

E2A and EBF Act in Synergy with the V(D)J Recombinase to Generate a Diverse Immunoglobulin Repertoire in Nonlymphoid Cells

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Summary

Immunoglobulin (Ig) and T cell receptor (TCR) genes are assembled during lymphocyte maturation through site-specific V(D)J recombination events. Here we show that E2A proteins act in concert with RAG1 and RAG2 to activate Ig V κ 1J but not Ig λ V λ III–J λ 1 rearrangement in an embryonic kidney cell line. In contrast, EBF, but not E2A, promotes V λ III–J λ 1 recombination. Either E2A or EBF activate IgH D μ 4J recombination but not V(D)J rearrangement. The Ig coding joints are diverse, contain nucleotide deletions, and lack N nucleotide additions. Ig κ VJ recombination requires the presence of the E2A transactivation domains. These observations indicate that in nonlymphoid cells a diverse Ig repertoire can be generated by the mere expression of the V(D)J recombinase and a transcriptional regulator.

Introduction

The ability of lymphocytes to recognize an enormous array of antigens is dependent on the successful rearrangement of the immunoglobulin (Ig) and T cell receptor (TCR) loci (Mombaerts et al., 1992; Shinkai et al., 1992). The Ig and TCR loci are assembled from variable (V), diversity (D), and joining (J) segments, during specific stages of lymphocyte development. V, D, and J DNA segments are flanked by recombination signal sequences (RSSs) that mark the border of the coding regions. Recombination is initiated and completed by two lymphocyte-specific proteins, RAG1 and RAG2, that recognize the RSSs and introduce highly specific breaks at the coding–signal boundaries (Schatz et al., 1989; Oettinger et al., 1990; van Gent et al., 1995). The broken ends are processed and joined with the contribution of the DNA break repair machinery, to create genes with

the ability to encode antigen receptors (Weaver et al., 1995).

The expression of RAG1 and RAG2 correlates with the onset of V(D)J recombination, and ectopic expression of RAG proteins allows site-specific recombination of the appropriate plasmid substrates both in vitro and in vivo (Schatz et al., 1989; Oettinger et al., 1990; van Gent et al., 1995). The restricted expression of the RAG genes in developing lymphocytes permits site-specific recombination only in B and T lineage cells. Additionally, within a lymphoid lineage, the assembly of antigen receptor genes is tightly regulated.

During B and T lineage maturation, rearrangement is developmentally regulated (reviewed by Sleckman et al., 1996). Ig heavy chain (IgH) gene rearrangement precedes Ig kappa (κ) and Ig lambda (λ) chain gene recombination (Yancopoulos and Alt, 1985). IgH gene rearrangement is also ordered: DJ rearrangement is initiated prior to the onset of recombination involving the variable regions. Ig light chain genes are assembled once a functional rearrangement at the IgH locus has been achieved. Rearrangement at these loci is usually initiated at the Ig κ locus, with λ joints being assembled predominately in lymphocytes that have failed to form a functional Ig κ gene product. Finally, allelic exclusion allows for the expression of only one functional rearranged IgH and one rearranged Ig light chain per individual B lineage cell.

Since the RAG proteins mediate all Ig and TCR gene assembly, it has been proposed that V(D)J recombination of these loci is regulated at the level of chromatin accessibility (Sleckman et al., 1996). A large body of evidence has indicated a strong correlation with transcription and rearrangement of an antigen receptor locus (Yancopoulos and Alt, 1985). Further evidence was provided by in vitro studies that showed that lineage specificity and temporal ordering of V(D)J recombination is reflected in the accessibility within the chromatin structure (Stanhope-Baker et al., 1996). Accessibility of the recombinase to its target sites is regulated, at least in part, by the Ig enhancers. Specific deletion of the IgH intronic enhancer results in a modest reduction of D to J rearrangement and a dramatic diminution in V to DJ rearrangements (Chen et al., 1993; Serwe and Sablitzky, 1993). Likewise, the Ig κ intronic enhancer is required for Ig κ rearrangement (Xu et al., 1996).

Multiple protein binding sites have been identified and characterized in each of the Ig enhancers. Many of the binding sites are not unique to specific Ig enhancers. Among the transcriptional regulators that bind Ig enhancer elements are the E2A proteins. The E2A gene encodes for two proteins, E12 and E47, that arise through alternative RNA splicing (Murre et al., 1989). E12 and E47 are differentially expressed in a wide variety of tissues; however, they exist as homodimers only in B lineage cells (Bain et al., 1993; Shen and Kadesch, 1995). B lineage cells in E2A-deficient mice are blocked at a stage prior to the onset of Ig gene rearrangement (Bain et al., 1994; Zhuang et al., 1994). E12 and E47 are required for the proper regulation of a wide variety of B

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lineage-specific genes, which include *RAG1*, *RAG2*, $\lambda 5$, *VpreB*, *Pax5*, and *EBF* (Schlissel et al., 1991; Choi et al., 1996; Sigvardsson et al., 1997; Kee and Murre, 1998). Additionally, E47 has been shown to be capable of inducing cross-lineage rearrangements, IgH DJ joints, when ectopically expressed in a pre-T cell line (Schlissel et al., 1991). A role of E2A proteins in site-specific recombination was further demonstrated in E2A-deficient mice in which TCR γ and δ rearrangements are perturbed (Bain et al., 1999).

In addition to E2A, another gene product, EBF, has been identified to be essential during the earliest stages of B cell development (Lin and Grosschedl, 1995). EBF is a transcriptional regulator that is expressed in various tissues, including olfactory neurons, adipocytes, and B lineage cells (Hagman et al., 1993; Wang and Reed, 1993). In addition to the *mb-1* gene, EBF regulates the expression of *B29*, $\lambda 5$, *VpreB*, *RAG1*, and *Pax5* (Sigvardsson et al., 1997; Kee and Murre, 1998; O'Riordan and Grosschedl, 1999). Recent studies have indicated that E2A and EBF act in concert to regulate $\lambda 5$, *VpreB*, and *RAG* expression (Sigvardsson et al., 1997; O'Riordan and Grosschedl, 1999). Lymphocytes in EBF-deficient mice are blocked at an early stage prior to the onset of Ig gene rearrangements (Lin and Grosschedl, 1995).

Here we show that two distinct classes of transcriptional regulators, E2A and EBF, have the ability to target the recombination machinery to Ig loci in nonlymphoid cells. Specifically, our observations show that overexpression of E2A and RAG1 and RAG2 activates Ig V κ 1J but not Ig λ VJ rearrangement. In contrast, EBF, but not E2A, promotes V λ III-J λ 1 recombination. Both E2A and EBF activate IgH D μ 4J but not V(D)J rearrangement. The Ig coding joints are diverse, contain nucleotide deletions, and lack N nucleotide additions. Since Ig κ VJ recombination requires the presence of the E2A transactivation domains, we suggest that E2A proteins promote rearrangement through activation of germline transcription and/or modulation of chromatin accessibility. These data indicate that the ectopic expression of a transcriptional regulator, either E2A or EBF, and the V(D)J recombinase is sufficient to generate a diverse Ig repertoire in nonlymphoid cells.

Results

E2A Promotes RAG1- and RAG2-Mediated Cleavage of Recombination Signal Sequences Localized to J Segments within the Ig κ Locus

The E2A proteins were originally identified as binding to E box sites present in the Ig κ chain enhancer (Murre et al., 1989). Since the Ig κ intronic enhancer is required for VJ rearrangement, we initiated our studies using this locus (Xu et al., 1996). To determine whether E2A proteins have the ability to allow accessibility of the recombinase to RSSs present in the Ig κ locus, a gene transfer approach was employed. BOSC 23 cells, a derivative of 293T, a human embryonic kidney cell line, were used as the recipient (Pear et al., 1993). This cell line can be transfected with very high efficiency, harbors the Ig loci in germline configuration, and expresses extremely low levels of E12 and E47 (data not shown). These properties

make this cell line uniquely suitable for the experiments described below.

BOSC 23 cells were transfected at high density, to ensure that 2 days posttransfection, most of the cells are in G1, the stage in which V(D)J recombination normally is initiated and completed. Genomic DNA was harvested 3 days posttransfection and analyzed for the presence of signal ends from J segments at the Ig κ locus using ligation-mediated PCR (LMPCR) (Figure 1A) (Stanhope-Baker et al., 1996). Briefly, a double-stranded asymmetric, oligonucleotide linker was ligated to genomic DNA isolated from transfected BOSC 23 cells. Genomic DNA was amplified by PCR using a linker- and a locus-specific primer as indicated. The amplified products were analyzed by Southern blotting using a 32 P end-labeled oligonucleotide probe (Figure 1A).

BOSC 23 cells transfected with *RAG1* and *RAG2*, in the absence of the E2A proteins, did not show detectable levels of double-stranded breaks (DSB), indicating that this region was not readily accessible to the recombination machinery (Figure 1B, lane 3). Likewise, the expression of E12 or E47 by themselves did not induce DSB (Figure 1B, lanes 4 and 6). However, in the presence of either E12 or E47 and RAG1 and RAG2, a significant level of signal ends was detectable (Figure 1B, lanes 5 and 7). The size of the amplified fragment, 155 bp, was as predicted. DSB were not detected in a reaction lacking T4 ligase, indicating that the amplified fragments required the presence of the linker (Figure 1B, lane 8). To further establish that the LMPCR assay detected the proper signal ends, purified BOSC 23 genomic DNA was treated with the restriction enzyme EcoICRI and then subjected to LMPCR. This enzyme fortuitously cleaves very close to the RSS heptamer that borders the J κ 1 segment and thus mimics cleavage at this RSS (Figure 1A). A fragment of the correct size, 152 bp, was observed (Figure 1B, lane 9). A separate PCR reaction, performed to amplify the *CD14* locus, showed that the DNA content was similar in each of the samples (data not shown). These data indicate that the E2A proteins have the ability to promote accessibility of the J κ 1 RSS to RAG1 and RAG2.

Activation of Ig κ VJ Rearrangement in Embryonic Kidney Cells by the E2A Proteins

The observation that E2A proteins have the ability to promote access of the recombinase to RSSs raised the possibility that BOSC 23 cells, ectopically expressing E2A and RAG1 and RAG2, harbor V κ J rearrangements. To examine this possibility, DNA was isolated from the transfectants and analyzed by PCR for the presence of VJ joints. The human Ig κ locus consists of 76 V segments and 5 J segments (for review, see Schable and Zachau, 1993). The V segments are divided into two homologous clusters, one harboring 40 V regions and being most proximal to the J segments and the other containing 36 V segments organized in an inverted orientation located 800 kb from the proximal cluster. The V regions can be divided into seven families, the largest of which is the V κ 1 family. To test for V κ 1J rearrangements, a forward V κ 1 primer, with specificity for the leader region of the V κ 1 family, and a degenerate reverse J κ primer, which recognizes all but the most distal of the J segments, were used (Feeney et al., 1997). The primers were tested on

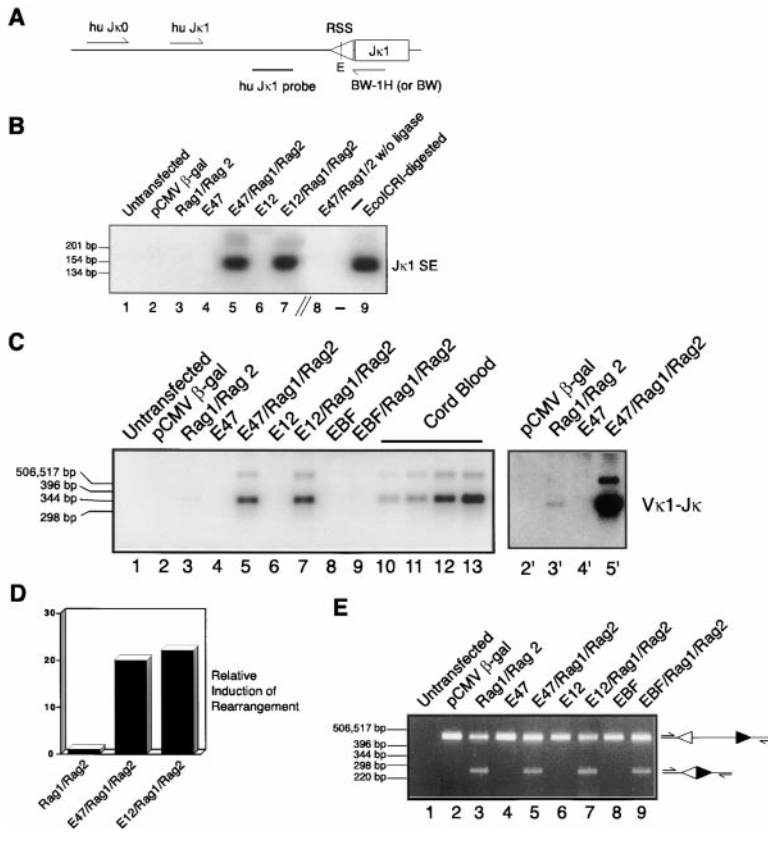


Figure 1. E2A and RAG1 and RAG2 Act in Concert to Promote Ig $V_{\kappa}1J$ Rearrangement in an Embryonic Kidney Cell Line, BOSC 23 (A) Diagram of the $J_{\kappa}1$ gene segment of the Ig κ locus. The positions of the PCR primers are indicated. The triangle represents the recombination signal sequence. E refers to the position of an EcoICRI site that cuts 11 bases upstream of the RSS cleavage site. (B) Genomic DNA from transfected BOSC 23 cells was analyzed for blunt signal ends (SE) 5' of $J_{\kappa}1$ using LM-PCR. Expression vectors used for transfection are indicated. Lane 8: genomic DNA was incubated in the absence of T4 ligase prior to PCR. Lane 9: BOSC 23 DNA was treated with EcoICRI, purified, and then diluted 1:500 with DNA from untransfected BOSC 23 cells before being subjected to the LMPCR assay. The BW primer was used as the reverse primer in this reaction only. (C) BOSC 23 cells were transfected with expression vectors containing E12, E47, EBF, RAG1, and RAG2. PCR was used to amplify 100 ng of DNA for $V_{\kappa}1-J_{\kappa}$ recombination using the $V_{\kappa}1$ and hu J_{κ} consensus primers for 26 cycles. PCR products were separated on a 1.5% agarose gel, transferred to Nytran Plus, and hybridized with a cloned $V_{\kappa}1/J_{\kappa}$ fragment. Lanes 10–13: PCR products performed on serial dilutions of cord blood DNA into BOSC 23 DNA. The amount of cord blood DNA used in each lane is as follows: lane 10, 2.5 ng; lane 11, 5 ng; lane 12, 25 ng; and lane 13, 50 ng. In the second panel, lanes 2–5 (designated as 2'–5') are shown in a long

exposure to better illustrate the rearrangements detectable in genomic DNA derived from transfectants expressing RAG1 and RAG2 by themselves.

(D) Histogram of relative levels of $V_{\kappa}1$ rearrangements from various transfections. The levels from at least five experiments were quantified by gel analysis with a Phosphor Imager. Within each experiment, levels of rearrangements were normalized to that seen with RAG1 and RAG2 alone (which was set to 1).

(E) DNA was isolated from transfected cells that had been cotransfected with the pJH200 plasmid. PCR was performed with the DR1 and NEB#1233 primers to amplify across the recombination signal sequences. PCR product from unrearranged plasmid is expected to be 457 bp, and the rearranged plasmid should show an amplified band of 268 bp. The schematic next to the gel illustrates the RSSs (triangles) and the primers (arrows).

genomic DNA isolated from human cord blood and efficiently amplified a target over a 20-fold dilution range (Figure 1C, lanes 10–13). When DNA was amplified from genomic DNA isolated from BOSC 23 cells, mock-transfected cells, or cells transfected with E2A alone, no product was detectable (Figure 1C, lanes 1, 2, 4 and 6). $V_{\kappa}1J$ rearrangements were detectable upon expression of RAG proteins by themselves (Figure 1C, lanes 3 and 3', and data not shown). Strikingly, BOSC 23 cells expressing RAG1 and RAG2 in the presence of either E12 or E47 showed relatively high levels of $V_{\kappa}1J$ rearrangements (Figure 1C, lanes 5 and 7). The fast migrating fragment is the result of amplification from the J_{κ} segment that has undergone rearrangement (e.g., $J_{\kappa}1$) (Figure 1C). Because the J_{κ} consensus primer will amplify four of the five J regions, the slower migrating fragment is the result of amplification from the J_{κ} segment immediately 3' to the J_{κ} that has undergone rearrangement. In the presence of E12 or E47, the level of VJ joints is increased by 20- to 25-fold as compared to RAG expression alone (Figure 1D). A separate PCR reaction, performed to amplify the *CD14* locus, showed that the DNA content was similar in each of the samples (data not shown).

To obtain an estimate of the frequency of rearrangement in the transfected cells, the hybridization signal was compared to that of DNA derived from human cord blood. The percentage of $V_{\kappa}1$ -positive B lineage cells in cells isolated from cord blood is, on average, 5%–10% (A. J. F., unpublished data). By comparing the hybridization signal of genomic DNA (100 ng) derived from the transfected cells to that of cord blood (2.5–50 ng), we estimate that 1%–2% of BOSC 23 cells harbor $V_{\kappa}1J$ joints upon expression of E2A, RAG1, and RAG2 (Figure 1C). In comparison, we estimate that approximately 0.05%–0.10% of BOSC 23 cells expressing RAG1 and RAG2 alone contain Ig κ VJ rearrangements. We also examined the ability of EBF to promote Ig κ recombination. EBF, unlike E2A, did not significantly enhance the ability of RAG1 and RAG2 to promote $V_{\kappa}1J$ rearrangement (Figure 1C, lane 9).

To ensure that RAG1 and RAG2 were equally active in the absence or presence of the E2A proteins, the plasmid substrate pJH200 was cotransfected. pJH200 contains two RSSs separated by a linker, which, upon rearrangement, will form a signal joint and remove approximately 190 bp. Rearrangements within pJH200 were analyzed by PCR using the appropriate primers

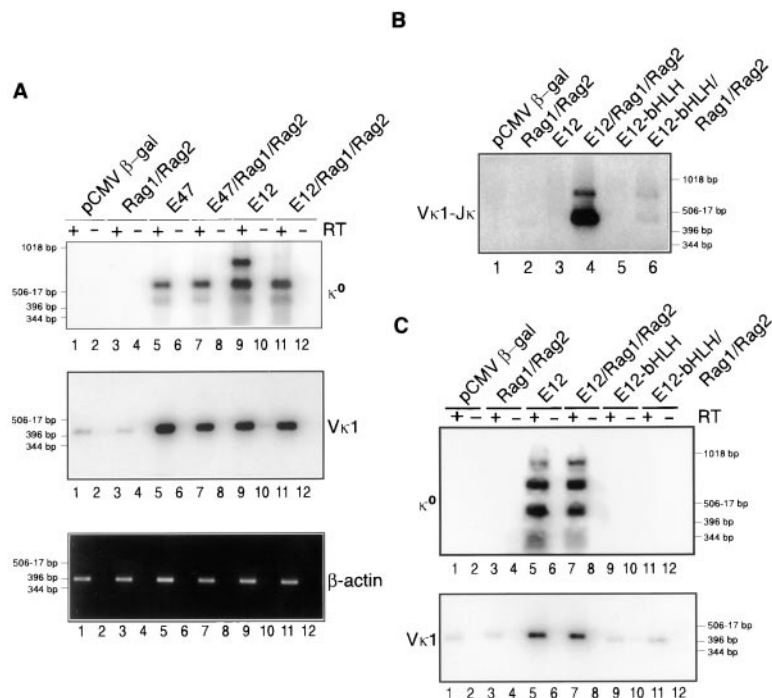


Figure 2. The E2A Activation Domains Are Required to Activate Ig κ Germline Transcription and V κ 1J Rearrangement

(A) RT-PCR was performed on RNA isolated from transfected BOSC 23 cells. The blot was hybridized with a κ° cDNA. The size of the PCR product is expected to be 643 bp. Slower migrating PCR products contain additional J κ segments. In the middle panel, RT-PCR was performed using the same cDNA as shown in the upper panel. The blot was probed with a cloned V κ 1 gene sequence (as in Figure 1C). In the bottom panel, RT-PCR was performed using the same cDNA with primers to amplify β -actin transcripts as a control. Ethidium bromide staining of a 1.5% agarose gel is shown. The band at 418 bp is of the expected size.

(B) PCR was performed on genomic DNA. The PCR was performed as in Figure 1C.

(C) RT-PCR to detect κ° and V κ 1 germline transcripts was performed on total RNA isolated from transfected BOSC 23 cells. The primers and probe used are as described in (A).

(van Gent et al., 1995). In contrast to the endogenous Ig κ locus, E2A proteins did not influence the ability of RAG proteins to promote rearrangement of the plasmid substrate (Figure 1E). Taken together, the data indicate that E2A acts in concert with RAG1 and RAG2 to specifically activate Ig V κ 1J joining in embryonic kidney cells.

Overexpression of E2A in Embryonic Kidney Cells Activates Ig κ Germline Transcription

To explore how the E2A proteins promote accessibility to both V and J segments, transfected BOSC 23 cells were examined for the presence of Ig κ germline transcripts (Thompson et al., 1992). RNA was isolated and analyzed by reverse transcription PCR (RT-PCR) using the appropriate primers. Ig κ germline transcripts (κ°) initiating 5' of the J κ cluster were activated by both E12 and E47 (Figure 2A). Germline transcripts initiating from the V κ 1 promoters were present at low levels in BOSC 23 cells that were mock transfected (Figure 2A). However, upon expression of either E12 or E47, germline transcripts initiating from the V κ 1 promoter were activated 40- to 100-fold (Figure 2A). RT-PCR using β -actin-specific primers was used as a control for RNA integrity and cDNA synthesis (Figure 2A). These observations indicate that ectopic expression of E2A in embryonic kidney cells activates Ig κ germline transcription. Since E2A binding sites are present in Ig V κ 1 promoters and in the Ig κ intronic enhancer, we propose that E2A proteins activate rearrangement by promoting accessibility of the recombinase to the RSSs.

The E2A Transactivation Domains Are Required to Promote Accessibility

The E2A proteins contain two highly conserved domains, AD1 and AD2, that are required for its ability to transactivate. Recent data has indicated that the AD1

domain interacts with the SAGA complex, an assembly of proteins that contains histone acetyltransferase (HAT) activity (Massari et al., 1999). To determine whether the E2A activation domains are essential for VJ rearrangement and germline transcription, a mutant form of E2A, lacking the transactivation domains but containing the minimal E12 bHLH domain (amino acids 510–636), was introduced into BOSC 23 cells (Kee and Murre, 1998). DNA was isolated from the transfectants and analyzed for the presence of Ig κ VJ joints. Whereas wild-type E12 activated Ig V κ 1J rearrangement, the mere presence of the E12 bHLH domain did not promote VJ recombination to significant levels (Figure 2B).

To examine whether the inability of the bHLH domain to activate Ig κ VJ rearrangement correlated with the absence of germline transcripts, RNA was analyzed by RT-PCR. E12 activated germline transcription as described above (Figure 2C). Deletion of the activation domains completely abrogated the ability of E12 to induce κ° and V κ 1 germline transcription (Figures 2B and 2C). These data indicate that the E2A transactivation domains are required to activate IgV κ 1J rearrangement as well as Ig κ germline transcription.

E2A or EBF Can Act in Synergy with the RAG Proteins to Activate IgH DJ

In addition to the Ig κ enhancers, functional E2A binding sites are present in both the intronic and 3' IgH enhancers. Additionally, ectopic expression of E47 has been shown to promote cross-lineage IgH DJ rearrangement in a pre-T cell line (Schlüssel et al., 1991). The human IgH locus contains more than 120 V regions, 27 D segments, and 6 J regions. There are seven families of V segments based on sequence homology. The D locus is grouped into seven families that are closely

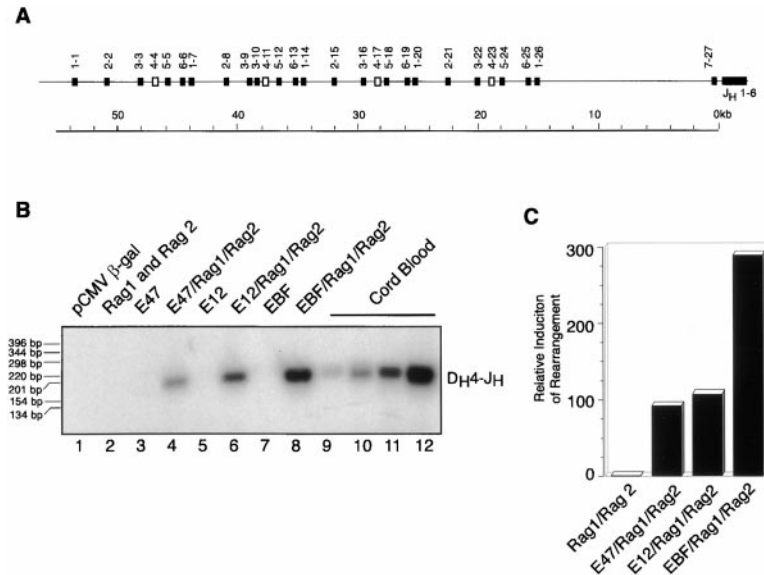


Figure 3. Either E2A or EBF Has the Ability to Act in Concert with RAG1 and RAG2 to Activate D_H4-J_H Rearrangement

(A) Schematic diagram of the human D_H locus, located downstream of the V_H6-1 region. The D segments are named with the following convention: the first number is the family, and the second is the position from the V_H6-1 segment. The D_H4 segments are denoted by open boxes. This map was adapted from the VBASE website: <http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html>.

(B) Either E2A or EBF and RAG1 and RAG2 act in concert to promote IgH D_H4-J rearrangements. PCR was performed on 200 ng of genomic DNA for 28 cycles. PCR products were run on a 2% agarose gel, transferred to Nytran Plus, and hybridized with a cloned D_H4-J_H1 fragment. Lanes 9-12 show a titration of cord blood DNA that was diluted back into BOSC DNA and contain 3.7 ng, 11 ng, 33 ng, and 100 ng, respectively. The expected size of the PCR product is approximately 187 bp.

(C) The results of at least three experiments were quantified, and the levels of D_H4-J_H rearrangements with E2A or EBF and RAG1 and 2 were compared to those with RAG1 and RAG2 alone (which was set to 1).

related in their coding sequence (Figure 3A). To determine whether either E2A or EBF have the ability to promote V(D)J recombination at the IgH locus, genomic DNA was isolated and analyzed for the presence of V(D)J and DJ joints (Szczepanski et al., 1999). Employing a V_H family-specific primer and a consensus J_H primer, no rearrangements to any of the V_H regions, including V_H1/7, V_H2, V_H3, V_H4, V_H5, and V_H6, were observed (data not shown). To examine for the presence of DJ joints, genomic DNA was amplified by PCR using a primer for the four D_H4 segments, located between 18-48 kb upstream of the J_H locus, and a consensus J_H primer (Figure 3B). Neither ectopic expression of E2A, EBF, nor the RAG proteins by themselves showed significant levels of DJ rearrangement (Figure 3B). In contrast, both E12 and E47 acted in synergy with RAG1 and RAG2 to activate DJ rearrangements in BOSC 23 cells (Figure 3B, lanes 4 and 6). Additionally, whereas EBF did not target the recombinase to the Ig V_κ1 locus, EBF activated IgH DJ rearrangement (Figure 3B, lane 8). These observations indicate that both E2A and EBF have the ability to target the recombinase to the IgH D-J region and that this causes an increase of IgH DJ rearrangements of more than 100-fold over that detected with RAG1 and RAG2 alone (Figure 3C).

EBF but Not E2A Targets the Recombinase to the Igλ Locus

The organization of the human Igλ locus is distinct from that of other Ig loci in several aspects. Approximately 30 functional V segments that belong to eleven separate families are localized over 1 Mb of DNA and organized into three clusters. Family members tend to be located within the same cluster (Figure 4A). Each of seven Jλ segments is paired with its own constant region as opposed to the Igκ locus in which five J segments are followed by a single constant region exon. A single Igλ

enhancer is located downstream of all the J/C regions, and thus the distance from many of the J segments is relatively large. We also note that *VpreB*, a surrogate light chain gene required for pre-B cell receptor function, and whose regulation has been shown to be influenced by EBF, is localized between the two most distal V region clusters (Figure 4A).

To examine whether E2A or EBF have the ability to promote Igλ VJ rearrangement, genomic DNA isolated from the transfectants was analyzed by PCR using the appropriate primers (M. van der Burg and A. W. L., unpublished data). Specifically, DNA was analyzed for the presence of VλIII-Jλ1 coding joints. Low levels of VλIII-Jλ1 coding joints were detectable in BOSC 23 cells transfected with RAG1 and RAG2 alone (Figure 4B, lane 2). Ectopic expression of E12 did not significantly enhance the ability of RAG1 and RAG2 to promote Ig VλIII-Jλ1 rearrangement. Strikingly, in the presence of EBF and RAG1 and RAG2, VJλ joints were readily detectable, approximately 25-fold higher as compared to DNA isolated from cells expressing E12, RAG1, and RAG2 (Figures 4B, lane 8, and 4C). These data indicate that distinct transcriptional regulators, EBF and E2A, have the ability to target the recombination machinery to specific Ig loci. E2A preferentially targets the recombination machinery to the IgH D_H4 and the IgVκ1 segments, whereas EBF allows accessibility of the recombinase to both the IgH and Igλ loci.

E2A and EBF Do Not Activate the Expression of PU.1, Pax5, Oct-2, and NF-κB in Embryonic Kidney Cells

The data described above raises the question whether E2A and EBF act directly or indirectly to regulate Ig rearrangement. It is conceivable that they function indirectly to activate the expression of other genes, whose products promote activation of Ig gene rearrangement.

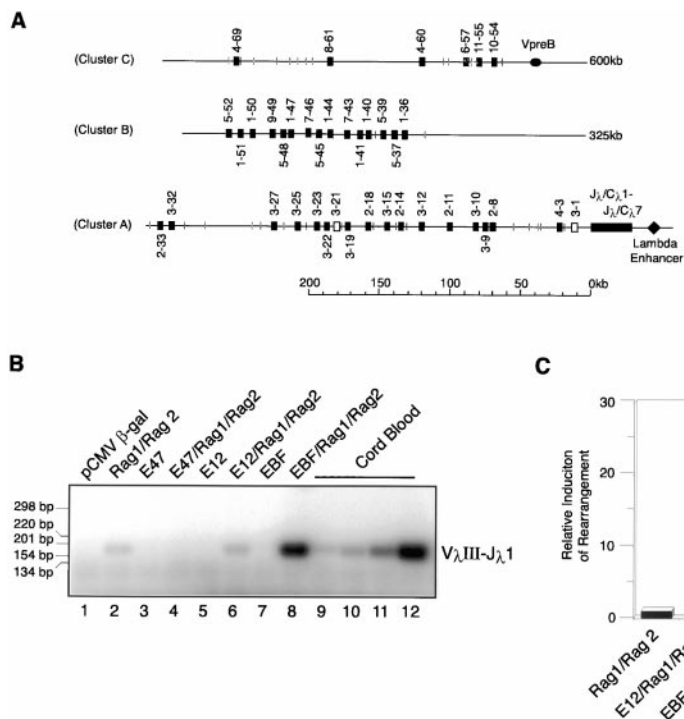


Figure 4. EBF and RAG1 and RAG2 Activate V_λIII-J_λ1 Recombination in BOSC 23 Cells

(A) A depiction is shown of the human Ig_λ locus. The V segments are named with the family number first followed by the position from the J/C regions. Two members of the V_λIII family that can be detected with the forward PCR primer are denoted as open boxes. This map was adapted from the VBASE website: <http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html>, and the IMGT website: <http://www.imgt.cines.fr:8104>.

(B) EBF and RAG proteins act in concert to promote Ig_λ VJ recombination in embryonic kidney cells. PCR products were run on a 2% agarose gel, transferred to Nytran Plus, and hybridized with a cloned V_λIII-J_λ1 fragment. PCR was used to amplify 200 ng of DNA for 26 cycles. Lanes 9–12 contain varying amounts of cord blood DNA diluted with BOSC 23 DNA: 3.7 ng, 11 ng, 33 ng, and 100 ng, respectively. The PCR product is of the expected size of approximately 168 bp.

(C) The blot in part (B) was quantified using a Phosphor Imager, and the levels of rearrangement plotted as compared to RAG1 and RAG2 alone.

A number of sites have been identified in the regulatory regions of the Ig enhancers that bind distinct classes of transcriptional regulators. Among the best characterized of these are E2A, EBF, Pax5, PU.1, Oct-2, and NF- κ B. To determine whether E2A or EBF have the ability to activate each other as well as induce the expression of Pax5, PU.1, Oct-2, and NF- κ B, nuclear extracts derived from transfected cells were examined by electrophoretic mobility shift assay (EMSA) and Western blotting (Figure 5). E2A and EBF DNA binding activity was readily detectable in cells transfected with either E2A or EBF (Figure 5A). We note that the relative levels of E2A and EBF DNA binding in pre-B cells (Nalm-6) is approximately 2- to 3-fold lower as compared to that of the transfectants (Figures 5A and 5B). E2A did not activate the expression of EBF, NF- κ B, Oct-2, PU.1, and Pax5 (Figure 5). Similarly, EBF did not induce the expression of E2A, Pax5, NF- κ B, Oct-2, and PU.1 (Figures 5A and 5C–5F). Thus, E2A and EBF do not have the ability to transiently activate the expression of this set of enhancer binding proteins in BOSC 23 cells. Since multiple E box sites are present in the IgH and Ig κ enhancers as well as in Ig κ promoter regions, we propose that E2A acts directly to control Ig rearrangement by promoting localized accessibility.

Analysis of the Coding Joints Isolated from Embryonic Kidney Cells Expressing Either E2A or EBF and the V(D)J Recombinase

To examine the nature of the Ig joints in the transfected cells, rearrangements were amplified by PCR and examined by heteroduplex analysis as well as DNA sequencing (Langerak et al., 1997). The V κ 1-J κ rearrangements from the E2A plus RAG transfectants were diverse and polyclonal, especially as compared to the rearrangements

seen in those cells that were transfected with RAG1 and RAG2 alone (data not shown). Over 65 recombinant clones were sequenced, and 45 of these were found to be unique (Table 1). Many distinct V κ 1 segments, interspersed throughout the proximal V κ locus, formed V κ 1J joints in the presence of E2A (Table 1 and Figure 6). Interestingly, only one VJ rearrangement utilizing a V segment from the distal locus, L24, was isolated (Figure 6A). Of the J κ segments, the J κ 1 segment was used with the highest frequency, followed by J κ 2 and J κ 4 (Figure 6B). While V κ 1 usage is distributed throughout the entire proximal variable segment cluster, the choice of the J κ segment appeared to be biased, either by location or by the sequences of the RSSs (Weber et al., 1994). J κ 1 is located most proximal to the V segments and was used in more than 62% of the rearrangements that were analyzed and twice as often as J κ 2. As expected, approximately one-third of the coding joints contained an open reading frame. Nearly all joints showed deletions of variable sizes. Although a few bases could not be assigned to germline sequences, obvious N nucleotide additions were lacking, indicating an absence of TdT activity. Thus, the expression of RAG proteins and a transcriptional regulator, E2A, is sufficient to generate a diverse repertoire of VJ κ joints, resembling those of B lineage cells.

Rearrangements of the Ig λ locus from EBF transfected cells were also cloned and sequenced (Table 1). Of the 20 clones that were sequenced, 6 unique coding joints were detected. As with the V κ rearrangements, the V_λIII coding joints mostly contained deletions of either or both the V and J coding ends. Half of the sequences showed an open reading frame. In contrast to the V κ sequences, all of the EBF Ig λ rearrangements used the same V gene segment, even though the primer could

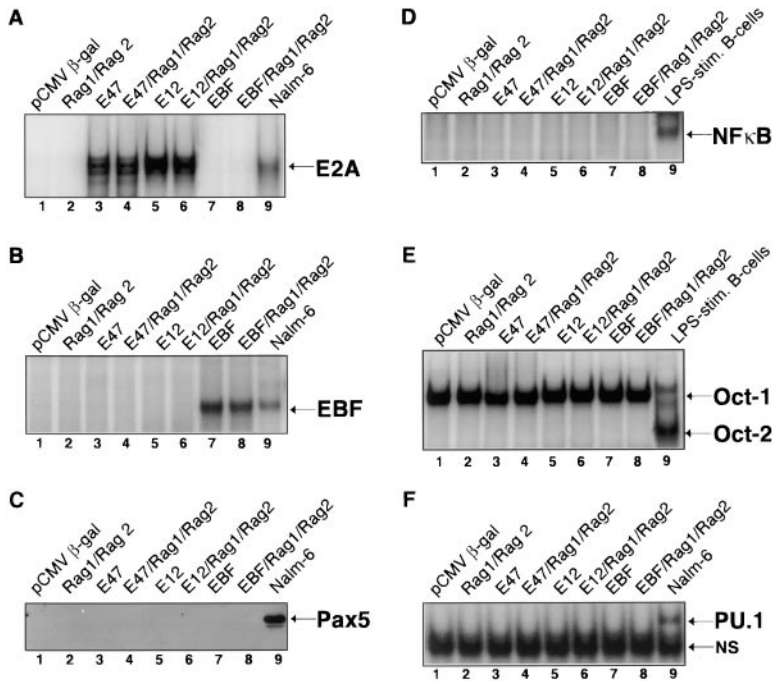


Figure 5. Ig Enhancer Binding Proteins Are Not Activated by the Ectopic Expression of Either E2A or EBF in BOSC 23 Cells

Nuclear extracts were prepared from transfected BOSC 23 cells and analyzed by EMSA and Western blotting for the presence of Ig enhancer-binding proteins.

(A) EMSA using the μ E5 binding site as a probe. The specificity of the binding complex was demonstrated using an anti-E2A antibody (data not shown).

(B) EMSA using a consensus EBF binding site as a probe.

(C) Western blot analysis of whole-cell extracts derived from transfected cells using an antibody with specificity for Pax5.

(D) EMSA was performed using the Ig κ NF- κ B site as a probe.

(E) EMSA for Oct binding proteins was performed with an octamer binding site. The slower migrating complex of Oct-1 is present in all of the extracts.

(F) EMSA using the μ B binding site derived from the IgH intronic enhancer as a probe. The complex was shown to be specific by shifting with an anti-PU.1 antibody (data not shown). NS refers to a nonspecific complex.

amplify rearrangements from at least one other V segment (Table 1 and data not shown).

To examine whether E2A and EBF differentially promote accessibility of the recombinase to distinct set of D_H4 segments, PCR fragments were cloned and sequenced (Table 1). Coding joints using the 4–17 and 4–23 D_H segments were detected in DNA from cells that had been transfected with E2A. DNA from EBF transfected cells contained rearrangements of the 4–4 and 4–23 segments (Table 1). All of the rearrangements contained deletions and many of the J_H segments were utilized (Table 1). We note that, overall, there was significantly less diversity among the coding joints isolated from the Ig λ and IgH loci, suggesting a lower frequency of rearrangements as compared to that of the Ig κ locus (our unpublished data).

Discussion

Our observations described here show that E2A proteins have the ability to activate rearrangement at the Ig κ but not at the Ig λ locus. In contrast, EBF allows Ig λ VJ but not Ig V κ 1J joining. Either E2A or EBF activate IgH DJ but not IgH V(D)J rearrangement. The coding joints are diverse and resemble those found in B lymphocytes. The striking finding is that a large degree of the variability normally associated with the antibody repertoire can be recapitulated in a nonlymphoid cell simply by the expression of a transcriptional regulator, either E2A or EBF, and the V(D)J recombinase.

E2A and Site-Specific Recombination

The data described here show that ectopic expression of E2A activates V(D)J joining in a nonlymphoid cell line.

Although these observations indicate that the expression of these transcriptional regulators act together with the RAGs to allow V(D)J rearrangements in nonlymphoid cells, they also indicate that RAG proteins by themselves have the ability to promote endogenous Ig gene rearrangement. This raises the question as to how RAG proteins by themselves have the ability to promote Ig rearrangement in nonlymphoid cells. In developing lymphocytes, the activity of the RAG proteins is tightly regulated during cell cycle progression (Schlüssel et al., 1993; Lin and Desiderio, 1994). Upon transient and overexpression in BOSC 23 cells, the regulation of RAG proteins might be perturbed allowing inappropriate access to the recombination locus. For example, it is conceivable that during the cell cycle, for example during DNA replication, RSSs become accessible to the high levels of RAG proteins that are transiently expressed in the transfectants.

The question arises whether the BOSC 23 cell line is unique. To this end, we performed similar experiments in the parent 293 cell line as well as the HeLa cell line (unpublished data). These cell lines were not transfected with the same efficiency as the BOSC 23 cell line. However, in these cell lines, E2A, RAG1, and RAG2 also synergized to promote V κ 1J rearrangement, albeit with significantly lower efficiency.

A role for E2A in Ig gene rearrangement is not unexpected. Previous studies have indicated that the Ig enhancers are essential for proper regulation of V(D)J recombination (Chen et al., 1993; Serwe and Sablitzky, 1993; Xu et al., 1996). The E2A proteins were originally identified as binding to the multiple E box sites present in the IgH and Ig κ enhancers. Additionally, a large subset of V κ regions contain a highly conserved E box immediately upstream of the TATAA box (Feeney et al., 1997;

Table 1. Sequences of Coding Joints in Transfected BOSC 23 Cells

	V- or D-segment	J-segment	V/D-segment used	J-segment used
A) V κ 1-J κ rearrangements in E2A/Rag1/Rag2 transfected cells	AAT AGT TAC CCT CC	TG TAC ACT TTT GGC CAA	L8	J1
	AAC AGT GCC CCT CC	-.GG ACG TTC GGC CAA	A20	J1
	TAC AAT TAC CCT CC	-.G ACG TTC GGC CAA	L11	J1
	TAT AGT TAC CCT CC	... ACT TTT GGC CAG	L9	J2
	AAT AGT TAC CCT CC	... ACT TTT GGC CAG	A30	J2
	AAT AGT TAC CCT CC	... P GGC CAG	L8	J2
	TAC AGT ACC CCC A	TG TAC ACT TTT GGC CAG	02/012	J2
	TAC AAT TAC CCT C	-G TGG ACG TTC GGC CAA	L11	J1
	AAT AGT TAC CCA C	-G TGG ACG TTC GGC CAA	A30	J1
	AAC AGT GCC CCT C	-G TGG ACG TTC GGC CAA	A20	J1
	AAC AGT GCC CCT C	.G TAC ACT TTT GGC CAG	A26	J1
	TAC AAT GCC CCT C	-G TGG ACG TTC GGC CAA	04/014	J1
	TAC AGT ACC CCA C	-G TGG ACG TTC GGC CAA	02/012	J1
	AAT AGT TAC CCT C	-G TGG ACG TTC GGC CAA	A30	J1
	AAT AGT TAC CCT C	...G ACG TTC GGC CAA	A30	J1
	TAC AAT TAC CCT C	...G ACG TTC GGC CAA	L11	J1
	TAT AGT TAC CCT C	...G ACG TTC GGC CAA	L9	J1
	TAT AGT TAC CCT C	...C ACT TTT GGC CAG	L9	J2
	AAC AGT GCC CCC ..	.G TAC ACT TTT GGC CAG	A20	J2
	AAT AGT TAC CCT C	...GG ACG TTC GGC CAA	A30	J1
	AAC AGT GCC CCTGG ACG TTC GGC CAA	A20	J1
	AAT AGT TAC CCT ..	TG TAC ACT TTT GGC CAG	A30	J2
	TAC AAT TAC CC ..	-G TGG ACG TTC GGC CAA	L11	J1
	TAT AGT TAC CC ..	.G TAC ACT TTT GGC CAG	L9	J2
	AAT AGT TAC CC ..	-G TGG ACG TTC GGC CAA	L8	J2
	AAT AGT TAC CC ..	.G TAC ACT TTT GGC CAG	L24	J1
	TAT AGT TAC CC TGG ACG TTC GGC CAA	L8	J1
	TAC AGT ACC CC TAC ACT TTT GGC CAG	02/012	J2
	AAT AGT TAC CCGG ACG TTC GGC CAA	A30	J1
	TAC AAT TAC CCAC ACT TTT GGC CAG	L11	J2
	AAT AGT TAC CCGG ACG TTC GGC CAA	A30	J1
	AAT AGT TAC CCG TTC GGC CAA	A30	J1
	AAT AGT TAC CCTTT GGC CAG	Vk431	J2
	AAT AGT TAC C ..	-G CTC ACT TTC GGC GGA	Vk431	J4
	TAC AGT ACC CTAC ACT TTT GGC CAG	02/012	J2
	AAT AGT TAC CCTC ACT TTC GGC GGA	Vk431	J4
	TAC AGT ACC CGG ACG TTC GGC CAA	02/012	J1
	TAC AAT GCC ..	-G TGG ACG TTC GGC CAA	04/014	J1
	AAT AGT T ..	-G TGG ACG TTC GGC CAA	L12A	J1
	AAT AGT TACG TTC GGC CAA	Vk431	J1
TAC AAT ..	-G TGG ACG TTC GGC CAA	04/014	J1	
TAC AAT ..	-C CTC ACT TTC GGC GGA	L11	J4	
AAC AGTG ACG TTC GGC CAA	A20	J1	
AAC AGG ACG TTC GGC CAA	A20	J1	
B) V λ III-J λ 1 rearrangements in EBF/Rag1/Rag2 transfected cells	GAC AGC AGC ACT GC	T TAT GTC TTC GGA ACT	3-1	J1
	GAC AGC AGC ACT GC	...T GTC TTC GGA ACT	3-1	J1
	GAC AGC AGC ACT G	...T CTC TTC GGA ACT	3-1	J1
	GAC AGC AGC ACTTAT GTC TTC GGA ACT	3-1	J1
	GAC AGC AGC ACTAT GTC TTC GGA ACT	3-1	J1
GAC AGC AGC ...	T TAT GTC TTC GGA ACT	3-1	J1	
C) D $_H$ 4-J $_H$ rearrangements in E2A/Rag1/Rag2 transfected cells	TAC GGT GGT AAC TCC	C TAC TGG TAC TTC GAT	4-17	J4
	TAC --- GGT GAC T	--- TGG TAC TTC GAT	4-23	J2
	TAC GGT GGT AAC TCG	C TAC TGG TAC TTC GAT	4-23	J2
	TAC GGT GGT AAC GG	... TGG TAC TTC GAT	4-23	J2
	TAC GGT GGT AAC TAA TAC TTC GAT	4-23	J1
TAC GGT GGT AAC TAC TGG TAC TTC GAT	4-23	J2	
TAC GGT GGT AACAC TGG TAC TTC GAT	4-23	J2	
D) D $_H$ 4-J $_H$ rearrangements in EBF/Rag1/Rag2 transfected cells	TAC --- AGT AAC TGG TAC TTC GAT	4-4	J2
	TAC --- AGT AAC TAC TGG TAC TTC GAT	4-4	J5
	TAC --- AGT AAC TGG TTC GAT	4-4	J5
	TAC GGT GGT AAC TCG	...TTC GAT	4-23	J4
	TAC GGT GGT AAC TCT GCT TTT GAT	4-23	J3
TAC GGT GGT AAC TAC TTT GAT	4-23	J4	

Coding joints in BOSC 23 cells transfected with either E2A or EBF and RAG1, and RAG2, are diverse and resemble those found in B lineage cells. Genomic DNA from transfected cells was amplified with primers specific for each locus. The resulting fragments were cloned and sequenced. The unique sequences are indicated here. Periods represent bases that have been deleted. Dashes indicate nucleotides that are not present in the germline sequence. Underlined bases are candidates for P nucleotide additions. Bold, italicized bases could not be matched to the germline DNA sequence.

(A) DNA sequences of Ig κ coding joints isolated from DNA derived from cells transfected with E2A, RAG1, and RAG2. The germline DNA sequences of the V κ L8 and J κ 2 are indicated.

(B) DNA sequences of V λ III-J λ 1 coding joints isolated from cells transfected with EBF/RAG1/RAG2. The germline sequences are indicated in reverse lettering.

(C) DNA sequences of D $_H$ 4-J $_H$ joints from cells transfected with E2A, RAG1, and RAG2. The germline sequences of 4-23 and J $_H$ 2 are indicated.

(D) DNA sequences of D $_H$ 4-J $_H$ coding joints derived from DNA from cells transfected with EBF and RAG1 and RAG2.

Bemark et al., 1998). We have shown that E2A activates germline transcription from these V κ 1 promoters. Although it remains to be determined that the E box sites are indeed essential for V(D)J rearrangement, they are reasonable candidates.

We have recently obtained additional evidence that E2A proteins in B lineage cells also regulate Ig κ VJ recombination. Overexpression of E12 in a pro-B cell line, expressing a functional IgH gene but carrying the Ig κ gene in germline configuration, promoted Ig κ rearrangement and showed high levels of Ig cell surface expression (Kee and C. M., unpublished data). Thus, an important role for E2A in promoting Ig κ VJ rearrangement in B lineage cells is likely.

E2A and EBF and IgH Rearrangement

The observations described here reveal that E2A or EBF also activate IgH DJ rearrangement in BOSC 23 cells. A role for E2A and EBF in DJ rearrangement is consistent with the presence of binding sites for these factors in the IgH enhancers. Five functional E box sites and two EBF binding sites have been identified in the IgH intronic enhancer (Lenardo et al., 1987; Åkerblad et al., 1996). Furthermore, E47, when transfected into a pre-T cell line, induced cross-lineage IgH DJ rearrangements and, when expressed in 3T3 cells, upregulated I $_{\mu}$ germline transcription (Schlüssel et al., 1991; Choi et al., 1996). We also note that lymphocyte development in E2A- and EBF-deficient mice is arrested at an early stage, prior

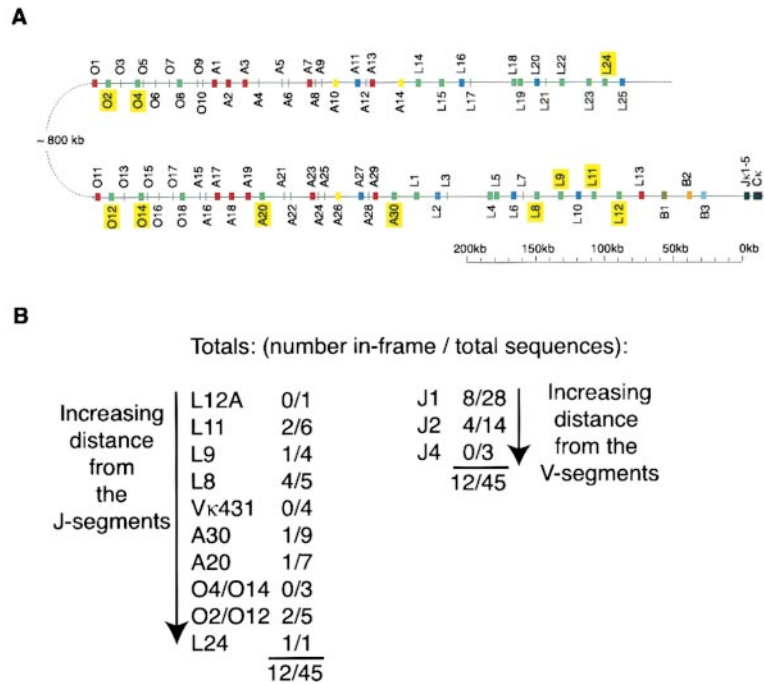


Figure 6. E2A Acts in Synergy with RAG1 and RAG2 to Generate a Diverse Igκ Repertoire

(A) A schematic representation of the human Igκ locus. The Vκ families are depicted by different colors as follows: Vκ1, green; Vκ2, red; Vκ3, blue; Vκ4, light blue; Vκ5, orange; Vκ6, yellow; and Vκ7, brown. This map is adapted from the V BASE website: <http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html>. Vκ segment rearrangements that have been detected by sequencing are highlighted in yellow.

(B) Diverse repertoire of coding joints. The Vκ431 segment is similar to L1, but it has not been determined if it is an allele of L1 or a separate gene. It is not listed on the map in Figure 6A (Cox et al., 1994; Klein and Zachau, 1995). Numbers indicate the number of in-frame joints and the total number of times a specific V region was isolated.

to the initiation of IgH DJ rearrangements (Bain et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995). Additionally, levels of germline transcripts are significantly lower or absent in cells isolated from both E2A- and EBF-deficient mice.

The data raise the question as to why E2A and/or EBF do not activate IgH V(D)J recombination. V(D)J rearrangements involve two steps, DJ followed by a V to DJ joining. It is likely that the rearrangement process in transiently transfected BOSC 23 cells is too inefficient to form V(D)J joints. Alternatively, additional transcriptional regulators might be required to activate IgH V(D)J rearrangement. It will be interesting to introduce additional transcriptional regulators, such as Pax5, ETS, Erg, and PU.1, in conjunction with E2A and/or EBF, into BOSC 23 cells and examine whether IgH V(D)J recombination can be achieved. Pax5, in particular, is an interesting candidate, since Pax5-null mutant B lineage cells show significant levels of DJ joints, whereas V(D)J rearrangements are severely perturbed (Nutt et al., 1997).

Regulation of Site-Specific Recombination during B Lineage Development

The rearrangement of Ig loci is regulated in a temporal fashion during B lineage development. For example, IgH gene rearrangement precedes that of Ig light chain recombination (Yancopoulos and Alt, 1985). This raises the question: how can the E2A proteins regulate both IgH DJ and Igκ VJ recombination? We would like to consider several possibilities. It is likely that additional transactivators and transcriptional repressors contribute to the proper temporal accessibility of these loci. For example, silencer elements may prevent the inappropriate rearrangement of the Igκ locus during the early stages of B lineage differentiation. Alternatively, it is possible that different levels and/or modified forms of E2A and EBF regulate the rearrangement of distinct Ig

loci at different stages. Higher levels of E2A may interact with relatively low affinity binding sites present in the Igκ enhancers. It will be important to determine the relative affinities for binding sites present in both the IgH and Igκ enhancers, as well as relative protein levels, and examine whether these regulators are differentially modified during early B cell progression.

Chromatin Accessibility and Recombination

The observations described here raise the question as to how transcriptional regulators act in concert with the RAG proteins to promote the joining of DNA segments that are separated by large distances. The E2A proteins contain three highly conserved domains: AD1, AD2, and the bHLH region. The bHLH domain is essential for DNA binding and dimerization but lacks the ability to trans-activate. The AD1 domain contains a highly conserved motif, LDFS, which activates transcription by direct recruitment of the SAGA complex, containing HAT activity (Massari et al., 1999). The activation domains of E2A also have the ability to interact with p300, also containing HAT activity (Qui et al., 1998). We propose that the E2A proteins recruit coactivators containing HAT activity to the relevant target sites. In such a model, the recruitment of p300 and/or SAGA would promote chromatin remodeling through histone modification. Such a model is consistent with the observation that in the absence of the transactivation domains, E2A does not have the ability to activate VJ recombination. Recent data has indicated that nucleosomal structures block access of RAG1 and RAG2 to the RSSs (Kwon et al., 1998; Golding et al., 1999). We suggest that the E2A proteins promote release from this repressive chromatin state, allowing recombination to be initiated and completed. Alternatively, it is conceivable that the activation of germline transcription by the E2A proteins is essential

to promote accessibility. E2A proteins may recruit co-activators containing HAT activity that promote accessibility of the recombination regions to both the transcription and recombination machinery.

Experimental Procedures

Cell Culture

BOSC 23 cells were grown as described (Pear et al., 1993).

DNA Constructs and Transfection Procedure

The pEBB RAG1 and pEBB RAG2 expression vectors have been previously described (Roman et al., 1997). All of the transcription factors and deletion constructs are cloned into the pH β APneo vector, which has been previously described (Kee and Murre, 1998). Calcium phosphate precipitation transfections were performed essentially the same as previously published (Pear et al., 1993). BOSC 23 cells were plated the night before the transfection at 5×10^6 cells per 10 cm dish, and 18–24 μ g of DNA total, including 6 μ g of each expression vector or carrier DNA, was used for each transfection. The cells were harvested approximately 3 days post-transfection.

Ligation-Mediated PCR

Genomic DNA was prepared by proteinase K treatment as previously described and dissolved in 10 mM Tris (pH 8.0) (Stanhope-Baker et al., 1996). This genomic DNA was then linker ligated and amplified exactly as described previously (Schlüssel et al., 1993; Stanhope-Baker et al., 1996) except that human-specific primers were used: hu κ 0, 5'-GGCCATAGACGTAATAATATTCTTCTTG-3'; hu κ 1, 5'-CATGCTATGTCAGAGAAGCCTCAGG-3'; hu κ 1probe, 5'-GTTCCCCTTGGTGAAGAAGGTTTCTG-3'. The PCR for CD14 was performed with primers specific for the human locus: huCD14-for, 5'-CAGAGGTTCCGAAGACTTATCGAC-3'; huCD14-rev, 5'-GTTATCTTTAGGTCCTCGAGCGTC-3'. Linker-ligated DNA (5 μ l) was amplified with the above CD14 primers for 24 cycles of 20 s at 94°C, 20 s at 65°C, and 40 s at 72°C.

Rearrangement and pJH200 Control PCRs

PCR was used to analyze 100–200 ng of genomic DNA, isolated from various BOSC 23 cells, in a 25 μ l reaction volume containing 10 mM Tris (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 100 ng of each primer, 200 μ M dNTPs, and 1 U of AmpliTaq Gold (PE Biosystems, Foster City, CA). PCR reactions were performed as follows: 8 min at 95°C, 26–28 cycles of 20 s at 95°C, 20 s at 61°C, 1 min at 72°C, followed by a 10 min extension at 72°C. The primers used are as follows: V κ 1, (Feeney et al., 1997); hu κ cons, 5'-ACGTTTGATCTCCAS YTTGGTCCC-3'; the Ig λ primers are unpublished results and the D_H and J_H primers have been published elsewhere (Szczepanski et al., 1999). Typically, 12 μ l of each PCR was analyzed on an agarose gel followed by ethidium bromide staining and/or Southern blotting.

To amplify rearrangements of the pJH200 plasmid, 100 ng of the same DNA used for genomic PCR was used in a PCR reaction as above but with the DR1 and NEB#1233 primers (van Gent et al., 1995) and 0.5 U of AmpliTaq Gold. The PCR program is as follows: 10 min at 95°C; 27 cycles of 15 s at 95°C, 15 s at 55°C, 30 s at 72°C; and 10 min at 72°C. Approximately 12 μ l was analyzed on a 2% agarose gel by ethidium staining.

Reverse Transcription PCR

Total RNA was prepared from BOSC 23 cells transfected, with the various expression constructs, by TRIzol according to manufacturer's instructions (GIBCO-BRL, Life Technologies, Rockville, MD). RNA was DNase I-treated for 15 min at room temperature, followed by addition of EDTA to 2.5 mM and heat inactivation for 10 min at 65°C. Reverse transcription using 2 μ g of this RNA was performed with Omniscript according to the manufacturer's instructions (Qiagen, Valencia, CA). Approximately 1/20th of the cDNA reaction was used in each PCR reaction as described above. The efficiency of each cDNA reaction was assayed by amplification of the β -actin transcripts with human-specific primers: huBact-for, 5'-GGATGATGATATCGCCGCG-3'; and huBact-rev, 5'-GGATAGCAACGTACA

TGGCTGGG-3'. Amplification was performed using the program: 3 min at 95°C; 20 cycles of 94°C for 15 s, 58°C for 15 s, 72°C for 45 s; and 10 min at 72°C.

PCR for κ ^o germline transcripts was performed as described above, except that AmpliTaq was used in place of AmpliTaq Gold. The following primers were used: hu5' κ ^o, 5'-CTGTCTTCAGATATGA GAGG-3'; huCk-rev, 5'-GATGAAGACAGATGGTGCAG-3'. The PCR amplification program is as follows: 3 min at 95°C; 35 cycles of 94°C for 20 s, 56°C for 20 s, 72°C for 60 s; 72°C for 10 min. The following primers were used for V κ 1 germline transcripts: huV κ -cons (Martin et al., 1991), 5'-GGTACCCAGTCTCCATCCTCCCTGTC-3'; L11-rev, 5'-CAGAGGTGACTTCGTGTCCCC-3'. The same PCR program as used for Ig κ rearrangements was used, except that 35 cycles were performed. Following PCR, 12 μ l of each reaction was run on a 1.5% agarose gel and analyzed by Southern blotting as described above.

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