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E2A Acts in cis in G₁ Phase of Cell Cycle to Promote Ig Gene Diversification

Munehisa Yabuki,*† Ellen C. Ordinario,2* W. Jason Cummings,* Monica M. Fujii,* and Nancy Maizels3*†

Rearranged Ig genes undergo diversification in sequence and structure initiated by the DNA deaminase, activation-induced deaminase. Ig genes must be transcribed for diversification to occur, but whether there are additional requirements for cis activation has not been established. Here we show, by chromatin immunoprecipitation, that the regulatory factor E2A associates with the rearranged Igκ gene in the chicken DT40 B cell line, which performs constitutive Ig gene diversification. By analysis of a DT40 derivative in which polymerized lactose operator tags the rearranged Igκ gene, we show that E2A must function in cis to promote diversification and that stimulation of diversification in cis depends on the E2A activation domains. By direct imaging, we show that λκ/E2A colocalizations are most prominent in G₁. We further show that expression of the E2A antagonist Id1 prevents λκ/E2A colocalizations in G₁ and impairs diversification but not transcription of λκ. Thus, E2A acts in cis to promote Ig gene diversification, and G₁ phase is the critical window for E2A action. The Journal of Immunology, 2009, 182: 408–415.

Three distinct processes diversify DNA sequence and structure of Ig genes that are producing functional Abs, to expand the repertoire and respond dynamically to infection by pathogens (1–4). In Ag-activated mammalian B cells, somatic hypermutation introduces nontemplated point mutations into rearranged and expressed V regions, and class switch recombination (CSR) juxtaposes a new constant region to the expressed V region. In chicken and other fowl, gene conversion expands a limited preimmune repertoire by using upstream pseudo-V(ψV) gene segments as templates for mutagenesis of rearranged and expressed V regions.

The regulated changes in genomic sequence and structure that take place at the Ig loci reflect both targeting of DNA damage to these genes and escape from faithful repair. Somatic hypermutation, CSR, and gene conversion are all initiated by the B cell-specific enzyme, activation-induced deaminase (AID; Refs. 5–8). AID deaminates cytosine to uracil, with clear preference for ssDNA (9–11). Transcription is prerequisite for diversification, which may reflect preference of AID for single-stranded substrates. Uracil in DNA is a common lesion that can be repaired faithfully by highly conserved and efficient pathways (12). However, the Ig loci can escape from faithful repair and undergo repair by error-prone pathways (13).

E2A, a member of the E family of basic-helix-loop-helix proteins, is a critical regulator of many aspects of lymphocyte development (14–19). E proteins dimerize to bind to the E-box motif, CANNTG, and their function is antagonized by Id proteins, which heterodimerize with E proteins to prevent DNA binding. In activated murine B cells, E2A regulates CSR as well as expression of the gene that encodes AID (20–22). There may be functional overlap between E2A and the related HEB and E2-2 proteins, which are also regulated by Id interactions and which may promote CSR in the absence of E2A (18). In chicken B cells, inactivation of the E2A gene impairs Igλ gene diversification but not transcription (23, 24); conversely, ectopic expression of E47 (one of two functionally equivalent isoforms encoded by E2A) promotes Igλ gene diversification but does not affect Igλ transcript levels (25).

The possibility that E2A might regulate Ig gene diversification by binding to sites in cis was first suggested by evidence that multimerized E-boxes stimulate hypermutation but not transcription of an Ig transgene in mice (26). This possibility has been further supported by the demonstration that multimerized E-boxes can promote Ig gene diversification but not transcription in chicken B cells (27). However, clear resolution of the question of whether E2A acts directly at the Ig genes to promote diversification has been difficult, for several reasons. E-boxes function as sites for E2A-dependent regulation only in specific contexts; therefore, the presence of an E-box does not guarantee E2A function at a site. The loose consensus and frequent occurrence of E-box motifs preclude mutational analysis of each individual site. In addition, because at some loci E2A is recruited by protein-protein rather than protein-DNA interaction, an E-box is not always prerequisite for E2A-dependent regulation (28).

We have now established that E2A acts in cis at the Ig genes to promote diversification, in experiments that take advantage of derivatives of the constitutively diversifying chicken B cell line, DT40, in which the rearranged Igλ allele is tagged with polymerized lactose operator (DT40 PolylacO-λκ). By chromatin immunoprecipitation (ChIP), we show that E2A associates with the rearranged but not unarranged Igλ allele in the parental line, DT40.
We demonstrate that in DT40 PolyLacO-\( \lambda _R \) cells, diversification is accelerated upon expression of an E47-lactose repressor (LacI) fusion protein, which explicitly tethers E47 to \( \lambda _R \), and that the stimulatory effect of E47-LacI expression is not evident in cells cultured with isopropyl-\( \beta \)-d-thiogalactopyranoside (IPTG); therefore, binding in \( cis \) is necessary to promote diversification. The activation domains of E47 are required for acceleration of diversification. By direct imaging of the rearranged \( \lambda _R \) gene in DT40 PolyLacO-\( \lambda _R \) GFP-LacI cells, we show that \( \lambda _R/E2A \) co-localizations predominate in \( G_1 \) phase and that expression of the E2A antagonist, Id1, impairs diversification and diminishes \( \lambda _R/E2A \) co-localizations specifically in \( G_1 \) phase but does not affect \( A \) transcript levels or localization of \( \lambda _R \) to active transcription factories. We conclude that E2A acts in \( cis \) in \( G_1 \) phase to promote Ig gene diversification.

Materials and Methods

Cell culture and gene targeting

DT40 and its derivative cell lines were maintained and transfected as described (29, 30). DT40 PolyLacO-\( \lambda _R \) was generated by gene targeting with a construct, pPolyLacO-\( \psi _{\lambda R} \), which carried a 3.8-kb PolyLacO fragment and homology arms designed for insertion between \( \psi _{\lambda 17} \) and \( \psi _{\lambda 20} \). To generate this targeting construct, pbLueprint KS (Stratagene) was engineered to contain two modified loxp recombination sites (7) at the \( XbaI \) and \( BamHi-PstI \) sites; a histidinol selection marker (scloned from a plasmid provided by Dr. Andrew Scharenberg, University of Washington, Seattle, WA) was inserted at the \( BamHi \) site; and the 3.8-kb PolyLacO fragment was subcloned from pAFS95-13 (provided by Dr. Aaron Straight, Stanford University, Stanford, CA) into the EcoRV site. A 2-kb fragment containing \( \psi _{\lambda 20} \) through \( \psi _{\lambda 23} \) and a 3.5-kb fragment containing \( \psi _{\lambda 13} \) through \( \psi _{\lambda 17} \) were amplified from DT40 genomic DNA using DyNAzyme (Finnzymes) and cloned into blunt-ended NotI and Xhol sites, respectively. The construct was verified by restriction analyses and partial sequencing. Plasmids carrying PolyLacO were propagated in recombination-deficient Escherichia coli strains SB122 (Invitrogen) or SURE2 (Strategene) to maintain PolyLacO. The construct was verified by restriction analyses and partial sequencing. Plasmids carrying PolyLacO were propagated in recombination-deficient Escherichia coli strains SB122 (Invitrogen) or SURE2 (Strategene) to maintain PolyLacO.

To insert PolyLacO in the \( \psi _{\lambda R} \) array, wild-type DT40 cells were transfected with the pPolyLacO-\( \psi _{\lambda R} \) construct; candidate clones were screened by Southern blotting; and genomic organization was verified by PCR. The sIgM loss assay was conducted as described (29, 34), and results were compared using the Mann-Whitney \( U \) test with the R software package (http://www.r-project.org). For cell cycle profiles based on DNA content, \( n \) = 100 exponentially growing cells were suspended in 0.1% Triton X-100, treated with 100 \( \mu \)g/ml RNase A and 50 \( \mu \)g/ml propidium iodide (PI), and analyzed as described (29). To assess cell cycle dependence of Id1 levels, DT40 cells were transfected with expression constructs of Id1-GFP or its derivative using a Nucleofector (Amaxa). At 24 h post-transfection, cells were fixed with Cytofix-Cytoperm solution (BD Biosciences) and treated with 100 \( \mu \)g/ml RNase A and 5 \( \mu \)g/ml PI; GFP intensity and DNA content were determined on a FACScan flow cytometer (BD Biosciences).

Expression constructs

The E47-LacI expression construct was generated by subcloning of E47 cDNA from the 5003 E47 plasmid (35; provided by Dr. Cornelis Murre, University of California, San Diego, CA) into the XbaI-BsrGI site of the p33 SS-GFP-LacI plasmid (provided by Dr. Andrew Belmont, University of Illinois, Urbana, IL). Activation domain (AD)-deleted mutants: E47\( A_1 \)LacI lacks residues 2-99; E47\( A_2 \)AD2-LacI lacks residues 325-432; and E47\( A_3 \)AD12-LacI lacks both AD domains. These mutants were made by first generating fragment(s) carrying deletions by PCR amplification and then exchanging these fragments with the corresponding regions of the parental E47-LacI plasmid. A DNA-binding mutant, E47\( R_{338}^W \)LacI, which carries an R338K mutation in the basic region, was made by QuikChange site-directed mutagenesis (Stratagene). The Id1 expression construct (36) was provided by Dr. Barbara Christy (University of Texas, San Antonio, TX). Id1-GFP was constructed by subcloning of Id1 cDNA into the Xhol site of a pEGFP-N1 vector (Clontech); and its derivative Id1\( Fmm \)-GFP, which carries R119G and I122V mutations in its canonical D-box motif (residues 119–126, RcsLexCV), was generated from Id1-GFP by QuikChange mutagenesis.

RT-PCR, Western blotting

For RT-PCR assays, IgA transcript was amplified with primers 5′-GTTCAG CAAACCCAGGAAAGAC-3′ and 5′-AATCCACAGTCTGGCGTCTG-3′; and β-actin were amplified with primers as described (37); E47 and E12 were amplified with published primers (37); HEB was amplified with 5′-TCAATATCGTGAGGGAGGC-3′ and 5′-ACTGGGATCATGGGAAGAGG-3′; and E2- was amplified with 5′-AATGCCTGAGGCTC TCAAA-3′ and 5′-TGGGGAATGTTGGGAAGAGG-3′. For Western blotting, whole-cell lysates (50 \( \mu \)g) were resolved by SDS-PAGE, blotted with anti-Id1 Ab (JC-FL; Santa Cruz Biotechnology), and detected using FluoroChem HD2 (Alpha Innotech).

Immunofluorescence staining and image analysis

To image PolyLacO, DT40 PolyLacO-\( \lambda _R \) cells were transfected with the GFP-LacI expression construct, p33 SS-GFP-LacI (from Dr. Andrew Belmont), which encodes LacI engineered to contain SV40 nuclear localization signals and lacking a sequence necessary for tetramer formation (38); or its derivative, red-fluorescent protein (RFP)-LacI, in which GFP was replaced with RFP (DsRed-monomer; Clontech). For immunostaining, cells (3–10\( \times 10^6 \)) were deposited onto glass slides using Cytospin 3 (800 rpm, 1 min; Shandon), fixed with 2% paraformaldehyde for 20 min, and stained as described previously (29). Primary Abs used were: anti-E2A (ab11787, 1:200; Abcam); anti-Pol II C-terminal domain phosphorylated at Ser5 (ab5131, 1:500; Abcam). Alexa Fluor 488- or -594-conjugated anti-mouse and -rabbit IgG (Molecular Probes) was used as secondary Abs. Fluorescent images were acquired using the DeltaVision deconvolution microscopy system (Applied Precision) and processed and analyzed with softWoRx (Applied Precision) and Imaris softwares (Bitplane). Fraction of colocalization was analyzed with Pearson’s \( \chi^2 \) test. Nuclear radii were calculated as the average of at least two independent measurements of diameter, divided by two. Cell cycle dependence of mean nuclear radius was determined independently for each cell line and proved to be relatively invariable. Standard values used to correlate nuclear radius to cell cycle were: \( G_1, r < 4 \mu m; G_2, r \geq 5.2 \mu m. \)

Results

E2A associates with the rearranged but not unrearranged Ig \( \lambda \) gene

Despite the considerable evidence for the importance of E2A in Ig gene diversification, this factor had not been shown to associate directly with the Ig \( \lambda \) genes. To test association of E2A with Ig\( \lambda \), we used anti-E2A Abs to immunoprecipitate chromatin from the chicken DT40 B cell line. This line was derived from a bursal lymphoma and carries out constitutive diversification of both Ig heavy and light chain genes by gene conversion. A search of the 11-kb chicken \( \lambda \) light chain locus identified >50 matches to the E2A consensus, CANNNTG: 17 in the region between \( \psi _{\lambda 17} \) and \( \psi _{\lambda 4} \), the most proximal of the upstream pseudogenes; 2 in the matrix attachment region in the J-C intron; and 6 in the \( E3 \) enhancer. In DT40 B cells, the functional allele has undergone VJ recombination early in B cell development, which deletes a 1.8-kb region to join the \( V \) and \( J \) segments, whereas the inactive \( \lambda \) allele
is unrearranged (Fig. 1A), allowing the two alleles to be readily distinguished by PCR. Following ChIP, recovery of the rearranged and unrearranged \(\lambda_R\) alleles was assayed relative to a control gene, \(OVA\). This showed that E2A was 17.6-fold enriched at the rearranged \(V_R\) allele, but not at the unrearranged \(V_U\) allele (Fig. 1B). Thus, E2A associates directly with the rearranged \(V_R\) allele.

**E2A acts in cis to regulate Ig\(\lambda\) diversification**

To ask whether E2A must bind in cis to promote diversification, we took advantage of a derivative of DT40, DT40 PolyLacO-\(\lambda_R\), in which PolyLacO has been inserted in the upstream nonfunctional pseudo-V regions which are templates for gene conversion. The rearranged and unrearranged alleles can readily be distinguished by PCR, using primers indicated by arrows. B, ChIP analysis of E2A enrichment at the rearranged \(\lambda_R\) and unrearranged \(\lambda_U\) loci in DT40 cells, relative to OVA gene control amplicon. Fold enrichment is shown below. NTC, No template control.
transfectants, although cultures of the latter line contained some sub-G1 (apoptotic) cells (Fig. 2D). Levels of Igλ transcripts were unaltered in the E47-LacI transfectants (Fig. 2E), as predicted by published results showing that E2A does not regulate Ig gene expression in chicken B cells (24, 25). Levels of AID transcripts were ~3-fold higher in the E47-LacI transfectants (Fig. 2E), as reported by others (25).

To ask whether E2A regulates diversification directly, via binding to Igλ, we cultured independent E47-LacI (n = 19) or GFP-LacI (n = 13) transfectants in the presence and absence of IPTG and determined clonal diversification rates using the sIgM loss fluctuation assay (29, 33, 34). This assay scores inactivating mutations regardless of whether they occur by gene conversion, point mutation, deletion, or insertion and thus quantitates initiating events independent of the outcome of mutagenesis. This analysis showed that the clonal rate of diversification was 4.5-fold higher in E47-LacI transfectants than in GFP-LacI controls (p = 0.019, Mann-Whitney U test; Fig. 2F). Culture with IPTG causes LacI to be released from PolylacO (33); therefore, in cells cultured with IPTG, E47-LacI does not bind specifically to the Igλ locus but is present throughout the nucleus. Culture with IPTG had no effect on GFP-LacI control transfectants but reduced diversification rates in DT40 PolylacO-λR E47-LacI cells to background levels (Fig. 2F). Thus, E47-LacI promotes diversification by acting in cis.

Accelerated diversification depends upon the E2A activation domains

We then asked which functions of E2A are necessary to promote Ig gene diversification by determining the effect on diversification rate of mutations in either activation domain of E2A, AD1, or AD2; or in the DNA-binding domain. We generated DT40 PolylacO-λR derivatives stably expressing E47-LacI mutants lacking AD1 (E47ΔAD1-LacI), AD2 (E47ΔAD2-LacI), or both (E47ΔAD1/2-LacI; Fig. 3A). Transfectants of E47ΔAD1-LacI were isolated with efficiencies comparable with those of the control transfectants; transfectants of E47ΔAD2-LacI and E47ΔAD1/2-LacI were at a slightly lower efficiency. Neither of these mutants accelerated Ig gene diversification (Fig. 3B). Thus, function of E47 in Ig gene diversification depends on the E2A activation domains.

We also attempted to generate transfectants expressing an E47-LacI mutant carrying a mutation in the basic region of E47 (R558K) which inactivates DNA binding (39). However, in each of two independent transfections, few transfectants were recovered (~15% compared with the control), suggesting that this mutation may have a dominant negative effect on cell survival. The effect of this mutation was not further analyzed.

E2A localizes to IgλR in G1 phase of cell cycle

In human B cells, receptor cross-linking in G1 can initiate somatic hypermutation, producing identifiable mutations within 90 min (40). Thus, it was of interest to determine the stage of cell cycle in which E2A acts at Igλ. This is commonly done by sorting cells stained with Hoechst 33342 for DNA content to enrich for cells in G1, S, or G2-M. However, although the rearranged and diversifying λR gene can readily be imaged as a bright dot in DT40 PolylacO-λR cells expressing GFP-LacI (33), following Hoechst 33342 staining and cell sorting, the fraction of cells exhibiting a clear fluorescent signal from the tagged gene diminished from the 90–95% routinely observed in unsorted cells to ~45%. Because such a loss in signal could bias results, we therefore determined cell cycle stage by a different approach. Analysis of Hoechst 33342-stained and sorted cells showed that nuclear size was significantly smaller in G1 than in G2-M cells (e.g., Fig. 4A). We therefore asked whether the nuclear radius (r) could be used to establish the stage of cell cycle, by measuring nuclear radii of G1 cells (n = 55) and G2-M cells (n = 55) from an exponentially growing DT40 PolylacO-λR population which had been stained with Hoechst 33342 and sorted based on DNA content. The mean nuclear radius of G1 cells was 3.8 ± 0.3 μm; that of G2-M cells was 5.4 ± 0.5 μm (Fig. 4B). Comparison of the G1:S:G2 cell ratios as determined by nuclear radius (3:6:1), and staining (2.7:5.5:1.7) further validated this approach. Thus, G1 cells were identified experimentally as r < 4 μm; and G2 cells as r > 5.2 μm.

Colocalizations of λR/E2A were readily identified by deconvolution microscopic analysis of DT40 PolylacO-λR GFP-LacI cells stained with anti-E2A Abs (e.g., Fig. 5A). λR/E2A colocalizations were evident in 26% of asynchronous cells (n = 227). Analysis of the cell cycle distribution of colocalizations showed that 45% of

FIGURE 3. Accelerated diversification depends upon the E2A activation domains. A, Schematic of wild-type E47 and its mutants. bHLH, basic-helix-loop-helix. B, Mean sIgM-loss of independent clonal DT40 PolylacO-λR cells expressing RFP-LacI (n = 17), E47-LacI (n = 10), E47ΔAD1-LacI (n = 22), E47ΔAD2-LacI (n = 9), or E47ΔAD1/2-LacI (n = 7), analyzed 3 wk posttransfection. Results shown are representative of two independent experiments. Values were normalized to DT40 PolylacO-λR RFP-LacI cells.

FIGURE 4. Nuclear radius correlates with cell cycle. A, Representative images of G1- and G2/M-enriched cells. G1 (left) and G2/M (right) cells were stained with Hoechst 33342 (10 μM; Molecular Probes) and then sorted based on DNA content. Bar, 10 μm. B, Representative cell cycle profile of DT40 PolylacO-λR cells. Mean radii ± SD are shown as horizontal bars within the representative profile. Dotted vertical lines indicate cutoffs for G1 and G2 used in experimental analyses: G1, r < 4 μm; G2, r > 5.2 μm.
Id1 expression inhibits Ig gene diversification and diminishes \( \lambda_n/E2A \) colocalizations

To ask whether \( \lambda_n/E2A \) colocalizations in G1 phase are critical to diversification, we determined the effect of Id expression on these colocalizations. Id antagonizes E2A, and expression in DT40 B cells of Id1 or Id3 has previously been shown to diminish Ig gene diversification (25). We generated stable DT40 PolyLacO-\( \lambda_n \) RFP-LacI Id1 transfectants, confirmed Id1 expression by Western blotting (Fig. 6A), and showed that Id1 expression did not alter the cell cycle profile (Fig. 6B). Assays of sIgM loss verified that Id1 expression diminished the clonal rate of Ig gene diversification in 18% of DT40 PolyLacO-\( \lambda_n \) RFP-LacI Id1 cells stained with Abs to active transcription factories (Fig. 6C). This is comparable with the cell cycle distribution. Thus, Id1 is transcribed throughout the cell cycle, but \( \lambda_n/E2A \) colocalizations predominates in G1 phase.

\[ \lambda_n/E2A \] colocalizations occurred in G1 cells, 38% in S cells, and 17% in G2 cells (Fig. 5B). Thus, there was an apparent excess of \( \lambda_n/E2A \) colocalizations in G1 (45%) relative to the fraction (25%) of G1 cells (\( p < 0.0001, \chi^2 \) test).

We also determined the cell cycle dependence of \( \lambda_n \) transcription, identifying active transcription factories by staining with Ab to phosphorylated Ser3 in the C-terminal domain of RNA polymerase II (P*-Pol II), a modification characteristic of elongating Pol II molecules (41). In asynchronous cell populations of DT40 PolyLacO-\( \lambda_n \) RFP-LacI cells, numerous active transcription factories could be identified throughout the nucleus, and \( \lambda_n/P*\)-Pol II colocalizations were readily observed in 19% of cells (\( n = 392; \) e.g., Fig. 5C). Analysis of the cell cycle distribution of \( \lambda_n/P*\)-Pol II colocalizations showed that 21% of colocalizations occurred in G1 cells; 58% in S cells; and 21% in G2 cells (Fig. 5D). This is comparable with the cell cycle distribution. Thus, \( \lambda_n \) is transcribed throughout the cell cycle, but \( \lambda_n/E2A \) colocalizations predominates in G1 phase.

Id1 expression affects the cell cycle distribution of \( \lambda_n/E2A \) colocalizations with respect to cell cycle

Quantification of \( \lambda_n/E2A \) colocalizations with respect to cell cycle showed that, in DT40 PolyLacO-\( \lambda_n \) RFP-LacI cells, 45% of colocalizations occurred in G1 phase, 38% in S phase, and 17% in G2 phase; and in DT40 PolyLacO-\( \lambda_n \) RFP-LacI Id1 cells, 10% of colocalizations occurred in G1 phase, 58% in S phase, and 32% in G2 phase. Thus, the most pronounced effect of Id1 expression was in G1 phase, when the fraction of \( \lambda_n/E2A \) colocalizations diminished from 45% in the parental line to 10% in Id1 transfectants (\( p < 0.0001, \chi^2 \) test). Given that total colocalizations were diminished 2-fold in Id1 transfectants, it was useful to plot those results in terms of the entire cell population. As shown in Fig. 7A, Id1 expression reduced \( \lambda_n/E2A \) colocalizations from 12% to 1.4% in G1 phase, or about 9-fold; but had a much more modest effect in S phase or G2. In contrast, the cell cycle profile of \( \lambda_n/P*\)-Pol II colocalizations was comparable in Id1 transfectants and the parental line: 21, 58, and 21% of colocalizations occurred in G1, S, and G2-M phase in the parental cell line, and 24, 60, and 16% in Id1 transfectants. As shown in Fig. 7B, in terms of the entire cell population, Id1 expression had essentially no effect in any stage of cell cycle. The absence of effect of Id1 expression on \( \lambda_n/P*\)-Pol II colocalizations is consistent with undiminished IgA transcript levels in DT40 PolyLacO-\( \lambda_n \) RFP-LacI Id1 transfectants (Fig. 6D).

Thus, Id1 expression affects the cell cycle distribution of colocalizations of \( \lambda_n \) with E2A, but not with P*-Pol II.

Id proteins are unstable, and they are targeted for degradation by a D-box motif conserved among Id family members (42). This raised the possibility that cell cycle-dependent regulation of Id1 levels could account for cell cycle dependence of \( \lambda_n/E2A \) colocalizations. To test this, we monitored expression levels in the course of cell cycle of wild-type Id1 and a derivative carrying a mutation in the D-box mutant, \( Id1^{Dbox} \), predicted to be unaffected by cell cycle-dependent destabilization. These proteins were transiently expressed as GFP fusions in DT40 cells.
and at 24 h posttransfection GFP intensity was determined with respect to cell cycle, as determined by PI staining. There was no apparent correlation of Id1-GFP levels with cell cycle or any difference between levels of Id1-GFP or Id1DBM-GFP in the course of the cell cycle (Fig. 7C). Thus, cell cycle dependence of Id1 levels does not account for diminished λR/E2A

and Id1 expression diminishes λR/E2A colocalizations. The colocalizations in DT40 PolyLacO-λR RFP-LacI cells and a derivative stably expressing Id1 (Id1 – and +, respectively) in each stage of cell cycle show the percent of total cells in which colocalizations were evident. Id1 expression diminished total colocalizations by about one-half. B, Effect of Id1 expression on cell cycle dependence of λR/P*-Pol II colocalizations. Details as in A. C, Fluorescence analysis of Id1-GFP levels in the course of the cell cycle. DT40 cells were transiently transfected with Id1-GFP or its derivative carrying mutations in the D-box, which determines cell cycle-dependent instability, and GFP intensity and DNA content were analyzed at 24 h posttransfection. Fold increases of mean GFP fluorescence intensity in Id1DBM-GFP relative to Id1-GFP is shown in the rightmost panel.
colocalizations in Id1 transfectants in G1 phase. Instead, G1 phase appears to be the critical window in which E2A promotes diversification.

**Discussion**

We have shown that E2A must act in cis to promote Ig gene diversification and that G1 phase is the critical window in which E2A functions in this process. Our experiments have examined A genes tagged with PolyLacO and imaged by binding to GFP-LacI or RFP-LacI. This provides a powerful approach for studying gene diversification. The tagged locus is visible in >90% of fixed cells, enabling analysis of colocalizations with factors involved in diversification. The ability to tether potential regulators and release by culture with IPTG makes it possible to study the effects of a factor at the IgA locus independent of its other targets. This is especially useful for a factor like E2A, which functions at the top of a large and complex regulatory hierarchy (43).

Our results establish that E2A directly regulates Ig gene diversification by physical association with the Ig loci. ChIP provided clear evidence for association of E2A with the rearranged λ5 allele in DT40 B cells. That E2A must function in cis was established by showing that the acceleration in diversification resulting from tethering E2A (E47-LacI fusion) to the IgA allele in DT40 PolyLacO-λ5 cells was not evident in cells cultured with IPTG, which releases LacI from LacO. E2A is best known as a transcriptional regulator, but E2A function in diversification did not reflect transcriptional activation at IgA, given that levels of IgA transcripts were not altered by ectopic expression of E47, confirming results of others (23, 25, 26). Function in diversification did require the E2A activation domains, AD1 and AD2. Moreover, expression of the E2A antagonist, Id1, diminished diversification and λ5/E2A colocalizations but did not affect λ5/P- Pol II colocalizations or IgA transcript levels. Thus, E2A is not required to recruit λ5 to transcription factories.

In addition to the direct cis regulation of Ig diversification by E2A, documented above, E2A could also stimulate Ig gene diversification indirectly, for example by regulation of AID expression. The AID gene was shown to be a target of transcriptional regulation by E2A in murine B cells (21), and we and others (25) have shown that ectopic expression of E2A increases AID transcript levels in DT40 B cells. This might contribute to or account for the modest increase in diversification evident in IPTG-cultured DT40 PolyLacO-λ5 GFP-LacI cells (Fig. 2F). Whereas E2A ablation has been reported not to diminish AID transcript levels in chicken B cells (24), other E proteins, such as HEB (Fig. 6D), might ensure a minimum level of AID expression in the absence of E2A. Alternatively, because Id1 expression did not affect AID expression (Fig. 6E), basal level AID expression may be mediated by factors other than E proteins.

Colocalizations of λ5 with E2A were most prominent in G1 phase. In contrast, localization of λ5 to active transcription factories was comparable with cell cycle distribution, suggesting that λ5 transcription occurs throughout the cell cycle. Id1 expression had the most pronounced effect on λ5/E2A colocalizations in G1 phase, and relatively minor effects at other stages of cell cycle. This suggests that λ5/E2A interaction in G1 is critical for initiation of diversification but does not address whether associations of E2A at other stages of cell cycle (which were not impeded by Id1) might also be important for completion of diversification. Id1 levels were constant throughout cell cycle; therefore, diminished λ5/E2A colocalizations in G1 phase caused by ectopic expression of Id1 does not reflect cell cycle dependence of Id1 levels. Id proteins heterodimerize with E proteins to inhibit DNA binding (14). Thus, the G1-specific effects of Id1 suggest that protein-protein interactions (e.g., Ref. 28), rather than DNA binding, may be critical to E2A associations with IgA at later stages of the cell cycle. This raises the possibility of distinct modes of E2A association with IgA during the cell cycle.

Additional lines of evidence support the view that diversification is initiated in G1 phase. Somatic hypermutation in the human BL2 cell line can be induced by in vitro stimulation that takes place only during G1 phase, and point mutations first become evident within 90 min of stimulation, when cells are still in G1 (40). In murine B cells activated for CSR, IgH colocalizations with factors that promote CSR are prominent in G1 phase (44); and DNA breaks at the S regions can be detected in G1 phase (45). DNA breaks have also been identified in later stages of cell cycle in hypermutating human B cell lines (46), but these proved to be AID independent (47).

We note that experiments thus far have not analyzed the course of diversification in a single cell. It is therefore possible that E2A may function in G1 phase to prepare a locus for events that occur later in cell cycle, or even during a subsequent cell cycle. E2A has been recently implicated in maintenance of histone H4 acetylation (24), and it is possible that E2A establishes a local chromatin environment favorable to AID attack or effective diversification in daughter cells.

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**Disclosures**

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**References**