

Selective Inhibition of Class Switching to IgG and IgE by Recruitment of the HoxC4 and Oct-1 Homeodomain Proteins and Ku70/Ku86 to Newly Identified ATTT *cis*-Elements*[§]

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Immunoglobulin (Ig) class switching is central to the maturation of the antibody response as IgG, IgA, and IgE are endowed with more diverse biological effector functions than IgM. It is induced upon engagement of CD40 on B lymphocytes by CD40L expressed by activated CD4⁺ T cells and exposure of B cells to T cell-secreted cytokines including interleukin-4 and transforming growth factor- β . It begins with germ line I_H-C_H transcription and unfolds through class switch DNA recombination (CSR). We show here that the HoxC4 and Oct-1 homeodomain proteins together with the Ku70/Ku86 heterodimer bind as a complex to newly identified switch (S) regulatory ATTT elements (SREs) in the I γ and I ϵ promoters and downstream regions to dampen basal germ line I γ -C γ and I ϵ -C ϵ transcriptions and repress CSR to C γ and C ϵ . This mechanism is inactive in the C α 1/C α 2 loci because of the lack of SREs in the I α 1/I α 2 promoters. Accordingly, in resting human IgM⁺IgD⁺ B cells, HoxC4, Oct-1, and Ku70/Ku86 can be readily identified as bound to the I γ and I ϵ promoters but not the I α 1/I α 2 promoters. CD40 signaling dissociates the HoxC4-Oct-1-Ku complex from the I γ and I ϵ promoter SREs, thereby relieving the I_H-C_H transcriptional repression and allowing CSR to unfold. Dissociation of HoxC4-Oct-1-Ku from DNA is hampered by CD153 engagement, a CD40-signaling inhibitor. Thus, these findings outline a HoxC4-Oct-1-Ku-dependent mechanism of selective regulation of class switching to IgG and IgE and further suggest distinct co-evolution and shared CSR activation pathways in the C γ and C ϵ as opposed to the C α 1/C α 2 loci.

In class switching, the Ig constant H chain μ (C μ) region is substituted with C γ , C α , or C ϵ . Class-switched Igs are a critical

feature of the high affinity late and memory antibody responses, because IgG, IgA, and IgE carry out those effector functions including binding to receptors for the Fc portion of γ , α , and ϵ H chains on phagocytic and proinflammatory cells and passage through “mucosae” that are required for the eradication of microbial pathogens. In general, Ig class switching occurs in germinal center (GC)¹ B cells upon stimulation by CD40L and cytokines expressed by activated CD4⁺ T cells. It is initiated by transcription of the intervening (I), switch (S), and C regions of the upstream (donor) and downstream (acceptor) C_H loci (1–3). Splicing of the nascent I_H-S-C_H RNA yields germ line I_H-C_H transcripts and probably couples transcription with the cleavage step of CSR, possibly through the action of activation-induced cytidine deaminase (AID) (4). CSR is effected through double-strand DNA breaks of the donor and acceptor S regions, excision of intervening DNA as a switch circle (SC) (2), and non-homologous end-joining (NHEJ) of the DNA ends (1–3) coupled with mismatch repair (5).

Germ line I_H-C_H transcription is driven by the I_H promoter lying upstream of each I_H region (6–8) upon CD40L-, IL-4-, and/or transforming growth factor- β -induced binding of the NF- κ B/c-Rel, Stat6, Smad, activating protein-1, or B cell lineage-specific activator protein transcription factors to I_H promoter-specific *cis*-elements (1, 6, 8–13). In CD40-induced (GC) B cells, germ line I_H-C_H transcription and CSR can be effectively down-regulated by bidirectional CD30:CD153-dependent signaling, which interferes with the recruitment of tumor necrosis factor receptor-associated factor molecules to CD40 and inhibits NF- κ B activation (14, 15). However, the regulation of germ line I_H-C_H transcription and CSR in general and in non-CD40-induced (pre-GC) B cells in particular remains to be defined.

We report here a mechanism of selective inhibition of class switching to IgG and IgE. This mechanism relies on the binding of HoxC4, Oct-1, and Ku70/Ku86 to newly identified SREs

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¹ The abbreviations used are: GC, germinal center; AID, activation-induced cytidine deaminase; C_H, heavy chain constant region; ChIP, chromatin immunoprecipitation assay; CHX, cycloheximide; CSR, class switch DNA recombination; HD, homeodomain; HIM, HD interaction motif; I_H, intervening region of C_H gene; Ig, immunoglobulin; NHEJ, non-homologous end-joining; S, switch (region); SC, switch circle; SRE, S-regulatory ATTT element; V_HDJ_H, variable/diversity/joining regions of heavy chain gene; IL, interleukin; GFP, green fluorescent protein; mAb, monoclonal antibody; GST, glutathione S-transferase; Ab, antibody; RT, reverse transcriptase; EMSA, electrophoretic mobility shift assay; DBB, DNA binding buffer; DAC, DNA affinity chromatography; mut, mutated; WT, wild type; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; RE, responsive element; ht, human trimeric; Stat, signal transducer and activator of transcription.

that exists in the $I\gamma$ and $I\epsilon$ but not in the $I\alpha 1/\alpha 2$ promoters. Such a binding dampens basal germ line $I\gamma$ -C γ and $I\epsilon$ -C ϵ transcription and represses CSR to C γ and C ϵ . It is potentiated by CD153 signaling and reversed by CD40 signaling. Thus, by selectively binding to the $I\gamma$ and $I\epsilon$ promoters, HoxC4, Oct-1, and Ku70/Ku86 differentially regulate class switching to IgG, IgE, and IgA and would minimize S region double-stranded DNA breaks, thereby contributing to the stability of the Ig H chain locus.

EXPERIMENTAL PROCEDURES

Human C γ , C α , and C ϵ Loci Sequence Analysis—S repeats and ATTT motifs were identified within the I_H promoters, S regions, and flanking areas using the Pustell dot-matrix comparison (16) and nucleotide subsequence search (MacVector™, Accelrys, Burlington, MA).

B Cell Preparation and Culture—The human monoclonal CL-01 IgM⁺IgD⁺ B cell line was reported previously (8, 15, 17–21). In the absence of stimulation, these cells maintain their IgM⁺IgD⁺ phenotype in culture but switch to all of the downstream isotypes upon exposure to CD40L and IL-4. In addition, they hypermutate the expressed I_H DJ_H genes and BCL6 when co-cultured with activated CD4⁺ T cells upon B cell receptor cross-linking (18–21). 7D7 and 4B6 are subclones of IgM⁺IgD⁺ CL-01 cells that were selected for spontaneous and ongoing switching to IgG, IgA, and IgE. Human peripheral blood IgM⁺IgD⁺ B cells and tonsil B cell subsets were separated as reported previously (21). B cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Sorting of GFP^{high} 7D7 and 4B6 B cells was performed using a Beckman-Coulter Altra cell sorter (Cornell University Weill Medical College Core Sorting Facility). Human IL-4 (Schering-Plough Corp., Kenilworth, NJ), htCD40L (Immunex Corp., Seattle, WA), anti-CD153 mAb (M81) (Immunex Corp.), okadaic acid (Sigma), and cycloheximide (Sigma) were added to cultures at 200 units/ml, 2 μ g/ml, 2 μ g/ml, 12 nM, and 100 μ g/ml, respectively.

Vectors—The $I\gamma 3$ -, $I\epsilon$ - and $I\alpha 1/\alpha 2$ -luciferase gene reporter pGL3 constructs were described previously (7, 8, 22). The $I\gamma 1$, $I\gamma 2$, and $I\gamma 4$ promoter cDNAs were PCR-amplified from *HindIII/BamHI* subclones of the respective human 5'-S γ regions (a gift from Dr. E. Max, Food and Drug Administration, Bethesda, MD) (23) and cloned into pGL3 vector (Promega, Madison, WI). Mutations replacing the 5', 3', or both 5'- and 3'- I_H promoter ATTT motifs to ggTT were introduced by PCR-based mutagenesis. The Ku70^{mutHIM} lacking the homeodomain (HD) interaction motif (HIM) (K595N and K596N) (24) was generated by overlap PCR assembly. cDNAs encoding human HoxC4 (a gift from Dr. P. Zhou, Cornell University, New York, NY), Oct-1 (a gift from Dr. R. Roeder, Rockefeller University, New York, NY), Ku70, Ku70^{mutHIM}, and Ku86 (a gift from Dr. J. Cartron, INSERM U76, Paris, France) were cloned into bicistronic pIRES2 vectors (Invitrogen) for gene overexpression experiments. Human HoxC4 and Oct-1 were also cloned into pcDNA3.1+ vectors (Invitrogen) for *in vitro* transcription and translation. HoxC4, Oct-1, Ku70, Ku70^{mutHIM}, and Ku86 cDNAs were cloned into the pGEX-6P1 vector (Amersham Biosciences) to generate the respective GST fusion proteins. The pBSKII plasmid (Stratagene Corp., La Jolla, CA) was used to demonstrate the binding of HoxC4, Oct-1, Ku70, and Ku86 to circular DNA containing the ATTT motif.

Antibodies—Anti-Ku70 (Ab-5), anti-Ku86 (Ab-2), and anti-Ku70/Ku86 (Ab-3) mAbs were from Lab Vision/NeoMarkers (Fremont, CA). Anti-Oct-1 (YL15) mAb was from Upstate Biotech (Waltham, MA); rabbit anti-Oct-1 Ab (sc-232) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-HoxC4 and anti-HoxC8 mAbs were from CRP Inc. (Berkeley, CA). Goat anti-Oct-2 (C-20) Ab was from Santa Cruz Biotechnology, Inc. Controls were MOPC-21 mouse IgG mAb (Sigma), rabbit, and goat polyclonal IgGs (Santa Cruz Biotechnology, Inc.).

Transfection and Gene Reporter Assays—CL-01 B cells were transfected with firefly luciferase gene reporter pGL3 vector (10 μ g) and *Renilla* luciferase gene control pRL-CMV vector (10 ng) (Promega). 7D7 and 4B6 B cells were transfected with the same reporter and control vectors together with expression vector(s) (2 μ g) as specified. Electroporation (525 V/cm, 950 microfarads) was performed in duplicates using a Gene Pulser II (Bio-Rad). Firefly and *Renilla* luciferase activities were measured as reported previously (8) at the specified times using a MLX microtiter plate luminometer (Thermo Labsystems, Chantilly, VA).

mRNA, cDNA, and RT-PCR—mRNA isolation, first strand cDNA synthesis, and RT-PCRs were performed as described previously (8). PCRs were made semi-quantitative by varying the number of ampli-

cation cycles and performing dilutional analysis to ensure a linear relationship between the amount of cDNA used and the intensity of the PCR product. HoxC4, Oct-1, and AID cDNAs were amplified using the following primers: HoxC4 forward, 5'-ATGGGATCATGAGCTCG-TATTTG-3'; HoxC4 reverse, 5'-CTATAACCTGGTAATGTCTCTGCG-3'; Oct-1 forward, 5'-ATGGGGAACAATCCGTCAGAAACCAGTAAA-3'; Oct-1 reverse, 5'-CTACTGTGCCTTGGAGCGGGTGGT-3'; AID forward, 5'-TGCTCTTCTCCGCTACATCTC-3'; and AID reverse, 5'-AACCTCATACAGGGGCAAAAGG-3'. The $I\gamma 3$ -C $\gamma 3$, $I\epsilon$ -C ϵ , $I\alpha 1/\alpha 2$ -C $\alpha 1$, V_H DJ_H-C γ , V_H DJ_H-C ϵ , V_H DJ_H-C $\alpha 1/\alpha 2$, Ku70, Ku86, and β -actin transcripts were RT-PCR amplified for 25 cycles using the primer pairs described previously (15). The PCR conditions were as follows: denaturation for 1 min at 94 °C, annealing for 1 min at 68 °C, and extension for 1 min at 72 °C. Before each RT-PCR, cDNAs were denatured for 5 min at 94 °C.

Electrophoretic Mobility Shift Assays (EMSAs) and Protein Purification—Cytoplasmic and nuclear protein extraction, probe labeling, EMSA, and supershift reactions were performed as reported previously (8). [³²P]ATP-labeled and cold I_H SRE double-stranded oligonucleotides encompassed the sequences depicted in Fig. 1B. The sequences of the S $\gamma 3$ and S ϵ SRE oligonucleotides were 5'-CAGCGGCAGACCA-GAAATGGGG-3' and 5'-GGGTTGGGGTGATTAAACTGAGT-3', respectively, encompassing the ATTT motif immediately 5' of the S $\gamma 3$ region and the first ATTT motif of the S ϵ region. The sequence of the Sp1 oligonucleotide was 5'-ATTCGATCGGGGCGGGCGAGC-3'. The SRE binding activity of CL-01 nuclear extracts (75 mg) was first enriched by 20–50% (NH₄)₂SO₄ precipitation, dialyzed against DNA binding buffer (DBB) (10 mM Tris-HCl, pH 7.6, 200 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol), and then fractionated on a Centricon concentrator using a 100-kDa molecular mass cut-off membrane (Millipore Corp., Bedford, MA). The >100-kDa fraction was applied to a Superose 6 gel filtration column (Amersham Biosciences), which was eluted at a 100 μ l/min rate with DBB containing 20% glycerol. Fractions (500 μ l) were collected and tested for SRE binding activity by EMSA. The SRE binding fractions were pooled and loaded onto a DAC column consisting of an agarose matrix bearing streptavidin (Pierce) and loaded with 5'-biotinylated pentamerized double-stranded $I\gamma 3$ 5'-SRE or mutSRE oligonucleotides (Qiagen Sciences, Germantown, MD). The column was washed with 200 mM KCl DBB containing 20% glycerol. The DNA-bound proteins were eluted using a 300–800 mM KCl gradient in DBB 20% glycerol and collected in 500- μ l fractions, which were monitored for SRE binding activity and protein content. Proteins were visualized in SDS-PAGE using the Rapid Silver Stain Plus kit (Bio-Rad). The fractions eluted at 300 mM KCl contained the strongest SRE binding activity and were pooled, concentrated on Centricon filters, and applied to SDS-PAGE. The resolved protein bands were stained by Coomassie Brilliant Blue G-250 (Bio-Rad), excised, and subjected to in-gel proteolysis by trypsin. Peptides mixtures were separated by a C8 reverse-phase column with a linear 0–60% CH₃CN gradient in 0.1% trifluoroacetic acid for 1 h. The molecular masses of the peptide mixtures were determined by MALDI-TOF mass spectrometry using a Voyager-DE short tandem repeat (PerSeptive Biosystems, Inc., Framingham, MA). The complete list of accurately measured masses of the tryptic peptides was used to search for protein candidates in the OWL protein sequence data base with the program ProFound (prowl.rockefeller.edu/cgi-bin/ProFound). Internal sequencing of tryptic peptides was performed as described previously (25).

Detection of Reciprocal Recombination SCs—Genomic DNA was extracted from B cells using the QIAamp DNA mini kit (Qiagen). Specific S γ -S μ , S ϵ -S μ , and S α -S μ reciprocal SCs were amplified from genomic DNA (500 ng) using nested PCRs and S μ , S γ -($\gamma 1$ –4), and S α -($\alpha 1/\alpha 2$) region-specific primers (17). PCR kinetics entailed a 1-min denaturation at 94 °C, a 1-min annealing at 68 °C, and a 4-min extension at 72 °C for two rounds of 30 cycles. Before each PCR, DNA was denatured for 5 min at 94 °C. The identity of PCR-amplified DNA was confirmed by Southern blot analysis using S μ -specific SC probes (17).

Phosphatase Treatment of Nuclear Extracts—Extracts prepared from freshly isolated human IgM⁺IgD⁺ B cells were incubated with 5 and 20 milliunits of acid phosphatase (Grade I, Roche Applied Sciences) in DBB in a final volume of 30 μ l at 25 °C for 10 min. A 10- μ l aliquot of the phosphatase-treated sample was then tested in each binding assay.

GST Proteins and Pull-down Assays—GST fusion proteins were expressed, purified using GSH-agarose beads according to the manufacturer's protocol (Amersham Biosciences), and analyzed for homogeneity by SDS-PAGE and silver staining. L-[³⁵S]Methionine-labeled proteins were translated using the TnT Quick coupled transcription/translation systems (Promega) method. For pull-down experiments, 5 μ l of *in vitro* translated protein was mixed with 50 μ g of nuclear extract and applied

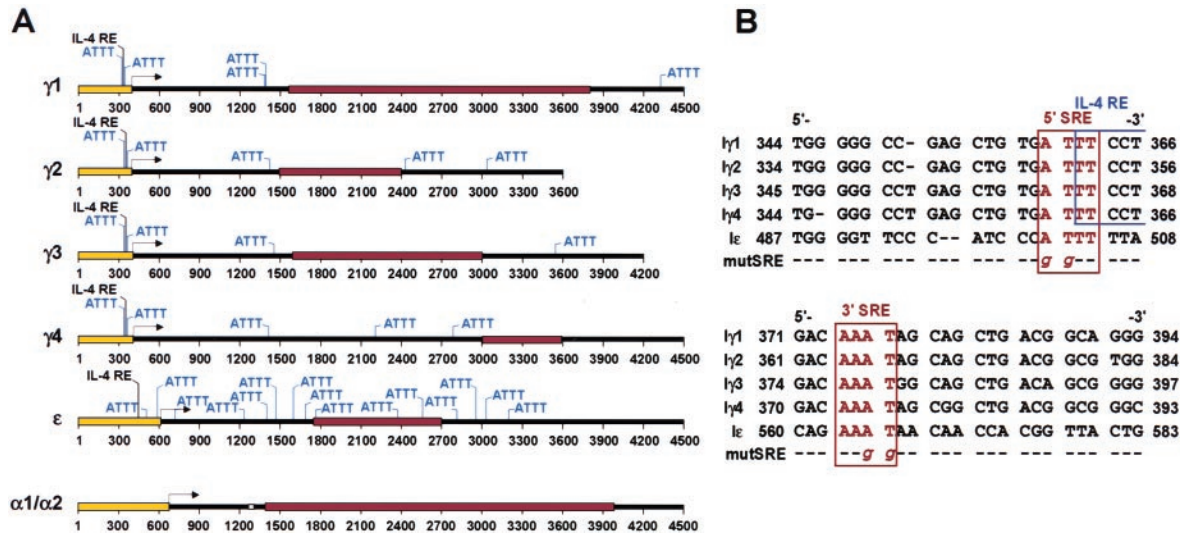
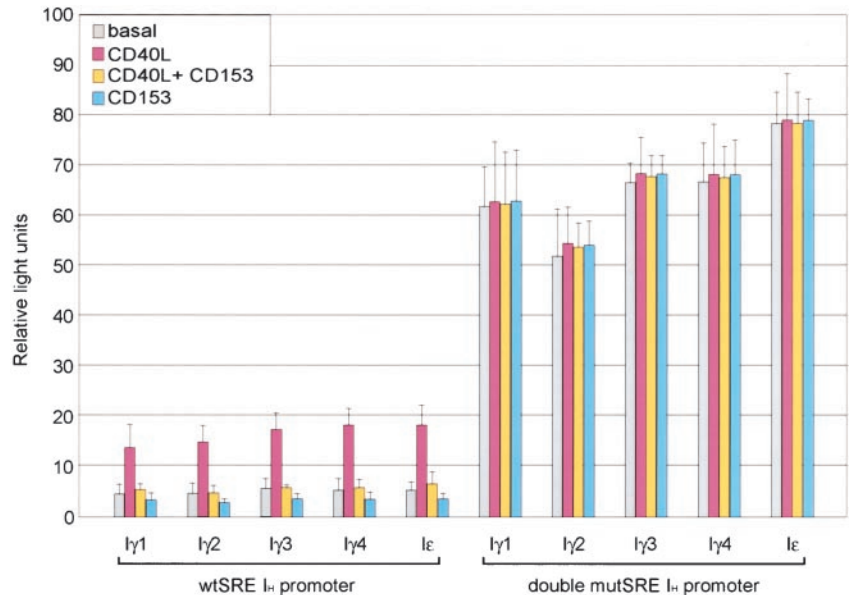


FIG. 1. ATTT SREs are found in the human $I\gamma$ and $I\epsilon$ promoters and related I_H -S regions but not in $I\alpha 1/2$ or $I\alpha 1/2$ -S $\alpha 1/2$. *A*, schematic depiction of the human $I\gamma 1$ -C $\gamma 1$, $I\gamma 2$ -C $\gamma 2$, $I\gamma 3$ -C $\gamma 3$, $I\gamma 4$ -C $\gamma 4$, $I\epsilon$ -C ϵ , and $I\alpha 1/2$ -S $\alpha 1/2$ DNA sequences. Yellow boxes denote promoters encompassing evolutionarily conserved sequence and flanking areas (8, 48–51). Red boxes denote S regions. Residue 1 in the γ sequences is as described by Mills *et al.* (23) and in the ϵ and α sequences is according to GenBankTM references below. Major initiation of transcription sites depicted by turned arrows is as follows: $\gamma 1$, 310; $\gamma 2$, 466; $\gamma 3$, 449; $\gamma 4$, 544; ϵ , 592; and $\alpha 1$, 748. I_H 5'-SRE (ATTT) and IL-4 RE-Stat6 (TTCNNNGAA) encompassed the following residue numbers: $I\gamma 1$, 360–363 and 362–371; $I\gamma 2$, 350–353 and 352–361; $I\gamma 3$, 362–365 and 364–373; $I\gamma 4$, 360–363 and 362–371; and $I\epsilon$, 502–505 and 438–447, respectively. The gray box within the $I\alpha 1/2$ -S $\alpha 1/2$ region denotes an unidentified area of ~200 bp as deduced from the equivalent mouse genomic DNA. Compilation is based on the GenBankTM sequences with accession numbers as follows: AL122127 ($\gamma 1$ and $\gamma 3$), U39934 ($\gamma 2$), HSG481A ($\gamma 4$), X56797 and J00222 (ϵ), L04540 and L19121 ($\alpha 1$), and L04541 ($\alpha 2$). *B*, SRE and flanking areas within the $I\gamma 1$, $I\gamma 2$, $I\gamma 3$, $I\gamma 4$, and $I\epsilon$ promoters. Upper cluster, 5'- I_H promoter SRE; lower cluster, 3'- I_H promoter SRE (residue numbers are indicated). Gaps (–) are used to align ATTT stretches. SRE and IL-4 RE-Stat6 are boxed. Mutations introduced in the different $I\gamma$ and $I\epsilon$ promoter-driven luciferase gene reporter pGL3 vectors are indicated by small italic letters. The position of residues identical in both WT and mutSRE sequences are underscored.

FIG. 2. ATTT SREs critically regulate $I\gamma$ and $I\epsilon$ promoter activity. CL-01 B cells were transfected with WT (*wtSRE*) or double mutSRE $I\gamma$ or $I\epsilon$ promoter-driven luciferase gene reporter pGL3 vectors and then cultured with or without htCD40L for 24 h in the presence or absence of agonistic anti-CD153 mAb M81. Luciferase activity was measured and expressed as relative light units. Values are means $\pm$ 1 S.D. of three experiments.



to GSH-agarose beads (20 μ l) equilibrated in binding buffer B (25 mM Tris-HCl, pH 7.9, 1 mM dithiothreitol, 150 mM NaCl, 0.01% Nonidet P-40). After 2 h at 4 $^{\circ}$ C, the beads were washed with buffer containing 150 mM KCl. Bound proteins were then eluted in SDS sample buffer, separated in SDS-PAGE, fixed, dried, and autoradiographed at -70° C.

Co-immunoprecipitation, Circular DNA Pull-down, and Immunoblotting Assays—Nuclear extracts (200 μ g of protein in 500 μ l of buffer B) were precipitated with indicated mAbs and Protein-G Plus-agarose (Santa Cruz Biotechnology, Inc.) or with CNBr-activated-Sepharose 4B beads (Amersham Biosciences) bearing circular pBSK vector (1 μ g of DNA/20 μ l of bead) (Stratagene Corp.). For circular DNA pull-down assay, buffer B was supplemented with 0.05 mg/ml poly(dI-dC)-poly(dI-dC) (Sigma) and WT or mutSRE oligonucleotides (50 ng). Pulled-down proteins were fractionated through 12% SDS-PAGE and transferred to nitrocellulose membranes. After blocking, these membranes were blotted with mAbs to HoxC4, Oct-1, Ku70, and Ku86, washed, and then

incubated with a horseradish peroxidase-conjugated rabbit Ab to mouse IgG (Santa Cruz Biotechnology, Inc.). After horseradish peroxidase addition, the specific proteins were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences). The whole cell extracts from tonsillar B cells were transferred to polyvinylidene difluoride membranes, blotted with mAbs to HoxC4, Ku70, Ku86, rabbit IgG to Oct-1, and actin (Sigma), washed, and then incubated with horseradish peroxidase-conjugated donkey Ab to mouse IgG or horseradish peroxidase-conjugated goat Ab to rabbit IgG (Santa Cruz Biotechnology, Inc.).

Chromatin Immunoprecipitation (ChIP) Assays—Freshly isolated circulating human IgM⁺IgD⁺ B cells (2.5×10^7) were treated with 1% formaldehyde for 10 min at room temperature to cross-link chromatin. After washing with cold phosphate-buffered saline containing protease inhibitors, chromatin was separated using nuclei-lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 M NaCl, 1% Triton-X-100, 0.5% sodium

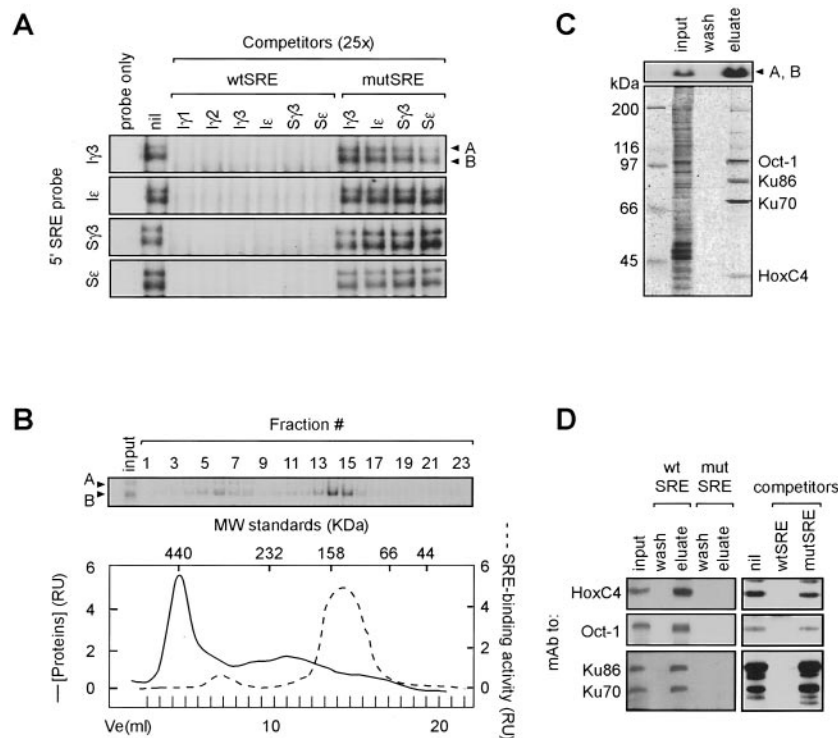


FIG. 3. I γ and I ϵ SREs recruit HoxC4 and Oct-1 HD proteins and the Ku70/Ku86 heterodimer. *A*, nuclear proteins from freshly isolated human peripheral blood IgM⁺IgD⁺ B cells specifically bind [γ -³²P]I γ 3 and [γ -³²P]I ϵ 5'-SRE as well as [γ -³²P]S γ 3 and [γ -³²P]S ϵ SRE probes. Efficient competition was achieved by a 25-fold molar excess of WT (*wtSRE*) but not mutant (*mutSRE*) cold oligonucleotides (I γ 4 competition is not depicted). Specific protein-DNA complexes A and B are indicated by arrowheads. *B*, purification of the SRE-binding proteins. Superose 6 gel filtration of the resolubilized (NH₄)₂SO₄ precipitate resulted in the elution of the SRE binding activity as analyzed using a [γ -³²P]I γ 3 5'-SRE probe in EMSA in two peaks, consisting of fractions 5 and 6 (~350 kDa) and fractions 14 and 15 (~150 kDa). *C*, SRE binding fractions were pooled and resolved by an I γ 3 SRE DAC column. The 300 mM KCl fractions (*eluate*) containing the strongest SRE binding activity (*top panel*) were pooled, concentrated, and resolved by SDS-PAGE for silver staining (*bottom panel*). The four major SRE-binding proteins were identified by MALDI-TOF mass spectrometry and internal peptide sequencing as follows: p100, Oct-1, internal peptide sequence (FKQRRRIKLGFQTQ), Swiss Protein Data Bank accession number P14859; p89, Ku86, internal peptide sequence (ALVLFGTGDTGTD), Swiss Protein Data Bank accession number P13010; p72, Ku70, peptide sequence (FNTSTGGLLLP), Swiss Protein Data Bank accession number P12956; and p34, HoxC4, peptide sequence (QGPGN-SRGGHGAQAG), Swiss Protein Data Bank accession number P09017. *D*, 200 mM KCl (*wash*) and 300 mM KCl DAC fractions (*eluate*) from either *wtSRE* or *mutSRE* affinity column (*left panel*) or nuclear proteins bound to immobilized circular plasmid DNA in the absence or presence of 100-fold excess WT or 5'-mutI γ 3 SRE oligonucleotides (*right panel*) were resolved by SDS-PAGE, and HoxC4, Oct-1, Ku70, and Ku86 proteins were detected by Western blotting using specific mAbs.

deoxycholate, 0.5% Sarkosyl, pH 8.0), re-suspended in immunoprecipitation buffer (20 mM Tris-HCl, 200 mM NaCl, 2 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitors), and sonicated to yield 500–1000-bp DNA fragments. These were precleared with agarose beads bearing protein A or G (Santa Cruz Biotechnology, Inc.) and then incubated with mAb to HoxC4, Ab to Oct-1, or mAb to Ku70/Ku86 overnight at 4 °C. The immune complexes were isolated using beads bearing protein A or G, eluted with “elution buffer” (50 mM Tris-HCl, 0.5% SDS, 200 mM NaCl, 100 μ g/ml proteinase K, pH 8.0), and then heated at 65 °C overnight to reverse cross-links. DNA was recovered by phenol extraction and ethanol precipitation and then solubilized in Tris-EDTA buffer. The recovered DNA was specified by cloning and sequencing of the PCR product amplified using the γ 3 promoter forward (281–300, 5'-TGGTGCCGCCAGTTTCAATC-3') and reverse (444–424, 5'-GTCTCAGCCCTTCTGTTGTG-3') primers, the I ϵ promoter forward (441–461, 5'-CCAAGAACAGAGAGAAAAGGG-3') and reverse (615–598, 5'-ATCAGGCTGGGAGAGTGAGTC-3') primers, or the I α promoter forward (172–193, 5'-ACAGGGTAGAGCAGGCACCTTG-3') and reverse (353–332, 5'-ATCAGGCTGGGAGAGTGAGTC-3') primers.

RESULTS

ATTT SREs Critically Modulate I γ and I ϵ but Not I α Promoter Activity—We have suggested the existence of a repression of germ line I γ 3-C γ 3 transcription in resting human IgM⁺IgD⁺ B cells (8) and tentatively ascribed it to I γ 3 promoter tetrameric ATTT SREs. In the I γ 1, I γ 2, I γ 3, and I γ 4 promoters, such SREs exist in two identical copies, one straddling the 5' boundary of the IL-4-responsive element Stat6-binding site (IL-4 RE-Stat6) and the other straddling the 3'

boundary of the IL-4 RE-Stat6. Two SREs were also identified in the I ϵ promoter, both of them 3' of the IL-4 RE-Stat6 (Fig. 1A). Additional ATTT motifs exist in the downstream I μ -S regions and flanking areas: 3, 3, 2, 3, and 13 in the γ 1, γ 2, γ 3, γ 4, and ϵ loci, respectively. No SRE was found in the I α 1/I α 2 promoters or I α -S α regions.

Transfection of human IgM⁺IgD⁺ CL-01 B cells, our model of inducible CSR and somatic hypermutation (8, 17, 19), with I γ or I ϵ promoter-driven luciferase gene reporter pGL3 vectors containing WT or mutated (ATTT to ggTT) *mutSRE*(s) was performed to analyze the role of these SREs in I μ promoter activity (Fig. 1B). Mutation of both the 5'- and 3'-SREs (double *mutSRE*) in the I γ and I ϵ promoters resulted in a 10- (I γ 2) to 15-fold (I ϵ) increase of basal reporter gene transcription and a 4- (I γ 2) to 6-fold (I ϵ) increase of human trimeric (ht) CD40L-induced transcription (Fig. 2). This possibly reflects the suboptimal induction of transfected CL-01 cells by soluble CD40L as underscored by our previous findings that similar culture conditions induce switching in approximately one-third of CL-01 cells (17). The double *mutSRE* also reverted the ability of CD153 signaling to reduce the basal (no reduction in double *mutSRE* versus a 25–40% reduction in WT SRE) and htCD40L-induced I γ and I ϵ promoter-driven reporter gene transcription (no reduction in double *mutSRE* versus a 79–84% reduction in WT SRE). Mutation of the 5'-SRE alone resulted in enhancement of basal and htCD40L-induced I γ or I ϵ promoter-driven

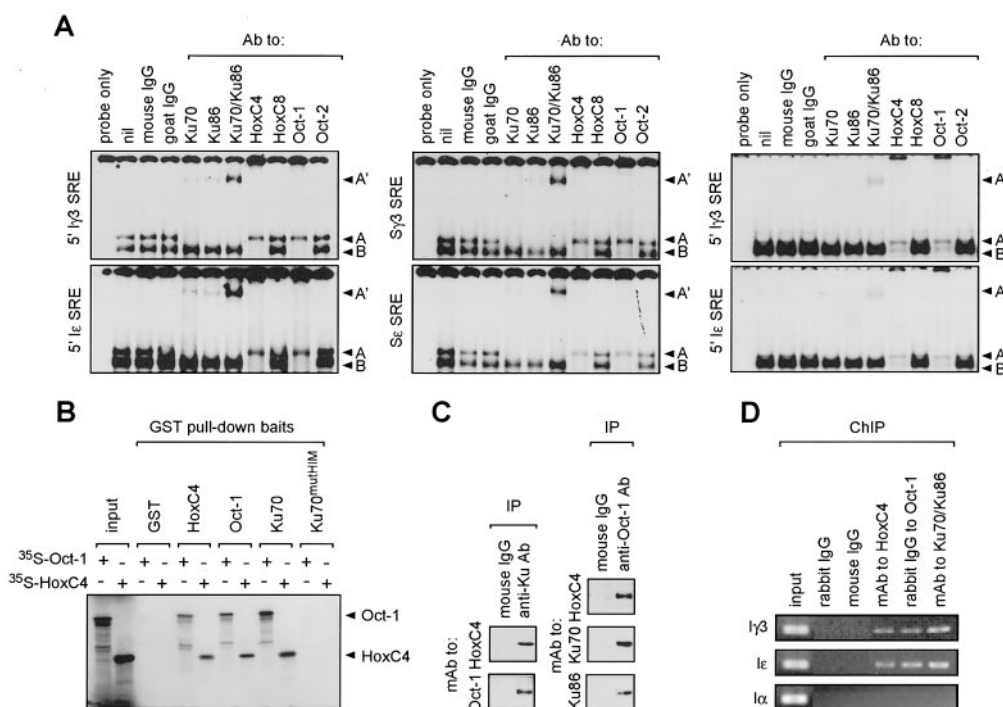


FIG. 4. HoxC4, Oct-1, and Ku70/Ku86 form a DNA-binding complex in B cell nuclei. A, composition of the Iγ3 and Iε 5'-SRE-binding protein complex as well as the Sγ3 and Sε SRE-binding protein complexes analyzed by supershift EMSA using antibodies (Ab) with the indicated specificities and nuclear extracts from freshly isolated human IgM⁺IgD⁺ B cells (left and central panels) and CL-01 IgM⁺IgD⁺ B cells (right panel). Arrowheads indicate complexes A and B as well as the supershifted complex A'. B, *in vitro* translated ³⁵S-labeled HoxC4 and Oct-1 bind to immobilized GST-HoxC4, GST-Oct-1, and GST-Ku70 but not GST or GST-Ku70^{mutHIM} fusion proteins. C, detection of nuclear HoxC4-Ku70/Ku86, Oct-1-Ku70/86, and HoxC4-Oct-1 complexes in CL-01 B cell nuclear extracts by co-immunoprecipitation and Western blotting. D, *in vivo* binding of HoxC4, Oct-1, and Ku70/Ku86 to Iγ and Iε but not Iα promoter DNA as assessed by ChIP assay. Cross-linked chromatin was precipitated from freshly isolated IgM⁺IgD⁺ B cells by Abs specific for human HoxC4, Oct-1, or Ku70/Ku86. The precipitated DNA was specified by PCR using the Iγ3, Iε, and Iα promoter primers. One of three ChIP assays is shown yielding comparable results.

transcription that was 28–56% lower than that of the double mutSRE (data of supplemental Fig. 2A versus those of Fig. 2). Finally, the absence of the ATTT SRE in Iα1/Iα2 was associated with a significantly higher basal activity of this promoter as compared with the Iγ1, Iγ2, Iγ3, Iγ4, and Iε promoters (supplemental Fig. 2B). Thus, the ATTT SREs critically mediate basal and CD153-induced inhibition of Iγ and Iε promoter-driven transcription.

Iγ and Iε SREs Recruit HoxC4, Oct-1, and Ku70/Ku86—EMSA were performed using radiolabeled oligonucleotides encompassing the Iγ3 or Iε 5'-SRE, the SRE immediately 5' of the Sγ3 region (Sγ3 SRE) or the most 5'-SRE in the Sε region (Sε SRE), and nuclear extracts from freshly isolated human peripheral blood IgM⁺IgD⁺ B cells to characterize the *trans*-factors specifically binding to the identified Iγ and Iε SREs. Two specific and closely migrating SRE-protein complexes (complexes A and B) were identified by all of the four probes (Fig. 3A). The formation of such complexes was inhibited by cold WT but not mutSRE (ATTT to ggTT) oligonucleotides containing the Iγ1, Iγ2, Iγ3, Iγ4, Iε, Sγ3, or Sε 5'- or 3'-SRE (data not shown). To analyze the composition of complexes A and B, nuclear extracts from CL-01 IgM⁺IgD⁺ B cells were subjected to sequential (NH₄)₂SO₄ precipitation, gel filtration, and SRE DNA affinity chromatography (DAC), which eventually yielded proteins of 100, 89, 72, and 34-kDa apparent molecular masses (Fig. 3, B–C). These proteins were identified as Oct-1, Ku86, Ku70, and HoxC4 by in-gel trypsin digestion, peptide mass fingerprinting, internal sequencing, and specific mAbs in eluates from a DAC column bearing SRE Iγ3 oligonucleotides (Fig. 3D, left panel). Additional proteins of 190, 144, and 120-kDa apparent molecular masses were also detected by silver staining, but they accounted for bands of minor intensity and were not sequenced (Fig. 3C). The binding of HoxC4,

Oct-1, and Ku70/Ku86 to DNA was mediated specifically by the SRE and was not attributed to "stickiness" of these proteins for free DNA ends as shown by the following: (i) the failure of HoxC4, Oct-1, and Ku70/Ku86 to bind to a mutSRE DAC column (Fig. 3D, left panel); (ii) the efficient pull-down of HoxC4, Oct-1, and Ku70/Ku86 by beads bearing circular pBSKII plasmid DNA containing 29 copies of the ATTT motif; and (iii) the inhibition of this pull-down by 100-fold molar excess of WT but not mutSRE oligonucleotides (Fig. 3D, right panel). Thus, whether in the I_H promoter or S area context, the ATTT SREs recruit the HoxC4 and Oct-1 HD proteins together with the Ku70/Ku86 heterodimer in a sequence-specific and DNA end-independent fashion.

HoxC4, Oct-1, and Ku70/Ku86 Form a DNA-binding Complex in B Cell Nuclei—The nature of the SRE-binding proteins was further verified by EMSAs utilizing nuclear extracts from freshly isolated IgM⁺IgD⁺ B cells, radiolabeled Iγ3 and Iε 5'-SRE as well as Sγ3 and Sε SRE probes, and specific anti-HoxC4, anti-Oct-1, anti-Ku70, anti-Ku86, and anti-Ku70/Ku86 mAbs. Anti-Ku70, anti-Ku86, and anti-Ku70/86 mAbs but not control HoxC8- or Oct-2-specific Abs-supershifted complex A, whereas anti-HoxC4 and anti-Oct-1 mAbs inhibited the formation of complex B (Fig. 4A). Comparable results were obtained in EMSAs utilizing Iγ3 and Iε 3'-SRE probes as well as Iγ1, Iγ2, or Iγ4 5'- and 3'-SRE probes (data not shown). Pull-down experiments using GST-HoxC4, GST-Oct-1, and GST-Ku70 fusion proteins, GSH-agarose beads, and *in vitro* translated ³⁵S-labeled HoxC4 and Oct-1 proteins premixed with freshly isolated IgM⁺IgD⁺ B cell nuclear extracts showed that HoxC4, Oct-1, and Ku effectively interact with one another in B cell nuclei. Both ³⁵S-labeled HoxC4 and ³⁵S-labeled Oct-1 bound to GST-HoxC4, GST-Oct-1, and GST-Ku70, indicating a significant self-association among HoxC4 and Oct-1 proteins as well

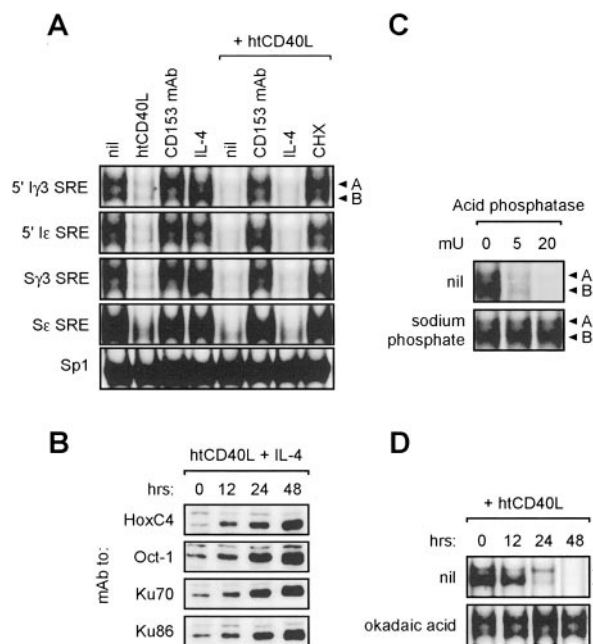


FIG. 5. CD40 signaling dissociates the HoxC4-Oct-1-Ku complex from SRE in a dephosphorylation-dependent fashion. *A*, freshly isolated IgM^+IgD^+ B cells were stimulated with nil, htCD40L, anti-CD153 mAb (M81), IL-4 alone, or with htCD40L in combination with anti-CD153 mAb, IL-4, or cycloheximide (CHX). After 48 h, nuclear proteins were extracted and tested for SRE binding activity by EMSA using the $[\gamma\text{-}^{32}\text{P}]\text{I}\gamma 3$ SRE, $[\gamma\text{-}^{32}\text{P}]\text{I}\epsilon$ 5' SRE, $[\gamma\text{-}^{32}\text{P}]\text{S}\gamma 3$ SRE, $[\gamma\text{-}^{32}\text{P}]\text{S}\epsilon$ SRE, or $[\gamma\text{-}^{32}\text{P}]\text{Sp1}$ probe. *B*, expression of HoxC4, Oct-1, and Ku70/Ku86 as detected by Western blotting and specific mAbs in nuclear extracts from freshly purified IgM^+IgD^+ B cells stimulated with htCD40L and IL-4 for the indicated time. *C*, acid phosphatase sensitivity of HoxC4-Oct-1-Ku binding to SRE. Nuclear extracts from freshly purified IgM^+IgD^+ B cells were incubated with indicated amounts of acid phosphatase in the absence or presence of 60 mM sodium phosphate for 10 min prior to the addition of $[\gamma\text{-}^{32}\text{P}]\text{I}\gamma 3$ SRE probe. Protein-DNA complexes were resolved on 7% polyacrylamide gel. *D*, freshly isolated IgM^+IgD^+ B cells were stimulated with htCD40L for the indicated times after incubation with 12 nM okadaic acid. Nuclear proteins were subsequently tested for SRE binding activity by EMSA using the $[\gamma\text{-}^{32}\text{P}]\text{I}\gamma 3$ SRE probe.

as direct physical interaction between HoxC4 and Oct-1, HoxC4 and Ku70, and Oct-1 and Ku70 (neither ^{35}S -labeled HoxC4 nor ^{35}S -labeled Oct-1 bound to GST alone) (Fig. 4B). Consistent with the critical role of the C-terminal HIM (24) in Ku70 binding, GST-Ku70^{mutHIM}, a GST fusion protein encoding Ku70 lacking its HIM, reacted with neither ^{35}S -labeled HoxC4 nor ^{35}S -labeled Oct-1. Also, a mAb that specifically recognizes the Ku70/Ku86 heterodimer interface (26) co-precipitated HoxC4 and Oct-1 from CL-01 nuclear extracts, and an anti-Oct-1 mAb co-precipitated HoxC4, Ku70, and Ku86 (Fig. 4C), indicating that Ku interacts with these HD proteins in B cell nuclei. Finally, ChIP assays in which the I γ and I ϵ promoter sequences were specified in the DNA that had been precipitated from freshly isolated IgM^+IgD^+ B cells by anti-Oct-1 Ab, anti-HoxC4 mAb, or anti-Ku mAb demonstrated direct binding of HoxC4-Oct-1-Ku to the I γ and I ϵ but not I α promoters (Fig. 4D). Thus, HoxC4, Oct-1, and Ku70/Ku86 can exist as discrete components of a HD-dependent nuclear complex and specifically bind to the I γ and I ϵ promoters and S region DNA *in vitro* and *in vivo*.

CD40 and IL-4 Signaling Dissociates HoxC4, Oct-1, and Ku70/Ku86 from SRE in a Dephosphorylation-dependent Manner—If binding of HoxC4-Oct-1-Ku to the I H promoter SREs is responsible for the basal repression of germ line I H -C H transcription, then htCD40L-induced germ line I H -C H transcription and subsequent CSR should entail the dissociation of the

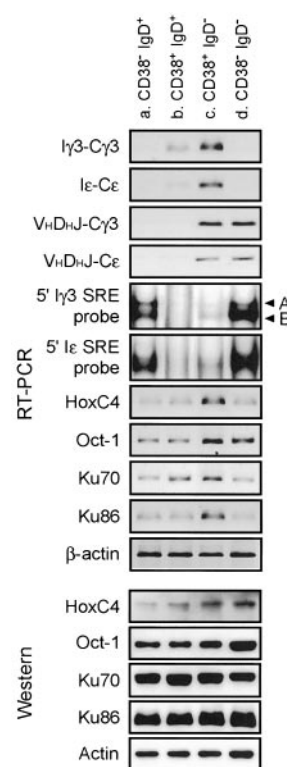


FIG. 6. *In vivo* dissociation of the HoxC4-Oct-1-Ku complex from SRE is associated with germ line I $\gamma 3$ -C $\gamma 3$ and I ϵ -C ϵ transcription and CSR to C $\gamma 3$ and C ϵ . Human tonsil B lymphocytes were separated into four discrete fractions representing sequential stages of differentiation: $\text{IgD}^+\text{CD}38^-$ naive pre-GC B cells (*a*), $\text{IgD}^+\text{CD}38^+$ early centroblasts (*b*), $\text{IgD}^-\text{CD}38^+$ centroblasts/centrocytes (*c*), and $\text{IgD}^-\text{CD}38^+$ memory B cells (*d*). These B cells were analyzed for their content in I $\gamma 3$ and I ϵ SRE-binding HoxC4-Oct-1-Ku complexes A and B as detected by EMSA utilizing 5'-SRE and 3'-SRE probes (data not shown). I $\gamma 3$ -C $\gamma 3$, I ϵ -C ϵ , V H DJ H -C $\gamma 3$, V H DJ H -C ϵ , HoxC4, Oct-1, Ku70, and β -actin transcripts were analyzed by specific RT-PCR. HoxC4, Oct-1, Ku70, Ku86, and actin proteins were detected by immunoblotting using specific mAbs.

HoxC4-Oct-1-Ku complex from SREs, and this dissociation should be prevented by physiological CD40-signaling inhibitors such as CD153 (15). EMSAs using I $\gamma 3$ and I ϵ promoter 5'-SRE as well as 5'-S $\gamma 3$ and S ϵ SRE probes showed that freshly isolated IgM^+IgD^+ B cells cultured for 2 days with either htCD40L alone or htCD40L and IL-4 but not an agonistic anti-CD153 mAb or IL-4 alone decreased the level of SRE-bound HoxC4-Oct-1-Ku complexes A and B by >95% (Fig. 5A). Comparable results were obtained utilizing I $\gamma 3$ and I ϵ 3'-SRE probes as well as I $\gamma 1$, I $\gamma 2$, or I $\gamma 4$ 5'- and 3'-SRE probes (data not shown). The htCD40L-induced dissociation of HoxC4-Oct-1-Ku from SREs was efficiently inhibited by cycloheximide, a protein synthesis inhibitor, or CD153 cross-linking, which has been shown to dampen germ line I H -C H transcription and repress CSR (15, 27). It was concomitant with increase of nuclear HoxC4, Oct-1, and Ku70/Ku86 proteins (Fig. 5B), suggesting a posttranslational modification in the htCD40L-induced dissociation of HoxC4-Oct-1-Ku from SRE. Incubation of nuclear extracts from freshly isolated IgM^+IgD^+ B cells with increasing amounts of acid phosphatase prior to the addition of the SRE probes and separation on native gel resulted in decreased SRE-binding by HoxC4-Oct-1-Ku (Fig. 5C). This was prevented by sodium phosphate, an inhibitor of acid phosphatase. Accordingly, pretreatment of B cells with okadaic acid, a Ser/Thr phosphatase inhibitor, efficiently abrogated the CD40-induced dissociation of HoxC4-Oct-1-Ku from the SRE (Fig. 5D).

To prove that *in vivo* activation of germ line I H -C H transcrip-

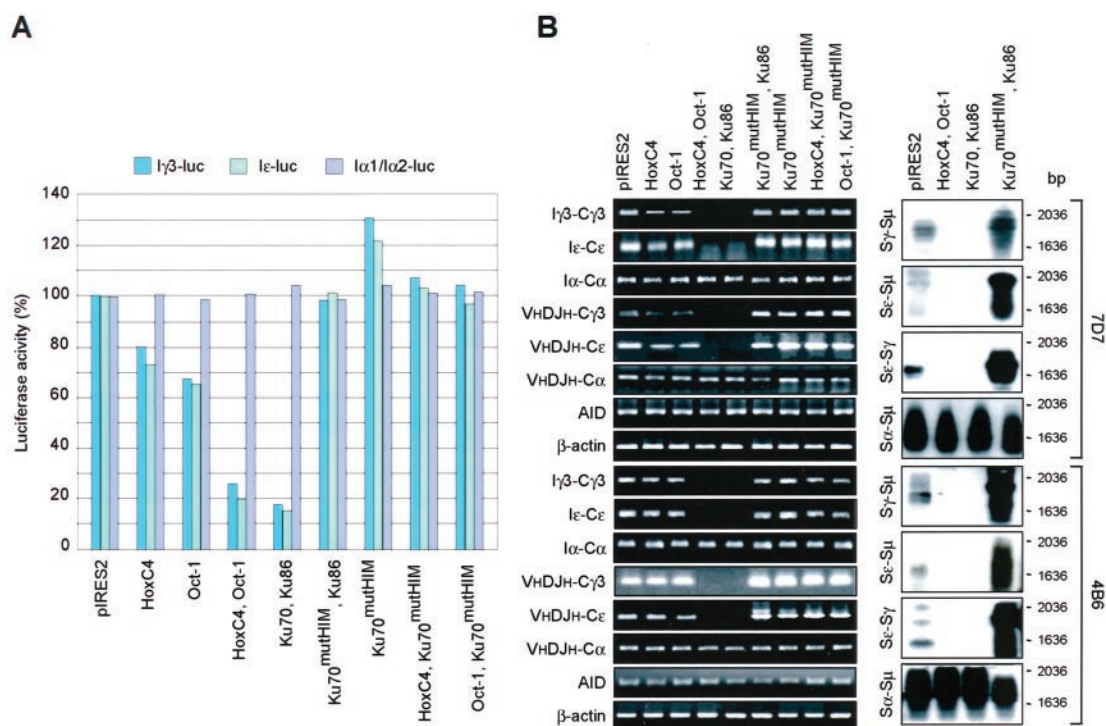


FIG. 7. Overexpression of HoxC4, Oct-1, and Ku70/Ku86 dampens germ line I γ -C γ and I ϵ -C ϵ transcription and represses CSR to C γ and C ϵ but not germ line I α -C α transcription and CSR to C α . A, 7D7 B cells were transiently co-transfected with pIRES2 expression constructs encoding the indicated proteins as well as with I γ 3, I ϵ , or I α 1/ α 2 promoter-driven luciferase (*luc*) gene reporter pGL3 vectors. Luciferase activity was measured after 24 h, normalized, and expressed as the percentage of the activity measured in cells transfected with empty pIRES2 vector. Comparable results were obtained in experiments involving I γ 1, I γ 2, or I γ 4 promoter-driven reporter vectors and 4B6 B cells (data not shown). B, 7D7 and 4B6 B cells were transfected with empty pIRES2 vectors or with pIRES2 expression vectors encoding the indicated proteins. Transfected cells were cultured in complete medium for 4 days. Sorted GFP^{high} cells were analyzed for expression of germ line I γ 3-C γ 3, I ϵ -C ϵ , and I α 1/2-C α 1/ α 2 transcripts; mature V_HDJH-C γ 3, V_HDJH-C ϵ , and V_HDJH-C α 1/ α 2 transcripts; and AID and β -actin transcripts (*left panels*) as well as S γ -S μ , S ϵ -S μ , and S α -S μ SCs (*right panels*). S γ -S μ , S ϵ -S μ , and S α -S μ SCs represent SCs from all of the four γ isotypes; S α -S μ SCs include SCs from both α 1 and α 2 isotypes.

tion and CSR, as that occurring in the GC of peripheral lymphoid organs, is associated with the CD40L-dependent dissociation of the HoxC4-Oct-1-Ku complex from the I_H promoter, we sorted human tonsil B lymphocytes into four fractions representing sequential stages of differentiation as follows: IgD⁺CD38⁻ naive pre-GC B cells; IgD⁺CD38⁺ early centroblasts; IgD⁺CD38⁺ centroblasts/centrocytes; and IgD⁻CD38⁻ memory B cells (17). Germ line I_H-C_H transcription is absent in pre-GC B cells, appears in early centroblasts, peaks in centroblasts/centrocytes, and is extinct in memory B cells, whereas mature V_HDJH-C γ and V_HDJH-C ϵ transcripts appear as a result of downstream CSR in centrocytes and are consistently expressed in memory B cells (17). Accordingly, pre-GC and memory B cells exhibited a strong SRE binding activity, which was consistent with the lack of I γ -C γ and I ϵ -C ϵ transcription in these lymphocytes (Fig. 6). In contrast, early centroblasts and centroblasts/centrocytes, which harbored germ line I γ -C γ and I ϵ -C ϵ as well as mature V_HDJH-C γ and V_HDJH-C ϵ (centroblasts/centrocytes only) transcripts, were devoid of HoxC4-Oct-1-Ku SRE binding activity. Consistent with the kinetics of *in vitro* induction by htCD40L and IL-4, CD38⁺ B cells up-regulated HoxC4, Oct-1, Ku70, and Ku86 transcripts. Up-regulation of the HoxC4 transcripts was reflected in the up-regulation of the related proteins, whereas the Oct-1, Ku70, and Ku86 proteins were abundant prior to up-regulation of the respective transcripts. Thus, CD40 and IL-4 signaling, which promotes germ line I_H-C_H transcription and triggers CSR to C γ and C ϵ , dissociates the HoxC4-Oct-1-Ku inhibitory complex from the SREs of the I γ and I ϵ promoters and 3'-flanking regions *in vitro* and *in vivo*.

Overexpression of HoxC4, Oct-1, and Ku70/Ku86 Represses CSR to C γ and C ϵ but Not C α —To prove that HoxC4, Oct-1,

and Ku70/Ku86 critically repress germ line I_H-C_H transcription as well as CSR in the C γ and C ϵ loci, we co-transfected 7D7 and 4B6 IgM⁺IgD⁺ B cells, both CL-01 cell subclones selected for spontaneous switching to IgG, IgA, and IgE, with a pIRES2 expression vector containing nil or cDNA encoding HoxC4, Oct-1, Ku70, Ku70^{mutHIM}, and/or Ku86 together with the I γ 3, I ϵ , or I α 1/ α 2 promoter-driven luciferase gene reporter pGL3 vector. Overexpression of HoxC4 or Oct-1 alone reduced only moderately the activity of co-transfected I γ 3 or I ϵ promoters, whereas overexpression of both HoxC4 and Oct-1 or Ku70/Ku86 reduced the I γ 3 or I ϵ promoter activity by up to 85% but had no effect on basal I α 1/ α 2 promoter activity (Fig. 7A). This was specific, because no inhibition could be measured when double mutSRE I γ 3 and I ϵ promoters were used (supplemental Fig. 7A). The C-terminal HIM was critically required, because overexpression of Ku70^{mutHIM} and Ku86 or Ku70^{mutHIM} alone failed to inhibit I γ 3 and I ϵ promoter-driven gene reporter transcription and ablated the HoxC4- or Oct-1-mediated inhibition of I γ 3 and I ϵ promoter activity.

The inhibition of I γ and I ϵ promoter-driven transcription by HoxC4, Oct-1, and Ku70/Ku86 reflects the ability of these *trans*-factors to effectively dampen endogenous germ line I_H-C_H transcription and repress CSR to C γ and C ϵ . 7D7 and 4B6 B cells were transfected with a bicistronic pIRES2 expression vector encoding GFP and HoxC4, Oct-1, Ku70, Ku70^{mutHIM}, and/or Ku86. After culture, B cells with high GFP expression were sorted and used as a source of mRNA and genomic DNA for the analysis of germ line I_H-C_H, mature V_HDJH-C_H (detected as FR3-C_H sequences) and AID transcripts as well as Sx-S μ , and Sx-S γ SCs. Overexpression of HoxC4 and/or Oct-1 or Ku70/Ku86 repressed endogenous I γ 3-

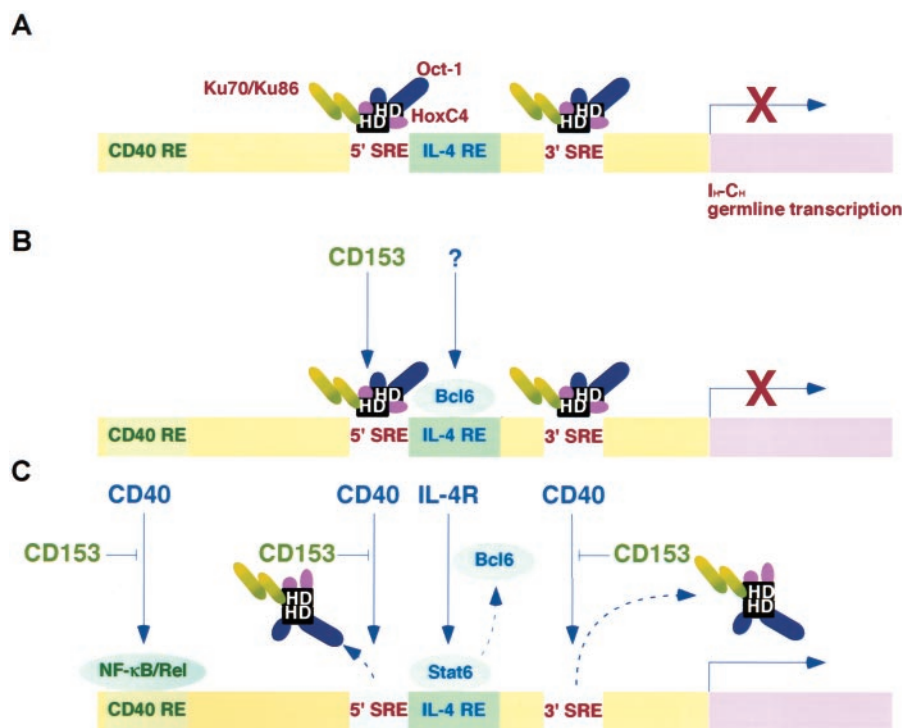


FIG. 8. Role of the Ku-HoxC4-Oct-1 complex in the regulation of CSR. A, HoxC4-Oct-1-Ku binds to the I γ and I ϵ promoter SREs in non-CD40-induced (pre-GC) B cells and exerts a basal transcriptional and recombinational repression on the C ϵ and C γ loci. Depicted is the 5'-SRE, IL-4 RE-Stat6, and 3'-SRE configuration of the human I γ 1, I γ 2, I γ 3, and I γ 4 promoters. In the I ϵ promoter, the IL-4 RE-Stat6 lies upstream of the two SREs and does not overlap with the 5'-SRE. B, the possible binding of Bcl6 as expressed in GC B cells to the IL-4 RE-Stat6 would further enhance the HoxC4-Oct-1-Ku complex-mediated inhibition. C, CD40 engagement and exposure to IL-4 would not only induce the binding of Stat6 to the IL-4 RE and displace Bcl6 from it, they would also dissociate HoxC4-Oct-1-Ku from the SREs, thereby lifting the inhibition off of the Ig H chain locus. This, together with the binding of NF- κ B to the CD40 RE, would allow I ϵ -C ϵ transcription and CSR to unfold. Engagement of CD153 by CD30 as expressed on activated suppressor T cells (15) inhibits not only the CD40-induced activation of NF- κ B/Rel but also the CD40-dependent dissociation of HoxC4-Oct-1-Ku from the SREs, thereby exerting a net repressive activity on I ϵ -C ϵ transcription and CSR.

C γ 3 and I ϵ -C ϵ transcripts, direct S μ $\rightarrow$ S γ , S μ $\rightarrow$ S ϵ , and sequential S γ $\rightarrow$ S ϵ CSR as indicated by the low levels of S γ -S μ , S ϵ -S μ , and S ϵ -S γ SCs, and mature V_HDJ_H-C γ 3 and V_HDJ_H-C ϵ transcripts. It did not affect germ line I α 1/I α 2-C α 1/C α 2, AID, and β -actin transcripts or CSR to C α 1/C α 2 as indicated by the normal level of S α -S μ SCs and mature V_HDJ_H-C α 1/C α 2 transcripts (Fig. 7B). Accordingly, it significantly decreased the concentration of IgG and IgE but not IgA in the culture fluids without affecting cell viability or proliferation (data not shown). Overexpression of HoxC4 or Oct-1 alone partially lowered the levels of germ line I γ 3-C γ 3 and I ϵ -C ϵ transcripts as well as mature V_HDJ_H-C γ 3 and V_HDJ_H-C ϵ transcripts, although in some experiments, a more profound repression was observed (data not shown). Consistent with the failure to repress basal I γ 3 and I ϵ promoter-driven reporter gene transcription, overexpression of Ku70^{mutHIM}/Ku86 failed to affect the level of endogenous I γ 3-C γ 3 and I ϵ -C ϵ transcripts. It also resulted in higher levels of mature V_HDJ_H-C γ 3 and V_HDJ_H-C ϵ transcripts as well as S γ -S μ and S ϵ -S μ and S ϵ -S γ SCs without affecting the level of S α -S μ SCs and mature V_HDJ_H-C α 1/ α 2 transcripts. An analysis of germ line and mature transcripts in the C γ 1 (supplemental Fig. 7B), C γ 2, or C γ 4 loci yielded comparable results (data not shown). Thus, HoxC4, Oct-1, and Ku70/Ku86 effectively repress germ line I γ -C γ and I ϵ -C ϵ transcription and CSR to C γ and C ϵ but not germ line I α -C α transcription and CSR to C α 1/C α 2 in a fashion that is dependent on the HIM of Ku70.

DISCUSSION

We have defined here a novel mechanism that inhibits class switching to IgG and IgE but not IgA. By identifying the key elements of this inhibitory mechanism, we provide the first

evidence for an important role of a Hox protein and a novel function for Oct-1 and Ku70/Ku86 in B cell differentiation. We show that HoxC4 and Oct-1 together with the Ku70/Ku86 heterodimer form a HD-dependent complex, which is recruited to ATTT motifs in the human I γ and I ϵ promoters to dampen germ line I γ -C γ and I ϵ -C ϵ transcription and repress direct and sequential CSR to C γ and C ϵ . The HoxC4-Oct-1-Ku-dependent inhibitory mechanism is operational in the presence of AID that plays a critical role in CSR (28) and would provide a threshold of transcriptional and recombinational inertia that must be overcome for effective initiation and unfolding of CSR, thereby contributing to the homeostasis of the H chain locus.

Hox proteins are phylogenetically conserved HD-containing *trans*-factors that serve principally as transcriptional repressors. They modulate transcription by binding to the HD-specific ATTT/A core-motif (29, 30) and critically regulate not only embryonic pattern formation, axis specification, and organogenesis (31) but also adult cellular processes including selective hematopoietic lineage differentiation and stem cell renewal (32). Oct-1 is a ubiquitous member of the POU (Pit, Oct, Une) family of transcription factors that regulates both general and cell type-specific genes (33) including V and C genes in the Ig locus (34). Ku70/Ku86, the ATP-dependent DNA helicase II subunits of the DNA-dependent protein kinase, serves as DNA end-binding and alignment factors in NHEJ DNA repair. NHEJ is critical not only in Ig V(D)J gene recombination and CSR but also in overall genome maintenance (3, 35).

The HoxC4-Oct-1-Ku complex may function as a common effector in the modulation of IgG and IgE class switching at different stages of the B cell natural history. In pre-GC and perhaps memory B cells, the complex would maintain the basal

repression of CSR in the C γ and C ϵ loci. The partial overlap of the 5'-SRE with the IL-4 RE-Stat6 (I γ) or the proximity of these *cis*-elements (I ϵ) would entail a complex regulation of the I γ and I ϵ promoters, allowing for competition and/or interplay among the respective *trans*-factors. In GC B cells, CD40 engagement and exposure to IL-4 induce the binding of NF- κ B to the CD40 RE and binding of Stat6 to the IL-4 RE. This would result in Bcl6 displacement (36) and dissociation of HoxC4-Oct-1-Ku from the SREs, which would in turn lift the inhibition off of the Ig H chain locus and activate germ line I γ -C γ and I ϵ -C ϵ transcription and CSR to C γ and C ϵ (Fig. 8). These processes are counteracted by a CSR inhibitory signal from B cell CD153 upon engagement by CD30 on suppressor T cells. Here, we demonstrate that CD153 signaling, which inhibits the CD40-induced activation of NF- κ B (14, 15), effectively prevents the CD40-dependent dissociation of HoxC4-Oct-1-Ku from SREs. Thus, in addition to repressing basal germ line I H -C H transcription and CSR in non-CD40-induced (pre-GC) B cells, HoxC4-Oct-1-Ku mediates the CD153-dependent inhibition of germ line I H -C H transcription and CSR in CD40-induced (GC) B cells.

Our findings suggest a role for a CD40 signaling-induced Ser/Thr phosphatase in the dissociation of the HoxC4-Oct-1-Ku complex from SREs. *In vitro* Ser/Thr-specific dephosphorylation of HoxC4-Oct-1-Ku abrogated their SRE binding, and pretreatment of freshly isolated B cells with okadaic acid prevented CD40-induced dissociation of HoxC4-Oct-1-Ku from SREs (Fig. 5D). A similar dephosphorylation-dependent regulation of Hox protein activity has been reported previously (37) in *Drosophila melanogaster*. The overexpression of HoxC4-Oct-1 and/or Ku70/Ku86 (Fig. 7) would probably overcome the dephosphorylating activity of endogenous phosphatase(s), thereby allowing for the expression of the CSR inhibitory activity by the repressor complex. Upon CD40-induced dissociation from SREs, the amount of HoxC4, Oct-1, and Ku proteins increased in cell nuclei (Fig. 5B), probably as a result of increased transcription and protein synthesis (HoxC4 and Oct-1) or cytoplasmic-to-nuclear translocation (Ku70/Ku86) (38), suggesting that these proteins play a role in later CSR-related events such as NHEJ (39) or regulate transcription of CSR-related genes by binding other HD recognition sequences.

For efficient inhibition of I γ and I ϵ promoter activity, HoxC4 and Oct-1 rely on the recruitment of the Ku70/Ku86 heterodimer. Overexpression of Ku70^{mutHIM} reverted HoxC4- and Oct-1-dependent I H promoter inhibition and enhanced basal promoter activity (Fig. 7, A and B), possibly through displacement of endogenous Ku70 and formation of Ku70^{mutHIM}/Ku86 heterodimers, which would effectively reduce the availability of the functional endogenous Ku70/Ku86 heterodimer but not associate with HD proteins. Importantly, forced expression of HoxC4 and Oct-1 as well as Ku70/Ku86 effectively repressed both germ line I γ -C γ and I ϵ -C ϵ transcription and CSR to C γ and C ϵ . Again, this repression was dependent on the ability of Ku70 to interact with HD proteins, because overexpression of the Ku70^{mutHIM}/Ku86 heterodimer failed to repress germ line I H -C H transcription and enhanced CSR to IgG and IgE as detected by the increased level of mature V H DJ H -C γ and V H DJ H -C ϵ transcripts and reciprocal SCs. The mechanism of Ku-dependent transcriptional inhibition was not addressed in this study. It may include DNA-dependent protein kinase-dependent phosphorylation of I H promoter- and/or S region-bound *trans*-factors (40), inhibition of histone acetyltransferases (41), or recruitment of histone deacetylases including Sir2-related proteins (42).

Inhibition of IgG and IgE class switching by HoxC4-Oct-1-Ku is consistent with the C γ - and C ϵ -shared CSR activation path-

way, which includes CD40 and IL-4R signaling, and possibly reflects the co-evolution of the C γ and C ϵ loci arising from the duplication of a single ancestral locus (43, 44). The lack of ATTT motifs within the I α 1/I α 2 promoters accounts for the failure of HoxC4-Oct-1-Ku to inhibit IgA class switching and emphasizes the difference in regulation (transforming growth factor- β *versus* IL-4) and ancestral origin (early *versus* late) of the C α 1/C α 2 *versus* the C γ and C ϵ loci. Also, it probably underlies the T cell CD40L independence and distinct anatomical compartmentalization of IgA-secreting cells, which are mainly CD5⁺ (B-1 lymphocytes). B-1 lymphocytes accumulate preferentially in the splanchic district and near external membranes and play a critical role in the first line of defense against microbial pathogens (45). Similar to B-1 cells, IgA appears early in phylogeny, emerging prior to IgG and IgE as the first of the "mature" isotypes in birds. The lack of regulation of IgA class switching by HoxC4-Oct-1-Ku may be compensated by other mechanisms. In the mouse, germ line I α -C α transcription and S μ $\rightarrow$ S α CSR are repressed by B cell lineage-specific activator protein, which binds to a specific *cis*-element within the I α promoter (46) or by the late SV40 factor, which binds to appropriately spaced CTGG repeats within S μ and S α regions, thereby recruiting histone deacetylases and the co-repressor Sin3A (47).

The inhibitory activity unveiled here is probably part of a broader regulation of the Ig H chain locus by HoxC4-Oct-1-Ku. Our preliminary experiments suggest that these *trans*-factors also regulate the human H chain 3'-hs1,2 enhancer element, which probably plays a role in Ig class switching.² Because of the wider recurrence of ATTT motifs in the human genome, HoxC4-Oct-1-Ku or other HD protein-Ku complexes could be involved in general transcriptional inhibition and anti-recombinogenic functions as part of the overall genomic caretaker activity as suggested by the extreme genomic instability of Ku70^{-/-} and Ku86^{-/-} mice (35). Dysregulation of the HoxC4-Oct-1-Ku-mediated inhibitory function could cause aberrant CSR and chromosomal translocation and contribute to B cell lymphomagenesis.

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