Localized Gene-specific Induction of Accessibility to V(D)J Recombination Induced by E2A and Early B Cell Factor in Nonlymphoid Cells

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Abstract

Accessibility of immunoglobulin (Ig) gene segments to V(D)J recombination is highly regulated and is normally only achieved in B cell precursors. We previously showed that ectopic expression of E2A or early B cell factor (EBF) with recombination activating gene (RAG) induces rearrangement of IgH and IgL genes in nonlymphoid cells. \textit{V/H9260} genes throughout the locus were induced to rearrange after transfection with E2A, suggesting that the entire \textit{Vk} locus was accessible. However, here we show that Ig loci are not opened globally but that recombination is localized. Gene families are interspersed in the D\textsubscript{H}, V\textsubscript{H9260}, and V\textsubscript{H9261} loci, and we show that certain families and individual genes undergo high levels of recombination after ectopic expression of E2A or EBF, while other families within the same locus are not induced to rearrange. Furthermore, in some families, induction of germline transcription correlates with the level of induced recombination, while in others there is no correlation, suggesting that recombination is not simply initiated by induction of germline transcription. The induced repertoire seen at 24 hours does not change significantly over time indicating the absence of many secondary rearrangements and also suggesting a direct targeting mechanism. We propose that accessibility occurs in a local manner, and that binding sites for factors facilitating accessibility are therefore likely to be associated with individual gene segments.

Key words: transcription factor • antibody diversity • recombination • antibody formation • Igs

Introduction

The antibody repertoire is highly diverse, and much of this diversity is due to the many V, D, and J gene segments in the genome. In each precursor lymphocyte, a unique combination of \textit{V\textsubscript{H}}, \textit{D\textsubscript{H}}, and \textit{J\textsubscript{H}} gene segments and \textit{V\textsubscript{L}} and \textit{J\textsubscript{L}} gene segments are joined by a process termed V(D)J recombination, in which recombination activating genes (RAG)*1 and RAG2 recognize the conserved recombination signal sequence adjacent to each segment, and join the segments to form a variable region exon (1). V(D)J recombination is highly regulated, in that VCR genes only recombine in T cells, and Ig gene segments only fully recombine in B cells (2). Rearrangement is also precisely ordered in that the H chain recombines before the L chain, and predominantly \textit{k} recombination precedes \textit{\lambda} recombination (1). The rearrangements at the TCR loci in T cells also display a very similar ordered rearrangement (3). In addition, some loci show control over the order of rearrangement of individual V genes or sets of genes. In the murine TCR C\gamma1 cluster, the most 5\textsuperscript{\prime} J\textsuperscript{\gamma} genes, \textit{V\gamma3}, rearranges first in fetal life, while late in gestation, the next most 5\textsuperscript{\prime} \textit{V\gamma} gene, \textit{V\gamma4}, becomes prominent (4). After birth, the predominant rearrangements are to the two most 5\textsuperscript{\prime} \textit{V\gamma} genes (5). Likewise, in the TCR-\alpha loci, the most 5\textsuperscript{\prime} J\textsuperscript{\alpha} genes recombine first, later followed by the more 3\textsuperscript{\prime} J\textsuperscript{\alpha} genes (6). Deletion of the TEA element 5\textsuperscript{\prime} of the J\textsuperscript{\alpha} cluster leads to a great reduction of the rearrangement of the 5\textsuperscript{\prime} J\textsuperscript{\alpha} genes, but does not affect rearrangement of the 3\textsuperscript{\prime} J\textsuperscript{\alpha} genes, thus showing that there is independent control over different portions of the locus (7).

The observation of this lineage-specific and locus-specific order of V(D)J recombination led to the proposal that the various receptor loci must become accessible at the ap-
ropriate stage of differentiation and in the appropriate lineage (1). This has been confirmed in lymphoid cells, where only the rearranging loci are accessible at any given stage in differentiation (8, 9). In recent years, it has become clear that chromatin restructuring plays a major role in this control of accessibility. Chromatin remodeling complexes have been described changing chromatin from inaccessible to accessible states, and histone acetylation and deacetylation is likely to play a key role in facilitating this process (10). However, it is not known which factors are involved in specifically regulating and targeting accessibility of the V, D, and J genes in the correct lineage and at the appropriate differentiation time.

The changes in accessibility and therefore chromatin structure also manifest itself in the appearance of germline transcripts, and a strong correlation exists between the onset of germline transcription and the subsequent V(D)J recombination at that locus (2). But although germline transcription has been shown to be required for Ig class switch recombination, its contribution to V(D)J recombination is still unclear (11, 12). It is still not known whether germline transcription is involved in actually opening the recombining loci or if it merely reflects the already open state of a previously altered chromatin structure.

Proteins that might participate in regulating accessibility include the E2A proteins E12 and E47. These basic helix-loop-helix proteins are encoded by the same E2A gene and are formed by alternative splicing (13). E2A proteins had originally been characterized by binding to E-box sites previously (24). The expression vectors pEBB-RAG1 and pEBB-RAG2 had been described previously (25). All transcription factors have been cloned in the pHBAPneo vector as described previously and were provided by B. Kee, University of California San Diego, La Jolla, CA (26). 10 µg of each expression vector was used per transfection and cells were harvested 1 or 3 days later for analysis of RNA and genomic DNA.

**Materials and Methods**

**Transfections.** The kidney epithelial cell line BOSC23 was transfected by calcium phosphate precipitation as described previously (24). The expression vectors pEBB-RAG1 and pEBB-RAG2 had been described previously (25). All transcription factors have been cloned in the pHBAPneo vector as described previously and were provided by B. Kee, University of California San Diego, La Jolla, CA (26). 10 µg of each expression vector was used per transfection and cells were harvested 1 or 3 days later for analysis of RNA and genomic DNA.

**Isolation of Genomic DNA.** DNA from transfected cells was isolated as described previously (27). PBLs were obtained from normal donors, and purified as described previously (28). Bone marrow pre-B cells (CD10+ surface Ig−) were isolated on a FACSVantage™ cell sorter after staining with FITC-anti CD10 (Becton Dickinson) and biotin-conjugated anti-IgM (BD PharMingen), followed by PE-streptavidin (Becton Dickinson). DNA was isolated from the cells and was further purified by phenol-chloroform extraction.

**RNA Isolation and Reverse Transcription PCR.** RNA from transfected cells was isolated using Trizol (GIBCO BRL) according to the manufacturer’s protocol, DNase treated and purified by phenol-chloroform extraction, and ethanol precipitation. 5 µg of each RNA were reverse transcribed (RT) using family-specific primers downstream of the RSS. An equal fraction of each cDNA reaction was then amplified by PCR with primers located in the leader and FR3 allowing to distinguish between the spliced and the unspliced transcript. Primers used were: VκI-RT 5’-GGCAGCCCAG CCTCACCACAT-3’; VκII-RT 5’-GAGCT-GCTCCCCAGACAAGCA-3’; VκI-L 5’-GGTCCAAGCT-
TAGCTCTCGGGGCT-3; VxI-FR3 5'-TTGAAAAATCTTCAGGCTGAG-3'; VxII-L 5'-TGCTAAGGCTTGCTGGCGCTGCAAATGC-3'; and VxII-FR3 5'-CCAACATCTCCAGCCTCCAC-3'. The control RT and PCR reactions for human \( \beta \)-actin were performed as described previously (20).

**PCR Analysis and Southern Blot Analysis.** 100 ng of genomic DNA from various transfection experiments was analyzed for induced recombination events by PCR using AmpliTaq Gold (PE Biosystems). Primers that detected the various V\( \gamma \) DNA from various transfection experiments was analyzed for in- 

\[
\begin{align*}
5' & \text{-CCCTGAGCGATTCTCTGGCTCCA-3'}; \\
5' & \text{-CCCCAAGCTTATGATTTATGAGGTCAGTAA-3'}; \\
5' & \text{-GCAGAAGCTTGGCCAGGCCCCTGTG-3'};\end{align*}
\]

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**Results**

**E2A Preferentially Targets Accessibility to the VxI Gene Family.** The human Igk locus contains 76 Vx and 5 Jx gene segments located in a distal and a proximal region, carrying 36 and 40 genes respectively, with 800 kb of intervening sequence between the two halves of the locus (21). 4 Vx families are used in the peripheral repertoire, VxI through VxIV. Importantly for our study, the VxI, II, and III genes are interspersed throughout the locus thus allowing us to investigate if accessibility is uniform throughout the locus. Therefore, we asked whether the various Vx families would be induced to rearrange in a similar frequency upon ectopic expression of E2A or EBF. The embryonic kidney epithelial cell line, BOSC23, was transiently transfected with expression vectors encoding E12, E47, or EBF, either alone or in combination with expression vectors encoding the recombinase enzymes, RAG1 and RAG2. DNA from transfected cells was isolated 3 d later, and amplified with Vx family-specific 5' primers and a consensus Jx 3' primer. PCR products were blotted, probed with Vx family-specific probes, and were quantitated using a PhosphorImager. To estimate the number of recombination events induced in each transfected sample, control PCR reactions were performed on known quantities of plasmids containing sequenced Vx–Jx fragments. To assure the linearity of amplification during the PCR, induced rearrangements for Vx families as well as the control plasmids were analyzed between 27 and 37 cycles and only the data points in the linear range were used for quantitation. Fig. 1 A shows a representative blot for VxI–Jx recombination. Cells transfected with expression constructs for either transcription factor in the absence of the RAG proteins did not show any recombination events. However, cells that had only been reconstituted with the RAG proteins alone in the absence of any transcription factor did result in very low levels of detectable VxI recombination in some transfections, while in other RAG-only transfecteds, VxI rearrangements were completely absent (Fig. 1 A). The number of recombination events detected per 100 ng of BOSC genomic DNA was then calculated. Fig. 1 B represents the calculated recombination events determined for the members of the Vx families. Fig. 1 B also indicates the number of rearranging genes in the proximal locus observed for each family. Since genes in the distal locus (located 1.4–1.9 Mb from the Jx cluster) seldom rearrange, we did not include them in the count of rearranging genes (28, 30). The number of unique out-of-frame sequences identified in PBLs are indicated, representing the nonselected repertoire of individual Vx families in vivo (28, 31).

It is most noticeable that VxI could be induced to undergo recombination at a much higher level than the other three families. Moderate levels of rearrangement were observed for VxIII, very low levels were detected for VxII, while no significant rearrangement of the single VxIV gene was observed. This is in contrast to the number of genes within the families and the level of recombination estimated for each family. The VxII family has more members
and as a family rearranges twice as often as VkIII based on nonproductive sequences in PBLs (30). However, transfected BOSC cells resulted in a fivefold lower level of recombination of VkII genes as compared with VkIII genes, and >30-fold lower rearrangement than VkI genes. Thus, although these Vk gene families are interspersed, the transcription factor induced level of rearrangement varies considerably for the 4 Vk families and also differs from their rearrangement in vivo (Fig. 1 B). Induction of recombination by EBF was observed, but was much lower than that induced by either form of E2A. To verify the low levels of rearrangement seen upon expression of EBF, we performed PCRs with 40 cycles of amplification. This indeed confirmed that EBF induced recombination in VkI, VkII, and VkIII (data not shown). At 40 cycles, we occasionally observed weak signals for VkIV rearrangement in E2A- or EBF-transfected cells, but overall levels of recombination were not significant.

**Vk Germline Transcription Does Not Always Correlate with Recombination Levels.** We next investigated whether the drastic difference in the level of recombination induced in our model system for the interspersed gene families VkI and VkII is correlated with their level of germline transcription. Total RNA was isolated from BOSC23 cells 3 d after transfection and cDNA prepared with family-specific primers located downstream of the RSS. Each cDNA was then PCR amplified with a combination of family-specific primers annealing in the leader and FR3 region. These primer sets allowed us to distinguish between unspliced and spliced transcripts. The resulting PCR products were blotted and probed with family-specific probes. Fig. 2 A shows the VkI transcripts detected in BOSC23 cells transfected with the indicated combinations of expression vectors. E47-transfected cells had much higher levels of germline VkI transcripts than EBF-transfected cells, as expected since there are no known EBF sites in VkI promoters (32). This correlates well with the observed level of VkI recombination. Furthermore, only a low level of transcription is observed in RAG-only transfected cells.

Transfected cells were also analyzed for VkII germline transcripts. A representative blot is shown in Fig. 2 B. Both E47 and EBF resulted in induction of germline transcripts, however the level of transcription was considerably higher in EBF transfecants, consistent with the presence of EBF sites in VkII promoters (32). Furthermore, the enhanced induction of transcription after EBF expression was mostly seen in the spliced isoform. Mock transfection and transfection with the RAG1/2 expression vectors alone resulted only in extremely low levels of transcription. These findings are in contrast to the observed levels of VkII–Jk recombination where neither E2A nor EBF could induce high levels of recombination.

**Vk and Jk Gene Utilization Is Biased in Vk–Jk Rearrangements.** The sequences of Vk–Jk rearrangements were analyzed from pooled PCR reactions of VkI and VkIII (Table I). Sequences were diverse, and all aspects of junctional diversity were normal. N regions were absent from all junctions as expected since TdT is not expressed in BOSC cells. Since the two isoforms of E2A resulted in similar recombination frequencies, sequences observed after either E12 or E47 transfection were combined for analysis. The genes are listed in their 5′ to 3′ order starting with the most distal
genes. VκI sequences observed upon E2A expression had been published (previously 20). VκI genes throughout the proximal region spanning 600 kb were found to have rearranged, although a small shift towards preferential use of the more proximal genes was observed in samples from E2A transfectants. A more prominent shift towards the 3' end of the locus was detected after EBF induced recombination although again genes throughout the proximal half were used.

Both transcription factors induced approximately equal ratios of utilization of the most Jκ-proximal VκIII gene, Vκ3-7 and the most 5' VκIII gene in the proximal half, Vκ3-20. Since the absolute number of recombination events observed after transient expression of EBF or E2A differed noticeably (Fig. 1 B), the similar gene usage indicated that the VκIII genes were similarly accessible even under conditions that resulted in low or high rearrangement levels. We had shown previously that Vκ3-20 (A27) rearranges more often than the other VκIII genes due to its unique RSS (33), so the overrepresentation of this gene in rearrangements recapitulates in vivo observations. However, the overuse of the most 3' proximal gene, Vκ3-7, as compared with the nonproductive rearrangements in the peripheral repertoire, demonstrated a slight 3' bias in induced rearrangements in the BOSC cells. In contrast to the usage of a variety of VκI and VκIII genes throughout the locus, we detected a strong bias in the usage of the most Vκ-proximal Jκ1 gene in cells transfected with either E2A or EBF (Table II). Our Jκ primer amplified Jκ1 and Jκ4 equally well, although mismatches may cause it to under represent Jκ2, 3, and 5. Nonetheless, since the entire Jκ locus occupies only 1.6 kb, the high frequency of Jκ1 utilization suggests targeted rearrangement of this most Vκ-proximal gene.

EBF Preferentially Induces Recombination of VαII Genes. The Igα locus contains ~70 Va genes which are organized in three clusters all upstream of seven Jκ-CA pairs (22). The Jκ-proximal A-cluster carries interspersed members of the VαII and VαIII family, while genes belonging to the VαI family are found further upstream in the B cluster. In the peripheral repertoire, VαII and VαI are the predominantly used V genes (34, 35). Our previous study had only analyzed rearrangements using VαII and Jκ1 segments. In this study we extended our analysis to include the VαI, VαII, and VαIII gene families, which together account for >90% of all Va genes expressed in the peripheral repertoire. We also used a primer detecting Jα2 and Jα3 since they are more frequently used in the peripheral repertoire than Jα1. Recombination of VαIII was strongest with EBF, but low levels of rearrangement were also induced by E2A (Fig. 3 A). Induction of VαII recombination by EBF was eightfold lower compared with VαIII. The low levels of rearrangement detected for VαIII after E2A expression were confirmed by extending the PCR reactions to 40 cycles of amplification (data not shown). Although there are about twice as many VαIII genes than there are VαII genes in the locus, the VαII family contributes at a twofold higher level to the expressed peripheral repertoire (Fig. 3 A). Induced recombination in BOSC cells however is clearly dominated by the VαIII family. In addition, we analyzed VαI-Jα rearrangements. The VαI genes contribute almost to the same extent to the peripheral repertoire as the VαII family. However, only negligible VαI rearrangement was detected in EBF transfected BOSC cells while no recombination was seen in E2A transfectants even at 40 cycles.
Three Distinct Genes Dominate the \(V_{\alpha}\) Recombination Repertoire. We had shown previously that EBF induced \(V_{\alpha}/J_{H}3\–J_{H}1\) rearrangements, but that only the most \(J_{H}\)-proximal gene, \(V_{\alpha}3-1\), recombined. However, in vivo \(J_{H}1\) contributes only a small fraction to the repertoire. We wished to confirm that the limited rearrangement of this \(V_{\alpha}\) gene was indeed due to limited accessibility of only the most proximal \(V_{\alpha}III\) gene. As in our previous study, the recombined \(V_{\alpha}III\) repertoire in BOSC cells transfected with either E2A or EBF was strongly dominated by \(V_{\alpha}3-1\), accounting for \(80\%\) of all \(V_{\alpha}III\) recombination events (data not shown).

This bias is not seen in the periphery, where \(V_{\alpha}3-1\) accounts for \(20\%\) of the repertoire (35). Sequence analysis of transfected BOSC cells further revealed that \(V_{\alpha}II\) rearrangements induced by EBF are dominated by \(V_{\alpha}2-8\) and \(V_{\alpha}2-14\) (data not shown). \(V_{\alpha}2-14\) is the single most frequently rearranging gene in vivo, rearranging \(5\–8\) times as often as \(V_{\alpha}3-1\), yet in BOSC cells \(V_{\alpha}3-1\) rearranged \(3\) times more often than \(V_{\alpha}2-14\). Thus, for the \(\lambda\) locus, the induction of rearrangement differs significantly from the in vivo rearrangement pattern.

Since our \(J_{\alpha}\)-specific PCR primer was designed to preferentially prime \(J_{\alpha}2\) and \(J_{\alpha}3\), we were unable to investigate the overall \(J_{\alpha}\) usage during these induced rearrangement events. However, all \(J_{\alpha}\) genes in \(V_{\alpha}–J_{\alpha}\) rearrangements from transfected samples used the more proximal \(J_{\alpha}2\) gene. This complete bias was not due to the \(J_{\alpha}\) primer used since sequences derived from PBL control DNA using the same primers displayed approximately equal usage of the two \(J_{\alpha}\) genes (data not shown).

**Table I.** \(\nu\kappa\) Gene Utilization in Transfected BOSC23 Cells, Peripheral Blood, and Cord Blood

| \(\nu\kappa\) Genes from distal half of locus combined | \(\nu\kappa\)I | \(\nu\kappa\)III |
|---|---|---|
| E2A | EBF | PBLs & CB |
| BOSC23 transfected with RAG1/2 and: | 1 (2%)<sup>b</sup> | 1 (3%) | 17 (11%)<sup>c</sup> |
| 1-39 (O12) | 5 (11%) | 41 (26%) |
| 1-37 (O14) | 3 (7%) | 8 (5%) |
| 1-33 (O18) | 1 (3%) | 28 (18%) |
| 1-27 (A20) | 7 (16%) | 11 (7%) |
| 1-17 (A30) | 9 (20%) | 4 (13%) | 9 (6%) |
| 1-16 (L1) | 3 (10%) | 14 (9%) |
| 1-13 (L4) | 3 (2%) |
| 1-12 (L5) | 1 (5%) | 2 (1%) |
| 1-9 (L8) | 5 (11%) | 3 (10%) | 2 (1%) |
| 1-8 (L9) | 4 (9%) | 3 (10%) | 14 (9%) |
| 1-6 (L11) | 6 (13%) | 4 (13%) | 3 (2%) |
| 1-5 (L12a) | 1 (2%) | 6 (19%) | 8 (5%) |
| 3-20 (A27) | 6 (40%) | 5 (42%) | 39 (38%) |
| 3-15 (L2) | 1 (7%) | 1 (8%) | 16 (16%) |
| 3-11 (L6) | 1 (7%) | 26 (25%) |
| 3-7 (L10) | 7 (46%) | 6 (50%) | 15 (15%) |

<sup>a</sup>np, only nonproductive (out-of-frame) sequences were analyzed as estimates of recombination frequency in vivo. CB, cord blood; PBL, peripheral blood lymphocyte.

<sup>b</sup>V\(\alpha\)/E2A values had been published previously (reference 20).

<sup>c</sup>Sequences are a combination of our unpublished and published data (reference 28) and the data of Foster et al. (reference 30).
tion events observed in peripheral lymphocytes (37). However, JH6, which contributes ~25% to the peripheral repertoire, was not amplified by our PCR primer.

EBF induced recombination in members of both the DH3 and DH4 gene family although levels were twofold higher for DH4 than for DH3 (Fig. 3B). This is in contrast to DH gene utilization in peripheral blood, where DH3 genes contribute almost fourfold more than DH4 genes (23, 36, 37). Similarly, the E2A proteins promoted recombination in members of both DH families, but at a three to fourfold lower level compared with the EBF transfectants.

**E2A and EBF Proteins Promote the Rearrangement of Different DH Genes.** We have previously had indications that the DH4 repertoire seen in transfected BOSC23 cells was more restricted in D gene usage than that observed in the periphery, particularly for the E2A-transfected cells, although only a small number of sequences had been analyzed (20). To determine whether this restriction holds up with more sequences and to determine if a similar change can be detected in the DH3 repertoire, we sequenced DH3–JH and DH4–JH recombination products. Several members of each family participated in DJ recombination when EBF was expressed in BOSC23 cells and the genes are located throughout the D-gene locus (Table III). In contrast, ectopic expression of either E12 or E47 showed almost exclusive use of the most proximal DH3-22 and DH4-23 genes. Comparison of our observed frequencies to a large database of VDJ rearrangements (23) revealed that the predominantly recombining genes in our in vitro system for both DH3 and DH4 differed from the ones found to contribute the most to the peripheral repertoire. The representation of individual DH genes in VDJ rearrangements may not, however, be reflective of the DH gene usage in DJH rearrangements since the DH segments in productive VDJ

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**Table II. Jκ Gene Utilization in Transfected BOSC23 Cells, Peripheral Blood, and Cord Blood**

| Corresponding Vk gene family/source of genomic DNA | VκI/E2A | VκI/EBF | PBL & CB np |
|---------------------------------------------------|---------|---------|------------|
| Jκ                                                |         |         | n = 54     |
| 1                                                 | 28 (62%)| 17 (57%)| 24%        |
| 2                                                 | 14 (31%)|         | 11%        |
| 3                                                 |         | 3 (10%) | 4%         |
| 4                                                 | 3 (7%)  | 10 (33%)| 59%        |
| 5                                                 |         |         | 2%         |

| VκIII/E2A | VκIII/EBF | PBL & CB np |
|-----------|-----------|-------------|
| Jκ        |           |             |
| 1         | 15 (100%) | 11 (92%)    | 39%        |
| 2         |           |             | 5%         |
| 3         |           |             | 5%         |
| 4         | 1 (8%)    |             | 47%        |
| 5         |           |             | 5%         |

* np, all sequences were obtained with the same VκI and Jκ primer combination; only nonproductive (out-of-frame) sequences were considered for analysis. CB, cord blood; PBL, peripheral blood lymphocyte.

Values from reference 20.

**Figure 3.** Levels of induced Vκ–Jκ and DH–JH recombination events after transient transfection. Induced recombination events for the members of several Vκ and DH gene families were analyzed as outlined in the legend to Fig. 1. Hybridization signals were quantitated, normalized to plasmid standards, and are represented as recombination events per 100 ng of transfected BOSC genomic DNA. (A) Levels of induced Vκ–Jκ rearrangements. The number of rearranging genes within each family are indicated. As an estimate of the contribution of each family to the peripheral repertoire, the number of nonproductive sequences identified in PBL (34) and their contribution within a large cDNA library (reference 35) are shown. (B) Levels of induced DH3 and DH4 rearrangements. The number of rearranging genes within each family and the contribution of each family to complete VDJ rearrangements isolated from peripheral blood (reference 23) are shown.
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Table III. D<sub>H</sub> Gene Utilization in Transfected BOSC23 Cells, Pre-B Cells, and Peripheral Blood

| Family | Genes | E2A | EBF | Pre-B | PBLs |
|--------|-------|-----|-----|-------|------|
|        |       | 2 (11%) | 4 (22%) | 4 (25%) | 42 (24%) |
| D<sub>H</sub> | 3-3 (DXP4) | 2 (11%) | 4 (22%) | 4 (25%) | 42 (24%) |
|        | 3-9 (DXP1) | 5 (28%) | 2 (13%) | 19 (11%) |
|        | 3-10 (DXP1') | 1 (8%) | 2 (11%) | 1 (6%) | 73 (41%) |
|        | 3-16 (D21/10) | 1 (8%) | 3 (19%) | 9 (5%) |
|        | 3-22 (D21/9) | 15 (79%) | 7 (39%) | 6 (38%) | 34 (19%) |
| D<sub>H</sub> | 4-4 (DA4) | 2 (4%) | 10 (32%) | 3 (8%) |
|        | 4-11 (DA1) | 2 (6%) | 3 (8%) |
|        | 4-17 | 2 (4%) | 5 (16%) | 4 (27%) | 21 (57%) |
|        | 4-23 | 44 (91%) | 14 (45%) | 11 (73%) | 10 (27%) |

*From 451 VDJ rearrangements analyzed by Corbett et al. (reference 23), 177 sequences used D<sub>H</sub>3 genes while only 37 used D<sub>H</sub>4 genes. Values in the table are represented as the percentage usage within each individual family. PBL, peripheral blood lymphocyte.

Discussion

In this study, we show examples of individual gene control of accessibility at the three Ig loci, with interspersed genes being induced to rearrange at very different frequencies after ectopic expression of E2A or EBF. We previously showed that E2A induces high frequencies of rearrangement of V<sub>k</sub> genes located throughout the locus in a non-lymphoid cell line. This suggested that E2A, perhaps by binding to the k enhancer, endowed accessibility to recombination to the entire V<sub>k</sub> locus. However, we now show that E2A induces much lower levels of recombination of the V<sub>k</sub>I gene family and only a moderate level of rearrangement of V<sub>k</sub>II genes, as compared with the levels of V<sub>k</sub>III genes. Since all three V<sub>k</sub> gene families are inter-
spersed within the locus, this data suggest that E2A preferentially targets recombination to the members of the V\textsubscript{H} family. Members of individual V and D gene families in general show \(>85\%\) identity in coding regions, and the flanking regions are also highly conserved, not surprisingly since gene families arose by gene duplication. Different V gene families display \(70\%\) identity. Thus, if accessibility is controlled on the level of individual genes, genes within a family would be more likely to be induced to rearrange by similar factors, and this is what we observed.

Previously, there was little data to suggest that control of accessibility would be so localized. There have been several examples of sequential accessibility of gene segments or gradients of rearrangement through a locus (5, 7, 38, 39). However, in all of these cases, the rearrangement proceeds from the most proximal to more distal genes. These observations have led to the chromosomal location hypothesis, which posits that the most proximal V and J segments will at least initially rearrange with the highest frequency, and that accessibility to V(D)J rearrangement may move directly down the locus (1). However, an indication that there may be control of V gene accessibility at the level of the individual gene came from the analysis of mice bearing a TCR-\gamma minilocus transgene in which 1 kb of promoter was switched between the V\gamma3 and V\gamma2 genes (40). The rearrangement frequency of the two genes in the adult thymus was reversed, indicating that the 5' flanking region of the two genes controlled their rearrangement pattern. This reversal in the rearrangement pattern of the two V genes in the adult thymus after the promoters were swapped could either reflect the ability of the respective promoters to promote germline transcription, or could indicate that there are sites in the upstream regions which control the local accessibility of the gene's RSS, possibly through the recruitment of chromatin-modifying proteins. In this regard, it has been shown that E2A can recruit the SAGA complex containing histone acetyltransferase, and acetylation of histones is associated with increased accessibility (41).

Table IV. \(J_{H}\) Gene Utilization in Transfected BOSC23 Cells and Peripheral Blood

| Family | Genes | \(D_{H3}/E2A\) | \(D_{H3}/EBF\) | \(D_{H3}/\text{pre-B}\) | PBLs np\(^{a}\) | \(J_{H6}\) corrected\(^{b}\) |
|--------|-------|---------------|---------------|----------------|----------------|----------------|
|        |       | \(n = 20\)   |               |                |                |                |
| J\(_{H}\) | 1     | 7 (37%)      | 2 (11%)       | 1 (6%)         | 4%             | 5%             |
|        | 2     | 4 (21%)      | 1 (6%)        | 3 (9%)         | 5%             | 6%             |
|        | 3b    | 7 (37%)      | 9 (50%)       | 2 (13%)        | 15%            | 20%            |
|        | 4b    | 1 (5%)       | 7 (39%)       | 2 (13%)        | 23%            | 20%            |
|        | 5b    | 2 (13%)      | 1 (7%)        | 4%             | 5%             |                |
|        | 6     | 23%          |               |                |                |                |

\(\text{np, only nonproductively rearranged sequences were considered for analysis. PBL, peripheral blood lymphocyte.}\)

\(^{a}\text{Values from reference 37.}\)

\(^{b}\text{PBL J}_{H}\) frequencies recalculated without \(J_{H6}\) since our \(J_{H}\) primer did not amplify \(J_{H6}\).
narration was detected. This indicates that germine transcription is not sufficient to induce V(D)J rearrangements, suggesting that the transcribed transcription factors may have functions other than promoting transcription during V(D)J recombination. The lower but detectable levels of VκII germine transcripts in E47 transfected cells may be due to the presence of E-boxes in some but not all members of the VκII family (21).

In the Vκ locus, EBF induces high levels of rearrangement of VκIII genes, vastly dominated by a single gene Vκ3-1, but lower levels of rearrangement are induced using VκII genes. The more distal VκI genes are not induced to rearrange at all, even though they contribute in large numbers to the peripheral repertoire. In the Dκ locus the average distance between any given Dκ3 gene segment and its adjacent Dκ4 gene is only ~1 kb. Expression of E2A or EBF together with the RAG proteins induced rearrangements of the Dκ3 and Dκ4 family, however E2A induced recombination levels were threefold lower than levels induced by EBF. Together the data obtained for the three different loci demonstrate that ectopic expression of E2A or EBF differentially induces rearrangement of interspersed gene families in a frequency unrelated to the recombination frequency of those families in vivo.

Sequence analysis of these rearrangements showed that the individual genes within a family were not equally induced to rearrange. V, D, and J genes rearrange at different frequencies in vivo. In some cases, this is due to the relative effectiveness of the RSS, which have much natural variation (43). In such situations, we would expect that the same nonrandom gene utilization that is caused by better RSS or less good RSS would be observed in individual gene rearrangements after ectopic expression of E2A or EBF, and that this would be independent of accessibility. Hence, we have compared the rearrangement frequency of individual genes induced by E2A or EBF to the frequencies observed in vivo in unselected out-of-frame rearrangements. In some cases, e.g., the VkIII genes or the VκI genes, the frequency of recombination of individual genes was similar to that observed in vivo, although in both cases, there was a slight skewing to more rearrangement of the J-proximal genes. In other cases, the frequencies were unlike those observed in the peripheral repertoire.

Previous indications for a gene-specific impact of E2A on V(D)J recombination came from studies performed on Vγ gene utilization during γδ T cell development, where it was shown that E2A-deficient mice undergo normal rearrangement of Vγ3Vδ1 receptors in fetal life, but do not show rearrangement of Vγ genes which normally undergo rearrangement in the adult thymus (44). Furthermore, ectopic expression of E2A can induce rearrangement of the other Vγ genes (45). E2A-deficient mice also display aberrant control of Vγ3Vδ1 rearrangement, in that it is not turned off in the adult thymus (44). This data suggests that E2A can play either positive or negative role in the control of gene rearrangement, and further suggests that specific V genes may require E2A for rearrangement, and others may be unaffected by E2A. Similarly, we have observed that genes within a locus which were induced by one transcription factor were different than those induced by other transcription factors. A striking example of this difference is the usage of all Dκ3 genes in rearrangements induced by EBF, whereas E2A dominantly induced rearrangement of the most proximal Dκ3 genes. In other cases, such as the VκIII family, all three transcription factors induced the same genes to preferentially rearrange.

In addition to preferential rearrangement of some gene families and of some individual genes by either EBF or E2A, in several loci we observed skewing toward rearrangement of the proximal genes. For example, expression of E2A proteins induced almost exclusive rearrangement of the most 3’ Dκ4 gene, Dκ4-23, and the most 3’ Dκ3 gene, Dκ3-22. Likewise, EBF or E47 induced rearrangement of the Igκ locus showed preferential usage of the most proximal Vκ3-1. Within the Jκ locus, Jκ1 was used in almost all rearrangements. This is in contrast to the peripheral repertoire, but the peripheral repertoire is likely to be affected by secondary rearrangements. In all the loci which we analyzed, secondary rearrangement of DH or VL elements upstream of existing rearrangements to downstream J segments can occur. Thus, the final peripheral repertoire will not necessarily reflect the initial frequency of rearrangement, and the higher frequency with which the more distal
Vk and Jk genes are detected in vivo may be due to secondary rearrangements or receptor editing. To address the question whether we would observe secondary rearrangements in our model system, D\(_{\alpha}4\)–J\(_{H4}\) recombination events from two different time points after transfection were compared. Our results indicate that there was no significant difference in gene segments used in recombination at day 1 compared with day 3. This excludes the possibility that the repertoire observed in BOSC cells 3 d after transfection was shaped significantly by secondary rearrangements. Consistent with this hypothesis, we did not detect any recombination events for VA1 genes, suggesting that the high frequency with which VA1 genes are seen in the peripheral repertoire is shaped by effects such as secondary recombination or receptor editing. Alternatively, it is also possible that the distinct clusters within the Ig\(_{\alpha}\) locus are separated by boundary elements that potentially could confine the first round of V(D)J recombination to the J proximal A-cluster and that this could not be overcome in our experimental system.

Based on the results presented here it can not be ruled out that the induced recombination events in BOSC cells may be due to indirect effects via upregulation of other gene products. However, the similarity of the repertoire 24 h after transfection to that after 72 h suggest a rather rapid induction of recombination after transfection. Assuming that recombination induced via secondary genes would be slower, one might expect a different repertoire or the absence of recombination at 24 h, which we did not observe. Therefore, we hypothesize a direct involvement of E2A and EBF in inducing accessibility for the recombination event. It is possible that the relevant E2A- or EBF-binding sites are indeed located in the promoter. However, most Vk promoters have potential E2A sites (21), but these genes are unequally induced to undergo recombination after transfection of E2A. Furthermore, only VkII genes, but none of the other Vk family genes, have known EBF sites in their promoter (32), yet EBF induces lower levels of rearrangement for VkII genes than for Vkl or VkIII genes. Thus, together with our observation that induced germline transcription does not uniformly correlate with recombination levels, we would speculate that the binding sites relevant to V(D)J recombination are not related to transcription, and that the E2A or EBF proteins may serve as a stationary docking sites for HAT-containing chromatin remodeling complexes. In this later case, the binding sites might be located outside the promoter region, maybe even closer to the RSS. Potential E2A sites can be found throughout the V and D loci, and some are indeed located near the RSS. Although it has been amply described that one reason for nonrandom gene segment usage in vivo is the naturally occurring variation in RSS (43), it is intriguing to speculate that perhaps the presence and/or relative position of binding sites for key transcription factors such as E2A and EBF may also influence the frequency of rearrangement of individual genes. Future studies will be aimed at determining whether the transcription factors act directly or indirectly on these genes, and where the relevant binding sites are located.

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