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Identification of E2A target genes in B lymphocyte development by using a gene tagging-based chromatin immunoprecipitation system

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The transcription factors encoded by the *E2A* gene are known to be essential for B lymphocyte development, and ectopic expression or gene inactivation studies have revealed several potential lineage-specific *E2A* target genes. However, it remains unknown whether these target genes are directly regulated by *E2A* at the transcriptional level. We therefore generated mice carrying an affinity-tagged *E2A* knock-in allele to provide a system for the direct elucidation of *E2A* target genes based on *E2A* binding to target regulatory regions. Abelson-transformed pre-B cell lines derived from these mice were used in chromatin immunoprecipitation experiments to identify regulatory sequences bound by *E2A* in the context of an early B lymphocyte environment. Significant *E2A* binding was detected at the promoters and enhancers of several essential B-lineage genes, including the $Ig\kappa$ intronic and 3' enhancers, $\lambda 5$ and *VpreB* surrogate light chain promoters, the *EBF* locus promoter region, and the *mb-1* (*Ig α*) promoter. Low levels of *E2A* binding were observed at several other lymphoid-restricted regulatory regions including the *Ig* heavy chain (*IgH*) intronic enhancer, the *IgH* 3' enhancers *hs3b/hs4*, the *RAG-2* enhancer, and the 5' regions of the *B29* and *TdT* loci. An *E2A* target gene, the predicted butyrophilin-like gene *NG9* (*BTL-1*), was also identified by using a chromatin immunoprecipitation-based cloning strategy. In summary, our studies have provided evidence that *E2A* is directly involved in the transcriptional regulation of a number of early B-lineage genes.

The development of lymphocytes from hematopoietic stem cells involves a series of highly regulated differentiation events that depend on the collaborative efforts of a number of transcription factors. These transcription factors coordinately regulate lymphopoiesis through the initiation, maintenance, and restriction of lineage-specific gene expression programs. The transcription factors encoded by the *E2A* gene are known to play critical roles in the regulation of lymphocyte development. *E2A* proteins are highly expressed in developing lymphoid cells (1, 2), and gene targeting studies have shown that *E2A* proteins are required for the initiation of B cell development in the bone marrow. Mice deficient in *E2A* demonstrate a complete and persistent block at the earliest stage of B cell development before the initiation of *Ig* heavy chain gene rearrangements, as well as a dramatic reduction in thymocyte number (3, 4).

The mammalian *E2A* gene encodes two major products, *E12* and *E47*, which are members of the basic helix-loop-helix (bHLH) family of transcription factors (5, 6). bHLH proteins are characterized by a conserved HLH dimerization domain and an adjacent basic region that mediates DNA binding (7, 8). *E12* and *E47* are generated by alternative splicing of adjacent exons encoding their bHLH domains and bind the consensus E-box sequence CANNTG as homodimers or as heterodimers with other bHLH transcription factors (5–7, 9, 10). As E-box binding factors, *E12* and *E47* are members of a subfamily of bHLH transcription factors denoted the E proteins (9). The E protein family also includes the transcription factors *E2-2* and HeLa E-box binding protein (HEB), which play important roles in lymphocyte development as well (11–14).

E2A proteins were initially characterized for their binding activity at the *Ig* heavy and light chain enhancers, which were subse-

quently shown to contain several functionally relevant *E2A* binding sites (10, 15–18). Ectopic expression of *E2A* in non-B cells was also found to induce germ-line *Ig* transcription and rearrangement, suggesting that *E2A* might play a key role in *Ig* gene regulation (19–21). More recent studies demonstrated that transfection of the kidney cell line BOSC23 with *E2A* and the RAG recombination machinery was sufficient to drive diverse recombination events at the *Ig* heavy and light chain loci (22, 23). Overexpression studies have also implicated *E2A* in the regulation of other genes involved in the initiation and maintenance of the B cell developmental program. Ectopic expression of *E12* in a macrophage line was shown to result in the induction of the B-lineage transcription factors *EBF* and *Pax5/BSAP*, as well as *IL-7R α* and *RAG-1* (24). Other potential *E2A* targets include several components of the pre-B cell and B cell receptor (BCR) complexes, including the surrogate light chains $\lambda 5$ and *VpreB* and the BCR signaling molecules *mb-1* (*Ig α*) and *B29* (*Ig β*) (25, 26). However, it remains unclear whether the lineage and stage-specific expression of these genes is directly regulated by *E2A* at the transcriptional level or, alternatively, is activated by other factors downstream of *E2A*.

Chromatin immunoprecipitation (ChIP) strategies have recently been used for more direct assessments of target gene regulation based on transcription factor binding to suspected regulatory regions. For example, ChIP has been successfully used in the verification and cloning of several *E2F* target genes in human cell lines (27). In yeast, ChIP-based analysis of transcription factor binding to relevant target sequences has been facilitated by the introduction of affinity tags to endogenous proteins by homologous recombination (28). We have therefore combined an *in vivo* murine gene-tagging approach with a ChIP assay to investigate both suspected and novel *E2A* target genes in lymphoid cells. Mice carrying a dual affinity-tagged *E2A* knock-in allele have been generated and used to derive pre-B cell lines that express a functional *E2A* fusion protein. This fusion protein provides a means for highly efficient immunoprecipitation of *E2A*-bound DNA fragments, which have been screened for the presence of several suspected *E2A* target sequences. We have used this approach to identify a subset of B-lineage genes whose regulatory regions are bound by *E2A* under physiological conditions, providing evidence for direct transcriptional regulation of these genes by *E2A*. The pre-B cell lines expressing affinity-tagged *E2A* have also been used in ChIP-based cloning of a previously uncharacterized *E2A* target gene.

Materials and Methods

***E2A^{FH}* Knock-In Mice.** The *E2A^{FH}* gene-targeting construct was generated by using an *E2A* genomic sequence isolated from the 129/sv strain. A 2-kb genomic fragment of homologous sequence was used in subcloning to create an in-frame fusion of the *E2A*

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Abbreviations: bHLH, basic helix-loop-helix; ChIP, chromatin immunoprecipitation; AMLV, Abelson murine leukemia virus.

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Table 1. Oligonucleotide primer pairs used for ChIP-PCR screening of putative E2A target genes (Regulatory region), ChIP-PCR enrichment confirmation of clones isolated as E2A-bound sequences (Clone), and RT-PCR analysis of Abelson cell line transcripts (Transcript)

Gene locus	Forward primer (5'–3')	Reverse primer (5'–3')
Regulatory region		
<i>Igk</i> intronic enhancer	CAG AGG GGA CTT TCC GAG AGG CC	ACC CTG GTC TAA TGG TTT GTA AC
<i>Igk</i> 3' enhancer	ATA GCA ACT GTC ATA GCT ACC GT	GCA GGT GTA TGA GGC TTT GGA AA
<i>mb-1</i> promoter	CCA CGC ACT AGA GAG AGA CTC AA	CCG CCT CAC TTC CTG TTC AGC CG
λ 5 promoter	GGG TTA AGA CAG GCA GCT GTG AG	CAA ACC CCA GGC TGT CTC TAG TT
5' <i>EBF</i> locus	GTG GGG TAC CAG CTG AAC TCC AC	CAG CTG AGC ATG TGT TTT AAT TG
<i>Pax5</i> promoter	ATG GAG GTT GCA ATT GAG TTG GG	ACA ATT CTG CCA AGC AAG TGG TT
<i>IgH</i> intronic enhancer	TCA GAA CCA GAA CAC CTG CAG CA	GGT GGG GCT GGA CAG AGT GTT TC
<i>IgH</i> 3' enhancer HS3a	GCT CTG GTT TGG GGC ACC TGT GC	GGG TAG GGC AGG GAT GCT CAC AT
<i>IgH</i> 3' enhancer HS3b	TGG TTT GGG GCA CCT CTG AG	GGG TAG GGC AGG GAT GTT CAC AT
<i>IgH</i> 3' enhancer HS4	GGG TAG ATG CAG CCT GTG TTC CG	GGA GTG TAG AGG AGA GCT GTG GC
<i>RAG-1</i> promoter	AGC CAG GTG CAG CTG GAG CTG GG	CAA CAT ATG CTG TCT ACT CTC TC
<i>RAG-2</i> promoter	TGA CTG GTA TCT CGG GAC TTA AC	GTG CCT ACA GAT GTT CCA GTG AG
<i>RAG-2</i> enhancer	GTC ACT TGG AAA CCA CGT GGT TA	TAG TGC ACA TGC TGC TGC TTA TA
5' <i>B29</i> locus	ACA TGC TGC CCA GGG TAG AGA TT	ATC TGG GAG CCC CTT AAA CAA CT
5' <i>TdT</i> locus	TGA GCT GGT TTT GTA ATT ATT AG	GAC TCA GGA AGA CGC ACA CAC CA
<i>CD19</i> promoter	CCT AAT GCT ATC CCC AGA TGA TA	TAA ATA TTT TTC AGA TGA GTG GG
<i>Oct-2</i> promoter	TGG CCT GAT AGT GTG GAA CTG TA	CGG CCA TTT GCA CAC AGC CAC GG
<i>VpreB</i> promoter	TGC CAA GCT GGC CAT GTG AAC AC	GAT GTT CCT CTA CCA TAT GTG AG
<i>CD5</i> promoter	CAT GTG AAT GGC CAG TGG GCA CT	CAG GCA GTG TGG GCC TGT GTC AC
Clone		
ChIP-1	CAC TTC TAA ACG GGC AGA CTT TA	GGA GGA AAT GCC AAG GCA CAT CC
ChIP-5	AAG CCA CAT GGG GAT CTC TCC CC	TCA CAA CTA ATC TGT TAC AAG TG
ChIP-6	ATT GTA GTG TCC CTG GGT GAG CA	AGA GAA TTG TCT TAG GAG TCA GA
ChIP-9	GGA GTG GCA GAG GTT AAG TAC CT	TTA CAG GGA ACC TGA GCC ACA AC
ChIP-11	ATG TTC CAA AGC CAA CAT GCA AC	TCA CAC ATG CAC ATA CAC GAA AA
ChIP-13	ATC TCT CTA GCT GAA ATT TAG AT	TTT CTA CCA CAA GCA TTA TGT TA
ChIP-17	GCA TAG GCA CTT ATG TAG TGT CC	TGC TTG AAT TAG CTC TTA TAG AA
ChIP-18	TGA GAG CTA GCA CTA CAG GCT TA	AGC ATT TAG GAA GAA GCA AAG GC
ChIP-20	ATG CCC TCT CCT GGT CTT TAT GG	CAG AAA TTT TCC TAA TAG GAC CT
ChIP-24	ATT TAT TGC TAA GAA GAC ATG CA	CAA ACA CAG TCT TTT GGC ACA TG
Transcript		
5' NG9	AAA GTC GGG GAA GAT GCC CTG CT	CGG CCT CCG TAC CCC TCC ATC GG
3' NG9	CTG GGC CAG GAG AAA ACA GCC CG	GGA AGT CGC CGC CTG TGG TTT TC
<i>Igk</i>	TCC ATC TTC CCA CCA TCC AG	GAT GTC TTG TGA GTG GCC TC
λ 5	TGT GAA GTT CTC CTC CTG CTG	ACC ACC AAA GTA CCT GGG TAG
μ	GGT GGC TTT GAA GGA ACA ATT CCA C	TCT GAA CCT TCA AGG ATG CTC TTG
EF1 α	AGT TTG AGA AGG AGG CTG CT	CAA CAA TCA GGA CAG CAC AGT C

carboxyl terminus with oligonucleotides encoding the following amino acid sequence: Ala-Gly-Asp-Tyr-Lys-Asp-Asp-Asp-Lys-Ala-Gly-His-His-His-His-His-Stop. A phosphoglycerate kinase promoter-driven neomycin resistance cassette (PGK-Neo) was inserted into a unique *Xba*I site downstream of the *E2A* poly(A) signal and between the homologous segments for positive selection of clones undergoing homologous recombination. A PGK promoter-driven thymidine kinase (PGK-TK) cassette was placed outside of the homologous targeting sequences to allow for negative selection against random nonhomologous recombination events. The final targeting construct was linearized and transfected into embryonic stem cells by electroporation. Transfected clones were cultured under double selection with gancyclovir and G418, and correct targeting of 27 of 95 clones was determined by PCR screening. Germ-line transmission was obtained from one of two injected clones, and mice carrying the *E2A^{FH}* allele were maintained in a specific pathogen-free environment at Duke University's animal facility.

Derivation of Abelson Pre-B Cell Lines. Abelson murine leukemia virus (AMLV) was prepared as supernatant from ABO10 cells, pretreated with polybrene, and added to *E2A^{FH/FH}* or *E2A^{loxP/loxP}* bone marrow cells cultured in RPMI 1640 media supplemented with 10% FBS, penicillin/streptomycin, β -ME, and gentamycin. After 3 wk, bulk cultures were subcloned to yield the Abelson lines *E2A^{FH1B}*, *E2A^{FH4}*, and *E2A^{loxP}* 1AB1.

Chromatin Extracts and Immunoprecipitations. Cells were fixed and washed for preparation of chromatin extracts essentially as reported by Fernandez *et al.* (29). Fixed cells were then sonicated (Fisher Scientific 550 tapered microtip probe, setting 4) for 20–25 cycles of

25 s on a cold block with 15 s cooling between each burst, to obtain 0.5- to 1.0-kb DNA fragments. Sonicates were centrifuged at $14,000 \times g$ for 5 min, and supernatants were harvested and stored at -80°C . ChIP procedures were adapted from those described by Fernandez *et al.* (29). Chromatin extracts (1–2 mg) from the *E2A^{FH1B}* pre-B cell line or the untagged control cell line AMLV-3B (derived from C57BL/6 bone marrow) were diluted 1:10 in ChIP buffer (140 mM NaCl/100 $\mu\text{g/ml}$ BSA/100 $\mu\text{g/ml}$ yeast tRNA/1% Triton X-100/1 mM PMSF) and incubated with 50 μl of anti-FLAG agarose for 2 h at 4°C , rotating slowly. The bound agarose beads were harvested by centrifugation ($14,000 \times g$ for 15 s) and washed three times in 1 ml of IP buffer, twice in 1 ml of IP buffer containing 500 mM NaCl, twice in 1 ml of wash buffer (10 mM Tris-HCl, pH 8.0/250 mM LiCl/1 mM EDTA), and three times in 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0/1 mM EDTA). Bound DNA was eluted, incubated at 65°C overnight to reverse cross-links, and then RNase-treated, deproteinized, and precipitated as described by Fernandez *et al.* (29). Processed DNA fragments were resuspended in 100 μl of distilled water. Cloning of immunoprecipitated DNA fragments was performed essentially as described by Weinmann *et al.* (27).

PCR Screening of Suspected Target Genes. A series of 4-fold dilutions of input chromatin and immunoprecipitated DNA from *E2A^{FH}* and control cell lines was PCR-amplified for 29–32 cycles (94°C , 1 min; 58°C , 1 min; 72°C , 1 min; with 2 min final extension at 72°C) in 20 μl PCR buffer containing 3 mM MgCl_2 and *Platinum Taq* polymerase (Invitrogen). The entire PCR samples were then resolved on 1.5% agarose gels and visualized by ethidium bromide staining. Relative band intensity was used in qualitative comparisons of

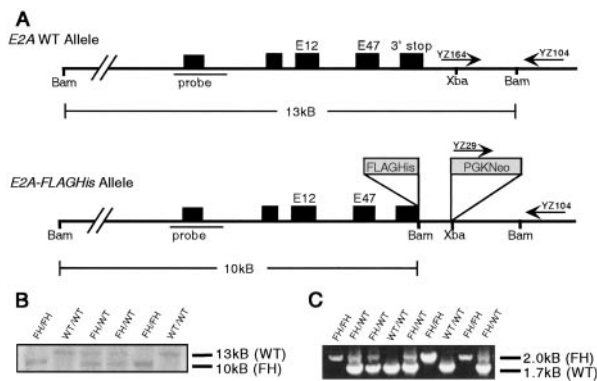


Fig. 1. Generation of dual affinity-tagged *E2A* knock-in allele. (A) Wild-type *E2A* locus and *E2A-FLAGHis* knock-in allele containing in-frame fusion of 3' *E2A* exon sequence with FLAG and hexahistidine affinity tags. (B) Southern blot genotyping confirmation of *Bam*HI-digested tail DNA from *E2A*^{FH/+} intercross pups. A 1.3-kb PCR-generated genomic *E2A* probe was used to detect the wild-type and knock-in alleles. (C) PCR genotyping of intercross pups by using YZ-104 (*E2A* antisense), YZ-29 (Neo sense), and YZ-164 (*E2A* sense) primers (31).

target sequence enrichment from ChIPs of *E2A*^{FH} vs. untagged control cell lines. All oligonucleotides used in PCR screening of immunoprecipitated DNA were 23-mers (IDT DNA) designed to amplify 150- to 200-bp regions surrounding potential *E2A*-binding sites within the regulatory regions of suspected target genes (Table 1, Regulatory region and Clone). In some cases, multiple primer sets were used to cover larger genomic sequences and potential regulatory regions; representative results for each of these regions are presented in Fig. 4.

Results

Generation of Affinity-Tagged *E2A*^{FH} Mice. We wanted to use a ChIP strategy to elucidate target genes that are directly regulated by *E2A* during lymphocyte development. Because the efficiency of immunoprecipitation-based studies can be limited by antibody specificity or affinity, we used an *in vivo* gene-tagging approach in generating an affinity-tagged *E2A* knock-in mouse model to facilitate the isolation of endogenous *E2A*-bound DNA sequences. Oligonucleotides encoding dual affinity tags were cloned into the 3' exon of the genomic *E2A* locus such that both E12 and E47 would be expressed as fusion proteins carrying carboxyl-terminal FLAG epitope and hexahistidine sequences (Fig. 1A). Proper targeting of the locus with the knock-in allele was confirmed by Southern blotting (Fig. 1B) and PCR genotyping (Fig. 1C). Bands representing the expected wild-type and *E2A*^{FH} alleles were detected, and normal distributions of each genotype were observed, indicating that the knock-in allele did not have an effect on neonatal survival.

***E2A*^{FH} Fusion Protein Supports Normal Lymphocyte Development.** B lymphocyte development is highly dependent on *E2A* protein dosage, such that even a 50% reduction in *E2A* protein activity translates into altered B cell development in the bone marrow (4, 13, 30). We therefore wanted to investigate B-lymphopoiesis in *E2A*^{FH} mice as a sensitive indicator of *E2A* fusion protein expression and function. Fluorescence-activated cell sorter (FACS; Becton Dickinson) analysis of bone marrow and splenocytes from wild-type, *E2A*^{FH/+} heterozygous, and *E2A*^{FH/FH} homozygous mice showed that lymphocyte development was normal in the knock-in mice. The *E2A*^{FH} allele led to no significant alterations in the relative percentages or numbers of pro- and pre-B cell populations (Fig. 2A) and mature IgM⁺ B cell populations in the bone marrow (Fig. 2B). Normal B and T lymphocyte profiles were also observed in the spleen (Fig. 2C and D) and thymus, and other hematopoietic populations in the bone marrow and spleen were also normal (data not shown). The fusion protein was therefore expressed from the

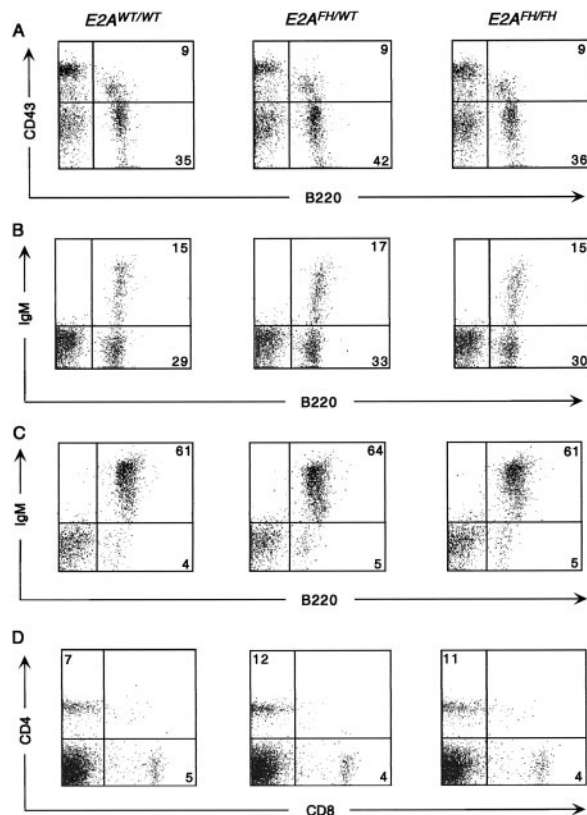


Fig. 2. Normal lymphocyte development in *E2A*^{FH} mice. Bone marrow (A and B) and splenocytes (C and D) from 3-wk-old wild-type (*E2A*^{WT/WT}), heterozygous (*E2A*^{FH/WT}), and homozygous (*E2A*^{FH/FH}) littermates were analyzed by FACS for expression of lymphocyte lineage surface markers by using the following fluorescent antibody conjugates: IgM-FITC, B220-APC, CD19-PE, CD4-FITC, and CD8-APC (Caltag, South San Francisco, CA); and CD43-PE (PharMingen). (A) Bone marrow staining for B220 and CD43. (B) Bone marrow staining for B220 and surface IgM. (C) Splenocyte staining for B220 and surface IgM. (D) Splenocyte staining for CD4 and CD8.

targeted locus and was functional in supporting normal lymphocyte development.

Abelson-Transformed Pre-B Cell Lines Express Functional *E2A*^{FH} Fusion Protein. To establish a culture system for studying *E2A* target sequences in the context of an early B cell environment, bone marrow was isolated from *E2A*^{FH/FH} mice and transformed with AMLV. The Abelson virus encodes the *v-Abl* oncogene and selectively targets developing pre-B cells for transformation. Primary transformants were established from *E2A*^{FH/FH} bone marrow cultures, and two subcloned lines, *E2A*^{FH1B} and *E2A*^{FH4}, were then derived for subsequent studies. These lines, as well as an untagged control Abelson line (*E2A*^{loxP} 1AB1) generated from the bone marrow of mice carrying a *loxP*-flanked *E2A* knock-in allele (*E2A*^{loxP/loxP}), displayed a typical B220⁺CD19⁺ pre-B cell phenotype (Fig. 3A; ref. 31). Anti-FLAG immunoprecipitations of *E2A* from nuclear extracts from *E2A*^{FH1B} or *E2A*^{loxP} control cells were then performed to verify the expression and functional utility of the *E2A*^{FH} fusion proteins. An anti-*E2A* Western blot of the immunoprecipitated extract confirmed that the fusion proteins were expressed and that affinity purifications could be performed by using the FLAG epitope tag (Fig. 3B). A band corresponding to the molecular mass of the immunoprecipitated *E2A* fusion protein (74 kDa) was also detected by colloidal staining of an SDS/PAGE gel (Fig. 3C). Electrophoretic mobility-shift analysis of nuclear extracts from the three Abelson clones (*E2A*^{FH1B}, *E2A*^{FH4}, and *E2A*^{loxP}) indicated that the *E2A* fusion protein bound to a μ E5 E-box

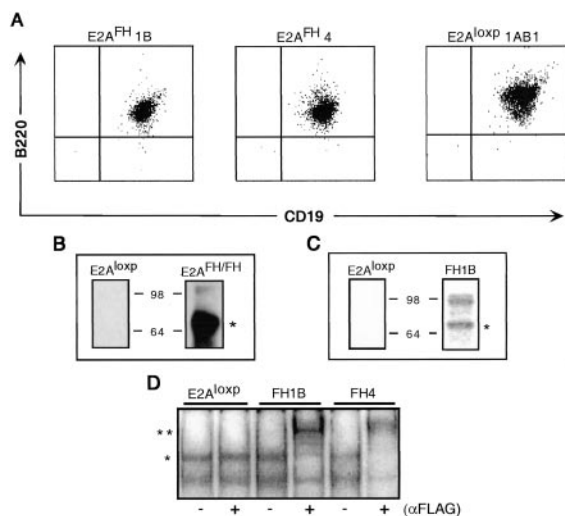


Fig. 3. Characterization of Abelson-transformed pre-B cell lines. (A) FACS analysis for expression of B-lineage markers CD19 and B220 on E2A^{FH} clone 1B, E2A^{FH} clone 4, and E2A^{loxP} control clone 1AB1. (B) Anti-E2A (G193-86, PharMingen) Western blot of elution fractions from anti-FLAG agarose affinity-purification of primary E2A^{loxP} and E2A^{FH/FH} transformant nuclear extracts (*, E2A monomer). (C) Colloidal Coomassie staining of FLAG peptide elution fractions from affinity-purification of subcloned Abelson lines. E2A monomer (*) is indicated, as well as a specific higher-molecular-weight band representing a commonly observed alternative isoform or modification of E2A. (D) Electrophoretic mobility-shift analysis on Abelson pre-B nuclear extracts for binding to radiolabeled μ E5 oligonucleotide (10). E2A in μ E5-binding complexes unshifted (*) or supershifted (**) with 1 μ l of anti-FLAG antibody.

oligonucleotide probe as expected and that the E2A^{FH}-DNA complex could be mobility-shifted with anti-FLAG antibody (Fig. 3D).

E2A Is Bound to a Subset of Lineage-Restricted Regulatory Regions. Phenotypic and biochemical analysis of the E2A^{FH} Abelson lines demonstrated that the E2A fusion protein was expressed, displayed normal DNA-binding characteristics *in vitro*, and could be immunoprecipitated with anti-FLAG antibody. The affinity-tagged protein should facilitate efficient immunoprecipitation of E2A-bound DNA sequences from cross-linked chromatin fragments. Chromatin extracts were therefore prepared from the E2A^{FH}1B or untagged control cell lines, and E2A-bound DNA fragments were obtained by anti-FLAG immunoprecipitation. These sequences were then screened for enrichment of promoter and enhancer regions within several potential lymphoid-restricted E2A target genes. Unfractionated input chromatin and immunoprecipitated DNA from the E2A^{FH}1B cell line and an untagged control cell line (AMLV-3B) were PCR-amplified by using primers surrounding the E-box sites within the regulatory elements of these genes (Table 1, Regulatory Region). Comparisons of the relative signals observed after amplification of immunoprecipitated DNA from tagged vs. control chromatin extracts allowed for qualitative determinations of whether each potential target sequence was enriched due to E2A binding.

Significant enrichment was observed for several suspected E2A targets, including the *Ig κ* intronic and 3' enhancers, the *mb-1* (*Ig α*) promoter, the λ 5 and *VpreB* promoters, and the 5' region of the *EBF* locus (Fig. 4A). Selective enrichment of these regulatory regions indicated E2A protein binding and suggested that E2A is likely to play a direct role in the transcriptional regulation of these genes during B cell development. Interestingly, several other potential E2A target sequences appeared to be only slightly enriched in the ChIP-PCR screen, including the *IgH* intronic and hs3b/hs4 3' enhancers, the *RAG-2* enhancer, and the 5' regions of the *B29*

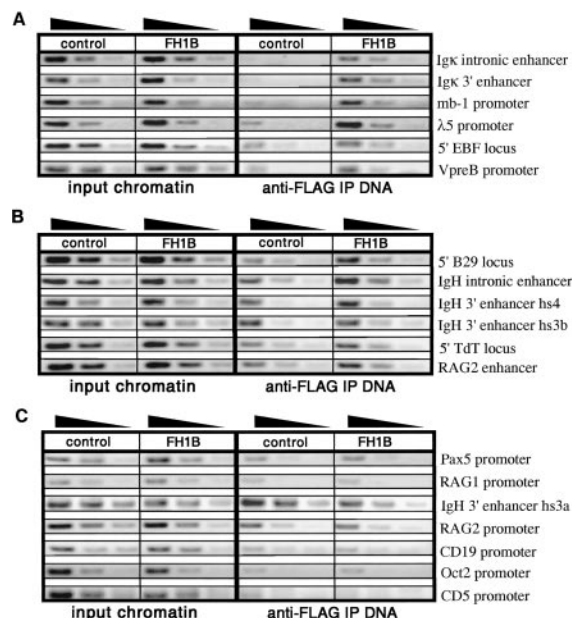


Fig. 4. ChIP-PCR screen for E2A binding to regulatory regions of potential target genes. A series of 4-fold dilutions of input chromatin and immunoprecipitated DNA from E2A^{FH}1B or untagged control pre-B cell lines were PCR-amplified by using primers specific for suspected E2A target regions (see Table 1, Regulatory region). Sequence enrichment in immunoprecipitated DNA from E2A^{FH}1B vs. control chromatin indicated E2A binding within genomic region. Each experiment was repeated at least three times, and results shown were reproducible for all targets. (A) Significant enrichment of the *Ig κ* enhancers, λ 5 and *VpreB* promoters, 5' *EBF* locus, and *mb-1* promoter was observed. (B) Low levels of enrichment were seen for the *IgH* intronic and hs3b/hs4 enhancers, 5' *B29* and *TdT* loci, and *RAG-2* enhancer. (C) No enrichment was indicated for the *Pax5*, *CD19*, *Oct-2*, *RAG-1*, *RAG-2*, and *CD5* promoters and the *IgH* hs3a enhancer.

and *TdT* loci (Fig. 4B). Meanwhile, no enrichment was observed for several other potential E2A targets, including the *IgH* 3' enhancer hs3a and the promoter sequences for *Pax5*, *RAG-1*, *RAG-2*, *Oct-2*, *CD5*, and *CD19* (Fig. 4C). The observation that only a subset of putative E2A targets was actually enriched provided support for both the selectivity and relevance of the assay because enrichment required more than just an accessible locus containing potential E2A binding sites.

Identification of Novel E2A Target Genes by ChIP-Based Cloning. Comparative PCR analysis provided a means for qualitative assessment of enrichment (and thus E2A binding) at a suspected target sequence. However, the regulatory regions identified as E2A targets by ChIP-PCR screening probably represent only a small subset of all genes subject to transcriptional regulation by E2A during B cell development. A more general approach was required to broaden the scope of the study to include the identification of novel E2A target genes. ChIP-based cloning strategies have recently been used in identifying novel target genes regulated by ubiquitous transcription factors such as E2F (27). We therefore used our E2A^{FH} pre-B cell ChIP system for the cloning and characterization of novel E2A target sequences. Immunoprecipitated DNA fragments from E2A^{FH}1B chromatin extracts were blunt-ended by polymerase treatment and ligated into pBluescript vector. The transformed clones were then screened by restriction digest, and 13 of 24 clones were found to contain inserts representing immunoprecipitated DNA fragments. These inserts were sequenced by using vector-specific primers, and the sequences obtained were then identified by BLAST or ENSEMBLE database searches for mouse genome matches. Ten of 13 sequences had single murine genome matches, whereas the remaining 3 yielded either no match or multiple genomic matches (Table 2). Sequence

Table 2. Summary of inserts isolated as E2A-bound sequences from ChIP-based cloning of novel E2A target genes

Clone	E boxes	Enriched	Chromosome	Gene	Family
ChIP-1	2	Yes	17	<i>NG9</i>	Butyrophilin-like
ChIP-2	0		Multiple hits		
ChIP-5	3	Yes	10	None	N/A
ChIP-6	0	No	9	Novel	PWWP domain
ChIP-8	0		Multiple hits		
ChIP-9	1	Yes	17	Novel	Zn/PHD finger
ChIP-11	3	Yes	4	Novel	TEF S-II homology
ChIP-13	1	Yes	6	Novel	Similar to K-ras
ChIP-14	2		No match		
ChIP-17	1	Yes	15	<i>CDH7</i>	Cadherin precursor
ChIP-18	2	Yes	5	Novel	Unknown
ChIP-20	2	Yes	8	<i>CSMD2</i>	C4B binding
ChIP-24	1	No	X	None	N/A

analysis of the 10 genomic clones indicated that 9 of 10 inserts contained at least one E box representing a potential E2A binding site. Importantly, eight of these nine sequences were subsequently found to be enriched by ChIP-PCR screening, providing strong confirmation that the majority of DNA fragments isolated by ChIP-based cloning represented actual E2A-bound sequences (Table 1, Clone; Table 2). Interestingly, seven of the eight sequences found to be enriched by ChIP-PCR were also located within predicted or known gene loci (Table 2).

The ChIP-1 insert yielded a perfect match to an intronic sequence of the putative mouse butyrophilin-like gene *NG9* (*BTL-II*), located within the extended *MHC II* locus (GenBank accession no. AF050157.1). The 428-bp sequence also contained two E-box sites, including one canonical E2A binding site, CAGGTG (Fig. 5A). The genomic region represented by the ChIP-1 clone was therefore selected for further analysis. Primers (I) specific for the sequences flanking the prominent ChIP-1 E box were used to confirm E2A-dependent enrichment of the region by PCR (Fig. 5A and B). Because the clone represented intronic sequence between central exons of the gene, two additional primer sets (II, III) were also designed to screen for enrichment of the putative promoter region of *NG9*. A fourth primer pair (IV) covering what was speculated to be a nonregulatory region upstream of the first *NG9* exon was used in ChIP-PCR screening as well. Interestingly, the genomic regions corresponding to both the ChIP-1 clone and the putative *NG9* promoter region were moderately enriched in immunoprecipitated DNA from E2A^{FH1B} chromatin (Fig. 5C). No significant enrichment of the nonregulatory region represented by primer pair IV was observed, supporting the specificity of the enrichment seen with the other primers.

The *NG9* gene contains several conserved domains characteristic of the butyrophilin gene family, including two Ig variable-like (IgV) domains and two Ig constant region-like (IgC) domains (Fig. 5A) (32). No functional information on the gene product is available, however, and little is known about its expression in lymphoid cells. Because E2A was shown to bind to putative regulatory regions of *NG9* and typically functions as a positive regulator of transcription, we wanted to evaluate *NG9* transcription in the cells from which the ChIP clone was isolated. cDNA from E2A^{FH1B} cells was therefore PCR-amplified by using primers specific for the 5' and 3' regions of the predicted *NG9* transcript, as well as primers to several other B-lineage genes (Table 1, Clone). *NG9* transcripts were detectable in E2A^{FH1B} cells along with *Igκ*, *λ5*, and *Iμ* transcripts, further supporting a role for E2A in the regulation of *NG9* expression (Fig. 5D).

Discussion

To our knowledge, there have been no previous studies in which an *in vivo* murine gene-tagging approach has been used to facilitate analysis of target genes regulated by an endogenously expressed transcription factor. The gene-tagging system provides

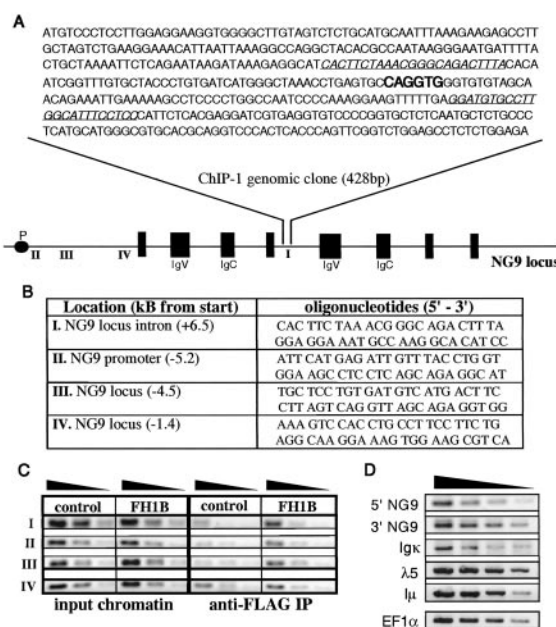


Fig. 5. E2A target gene isolated by ChIP-based cloning. Immunoprecipitated DNA fragments from E2A^{FH1B} chromatin extract were cloned, sequenced, and identified by mouse genome database search (see Table 2). (A) ChIP-1 clone insert with an exact match to an intronic region within the putative butyrophilin-like gene *NG9*. Clone-specific primers (I; in italics) surrounding the consensus E box (CAGGTG) were used to confirm E2A binding by ChIP-PCR. (B) Primers to the ChIP-1 insert (I), the predicted promoter region (II and III) of the *NG9* locus, and a region upstream of the transcription start site (IV). (C) ChIP-PCR screen for E2A binding at the ChIP-1 insert genomic sequence and *NG9* 5' regulatory regions. (D) Semiquantitative RT-PCR analysis of four 3-fold dilutions of E2A^{FH1B} cDNA for the predicted *NG9* transcript, along with relevant B-lineage genes (*Igκ*, *λ5*, *Iμ*) and a loading control (EF1α) (see Table 1, Clone). Thirty cycles (21 cycles for EF1α) of PCR amplification (94°C, 30 s; 57°C, 30 s; 72°C, 30 s, with 1 min extension at 72°C) were performed in PCR buffer containing 3 mM MgCl₂ and *Platinum Taq* polymerase (Invitrogen).

a distinctive alternative to the use of ectopically expressed proteins and also bypasses the challenges of low antibody affinity and specificity that can sometimes interfere with traditional protein isolation approaches. Similarly, to our knowledge there have been no previous studies to directly characterize E2A binding to a number of lymphoid lineage-restricted regulatory regions at the chromatin level. Here, we have demonstrated E2A binding to regulatory regions within a select subset of genes required for B lymphocyte development. Previous ectopic expression and promoter-analysis studies have implicated E2A in the regulation of many of these target genes, including the *Ig* heavy and *κ* light chains, surrogate light chains *λ5* and *VpreB*, and the transcription factor *EBF* (22, 24, 26). Significant enrichment of these sequences by E2A-ChIP provides more conclusive evidence for direct transcriptional regulation by E2A. Binding of E2A to 5' regulatory regions within the *EBF* locus is also in agreement with recent studies by Smith *et al.* (33), who have characterized a potential E2A binding site within the newly identified *EBF* promoter region. We were also intrigued to see strong enrichment of the *mb-1* (*Igα*) promoter region, because this observation provided direct evidence for the regulation of yet another component of the B cell receptor complex by E2A. Although *mb-1* has been implicated as a potential E2A target in genetic studies, it has generally been considered a primary target for other transcription factors, such as Pax5/BSAP, Ets, and Oct-2 (34, 35). However, our data on E2A binding to the *mb-1* promoter supports unpublished work by Hagman *et al.* demonstrating occupation of an *mb-1* promoter E-box site by DNA

footprinting analysis (J. Hagman, personal communication). These studies provide further insight into the collaborative regulation of gene expression by E2A and other transcription factors during B-lymphopoiesis.

We consistently observed that certain regulatory regions (*Igκ* enhancers, *mb-1* promoter, 5' *EBF* locus, and $\lambda 5$ and *VpreB* promoters) are significantly enriched after E2A-DNA immunoprecipitations, whereas other putative targets (*IgH* intronic and *hs3b/hs4* enhancers, 5' *TdT* and *B29* loci, and *RAG-2* enhancer) show only very low levels of enrichment. We have also evaluated a group of lymphoid lineage-restricted genes that show no detectable enrichment in our ChIP-PCR assay, including the *IgH* 3' enhancer *hs3a* and the promoters for *Pax5*, *RAG-1*, *RAG-2*, *CD19*, *Oct-2*, and *CD5*. The lack of enrichment of these sequences further substantiates the specificity of the ChIP-PCR system as a means for elucidation of direct vs. indirect E2A target genes. Although the transcription of these genes has been shown to be affected by E2A activity, previous studies have provided no significant evidence for direct transcriptional regulation by E2A. Our data suggests that *Pax5*/BSAP, *Oct-2*, *CD19*, and *RAG-1* may be targets for transcriptional regulation by other genes downstream of E2A. This possibility is supported by previous observations that ectopic expression of EBF in a macrophage line leads to activation of a subset of E2A-responsive genes including *Pax5* (24). E2A-mediated activation of the *EBF* locus may therefore play a role in establishing the hierarchy of transcription factors at the earliest stages of B-lymphopoiesis, with EBF subsequently contributing to the induction of *Pax5* and other downstream genes.

Perhaps the most surprising data obtained from the ChIP-PCR screen were the minimal enrichment observed for the *IgH* enhancers. The presence of abundant *I μ* transcripts in the pre-B cell lines indicates that the *IgH* locus is accessible and transcriptionally active. The *IgH* enhancers also contain well-characterized E-box sites, which are required for normal Ig gene transcription and rearrangement (15, 18). However, previous transfection and DNase hypersensitivity studies have shown that only one of the 3' *IgH* enhancers, *hs4*, is thought to be active at the early stages of B cell development (36, 37). Interestingly, *hs4* was the most prominently enriched of the 3' enhancers, with its most proximal enhancer, *hs3b*, showing slightly lower enrichment. No detectable enrichment of *IgH* enhancer *hs3a* was observed, which is in agreement with previous data suggesting that this enhancer is only functional in activated mature B cells (reviewed in ref. 37). Here, we must also emphasize that a lack of significant E2A binding at any given regulatory region in the pre-B cell lines does not exclude the possibility of direct transcriptional regulation by E2A. Alternatively, certain regulatory regions may be bound by E2A only at restricted developmental stages during B cell development and/or activation. This caveat may be particularly relevant in the analysis of genes involved in Ig rear-

angement, such as *RAG-1* and *RAG-2*, *TdT*, and the Ig genes themselves. The tight and highly ordered developmental regulation of these loci during B-lymphopoiesis may involve significant E2A binding only during specific phases of Ig gene rearrangement, assembly, and expression. Future studies on E2A-bound regulatory regions in primary cells at different stages of development should provide further insight on these dynamic regulatory processes.

A ChIP-based cloning strategy was used to isolate several novel E2A-bound sequences, including an intronic region within the novel E2A target gene *NG9* (also known as *BTL-II* for butyrophilin-like *MHC class II*-associated). E2A binding at E-box-containing genomic regions across this locus was subsequently investigated by sequence analysis and ChIP-PCR screening. The *NG9* locus lies downstream from the *E α* locus and upstream from a cluster of other butyrophilin-like genes within the extended *MHC II* locus (32). As with other butyrophilin genes, each conserved domain of *NG9* is encoded by a separate exon, providing support for the notion that the butyrophilin gene clusters arose through exon shuffling and duplication. However, *NG9* lacks both the transmembrane domain and the conserved carboxyl-terminal B30.2 domain of the traditional butyrophilins. Interestingly, sequencing data from multiple cell lines indicates that the *NG9/BTL-II* locus is also highly polymorphic with respect to HLA haplotype (32). These polymorphisms translate into at least five different *NG9* alleles and may have been generated and maintained during the diversification of the *MHC/HLA* loci. Although *NG9* transcripts have previously been detected in skeletal muscle and a number of gut tissues, to our knowledge no previous work has evaluated potential regulatory regions and characterized *NG9* transcription specifically in lymphoid cells (32). A number of other potential target sequences were also isolated by ChIP-based cloning, most of which appear to be bona fide E2A-bound targets based on their enrichment and their location within known or predicted gene loci. The ChIP-PCR screening strategy used in characterizing E2A binding at the *NG9* locus should also prove quite useful in evaluating these and other large genomic regions for transcription factor binding *in vivo*. In future studies, we will be combining the ChIP-based cloning system with gene array analysis on E2A-deficient cell lines to characterize additional E2A target genes. This powerful two-tiered approach will allow for the elucidation of target genes based on both E2A binding and the requirement for E2A in the normal expression of these genes.

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