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Transcription of Germ Line Vα Segments Correlates with Ongoing T-Cell Receptor α-Chain Rearrangement

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M14T is a virally transformed immature T-cell line which continues to rearrange its T-cell antigen receptor (TCR) α-chain genes in vitro and thus represents a dynamic system for studying TCR assembly. In an effort to investigate whether the TCRα locus is accessible for V(D)J rearrangement events, we examined M14T cells for the presence of germ line TCRα transcripts. Several unrearranged Vα segments were found to be transcriptionally active in M14T cells. By comparison, germ line Vα transcripts are absent in nonlymphoid and pro-T-cell lines and barely detectable in mature T-cell lines, suggesting that this phenomenon is likely stage and tissue specific. We demonstrate a perfect correlation between transcriptionally active Vα segments and their involvement in ongoing Vα-to-Jα rearrangements. In addition, data suggesting that the unrearranged Jα locus is also transcriptionally active in the M14T line are presented. Furthermore, the recombination-activating genes *RAG-1* and *RAG-2* are differentially expressed, with *RAG-2* detectable only by polymerase chain reaction, implying that very low levels of one of these gene products are sufficient to complement the other to facilitate VJ rearrangements. These findings provide the first direct evidence for an accessibility model of antigen receptor rearrangement in T cells.

Genes encoding antigen receptors (immunoglobulins [Ig] in B cells and T-cell receptors [TCR] in T cells) are assembled from multiple variable (V), joining (J), and (in some cases) diversity (D) gene segments which somatically rearrange upstream of a nonvariable constant (C) gene segment during early lymphocyte development (for reviews, see references 49 and 22). Rearrangements of V, D and J (or V and J) segments at both Ig and TCR loci are mediated by a common, site-specific V(D)J recombinase activity which recognizes highly conserved recombination signal sequences (RSSs) flanking all such gene segments (22, 49, 53). Two lines of evidence indicate that antigen receptor assembly is tissue and stage specifically regulated. First, whereas antigen receptors are rearranged and expressed only in lymphocytes, B cells never completely rearrange TCR genes, nor do T cells completely rearrange Ig genes. Second, antigen receptor rearrangement occurs in ordered stages; D-to-J rearrangement occurs first and is followed by V-to-DJ rearrangement. In pre-B cells, V(D)J rearrangement at the Ig heavy-chain (IgH) locus precedes rearrangement at the Ig light-chain (IgL) locus (for a review, see reference 1). Similarly, rearrangement at the TCRβ locus precedes rearrangement at the TCRα locus in pre-T cells (4). The rearrangements of Ig and TCR V gene segments are mediated in part by the synergistic interaction of the products of the recombination activating genes *RAG-1* and *RAG-2* (33, 40), which are expressed early in B- and T-cell development.

It has been proposed that tissue- and stage-specific control of Ig gene assembly in B cells is achieved by regulating the accessibility of component germ line gene segments to the common V(D)J recombinase (1, 51, 52). Evidence supporting an accessibility model comes from the detection of germ line (i.e., sterile) transcripts emanating from unrearranged V_H segments at the IgH locus prior to or during the period of active V_H-to-DJ_H rearrangement (25, 51). Furthermore, germ line V_H transcription disappears as soon as a productively rearranged IgH μ chain is expressed. The precise timing of the transcriptional activation of the germ line V_H segments is believed to reflect or establish a state of open chromatin which is permissive (i.e., accessible) to V_H rearrangement, while the absence of V_H transcripts reflects a closed (inaccessible) V_H locus. In addition, the appearance of unrearranged Jκ-Cκ transcripts clearly precedes Vκ-to-Jκ rearrangement at the IgL κ locus (33). Indeed, it was recently demonstrated that the frequency of Vκ-to-Jκ rearrangement correlates with the rate of germline Jκ-Cκ transcription (41). Similarly, sterile Vκ transcripts have been detected in both mouse and human pre-B lines which undergo Vκ-to-Jκ rearrangements in vitro (31, 42). With regard to potentially similar events preceding the assembly of functionally rearranged TCR V regions, incompletely rearranged Dβ-Jβ transcripts at the TCRβ locus were reported to appear during normal pre-T-cell development (44).

M14T is a $CD4^-$ $CD8^-$ $TCR\alpha\beta^+$ immature murine T-cell line obtained by retrovirus transformation of thymic lymphocytes (36). M14T has a primary Vα-Jα rearrangement on each of its TCRα alleles yet continues to undergo Vα-to-Jα rearrangements (i.e., secondary rearrangements) on both alleles during propagation in culture (13, 30). These rearrangements involve upstream germ line Vα segments joining to downstream Jα segments, deleting the primary Vα-Jα complex (13). Several recent studies suggest that secondary Vα-to-Jα rearrangements at the TCRα locus occur frequently in normal thymocytes and may represent physiologically important events during T-cell development (34, 38, 45, 48).

The ongoing TCR α-chain rearrangement phenomenon in M14T makes this cell line a valuable tool for studying TCR assembly and expression at the early stages of T-cell development. To investigate whether TCR rearrangement is consistent with an accessibility mechanism, M14T cells were

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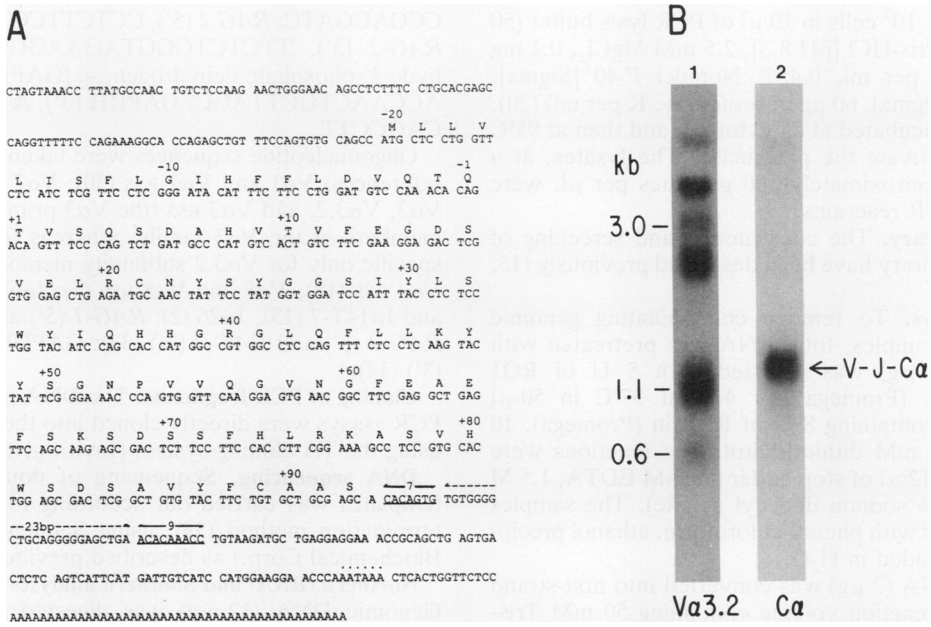

FIG. 1. Sequence analysis and expression of a germ line Vα3.2 gene. (A) Nucleotide sequence of a germ line Vα3.2 transcript in M14T cells, as determined from the cDNA clone pJF1A. Predicted amino acid sequence and RSS (heptamer-7/nonamer-9) sequences are indicated. (B) Northern blot analysis of 10 μg of M14T poly(A)$^+$ RNA. In lane 1, the Northern blot was screened with the entire Vα3.2 cDNA; in lane 2, the blot was stripped and reprobed with a Cα probe. The size of a mature α-chain transcript is indicated.

examined for the presence of germ line Vα and Jα sterile transcription. We show that the transcription of germ line Vα segments is strongly correlated with their proclivity for undergoing secondary Vα-Jα rearrangements in M14T cells. Our results suggest that, analogous to Ig gene assembly, transcriptional activation of unrearranged, component TCR gene segments may also be a prerequisite for their subsequent rearrangement. These findings represent the first direct evidence for an accessibility model of antigen receptor rearrangement in T cells.

MATERIALS AND METHODS

Cell lines and tissue culture. NIH 3T3 fibroblasts and S49 and CTLL-2 mature T-cell lines were obtained from the American Type Culture Collection; 1010 (37) and C6VLB pre-T lines were obtained from James Allison (University of California, Berkeley). AJ95 is a surface IgM-positive B-cell line obtained from Jon Braun (University of California, Los Angeles) (8), and MPC-11 is a plasma cell tumor line (10). NIH 3T3 cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% calf serum and antibiotics. All other lymphoid lines described in this study were maintained in RPMI 1640 containing 2 mM glutamine, 50 μM 2-mercaptoethanol, antibiotics, and either 10% fetal calf serum or 10% horse serum, depending on the cells' requirements. Forty percent (vol/vol) of the culture medium for CTLL-2 cells contained conditioned medium from concanavalin A-stimulated rat splenocyte cultures (Rat T-cell Polyclone; Collaborative Research, Inc.). Cells were routinely grown at 37°C in an air-plus-5% CO_2 atmosphere.

M14T cells (13, 30) were grown in DMEM containing 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM glutamine, and antibiotics. M14T sublines were cloned at limiting dilution in 96-well microtiter plates (Costar, Cambridge, Mass.) in DMEM medium (supplemented as described above). Cells (0.2 per well) were seeded, and only clones from plates containing less than 30 positive wells were used.

Total RNAs from pre- and pro-T lines FTH5 (35), FT612 (35), and LB6 were gifts from Ronald Palacios (Basel, Switzerland).

DNA probes. The DNA probes used in this study were a 225-bp *Fnu*4HI Vα3.2 fragment from a PCR (polymerase chain reaction)-amplified Vα-Jα secondary rearrangement (amplified from the M14T-1 subline) (13), a 625-bp *Eco*RI fragment from pJF1A (Fig. 1) containing the entire Vα3.2 sterile cDNA, a 151-bp PCR-amplified Jα6.19-Cα cDNA fragment (13), a 100-bp *Fnu*4HI PCR-amplified Jα14T-1 cDNA fragment (13), a 110-bp *Rsa*I PCR-amplified Jα14T-7 cDNA fragment (13), a 400-bp *Rsa*I-*Spe*I fragment of the Vα4 cDNA clone A26 (30), a 1.1-kb *Eco*RI fragment of the TCRα cDNA clone T 1.2 containing the Cα region (7) (a gift from Michael Steinmetz), a 824-bp *Bgl*I-*Hin*dIII fragment of the *RAG-1* cDNA clone M6 (40), and a 2.2-kb *Not*I fragment of the *RAG-2* cDNA clone MR2-1 (33) (both *RAG-1* and *RAG-2* cDNA clones were a gift from David Schatz). Other Vα gene probes (kindly provided by Lee Hood) included a 451-bp *Hin*dIII-*Eco*RI fragment of the pUC-Vα1 clone, a 251-bp *Hin*dIII-*Eco*RI fragment of the pUC-Vα2 clone, and a 296-bp *Hin*dIII-*Eco*RI pUC-Vα8.3 clone.

Mapping of Jα segments. The germ line locations of Jα14T-1 and Jα14T-7 (13) were determined by using the two Jα regions as probes to screen BALB/c germ line cosmid clones which span the unrearranged Jα locus (pJA73 and pJA74, gifts from Marie Malissen) (28). The two Jα segments were subsequently mapped more precisely within the pJA73 clone (11).

Preparation of nucleic acids. Total RNA and poly(A)$^+$ RNA were prepared exactly as described previously (30). Total genomic DNA was isolated from tissue culture cells by using proteinase K as described previously (15). For PCR assays on small numbers of cells, DNA lysates were pre-

pared by lysing 5×10^3 cells in 10 μl of PCR lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.1 mg of gelatin [Sigma] per ml, 0.45% Nonidet P-40 [Sigma], 0.45% Tween 20 [Sigma], 60 μg of proteinase K per ml) (20). The lysates were incubated at 55°C for 1 h and then at 95°C for 10 min to inactivate the proteinase. The lysates, at a concentration of approximately 500 genomes per μl, were used directly in PCR reactions.

M14T cDNA library. The construction and screening of the M14T cDNA library have been described previously (13, 30).

RNA PCR assays. To remove contaminating genomic DNA from RNA samples, total RNA was pretreated with DNase. RNA (20 μg) was digested with 5 U of RQ1 RNase-free DNase (Promega) for 4 h at 37°C in 50-μl reaction volumes containing 8 U of RNasin (Promega), 10 mM $MgCl_2$, and 1 mM dithiothreitol. The reactions were stopped by adding 12 μl of stop buffer (50 mM EDTA, 1.5 M sodium acetate, 1% sodium dodecyl sulfate). The samples were then extracted with phenol-chloroform, ethanol precipitated, and resuspended in H_2O.

Total cellular RNA (2 μg) was converted into first-strand cDNA in a 20-μl reaction volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each dATP, dCTP, dGTP, and dTTP, 0.1 mM random hexamers (Pharmacia), 12 U of RNasin, and 200 U of mouse mammary tumor virus reverse transcriptase (Bethesda Research Laboratories). The samples were incubated at 42°C for 2 h and then heat inactivated at 95°C for 5 min. At room temperature, reaction volumes were increased to 100 μl in 50 mM KCl–10 mM Tris-HCl (pH 8.4)–2 mM $MgCl_2$–200 μg of gelatin per ml–0.2 μM each primer–1 U of *Taq* polymerase (Perkin-Elmer-Cetus Corp.). The samples were subjected to 25 rounds of amplification as follows: 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C. The final cycle was followed by a single period at 72°C for 5 min. An aliquot from each sample (5 μl) was fractionated on an 8% polyacrylamide gel, and the PCR products were directly visualized after staining of the gel with ethidium bromide and UV illumination.

DNA PCR assays. A typical PCR reaction mixture contained either 300 ng of genomic DNA or 1 μl (500 genomes) of cell lysate in a reaction volume of 50 μl containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 200 μg of gelatin per ml, 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dTTP, 0.1 mM dGTP, 0.1 μM each oligonucleotide primer, and 1 U of *Taq* polymerase. The samples were amplified for 30 cycles, using parameters described above for RNA PCR assays. An aliquot from each reaction was fractionated on an 8% polyacrylamide gel, electroblotted onto a nylon membrane (see below), and screened with a ^{32}P-labeled probe.

Oligonucleotide primers. Oligonucleotides were synthesized at the Department of Microbiology, State University of New York at Stony Brook. The sequences are presented 5′ to 3′. The following primers were used: Vα1, CTCATTGTC CCAGAGGGAGC; Vα1 RSS, GGTTTACATACAGGCTGC; Vα2, TGGCCAGCAGCAGGAGAAAC; Vα2 RSS, TGCAG GTTTGGGTATGGGCT; Vα3, TCTCTGCAGCTGAGAT GCAA; Vα3.2, TGGTACATCCAGCACCATGG; Vα3 RSS, TGTGTTCAGCTCCCCCTGCA; Vα4, AGAGGGTTTGAA GCCACATA; Vα4 RSS, CTGCAGTTTCTGCCACTG; Vα8, TGCCTGTGATGCTGAACTGC; Vα8 RSS, CTGCACCCT TGGTTCATGTG; Jα6.19, TGCATAAACTTGGAGTCT; Jα72, AAGTTTGTAGCCCATGTT; Jα14T-1, CAACTTAT TGAAGCTACC; Jα14T-7, AGGTTTGTAGTTTCCTCC; Jα26, TAATTTACCACTTAGTCC; Cα, CTGTCCTGAGA CCGAGGATC; *RAG-2* (5′), CCTCTTCGTTATCCAGCTAC; *RAG-2* (3′), TTCTCTGGGTAGAAGGCATG; glutaraldehyde 3′-phosphate dehydrogenase (GAPDH) (5′), TGCACC ACCAACTGCTTAGC; GAPDH (3′), ATGAGCTTGCCCA CAGCCTT.

Oligonucleotide sequences were taken from the following references: Vα1 and Vα1 RSS (50); Vα2 (19); Vα2 RSS (44); Vα3, Vα3.2, and Vα3 RSS (the Vα3 primer is specific for all members of the Vα3 family, whereas the Vα3.2 primer is specific only for Vα3.2 subfamily members) (13); Vα4 (30); Vα4 RSS (18); Vα8 and Vα8 RSS (5); Jα6.19, Jα72, Jα14T-1, and Jα14T-7 (13); Jα26 (2); *RAG-1* (5′) and *RAG-1* (3′) (40); *RAG-2* (5′) and *RAG-2* (3′) (33); GAPDH (5′) and GAPDH (3′) (14).

Cloning of PCR fragments. The DNA products of selected PCR assays were directly cloned into the plasmid pCR2000, using the TA cloning system (Invitrogen).

DNA sequencing. Sequencing of double-stranded DNA templates was carried out according to the dideoxy-chain termination method (39), using Sequenase (United States Biochemical Corp.) as described previously (13, 30).

Northern (RNA) and Southern analyses and electroblotting. Genomic DNA (12 μg) was digested with the indicated enzymes, electrophoresed in 1% agarose gels, and transferred to Nytran filters (Schleicher & Schuell, Keene, N.H.). Poly(A)$^+$ RNA (10 μg) was electrophoresed in 1% agarose gels containing 6% formaldehyde and also transferred to Nytran filters. Hybridizations were performed at 42°C in a 50% formamide buffer for 18 h as previously described (29).

DNA fragments fractionated by polyacrylamide gel electrophoresis were transferred to Nytran filters, using a Trans-Blot semidry electrophoretic transfer cell (Bio-Rad). After electrophoresis, the acrylamide gel was soaked in 1× TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA) for 30 min along with six sheets of Whatman 3MM paper and a Nytran filter. The acrylamide gel was placed on top of the Nytran membrane and layered between the saturated Whatman paper (three sheets on top and three below). The sandwiched gel was then placed in the transfer cell (cathode side down) and transferred at 10 to 15 V for 30 min. The membrane was removed from the sandwich, placed on a sheet of Whatman 3MM paper saturated with 0.4 N NaOH for 10 min (DNA exposed surface up), and then rinsed in 3× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 10 min. The membrane was then baked at 80°C for 1 h under vacuum and hybridized as described above.

Nucleotide sequence accession number. The unrearranged Vα3.2 cDNA sequence is in the GenBank data base under accession number M76612.

RESULTS

Unrearranged Vα3.2 gene expression. To determine whether other germ line members of the Vα3 family in addition to a Vα3.1 gene (13) were transcriptionally active in M14T, we screened an M14T cDNA library for clones that would hybridize to a Vα3.2 subfamily probe but not cross-hybridize to a Cα probe. A 621-bp cDNA clone, designated pJF1A, was isolated; its sequence is presented in Fig. 1A. According to the nomenclature put forth by Tan et al. (46), pJF1A is clearly a member of the Vα3.2 subfamily. The conserved heptamer-spacer-nonamer RSS, which mediates Vα-to-Jα recombinations and is normally deleted during the assembly of a complete α-chain gene, is retained in the pJF1A clone. Hence, this cDNA is derived from a transcript containing unrearranged Vα coding and 3′ flanking noncod-

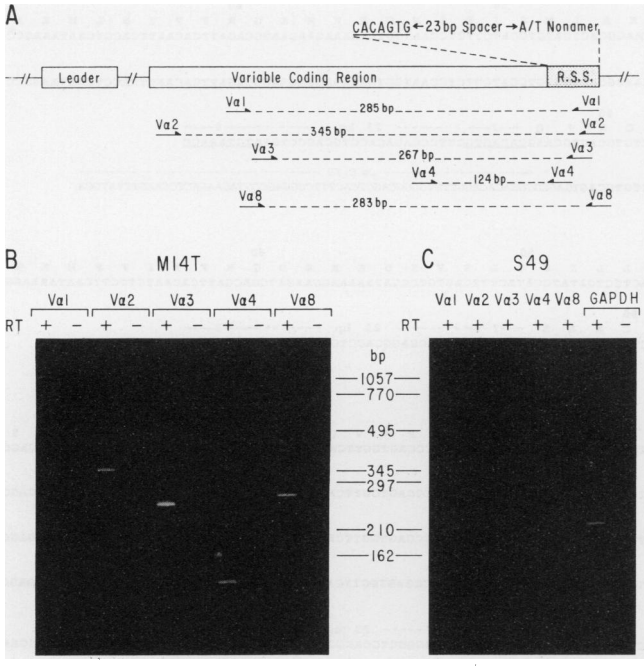

FIG. 2. Detection of germ line Vα transcripts in M14T by PCR. (A) Strategy used to detect germ line Vα transcripts in M14T. Primers specific for the Vα coding region and RSSs of four different Vα families were used to amplify first-strand cDNA prepared from M14T total RNA (see Materials and Methods). As a positive control, primers specific for the Vα3 coding sequence and a Vα3 RSS were also used. The expected PCR products of this assay are shown. (B and C) One-twentieth of each PCR reaction mixture was electrophoresed on an 8% acrylamide, and the PCR products were detected after ethidium bromide staining. To rule out genomic DNA contamination, identical PCR reactions were performed in the absence of reverse transcriptase (−RT lanes). As a positive control for the mature T-cell line S49 PCR assay (C), primers specific for the ubiquitously expressed GAPDH gene were used (14).

ing sequences. The presence of conserved cysteine residues and N-glycosylation sites (16) suggests that pJF1A does not represent a Vα pseudogene.

When the entire Vα3.2 cDNA is used as a probe to screen an M14T Northern blot, six different-size RNA transcripts are revealed (Fig. 1B). The bands at 1.1 and 3 kb cohybridize to a Vα3.1 probe (data not shown) and hence probably represent germ line transcription from the previously identified homologous Vα3.1 gene segment (13). However, the bands at 4.3, 3.6, 2.5, and 0.6 kb (Fig. 1B) appear to be specific for the Vα3.2 subfamily, since none of these bands are evident with a Vα3.1 probe (13). The different-size Vα3.2 transcripts may indicate more than one transcriptional start site or more than one polyadenylation site for a single Vα3.2 germ line segment. Alternatively, they may represent unrearranged Vα transcription emanating from several members of the Vα3.2 gene family.

Detection of germ line Vα transcription by PCR. To test the hypothesis that other germ line Vα segments are transcriptionally active in M14T, we used a sensitive PCR expression assay (Fig. 2A). Primers specific for the Vα coding region and the RSSs of four different Vα families (Vα1, -2, -4, and -8) were used to amplify first-strand cDNA prepared from M14T total RNA. Since one of the primers is located in the RSS, only transcripts from unrearranged Vα segments should be amplified. As a positive control, one primer specific for both Vα3.1 and Vα3.2 coding sequences and a primer specific for the Vα3 RSS were used. Our choice of which specific Vα families to assay was based on two criteria: (i) the availability of previously reported germ line Vα sequences (5, 13, 18, 19, 45, 50) and (ii) previous M14T Southern blot data indicating which Vα segments were still in germ line configuration (data not shown) (20). The expected PCR products of this assay are shown in Fig. 2A.

As shown in Fig. 2B, sterile transcripts emanating from unrearranged Vα1, Vα2, Vα3, Vα4, and Vα8 germ line segments were amplified from M14T RNA. By comparison, no sterile transcripts were observed in the mature T-cell line S49 (Fig. 2C). To rule out the possibility that the M14T PCR products were amplified from contaminating genomic DNA (and not from RNA transcripts), identical PCR reactions were performed in the absence of reverse transcriptase. Under these conditions, no sterile transcripts were detected (Fig. 2B, −RT lanes). The putative Vα1, Vα2, Vα4, and Vα8 PCR products displayed in Fig. 2B were cloned and sequenced (Fig. 3). As expected, the sequence data verify that the M14T PCR products encode sterile Vα transcripts.

Tissue-specific and developmentally regulated germ line Vα expression. To address the tissue and developmental specificity of germ line Vα expression, we examined several murine lymphoid and nonlymphoid cell lines for the presence of sterile Vα transcripts. This analysis was performed by revealing electrophoresed PCR products with ethidium bromide (Fig. 2B) to allow for a direct comparison with the levels of sterile Vα transcripts found in M14T cells. Table 1 indicates that germ line Vα expression is restricted to the T-lymphoid lineage. Germ line Vα transcripts were absent in pro-T cells (the most immature T-lymphoid-committed stage, since TCRα, -β, -γ, and -δ genes are all in germ line configuration) (35), whereas they were present in immature cells. The M14T line likely represents a transformed immature T cell (i.e., pre-T) due to the absence of CD4 and CD8 expression and the presence of *RAG-1* and *RAG-2* (see below) and c-*myb* (data not shown) transcripts, which are all characteristic of immature T cells (14, 36, 43). Therefore, the activation of germ line TCR Vα expression likely occurs at an early stage of T-cell development, with the highest levels in cells actively undergoing Vα-to-Jα joining (i.e., akin to the M14T line). The absence of sterile Vα transcripts in the mature lines S49 and CTLL-2 (except a very weak Vα2 signal) suggests that germ line Vα expression may be downregulated at the mature T-cell stage.

Detection of transcription from the unrearranged Jα-Cα locus. During B-cell development, germ line Jκ-Cκ transcription appears to be specifically induced prior to Vκ-Jκ rearrangement (33). Furthermore, Schlissel and Baltimore have recently demonstrated that the frequency of Vκ-to-Jκ rearrangement correlates with the rate of germ line Jκ-Cκ transcription (41).

To test whether the unrearranged M14T Jα-Cα locus is transcriptionally active, first-strand cDNA prepared from M14T total RNA was PCR amplified, using a 5′-RSS-Jα6.19 primer (primer X; Fig. 4) along with a Cα primer. Assuming proper splicing on a putative unrearranged Jα6.19 transcript between the Jα6.19 segment and the first Cα exon (Fig. 4), a 140-bp PCR product is expected with this assay. The Jα6.19 segment was specifically chosen for this assay because of the availability of germ line Jα sequence data essential for primer synthesis (27). Since primer X is specific for the 5′ germ line portion of Jα6.19, only transcripts encoding the unrearranged Jα6.19-Cα splice will be detected. Figure 5 demonstrates that unrearranged Jα6.19 transcripts are

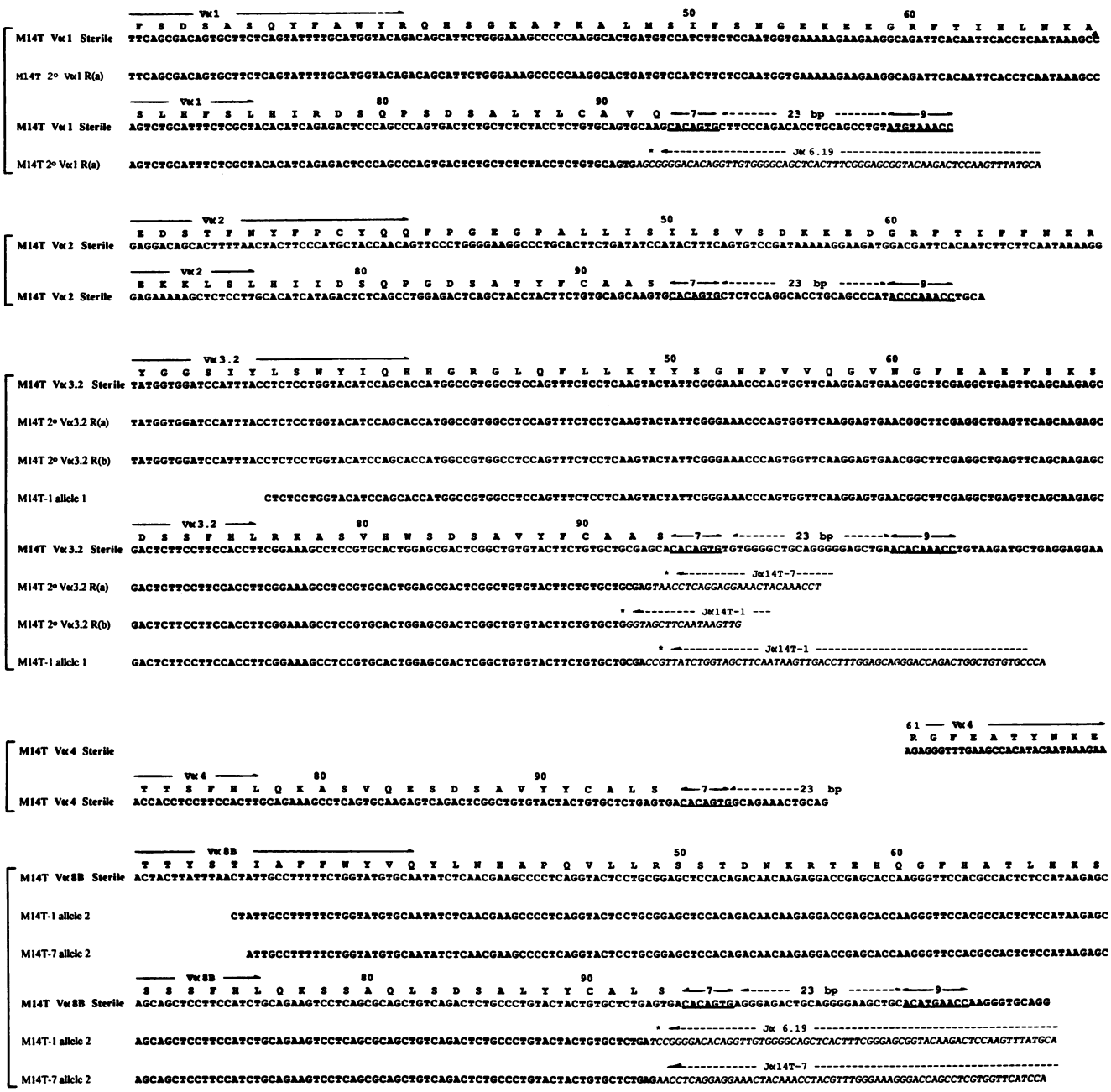

FIG. 3. Sequence comparison of sterile Vα transcripts and secondary Vα-Jα rearrangements. The sterile Vα1, Vα2, Vα4, and Vα8 unrearranged transcripts detected by PCR (Fig. 2B) were cloned, and the sequences are shown. The sterile Vα3.2 transcript shown is a partial sequence from Fig. 1A. Secondary Vα-Jα rearrangements in M14T cells detected by PCR (Fig. 6B, lanes 1 and 3) were randomly cloned, and the sequences are shown [2°Vα1 R(a), 2°Vα3.2 R(a), and 2°Vα3.2 R(b)] in comparison with the sterile Vα transcripts. In addition, the sequences of secondary Vα-Jα rearrangements found in the M14T-1 and M14T-7 sublines (14) are compared with those of the sterile Vα transcripts.

present in M14T, thus supporting our contention that the accessibility of the TCRα locus is strongly associated with its rearrangement. It could be argued that the unrearranged Jα6.19-Cα complex detected in Fig. 5 is simply an aberrant splice of the primary Vα8-Jα72 rearranged transcript on TCRα allele 2 (Fig. 4). This would seem unlikely in light of data with the Igκ locus showing that Cκ splicing occurs exclusively to the 5'-most (rearranged) Jκ segment for transcripts initiating upstream of Ig Vκ-Jκ rearrangements (21, 26). Furthermore, EL4 and CTLL-2, both of which have undergone primary Vα-Jα rearrangements and express a mature TCRα transcript, fail to produce the unrearranged transcription product (Fig. 5).

Correlation of germ line Vα transcription and ongoing TCRα rearrangements. To better establish the correlation between germ line transcription of Vα and Jα segments in M14T with its ability to undergo in vitro recombination events, we used PCR to assay M14T genomic DNA for

TABLE 1. Detection of germ line Vα transcripts by PCR[a]

Cell line	Intensity of PCR signal[b]					
	Vα1	Vα2	Vα3	Vα4	Vα8	GAPDH
Fibroblast						
NIH 3T3	−	−	−	−	−	+
B cell						
AJ95	−	−	−	−	−	+
MPC11	−	−	−	−	−	+
Pro-T cell						
FTH5	−	−	−	−	−	+
FT612	−	−	−	−	−	+
Pre-T cell						
M14T	+	++	+++	++	++	+
LB6	−	+/−	+/−	−	−	+
1010	+/−	−	−	−	−	+
C6VLB	−	−	−	−	−	+
Mature T cell						
S49	−	−	−	−	−	+
CTLL-2	−	+/−	−	−	−	+

[a] The cell lines listed were examined for germ line Vα transcription by using the PCR expression assay exactly as described in the legend to Fig. 2 and Materials and Methods. PCR products were also revealed by ethidium bromide staining as in Fig. 2.

[b] Sterile transcription is indicated as detectable (+), not detectable (−), +/− barely detectable (+/−), or also detectable by Northern blot (+++). GAPDH primers (14) were used as a positive control.

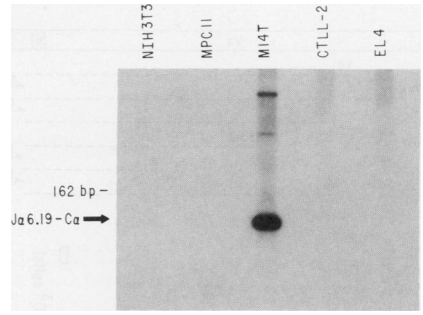

FIG. 5. Detection of unrearranged Jα-Cα transcripts. Total RNA from each cell line was converted into first-strand cDNA and PCR amplified by using a 5′-RSS-Jα6.19 primer (primer X; Fig. 4) along with a Cα primer. One-twentieth of each PCR reaction mixture was electrophoresed on an 8% acrylamide gel, electroblotted onto a nylon membrane, and hybridized with a Jα6.19 probe.

secondary Vα-Jα rearrangements. Primers specific for the transcriptionally active Vα segments (Fig. 4) were used together with a cocktail of primers specific for all known Jα segments remaining at the M14T TCRα locus (primers A to E; Fig. 4). The expected products of this assay are shown in Fig. 6A. Figure 6B shows that genomic DNA prepared from the primary M14T line displayed between three to six secondary rearrangements involving germ line Vα segments known to be transcriptionally active. These data strongly support our contention that transcriptional activation of germ line TCR gene segments correlates with and may therefore be a prerequisite to their rearrangement. We also found that M14T cells continuously cultured in vitro for several months began to lose Vα1-Jα heterogeneity compared with M14T cells of the primary culture (compare lane 1 in Fig. 6B with lane 6 in Fig. 6C). However, when subclones derived from the continuously propagated M14T cells were assayed by PCR, new Vα1-Jα rearrangements were observed (compare lanes 1 in Fig. 6B with lanes 7 and 8 in Fig. 6C). This result confirms that secondary TCRα locus rearrangements are ongoing in the M14T line.

In contrast to Vα1-Jα complexes, we found that M14T cells maintained in culture for several months continue to

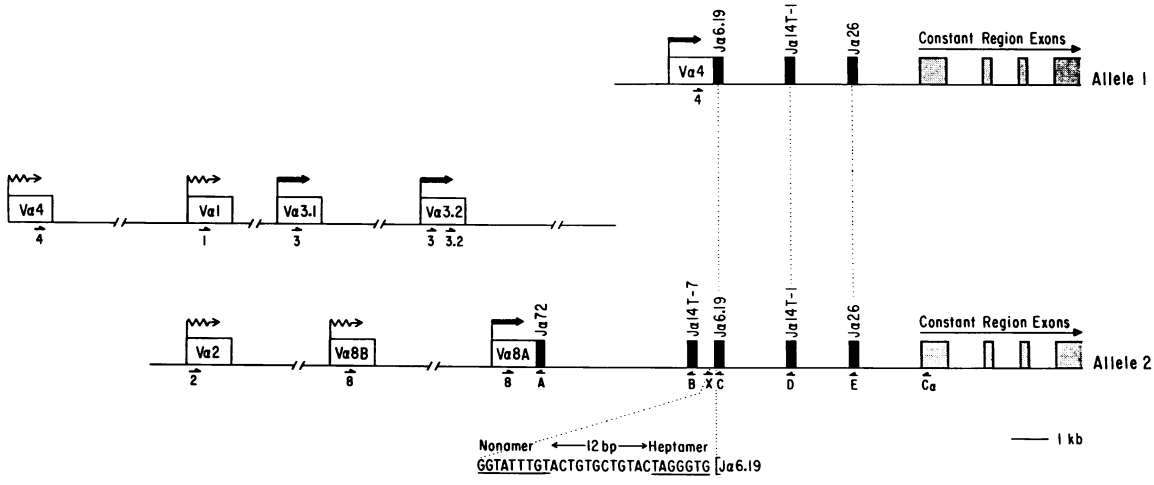

FIG. 4. Schematic representation of the TCRα locus in M14T. Black boxes juxtaposed to white boxes represent primary Vα-Jα rearrangements on alleles 1 and 2 (14). Unrearranged white boxes represent transcriptionally active germ line Vα segments which reside 5′ of the primary rearrangements. Heavy black arrows above the Vα genes indicate transcription detectable by Northern blot; zigzag arrows indicate transcription detectable by PCR. Short horizontal arrows beneath the Vα, Jα, and Cα genes indicate specific PCR primers. The Vα segments on the center line indicate germ line Vα families which have members on both chromosomal homologs. Double diagonal lines indicate that the precise orientations and distances of the germ line Vα segments relative to the primary Vα-Jα complexes are unknown. The germ line positions of Jα6.19, Jα26, and Jα72 were determined previously (50). The germ line positions of Jα14T-1 and Jα14T-7 (13) were determined by mapping the two genes within the cosmid clone pJA73 (50) as described in Materials and Methods. The sizes of the Vα and Jα boxes are not to scale.

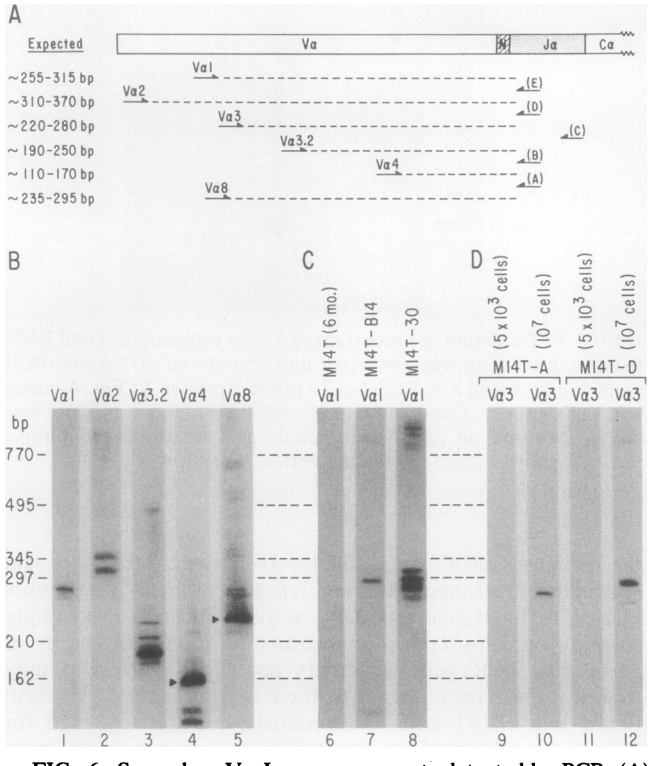

FIG. 6. Secondary Vα-Jα rearrangements detected by PCR. (A) Strategy to amplify secondary Vα-Jα rearrangements in M14T. Genomic DNA from M14T cells was PCR amplified by using primers specific for the transcriptionally active germ line Vα segments (Fig. 4) together with a cocktail of primers specific for all known Jα segments left at the TCRα locus in M14T (primers A to E; Fig. 4). The expected products are shown. (B) Genomic DNA from M14T primary culture cells was PCR amplified as described above. One-fiftieth of each separate Vα-specific PCR reaction mixture was electrophoresed on an 8% acrylamide gel and electroblotted onto a nylon membrane. Each membrane was then screened with the appropriate Vα probe (indicated above each line). Arrowheads indicate primary Vα-Jα rearrangements. (C) Vα1-Jα secondary rearrangements were assayed by PCR, using genomic DNA from M14T cells maintained continuously in culture for more than 6 months or from subclones (B14 and 30) made from these cells. (D) M14T sublines cloned by limiting dilution were assayed for Vα3-Jα secondary rearrangement at different stages of clonal expansion.

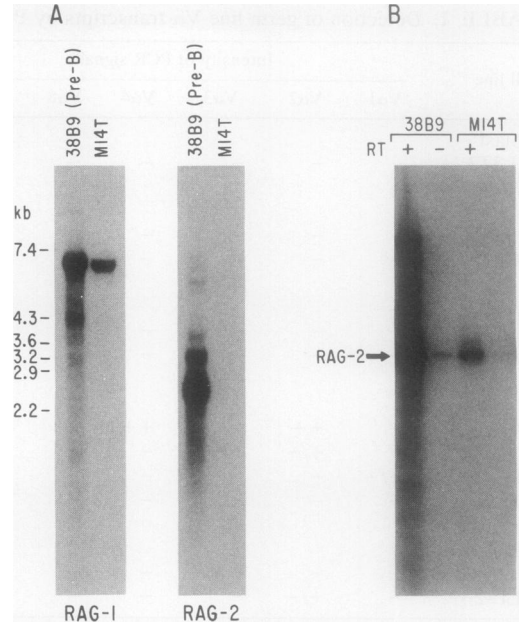

FIG. 7. Expression of *RAG-1* and *RAG-2* in M14T cells. (A) Northern blot analysis of 10 μg of poly(A)$^+$ RNA from 38B9 and M14T, hybridized to a *RAG-1* probe; and a *RAG-2* probe, as indicated. (B) *RAG-2* expression in M14T cells determined by PCR. First-strand cDNA prepared from 38B9 and M14T total RNA was PCR amplified by using *RAG-2*-specific primers. One-twentieth of each reaction mixture was run on an acrylamide gel, electroblotted onto a nylon membrane, and screened with a *RAG-2* probe. Negative control reactions were performed in the absence of reverse transcriptase (−RT).

display secondary Vα3-Jα rearrangements. To verify that these complexes arise from ongoing Vα3 rearrangements, two M14T sublines were cloned by limiting dilution and assayed for Vα3 rearrangements at both early and late stages of clonal expansion. We found no Vα3 rearrangements in subclones A and D after expansion to 5,000 cells (Fig. 6D, lanes 9 and 11; parental Vα-Jα complexes were detected in these cells by PCR [data not shown]). However, after expansion to 10^7 cells, subclones A and D now display new, distinct Vα3-Jα rearrangements (Fig. 6D, lanes 10 and 12), again confirming that such secondary TCRα gene rearrangements are ongoing in M14T cells.

To directly confirm the correlation between germ line transcription and subsequent recombination in M14T, we randomly cloned and sequenced some of the secondary Vα-Jα rearrangements displayed in Fig. 6B. We compared these sequences with those of the sterile Vα transcripts (Fig. 3). We also compared the sequences of previously reported secondary Vα-Jα rearrangements found in M14T sublines (13) with those of the sterile Vα transcripts. Alignment of the Vα1, Vα3.2, and Vα8 sterile transcripts with Vα1-Jα, Vα3.2-Jα, and Vα8-Jα secondary joins, respectively, indicated that the sequences are identical up to the site of Vα-to-Jα joining (Fig. 3). Thus, we find a perfect correlation between the transcription of a Vα segment and its ability to rearrange. We (12) and others (20) have determined that only a single unrearranged member of the Vα2 gene family exists in M14T (its allelic homolog and all other members of the Vα2 gene family have been deleted). Thus, although we show no Vα2-Jα sequence data, the secondary Vα2-Jα rearrangements (Fig. 6B, lane 2) must directly involve the transcriptionally active Vα2 segment.

Expression of *RAG-1* and *RAG-2* in M14T. The specific components of the V(D)J recombination machinery have not been analyzed biochemically, but the products of two recently isolated genes are believed to play crucial roles. *RAG-1* and *RAG-2*, which are predominantly expressed in pre-T and pre-B cells, can activate V(D)J recombination in fibroblasts and are believed to be directly involved in the reaction (33, 40). Since Vα-Jα rearrangements are ongoing in M14T cells, we examined them for *RAG-1* and *RAG-2* expression. Northern blots containing M14T poly(A)$^+$ RNA were screened with *RAG-1* and *RAG-2* probes. The pre-B line 38B9, previously shown to express high levels of *RAG-1* and *RAG-2* (33), was used as a positive control. Figure 7A reveals that M14T expresses *RAG-1* but surprisingly not *RAG-2*, which is somewhat unexpected for a prelymphoid line (33). Either *RAG-1* or *RAG-2* induced V(D)J recombinase activity in fibroblasts but at an extremely low frequency which was only revealed after stringent genetic selection

(33). It remains unclear whether expression of one *RAG* gene was sufficient to generate enough recombinase activity for V(D)J joining or whether a negligible amount of endogenous *RAG* gene activity was acting in concert with the transfected gene in these experiments (33). Indeed, *RAG-2* may be expressed in M14T cells, albeit at very low levels. To test this possibility, we assayed M14T for *RAG-2* expression using a sensitive PCR assay. First-strand cDNA prepared from M14T RNA was PCR amplified by using *RAG-2*-specific primers. As shown in Fig. 7B, the more sensitive PCR assay revealed that *RAG-2* was expressed by the M14T line.

DISCUSSION

The TCR α-chain locus has a unique germ line organization with a great potential for rearrangement diversification. An estimated 50 to 100 Jα segments are spread out over 65 kb upstream of the Cα region (16, 50), whereas at other antigen receptor loci, a small number of J elements are clustered a short distance 5′ of the C region. In addition, the germ line TCRα locus contains as many as 100 Vα segments, whereas other TCR loci have far fewer V elements (22, 4, and 7 for the TCRβ, -γ, and -δ loci, respectively) (4, 22). Furthermore, we and others have shown that (i) allelic exclusion is not operative via DNA rearrangement at the TCRα locus (3, 13, 23, 24, 28, 29, 34, 47) and (ii) secondary Vα-to-Jα rearrangements can occur by a loop-out mechanism joining upstream Vα segments to downstream Jα segments, deleting preexisting Vα-Jα rearrangements (13, 30, 34, 38, 45, 48). The fact that secondary Vα-Jα rearrangements occur in normal thymocytes suggests that they are physiologically important recombination events which function to increase the diversity of the TCR repertoire (13, 38).

In this study, several unrearranged Vα gene segments were found to be transcriptionally active in M14T, an immature T-cell line which actively undergoes secondary Vα-to-Jα rearrangements in cell culture. By comparison, germ line Vα transcripts are absent in nonlymphoid and pro-T-cell lines and are generally not detectable in mature T-cell lines, suggesting that germ line Vα expression is tissue and developmental stage specific. In the M14T line, germ line transcripts emanating from an unrearranged Vα3.2 gene were readily detectable by Northern blot analysis, whereas sterile transcripts for unrearranged Vα1, Vα2, Vα4, and Vα8 gene segments were detected only by PCR. In addition, germ line transcription emanating from the unrearranged Jα-Cα locus was detected in M14T in a PCR assay. The latter transcripts appear to initiate downstream of a primary Vα-Jα rearrangement and result in an unrearranged Jα segment spliced to the Cα exon. The absence of these transcripts in nonlymphoid and mature T-cell lines again suggests tissue and stage specificity. M14T Northern analysis with a Cα probe reveals no abundantly expressed truncated messages (Fig. 1B), suggesting that the sterile Jα-Cα transcripts are transcribed at low levels. However, several different species of abundantly expressed unrearranged Jα-Cα transcripts have been observed in human lymphoid cell lines, strongly suggesting that promoters reside upstream of germ line Jα segments (3).

The appearance of germ line TCR transcripts in M14T is entirely consistent with an accessibility model of antigen receptor rearrangement proposed by Alt et al. for B-lymphoid cells (1). PCR rearrangement assays and sequence analyses conclusively demonstrate that the transcriptionally active Vα and Jα segments are the identical ones involved in secondary Vα-to-Jα rearrangement events in the M14T line. Moreover, the presence of sterile transcripts at an early stage of T-cell development is consistent with the timing of TCR gene rearrangements (22). In other work, we find that the methylation status of the Vα3.2 alleles in M14T cells concurs with the transcriptional competence of these V gene segments (data not shown). The Vα3.2 genes in M14T are hypomethylated, while their counterparts in nonlymphoid cells are hypermethylated and partially methylated in mature T cells, respectively (data not shown). Albeit more indirect that the transcriptional evidence presented here, these other observations also support our contention that Vα genes maybe preferentially accessible at an early stage in T-cell development. The transcription of TCR V genes is differentially controlled before and after their rearrangement, presumably by novel, developmental stage-specific transcription factors. Subcloning experiments with M14T cells reveal a high frequency of secondary Vα3-Jα rearrangements relative to other Vα families, suggesting that germ line Vα segments expressed at high levels may be preferentially utilized in secondary rearrangement events (12). While the accessibility model was initially proposed to explain Ig gene assembly (51), the data presented here provide the first direct evidence for an accessibility model of TCR gene rearrangement suggesting that this mode of DNA recombination is conserved for antigen receptor assembly in B and T lymphocytes.

In addition to the cell-type-specific requirements for unique V region family rearrangements in B- or T-lineage cells, the products of the *RAG-1* and *RAG-2* genes are also essential for mediating V(D)J assembly in both early B and T cells (33, 40). Pre-B-cell lines, which express sterile VH transcripts and high levels of *RAG-1* and *RAG-2*, yield occasional IgH V segment rearrangements (1, 33, 40). However, the low frequency of secondary TCR Vα gene rearrangements in M14T cells occurs in the presence of discordant *RAG* gene expression. This is reflected by a moderate amount of *RAG-1* and very low levels of *RAG-2*. Given the synergistic effects of the two *RAG* gene products in mediating V(D)J joinings (33), this later observation may in part explain the low frequency of secondary Vα rearrangements in M14T cells, especially for the germ line context Vα segments expressed at high levels. Future experiments will be directed toward addressing this issue by modulating unrearranged Vα and/or *RAG* gene expression via external stimuli or by directly introducing exogenous *RAG* gene expression vectors into the M14T line and assessing their qualitative and quantitative effects on secondary Vα-to-Jα rearrangements.

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