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Novel B-Cell Precursors Blocked at the Stage of DJ_H Recombination

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Abelson murine leukemia virus-transformed cells have provided the principal model for study of the early events in immunoglobulin gene rearrangements. In this communication, we describe a new type of Abelson virus-transformed pre-B-cell line that is arrested at the DJ_H stage of the recombination process. These cells differ from other pre-B transformants with respect to two properties associated with the immunoglobulin rearrangement process. First, in contrast to cell lines undergoing V_H-to-DJ_H joining in vitro, none of these cell lines contained detectable levels of RNAs transcribed from their unrearranged V_H genes. Second, only some of the cell lines recombined exogenous heptamer-nonamer sequences, indicating that many of them have lost at least a portion of the enzymatic machinery that mediates recombination. The correlation between the absence of unrearranged V_H RNAs and the inability to rearrange endogenous immunoglobulin gene segments suggests that V_H gene transcription is required both to maintain an active recombination system and for the final step in variable-region formation.

Early B-cell differentiation is characterized by assembly of a functional heavy-chain gene involving two recombinatorial events between three separate elements, V_H, D, and J_H (reviewed in reference 37). Studies of Abelson murine leukemia virus (Ab-MLV)-transformed and other tumor cells have revealed that the recombination process is ordered, with D-to-J_H recombination preceding V_H-to-DJ_H recombination (reviewed in reference 1). The mechanism directing the ordered rearrangement of immunoglobulin genes is not understood. Accessibility of the participating gene segments to the enzymes and factors involved in recombination is likely to play a key role in the process (4, 39). Consistent with this notion, both unrearranged V_H segments and rearranged DJ_H segments are transcribed in early B-cell precursors that are in the process of V_H-to-DJ_H joining and RNAs transcribed from unrearranged V_H segments are prominent in tissues rich in early B-cell precursors (2, 40).

Analysis of immunoglobulin structure and expression has identified two types of Ab-MLV-transformed pre-B cells. Cell lines of the first type are most often derived from fetal liver and usually contain only 1 to 10% cytoplasmic μ-positive cells (38). These cells have undergone DJ_H rearrangements and continue the rearrangement process in vitro as evidenced by the accumulation of V_H-to-DJ_H and D replacement rearrangements in the cells (3, 11, 23, 24). This type of continually rearranging cell line will be referred to here as DJ-R (rearranging). In contrast, nearly all the cell lines derived from adult bone marrow that have been analyzed have completed $V_H DJ_H$ rearrangement at least on one allele (3). While most of these cell lines contain 50 to 100% cytoplasmic μ-positive cells, some of them are μ negative because the imprecision inherent to the rearrangement process has created nonsense or missense mutations (reviewed in reference 1; 3).

Because previous analyses of Ab-MLV-transformed cells derived from adult tissues have centered on μ-positive cells (3), we examined the immunoglobulin gene structure in a panel of μ-negative transformants derived from the bone marrow of normal adult mice. This study revealed a new

type of μ-negative Ab-MLV-transformed B-cell precursor that has undergone only DJ_H joining and does not undergo further rearrangement in vitro at an appreciable frequency. These cell lines are similar to other Ab-MLV-transformed cells with respect to expression of differentiation markers associated with early B-lineage cells (20; reviewed in reference 25). However, only some of these cell lines possess an active recombination system as judged by their ability to recombine exogenous signal sequences. In addition, although all the cell lines contain RNAs transcribed from their DJ_H units, none of these cell lines contain detectable levels of RNAs transcribed from unrearranged V_H genes. This new cell type, arrested at the stage of DJ_H rearrangement, provides a unique model system to study the regulation of the two recombinatorial events in immunoglobulin heavy-chain gene assembly.

MATERIALS AND METHODS

Cell lines. Transformed pre-B-cell clones were derived from normal adult bone marrow (1881, 192-11, 298-13, 298-18, 298-26, 300-3, 300-9, 300-10, 300-16, 300-19, 300-25, 300-26, 300-30, 300-31, 300-35, C-9, C-11, 480-35, 480-36, 480-39, and 481-29), from bone marrow of adult mice treated with 250 mg of cyclophosphamide per kg of body weight (14-4, 2-1-1, 2-10-2, 2-3-1, 3-1-1, 14-1, 7-8, 2-6-2, 21-7, 2-11-2, 21-10, 21-6, 7-4, and 2-3-2), or from fetal liver (22D6, 22D10, 38B9, 40E1, 40E3, and 41B1) as previously described (26). All the cell lines classified in the Results section as DJ-R contain a single, unique Ab-MLV integration except for 2-1-1, which contains two molar integration sites (3; data not shown). All DJ-F cells contain an intact J_H-$C_μ$ intron in the vicinity of the EcoRI site 3' of J_{H4} (data not shown). Established cell lines were maintained in RPMI 1640 medium supplemented to contain 10% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol. Some cell lines were subcloned by plating the cells at a density of 0.3 cells per well in flat-bottomed 96-well plates (Becton Dickinson Labware, Oxnard, Calif.). After 10 days, subclones were transferred first to 24-well plates and then to 60-mm plates and maintained in the same fashion as other transformants (27).

Immunofluorescence analysis. Expression of cytoplasmic μ protein was determined by staining with a fluorescein iso-

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thiocyanate-conjugated goat anti-mouse immunoglobulin M (IgM) antibody (Organon Teknika, Malvern, Pa.) (38). In most cases, the synthesis of μ was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of [^{35}S](B220)-labeled immunoprecipitates (28). Expression of ly5(B220) and the BP1 determinant was monitored by surface immunofluorescence (33) with appropriate antibodies. The mouse monoclonal IgG2a antibody BP1 (9) (gift of M. D. Cooper, University of Alabama at Birmingham), the rat monoclonal anti-ly5 IgM antibody RA3.3A1/6.1 (7), and the biotinylated monoclonal rat anti-Thy1.2 antibody 30-H12 (16) were used in conjunction with goat anti-mouse IgG2a, goat anti-rat IgM (Southern Biotechnology Associates), and fluorescein isothiocyanate-avidin (E-Y Laboratories, Inc.), respectively.

DNA and RNA analysis. DNA and RNA were obtained by the method of Chirgwin and co-workers (6). Briefly, cells were lysed in a 4 M guanidinium lysis buffer, and the cell lysates were centrifuged through a 5.7 M CsCl gradient. DNA was obtained by collecting the viscous material just above the CsCl cushion and was recovered by precipitation in 2 volumes of 95% ethanol. The DNA was washed with 70% ethanol and suspended in 10 mM Tris–1 mM EDTA (pH 8.0). Total cellular RNA was obtained from the same CsCl gradient by suspending the pellet at the bottom of the tube following centrifugation (6). DNA from subclones was prepared by adding lysis buffer (10 mM EDTA, 10 mM Tris [pH 7.4], 25 mM EDTA, 1% sodium dodecyl sulfate) to approximately 5 × 10^5 cells and incubating the mixture with 50 μg of proteinase K per ml for 1 h (W. Frankel, Ph.D. thesis, Albert Einstein College of Medicine, Bronx, N.Y.) The lysates were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with ether and precipitated in 0.5 M ammonium acetate and 2.5 volumes of 95% ethanol at room temperature. The DNA was then washed twice in 70% ethanol, and the total yield was used in a single restriction digestion without quantitation.

The DNAs were digested with EcoRI (New England BioLabs, Inc., Beverly, Mass.), fractionated through 0.8% agarose, and transferred to GeneScreen Plus membranes (Dupont, NEN Research Products, Boston, Mass.). The membranes were hybridized as recommended by the supplier to J$_H$ and 5' D probes (3) (gifts of F. Alt, Columbia University) that had been labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Dupont, NEN Research Products) by the random oligonucleotide priming method (10). The RNAs were fractionated through formaldehyde-agarose gels and transferred either to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) or to GeneScreen Plus. The membranes were hybridized as recommended by the suppliers to a J558 V$_H$ probe (5) (gift of P. Brodeur, Tufts University School of Medicine), and to oligonucleotide probes directed against J$_{H2}$, J$_{H3}$, and J$_{H4}$ or the lambda 5 gene (30). The J558 probe was labeled by the random priming method (10), while the oligonucleotides were labeled with polynucleotide kinase and [γ-^{32}P]ATP (7,000 Ci/mmol; Dupont, NEN Research Products) (36).

Assay for recombination activity. The plasmid pJH201 (12) (gift of M. Gellert and M. Lieber, National Institutes of Health) was introduced into 2 × 10^6 cells as described previously (12). Following transfection, the cells were plated in 0.5 ml of growth medium in 24-well plates. A small-scale plasmid preparation protocol (19) was used to recover the plasmid DNA from the cells 48 h after transfection. The DNAs were digested or mock digested with DpnI, and 1 μl of the mixture was used to transform 20 μl of competent Escherichia coli DH5α cells (Bethesda Research Laborato-

ries, Inc., Gaithersburg, Md.). The cells were then plated onto agar plates containing either 100 μg of ampicillin per ml or 100 μg of ampicillin and 11 μg of chloramphenicol per ml, and the number of colonies growing on each type of plate was counted 18 to 20 h later. The ratio of colonies in the two types of plates was used to calculate the frequency of recombination. Most cell lines were transfected two to three times, and because similar recombination frequencies were obtained from each preparation, the data from individual experiments were combined.

In the initial experiments, the proportion of ampicillin-resistant colonies obtained from DpnI and mock-digested DNAs was used to measure the frequency of replicated molecules in each cell line (17). Subsequent experiments used only undigested DNA, and the number of colonies obtained was adjusted to reflect the frequency of recombinants among replicated molecules. In several cases, including those involving cells classified as DJ-F, representative chloramphenicol-resistant colonies were analyzed to examine the authenticity of the recombination event. For this analysis, DNA from chloramphenicol-resistant colonies was digested with HgiAI and fractionated through a 1.2% agarose gel to detect unique fragments diagnostic of heptamer-nonamer-mediated recombination (12). In all cases, a pattern consistent with heptamer-nonamer-mediated recombination was observed.

RESULTS

Several μ-negative Ab-MLV transformants have a unique immunoglobulin gene structure. The immunoglobulin heavy-chain gene structure in a panel of 11 clonally derived, μ-negative, Ab-MLV-transformed lymphoid cells derived from normal adult mouse bone marrow was examined by Southern blotting. First, EcoRI-digested DNAs were analyzed with a J$_H$-region probe to identify rearranged variable-region fragments. Then the DNAs were reprobed with a mixture of 5'-flanking D-region probes to assess the presence and linkage of the DJ$_H$ segments to V$_H$ segments. Immunoglobulin heavy-chain genes that have completed variable-region assembly have deleted all D-related fragments and thus do not hybridize to this probe mixture (3, 27). In contrast, heavy-chain genes that have only completed D-to-J$_H$ joining contain rearranged D-related fragments that comigrate with the rearranged J$_H$ fragments. Thus, this assay, which has been validated by cloning and analysis of 27 heavy-chain gene segments (3), can be used to assess the nature of immunoglobulin heavy-chain gene rearrangements.

Analysis of the panel of μ-negative cells revealed that, like virtually all Ab-MLV transformants analyzed to date (reviewed in reference 1), all the cell lines contained rearranged J$_H$ fragments on both alleles (data not shown). In addition, 4 of the 11 cell lines had at least one rearrangement that was not associated with a rearranged D-related gene segment, indicating the presence of a V$_H$DJ$_H$ joint at that allele. A representative cell line in which both J$_H$ fragments are rearranged and all D-related fragments are deleted is shown in Fig. 1B. In contrast to this pattern, 7 of the 11 μ-negative cell lines analyzed had undergone only DJ$_H$ rearrangements on both alleles. Representatives of this type of cell line are shown in Fig. 1G through K. In this respect, these cell lines are similar to the predominant type of cell line isolated from fetal liver (3) or cyclophosphamide-treated bone marrow (L. Ramakrishnan and N. Rosenberg, manuscript in preparation). However, in contrast to these previously described cells, the DJ$_H$ cells in the bone marrow panel lacked multiple
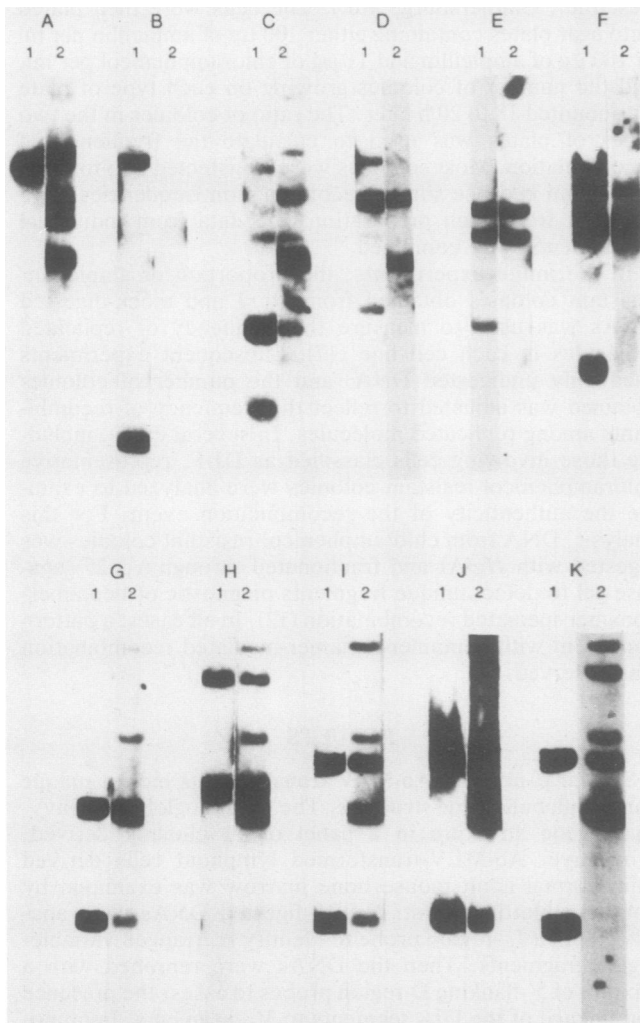
FIG. 1. Immunoglobulin gene structure in pre-B-cell lines. Southern analysis of 10 μg of EcoRI-digested genomic DNA was performed first with a J_H probe (lanes 1) and subsequently with a mixture of the 5' D-flanking probes 5'DSP2 and 5'DFL16 (lanes 2) (3). Liver DNA (A); DNA from 300-16, a representative VDJ cell line (B); DNA from 2-1-1, 14-4, 2-10-2, and 481-29, representative DJ-R cell lines (C through F, respectively); and DNA from 300-3, 300-30, 300-31, 300-25, and 300-10, representative DJ-F cell lines (G through K, respectively) are shown.

CLONE	GENE STRUCTURE	FREQUENCY REARRANGED	FREQUENCY IG POSITIVE
481-29	DJ-R	18/33	7/10
2-6-2	DJ-R	8/12	NT
300-31	DJ-F	1/29	0/30
300-35	DJ-F	0/29	0/30

FIG. 2. Immunoglobulin gene structure in subclones of DJ-R and DJ-F cell lines. Southern analysis of genomic DNA from subclones was done with a J_H probe. DNA was obtained by a small-scale preparation technique (Frankel, Ph.D. thesis), digested with EcoRI, and used for the analysis without quantitation. Analysis of representative subclones from the DJ-R cell line 481-29 (A) and two DJ-F cell lines, 300-31 and 300-35 (B and C respectively), is shown. L, Liver DNA; P, DNA from parental cell line.

submolar J_H fragments such as those evident in lanes 1 of Fig. 1C through F. As shown by others (3, 23, 24), such fragments represent new DJ_H or V_HDJ_H recombinations in subpopulations of clonal DJ-R cell lines, and their absence in the cell lines analyzed in Fig. 1G through K suggests that these cells may have ceased rearrangement at the stage of DJ_H recombination. We have termed this type of cell line DJ-F (fixed).

D replacements and V_H-to-DJ_H rearrangements do not occur frequently in DJ-F cells. The presence of cytoplasmic μ-positive cells in a DJ-R population is a sensitive indicator of ongoing formation of productive heavy-chain variable-region genes, and a typical DJ-R cell population contains 1 to 10% μ-positive cells (3, 37; unpublished data). Analysis of cytoplasmic μ production in all seven of the DJ-F cells revealed that fewer than 1 in 2,000 cells in each population synthesized μ, suggesting that, consistent with the Southern
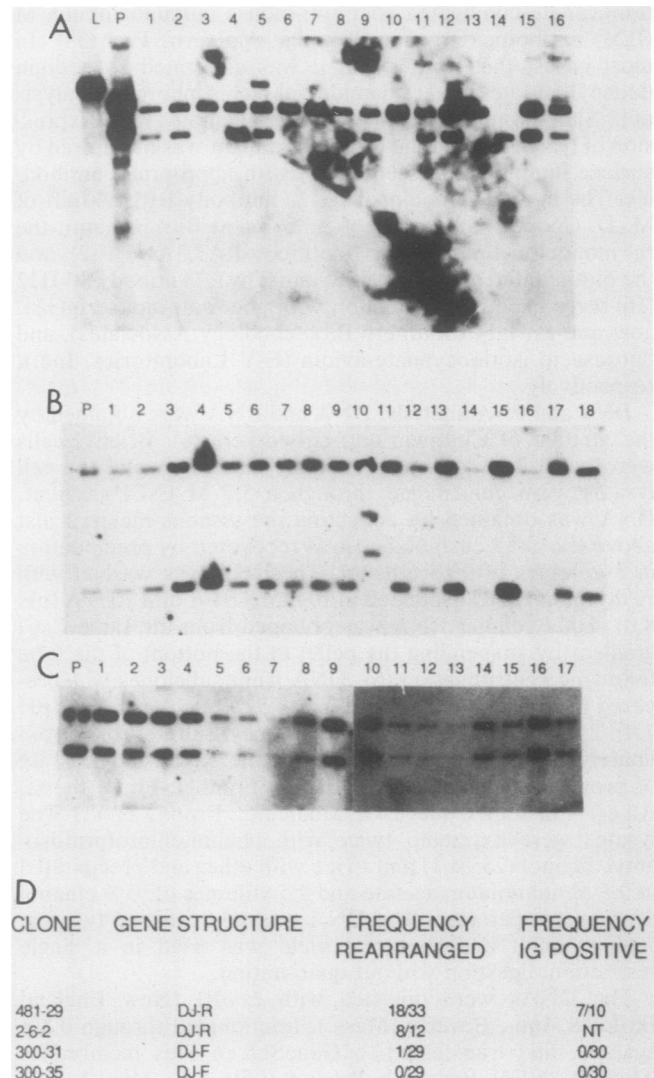
analysis, productive V_HDJ_H rearrangements were not being formed in culture. However, the DJ-F cells could have been undergoing a very high frequency of either nonproductive V_HDJ_H rearrangements or D replacement rearrangements (23, 24). Neither of these types of rearrangements would give rise to cytoplasmic μ-positive cells, and the presence of a large number of different rearrangements might not be detected in the Southern blotting assay.

To determine whether the DJ-F cell lines were actually undergoing rearrangements at a high frequency, we prepared subclones of two representative DJ-F and DJ-R cell lines. DNA from randomly chosen subclones was examined for changes in immunoglobulin gene structure. As expected, subclones with new J_H rearrangements were detected in both DJ-R populations (Fig. 2D). In the representative

sample shown (Fig. 2A), new J_H fragments were detected in some clones (lanes 5, 7, 8, 11, 15, and 16), while others had lost a parental J_H fragment without acquiring a new one (lanes 3, 6, 10, 13, and 14). In contrast, only one subclone (Fig. 2B, lane 10) from both of the DJ-F cell lines examined (Fig. 2B and C) had undergone a change in J_H gene structure. In this case, only one of the new fragments was associated with a newly rearranged D-related fragment, suggesting that the second was a $V_H DJ_H$ recombination. The possibility that this rearrangement reflected a deletion affecting the *Eco*RI site in J_H-C_μ was not examined because all the subclones were analyzed by small-scale DNA preparations. However, assuming that this rearrangement represented a V_H-to-DJ_H joining event, the frequency of rearrangement in the DJ-F cells examined is at least 15 times lower than in the DJ-R cells (Fig. 2D).

Consistent with the results obtained in the Southern blotting, immunofluorescence analyses revealed that 7 of the 10 subclones examined from one of the DJ-R cells expressed μ (Fig. 2D). Indeed, those subclones in which rearrangement appeared to be most active based on the results of the Southern analysis contained 1 to 10% μ-positive cells. This frequency is consistent with the idea that many new rearrangements are occurring, a fraction of which lead to immunoglobulin production. In contrast, none of the subclones from either of the DJ-F cell lines expressed μ protein (Fig. 2D).

DJ-F cells resemble B-lineage precursors. Previously characterized Ab-MLV-transformed lymphoid cells share a number of differentiation markers with normal pre-B cells (reviewed in reference 25). However, DJ_H rearrangements have been observed in some Ab-MLV-induced thymic tumors (8) and in other cells of the T and myeloid lineages (13, 14), raising the possibility that the DJ-F cells are not related to cells of the B lineage. To address this issue, we examined expression of the B-lineage-specific form of ly5 (7) and the Thy1 (16) and BP1 determinants (9) by immunofluorescent staining. As expected for cells related to early B-cell precursors, all the DJ-F cells expressed ly5(B220) and BP1 and none of them expressed Thy1 (Table 1). This pattern was indistinguishable from that observed with DJ-R and VDJ transformants.

In addition to differences in expression of surface markers, hematopoietic cells can be distinguished based on the expression of particular lineage-specific genes. One such differentiation marker is expression of lambda 5. This gene is related to the light-chain locus (30) and appears to be expressed exclusively in early cells of the B lineage (31). To determine whether DJ-F cells expressed lambda 5, total cellular RNA from a panel of cell lines was analyzed by Northern blotting. All the DJ-F cells expressed the 1.2-kilobase lambda 5 RNA (Fig. 3, lanes 4 to 11) at levels comparable to those of the DJ-R and VDJ cell lines (Fig. 3, lanes 1 to 3 and 12 to 15, respectively). As expected, the mature B-cell lines A105 (J. Runnels and N. Rosenberg, unpublished data) and WEHI-231 (39) and the hybridoma TIB-97 (21) did not express this RNA (Fig. 3, lanes 15 to 17). Consistent with their relationship to cells of the B lineage, Southern analysis revealed that none of the cell lines had rearranged their T-cell-receptor β or γ1 and γ2 genes (Table 1; data not shown). These analyses coupled with the analysis of surface antigen expression demonstrate that the DJ-F cells are related to cells of the B-lymphocyte lineage.

DJ-F cells lack unrearranged V_H RNAs. Transcription of unrearranged V_H genes is prominent in late gestation fetal liver and has been observed in all DJ-R-transformed cells

TABLE 1. Characteristics of DJ-F cell lines[a]

Characteristic	Gene structure[b]		
	DJ-F	DJ-R	VDJ
μ Expression	0/7	10/10	5/12
B220 expression	7/7	12/12	7/7
BP1 reactivity	7/7	6/14	11/12
Thy1 expression	0/7	1/12	0/3
λ5 RNA	7/7	4/4	4/4
TCRβ rearranged	0/6	0/3	0/3
TCRγ1, γ2 rearranged[c]	0/6	0/1	0/3
J558 V_H RNAs	0/7	12/12	4/6

[a] DJ-F transformants had <0.01% μ-positive cells, most DJ-R transformants had 1 to 10% μ-positive cells, and VDJ transformants had either 20 to 100% or <0.01% μ-positive cells. In B220, 100% of the cells in all the populations expressed the determinant. Populations containing >10% BP1-reactive cells were considered positive. For Thy1, 10% of the cells in the positive cell line reacted with the antibody. The DJ-F cell lines studied were 300-3, 300-10, 300-25, 300-26, 300-30, 300-31, and 300-35, except that TCR receptor gene structure was not examined in 300-26. The DJ-R cell lines examined for cytoplasmic μ expression were 38B9, 22D6, 41B1, 40E3, 2-10-2, 2-3-2, 2-6-2, 21-10, 192-11, and 481-29; B220 and Thy1 expression were examined in 22D10, 40E1, 38B9, 22D6, 41B1, 40E3, 2-10-2, 2-11-2, 2-3-2, 2-1-1, 21-10, and 192-11; BP1 expression was examined in the same group and also in 2-6-2 and 21-7; lambda 5 expression was examined in 14-4, 300-19, 481-29, and 298-13; TCRβ genes were examined in 481-29, 2-1-1, and 192-11; TCRγ genes were examined in 192-11; J558 RNAs were 192-11, 481-29, 2-10-2, 2-11-2, 2-1-1, 2-3-2, 21-7, 21-10, 14-4, 21-6, 300-19, and 298-13. The VDJ cell lines examined for cytoplasmic μ were 2-3-1, 14-1, 3-3-1, 298-18, 300-16, 7-4, 1881Y, C-11, C-9, 298-26, 480-36, 480-39; lambda 5 was examined in 2-3-1, 7-8, 14-1, 3-3-1; Thy1 was examined in 298-18, 1881Y, and 480-36; B220 was examined in 2-3-1, 298-18, 300-16, 7-4, 1881, 480-36, 480-39; BP1 was examined in the same panel plus 7-8, 3-1-1, 298-26, 300-9 and 480-35; J558 RNAs were examined in 7-8, 14-1, 7-4, C-11, C-9, and 298-26; TCR genes were examined in 298-18, 300-16, and 298-26.

[b] Number of cell lines with characteristic/number of cell lines tested.

[c] TCR, T-cell receptor.

and in many transformants that have completed $V_H DJ_H$ rearrangement (41). DJ-R cells also express RNAs containing DJ_H and C_μ sequences that initiate 5' of the rearranged D element (2, 22). Other studies have suggested that transcriptional activity is associated not only with variable-region formation (4) but also with heavy-chain class switching (18, 35, 42). Thus, in several instances, transcription of a region and recombination in that area are at least temporally associated.

To determine whether the levels of RNAs from the variable region in the DJ-F cells were similar to those in DJ-R cells, we analyzed total cellular RNAs from the DJ-F cells and a panel of other B-lineage cells for the presence of unrearranged V_H RNAs and RNAs transcribed from the J_H region. All the DJ-R cell lines examined expressed RNAs of the appropriate size for unrearranged V_H genes of the J558 family (Fig. 4A, lanes 1, 8, and 9). An RNA of appropriate size for full-length γ2b mRNA was detected in MPC-11 (lane 10), a myeloma which expresses a member of this V_H family (5), and consistent with published data (41), the mature B-cell lymphoma WEHI-231 (39) did not express unrearranged V_H RNAs. In contrast to the DJ-R cell lines, none of the DJ-F cell lines expressed these genes at a detectable level (Fig. 4A, lanes 2 to 7 and 12). However, analysis of the same filter with a mixture of oligonucleotides specific for J_H-coding sequences revealed that all the DJ-F cells expressed RNAs from the DJ_H region (Fig. 4B, lanes 2 to 7 and 12) and that the levels were at least as great as those seen in the DJ-R samples (lanes 1, 8, and 9). These RNAs are of the appropriate size for the truncated μ RNAs that have been observed in DJ-R cell lines (2, 22). These data suggest that recombination in the DJ-F cells is limited by either the
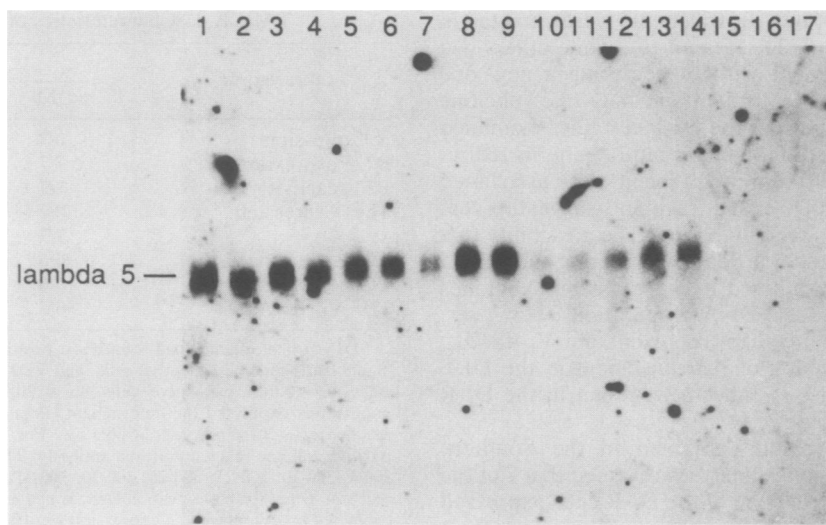
FIG. 3. Expression of lambda 5 RNA. Northern (RNA) analysis of 20 µg of total RNA was performed with an oligonucleotide probe homologous to lambda 5 coding sequences (30). RNAs from the DJ-R cell lines 300-19 (22), 481-29, and 298-13 (22) (lanes 1 to 3, respectively); from the DJ-F cell lines 300-25, 300-35, 300-3, 300-30, 300-26, and 300-10, and 300-31 (lanes 4 to 10, respectively); and from the VDJ cells 3-1-1, 14-1, 2-3-1, and 7-8 (lanes 11 to 14, respectively) were analyzed. RNAs from mature cells of the B lineage (A105, WEHI-231, and TIB-97) were analyzed in lanes 15 to 17, respectively.

decreased transcriptional accessibility of the V_H gene segments or the low level of these products. As judged by the presence of RNAs that hybridize with J_H-region probes, the DJ_H regions of these cells appear to be accessible for transcription and presumably recombination.

Some DJ-F cells recombine an exogenous recombination substrate. The lack of transcriptional activity at the V_H locus in DJ-F cells suggested that the configuration of these genes was limiting their recombination potential. However, an inactive recombination system could also be limiting rearrangement. To distinguish between these possibilities, we assessed the ability of the DJ-F cells to rearrange a substrate containing heptamer-nonamer recognition sequences with an extrachromosomal substrate in a transient transfection assay (12). This assay employs the pJH201 plasmid, which carries the genes for ampicillin and chloramphenicol resistance, the *E. coli* and polyomavirus origins of replication, and heptamer-nonamer recognition elements. Recombination of the heptamer-nonamer sequences activates expression of the chloramphenicol resistance gene, which is monitored following transformation of *E. coli*. Although levels of activity in transformed pre-B-cell lines vary from 0.5 to 30% and do not correlate precisely with differentiation as judged by immunoglobulin gene structure or synthesis (17), all cell lines of this type and early T cells contain the activities required to rearrange this plasmid, while other hematopoietic and non-hematopoietic cells do not (17).

Transfection of VDJ and DJ-R cells with pJH201 revealed that these cells contained recombination activities similar to those reported for such cell lines (Table 2) (17). Six of seven DJ-F cell lines were analyzed in the recombination assay; the seventh cell line, 300-3, did not take up sufficient DNA to be tested. Among those studied, at least two categories were found (Table 2). Two of the cell lines did not have detectable activity, and the frequencies obtained for two others, 300-25 and 300-35, were in the range of those found in some cell lines of myeloid origin that have been considered to be greatly reduced levels by others (17). Thus, the block in rearrangement in these four cell lines may reflect the loss of an active recombination system. However, two of the cell
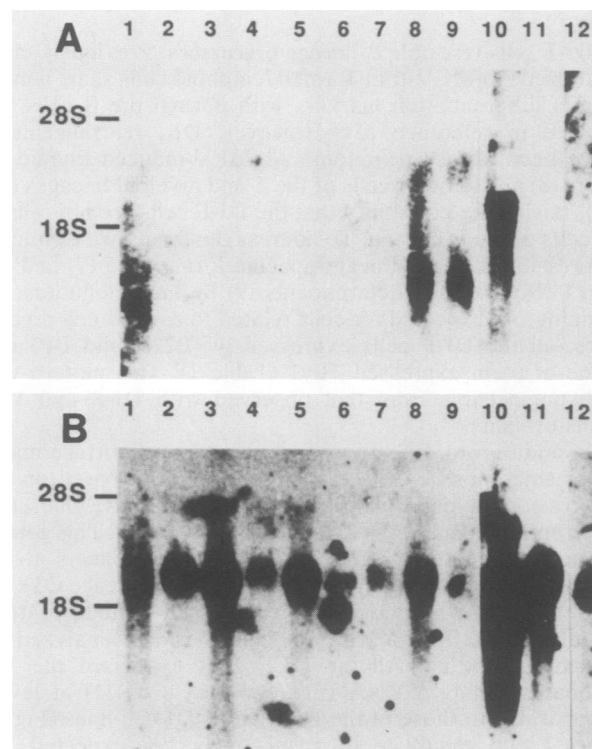

FIG. 4. Expression of unrearranged V_H RNAs in DJ-F cell lines. Northern analysis of 20 µg of total RNA was performed with a J558 V_H probe (5) (A). RNAs in lanes 1, 8, and 9 are from the DJ-R cell lines 481-29, 14-4, and 3-3-1, respectively; lanes 2 to 7 and lane 12 contain RNAs from the DJ-F cell lines 300-3, 300-30, 300-25, 300-35, 300-31, 300-10, and 300-26, respectively. Lane 10 contains RNA from the γ2b-producing myeloma cell line MPC 11 that expresses a J558 V_H gene, and lane 11 contains RNA from the B-cell lymphoma WEHI-231 that does not express a J558 family V_H gene. The J558 V_H probe was washed off, and the filter was reprobed with a mixture of oligonucleotide probes directed against the J_{H2}-, J_{H3}-, and J_{H4}-coding regions (B). This mixture will not detect RNAs in which J_{H1} sequences are used.

TABLE 2. Recombination activity in DJ-F cell lines[a]

Gene structure	Cell line	No. of Amp^r colonies	No. of Amp^r-Cam^r colonies	$R \times 100$[b]
VDJ	1881Y	5,493	251	4.5
	298-26	832	28	3.7
DJ-R	2-11-2	254	8	3.1
	2-10-2	1,178	13	1.1
	2-1-1	436	23	5.3
	22D6	181	6	3.3
	300-19	1,941	77	4.0
DJ-F	300-31	3,956	0	<0.025
	300-26	1,656	0	<0.060
	300-35	4,474	6	0.13
	300-25	2,695	4	0.14
	300-30	991	28	2.8
	300-10	1,084	166	15.3

[a] Amp^r, Ampicillin resistant; Cam^r, chloramphenicol resistant.
[b] $R \times 100 = (\text{Amp}^r\text{-Cam}^r \text{ colonies/Amp}^r \text{ colonies}) \times 100$.

lines, 300-10 and 300-30, retained levels of activity similar to those found in most transformed pre-B cells, suggesting that recombination of the endogenous genes in these cells is not limited by an inactive recombination system.

DISCUSSION

Ab-MLV-transformed lymphoid cells have provided one of the principal model systems for analysis of early B-cell differentiation (reviewed in reference 1). The DJ-F cells described here define a new type of transformant that is isolated at a high frequency from normal adult mouse bone marrow. The tissue-specific distribution of these transformants and those of the DJ-R and VDJ type (3; Ramakrishnan and Rosenberg, in preparation) is consistent with the notion that the phenotype of the transformant reflects the predominant types of target cell present in the tissue. This observation and the fact that VDJ and DJ-R transformants appear to be accurate representations of early B-cell precursors (reviewed in reference 1) suggests that normal lymphoid cells of DJ-F type exist as well. Presumably, these cells would arise during the course of normal differentiation and be dead-end by-products of the pathway eliminated through either senescence or some active mechanism.

Although the DJ-F cells studied here are transformed, it is unlikely that the transformation process itself arrests differentiation in these cells. Although the direct effect of v-abl expression on rearrangement awaits analysis of normal cells or cells transformed by temperature-sensitive mutants of Ab-MLV, DJ-R transformants display all the characteristics of typical Ab-MLV-transformed cells and continue rearrangement in vitro (3, 23, 28, 38). The DJ-R cells, already known to be capable of generating VDJ cells in vitro (23, 24), may give rise to DJ-F cells as well. Preliminary analysis of one typical DJ-R cell line has revealed that a clone lacking submolar J_H fragments does not have detectable levels of unrearranged V_H gene RNAs, while a second clone which has undergone V-to-DJ$_H$ rearrangement retains expression of those RNAs (unpublished data).

DJ-F cells fail to undergo both V_H gene appendage and secondary D gene replacements at a significant frequency. Secondary D rearrangements, observed in DJ-R cells at approximately the same frequency as V_H rearrangements (24), involve replacement of the existing DJ$_H$ units with new units formed by recombination of a 5' D with 3' J$_H$ segment.

All the DJ-F cells retain large numbers of V_H genes (data not shown) and multiple D segments 5' of their J$_H$ rearrangements and many of them retain at least one 3' J$_H$ segment, indicating that the elements needed to participate in both types of recombinations are present. Because D replacements often involve use of the 5'-most D segment (24), the fact that most DJ-F cells retain some D segments suggests that rearrangement in the DJ-F cells ceased after the initial recombination. Thus, our data suggest that both V_H-to-DJ$_H$ rearrangements and secondary DJ$_H$ rearrangements require the same set of signals.

The failure of DJ-F cells to undergo further rearrangement suggests that they lack some of the cellular factors required for the process. It is likely that the cell lines which recombine an introduced extrachromosomal recombination substrate retain the subset of these signals that are required for recombination of heptamer-nonamer units but lack those specifically required for rearrangement of endogenous gene elements. One of these signals may be reflected by the decreased expression of unrearranged V_H RNAs. The absence of these RNAs in DJ-F cells coupled with their prominence in other transformants and in tissues where B-cell generation is ongoing (41) suggests that transcription of these regions is functionally important in variable-region assembly. Further experiments are needed to determine whether the presence of these RNAs merely reflects a chromatin configuration that is compatible with recombination (reviewed in reference 1) or whether they or the putative peptide products specified by them play an active role in recombination.

The signals required for transcription of unrearranged V_H genes have not been defined. If at least some of these genes are expressed from the V_H promoter that is used after rearrangement (41), the tissue-specific factors important for transcription of fully rearranged immunoglobulin genes (15, 34) may mediate expression of the unrearranged genes. These factors appear to be present in DJ-F cells because cells transfected with a fully rearranged μ gene express it at high levels (unpublished data). Alternatively, because lipo-polysaccharide treatment decreases the level of unrearranged V_H RNAs (41) but stimulates the levels of octamer-binding factor (34), it is possible that expression of unrearranged V_H RNAs is mediated by different factors. In either case, DJ-F cells may lack a distinct signal that allows, either directly or indirectly, the transcription of unrearranged V_H genes. Such a factor might be involved in transcription or be important in altering the chromatin structure of the V_H region in preparation for recombination.

Some DJ-F cells fail to recombine the extrachromosomal recombination substrate at detectable levels or do so at levels lower than those reported for early B- and T-cell lines (17). Because these cells have completed the first step in rearrangement, it is likely that the entire recombination system was once active but has been turned off prematurely. The absence of the basic components necessary to mediate heptamer-nonamer recombination probably plays a major role in arresting rearrangement in these cells. The nature of these components has yet to be defined, but the observation that a single DNA segment can confer recombination activity to fibroblast cells (32) suggests a central role for a single factor.

Whether the absence of detectable V_H RNAs is related to the loss of the recombination system cannot be determined from these experiments. However, the fact that some early B-cell lines which have completed both heavy- and light-chain gene assembly retain the ability to recombine the

substrate (17) but do not express unrearranged V_H RNAs (41) suggests that the recombination machinery is lost gradually. This hypothesis could be extended to suggest that the DJ-F cells which continue to rearrange the recombination substrate but lack detectable V_H RNAs are still in the process of turning off the recombination system, while those which lack both activities have completed the process. Consistent with this idea, both a DJ-F and VDJ subclone derived from a DJ-R clone rearrange the exogenous substrate, but only the parental DJ-R clone and the VDJ subclone express unrearranged V_H RNAs (unpublished data).

Irrespective of the relationship between unrearranged V_H gene transcription and recombination activity, the DJ-F cells represent a unique model system in which this and other features controlling immunoglobulin gene assembly can be explored.

ACKNOWLEDGMENTS

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