E2A activity is induced during B-cell activation to promote immunoglobulin class switch recombination

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The basic helix–loop–helix protein, E2A, is required for proper early B lymphopoiesis. Specifically, in E2A-deficient mice, B-cell development is blocked at the progenitor stage prior to the onset of immunoglobulin (Ig) V(D)J recombination. Here, we demonstrate that E2A plays an additional role during peripheral B lymphopoiesis. Upon activation of primary mature B lymphocytes, both E2A protein levels and DNA-binding activity are induced. Furthermore, we show that mature B cells, expressing a dominant-negative E2A antagonist, proliferate normally in response to mitogenic signaling and appropriately express the early and late activation markers CD69, CD44, IgD and B220. However, in the absence of E2A activity, B lymphocytes are blocked in their ability to express secondary Ig isotypes. We demonstrate that the defect lies at the level of DNA rearrangements between the Ig switch regions. These data suggest that E2A is an essential target during B-cell activation and its induction is required to promote Ig class switch recombination.

Keywords: B-cell activation/E2A/helix–loop–helix/immunoglobulin class switch recombination/isotype switching

Introduction

The humoral immune response involves the activation and differentiation of mature B lymphocytes into high affinity antibody-secreting effector cells. B-cell activation is initiated by antigen binding to surface immunoglobulin (sIgM), which signals naive cells to enter the cell cycle. Upon immunization with T cell-dependent antigens, B cells differentiate along one of two developmental pathways. Activated B cells can either differentiate to become low affinity plasmacytes or, alternatively, migrate to form germinal centers. In germinal centers, B cells, with the help of follicular dendritic cells and antigen-primed T cells, undergo clonal proliferative expansion, isotype switching and somatic hypermutation (MacLennan, 1994). These B cells then function as either memory B cells or high affinity antibody-secreting cells.

Recent studies have indicated the presence of a transcriptional activator in germinal centers, the E2A proteins (Roberts *et al.*, 1993; Goldfarb *et al.*, 1996; Rutherford

and LeBrun, 1998). The E2A gene products are class I basic helix–loop–helix (HLH) proteins, which are characterized by their broad tissue distribution and their ability to form homodimers as well as heterodimers with tissue-restricted, class II HLH members (Murre *et al.*, 1989b, 1994; Lassar *et al.*, 1991). Class I members, also referred to as E proteins, bind to E-box sites, originally identified in the immunoglobulin enhancers and now known to be present in the promoters and enhancer of various tissue-specific genes (Church *et al.*, 1985; Ephrussi *et al.*, 1985; Murre *et al.*, 1994).

The E2A gene codes for two proteins, E12 and E47, which arise through differential splicing of the exon encoding the HLH domain (Murre *et al.*, 1989a; Sun and Baltimore, 1991). The two proteins share identical N-terminal transactivation domains but have distinct dimerization and DNA-binding capabilities (Murre *et al.*, 1989a; Aronheim *et al.*, 1993; Quong *et al.*, 1993; Massari *et al.*, 1996). In many tissue types, E2A proteins have been shown to activate gene transcription by forming heterodimers with tissue-specific HLH proteins. In contrast, in B lymphocytes, the active DNA-binding complex consists of E47 homodimers (Murre *et al.*, 1991; Bain *et al.*, 1993). Targeted disruption of the E2A gene has revealed a crucial role for E2A in early B-lineage development. B-cell development is arrested at the early progenitor stage prior to IgH gene rearrangements and the expression of a number of B lineage-specific genes (Bain *et al.*, 1994; Zhuang *et al.*, 1994). Furthermore, reconstitution of normal numbers of peripheral mature B cells requires the presence of both E12 and E47 proteins (Bain *et al.*, 1997). Recent evidence has suggested an additional role for the E2A proteins in germinal centers (Roberts *et al.*, 1993). Analysis of human peripheral lymphoid tissues showed high levels of E2A protein expression in the highly proliferative dark zones of germinal centers (Goldfarb *et al.*, 1996; Rutherford and LeBrun, 1998).

Here, we have analyzed the regulation and role of E2A in primary mature B lymphocytes. Naive mature B cells show very low E2A protein levels and E2A DNA-binding activity. However, upon B-cell activation, E2A activity is strongly induced. B lymphocytes lacking E2A activity have lost the ability to undergo class switch recombination (CSR) due to an inability to rearrange Ig switch DNA segments. These data suggest that proper CSR requires the presence of the E2A proteins.

Results

E47 expression in normal B cells is induced upon stimulation with LPS

High levels of E2A proteins have been reported in cell lines characteristic of the mature B and plasma cell stages (Murre *et al.*, 1991; Jacobs *et al.*, 1993). However,

Fig. 1. Induction of E47 in LPS-activated B cells. Unstimulated (A) and 48 h LPS-activated splenic B cells (B) were fixed and stained for E47 proteins (green). The cells were also stained and visualized using the Hoechst DNA dye to indicate nuclear localization (C and D).

immunohistochemical staining of human lymph tissues showed strong staining primarily in the dark zones of germinal centers, suggesting that activated germinal center cells possess particularly high levels of E2A (Goldfarb *et al.*, 1996; Rutherford and LeBrun, 1998). To examine the expression pattern of E2A in normal B-lineage cells, purified splenic B lymphocytes from wild-type C57BL/6 mice were isolated and analyzed for the presence of E2A by immunofluorescence (Figure 1). Staining with an E47-specific antibody showed detectable but low levels of E47 proteins in unstimulated splenic B cells (Figure 1). In contrast, mature B cells stimulated with lipopolysaccharide (LPS) revealed strong punctate nuclear staining, indicating that E47 protein levels are significantly increased in response to LPS (Figure 1).

E2A protein levels and DNA-binding activity are strongly induced upon B-cell activation

In order to examine E2A induction further in activated B lymphocytes, purified splenic B cells were stimulated with various mitogens in the presence or absence of the cytokine interleukin-4 (IL-4) (Figure 2). Nuclear extracts were prepared from naive and activated B cells and analyzed for the presence of the E12 and E47 proteins by Western blotting (Figure 2A). E12 and E47 were present at very low levels in naive mature B cells (Figure 2A, lane 1). Upon activation with LPS, both proteins were significantly induced, consistent with the data described above (Figure 2A, lane 6). Interestingly, treatment with IL-4 or anti-CD40 alone did not affect E2A protein levels, and only a modest increase could be detected upon

stimulation with anti-IgM alone (Figure 2A, lanes 3–5). In contrast, activation through ligation of CD40, CD38 or surface IgM in conjunction with IL-4 strongly induced E2A protein levels (Figure 2A, lanes 7–9).

To determine if E2A DNA binding was induced, nuclear extracts were analyzed by electrophoretic mobility shift assay (EMSA), using the μ E5 or octamer sites present in the IgH intronic enhancer as probes (Figure 2B). Octamer DNA binding was not altered upon mitogen stimulation (data not shown). E2A DNA-binding activity could be detected, albeit at low levels, in unstimulated B cells (Figure 2B, lane 1). Stimulation with either IL-4, anti-IgM or anti-CD40 alone did not increase E2A binding activity (Figure 2B, lanes 3–5). However, consistent with the observed E2A protein levels, the combinations of IL-4 with anti-IgM, anti-CD40 or anti-CD38 strongly induced E2A DNA binding, as did treatment with LPS in the presence or absence of IL-4 (Figure 2B, lanes 6–10).

In mature B-cell lines, the predominant E-box-binding species is comprised of E47 homodimers (Murre *et al.*, 1991; Bain *et al.*, 1993; Shen and Kadesch, 1995). In contrast, E12 has been shown to bind poorly as a homodimer due to an inhibitory domain N-terminal to its basic region (Sun and Baltimore, 1991). Since both E12 and E47 were up-regulated upon B-cell activation, we examined which proteins were binding in the induced complexes. Nuclear extracts were incubated with monoclonal antibodies specific for the class I HLH members prior to EMSA analysis. Addition of antibodies specific for E12 did not interfere with DNA-binding activity (Figure 2C, compare lanes 1–5 with lanes 6–10). In

Fig. 2. E2A protein levels and DNA-binding activity are induced in activated primary B lymphocytes. (A) Western blot analysis showing E12 and E47 protein levels in nuclear extracts derived from naive and activated B-lineage cells. Nuclear extracts were prepared from primary B-cell cultures stimulated for 48 h with the indicated mitogens. (B) E2A DNA-binding activity in naive and activated B cells. Nuclear extracts derived from naive and activated B cells were analyzed by EMSA using the μ E5 site as a probe. NS refers to a non-specific complex. (C) E47 DNA-binding activity is induced in activated B lymphocytes. Nuclear extracts were analyzed by EMSA analysis using the μ E5 site as a probe in the presence or absence of monoclonal antibodies with specificity for either E12 or E47. (D) Purified populations of Th1 and Th2 effector T cells were generated from CD4⁺ T cells and analyzed for cytokine expression. (E) Western blot analysis of E47 and E12 expression levels in nuclear extracts derived from naive B lymphocytes (lanes 1 and 3) and B cells activated with either Th1 (lane 4) or Th2 (lane 5) effector cells in the presence of specific antigen. Nuclear extract from LPS-activated B cells is included as a control (lane 2).

contrast, antibodies with specificity for E47 completely disrupted binding activity in each of the complexes, indicating the presence of E47 (Figure 2C, compare lanes 1–5 with lanes 11–15). Furthermore, incubation with an antibody with specificity for the E proteins E12, HEB and E2-2, but not E47, confirmed that E47 was binding as a homodimer in activated B cells (data not shown). These results indicate that E2A lies downstream of multiple B-cell activation signaling pathways, which synergize to induce E2A protein levels and DNA-binding activity.

Activation through cognate B–T cell interactions induces E2A expression in primary B lymphocytes

To examine whether T lymphocytes have the ability to signal B cells to induce E2A expression, naive B cells were activated by co-culturing directly with purified polarized populations of CD4⁺ effector T helper cells (Th1 or Th2 effectors) in the presence of specific peptide antigen

(PCCF). To establish these populations, naive CD4⁺ T cells were isolated from AND TCR transgenic mice, and cultured with peptide and antigen-presenting cells (APCs) along with various cytokines and cytokine antagonists to direct polarization to either Th1 or Th2 effector T-cell populations (Swain, 1994). Proficient polarization was achieved; Th1 effectors secreted exclusively interferon- γ (IFN- γ) and IL-2, whereas Th2 effectors secreted IL-4 and IL-5 (Swain, 1994) (Figure 2D). Furthermore, to provide physiological signaling through the B-cell receptor, HEL Tg naive B cells and HEL protein were used for the B–T cell cultures. Nuclear extracts were isolated from naive and activated B cells and analyzed for E12 and E47 protein levels. Both Th1- and Th2-activated B cells induced E2A protein levels (Figure 2E). Western blots using specific antibodies showed that both gene products, E12 and E47, were induced at levels similar to those observed with LPS stimulation (Figure 2E;

Fig. 3. Kinetics of E2A DNA-binding activity and protein expression. Nuclear extracts and RNA were prepared from activated B lymphocytes at the indicated times. (A) EMSA analysis of nuclear extracts using the μ E5 site as a probe. The complex containing E2A DNA-binding activity is indicated. (B) Western blot analysis of E47 expression from nuclear extracts as described in (A). (C) Northern blot analysis showing the kinetics of E2A mRNA expression. The blot was reprobed for the ribosomal gene L32. E2A mRNA levels were quantitated and normalized to L32. Relative E2A mRNA levels at each time point are indicated.

compare lane 2 with lanes 4 and 5). These data demonstrate that both Th1 and Th2 effector cells have the ability to induce E2A expression during cognate B–T cell interactions. Furthermore, these data suggest that E2A induction during a T-dependent response does not require IL-4 signaling exclusively, but can be mediated by other cytokines such as IFN- γ and IL-2.

Kinetics of E2A induction

To determine the kinetics of E2A induction during B-cell activation, unprimed splenic B cells were activated with anti-IgM plus IL-4, and nuclear extracts and RNA were prepared at different times ranging from 0 to 48 h post-stimulation. E2A DNA-binding activity was induced by 24 h post-activation and increased further by 48 h post-stimulation (Figure 3A). Additional samples prepared at 72 and 96 h post-activation revealed that E2A DNA-binding activity peaked at 48 h and remained elevated in the presence of mitogens (data not shown). Western blot analysis of nuclear extracts revealed a corresponding increase in E47 protein levels at 24 and 48 h post-activation (Figure 3B). These data suggest that E2A DNA-binding activity correlates with accumulated E47 protein levels. To examine whether this increase was due to increases in E2A mRNA transcription, RNA from each

of these time points was analyzed by Northern blotting (Figure 3C). Interestingly, E2A mRNA levels remained relatively constant, with a nominal increase of 1.4-fold by 48 h (Figure 3C). In addition, analysis of RNA from cultures activated with the various mitogens, presented in Figure 2, also did not show increased levels of E2A transcripts (data not shown). Thus, E2A is regulated post-transcriptionally during B-cell activation, resulting in increased DNA-binding activity.

Ectopic expression of Id3 inhibits E2A DNA-binding activity in primary activated B cells

The data described above demonstrate that E2A is a common downstream target of activated B-cell signaling pathways, consistent with the presence of high levels of E2A expression in germinal centers. These data raise the question of the nature of the role of the E2A proteins in activated mature B cells. B lymphocytes in E2A null mutant mice are arrested early in development prior to the development of mature B-lineage cells. Therefore, in order to address the role of E2A in activated B cells, we utilized retrovirus-mediated gene transfer to ectopically express Id3, a dominant-negative antagonist of E2A. The full-length cDNA for murine Id3 was subcloned into a retroviral vector directly upstream of an internal ribosome entry site (IRES) followed by the coding sequence for green fluorescent protein (GFP) (Heemskerk *et al.*, 1997) (Figure 4A). The IRES enables both Id3 and GFP to be translated individually from the same bicistronic viral RNA message. Thus Id3 transductants could be monitored by analyzing for GFP expression using flow cytometry. As controls, the full-length cDNA for Id3 in the antisense orientation (AS3) or vector alone was used with equivalent results. For simplicity, only the AS3 results are shown.

Id3 has been shown to block the DNA-binding and transcriptional activity of the E proteins *in vitro* and *in vivo*, by virtue of the fact that it can heterodimerize with E proteins but lacks a DNA-binding domain (Christy *et al.*, 1991; Loveys *et al.*, 1996; Heemskerk *et al.*, 1997). To demonstrate that the forced expression of Id3 indeed functioned as a dominant-negative regulator of E2A during normal B-cell activation, splenic B cells were isolated from wild-type C57BL/6 mice and activated with LPS (see the schematic in Figure 4B). At 24 h post-stimulation, the cells were transduced with retroviral supernatants encoding Id3–GFP or control antisense Id3–GFP and cultured for 2 additional days in the presence of mitogen. Three days post-stimulation, the cultures were sorted for GFP expression by flow cytometry and whole-cell extracts were prepared from each GFP-positive population (Figure 4B). As expected, Western blot analysis showed high levels of induced E47 proteins in both extracts (Figure 4C). EMSA analysis using the μ E5 probe showed strong E2A binding in the extract from control (AS3) transduced cells (Figure 4D). In contrast, E2A DNA binding could not be detected in extracts from Id3-transduced cells (Figure 4D). As a control, equivalent amounts of an upstream stimulating factor (USF)-containing complex are detected in both extracts using the unrelated μ E3 site as a probe (Figure 4D). Thus, retroviral expression of Id3 in activated B cells blocks E2A DNA-binding activity.

Fig. 4. Expression of the dominant-negative inhibitor Id3 disrupts E2A DNA-binding activity in primary activated B cells. **(A)** Schematic representation of the Id3-GFP retroviral construct. The gene for GFP is indicated downstream as a shaded box. An IRES which allows for translation of both genes is indicated. **(B)** Schematic diagram of the protocol used to infect activated B lymphocytes. **(C)** Western blot analysis of E47 protein levels. Splenic B cells were activated by LPS, transduced with either Id3-GFP (Id3) or control antisense Id3-GFP (AS3) and sorted for GFP expression 3 days post-activation. Whole-cell extracts were prepared from the populations and analyzed for the presence of E47. **(D)** Id3 inhibits E2A DNA-binding activity in primary activated B cells. Whole-cell extracts as described in (C) were analyzed by EMSA using the μ E5 and μ E3 sites as probes. The E2A- and USF-containing complexes are indicated. NS refers to a non-specific DNA-binding complex.

Loss of E2A activity does not perturb B-cell growth

To determine whether E2A activity is required for the growth and/or viability of normal B cells during mitogenic activation, cells were stimulated with anti-CD40 plus IL-4, infected with retrovirus and analyzed by flow cytometry 2–5 days post-transduction. Aliquots analyzed each day revealed comparable numbers of live cells from both cultures (Figure 5A). Furthermore, the fraction of GFP-positive cells was similar, peaking at ~45% for both control and Id3-transduced populations (Figure 5A). Thus, expression of Id3 does not inhibit the growth of activated B lymphocytes.

To ascertain further whether expression of Id3 perturbs cell proliferation during B-cell activation, naive B cells were labeled with a Bodipy Red analog of CFSE (BRSE). The fluorescent dye BRSE, like CFSE, attaches to proteins in the cell membrane. Its mean fluorescence decreases ~2-fold with each consecutive cell division, corresponding to each cell retaining one-half of the initial amount of BRSE-labeled membranes. Analysis of control versus Id3-

Fig. 5. Expression of Id3 in activated B lymphocytes does not interfere with cell growth. **(A)** Aliquots taken from activated B-cell cultures infected with Id3 or antisense Id3 virus were analyzed by flow cytometry for viability on the basis of forward side scatter (F/S). The relative number of viable cells is depicted on the left graph. The percentage of GFP-positive cells in each population on days 2–5 is shown in the right panel. **(B)** BRSE-labeled naive B cells were activated with anti-CD40 plus IL-4, transduced with either control or Id3 virus and analyzed by flow cytometry. Shown on the left are GFP and BRSE fluorescence from control and Id3-transduced cultures analyzed during days 0–5 post-infection. Viable cells were gated by F/S scatter. In the right panels, histograms are shown indicating the relative BRSE fluorescence in the GFP-positive populations. The progression of cell division is represented by the sequential 2-fold reduction in the mean BRSE fluorescence. Vertical dashed gray lines indicate the positions of each consecutive cell division. Control antisense Id3-GFP+ (AS3) = shaded peaks, Id3-GFP+ (Id3) = solid dark line; the peak indicated by the dotted line represents the mean BRSE fluorescence from day 0 labeled cells.

transduced activated cultures revealed that Id3-expressing cells underwent normal successive cell divisions (Figure 5B). Furthermore, comparison of control versus Id3-expressing cells revealed that both populations underwent the same number of divisions (4–7 cell divisions) in a temporally identical manner and with strikingly similar proportions (Figure 5B). These data demonstrate that the loss of E2A activity through the ectopic expression of Id3 does not perturb activated B-cell growth.

Fig. 6. Analysis of early and late markers in control and Id3-expressing activated B lymphocytes. Primary B cells were stimulated with anti-CD40 plus IL-4 and transduced with either control or Id3-GFP virus. Cells were stained on day 5 with the following antibodies: CD69-PE, CD44-PE, B7.2-PE, IgD-PE, B220-PE, IgM-PE or isotype control-PE and analyzed by flow cytometry for GFP and cell surface marker expression. Cell surface expression levels of naive cells (top row), activated B cells expressing control antisense Id3-GFP+ (AS3) (middle row), and Id3-GFP+ (Id3) expressers (bottom row) analyzed 5 days post-infection are shown. The open histogram represents isotype control. The shaded histogram indicates specific staining.

Expression of early and late markers in activated B cells expressing the E2A antagonist Id3

To determine whether the absence of E2A activity leads to abnormalities in B-cell activation, control and Id3 transductants were analyzed for the expression of both early and late activation markers. Splenic B cells were isolated from wild-type C57BL/6 mice and cultured with anti-CD40 plus IL-4. At 24 h post-stimulation, the cultures were transduced and cultured for 4 additional days in the presence of mitogen (see the schematic in Figure 4B). At 5 days post-activation, the cultures were analyzed by flow cytometry for GFP expression and for the presence of early activation markers, including CD69, B7.2 and CD44. B cells activated with anti-CD40 plus IL-4 and transduced with antisense Id3-GFP virus showed normal induction of CD69, CD44 and B7.2 expression (Figure 6). Similarly, Id3-expressing cells induced expression of the activation markers CD69, CD44 and B7.2 (Figure 6). Interestingly, Id3 transductants showed higher levels of B7.2 expression, suggesting that E2A may regulate B7.2 gene expression.

Decreased expression of other markers has been associated with changes that occur during the later stages of B-cell activation (Hodgkin *et al.*, 1996; Hasbold *et al.*, 1998). These include the markers IgD, B220 and IgM. By day 5, IgD expression is down-regulated in nearly all activated B cells, B220 expression is lowered in a small subpopulation of cells and IgM expression shifts from high to medium levels (Hodgkin *et al.*, 1996; Hasbold *et al.*, 1998). To examine further whether Id3-expressing cells are blocked during later stages of B-cell activation, we analyzed for the expression of IgD, B220 and IgM. Each of the markers was down-regulated appropriately (Figure 6). We noted, however, that a larger percentage of Id3 transductants had reduced levels of IgM expression. These data suggest that E2A may regulate IgM transcription, consistent with the presence of multiple E2A-binding sites present in the Ig intronic and 3' enhancers (Ephrussi

et al., 1985; Murre *et al.*, 1989a; Pettersson *et al.*, 1990). Taken together, these data indicate that B cells devoid of E2A activity have altered levels of B7.2 and IgM expression, but regulate the early and late activation markers CD69, CD44, IgD and B220 normally.

Ectopic expression of Id3 during B-cell activation perturbs Ig isotype switching

Upon activation, B lymphocytes differentiate to produce alternative secondary Ig isotypes. Ig isotype switching or CSR is achieved by the replacement of the IgM constant region with that of another Ig isotype, through a DNA rearrangement event involving switch regions (S) located 5' to the constant domains (Shimizu and Honjo, 1984). *In vitro*, isotype switching can be induced by activating B-lineage cells with the appropriate combinations of mitogens and cytokines (Snapper and Mond, 1993). For example, the presence of LPS allows switching from IgM to IgG3 and IgG2b (Gronowicz *et al.*, 1979; Bergstedt-Lindqvist *et al.*, 1981). LPS in combination with transforming growth factor- β (TGF- β) promotes switching to IgG2b and IgA (McIntyre *et al.*, 1993). Alternatively, the presence of IL-4 in conjunction with either LPS or anti-CD40 allows isotype switch recombination to IgG1 and to a lesser extent to IgE (Snapper and Paul, 1987; Maliszewski *et al.*, 1993).

To examine a potential role for E2A in promoting CSR, Id3-transduced populations were analyzed for their ability to promote isotype switching. B lymphocytes were cultured with anti-CD40 plus IL-4, LPS plus TGF- β or LPS alone and analyzed 5 days post-stimulation for cell surface expression of IgG1, IgG2b and IgG3, respectively (Figure 7A and B).

GFP/IgG1-double-positive cells from control populations were readily detectable in B cells activated with anti-CD40 and IL-4 (Figure 7A and B, top panels). In contrast, cells expressing Id3 showed significantly reduced

Fig. 7. Enforced expression of the E2A antagonist protein Id3 inhibits Ig isotype switching. (A) B-lineage cells were activated with the indicated mitogens and transduced with either Id3-GFP or control antisense Id3-GFP virus. At 5 days post-activation, cells were stained with biotinylated antibodies against IgG1 (anti-CD40 plus IL-4), IgG2b (LPS plus TGF- β) or IgG3 (LPS), followed by staining with streptavidin-PE, and analyzed by flow cytometry for GFP and Ig isotype expression. Percentages of the viable populations in each quadrant are shown. (B) Histogram representation of the data shown in (A) for the GFP-positive populations.

numbers of surface IgG1-positive cells (compare 25% IgG1+ for the antisense with 1.6% for Id3; Figure 7A and B, top panels). Cultures activated with either LPS plus TGF- β or LPS alone showed that Id3 perturbed switching to the IgG2b and IgG3 isotypes as well (Figure 7A and B, middle and lower panels). IgG2b-positive cells were reduced from 12% in the control to 4% in cells that express Id3. Likewise, switching to IgG3 was reduced from 6.6 to 1.1% (Figure 7A and B, middle and lower panels). At least three independent experiments were performed for each isotype with similar results (data not shown). Furthermore, the inhibition of isotype switching was independent of the mitogens that were used. For example, cultures activated with LPS plus IL-4, which also promotes switching to IgG1, displayed similar disruption of isotype switching in Id3 transductants (data not shown). In addition, to eliminate the possibility that the inhibition of isotype switching was due to Id3 protein expression rather than to its E2A antagonistic properties, we ectopically expressed Id2 in activated B cells. Like Id3, Id2 can antagonize E2A activity via its HLH domain which lacks a basic region; however, outside of the HLH region, the two are highly divergent (Riechmann *et al.*, 1994). As expected, isotype switching was inhibited similarly in Id2 transductants, suggesting that the block in isotype switching is due to the perturbation of E2A activity (data not shown). Taken together, these data suggest an essential role for E2A in promoting immunoglobulin isotype switching.

Class switch DNA rearrangements are perturbed in activated B cells expressing the HLH inhibitor protein Id3

The data described above indicate that activated B cells expressing Id3 are unable to express secondary Ig isotypes.

To examine further how class switching is perturbed, we assayed for S μ -S γ 1 DNA rearrangements (Figure 8A). CSR events do not occur at sequence-specific sites, but are distributed throughout the switch regions (which vary from 1 to 10 kb in length). To assay for CSR, we used a recently described strategy designated as digestion-circularization PCR (DC-PCR) (Figure 8A) (Chu *et al.*, 1992, 1993). DC-PCR allows for the direct measurement of all S μ -S γ 1 rearrangements by detection of a uniform length product regardless of the location of the breakpoints (Figure 8A).

Transduced activated B cells were sorted for GFP expression. Genomic DNA was then prepared from the sorted populations and analyzed by DC-PCR. Five-fold dilutions made of each DNA sample, derived from two independent experiments, were amplified (Figure 8B). In addition, to check for the integrity of the DNAs, the non-rearranging n-acetylcholine receptor (nAChR) locus was used as a control (Figure 8). S μ -S γ 1-specific joints were present at significant levels in DNA isolated from control GFP-positive cells (Figure 8B, lanes 4, 5, 10 and 11). In contrast, B cells expressing Id3 showed significantly reduced levels of the recombination joints (Figure 8B; compare lanes 4 and 5 with lanes 7 and 8; and lanes 10 and 11 with lanes 13 and 14). Thus, ectopic expression of Id3 in activated B cells inhibits CSR.

Ig isotype switching is preceded by the presence of germline transcripts originating from the targeted locus (Figure 8C). To investigate whether early events during CSR are perturbed in cells lacking E2A activity, we assayed for the presence of sterile I γ 1 or I γ 3 transcripts. RNA was prepared from sorted populations and analyzed by RT-PCR for the presence of germline transcripts (Figure 8D). Germline transcripts were readily detectable in control-infected activated B cells (Figure 8D, lanes 2

Fig. 8. Analysis of Sμ-Sγ1 DNA rearrangements and germline transcripts in Id3-transduced activated B cells. **(A)** Diagram of the digestion-circularization PCR (DC-PCR) assay for detecting Sμ-Sγ1 DNA rearrangements from genomic DNA. **(B)** Primary B cells were activated using anti-CD40 plus IL-4 and transduced with either control antisense Id3 (AS3) or Id3 (Id3) virus. Genomic DNA was isolated from cells sorted for GFP expression 5 days post-activation and analyzed by DC-PCR. DNA from naive and anti-CD40 plus IL-4-activated B cells were included as negative and positive controls, respectively. Five-fold dilutions of each digested/ligated DNA sample were amplified. The Sμ-Sγ1 and control nAChR products are indicated. **(C)** Schematic diagram of the germline Igγ1 locus. Sterile germline transcripts detected prior to isotype switching are depicted. **(D)** B cells were activated with anti-CD40 plus IL-4 or with LPS alone and transduced with either Id3-GFP (Id3) or control antisense Id3-GFP (AS3) virus. At 3 days post-stimulation, RNA was derived from cells which were sorted for GFP expression. RNA from naive B cells was included as a negative control for Iγ1 and Iγ3 germline transcripts. Germline Iγ1 and Iγ3 transcripts were detected by semi-quantitative RT-PCR followed by Southern blot using Iγ1- or Iγ3-specific internal probes. B cell-specific Igβ mRNA (B29) was used to normalize each RT-PCR. Under the experimental conditions used, signal intensity was proportional to the amount of input RNA.

and 5). Interestingly, Id3-expressing cells also displayed clear induction of these transcripts, albeit at reduced levels (Figure 8D, lanes 3 and 6). These data suggest that germline transcription is not sufficient to promote isotype switching and that E2A plays a fundamental role downstream of germline transcription but prior to switch rearrangements. In summary, B-lineage cells that lack E2A DNA-binding activity have the ability to induce germline transcription but are deficient in their ability to promote CSR.

Discussion

Previous data have demonstrated that the E2A proteins are required during the early stages of B-lineage development (Bain *et al.*, 1994; Zhuang *et al.*, 1994). E12 and E47 have been shown to regulate the expression of B-lineage genes, including λ5, EBF, TdT and RAG-1 (Schlissel *et al.*, 1991; Choi *et al.*, 1996; Bain *et al.*, 1997; Sigvardsson *et al.*, 1997; Kee and Murre, 1998). In addition, E2A

has been implicated in the regulation of Ig rearrangements in cell lines (Schlissel *et al.*, 1991; Goldfarb *et al.*, 1996). The data presented here suggest a novel role for E2A during peripheral B-cell differentiation. We demonstrate that induction of the E2A proteins occurs in response to B-cell activation and that B lymphocytes lacking E2A activity are disrupted in Ig CSR.

E2A is a downstream target during peripheral B-cell activation, and disruption of E2A function perturbs immunoglobulin isotype switching

That the E2A proteins play a role in the B lymphocyte immune response is clear. We demonstrate here that cognate B-T cell interactions in the presence of specific peptide lead to strong induction of E2A protein levels. Examination of individual mitogenic signals *in vitro* reveals that weak mitogenic stimuli such as IL-4, anti-IgM or anti-CD40 alone are insufficient to induce high levels of E2A expression. In contrast, we show further that treatment with combinations of these stimuli, which

are known to elicit strong B-cell activation responses, results in the synergistic induction of E2A protein levels and DNA-binding activity. These data are consistent with E2A protein expression being highest in the dark zones of germinal centers. The highly proliferative dark zones are where Ig isotype switching occurs and where combined signaling mediated by engagement of sIg, CD40 and cytokine receptors is required. Interestingly, neither E2A protein levels nor DNA-binding activity are induced during peripheral T-lymphocyte activation (data not shown). Thus the induction of E2A in response to activation signaling is specific to the B-cell lineage and is consistent with its role in B-lineage-specific events such as CSR.

The data presented here also suggest an essential role for the E2A proteins in regulating Ig isotype switching. Expression of the E2A antagonist protein, Id3, in activated B cells interfered with IgG1, IgG2b and IgG3 isotype class switching. Consistent with these results, it has been reported that overexpression of Id1 in a cell line undergoing spontaneous Ig heavy chain class switching inhibited cell surface expression of IgA (Goldfarb *et al.*, 1996). It is conceivable that E2A is required for switching to all isotypes; however, this remains unclear as we were unable to achieve high levels of switching to other isotypes using this assay.

Although it is plausible that the block in isotype switching is unrelated to the observed disruption of E2A activity in our activated B cells, we feel this is highly unlikely. First, ectopic expression of two independent E2A antagonists gave the same phenotypic inhibition of isotype switching. Secondly, although the Id proteins potentially can target the other E-box-binding proteins, HEB and E2-2, the active E-box-binding complex in mature B-lineage cells has been shown to consist of an E47 homodimer (Murre *et al.*, 1991; Bain *et al.*, 1993; Shen and Kadesch, 1995). Consistent with this, we demonstrate here that activation of primary B lymphocytes induces an E47 homodimer complex. Thirdly, HEB and E2-2 are not expressed in human germinal center cells, the site of isotype switching, whereas the E2A proteins are highly expressed (Goldfarb *et al.*, 1996). However, we cannot rule out the possibility that the Id proteins are acting on an as yet unidentified E protein which fails to bind to our E-box site.

Potential mechanism for E2A in isotype switching

In response to mitogenic stimulation, Id3-transduced cells proliferate normally and express many of the markers characteristic of B-cell activation. These data show that B-cell activation can proceed in the absence of E2A DNA-binding activity but that CSR recombination is severely perturbed. The data further show that the defect lies at the level of DNA rearrangements, since genomic DNA isolated from Id3-expressing cells lacks significant levels of $\text{S}\mu\text{-S}\gamma 1$ joints. This then raises the question of how E2A regulates the excision/recombination of DNA during isotype switching. One intriguing possibility is that E2A may be involved directly in the induction and/or activation of the switch recombinase. However, these studies await the identification of the switch recombinase.

Another potential mechanism is that the E2A proteins promote accessibility of the various loci to the switch recombination machinery. For example, E2A may induce

germline transcription, thereby promoting accessibility. However, we demonstrate here that significant levels of sterile $\text{I}\gamma 1$ and $\text{I}\gamma 3$ transcripts are present in Id3-transduced cells. In fact, these data confirm that the presence of germline transcripts is not sufficient to allow CSR in activated B-lineage cells and suggest that E2A performs a unique function in CSR (Snapper *et al.*, 1997). We would like to consider an alternative model in which E2A proteins regulate locus accessibility through the recruitment of cofactors which have the ability to acetylate histones. Such a role for E2A has been proposed in various aspects of both Ig and $\gamma\delta\text{TCR}$ site-specific V(D)J recombination (Bain *et al.*, 1999). Recently, both biochemical and genetic evidence have shown that E2A directly associates with the transcriptional co-activator complex, designated SAGA (Massari *et al.*, 1999). The SAGA complex contains histone acetyltransferase activity and has been implicated directly in chromatin remodeling, through histone modification (Grant *et al.*, 1997, 1998).

Nucleoprotein complexes have been reported which bind to sites present in all four Ig γ switch regions in locations where switch recombination break points are clustered (Kenter *et al.*, 1993). One such complex, the SNAP complex, has been shown to contain E47 or a related polypeptide (Ma *et al.*, 1997). From these data, we would like to propose a model in which E2A proteins regulate accessibility to specific loci, during CSR and V(D)J rearrangement, by recruitment of co-activators that have the ability to modulate chromatin structure.

Materials and methods

Isolation and activation of naive splenic B cells

Primary mature B lymphocytes were purified and cultured as previously described (Croft and Swain, 1991). Briefly, spleens were isolated from wild-type C57BL/6 mice (Jackson Labs) into Hank's balanced salt solution (HBSS) with 2% fetal calf serum (FCS), gently teased apart and the debris allowed to settle out on ice for 5 min. Cells were washed once with HBSS (2% FCS) and the T cells were removed by complement lysis using anti-CD4-, anti-CD8- and anti-Thy1.2-specific antibodies. Following complement lysis, the cells were washed once with HBSS (2% FCS) followed by two more washes with complete RPMI medium (RPMI 1640 plus 200 U/ml penicillin, 200 $\mu\text{g}/\text{ml}$ streptomycin, 4 mM L-glutamine, 10 mM HEPES pH 7.3 and 50 μM β -mercaptoethanol). Purity was analyzed by flow cytometry and was routinely >90% B220+. Cells were stimulated in complete medium at 1×10^6 cells/ml with the indicated stimuli at the following final concentrations: 2.5% IL-4; 10 $\mu\text{g}/\text{ml}$ anti-IgM [F(Ab')₂ fragment to mouse IgM; Cappel]; 2.5 $\mu\text{g}/\text{ml}$ anti-CD40 (gift from M.Howard); 25 $\mu\text{g}/\text{ml}$ LPS (Sigma); 2.5 $\mu\text{g}/\text{ml}$ anti-CD38 (gift from M.Howard); and 1 ng/ml TGF- β . Cells were harvested at 48 h post-stimulation or at the times indicated, washed and used for immunofluorescence, nuclear extract preparation or RNA preparation.

Immunofluorescence

In order to avoid cross-reactivity between the secondary antibody and endogenous murine immunoglobulins, the monoclonal murine anti-E47 antibody (32.1) was biotinylated (as per the manufacturer's instructions; Pharmingen). Naive and 48 h LPS-stimulated cells were spun down and washed in cold $1 \times$ phosphate-buffered saline (PBS). The following steps were performed at room temperature. Cells were allowed to settle on polylysine- (Sigma) coated coverslips for 10 min, washed once with $1 \times$ PBS and then fixed in 4% formaldehyde (diluted in $1 \times$ PBS from a 16% MeOH-free stock; Polysciences, Inc., catalog no. 18814) for 10 min. Cells were then permeabilized with 100% methanol for 2 min followed by four washes with SolnA [0.1% bovine serum albumin (BSA) in $1 \times$ PBS + 0.02% sodium azide] and one wash with 10% normal goat serum in SolnA (NGS/SolnA). Coverslips were incubated with the primary antibody in NGS/SolnA for 1 h followed by four washes with SolnA followed by incubation with secondary antibody streptavidin-

fluorescein isothiocyanate (FITC) (Pharmingen) at 10 µg/ml in NGS/SolnA for 1 h. Cells were washed four times with SolnA then mounted on slides in mount [containing 90% glycerol and two crystals of P-phenylene-diamine (PPD) (Sigma, catalog no. P-6001) per 10 ml mount and 0.5 µg/ml Hoechst DNA dye to visualize nuclear staining], sealed with nail varnish and stored at -20°C or analyzed directly by microscopy.

Activation of B cells by Th1 and Th2 T cells

Polarized Th1 and Th2 effector T cell populations were generated from AND (pigeon cytochrome *c*-specific TCR transgenic) mice as previously described (Swain, 1994). Naive, resting B cells (purified by antibody + complement lysis followed by collection of the low density fraction from Percoll gradients) from MD4 Ig BCR Tg mice (HEL-specific) were isolated and cultured (5×10^5 /ml) with mitomycin C-treated Th1 or Th2 effector cells at a 1:1 ratio in T75 flasks. For mitomycin C treatment, T cells were treated with a 1/10 dilution in a 1 ml volume for 20 min at 37°C. The T cells were then washed three times in complete medium and added to flasks containing the B cells plus Ag (Ag = PCCF, i.e. pigeon cytochrome *c* peptide at 5 µM and HEL at 10 µg/ml). Cells were incubated for 3 days, at which time >95% of the cells were CD19⁺ and the B cells were highly activated as determined by flow cytometry. The cytokine expression assay for determining the efficiency of Th1 and Th2 polarization was described previously (Swain, 1994). Here 1 U of IL-4 is equivalent to 1 pg and 1 U of IFN-γ is equivalent to 1 ng.

Western blot analysis

Nuclear extract (12.5 µg) or 20 µg of whole-cell extract was resolved on 8% SDS-polyacrylamide gels and transferred to Immobilon membrane (Millipore) as previously described (Bain *et al.*, 1997). The anti-E47 antibody (32.1, Pharmingen) was used at a dilution of 1:250 (~3 µg/ml), and the anti-E12 antibody (382.6, Pharmingen) at a dilution of 1:500 (~1.5 µg/ml) followed by visualization using a goat anti-mouse-horseradish peroxidase (HRP) secondary antibody (Gibco-BRL, 1:15 000 dilution) and ECL developing reagents (as per the manufacturer's instructions; Amersham).

Electrophoretic mobility shift assay (EMSA)

Nuclear and whole-cell extracts were prepared by a modified (Dignam *et al.*, 1983) protocol as previously described, with the exception that whole-cell extracts were lysed directly in solution C containing 1% NP-40 (Bain *et al.*, 1993). Nuclear extract (5–10 µg) or whole-cell extract (10–20 µg) was incubated with ³²P-labeled oligonucleotides containing the wild-type monomeric µE5 E2-box sequence, the µE3 sequence or the octamer sequence from the immunoglobulin heavy chain enhancer. In cases where antibodies were added, extracts were pre-incubated with the indicated antibody (anti-E12, 34.1 or anti-E47, 32.1 antibodies; Pharmingen) for 10 min at room temperature prior to incubation with the probe.

Northern blot analysis

Total RNA was isolated from naive and activated B cells via Trizol (as per the manufacturer's instructions; Gibco-BRL) or by RNeasy (as per the manufacturer's instructions; Qiagen). Northern blots and hybridization were performed as described (Maniatis *et al.*, 1982). Probes were 300 bp–1 kb fragments derived from the cDNA clones of the murine Id3 (from ATCC), E2A and L32 genes. The ribosomal gene L32 was used as a control for total RNA loading.

Generation of viral supernatants and transduction of primary B lymphocytes

The full-length cDNA for murine Id3 was cloned in both orientations into the polylinker of retroviral vector LZRSpBMN-linker-IRES-EGFP (Nol1-) (S-003) (Heemskerk *et al.*, 1997) obtained from Hergen Spits (The Netherlands Cancer Institute). Both the Id3 and antisense Id3 constructs were then transfected into the ΦNX-eco packaging line (Kinsella and Nolan, 1996) obtained from Garry Nolan (Stanford University) by calcium phosphate precipitation in complete Dulbecco's modified Eagle's medium (DMEM; DME high glucose, 10% FCS, L-glutamine, penicillin and streptomycin). At 24 h post-transfection, the cells were washed once with and changed to complete RPMI medium. Supernatants were harvested after an additional 24 and 48 h, with fresh complete RPMI medium being added after the first viral supernatant harvest. Viral supernatants were stored in aliquots at -80°C.

Primary B cells were isolated and activated with the indicated mitogens as described above. At 24 h post-stimulation, the appropriate viral supernatants were thawed and incubated on ice for 10 min in the

presence of 10 µg/ml DOTAP (Boehringer Mannheim). The B cells were counted, spun down then resuspended in the viral supernatant + DOTAP at a concentration of $0.5\text{--}1 \times 10^6$ cells/ml of supernatant-DOTAP mixture. Cells were placed in 6- or 24-well tissue culture plates and spun at 2500 r.p.m. (927 g) for 90 min at 32°C in a Sorvall RT 6000D centrifuge using a H1000B rotor. Afterwards, half of the supernatant was removed and fresh complete RPMI medium plus fresh mitogens (to give a final concentration of 1×) were added and the cells returned to the incubator (37°C 5% CO₂) for an additional 1–4 days.

BRSE labeling of primary B cells

The Bodipy Red succinimidyl ester dye (referred to here as BRSE) was purchased from Molecular Probes [catalog no. D2219, chemical name: 4,4-difluoro-5-(2-thienyl)-4-bora-3a,4-adiazas-indacene-3-propionic acid, succinimidyl ester]. BRSE is analogous to the more commonly used CFSE membrane dye, but fluoresces in the FL2 channel allowing for the simultaneous monitoring of GFP-positive (FL1 channel) and BRSE fluorescence. Splenic B cells were purified as described above, washed once with 1× PBS and labeled in 1× PBS containing 2 µM BRSE at 37°C for 15 min at a cell concentration of 1×10^7 cells/ml. The BRSE-labeled cells were subsequently washed twice with complete RPMI medium then stimulated in culture as described above with anti-CD40 plus IL-4.

Flow cytometric analysis

To analyze for the expression of surface Ig antibody isotypes and cell surface molecules, cells were stained and analyzed using a FACscan (Becton-Dickinson Immunocytometry Systems, San Jose, CA) as previously described (Bain *et al.*, 1997). Live cells were gated based on forward and side scatter. The following antibodies were used (all from Pharmingen, San Diego, CA): CD16/CD32 (Fc block), anti-CD69-phycoerythrin (PE), anti-CD44-PE, anti-B7.2-PE, anti-IgD-PE, anti-B220-PE, anti-IgM_b-PE, anti-IgG1-biotin, anti-IgG2b-biotin, anti-IgG3-biotin and streptavidin-PE.

Digestion-circularization PCR (DC-PCR) analysis of Sμ-Sγ1 genomic DNA rearrangements

DC-PCR was performed as described in Chu *et al.* (1992, 1993). Briefly, 1–2 µg of genomic DNA was digested overnight with *Eco*RI (Gibco-BRL), incubated at 70°C for 20 min to heat-inactivate the *Eco*RI enzyme and then ligated overnight at 16°C with T4 DNA ligase (NEB 400U/rxn) under dilute conditions (1.8 µg/ml) to promote recircularization. Five ng and subsequent 5-fold dilutions (i.e. 5, 1 and 0.2 ng) of each DNA sample were amplified using µ-, γ1- and nAChR-specific primers. Each reaction contained genomic DNA, 1× PCR buffer (10 mM Tris pH 8.3, 50 mM KCl from Perkin-Elmer), 2 mM MgCl₂, 10 pmol of each primer, 0.5 µl dCTP (5 µCi; ICN 3000 Ci/mmol), 0.2 U of Amplitaq Gold (Perkin Elmer) and 200 µM dNTPs in a 20 µl volume. Amplification was as follows: one cycle at 95°C for 7 min; five cycles at 95°C for 15 s, 65°C for 1.5 min and 72°C for 1 min; 35 cycles at 95°C for 15 s, 68°C for 1.5 min and 72°C for 1 min; and a final cycle at 72°C for 10 min. PCR products were analyzed by standard polyacrylamide gel electrophoresis (8%) and visualized by autoradiography. Primer sequences for Sμ-Sγ1 were S3, 5'-GGAGACC-AATAATCAGAGGGAAG-3'; and S4, 5'-GAGAGCAGGGTCTCC-TGGGTAGG-3'. These are the same S3 and S4 primers described in Casellas *et al.* (1998), which are also identical to those described in Chu *et al.* (1992) called S1 and S2 with the exclusion of the 5' restriction sites. nAChR primers were A3, 5'-GGACTGCTGTGGGTTT-CACCCAG-3'; and A4, 5'-GCCTTGCTTGCTTAAGACCCTGG-3'. These are the same as the A3 and A4 primers described in Casellas *et al.* (1998), which are also identical to those described in Chu *et al.* (1992) called A1 and A2 with the exclusion of the 5' restriction sites. A [³²P]ATP-labeled 1 kb ladder (as per the manufacturer's instructions; Gibco-BRL) was used to verify the correct molecular weight of the generated products.

Semi-quantitative RT-PCR for detecting Ig switch germline Iγ1 and Iγ3 transcripts

Primary B cells were activated and infected with Id3 and control virus as described above. At 3 days post-activation, cultures were sorted based on GFP-positive expression and total RNA was prepared from these populations via RNeasy (as per the manufacturer's instructions; Qiagen). Iγ1 and Iγ3 transcripts were amplified using a coupled RT-PCR assay (Sellner and Turbett, 1998). Briefly, each reaction contained final concentrations of 67 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100, 6 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.1 mM dNTPs,

1 mM MgCl₂, 50 ng of each primer and 0.5 U of AMV reverse transcriptase (Promega) in a final volume of 25 µl. Coupled RT-PCR conditions were as follows: one cycle at 42°C for 30 min; one cycle at 95°C for 5 min; 18 cycles (17 for B29) of 95°C for 45 s, 55°C for 45 s and 72°C for 75 s; with a final extension step of 10 min at 72°C. Primer sequences are as follows: 5'Iy1, 5'-CAGCCTGGTGTCACTAG-3'; 3'Iy1, 5'-GCAAGGGATCCAGATTCCAG-3'; 5'Iy3, 5'-CAAGTG-GATCTGAACACA-3'; 3'Iy3, 5'-GGCTCCATAGTTCATT-3'; 5'B29, 5'-CTGGTGCTGTCTTCCATGC-3'; and 3'B29, 5'-GATGAGGAGGG-TCTGGATCA-3'. PCR products were separated on 2% agarose gels, transferred to a nylon membrane and analyzed by Southern blotting using Iy1- and Iy3-specific internal probes. Iy1 and Iy3 Southern probes were generated by coupled RT-PCR using internal 5' primers coupled with the appropriate 3' primer above; sequences are: inner5'Iy1, 5'-ATGTAGAGCTGCACACCCCA-3'; and inner5'Iy3, 5'-GATA-TATCAGGATACCT-3'.

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References

- Aronheim, A., Shiran, R., Rosen, A. and Walker, M.D. (1993) The E2A gene product contains two separable and functionally distinct transcription activation domains. *Proc. Natl Acad. Sci. USA*, **90**, 8063–8067.
- Bain, G., Gruenwald, S. and Murre, C. (1993) E2A and E2-2 are subunits of B-cell-specific E2-box DNA-binding proteins. *Mol. Cell. Biol.*, **13**, 3522–3529.
- Bain, G. *et al.* (1994) E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell*, **79**, 885–892.
- Bain, G., Maandag, E.R., ten Riele, H., Feeney, A., Sheehy, A., Schlissel, M., Shinton, S., Hardy, R. and Murre, C. (1997) Both E12 and E47 allow commitment to the B cell lineage. *Immunity*, **6**, 145–154.
- Bain, G., Romanow, W.J., Albers, K., Havran, W.L. and Murre, C. (1999) Positive and negative regulation of V(D)J recombination by the E2A proteins. *J. Exp. Med.*, **189**, 289–300.
- Bergstedt-Lindqvist, S., Fernandez, C. and Severinson, E. (1981) A synergistic polyclonal response to dextran sulphate and lipopolysaccharide: immunoglobulin secretion and cell requirements. *Scand. J. Immunol.*, **15**, 439–448.
- Casellas, R. *et al.* (1998) Ku80 is required for immunoglobulin isotype switching. *EMBO J.*, **17**, 2404–2411.
- Choi, J., Shen, C.-P., Radomska, H., Eckhardt, L. and Kadesch, T. (1996) E47 activates the Ig-heavy chain and TdT loci in non-B cells. *EMBO J.*, **15**, 5014–5021.
- Christy, B., Sanders, L., Lau, L., Copeland, N., Jenkins, N. and Nathans, D. (1991) An Id-related helix–loop–helix protein encoded by a growth factor-inducible gene. *Proc. Natl Acad. Sci. USA*, **88**, 1815–1819.
- Chu, C.C., Paul, W.E. and Max, E.E. (1992) Quantitation of immunoglobulin µγ1 heavy chain switch region recombination by a digestion-circularization polymerase chain reaction method. *Proc. Natl Acad. Sci. USA*, **89**, 6978–6982.
- Chu, C.C., Max, E.E. and Paul, W.E. (1993) DNA rearrangement can account for *in vitro* switching to IgG1. *J. Exp. Med.*, **178**, 1381–1390.
- Church, G.M., Ephrussi, A., Gilbert, W. and Tonegawa, S. (1985) Cell type specific contacts to immunoglobulin enhancers in nuclei. *Nature*, **313**, 798–801.
- Croft, M. and Swain, S.L. (1991) B cell response to T helper cell subsets. II. Both the stage of T cell differentiation and the cytokines secreted determine the extent and nature of helper activity. *J. Immunol.*, **147**, 3679–3689.
- Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.
- Ephrussi, A., Church, G.M., Tonegawa, S. and Gilbert, W. (1985) B-lineage-specific interactions of an immunoglobulin enhancer with cellular factors *in vivo*. *Science*, **227**, 134–140.
- Goldfarb, A.N., Flores, J.P. and Lewandowska, K. (1996) Involvement of the E2A basic helix–loop–helix protein in immunoglobulin heavy chain class switching. *Mol. Immunol.*, **33**, 947–956.
- Grant, P.A. *et al.* (1997) Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes Dev.*, **11**, 1640–1650.
- Grant, P.A., Sterner, D.E., Duggan, L.J., Workman, J.L. and Berger, S.L. (1998) The SAGA unfolds: convergence of transcription regulators in chromatin-modifying complexes. *Trends Cell Biol.*, **8**, 193–197.
- Gronowicz, E.S., Doss, C., Assisi, F., Vitetta, E.S., Coffman, R.L. and Strober, S. (1979) Surface Ig isotypes on cells responding to lipopolysaccharide by IgM and IgG secretion. *J. Immunol.*, **123**, 2049–2056.
- Hasbold, J., Lyons, A.B., Kehry, M.R. and Hodgkin, P.D. (1998) Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4. *Eur. J. Immunol.*, **28**, 1040–1051.
- Heemskerck, M.H., Blom, B., Nolan, G., Stegmann, A.P., Bakker, A.Q., Weijer, K., Res, P.C. and Spits, H. (1997) Inhibition of T cell and promotion of natural killer cell development by the dominant-negative helix–loop–helix factor Id3. *J. Exp. Med.*, **186**, 1597–1602.
- Hodgkin, P.D., Lee, J.H. and Lyons, A.B. (1996) B cell differentiation and isotype switching is related to division cycle number. *J. Exp. Med.*, **184**, 277–281.
- Jacobs, Y., Vierra, C. and Nelson, C. (1993) E2A expression, nuclear localization and *in vivo* formation of DNA- and non-DNA-binding species during B-cell development. *Mol. Cell. Biol.*, **13**, 7321–7333.
- Kee, B.L. and Murre, C. (1998) Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix–loop–helix transcription factor E12. *J. Exp. Med.*, **188**, 699–713.
- Kenter, A.L., Wuerffel, R., Sen, R., Jamieson, C.E. and Merkulov, G.V. (1993) Switch recombination breakpoints occur at nonrandom positions in the Sg tandem repeat. *J. Immunol.*, **151**, 4718–4731.
- Kinsella, T.M. and Nolan, G.P. (1996) Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum. Gene Ther.*, **7**, 1405–1413.
- Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991) Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins *in vivo*. *Cell*, **66**, 305–315.
- Loveys, D.A., Streiff, M.B. and Kato, G.J. (1996) E2A basic-helix–loop–helix transcription factors are negatively regulated by serum growth factors and by the Id3 protein. *Nucleic Acids Res.*, **24**, 2813–2820.
- Ma, L., Hu, B. and Kenter, A.L. (1997) Ig Sg-specific DNA binding protein SNAP is related to the helix–loop–helix transcription factor E47. *Int. Immunol.*, **9**, 1021–1029.
- MacLennan, I.C. (1994) Germinal centers. *Annu. Rev. Immunol.*, **12**, 117–139.
- Maliszewski, C.R., Grabstein, K., Fanslow, W.C., Armitage, R., Spriggs, M.K. and Sato, T.A. (1993) Recombinant CD40 ligand stimulation of murine B cell growth and differentiation: cooperative effects of cytokines. *Eur. J. Immunol.*, **23**, 1044–1049.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Massari, M., Jennings, P. and Murre, C. (1996) The AD1 transactivation domain of E2A contains a highly conserved helix which is required for its activity in both *Saccharomyces cerevisiae* and mammalian cells. *Mol. Cell. Biol.*, **16**, 121–129.
- Massari, M.E., Grant, P.A., Pray-Grant, M.G., Berger, S.L., Workman, J.L. and Murre, C. (1999) A conserved motif present in a class of helix–loop–helix proteins activates transcription by direct recruitment of the SAGA complex. *Mol. Cell*, **4**, 1–11.
- McIntyre, T.M., Klinman, D.R., Rothman, P., Lugo, M., Dasch, J.R., Mond, J.J. and Snapper, C.M. (1993) Transforming growth factor β1 selectivity stimulates immunoglobulin G2b secretion by lipopolysaccharide-activated murine B cells. *J. Exp. Med.*, **177**, 1031–1037.
- Murre, C., McCaw, P.S. and Baltimore, D. (1989a) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD and myc proteins. *Cell*, **56**, 777–783.
- Murre, C. *et al.* (1989b) Interactions between heterologous helix–loop–helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell*, **58**, 537–544.

- Murre,C., Voronova,A. and Baltimore,D. (1991) B-cell- and myocyte-specific E2-box-binding factors contain E12/E47-like subunits. *Mol. Cell. Biol.*, **11**, 1156–1160.
- Murre,C. *et al.* (1994) Structure and function of helix–loop–helix proteins. *Biochim. Biophys. Acta*, **1218**, 129–135.
- Pettersson,S., Cook,G.P., Bruggemann,M., Williams,G.T. and Neuberger,M.S. (1990) A second B cell-specific enhancer 3' of the immunoglobulin heavy-chain locus. *Nature*, **344**, 165–168.
- Quong,M.W., Massari,M.E., Zwart,R. and Murre,C. (1993) A new transcriptional activation motif restricted to a class of helix–loop–helix proteins is functionally conserved in both yeast and mammalian cells. *Mol. Cell. Biol.*, **13**, 792–800.
- Riechmann,V., Cruchten,I.V. and Sablitzky,F. (1994) The expression pattern of Id4, a novel dominant-negative helix–loop–helix protein, is distinct from Id1, Id2 and Id3. *Nucleic Acids Res.*, **22**, 749–755.
- Roberts,V.J., Steenbergen,R. and Murre,C. (1993) Localization of E2A mRNA expression in developing and adult rat tissues. *Proc. Natl Acad. Sci. USA*, **90**, 7583–7587.
- Rutherford,M.N. and LeBrun,D.P. (1998) Restricted expression of E2A protein in primary human tissues correlates with proliferation and differentiation. *Am. J. Pathol.*, **153**, 165–173.
- Schlissel,M., Voronova,A. and Baltimore,D. (1991) Helix–loop–helix transcription factor-E47 activates germ-line immunoglobulin heavy-chain gene transcription and rearrangement in a pre-T-cell line. *Genes Dev.*, **5**, 1367–1376.
- Sellner,L.N. and Turbett,G.R. (1998) Comparison of three RT–PCR methods. *BioTechniques*, **25**, 230–234.
- Shen,C.P. and Kadesch,T. (1995) B-cell-specific DNA binding by an E47 homodimer. *Mol. Cell. Biol.*, **15**, 4518–4524.
- Shimizu,A. and Honjo,T. (1984) Immunoglobulin class switching. *Cell*, **36**, 801–803.
- Sigvardsson,M., O'Riordan,M. and Grosschedl,R. (1997) EBF and E47 collaborate to induce expression of the endogenous immunoglobulin surrogate light chain genes. *Immunity*, **7**, 25–36.
- Snapper,C.M. and Mond,J.J. (1993) Towards a comprehensive view of immunoglobulin class switching. *Immunol. Today*, **14**, 15–17.
- Snapper,C.M. and Paul,W.E. (1987) B cell stimulatory factor-1 (interleukin 4) prepares resting murine B cells to secrete IgG1 upon subsequent stimulation with bacterial lipopolysaccharide. *J. Immunol.*, **139**, 10–17.
- Snapper,C.M., Marcu,K.B. and Zelazowski,P. (1997) The immunoglobulin class switch: beyond 'accessibility'. *Immunity*, **6**, 217–223.
- Sun,X.-H. and Baltimore,D. (1991) An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. *Cell*, **64**, 459–470.
- Swain,S.L. (1994) Generation and *in vivo* persistence of polarized Th1 and Th2 memory cells. *Immunity*, **1**, 543–552.
- Zhuang,Y., Soriano,P. and Weintraub,H. (1994) The helix–loop–helix gene E2A is required for B cell formation. *Cell*, **79**, 875–884.

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