

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

Peter Goebel,¹ Noel Janney,¹ Joaquín R. Valenzuela,¹
William J. Romanow,² Cornelis Murre,² and Ann J. Feeney¹

¹The Scripps Research Institute, Department of Immunology IMM-22, La Jolla, CA 92037

²Department of Biology, University of California at San Diego, La Jolla, CA 92093

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. V κ I genes throughout the locus were induced to rearrange after transfection with E2A, suggesting that the entire V κ 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_H, V κ , and V λ 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 V_H, D_H, and J_H gene segments and V_L and J_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 TCR 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 κ recombination precedes λ 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 γ 1 cluster, the most J-proximal V gene, V γ 3, rearranges first in fetal life, while late in gestation, the next most 5' V γ gene, V γ 4, becomes prominent (4). After birth, the predominant rearrangements are to the two most 5' V γ genes (5). Likewise, in the TCR- α loci, the most 5' J α genes rearrange first, later followed by the more 3' J α genes (6). Deletion of the TEA element 5' of the J α cluster leads to a great reduction of the rearrangement of the 5' J α genes, but does not affect rearrangement of the 3' J α 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-

Address correspondence to Ann J. Feeney, The Scripps Research Institute, Dept. of Immunology IMM-22, 10550 North Torrey Pines Rd., La Jolla, CA 92037. Phone: 858-784-2979; Fax: 858-784-9190; E-mail: feeney@scripps.edu

*Abbreviations used in this paper: EBF, early B cell factor; RAG, recombination activating gene; RT, reverse transcription.

appropriate stage of differentiation and in the appropriate lineage (1). This had 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 differentiative 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 present in the Ig κ enhancer as well as the IgH enhancer. The observations that the presence of the enhancers is necessary for efficient V(D)J recombination to occur (14, 15), and that B cell development is arrested in E2A knockout mice before onset of D-to-J rearrangement (16, 17), makes E2A a candidate as potential regulatory cofactor for recombination. In fact, it was previously shown that the overexpression of E47 in a pre-T cell line could induce IgH DJ recombination (18). Another protein that is essential for B cell differentiation, and that is B lineage specific in expression, is early B cell factor (EBF). EBF-deficient mice display a phenotype similar to the E2A-deficient mice, with B cell development being arrested before D-J recombination (19).

Recently we have shown that ectopic expression of E2A or EBF together with RAG1 and RAG2 can induce V(D)J recombination in a nonlymphoid cell line (20). While both transcription factors induced D_H4-J_H recombination, V κ I-J κ recombination was only seen with E2A, and V λ III-J λ I rearrangements were only detected in EBF transfectants. V κ I rearrangements used V κ genes throughout the locus, suggesting global accessibility of the entire V κ locus. However, the frequency with which some other individual genes rearranged differed from that described for the peripheral repertoire. The members of the D_H, V κ , and V λ gene families are interspersed at their respective loci (21–23). The goal of the present study therefore was to determine whether E2A and/or EBF induced accessibility of the entire loci, or if there was evidence for partial induction of

accessibility. Here we present data suggesting that the loci are not uniformly accessible. For example, the levels of recombination for V κ II genes are vastly reduced compared with V κ I, even though these genes are interspersed. Interestingly, the V κ I and V κ II gene family both showed induced levels of germline transcription, but V κ I transcription was highest after E2A transfection, while V κ II transcripts were best induced after EBF expression. Thus, germline transcription correlated well with recombination for V κ I, but not for V κ II.

We also show that while expression of EBF induced rearrangement of almost all D_H3, D_H4, and J_H genes throughout the locus, E2A-activated rearrangement exhibited a strong bias for the most J_H-proximal D_H genes and preferential usage of the 5' located J_H genes. Furthermore, the induced D_H4-J_H repertoire was diverse at 24 h after transfection and similar to that seen at 72 h, implying the absence of much secondary rearrangement in our *in vitro* model system. Within the λ locus, we detected recombination of the V λ II family, but at a much lower level than V λ III genes even though these two families are interspersed, and we did not observe significant levels of rearrangement of the more distal V λ I genes even though both V λ I and V λ II genes are seen at a much high frequency than V λ III genes in PBLs. These data suggest that accessibility may be endowed in a local manner, and that neighboring genes are not equally accessible to V(D)J recombination.

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 pH β APneo vector as described previously and were provided by B. Kee, University of California San Diego, La Jolla, CA (26). 10 μ g of each expression vector was used per transfection and cells were harvested 1 or 3 d 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 μ g 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 are: V κ I-RT 5'-GGCAGCCCAGCCTCACACAT-3'; V κ II-RT 5'-GAGCT-GCTCCCCAGACAAGCA-3'; V κ I-L 5'-GGTCCAAGCT-

TAGCTCCTGGGGCT-3'; V κ I-FR3 5'-TTGCAAAATCT-TCAGGCTGCAG-3'; V κ II-L 5'-TGCTAAGCTTCTGGGG-CTGCTAATGC-3'; and V κ II-FR3 5'-CCAACATCCTCAG-CCTCCAC-3'. The control RT and PCR reactions for human β -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 κ , V λ , and D $_H$ gene segments were designed to amplify almost all, if not all, members of the different gene families. Primers D $_H$ 3 and D $_H$ 4 were published previously and had been used in our earlier study (29). The J λ primer was designed to specifically recognize J λ 2 and J λ 3. Primers used are as follows: V κ I 5'-GGTCCAAGCT-TAGTCTCCTGGGGCT-3'; V κ II 5'-CTAGAAGCTTCTCC-CTGCCCGTACCCYTGGA-3'; V κ III 5'-AGCTAAGCT-TCCCTCCTGCTACTCT-3'; V κ IV 5'-GTATAAGCTTTGT-CCATTACTGACT-3'; J κ 5'-ACGTTTGAATTCCACCT-TGGTCCC-3'; D $_H$ 3 5'-CCTCCTCMGGTCAGCCCYGG-ACAT-3'; D $_H$ 4 5'-CCCAGGACGCAGCACCRCTGTCAA-3'; J $_H$ 5'-TGTGGAATTCACCTGAGGAGACGGTGACCA-3'; V λ I 5'-CAGGAAGCTTCCCCAACTCCTCATC-3'; V λ II 5'-CCCCAAGCTTATGATTTATGAGGTGAGTAA-3'; V λ III 5'-GCAGAAGCTTGGCCAGGCCCTGTG-3'; and J λ 5'-TAGGGAATTCAGCTTGGTCCCTCC-3'. PCR reactions for individual gene families were performed by using a family specific 5' primer in combination with a common J-gene 3' primer. A positive control PCR was included in each set using genomic DNA from PBLs. For standardization, PCR reactions were performed on pools of plasmids containing cloned and sequenced recombined fragments. Each pool contained known amounts and therefore known copy numbers of representative mixtures of genes belonging to the same family. Serial dilutions of these plasmids were analyzed in the same linear amplification range as the experimental DNA samples to allow calculation of recombination events in experimental samples. The PCR reaction conditions were as follows: 10 min at 95°C followed by 27–40 cycles of 20 s at 94°C, 30 s at 58°C, and 90 s at 72°C. Aliquots were taken from each PCR reaction in intervals of three cycles. The samples were transferred onto Hybond N⁺ nylon membrane (Amersham Pharmacia Biotech), hybridized with [³²P]-labeled gene family-specific oligonucleotide probes, and quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics). The signals obtained from BOSC genomic DNA were normalized to the signals from the plasmids and are presented as number of recombination events per 100 ng of BOSC DNA. Family-specific probes are as follows: V κ I 5'-TCTGCATCTGTAGGAGACAGAGTCACCATC-3', V κ II 5'-GGCCAGTCTCCACAGCTCCTGATC-3', V κ III 5'-GC-CACCCGTCTTTGTCTCCAG-3', V κ IV 5'-CGTGATG-ACCCAGTCTCCAGACTCCCTGGCTGTGTCTC-3', D $_H$ 3 5'-TGAGGTCTGTGCTCACTGTGGTATTACGAT-3', D $_H$ 4 5'-CCTRCTGTGTGACTACRGTAACTAC-3', V λ I/II 5'-TTCTCTGGCTCCAAGTCTGGCA-3', V λ III 5'-GGGAT-CCCTGAGCGATTCTCTGGCTCCA-3'.

Sequence Analysis. To obtain a representative sequence population from amplified products, aliquots from 4–6 individual PCR reactions were pooled and ligated into the TOPO TA cloning vector (Invitrogen). Bacterial colonies were screened by colony hybridization using the same probes as used for the Southern blot analysis. Positive clones were grown and minipreped. The PCR inserts were sequenced using the Sequenase 2.0 sequencing kit (Amersham Pharmacia Biotech).

Results

E2A Preferentially Targets Accessibility to the V κ I Gene Family. The human Ig κ locus contains 76 V κ and 5 J κ 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 V κ families are used in the peripheral repertoire, V κ I through V κ IV. Importantly for our study, the V κ I, 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 V κ 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 V κ family-specific 5' primers and a consensus J κ 3' primer. PCR products were blotted, probed with V κ 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 V κ -J κ fragments. To assure the linearity of amplification during the PCR, induced rearrangements for V κ 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 V κ I-J κ 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 V κ I recombination in some transfections, while in other RAG-only transfectants, V κ I 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 V κ 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 J κ 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 V κ families in vivo (28, 31).

It is most noticeable that V κ I could be induced to undergo recombination at a much higher level than the other three families. Moderate levels of rearrangement were observed for V κ III, very low levels were detected for V κ II, while no significant rearrangement of the single V κ IV 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 V κ II family has more members

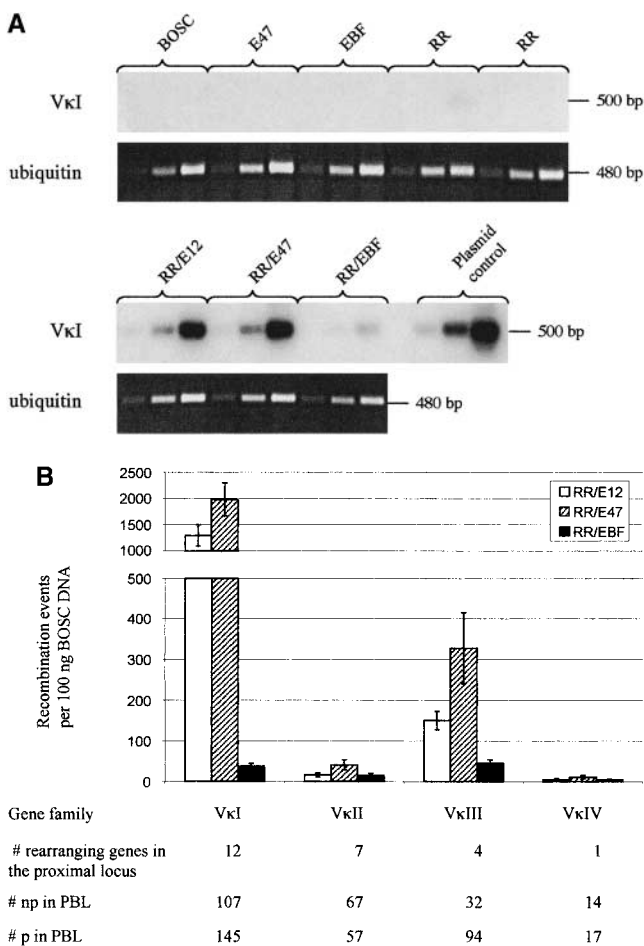


Figure 1. Southern blot analysis and quantitation of PCR amplified Vκ recombination products. (A) A representative blot for the amplification of the VκI family is shown. Aliquots were taken after 28, 31, and 34 cycles of amplification. The top panel shows samples from cells transfected with E47, EBF, or with RAG1 and RAG2 (RR) alone compared with mock-transfected BOSC23 cells. The bottom panel shows DNA from cells transfected with each transcription factor together with the RAG proteins. The bottom panel also shows amplification obtained from 10 fg of a plasmid control sample used for quantitation. The equality of genomic DNA used in each sample was confirmed by amplification of the same genomic DNA samples with primers for the ubiquitin-conjugating enzyme, shown under each lane of the blot, taken at 25, 28, and 31 cycles. (B) Levels of induced Vκ-Jκ rearrangements. Hybridization signals from PCR products in the linear amplification range were quantitated by PhosphorImager and normalized to the plasmid standards. Samples from cells that had only been transfected with the transcription factors alone did not result in measurable PCR products and were excluded from quantitation. Normalized values are expressed as the number of recombination events detected per 100 ng of transfected BOSC genomic DNA. The bar graph shows the average ±SEM of the normalized signals. The numbers under each family represent the number of recombining genes in the proximal half of the Vκ locus seen in large databases. The second and third row of numbers represents nonproductive (estimates of recombination frequency) and productive (expressed repertoire) sequences in vivo (references 28 and 30).

and as a family rearranges twice as often as VκIII based on nonproductive sequences in PBLs (30). However, transfected BOSC cells resulted in a fivefold lower level of recombination of VκII genes as compared with VκIII genes,

and >30-fold lower rearrangement than VκI genes. Thus, although these Vκ gene families are interspersed, the transcription factor induced level of rearrangement varies considerably for the 4 Vκ 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 VκI, VκII, and VκIII (data not shown). At 40 cycles, we occasionally observed weak signals for VκIV rearrangement in E2A- or EBF-transfected cells, but overall levels of recombination were not significant.

Vκ 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 VκI and VκII 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 VκI transcripts detected in BOSC23 cells transfected with the indicated combinations of expression vectors. E47-transfected cells had much higher levels of germline VκI transcripts than EBF-transfected cells, as expected since there are no known EBF sites in VκI promoters (32). This correlates well with the observed level of VκI recombination. Furthermore, only a low level of transcription is observed in RAG-only transfected cells.

Transfected cells were also analyzed for VκII 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 transfectants, consistent with the presence of EBF sites in VκII 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 VκII-Jκ recombination where neither E2A nor EBF could induce high levels of recombination.

Vκ and Jκ Gene Utilization Is Biased in Vκ-Jκ Rearrangements. The sequences of Vκ-Jκ rearrangements were analyzed from pooled PCR reactions of VκI and VκIII (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

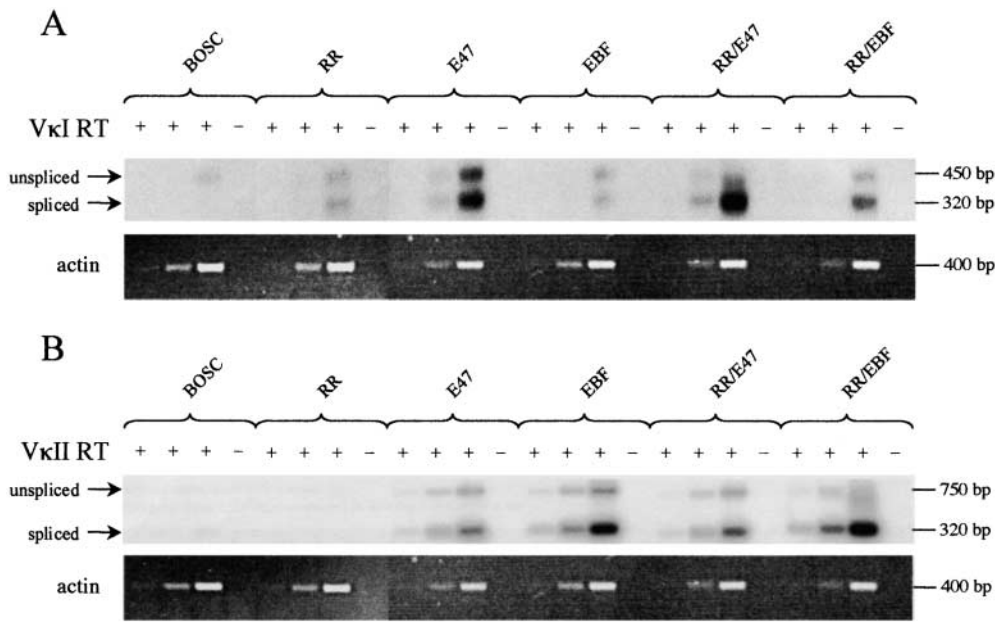


Figure 2. Induction of VκI and VκII germline transcription after transient transfection of BOSC23 cells. (A) RNA was isolated from cells transfected with the indicated expression vectors or from mock-transfected BOSC cells. cDNA was prepared with a VκI family-specific primer located downstream of the RSS and amplified with primers in the leader and FR3. This results in amplification of unspliced and spliced transcripts as indicated on the blot. Aliquots taken from each PCR at consecutive intervals (25, 28, and 31 cycles) are indicated with RT⁺. One aliquot from each RT⁻ control PCR taken at 31 cycles was also included to test for possible genomic contamination in the RNA preparation. The equal input amounts of RNA was confirmed by actin PCR (25, 28, and 31 cycles), shown for each

set beneath the blot. (B) RNA from the same transfected cells was also analyzed for the presence of VκII germline transcripts. The analysis was performed as described in A, using primer combinations specific for VκII germline transcripts but aliquots were taken at 34, 37, and 40 cycles. As for VκI, the unspliced and spliced transcripts were detected and are indicated on the blot. Actin PCRs confirmed equal input amounts of RNA.

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 VλIII Genes. The Igλ locus contains ~70 Vλ genes which are organized in three clusters all upstream of seven Jλ-Cλ pairs (22). The Jλ-proximal A-cluster carries interspersed members of the VλII and VλIII family, while genes belonging to the VλI family are found further upstream in the B cluster. In the peripheral repertoire, VλII and VλI are the predominantly used V genes (34, 35). Our previous study had only analyzed rearrangements using VλIII and Jλ1 segments. In this study we extended our analysis to include the VλI, VλII, and VλIII gene families, which together account for >90% of all Vλ genes expressed in the peripheral repertoire. We also used a primer detecting Jλ2 and Jλ3 since they are more frequently used in the peripheral repertoire than Jλ1. Recombination of VλIII was strongest with EBF, but low levels of rearrangement were also induced by E2A (Fig. 3 A). Induction of VλII recombination by EBF was eightfold lower compared with VλIII. The low levels of rearrangement detected for VλIII 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 VλIII genes than there are VλII genes in the locus, the VλII 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 VλIII family. In addition, we analyzed VλI-Jλ rearrangements. The VλI genes contribute almost to the same extent to the peripheral repertoire as the VλII family. However, only negligible VλI rearrangement was detected in EBF transfected BOSC cells while no recombination was seen in E2A transfectants even at 40 cycles.

Table I. *Vκ Gene Utilization in Transfected BOSC23 Cells, Peripheral Blood, and Cord Blood*

		BOSC23 transfected with RAG1/2 and:		
		E2A	EBF	PBLs & CB np ^a
VκI	Genes from distal half of locus combined	1 (2%) ^b	1 (3%)	17 (11%) ^c
	1-39 (O12)	5 (11%)		41 (26%)
	1-37 (O14)	3 (7%)	5 (16%)	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%)
	VκIII	Genes from distal half of locus combined		
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%)

^anp, only nonproductive (out-of-frame) sequences were analyzed as estimates of recombination frequency in vivo. CB, cord blood; PBL, peripheral blood lymphocyte.

^bVκI/E2A values had been published previously (reference 20).

^cSequences are a combination of our unpublished and published data (reference 28) and the data of Foster et al. (reference 30).

Three Distinct Genes Dominate the Vλ Recombination Repertoire. We had shown previously that EBF induced VλIII–Jλ1 rearrangements, but that only the most Jλ-proximal gene, Vλ3-1, recombined. However, in vivo Jλ1 contributes only a small fraction to the repertoire. We wished to confirm that the limited rearrangement of this Vλ gene was indeed due to limited accessibility of only the most proximal VλIII gene. As in our previous study, the recombined VλIII repertoire in BOSC cells transfected with either E2A or EBF was strongly dominated by Vλ3-1, accounting for ~80% of all VλIII recombination events (data not shown). This bias is not seen in the periphery, where Vλ3-1 accounts for <20% of the repertoire (35). Sequence analysis of transfected BOSC cells further revealed that VλII rearrangements induced by EBF are dominated by Vλ2-8 and Vλ2-14 (data not shown). Vλ2-14 is the single most frequently rearranging gene in vivo, rearranging 5–8 times as often as Vλ3-1, yet in BOSC cells Vλ3-1 rearranged ~3 times more often than Vλ2-14. Thus, for the λ locus, the induction of rearrangement differs significantly from the in vivo rearrangement pattern.

Since our Jλ-specific PCR primer was designed to preferentially prime Jλ2 and Jλ3, we were unable to investigate

the overall Jλ usage during these induced recombination events. However, all Jλ genes in Vλ–Jλ rearrangements from transfected samples used the more proximal Jλ2 gene. This complete bias was not due to the Jλ primer used since sequences derived from PBL control DNA using the same primers displayed approximately equal usage of the two Jλ genes (data not shown).

E2A and EBF Induce Rearrangement of D_H3 and D_H4 Gene Families. The first step during V(D)J recombination is the joining of one D_H gene segment to one of six J_H segments. The main part of the human D_H gene cluster, located at the 3' end of the IgH locus, spans 39 kb and contains 26 segments, arranged in four repeats of single members that can be grouped into six families, D_H1 through D_H6 (23). 15 kb further 3' is the single D7-27. We chose to compare the induction of recombination of D_H3, which represents the most dominant D_H family in the peripheral repertoire (23, 36, 37), to that of D_H4, which we showed previously to be induced to rearrange by EBF and E2A (20). The J_H primer used in our analysis had a perfect match to J_H1, J_H4, and J_H5, but contained mismatches to the remaining three J_H genes. J_H4 alone has been reported to account for about half of all recombina-

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

		Corresponding V κ gene family/source of genomic DNA		
		V κ I/E2A	V κ I/EBF	PBLs & CB np ^a
		<i>n</i> = 54		
J κ	1	28 (62%) ^b	17 (57%)	24%
	2	14 (31%)		11%
	3		3 (10%)	4%
	4	3 (7%)	10 (33%)	59%
	5			2%
		V κ III/E2A	V κ III/EBF	PBL & CB np
		<i>n</i> = 62		
J κ	1	15 (100%)	11 (92%)	39%
	2			5%
	3			5%
	4		1 (8%)	47%
	5			5%

^anp, all sequences were obtained with the same V κ I and J κ primer combination; only nonproductive (out-of-frame) sequences were considered for analysis. CB, cord blood; PBL, peripheral blood lymphocyte.

^bValues from reference 20.

tion events observed in peripheral lymphocytes (37). However, J_H6, which contributes ~25% to the peripheral repertoire, was not amplified by our PCR primer.

EBF induced recombination in members of both the D_H3 and D_H4 gene family although levels were twofold higher for D_H4 than for D_H3 (Fig. 3 B). This is in contrast to D_H gene utilization in peripheral blood, where D_H3 genes contribute almost fourfold more than D_H4 genes (23, 36, 37). Similarly, the E2A proteins promoted recombination in members of both D_H families, but at a three to fourfold lower level compared with the EBF transfectants.

E2A and EBF Proteins Promote the Rearrangement of Different D_H Genes. We have previously had indications that the D_H4 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 D_H3 repertoire, we sequenced D_H3–J_H and D_H4–J_H 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 D_H3–22 and D_H4–23 genes. Comparison of our observed frequencies to a large database of VDJ rearrangements (23) revealed that the pre-

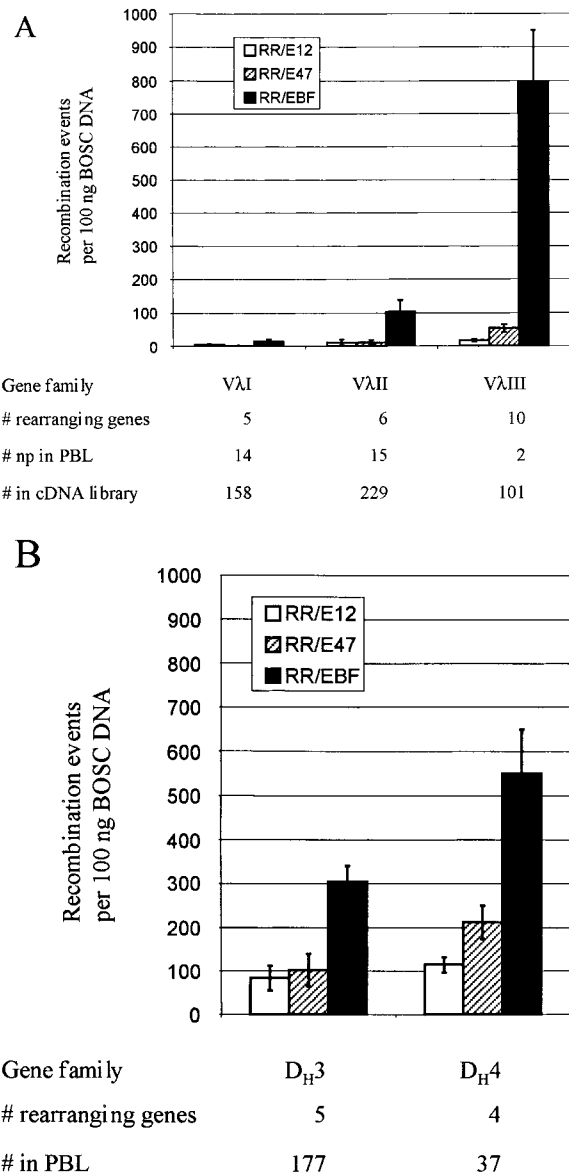


Figure 3. Levels of induced V λ –J λ and D_H–J_H recombination events after transient transfection. Induced recombination events for the members of several V λ and D_H gene families were analyzed as outlined in the legend to Fig. 1. Hybridization signals were quantitated, normalized to plasmid standards, and are represented as recombination events per 100 ng of transfected BOSC genomic DNA. (A) Levels of induced V λ –J λ 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 D_H3 and D_H4 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.

dominantly recombining genes in our in vitro system for both D_H3 and D_H4 differed from the ones found to contribute the most to the peripheral repertoire. The representation of individual D_H genes in VDJ rearrangements may not, however, be reflective of the D_H gene usage in DJ_H rearrangements since the D_H segments in productive VDJ

Table III. *D_H Gene Utilization in Transfected BOSC23 Cells, Pre-B Cells, and Peripheral Blood*

Family	Genes	BOSC23 transfected with RAG1/2 and:			
		E2A	EBF	Pre-B	PBLs
D _H 3	3-3 (DXP4)	2 (11%)	4 (22%)	4 (25%)	42 (24%) ^a
	3-9 (DXP1)		5 (28%)	2 (13%)	19 (11%)
	3-10 (DXP'1)	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 _H 3	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%)

^aFrom 451 VDJ rearrangements analyzed by Corbett et al. (reference 23), 177 sequences used D_H3 genes while only 37 used D_H4 genes. Values in the table are represented as the percentage usage within each individual family. PBL, peripheral blood lymphocyte.

rearrangements have potentially been subject to selection. Therefore, we analyzed sequences amplified from genomic DNA from sorted sIg⁻ pre-B cells (Table III). These sequences showed that all D_H3 genes could recombine at frequencies approximating that seen in the EBF-transfected cells. Surprisingly, D_H4 recombination in pre-B cells was biased toward the 3' located genes. Therefore, our data show that EBF induces rearrangement of a variety of D_H genes located throughout the locus, whereas E2A almost exclusively permits rearrangement of the most J-proximal D_H4 gene and strongly biases the D_H3 gene utilization.

Most J_H Genes Are Used in Rearrangements Induced by EBF while Rearrangements Induced by E2A only Use the Most Proximal J_H Genes. The H chain locus carries six J gene segments in a region of 2.3 kb located and the individual J_H genes are separated by intervening DNA of 200–600 bp. To determine the impact the transcription factors may have on the J_H gene accessibility, our DJ sequences were analyzed for their J_H gene usage (Table IV). The values indicate a strong bias for genes located at the 5' end of the J_H cluster in E2A-transfected cells. Expression of EBF on the other hand resulted in an increased participation of genes from the 3' end. Since our J_H primer could bias the apparent J_H usage, we analyzed the J_H usage in our control PCR reactions from sIg⁻ pre-B cells. To compare the percent usage observed in our system to that of the peripheral repertoire, published frequency values (37) were recalculated to exclude J_H6, which had not been seen in our analysis due to primer disfavor. Comparison between the J_H usage in EBF transfectants and the J_H usage in vivo indicates full accessibility to all J_H genes in cells expressing EBF. Thus, just as we observed that a wide range of D_H genes located throughout the locus were rearranged upon expression of EBF, many J_H genes are used in these rearrangements. In contrast, E2A preferentially induces rearrangement of the proximal D_H and J_H genes.

D_H-J_H Recombination Is Heterogeneous as Early as 24 h After Transfection. Heavy and light chain loci can undergo secondary rearrangements in vivo, recombining upstream D- or V-gene segments respectively to downstream J-gene segments, thus skewing the gene utilization to more distal located gene segments. Therefore, we asked if the contribution of individual genes changes over time in transfected BOSC23 cells, thereby altering the frequency with which gene segments are observed. We chose to compare the D_H4-J_H rearrangements in genomic DNA harvested from cells either 24 or 72 h after RAG1/RAG2/EBF transfection since a wide array of D_H and J_H genes are seen at day 3 after transfection (Fig. 4). Although we detected a somewhat higher frequency of rearrangement of D_H4-23 to J_H2 and J_H3 in day 1 genomic DNA, the contribution of individual D_H4 and J_H gene segments does not change significantly at the later time point. The pattern of individual gene usage also suggesting that the sequences observed in day 3 genomic DNA are not predominantly due to secondary recombination events.

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 VκI genes located throughout the locus in a non-lymphoid cell line. This suggested that E2A, perhaps by binding to the κ enhancer, endowed accessibility to recombination to the entire Vκ locus. However, we now show that E2A induces much lower levels of recombination of the VκII gene family and only a moderate level of rearrangement of VκIII genes, as compared with the levels of VκI genes. Since all three Vκ gene families are inter-

Table IV. *J_H Gene Utilization in Transfected BOSC23 Cells and Peripheral Blood*

Family	Genes	Corresponding D _H gene family/source of genomic DNA				J _H 6 corrected ^c
		D _H 3/E2A	D _H 3/EBF	D _H 3/pre-B	PBLs np ^a	
J _H	1	7 (37%)	2 (11%)	1 (6%)	4%	5%
	2	4 (21%)		1 (6%)		
	3b	7 (37%)	9 (50%)	4 (25%)	8%	
	4b	1 (5%)	7 (39%)	8 (50%)	50%	
	5b			2 (13%)	15%	
	6				23%	
<i>n</i> = 20 ^b						
Family	Genes	D _H 4/E47	D _H 4/EBF	D _H 4/pre-B	PBLs np	J _H 6 corrected
J _H	1	26 (54%)	11 (35%)		4%	5%
	2	14 (29%)	3 (10%)	3 (21%)		
	3b	6 (12%)	3 (10%)	3 (21%)	8%	10%
	4b	2 (4%)	10 (32%)	7 (50%)	50%	65%
	5b		4 (13%)	1 (7%)	15%	20%
	6				23%	
<i>n</i> = 20						

^anp, only nonproductively rearranged sequences were considered for analysis. PBL, peripheral blood lymphocyte.

^bValues from reference 37.

^cPBL J_H frequencies recalculated without J_H6 since our J_H primer did not amplify J_H6.

dispersed within the locus, this data suggest that E2A preferentially targets recombination to the members of the VκI 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-γ minilocus transgene in which ~1 kb of promoter was switched between the Vγ3 and Vγ2 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 re-

versal 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).

The possible correlation between germline transcription and induced recombination was also addressed. It had previously been reported that the level of recombination can correlate with the level of germline transcription (3, 12, 42). Therefore, we compared the levels of germline transcription for the highly recombining VκI gene family to the low recombining VκII family. In accordance with the E-box binding sites in all VκI promoter regions (21), high levels of VκI germline transcripts were observed after E47 expression, while EBF expression resulted in only low levels of transcription. Thus, for members of the VκI family a good correlation between germline transcription and induced recombination was observed. However, this correlation did not hold true for the VκII gene family. VκII promoters have been shown to have EBF binding sites (32), and we observed that EBF indeed induces significant levels of germline transcription. However, despite this high level of transcription, only very low levels of VκII-Jκ recombina-

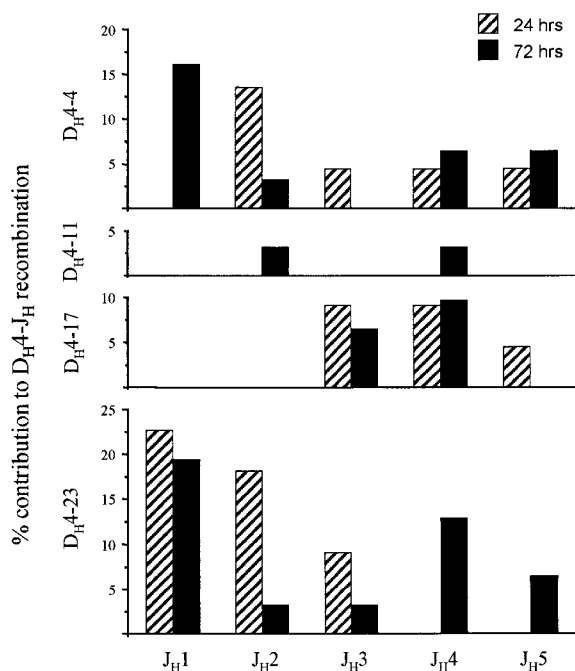


Figure 4. Frequency of EBF-induced D_H - J_H recombination events 24 and 72 h after transfection. Genomic DNA from transfected BOSC cells was harvested either 24 or 72 h after transfection with RAG1, RAG2, and EBF expression vectors. D_H - J_H recombination products were amplified as outlined in the legend to Fig. 1 and the resulting PCR products were cloned and sequenced. Data were derived from 31 d 3 sequences and 21 d 1 sequences. The bar graphs represent the percent contribution of individually detected D_H - J_H recombination events to all the sequences for that particular time point.

nation was detected. This indicates that germline transcription is not sufficient to induce V(D)J rearrangements, suggesting that the transfected transcription factors may have functions other than promoting transcription during V(D)J recombination. The lower but detectable levels of $V\kappa$ II germline transcripts in E47 transfected cells may be due to the presence of E-boxes in some but not all members of the $V\kappa$ II family (21).

In the $V\lambda$ locus, EBF induces high levels of rearrangement of $V\lambda$ III genes, vastly dominated by a single gene $V\lambda$ 3-1, but lower levels of rearrangement are induced using $V\lambda$ II genes. The more distal $V\lambda$ I genes are not induced to rearrange at all, even though they contribute in large numbers to the peripheral repertoire. In the D_H locus the average distance between any given D_H 3 gene segment and its adjacent D_H 4 gene is only ~ 1 kb. Expression of E2A or EBF together with the RAG proteins induced rearrangements of the D_H 3 and D_H 4 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 $V\kappa$ III genes or the $V\kappa$ I 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 $V\gamma$ gene utilization during $\gamma\delta$ T cell development, where it was shown that E2A-deficient mice undergo normal rearrangement of $V\gamma$ 3 $V\delta$ 1 receptors in fetal life, but do not show rearrangement of $V\gamma$ genes which normally undergo rearrangement in the adult thymus (44). Furthermore, ectopic expression of E2A can induce rearrangement of the other $V\gamma$ genes (45). E2A-deficient mice also display aberrant control of $V\gamma$ 3 $V\delta$ 1 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 D_H genes in rearrangements induced by EBF, whereas E2A dominantly induced rearrangement of the most proximal D_H genes. In other cases, such as the $V\kappa$ III 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' D_H 4 gene, D4-23, and the most 3' D_H 3 gene, D3-22. Likewise, EBF or E47 induced rearrangement of the $Ig\lambda$ locus showed preferential usage of the most proximal $V\lambda$ 3-1. Within the $J\kappa$ locus, $J\kappa$ 1 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 recombination of D_H or V_L 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

V κ and J κ 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_H4–J_H 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 V λ I genes, suggesting that the high frequency with which V λ I 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 λ 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 V κ promoters have potential E2A sites (21), but these genes are unequally induced to undergo recombination after transfection of E2A. Furthermore, only V κ II genes, but none of the other V κ family genes, have known EBF sites in their promoter (32), yet EBF induces lower levels of rearrangement for V κ II genes than for V κ I or V κ III 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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