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Continuing rearrangement of immunoglobulin and T-cell receptor genes in a Ha-*ras*-transformed lymphoid progenitor cell line

(DNA rearrangement/lymphocyte differentiation/retroviral transformation)

ALESSANDRO ALESSANDRINI*, JACALYN H. PIERCE†, DAVID BALTIMORE‡§, AND STEPHEN V. DESIDERIO*

*Howard Hughes Medical Institute Laboratory of Genetics and Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; †Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD 20205; ‡Whitehead Institute for Biomedical Research, Cambridge, MA 02142; and §Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

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ABSTRACT **The arrangement of immunoglobulin genes has been examined in a series of lymphoid cell lines transformed with the Harvey murine sarcoma virus. One cell line, HAFTL-1, expresses antigenic markers characteristic of B-lymphoid cells and undergoes frequent rearrangement at the J_H locus (where J = joining and H = heavy chain) during propagation in culture. By molecular cloning and nucleotide sequence determination, these rearrangements were found to represent the earliest postulated step in heavy chain gene assembly: the joining of a diversity (D) segment to a J_H segment. The HAFTL-1 cell line also undergoes infrequent D_β-to-J_β joining at the T-cell receptor β locus in culture. The observations presented here suggest that the HAFTL-1 cell line represents the early stage of B-cell differentiation at which immunoglobulin gene rearrangement is initiated.**

The variable regions of immunoglobulin heavy chain genes are encoded in the germ line by three discrete elements: V_H, D, and J_H (where V = variable, D = diversity, J = joining, and H = heavy chain). During the differentiation of B lymphocytes, individual segments from each group are joined to form a complete V_H–D–J_H variable region (1–8). The organization of the T-cell receptor β-chain gene family resembles that of the immunoglobulin heavy chain locus (9–12). The variable region of the T-cell receptor β chain is encoded by three DNA segments—V_β, D_β, and J_β—that are joined during T-cell differentiation. Unrearranged immunoglobulin and T-cell receptor gene segments are accompanied by similar, conserved sequence elements that lie near the sites of recombination; these elements have been proposed to mediate rearrangement (2, 3, 9, 10, 13, 14).

Transformation of cells from murine bone marrow or fetal liver with the Abelson murine leukemia virus (Ab-MuLV) generates permanent lymphoid cell lines that represent analogues of cells at early stages of B-cell differentiation (15–18). The most immature Ab-MuLV transformants are cell lines in which DJ_H joining has occurred on both chromosomes and in which V_H-to-DJ_H rearrangement occurs spontaneously in culture (17, 19–21). Infection of murine bone marrow or fetal liver cells with the Harvey murine sarcoma virus (Ha-MSV) has also been found to yield permanent lymphoid cell lines (22). These cell lines express characteristic B-lymphoid antigenic markers suggesting that they are members of the B-cell lineage (22, 23). All Ha-MSV-transformed cell lines are null with respect to expression of immunoglobulin heavy or light chain, unlike Ab-MuLV-transformed bone marrow cell lines, at least half of which express μ chain (17). Taken together, these observations suggest that Ha-MSV and Ab-MuLV are capable of transforming overlapping sets of B-lymphoid cells and that Ha-MSV-transformed cell lines may include earlier stages of B-cell differentiation than are represented among Ab-MuLV transformants. To test this hypothesis, we have examined the arrangement of immunoglobulin gene segments in a series of Ha-MSV-transformed cell lines. Here we present evidence that one cell line, HAFTL-1, actively recombines D segments to J_H segments when propagated in culture; this is the earliest postulated step in immunoglobulin gene assembly. In addition, we show that this cell line undergoes infrequent D_β-to-J_β joining at the T-cell receptor β locus in culture.

MATERIALS AND METHODS

Cell Lines. Ha-MSV-transformed cell lines were produced as described by Pierce and Aaronson (22). Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 μM 2-mercaptoethanol. Cells were cloned by limiting dilution at a concentration of 0.2 cell per microtiter well.

Preparation and Analysis of Genomic DNA. Genomic DNA was prepared as described (24). DNA probes were labeled by nick-translation with ^{32}P to a specific activity of $3–5 \times 10^8$ cpm/μg, as described by Rigby *et al.* (25). Hybridization reactions were undertaken in 50% formamide, 0.6 M NaCl, 0.075 M sodium citrate, 0.065 M KH_2PO_4, 0.005 M Na_2-EDTA, 0.02% (each) bovine serum albumin, Ficoll, and polyvinylpyrrolidone 360, 100 μg of denatured salmon sperm DNA per ml, and 10^6 cpm of heat-denatured probe per ml for 16 hr at 42°C. Filters were washed in 0.3 M NaCl, 0.03 M sodium citrate, and 0.1% $NaDodSO_4$ at 68°C for 1 hr; ^{32}P was detected by autoradiography.

DNA Hybridization Probes. Probes derived from the J_H–C intron (where C = constant) (J_H-specific probe) and the 5′ flanking sequences of D_{FL16} and D_{SP2} gene segments (D-specific probe) were prepared from the plasmids pJ_H800, pJ_H38B9-7.1, and p40E4-2-5.2 as described by Alt *et al.* (19). The T-cell receptor $C_\beta 1$ probe is a cDNA clone representing the 3′ portion of the $C_\beta 1$ gene. To prepare the probe, the plasmid pYTJ70 (a gift of Tak Mak) was cut with the *Pst* I restriction enzyme and the resulting 0.8-kilobase (kb) DNA fragment was purified by electrophoresis through 0.8% agarose. The location of the probe with respect to the T-cell receptor C_β locus is indicated in Fig. 4*A*.

Isolation and Characterization of Genomic DNA Clones. Genomic DNA from the HAFTL-1-14-6 cell line was digested with *Eco*RI and fractionated by electrophoresis through a preparative 0.8% agarose gel (Bull's Eye preparative gel unit, Hoefer, San Francisco). Selected fractions were pooled and DNA was ligated to Charon 30 DNA (26), which had previously been digested with *Eco*RI and dephosphorylated with

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Abbreviations: Ab-MuLV, Abelson murine leukemia virus; Ha-MSV, Harvey murine sarcoma virus; H, heavy chain; J, joining; D, diversity; V, variable; C, constant; kb, kilobase(s).

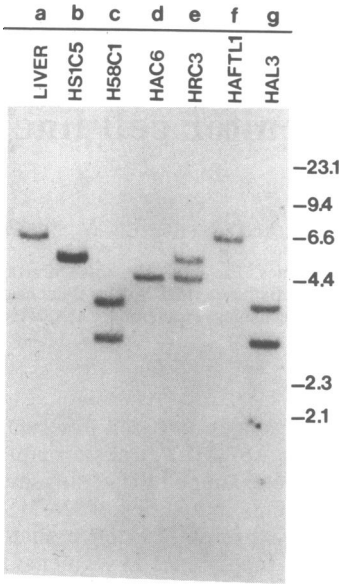

FIG. 1. Analysis of J_H-associated rearrangements in Ha-MSV-transformed cell lines. DNA (12 μg) was digested with *Eco*RI, fractionated by electrophoresis in 0.8% agarose, transferred to nitrocellulose, and assayed for hybridization to a DNA probe specific for the J_H–C_μ intron (see ref. 19 and Fig. 3*A*). The positions of DNA standards are indicated in kb. Lane a, DNA from BALB/c liver; lanes b–g, DNA from Ha-MSV-transformed cell lines as indicated.

calf intestinal phosphatase. The products of ligation were encapsidated *in vitro*; the packaging extract (Gigapack, Promega Biotec, Madison, WI) was used according to the supplier's instructions. The resultant bacteriophage were propagated in the *Escherichia coli* strain C600. Plaques were screened by the method of Benton and Davis (27). Bacteriophage were isolated by three additional rounds of plaque purification.

Fractionation by genomic DNA from HAFTL-1-14-9, construction of a size-selected library, and screening of recombinant plaques were performed as described above. Selected DNA fractions were cloned into the *Eco*RI site of the λgt10 vector (28).

RESULTS

Unrearranged J_H Locus in a Ha-MSV-Transformed Cell Line. We initially examined six cell lines, produced by Ha-MSV transformation of adult bone marrow (HSIC5, H58C1, HAC6, HRC3, and HAL3) or fetal liver (HAFTL-1) from NFS mice. These cell lines produce no immunoglobulin, express the B-cell lineage markers Ly 5 (B220) and Lyb 2, and do not express Thy-1, a marker found on all T cells (23). To examine the arrangement of immunoglobulin J_H gene segments in these cell lines, genomic DNA was digested with *Eco*RI, fractionated by electrophoresis in agarose, transferred to nitrocellulose, and hybridized to a J_H-specific probe (ref. 19 and Fig. 3*A*). All the bone marrow-derived cell lines analyzed had undergone rearrangement at both J_H alleles, as evidenced by loss of the unrearranged 6.2-kb J_H-containing fragment and the appearance of novel J_H-containing fragments (Fig. 1, lanes a–e and g). The HAFTL-1 cell line, in contrast, revealed a predominant J_H-containing fragment that comigrated with the unrearranged fragment found in liver

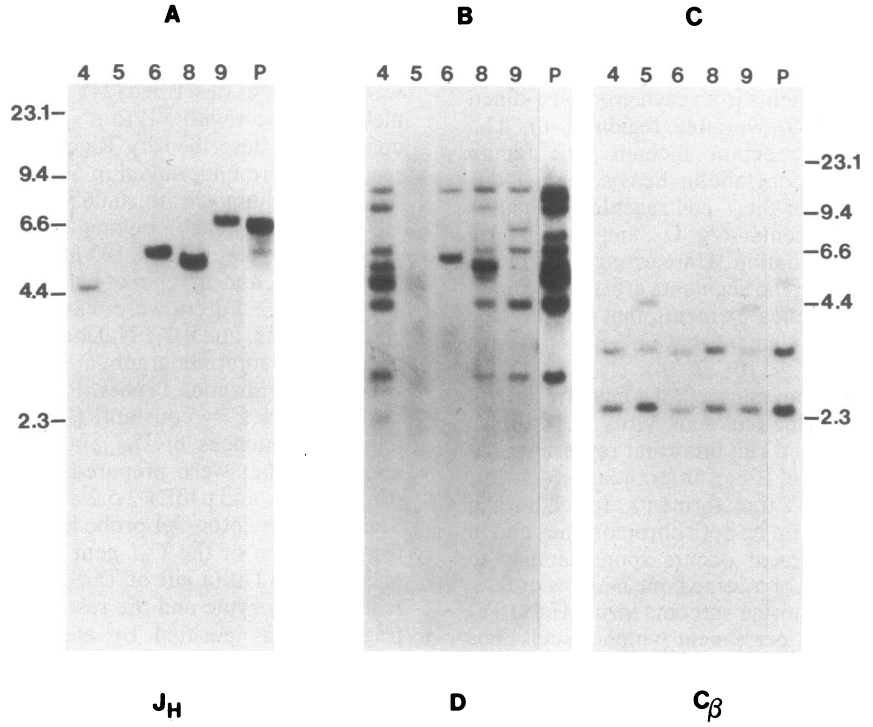

FIG. 2. Immunoglobulin and T-cell receptor gene rearrangements in subclones of the HAFTL-1-14 cell line. The primary subclone HAFTL-1-14 was derived from the HAFTL-1 cell line by limiting dilution to a concentration of 0.2 cell per microtiter well. This primary subclone was carried for 2 weeks in culture and secondary clones were obtained by limiting dilution as described above. Genomic DNA was digested with *Eco*RI and assayed for rearrangements by hybridization to DNA probes. (*A*) J_H-associated rearrangements. The filter was hybridized to the J_H probe and washed. The positions of DNA standards are indicated in kb. Lane P, DNA from HAFTL-1-14; lanes 4, 5, 6, 8, and 9, DNA from HAFTL-1-14-4, HAFTL-1-14-5, HAFTL-1-14-6, HAFTL-1-14-8, and HAFTL-1-14-9, respectively. (*B*) D-associated rearrangements. The filter was hybridized to a mixture of two DNA probes derived from the 5′ flanks of D_{FL16} and D_{SP2} gene segments; these probes are described in ref. 19. Hybridization was performed as described in *Materials and Methods* except that the filter was washed in 0.015 M NaCl, 0.0015 M sodium citrate, and 0.01% $NaDodSO_4$. Lanes are marked as in *A*. (*C*) C_β-associated rearrangements. The filter used in *B* was washed in 0.5 M NaCl, 0.05 M NaOH, and 0.002 M Na_2EDTA for 30 min at 28°C to remove the D probe. The filter was neutralized and hybridized to the T-cell receptor $C_\beta 1$ probe (see *Materials and Methods* and Fig. 4*A*). Lanes are marked as in *A*. The positions of DNA standards are marked in kb.

DNA (Fig. 1, lanes a and f). Similar analysis of HAFTL-1 DNA with *Hin*dIII or *Bam*HI showed this fragment to be identical to the unrearranged J_H-containing fragment in NFS liver DNA (data not shown).

The HAFTL-1 Cell Line Undergoes D-to-J_H Rearrangement in Culture. When the autoradiogram shown in Fig. 1 was exposed for longer times, faint additional bands were seen to hybridize to the J_H probe in the lane containing HAFTL-1 DNA. This suggested that the HAFTL-1 cell line was capable of undergoing D-to-J_H rearrangement during propagation in culture. To test this hypothesis, a clonal cell line, HAFTL-1-14, was isolated from HAFTL-1 by limiting dilution. This cell clone was shown to be unrearranged at the J_H locus (data not shown). The HAFTL-1-14 cell line was carried for 2 weeks in culture and subclones were obtained. The Ha-MSV proviral integration pattern in these isolates was identical, demonstrating that they were indeed of clonal origin (data not shown). DNA from secondary cell clones was digested with *Eco*RI and assayed for D-to-J_H rearrangement by hybridization to J_H- and D-specific (19) probes (Fig. 2 *A* and *B*). Several of these clones had lost the unrearranged J_H-containing fragment and had acquired novel fragments (Fig. 2*A*, subclones 4, 6, 8, and 9). These J_H-containing fragments also hybridized to the D probe (Fig. 2*B*, subclones 4, 6, 8, and 9), suggesting that they might represent DJ_H rearrangements. In addition, J_H-associated rearrangements were accompanied by deletion of one or more D-containing fragments (Fig. 2*B*, subclones 4, 6, 8, and 9). This is consistent with the joining of the 3′ end of a D segment to the 5′ end of a downstream J_H segment and concomitant loss of intervening DNA. No rearrangement of κ or λ genes was detected (data not shown).

We observed only a single J_H-containing fragment in these subclones and the complete loss of some D-containing fragments upon rearrangement. The most likely explanation is that loss of D and J_H sequences from the opposite chromosome occurred before the isolation of subclones. One subclone showed complete loss of J_H- and D-containing fragments (Fig. 2 *A* and *B*, subclone 5), suggesting that it had undergone extensive deletion of sequences from the remaining heavy chain allele. Extensive deletion of DNA from one heavy chain locus is not an obligatory feature of rearrangement in the HAFTL-1 cell line, however, because other sets of subclones made from this cell line fail to show such deletion, yet undergo similar J_H-associated rearrangement (data not shown).

To directly demonstrate the occurrence of D-to-J_H joining in the HAFTL-1-14 cell line, we carried out molecular cloning of the J_H-associated rearrangement from HAFTL-1-14-6. The restriction map of this fragment and its relationship to the J_H region in BALB/c germ-line DNA is shown in Fig. 3*A*. Comparison of the two restriction maps revealed that rearrangement had occurred near J_H2. We therefore determined the DNA sequence of this region. From this analysis it was apparent that the J_H-containing *Eco*RI fragment from the HAFTL-1-14-6 cell line had arisen by recombination between an FL16-type D segment and J_H2 (Fig. 3*B*). The 5′ flank of the D region and part of its coding sequence are identical to the sequence of $D_{FL16.1}$ but, as is typical of DJ_H joints (8), some nucleotides were apparently lost from the D and J_H coding sequences. In addition, four nucleotides not represented in the $D_{FL16.1}$ or J_H2 germ-line sequences were found at the D–J_H junction. These nucleotides probably constitute an N region (20, 32), although it is also possible that

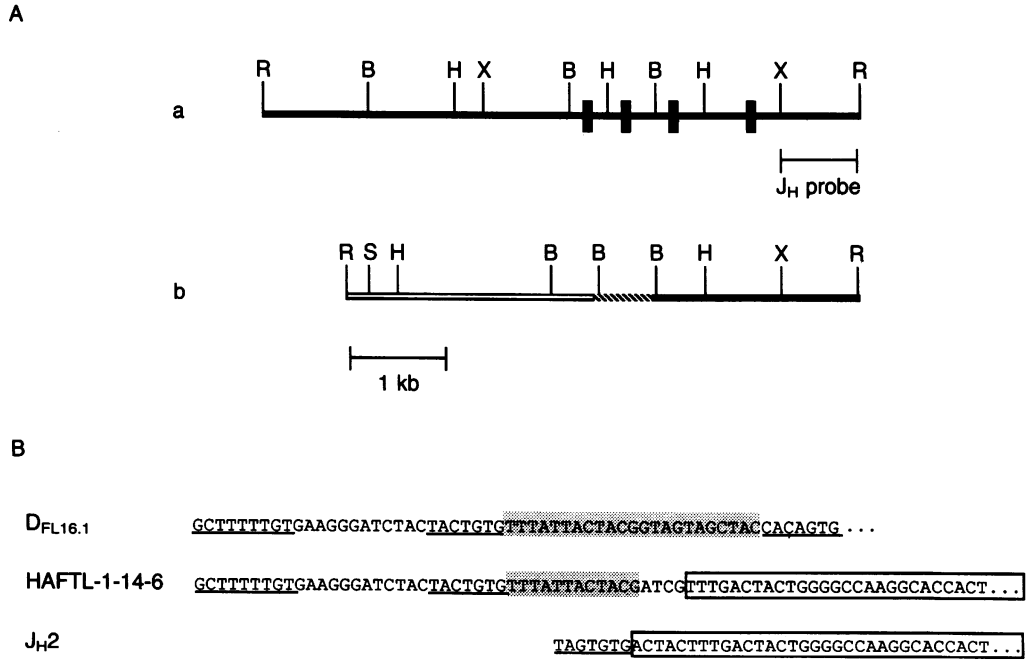

FIG. 3. D-to-J_H joining in the HAFTL-1-14-6 cell line. A size-selected genomic DNA library for the HAFTL-1-14-6 cell line was constructed in Charon 30 and screened for hybridization to the J_H probe. The J_H probe was labeled with ^{32}P by the method of Feinberg and Vogelstein (29). After initial propagation in Charon 30, the 5.5-kb J_H-containing fragment was subcloned into the *Eco*RI site of pGEM-2 (Promega Biotec). The recombination junction was localized by restriction mapping to the 600-base-pair *Bam*HI fragment indicated in *A*. This was subcloned into M13mp19 (30) and the nucleotide sequence across the joint was determined by the method of Sanger *et al.* (31). (*A*) Map of the recombinant from HAFTL-1-14-6. a, Map of the 6.2-kb J_H-containing *Eco*RI fragment from the BALB/c germ line (3), shown for comparison. b, Map of the 5.5-kb J_H-associated rearrangement from HAFTL-1-14-6. The solid line represents the region of identity with the BALB/c germ-line map; the open bar represents the minimal extent of novel DNA sequence; the hatched bar designates the localization of the recombinant junction. Restriction enzymes are abbreviated as follows: R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; X, *Xba* I; S, *Sac* I. (*B*) Nucleotide sequence of the $D_{FL16.1}$–J_H2 fusion in HAFTL-1-14-6. Top line and bottom line, partial sequences of the germ-line $D_{FL16.1}$ (8) and J_H2 (3, 7) segments. Middle line, sequence at the recombinant junction of clone HAFTL-1-14-6. Sequences derived from the J_H coding region are boxed; sequences derived from the D coding region are stippled. Conserved heptamer and nonamer sequences are underlined.

the germ-line sequences of NFS and BALB/c gene segments differ at these positions.

Continuing D_β-to-J_β Rearrangement at the T-Cell Receptor β Locus in the HAFTL-1-14 Cell Line. Because of the structural similarity between immunoglobulin heavy chain genes and T-cell receptor β-chain genes, we asked whether D_β-to-J_β rearrangement could also occur during propagation of the HAFTL-1-14 cell line. The assay employed a cDNA probe homologous to the 3′ end of $C_\beta 1$ (ref. 33 and Fig. 4*A*). This probe detects two *Eco*RI fragments of about 2 and 3 kb in BALB/c germ-line DNA (10). When genomic DNA from the HAFTL-1-14 cell line and its subclones was assayed, the pattern of hybridization in the parental cell line and in most subclones was identical to that of germ-line DNA (Fig. 2*C*, lanes 4, 6, 8, and P). Two subclones, however, showed a novel fragment of about 4 kb and a decrease in the intensity of the 3-kb fragment. These observations suggested that the new fragments arose by recombination between $D_\beta 2.1$ and a $J_\beta 2$ segment (12).

To determine the structure of the C_β-associated rearrangement in HAFTL-1-14-9, the 4-kb *Eco*RI fragment was molecularly cloned. The restriction map of this fragment is compared to the restriction map of a portion of the C_β locus in Fig. 4*A*; the rearrangement apparently involved a deletion spanning the *Eco*RI restriction site between the $D_\beta 2.1$ segment and the $J_\beta 2$ cluster. DNA sequence analysis revealed that this deletion was the result of recombination between $D_\beta 2.1$ and $J_\beta 2.5$ (Fig. 4*B*). The junction formed during D_β-to-J_β joining was similar in two respects to the junction formed during D-to-J_H joining. First, several nucleotides were lost from the 3′ end of the $D_\beta 2.1$ coding region. Second, two nucleotides not present in the BALB/c germ-line sequences were found at the D_β–J_β junction; these most likely represent an N region.

DISCUSSION

The assembly of immunoglobulin genes occurs by means of an ordered program of recombination between gene segments (17–20, 36, 37). The immunoglobulin gene rearrangements observed in the HAFTL-1 cell line represent the earliest postulated step in this program: the joining of a D segment to a J_H segment. We failed to observe rearrangement of light chain gene segments in this cell line. These observations, together with the presence of antigenic markers characteristic of the B lineage, suggest that the HAFTL-1 cell line represents the early stage of B-cell differentiation at which immunoglobulin gene rearrangement is initiated. This cell line may not be absolutely frozen at the D-to-J_H joining stage, however, because we have observed secondary rearrangement of a DJ_H-containing fragment in some progeny cells (unpublished results). In its ability to undergo continuing rearrangement of immunoglobulin gene segments in culture, the cell line resembles many Ab-MuLV-transformed lymphoid cell lines (17, 18). All Ab-MuLV-transformed cell lines, however, represent stages of B-cell ontogeny that follow D-to-J_H joining (19, 20, 38). The observations presented here support the notion that Ha-MSV and Ab-MuLV transform overlapping but ontogenically distinct sets of lymphoid cells (22).

The putative recombinational signal sequences of immunoglobulin and T-cell receptor gene segments are virtually identical (2, 3, 9, 10, 13, 14) and are apparently able to mediate joining of T-cell receptor gene segments in B-lymphoid cells (39). These observations have suggested that

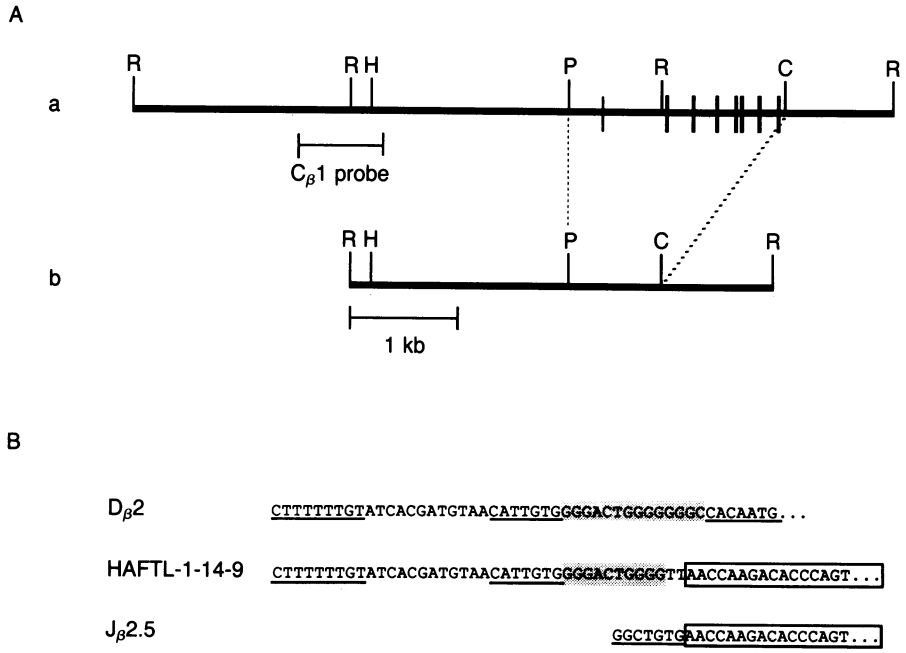

FIG. 4. D_β-to-J_β joining in the HAFTL-1-14-9 cell line. A size-selected genomic DNA library for the HAFTL-1-14-9 cell line was constructed in λgt10 and screened for hybridization to the $C_\beta 1$ probe. After initial propagation in λgt10, the 4.0-kb C_β-containing fragment was subcloned into the *Eco*RI site of pUC13 (34). The region spanning the recombinant junction was localized by restriction mapping and the nucleotide sequence across the joint was determined by the method of Maxam and Gilbert (35). (*A*) Map of the recombinant from HAFTL-1-14-9. a, Restriction map of a portion of the β-chain locus, encompassing $C_\beta 1$, $D_\beta 2.1$, and the $J_\beta 2$ cluster (12), for comparison. The position of the $D_\beta 2.1$ sequence is marked with a narrow vertical line; the positions of the $J_\beta 2$ segments are marked with heavy vertical lines. The region represented by the $C_\beta 1$ probe is indicated. b, Map of the 4.0-kb rearrangement from HAFTL-1-14-9. The dotted lines indicate the occurrence of a deletion within the germ-line *Pvu* II–*Cla* I fragment spanning the *Eco*RI site. Restriction enzymes are abbreviated as follows: R, *Eco*RI; H, *Hin*dIII; P, *Pvu* II; C, *Cla* I. (*B*) Nucleotide sequence of the $D_\beta 2.1$–$J_\beta 2.5$ joint in HAFTL-1-14-9. Top line and bottom line, partial sequences of the germ-line $D_\beta 2.1$ (12) and $J_\beta 2.5$ (10) segments. Middle line, nucleotide sequence at the recombinant junction of HAFTL-1-14-9. Sequences derived from the J_β coding region are boxed; sequences derived from the D_β coding region are stippled. Conserved heptamer and nonamer sequences are underlined.

the mechanisms of immunoglobulin and T-cell receptor rearrangement are similar (9, 12, 39). We therefore propose that the continuing rearrangement of immunoglobulin and T-cell receptor genes that we have observed in the HAFTL-1 cell line is catalyzed by a common apparatus that is active in this cell line. Rearrangements of immunoglobulin and T-cell receptor gene segments are not mutually exclusive events, as evidenced by the presence of DJ_H and $D_\beta J_\beta$ rearrangements in the HAFTL-1-14-9 cell clone. Furthermore, the rearrangement of T-cell receptor gene segments in the HAFTL-1 cell line is not accompanied by the acquisition of T-cell markers or the loss of B-cell markers; in particular, the HAFTL-1-14-9 subclone homogeneously expresses the B-lymphoid marker Lyb 2 (J.H.P., H. C. Morse III, and S.V.D., unpublished observations). Thus, rather than representing a common B- and T-cell precursor, the HAFTL-1 cell line appears to be an analogue of a very early B-cell progenitor. The presence of "inappropriate" gene rearrangements in members of the T- and B-cell lineages is not unprecedented. Partial rearrangements of immunoglobulin heavy chain genes have been frequently found in T-lymphoid cell lines and tumors (6, 40–42). Likewise, rearranged T-cell receptor genes have been recently found in B-lymphoid cells (43, 44). The spontaneous rearrangement of T-cell receptor β-chain gene segments in the HAFTL-1 cell line prompted us to screen 18 B-cell hybridoma lines for such rearrangements; $J_\beta 2$-associated rearrangements were found in two of these cell lines (A.A., P. Gearhart, and S.V.D., unpublished observations).

We observed T-cell receptor gene rearrangement in two of nine subclones of the HAFTL-1-14 cell line, whereas rearrangement at the immunoglobulin H chain locus was observed in eight of the nine subclones. This difference is consistent with the B-lymphoid character of the cell line. It has been proposed that the ability of a gene segment to undergo rearrangement is in part modulated by the accessibility of the segment to the recombinational apparatus (45). If this is true, then it may be possible to demonstrate other differences between the immunoglobulin and T-cell receptor loci in the HAFTL-1 cell line, for example, in their levels of transcription or in their susceptibility to nuclease digestion.

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