three positions) oligonucleotides homologous to conserved framework region 3 (FR3) sequences of three V_H gene families (V_H7183, V_H558, and V_HQ52) and the J3 primer. This results in amplified VDJ rearrangements of ~1,058, ~741, or ~358 nucleotides. PCR products were detected and quantified by Southern blotting and hybridization with appropriate Ig gene probes.

Both DJ and VDJ rearrangement result in loss of the Mu0 sequence and its amplification product. Likewise, any V to DJ rearrangement event results in loss of all the D_H L primer target sequences and amplified DJ fragments.

RNA PCR Assays. Constant region (Mu0, DC_H, and C_κ) RNA PCR assays were performed on cDNA made by random priming of total RNA samples (14). 3 μg of RNA was reverse transcribed in a reaction containing 200 U cloned Moloney leukemia virus reverse transcriptase (Bethesda Research Laboratories), 5 pmol random hexamers (Pharmacia Fine Chemicals, Piscataway, NJ), 10 mM Tris, pH 8.4, 50 mM KCl, 2.0 mM $MgCl_2$, 100 μg/ml BSA (Bethesda Research Laboratories), 5 mM DTT, and 20 U RNasin (Boehringer Mannheim Biochemicals, Indianapolis, IN) in a final volume of 20 μl. The reaction was incubated for 1 h at 42°C then heat-inactivated at 75°C for 10 minutes. Samples were kept on ice or stored frozen at −20°C until evaluation by PCR.

V_H and V_κ PCR assays, which do not span introns, required pretreatment of purified RNA with DNase to eliminate contamination with genomic DNA. 10 μg of RNA was digested with 2 U RQ1 RNase-free DNase (Promega Biotec, Madison, WI) for 10 min at 37°C in a 15-μl reaction containing 1 U/μl RNasin (Boehringer Mannheim Biochemicals), 10 mM Tris, pH 7.5, 2 mM $MgCl_2$, and 5 mM DTT. The DNase was heat-inactivated at 75°C for 10 min. 3 μg of DNase-treated RNA was reverse transcribed in a 20-μl reaction as above, except that the random hexamers were replaced with 250 ng of each 3′ reverse transcription primer (degenerate oligonucleotides homologous to the heptamer-nonamer region immediately 3′ to the V genes). The cDNA samples were kept on ice or stored frozen at −20°C until used for PCR.

2 μl of cDNA was used in a 50-μl PCR reaction in the same buffer and under the same conditions as for DNA (above). Each transcript was analyzed with specific primers (see below) in a separate reaction. The amplified products were subjected to electrophoresis on agarose gels and blot-transferred to nylon membranes. The probes used for blot hybridization came from gel-purified control amplification reactions that were labeled by standard methods (15).

PCR and Reverse Transcription Primers. The following primers were used in these experiments: V_HB: GGWCTGASGACGTCRAMSTGGA; V_H558R: GCTGCAGYTGAGYTGCATGWAGGC; V_HRT: TGWGGTWSYMWCACTGTG; V_κB: GACATTCAGCTGACCCAGTCTCCA; V_κR: GTYCCWGAYCCACTGCCACTGAASC; V_κRT: GGCCCGGGTTTWTGTTMWGRBYTGTAKCACAGTG; Mu0: CCGCATGCCAAGGCTAGCCTGAAAGATTACC; V_H558: CGAGCTCTCCARCACAGCCTWCATGCARCTCARC; V_H7183: CGGTACCAAGAASAMCCTGTWCCTGCAAATGASC; V_HQ52: CGGTACCAGACTGARCATCASCAAGGACAAYTCC; D_H R: TTTTGYTGMTGGATATAKCACTGAG; D_H L: GGAATTCGMTTTTTGTSAAGGGATCTACTACTGTG; J3: GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG; J4: TCCCTCAAATGAGCCTCCAAAGTCC; C_H: ATGCAGATCTCTGTTTTTGCCTCC; K^o: TCCACGCATGCTTGGAGAGGGGGTT; C_κ: GTCCTGATCAGTCCAACTGTTCAG.

The sequences are presented 5′ to 3′. Degenerate nucleotide positions are coded as follows: R = A or G; Y = C or T; S = C or G; M = A or C; K = G or T; W = A or T; B = C, G or T. VB and V_HB were adapted from reference 16. Most PCR primers contain a restriction enzyme linker sequence at their 5′ end and ~25 nucleotides of target homology.

RNase Protection Assay for Oct-2. Oct-2 mRNA levels were measured by RNAase protection, using a probe derived from the 5′ end of a murine Oct-2 cDNA. The probe spans the region homologous to nucleotides 436–547 in the human cDNA sequence (17). 30 μg of total cytoplasmic RNA was used in each experiment. As a control for the amount of RNA present in each hybridization, a parallel experiment was performed using an γ-actin probe. The amount of probe protected in each experiment was quantified using a blot analyzer (model 603; Betagen, Natick, MA).

Results

H Chain Gene Rearrangements in Cultured Cells. We devised a series of assays to sensitively detect H chain gene rearrangements in a cell population using the PCR. Fig. 1 indicates the positions of the various PCR primers used to detect

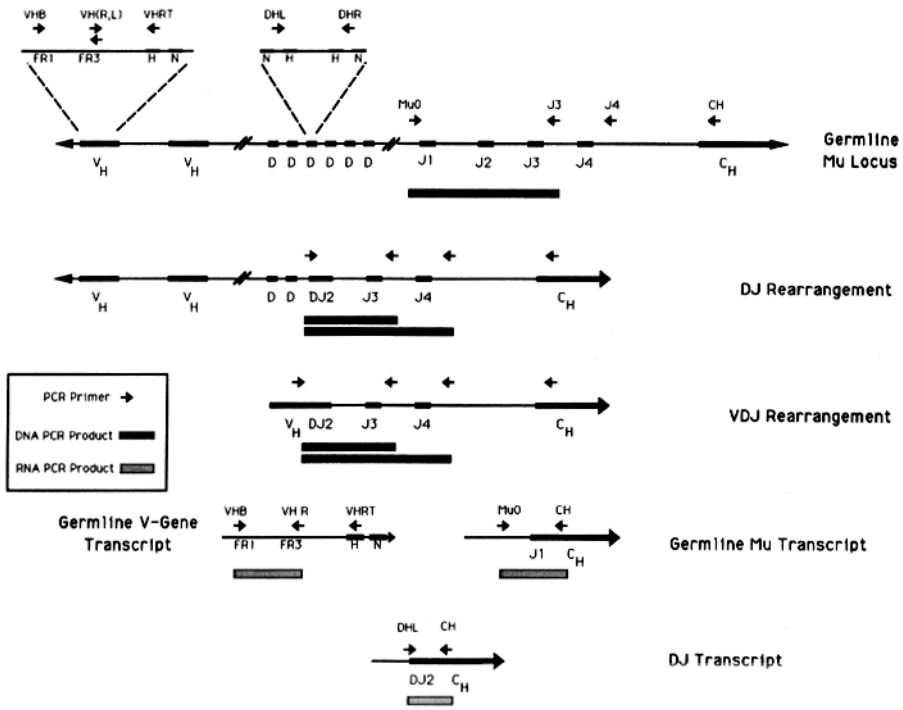

Figure 1. Diagram of the various PCR assays used to evaluate Ig H chain gene rearrangement and germline gene transcription. The upper line is a schematic map of the germline Ig H chain locus (not drawn to scale). Subsequent lines depict various partially or fully rearranged alleles or processed germline gene transcripts. The short arrows show the positions of PCR primers and the shaded bars represent predicted PCR products. *H* and *N* represent the conserved heptamer and nonamer recombination signal sequences. The sizes of the expected PCR products and the sequences of the PCR primers are as shown in Materials and Methods.

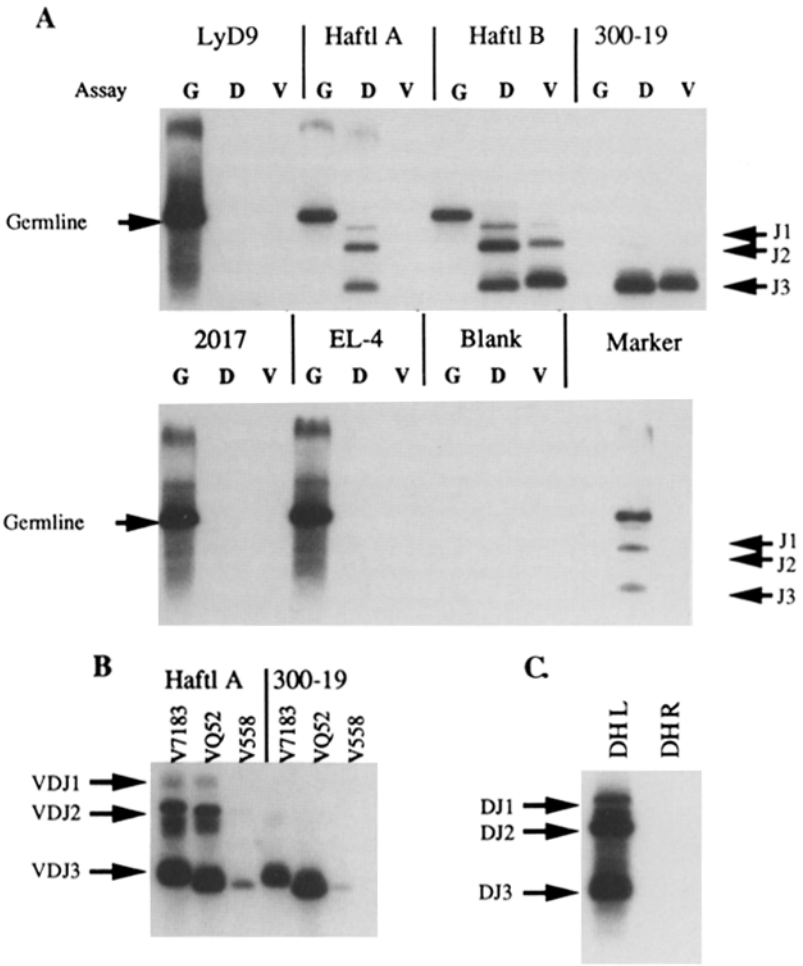

Figure 2. DNA PCR assays of germline and rearranged Ig H chain genes in cultured cells active for Ig gene rearrangement. (*A*) An autoradiogram of germline (*G*), DJ (*D*), and VDJ (*V*) rearrangement assays performed on the indicated pre-B and T cell lines is shown. The positions of PCR fragments corresponding to DJ or VDJ rearrangements involving J_H1, J_H2, and J_H3 are indicated. The lanes labeled *Blank* are the products of control amplification reactions containing no template. (*B*) The cell lines HAFTL A and 300-19 were assayed by PCR for VDJ rearrangements using V gene family–specific V_H primers. The primers and positions of migration of VDJ1, VDJ2, and VDJ3 PCR products are indicated. (*C*) DJ rearrangement PCR assays were performed on DNA from HAFTL cells using either DH L or DH R as the 5′ primer. The various DJ rearrangements are indicated.

germline alleles and DJ and VDJ gene rearrangements, and shows diagrammatically several of the possible PCR products. In their unrearranged states, sequences homologous to the various V, D, and constant region PCR primers are too far apart to allow efficient amplification. Rearrangements of most D segments or many members of three V_H gene families (V_H558, V_H7183, V_HQ52) to J_H 1, 2, or 3 result in distinguishable amplified products. By limiting the number of amplification cycles (9), these assays can provide a measure of the relative frequencies of different rearrangement events in actively rearranging cell populations.

We used these PCR assays to assay for H chain gene rearrangements in HAFTL A and HAFTL B, independent, cloned, H-ras-transformed pre-B cell lines (Fig. 2 *A*). The presence of a hybridizing amplified germline fragment as well as several fragments of predicted lengths using both V_H and D_H primers showed that DJ and VDJ rearrangements must have occurred during the growth of these clonal cell lines in culture. Single cell clones of HAFTL A, cultured for several months, display a rearrangement pattern similar to the parental line (data not shown), indicating that rearrangement is ongoing. LyD9 (4) is a $B220^+$ pro-B cell line that under certain conditions is capable of differentiation into mature, surface Ig^+ B cells. PCR analysis detected only germline H chain alleles. The Abelson virus–transformed pre-B cell line 300-19 (6) was initially reported to have the genotype DJ/DJ. PCR analysis demonstrated DJ3 and VDJ3 gene rearrangements. Our assay detected infrequent DJ rearrangement events (faint DJ signals; Fig. 2 *A*) in the pre-T cell line 2017 (18), a phenomenon previously reported in other T cells. A variety of cells failed to show any rearrangement, including EL-4 (T cell), BAF3 (pro-B; data not shown), and 3T3 (fibroblast; data not shown).

In agreement with previously reported findings in both transformed cells and primary tissue (19), PCR analysis (Fig. 2 *B*) revealed that pre-B cells rearrange different V_H gene families at distinct frequencies corresponding to their distance from the constant region gene cluster. We assayed V to DJ rearrangement in HAFTL A and 300-19 cells using the J3 primer with each of three degenerate V_H gene family oligonucleotide primers in separate reactions. As shown in Fig. 2 *B*, hybridizing fragments representing V_H7183 and V_HQ52 rearrangements were much more intense than those obtained using the V_H558 primer, despite the fact that members of the more distal V_H558 gene family are much more numerous.

We found that D to J rearrangements are asymmetric. Symmetrical hepatamer-12 nucleotide spacer-nonamer sequences lie both 5′ and 3′ to each identified D gene (20, 21). Previous studies have shown that rearrangement can occur with deletion or inversion of the intervening DNA (22, 23). PCR analysis using primers specific for either the 5′ or 3′ D_H recombination signal sequences (Fig. 2 *C*) revealed the nearly exclusive use of the 3′ sequence for D to J rearrangement in HAFTL A cells, although faint signals could be seen in the D_H R lane on lengthy exposure. We cannot as yet offer a mechanistic explanation of this observation.

As noted above, the recombination signal sequences flanking the D genes are symmetrical. If template accessibility alone

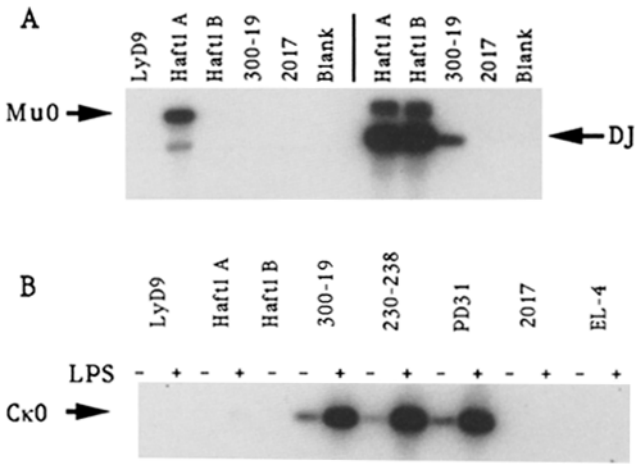

Figure 3. RNA PCR evaluation of germline Ig H chain or κ L chain constant region transcription. (*A*) RNA from the indicated cell lines were assayed for germline Mu (*Mu0*) and partially rearranged DJ allele transcripts by PCR. The positions of the various PCR products are indicated. (*B*) RNA from the indicated cell lines, grown in the presence (+) or absence (−) of LPS, were assayed for germline κ constant region transcription. The position of the germline C_κ transcript is indicated.

dictates Ig gene rearrangement, one might expect to detect V to D rearrangements in cells that transcribe unrearranged V_H genes (see below) and carry out DJ rearrangement. However, we were unable to detect any V to D rearrangement in a wide variety of pre-B cells using the three V_H gene PCR primers and the D_H R primer (data not shown), indicating a level of control beyond that of accessibility alone.

This sensitive, population-based PCR analysis of H chain gene rearrangement confirms and extends previous data. The DJ rearrangements previously reported in HAFTL and 300-19 were confirmed, but previously unrecognized VDJ alleles were detected, showing that these cells are not strictly blocked in progression through the rearrangement process. The preference for rearranging Cμ-proximal V_H genes was definitely shown here. The polarity of D to J rearrangements and the lack of V to D events show that complex controls direct the rearrangement process.

Germline Transcripts of Ig Constant Region Genes. To test the hypothesis that transcriptional activity is required for Ig gene rearrangement, we surveyed a variety of lymphoid cell lines, whose potential for gene rearrangement we had demonstrated, for the presence of transcripts of unrearranged or partially rearranged (DJ) H and κ L chain alleles. RNA was reverse transcribed using random hexameric primers and then amplified with primers located such that they span the J-Cμ intron (Fig. 1) or the J-Cκ intron. The sizes of the PCR products we detected corresponded exactly to transcripts that initiated upstream of the 5′ PCR primer (Mu0, D_H L, or K^o), and were properly spliced from the first J gene to the first constant region exon (Fig. 3).

We detected an amplified transcript from the completely unrearranged H chain gene in HAFTL cell lines, but not in the pro-B cell line LyD9 or in the pre-T cell line 2017 (Fig. 3 *A*). It was transcribed, however, in EL-4 T cells (data not shown). Partially rearranged DJ alleles, previously shown to

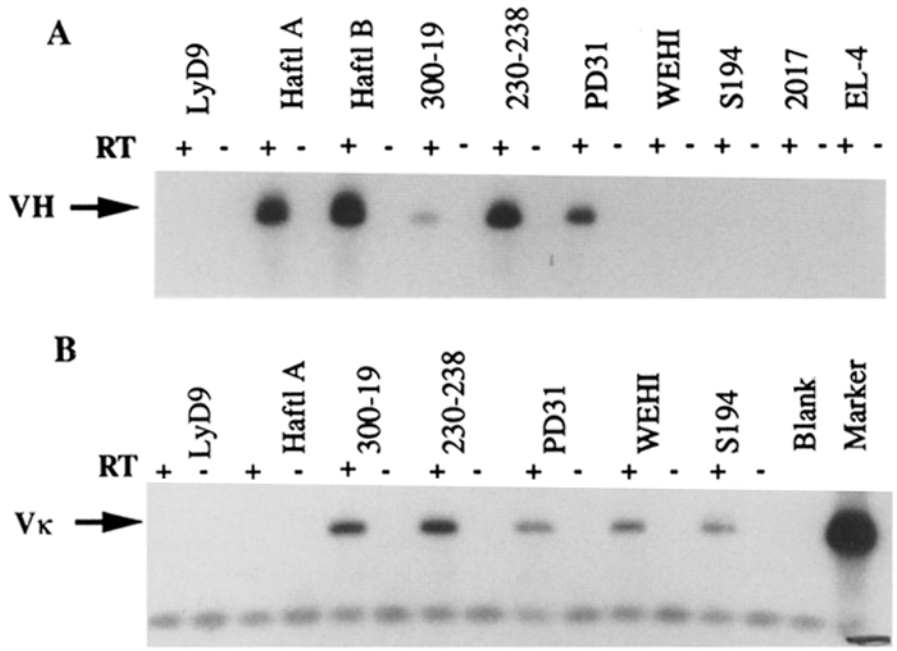

Figure 4. RNA PCR assays for the presence of germline V_H and V_κ gene transcripts. RNA samples from the indicated cell lines were assayed for germline V_H (*A*) and germline V_κ (*B*) transcription. Samples indicated by + and − either did or did not contain reverse transcriptase in the cDNA synthesis step of the assay. The lane labeled *Blank* is a control reaction that contained no template.

be transcriptionally active by Reth and Alt (24), were transcribed whenever present in pre-B cells (see below).

We could detect an amplification product corresponding to a spliced germline κ constant region transcript (25, 26) constitutively in all pre-B and B cells at or beyond the stage of development represented by 300-19 cells (Fig. 3 *B*). This transcript was weakly inducible by LPS treatment at an earlier stage (HAFTL) but not in LyD9 cells. Intense PCR signals from treated cultures (Fig. 3 *B*) showed that LPS strongly induced the germline C_κ transcript in most pre-B cells, as had been reported previously (27). Germline κ transcripts were not induced in T cells treated with LPS (Fig. 3) or with PMA and PHA (data not shown), treatments known to induce the κ enhancer binding protein NF-κB in these cells (28). The presence of these germline transcripts correlated with competence for Ig gene rearrangement. Each cell line capable of rearranging a given locus made a readily detectable germline transcript of that locus (see Discussion).

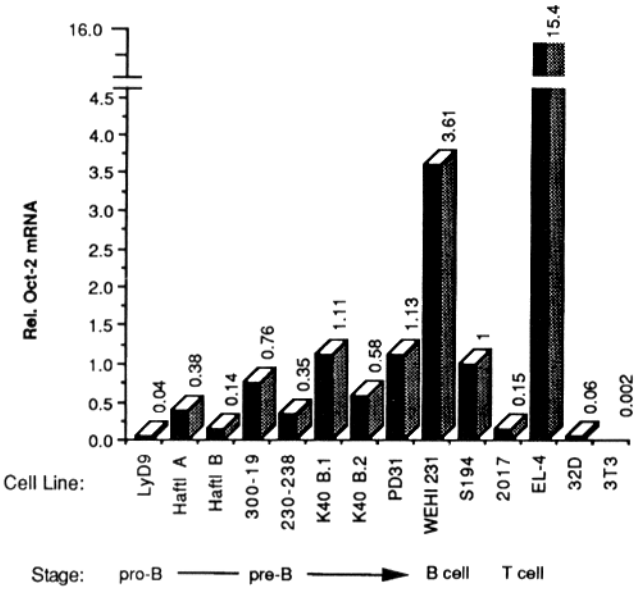

Figure 5. Relative levels of *Oct-2* mRNA in various lymphoid cell lines. RNA from the indicated cell lines was assayed for *Oct-2* sequences and actin mRNA by RNase protection. Radioactivity was quantified using a blot analyzer (Betagen) and normalized based on the assumption of constant levels of actin mRNA. The amounts of *Oct-2* message, relative to that present in the B cell S194, are shown. The results shown for HAFTL A and HAFTL B are the averages of three and two independent assays, respectively. The remaining assays were single determinations.

Unrearranged Variable Gene Transcription. Yancopoulos and Alt (29) previously reported the existence of transcripts of unrearranged H chain variable genes in both cultured pre-B cells and mouse fetal liver. We designed PCR assays to detect these V_H transcripts (Fig. 1) as well as possible transcripts of unrearranged κ L chain variable genes to further test the relationship between transcription and Ig gene rearrangement. The primers used for the reverse transcription step of the assays were degenerate oligonucleotides with homology to the heptamer-spacer region 3′ to the V_H558 family of genes or to a majority of the V_κ genes. Therefore, only unrearranged gene transcripts could be reverse transcribed. We then amplified the cDNA using the complement of the V_H or V_κ (9) DNA amplification primers and a second degenerate primer, V_HB or $V_\kappa B$, complementary to sequences at the 5′ end of the V_H and V_κ gene families (16). Since these assays do not cross intron/exon boundaries, we performed control reactions that lacked reverse transcriptase (Fig. 4) to eliminate DNA contamination as an artifactual source of PCR amplification products. None of the RNA samples produced an amplified product in the absence of reverse transcriptase. We could first detect V_H transcripts in HAFTL cells, and they were present in all the pre-B cells we assayed (Fig. 4 *A*). They were absent, how-

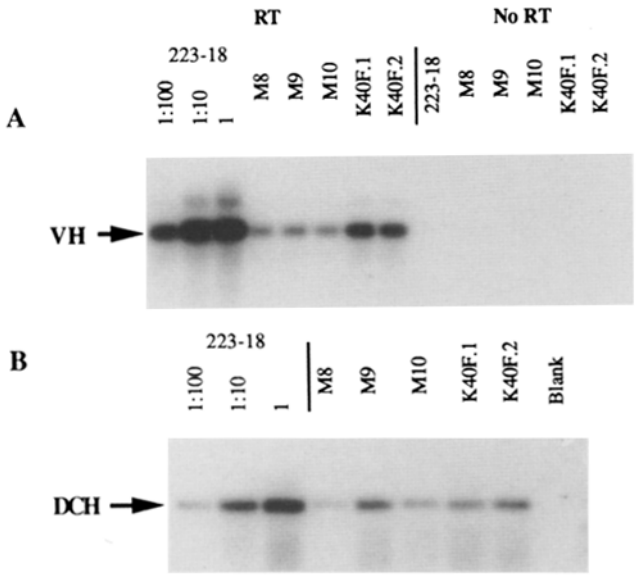

Figure 6. Germline V_H and DJ transcription in H chain–positive and –negative pre-B cell lines. We analyzed, by RNA PCR, the amounts of germline V_H (*A*) and DJ (*B*) transcription in the indicated pre-B cell lines. The quantitative nature of the assays was demonstrated by assaying several dilutions of 223-18 RNA (lanes labeled *1:100*, *1:10*, and *1*). The lanes labeled *No RT* are control reactions from which RT was omitted. The lanes labeled *Blank* were control reactions containing no template. The position of the amplified DJ transcript is labeled DCH.

ever, from the two mature B cell lines studied, as well as from T cells. Unrearranged V_κ transcripts were absent from LyD9 and HAFTL cells, but present in the remaining pre-B and mature B cells. V_κ transcripts were absent from the two T cell lines tested. Cloning and sequencing of amplified V_κ transcripts showed that they initiate near the same promoter sequence used by rearranged κ genes (data not shown). It is of note that these two transcription units, V_H and V_κ, contain conserved octamer sequences (30; see below) in their promoters but were differentially regulated.

Oct-2 mRNA Levels during B Cell Development. The lymphoid-specific transcription factor Oct-2 binds in vitro to a conserved octameric DNA sequence in the V_H and V_κ promoters and the H chain enhancer (31). To determine whether quantitative modulation of Oct-2 levels might account for differential regulation of these octamer-containing transcription units, we measured the relative amounts of Oct-2 mRNA by RNAse protection in a variety of pre-B, B, and T cells. Differences in Oct-2 mRNA levels, while extensive, did not correlate with the regulation of the V_H, V_κ, μ germline, or DJ transcripts (Fig. 5). EL-4 T cells, which had high levels of Oct-2 mRNA but no detectable V_H or V_κ transcripts, did, however, transcribe the germline H chain locus. Mature B cells had significant amounts of Oct-2 mRNA and they expressed V_κ but not V_H transcripts. The situation is reversed in HAFTL cells, which had modest Oct-2 mRNA levels and V_H but not V_κ transcripts. HAFTL cells also transcribed the germline H chain gene, presumably with the involvement of the octamer-containing H chain enhancer. Therefore, other factors, possibly acting in conjunction with Oct-2, must be responsible for determining the pattern of V_H, V_κ, DJ, and germline μ gene transcription during development.

A Productive H Chain Gene Rearrangement Does Not Affect Germline H Chain Gene Transcription or Rearrangement. In general, B cells have been found to express only one functionally rearranged H chain and one functionally rearranged L chain gene (10, 11). Various investigators have hypothesized that allelic exclusion is a regulated phenomena (3). That is, pre-B cells that have productively rearranged an Ig locus inactivate the allelic locus for further rearrangement. Furthermore, it has been proposed that template accessibility, as reflected by transcriptional activity of unrearranged gene segments, determines rearrangement; actively transcribed genes rearrange and, similarly, genes that are not transcribed do not rearrange. To test these hypotheses, we examined a variety of cell lines with productive H chain gene rearrangements for their levels of unrearranged gene transcription and the presence of ongoing H chain locus rearrangement.

We studied Abelson virus–transformed pre-B cell lines with the following H chain genotypes: 223-18 VDJ^+/DJ (+ and – indicate productively and nonproductively rearranged alleles); M8 VDJ^-/DJ; M9 VDJ^+/DJ; M10 VDJ^-/DJ; K40F.1 VDJ^-/DJ; and K40F.2 VDJ^+/DJ. K40F.2 was obtained by sib-selection for cytoplasmic μ fluorescence from a population of K40F.1 (Alan Shapiro and Anthony DeFranco, University of California, San Francisco; unpublished data). PCR analysis showed that K40F.2 was VDJ^+ by virtue of V gene replacement (see below).

RNA PCR assays of DJ and V_H transcripts showed no consistent difference in the amount of DJ or unrearranged

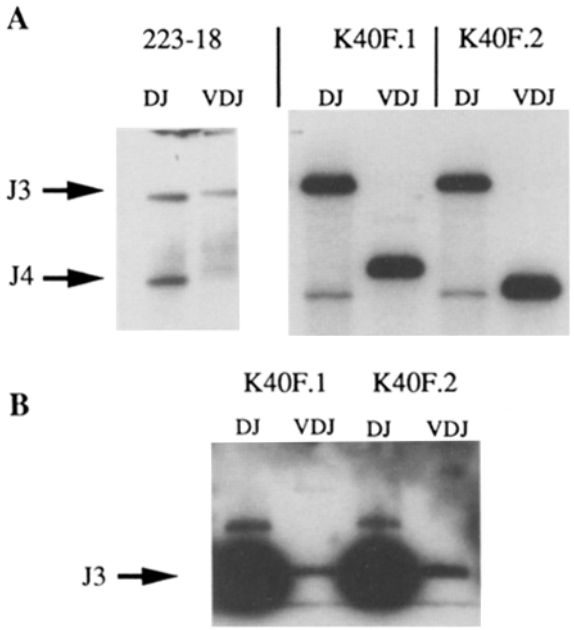

Figure 7. DJ and VDJ PCR assays on various H chain–positive and –negative pre-B cell lines. (*A*) The J4 3′ PCR primer (Fig. 1) was used to assay the indicated cell lines for rearrangement events involving J_H3 and J_H4 (*B*). The J3 3′ PCR primer was used to examine DJ3 and VDJ3 rearrangements in the indicated cell lines.

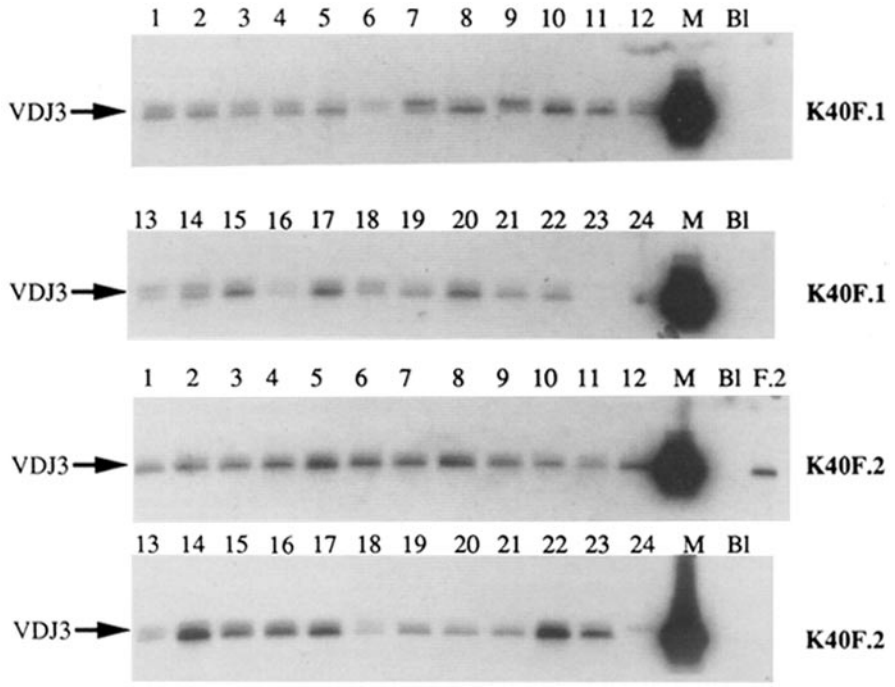

Figure 8. VDJ3 rearrangements in limiting dilution subclones of H chain–positive (K40F.2) and –negative (K40F.1) cell lines. The PCR assay of VDJ3 rearrangements in 24 subclones of each cell population is shown with the position of the VDJ3 signal indicated. Lanes labeled *M* contain positive control reactions, *Bl* contain no template controls, and *F.2* contain an assay of the parental K40F.2 cell population.

V_H transcripts between cell lines that produce H chain protein and those that do not (Fig. 6). The assay provides quantitative information because increasing amounts of 223-18 RNA resulted in increasing amounts of amplified product (Fig. 6, *A* and *B*). Therefore, the presence of a productive H chain gene rearrangement does not affect the regulation of unrearranged gene transcription in Abelson virus–transformed pre-B cell lines.

We next asked whether productive rearrangement of the H chain locus inactivated further H chain gene rearrangement in these cell lines. We used a 3′ primer (J4; Fig. 1) downstream of J_H4 to look at rearrangement events involving J_H3 and J_H4. Despite being a cloned, H chain–positive cell line with the reported genotype DJ3/VDJ3, 223-18 had readily detectable levels of DJ4 and VDJ4 rearrangements in addition to its productively rearranged VDJ3 allele (Fig. 7 *A*). PCR analysis showed that both K40F.1 and K40F.2 had the genotype DJ3/VDJ4 (Fig. 7 *A*). The amplified VDJ4 allele in K40F.2 was slightly shorter, leading us to conclude that either a V gene replacement event (32) or a small deletion

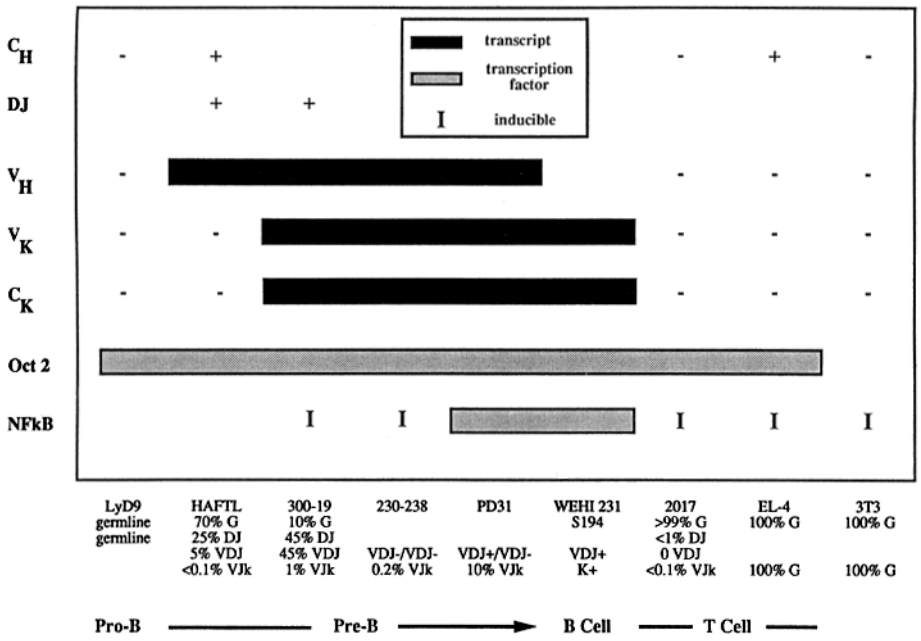

Figure 9. Germline Ig gene transcription and rearrangement in cell lines representing various stages of B cell development. The approximate distribution of gene rearrangement in H and L chain loci, as determined by PCR, is indicated beneath each cell line. The amounts of Oct-2 and NF-κB vary in the different cell lines. The C_κ transcript is constitutive in the indicated cell lines, but markedly inducible in 300-19, 230-238, and PD 31. Data for NF-κB are from both our own work and that of others (19, 36–41).

was responsible for K40F.2's H chain positivity. The presence of faint amplified DJ4 fragments in both siblings indicated remarkably similar rates of ongoing DJ rearrangement with the pre-existing DJL3 allele being replaced by DJ4 rearrangements in some chromosomes (Fig. 7 *A*). A closer look at the DJ3 allele using the J3 3′ PCR primer showed that V to DJ joining also occurred (Fig. 7 *B*, lanes labeled *VDJ*) despite the presence of a productive VDJ4 rearrangement.

To compare the rates of V to DJ rearrangement in these sibling cell lines differing only in the functional nature of their VDJ4 rearrangement, we analyzed 24 limiting dilution subclones of each line for the presence of V to DJ3 rearrangement. All of the subclones had roughly similar amounts of VDJ rearrangement regardless of the preexistence of a functionally rearranged H chain gene (Fig. 8).

We conclude from these observations that Abelson virus–transformed pre-B cells do not demonstrate allelic exclusion of H chain gene rearrangement and that H chain protein synthesis does not affect unrearranged H chain gene transcription.

Discussion

Lymphoid development consists of a cascade of regulated, and apparently interdependent, transcriptional activation and gene rearrangement events. During their development, committed progenitor cells give rise to Ig surface–positive B cells over the course of three to five cell divisions (33). Abelson virus, and other agents, can transform committed cells into continuously growing cell lines that display indefinitely the characteristics of pre-B cells. We had previously seen that individual lines differ in phenotype and comprise a series of cells that define steps in the development of the lineage (2).

With the vastly more sensitive PCR assay and with the ability to assay many more events of immunodifferentiation, we now see that each transformed cell line represents a wider range of stages than was previously evident and that whereas developmental events are initiated in a time progression, there is little termination of the events (Fig. 9). Thus, these cell lines neither display allelic exclusion nor do they terminate V_H transcription even if they contain and express a productive VDJ allele. We conclude that transformed pre-B cells are valuable for studying the initiation of various stages of immunodifferentiation but not necessarily their further regulation. We can find a perfect correlation between the initiation of transcription of a gene segment and its ability to be rearranged as was implied by previous, more fragmentary evidence (Fig. 9) (9, 24, 29, 34).

The Control of κ Gene Transcription and Rearrangement. This study has provided the first data on the initiation of transcription around the κ L chain locus. Both V_κ and C_κ transcription are undetectable in the HAFTL cell lines, the earliest stage we have that actively rearranges H chain loci, and become evident simultaneously in 300-19 and 230-238, lines that represent more mature stages in the lineage. These events correlate to the appearance of active NF-κB in the cell nucleus, which could direct C_κ transcription by binding to its site in the intron κ enhancer. The V_κ genes, however, have no known NF-κB sites in their promoters. Oct-2, a protein of the B cell lineage, is present in HAFTL cells and therefore does not correlate to κ gene segment transcription. It also does not correlate with either V_H or $C\mu$ transcription (Fig. 9), and therefore does not appear to have a determinative role in any of the regulatory events we have studied.

The Relationship between Transcription and Ig Gene Rearrangement. Our data support the notion that the transcriptional activity of germline Ig genes either causes or reflects local changes in chromosome structure that play a role in targeting the rearrangement process. All rearranging loci in cultured pre-B cells are transcribed before or coincident with their rearrangement (Fig. 9). Furthermore, DJ, but never VDJ, rearrangement occurs in some T cells (20), and we found that some T cells transcribe the germline H chain constant region gene but not the V_H locus.

We can suggest several ways in which transcription and recombination might be related. The recombination apparatus might interact directly with specific transcription factors or recognize DNA sequences made accessible by the presence of transcription complexes. Alternatively, it might utilize germline transcripts in the rearrangement mechanism. Conversely, it is possible that transcription is the result of recombinase interaction with various loci and the structural changes these interactions might induce. This is somewhat less likely in our view since the appearance of germline C_κ transcripts clearly precedes κ gene rearrangement and increased κ transcription correlates with an increased rate of κ gene rearrangement (9).

While transcriptional activation provides a satisfying explanation for the targeting of rearrangement, the present study does not allow us to make conclusions regarding the potential role of transcription in the inactivation of H chain gene rearrangement because we have demonstrated that H chain–positive cell lines continue to rearrange the H chain locus.

H Chain Protein and the Regulation of Gene Rearrangement. H chain protein is first detected in the pre-B cell lineage before the activation of κ gene rearrangement (2). Furthermore, no reports exist of mature B cells that produce two H chain proteins carrying different idiotypes (allelic exclusion; 10, 11). These facts have led to the suggestion that H chain protein might serve a central regulatory role in the B cell lineage, signalling the cessation of H chain gene rearrangement and the activation of L chain rearrangement (3, 6, 8). One report suggested that a transfected, protein-producing H chain gene could arrest V to DJ joining and activate κ rearrangement in the cell line 300-19 (8). A second report (29) showed that germline V_H gene transcription decreased in transformed cells containing a functional H chain gene rearrangement.

We compared Abelson virus–transformed pre-B cells of similar maturity that differed only in the outcome of their H chain gene rearrangement: 223-18, M9, and K40F.2 produced H chain protein, whereas M8, M10, and K40F.1 did not. Our RNA PCR assays failed to reveal a difference in germline V_H or DJ transcription among these cell lines (Fig. 6). Moreover, H chain–producing cell lines continued to rearrange the H chain locus (Figs. 7 and 8). We conclude that

H chain protein does not regulate unrearranged H chain gene transcription or rearrangement in Abelson virus–transformed pre-B cells. The role of H chain protein in the regulation of Ig gene rearrangement in nontransformed cells remains to be elucidated.

These results are in apparent contradiction with those obtained from several sets of experiments involving Ig H chain transgenic mice where inhibition (albeit incomplete) of endogenous H chain gene rearrangement was observed (reviewed in reference 35). The transgenic experiments were analyzed either by using Abelson virus or hybridoma technology to generate pre-B and B cell clones and by determining their genotypes by blotting or by assessing endogenous antibody production immunologically. They clearly indicated that transgenic expression of one complete H chain gene can partially inhibit rearrangement of the endogenous H chain gene segments. While a variety of interpretations of both our experiments and the H chain transgenic experiments are possible, the transgenic results are particularly clear, and it remains to be shown why the Abelson virus transformants do not behave as might have been predicted from them.

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