The immunoglobulin heavy chain (IgH) 3′ regulatory region modulates IgH locus transcription, upon induction by specific trans-acting factors, and plays a significant role in class switch DNA recombination (CSR) and, perhaps, somatic hypermutation (SHM). CSR and SHM are central to the maturation of the antibody response. In contrast to the single 5′-hs3a-hs1,2-hs3b-hs4-3′ mouse IgH 3′ regulatory region, the human IgH 3′ regulatory region exists as a 5′-hs3a-hs1,2-hs4-3′ cluster duplicated 3′ of Cε1 and Cε2. We show here that the human hs1,2 element is the strongest enhancer of transcription, as directed by a Vδ1 or the ECS-Iγ3 promoter, thereby suggesting a dominant role for hs1,2 over hs3 and hs4 in the overall activity of the 3′ regulatory region. Within hs1,2, we identified three regions (1, 2, and 3) that are all necessary, but individually not sufficient, for enhancement of transcription. In region 2, a HoxC4 site and a HoxC4/embedded octamer (HoxC4/Oct) site are conserved across human, mouse, rat, and rabbit. These two sites recruit HoxC4 and Oct-1/Oct-2, which act synergistically with the Oca-B coactivator to effect the full hs1,2-enhancing activity. HoxC4, Oct-1/Oct-2, and Oca-B recruitment is negligible in pro-B cells, moderate in pre-B cells, and maximal in germinal center B cells and plasma cells, where HoxC4, Oct-2, and Oca-B expression correlates with hs1,2 activation and ongoing CSR. The hs1,2-mediated enhancement of VγH and CγH promoter-driven transcription as induced by HoxC4 and Oct-1/Oct-2 suggests an important role of these homeodomain proteins in the overall regulation of the IgH locus expression.

Gene transcription of the Ig heavy (H) and light (L) chain locus proceeds in a lymphoid-restricted and developmental stage-specific fashion, leading to Ig V(D)J recombination and the emergence of mature B cells expressing unique receptors for antigen. Upon encounter with antigen, B cells undergo somatic hypermutation (SHM) and class switch DNA recombination (CSR) to produce affinity-mature, isotype-switched antibodies. Like V(D)J recombination, SHM and CSR are critically dependent on transcription, as driven by three main cis-regulatory regions: the promoter upstream of each V gene (V), the IgH evolutionarily conserved sequence (ECS)-interrupting (I) region promoter, which is located upstream of each constant region (CγH) exon cluster, and the IgH intrinsic enhancer (iEγ2) (1–4). The identification of an additional cis-regulatory region was suspected after mouse cell lines lacking the iEγ2 enhancer were found to still effectively transcribe IgH genes and a mouse cell line containing an intact iEγ2 enhancer but showing a large deletion of sequence downstream of the Ca gene showed decreased IgH transcription (5, 6).

Indeed, a second B cell-specific regulatory region was identified ∼25 kb downstream of the rat Ca gene and 16 kb downstream of the mouse Ca gene, with 82% sequence identity (7–9). The murine 3′ regulatory region consists of five B cell–specific DNase I hypersensitivity sites, each characterized as an IgH 3′ enhancer (3′EH): hs3a, hs1,2, hs3b, and hs4, with hs1,2 lying at the center of a region of symmetry flanked by inverted repeat sequences (6). hs1–4 collectively function as a locus control region (LCR) (5, 6), as suggested by the position-independent and copy number–dependent deregulation of c-MYC expression in plasmacytoma cells transfected with a hs1,2-hs3b-hs4-linked c-MYC construct (10). Additional sequences may be required to allow the murine hs1–4 enhancers to act as a classical LCR, as in transgenic mice harboring a Vδ1 promoter-β-globin reporter gene linked to the Ig 3′EH regulatory region, transgene expression was strictly confined to B cells, and reporter gene activity was integration-independent but not copy number–dependent (11).

A role for the mouse hs1,2 and hs3a enhancers in CSR to IgG2b and IgE has been suggested by Cre/loxP gene targeting experiments (12). These extended previous findings obtained by replacement of DNA encompassing the mouse hs1,2 element with the neomycin (neo) gene (13). Further experiments involving hs3b and hs4 and Cre/loxP knockouts showed severe impairment of germ-line IgH-CγH transcription and CSR to IgG2a, IgG2b, IgG3, IgE, and IgA, indicating that the distal portion of the regulatory region is required for CSR to most isotypes (14).
The role of the 3′ regulatory region in SHM awaits better definition. The use of a transgenic construct containing murine hs1,2 suggested that this element does not play a role in SHM, even when coupled with the iκB enhancer (15). In contrast, the use of a transgenic construct containing the distal hs3b and hs4 elements, but not hs1,2, resulted in an increase in the SHM level of transgenomes driven by a V H promoter, pointing to a role for hs3b and hs4 in SHM (15, 16). However, more recent experiments have indicated that hs3b and hs4 are dispensable for SHM and V pI D M1 gene assembly (17).

The human IgH 3′ regulatory region comprises the B specific DNase I hypersensitivity sites hs1,2, hs3, and hs4, which are arranged in the 5′-hs3-hs1,2-hs4 3′ sequence and duplicated as discrete enhancer clusters 3′ of Cα1 and 3′ of Cα2 (supplementary Fig. S1), with the Cα1 and Cα2 hs1,2 sequences inverted with respect to each other (18, 19). Like their murine counterparts, the human 3′ E H hs1,2 and hs4 elements are induced by the Oct-2 transcription factor (trans-factor) and its interacting coactivator Oca-B (6). In contrast to the mouse, negative regulatory mechanisms would not be conserved, as binding sites for B cell-specific activator protein (BSAP), a repressor of the mouse 3′ E H hs1,2, are lacking in human hs1,2 (6). Human 3′ E H elements have been shown to enhance transcription as driven by a mouse Vκ or Vλ promoter or a human E-CS1 or E-CS1′3′ promoter (18–21). Like its murine counterpart, the human 3′ E λ regulatory region likely functions as an LCR and may play a role in V λ gene rearrangement, CSR, and SHM (2, 6).

To identify the elements involved in the induction of the human IgH 3′ regulatory region, we created hs1,2, hs3, and hs4 enhancer-DNA constructs and 5′, 3′, and internal deletion hs1,2 mutants, and inserted them into luciferase (luc) reporter gene vectors driven by a human V H or E-CS1′H promoter. By bearing the luc gene downstream of the promoter and upstream of hs1,2, hs3, or hs4, these vectors mimicked the physiological promoter-gene-enhancer structure found in the IgH locus. In addition, we generated hs1,2 enhancer constructs containing mutations of selected cis-elements and enforced expression of respective trans-factors. Finally, to determine the stages of B cell ontogeny at which the human hs1,2 is activated, we transfected pro-B cells, pro-B cells, early and late germinal center B cells, and plasma cells with a reporter gene vector containing a fully inducible hs1,2 enhancer construct, as driven by the human β-globin promoter. We then measured the enhancement of such a promoter activity and analyzed it with the levels of endogenous germ-line H C T1 and mature V pI D M1-C λ transcripts, the expression of trans-factors targeting hs1,2, and the formation of specific nucleoprotein complexes.

**EXPERIMENTAL PROCEDURES**

**Human IgH 3′ Regulatory Region**—The human hs1,2, hs3, and hs4 DNA sequences were from the 3′ E H cluster lying 3′ of the Cα2 exon (19). The 1079-bp hs1,2 DNA spans residues 222–1400 according to the nomenclature of this genomic clone (GenBankTM accession no. AF137724 and U84574); the 695-bp hs3 DNA spans residues 526–1221 (GenBankTM accession no. AF013719 and Y14406); and the 426-bp hs4 DNA spans residues 1–426 (GenBankTM accession no. AF013726). The hs1,2 region 1 G-rich repeats were identified by Pustell DNA matrix analysis (22), enabling the search for regions of high similarity between two nucleic acid sequences using a dot matrix plot. The human, rat, and rabbit hs1,2 region 2 sequences were compared using the ClustalW algorithm (23) allowing for multiple alignments of nucleotide sequences. All sequence comparisons were implemented by MacVector, version 6.3.3 (Accelrys Inc., San Diego, CA). The identification of putative cis-regulatory binding sites was performed using MatInspector (www.genomatix.de/cgibin/matinspector/matinspector.pl), which utilizes the TRANSFAC library of matrices (www.gene-regulation.de/) to locate consensus matches in nucleotide sequences (24).

**Vectors**—The pGL3-Basic luc-reporter gene vector (Promega Corp., Madison, WI) was modified by inserting three different promoter sequences between the SacI and BglII restriction sites: −449+265 E-CS1′H3′ promoter DNA (GenBankTM accession no. S79588) (25). V pI promoter DNA isolated from the IgG, mAb57-producing cell line (26, 27), or the human β-globin promoter DNA (GenBankTM accession no. U01317). The pGL3 vectors containing the V pI, E-CS1′H3′ or β-globin promoter were further modified by inserting between the BamHII/Sall restriction sites the hs3, hs1,2, and hs4 elements, resulting in the positioning of each 3′-2 kb downstream of the respective promoter and immediately flanking the firefly luc gene. To generate sequential 5′- and 3′-end truncation mutants, hs1,2 was PCR-amplified using appropriate primers with BamHII/Sall overhangs. Internal hs1,2 deletion mutants were generated by first amplifying 5′- and 3′-halves, minus the targeted sequence, ligating the two fragments, and re-amplifying the “complete” transcription of the 5′ and 3′ PCR fragments and immediately flanking the targeted motifs generated site-targeted mutations of the 5′-HoxC4 and 3′-HoxC4/Oct-binding sites. The 5′ HoxC4 site (ATTTT, residues 715–718) was replaced by cggg. The HoxC4/Oct site (ATTTGCGAT, residues 773–780) was replaced with a KpnI restriction site. The double mutant hs1,2 DNA was generated using the single 5′ HoxC4 and 3′ HoxC4/Oct mutants as templates. All digested PCR products were gel-purified and subcloned into the pGL3 vectors driven by the V pI or E-CS1′H3′ promoter.

For enforced expression studies, cDNA encoding human HoxC4, Oct-1, Oct-2, or Oca-B was subcloned into pcDNA3.1 vectors using the pcDNA3.1/V5-His TOPO TA expression kit (Invitrogen, Carlsbad, CA). The pcDNA3.1 expression vector contains a cytomegalovirus promoter for high level expression and a T7 promoter for in vitro translation using the T7 T Quick Coupled Translation/Translation System (Promega Corp.). The expression vector encoding the dominant negative HoxC4 lacking the homeodomain was described (28). The glutathione S-transferase (GST) fusion proteins were generated by subcloning human HoxC4, Oct-1, Oct-2, and Oca-B cDNA into the pGEX-6P-1 vector as described previously (28). GST fusion proteins were expressed in BL21 bacteria, purified using GSH-agarose beads according to the manufacturer’s protocol (Sigma-Aldrich) and analyzed for homogeneity by SDS-PAGE and silver staining. Proteins were eluted in 15 mM reduced glutathione in 50 mM Tris buffer, pH 8.0. Restriction enzyme mapping and DNA sequencing were used to verify all of the correct plasmids.

**Cell Lines**—RS4;11 is a human cytoplasmic and surface IgM′-pro-B cell line (29). Nalm-6 is a human cytoplasmic IgM′- and surface IgM′-pre-B cell line (30). 4B6 is an IgM′, IgD′ cell line with an early germinal center phenotype and undergoes spontaneous switching to IgG, IgE, and IgA (28). It was derived from our inbred C57BL/10J IgM′, IgD′-B cell line (31–33) by sequential subcloning and selection for IgG, IgA, and IgE secretion. HS Sultan is a human IgG′κ Burkitt’s lymphoma with a late germinal center phenotype (34). U266 is a human myeloma IgE′κ cell line (35). All cell lines were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Hyclone), 2 mM glutamine, and 10% heat-inactivated fetal calf serum in a 5% CO2 incubator at 37°C in a humidified atmosphere. For labor-intensive experiments, colonies were plated in complete media and cultured for 2–10×103 days. For cell lines (10×106), PBL, or BM, cells were cultured for 24–48 h. HoxC4 transfections were performed using an MLX microtiter plate luminometer (Thermo Labsystems, Beverly, MA).

**Antibodies**—mAbs to Ku70 (Ab-5), Ku86 (Ab-2), and Ku70/Ku86 (Ab-7) were from Lab Vision/NeoMarkers (Fremont, CA). The anti-Oct-1 (YL 15) mAb was from Upstate Biotech (Charlottesville, VA) and the anti-HoxC4 mAb from Covance (Princeton, NJ). The rabbit antibodies to Oct-1 (C-20), Oct-2 (C-20), and Oca-B (C-20) as well as rabbit IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG, goat anti-mouse IgG, and donkey anti-goat IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**Transfection Assays**—B cells (5×105) in 0.5 ml of medium in an 0.4-cm cuvette were electroporated using Gene Pulser II (Bio-Rad). All electroporations were performed in duplicate, adhering to the following parameters: 486 cells, 210 V, 950 μF; HS Sultan cells, 290 V, 900 μF; and U266 cells, 250 V, 1050 μF. For luc-reporter transient transfections, 10 μg of pGL3-promoter-only and/or pGL3-enhancer-promoter plasmid DNA and 10 ng Renilla prl-TK control vector (as an internal control for transfection efficiency) were used. For enforced expression studies, pcDNA3.1 expression vector encoding Oct-1, Oct-2, and/or HoxC4 was cotransfected with the pGL3-promoter-only vector containing a cytomegalovirus promoter, as described previously (28). Briefly, plasmid DNA (2 μg) was gently mixed with Cellfectin following the manufacturer’s instructions (Invitrogen). The pGL3 reporter construct contains a reporter gene for luciferase that is driven by the V pI promoter and a reporter gene for Renilla that is driven by a T7 promoter. Cell transfections were performed using Cellfectin following the manufacturer’s instructions (Invitrogen). B cells (5×105) were cultured for 24–48 h. HoxC4 transfections were performed in 1× passive lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using an MLX microtiter plate luminometer (Thermo Labsystems, Beverly, MA).

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ish peroxidase-conjugated goat IgG to mouse IgG and rabbit IgG were also from Santa Cruz Biotechnology, Inc. The rabbit IgG to Stat-1 was from Transduction Laboratories (San Diego, CA). The mouse IgG and mAbs to β-actin were from Sigma-Aldrich.

EMSA—Nuclear and cytoplasmic extracts from mammalian cells were prepared using a micro-procedure involving hypotonic lysis followed by high salt nuclei extraction (28). Whole-cell extracts were prepared using extraction buffer (20 mM HEPES, 0.5% Nonidet P-40, 2 mM EDTA, 0.3 M KCl, 1 mM dithiothreitol, 5% glycerol, pH 7.5) and a mixture of protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitors), and sonication.

Western Blot Analysis—Whole human B cell extracts (25 μg) were fractionated through 10% SDS-PAGE. Proteins were blotted onto polyvinylidene difluoride membranes (Bio-Rad) overnight (30 V) at 4 °C and fractionated through 10% SDS-PAGE. Proteins were blotted onto polyvinylidene difluoride membranes (Bio-Rad) overnight (30 V) at 4 °C and stained with 0.1% Coomassie blue. The membranes were washed with water and dried before autoradiography.

Reverse Transcription PCRs—Total RNA was extracted from human B cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). First strand cDNA syntheses were performed using SuperScript II reverse transcriptase (Invitrogen) with oligo(dT)12-18 as primer. HoxC4, Oct-1, Oct-2, Oca-B, and β-actin cDNAs were amplified using the following primers: HoxC4 forward, 5’-ATGGGATCATGAGCTCGTATTTG-3’; HoxC4 reverse, 5’-CTATAAACCTGGTAATGTCCTCTGC-3’; Oct-1 forward, 5’-ATGGGGACACCATCGTCAGAAACCAGTAAA-3’; Oct-1 reverse, 5’-TCTTGTTGCCCCTGGAGGCTGTGTTG-3’; Oct-2 forward, 5’-CTACAACCTGGACATCGTGGGACT-3’; Oct-2 reverse, 5’-TCCTTCTTCGCAAACAGC-3’; β-actin forward, 5’-GTACCACTGGCATCGTGATGGACT-3’; and β-actin reverse, 5’-ATTCACACCGGAGTACTTGCGTCA-3’. Germ-line IGH-CH3, -ACT, and mature VDJ-Cγ transcripts were detected using specific primers, as reported (28). Before each reverse transcription PCR, cDNAs were denatured for 5 min at 94 °C. PCRs involved denaturation (1 min at 94 °C), annealing (1 min at 68 °C), and extension (1 min at 72 °C).

Western Blot Analysis—Whole human B cell extracts (25 μg) were fractionated through 10% SDS-PAGE. Proteins were blotted onto polyvinylidene difluoride membranes (Bio-Rad) overnight (30 V) at 4 °C and then detected by primary (1:250 to 1:1000) and secondary (1:2500) antibodies. After washing with phosphate-buffered saline-Tween (0.05%), bound horseradish peroxidase-conjugated antibodies were detected using Enhanced Chemiluminescence Plus reagents (Amersham Biosciences).

Chromatin Immunoprecipitation (ChIP) Assays—B cells (2.5 × 107) were treated with 1% formaldehyde for 10 min at room temperature to cross-link chromatin (36). After washing with cold phosphate-buffered saline containing protease inhibitors, chromatin was separated using nuclei-lysis buffer (10 mM Tris-HCl, 0.5 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.5% sodium deoxycholate, 0.5% sarkosyl, pH 8.0), resuspended in IP-1 buffer (20 mM Tris-HCl, 0.2 mM NaCl, 2 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitors), and soni-
Activation of IgH hs1,2 by HoxC4

hs1,2 Is the Strongest Enhancer in the Human Cα2 3′ Regulatory Region—To address the function of the human IgH 3′ regulatory region, we analyzed the ability of the Cα2 hs1,2, hs3 and hs4 elements to enhance transcription of a luciferase (luc) reporter gene vector, as driven by a human VH1 promoter, the human ECS-I promoter, or Ig-irrelevant human \( \delta \)-globin promoter) increase in Luc activity; hs4 was second, yielding a 46-fold (VH1 promoter), 10-fold (ECS-I\( \gamma \) promoter), and 15-fold (\( \beta \)-globin promoter) increase in Luc activity, whereas hs3 was the weakest of the three elements, yielding 6-fold (VH1 promoter), 2-fold (ECS-I\( \gamma \) promoter), and 2-fold (\( \beta \)-globin promoter) increase in Luc activity (Fig. 1).

hs1,2 Contains Three Regions with Multiple cis-Elements—Because of its dominant activity, we analyzed hs1,2 for putative trans-factor-binding motifs and segregated it into three regions (1, 2, and 3) based on the identified cis-elements (Fig. 2). Region 1 encompasses residues 452–669 and comprises four highly G-rich –53-bp tandem arrayed segments (G-rich repeats 1–4 (GRR)), which have been suggested to display some polymorphism (19, 37). In each GRR, we identified two Hox-binding sites (ATTT, 5′-GGGGYNNCCY consensus) site. Region 2, originally classified as an ECS core (19), encompasses residues 684–818. In this region, we identified two Hox-binding sites (ATTG, 5′ at residues 715–718 and 3′ at residues 773–780), in addition to the previously identified E-Box (characterized as a \( \mu \)E5 site, CANNTG, residues 657–698), Oct (ATTTGCAT, residues 773–780), and AP-1 (TGAGTC, residues 809–815)-binding sites (19). We found that these ATTG sites recruit HoxC4 (not shown). We determined that the 3′ HoxC4-binding site is embedded within the 5′ portion of the Oct site and therefore designated it as HoxC4/Oct site. Region 3 encompasses residues 819–1133 and contains eight putative cis-elements: a GATA (WGATAR consensus) site, an E-Box site, two Myb (YAACKG consensus) sites, two Ikaros (GGGAA consensus), and one \( \kappa \)B (GGGGYNNCCY consensus) site.

RESULTS

hs1,2 [Boxed] within each region. The 3′ HoxC4/Oct-, Myb-, and AP-2-binding sites match the respective consensus in the reverse orientation. See “Results” for consensus sites.
Fig. 3. hs1,2 region 2 and the 5' HoxC4 and 3' HoxC4/Oct cis-elements are conserved across species. ClustalW-formatted alignments of human, mouse, rat, and rabbit 3' E, hs1,2 regions 1, 2, and 3. The Co1 hs1,2 element contains only one GRR, whereas the Co2 hs1,2 element contains four GRRs. Residue numbers are as follows: human Co1, 452–506 (region 1), 507–641 (region 2), and 642–956 (region 3) (GenBank accession numbers AF013722 and U84575); human Co2, 452–669 (region 1), 684–818 (region 2) and 819–1133 (region 3) (GenBank accession no. AF013724, U84574); mouse, 498–711 (region 1), 851–988 (region 2), and 1098–1394 (region 3) (GenBank accession no. X62778); rat, 328–536 (region 1), 645–774 (region 2), and 1059–1353 (region 3) (GenBank accession no. A28224); and rabbit, 372–592 (region 1), 605–733 (region 2), and 738–1018 (region 3) (GenBank accession no. AF314408). Gaps in the sequence for each species are as follows: human Co1, none; human Co2, 669–683; mouse, 712–761, 777–850, and 989–1097; rat, 527–644 and 775–1058; and rabbit, 593–604 and 734–737. All cis-elements are boxed. Note the identity of the ATTG and ATTGGTAC sites across human, mouse, rat, and rabbit. Also, note the lack of BSAP sites in the human.
Regions 1, 2, and 3 are necessary but individually are not sufficient for transcription enhancement. Human 4B6 B cells were transfected with the indicated pGL3 luc-reporter gene vectors to measure the contribution of different DNA regions to the overall hs1,2-enhancing activity. Sequential 5’- and 3’-end truncation, internal deletion, and subfragment mutants of the hs1,2 sequence were created and subcloned into the pGL3 vector driven by VH1 or ECS-I’y promoter. The basal enhancing activity was determined and expressed as relative light units. Data are the mean values of three independent experiments plus standard deviations.

FIG. 4. Regions 1, 2, and 3 are necessary but individually are not sufficient for transcription enhancement. Human 4B6 B cells were transfected with the indicated pGL3 luc-reporter gene vectors to measure the contribution of different DNA regions to the overall hs1,2-enhancing activity. Sequential 5’- and 3’-end truncation, internal deletion, and subfragment mutants of the hs1,2 sequence were created and subcloned into the pGL3 vector driven by VH1 or ECS-I’y promoter. The basal enhancing activity was determined and expressed as relative light units. Data are the mean values of three independent experiments plus standard deviations.

Fig. 4. Regions 1, 2, and 3 are necessary but individually are not sufficient for transcription enhancement. Human 4B6 B cells were transfected with the indicated pGL3 luc-reporter gene vectors to measure the contribution of different DNA regions to the overall hs1,2-enhancing activity. Sequential 5’- and 3’-end truncation, internal deletion, and subfragment mutants of the hs1,2 sequence were created and subcloned into the pGL3 vector driven by VH1 or ECS-I’y promoter. The basal enhancing activity was determined and expressed as relative light units. Data are the mean values of three independent experiments plus standard deviations.

and two AP sites (an AP-4 (CAGCTG consensus) and an AP-2 (GSSWOSGCC consensus)). Regions 1 and 3 are only 47 and 39% identical, respectively, in human, mouse, rat, and rabbit (Fig. 3). In contrast, region 2 is 87% identical overall and 100% identical in the identified HoxC4 and HoxC4/Oct elements across human, mouse, rat, and rabbit.

Regions 1, 2, and 3 Are All Necessary for Co2 hs1,2 Activation—To determine the minimal sequence responsible for mediating hs1,2 activation, we generated 5’- and 3’- truncation and internal deletion mutants and inserted them in luc-reporter gene vectors, which were then used to transfect 4B6 cells and to measure the enhancement of transcription, as driven by a VH1 or the ECS-I’y3 promoter. The transcription-enhancing activity mediated by the full-length Co2 hs1,2 construct (322–1400) was referred to as 100% and used as the term of comparison for all other constructs (Fig. 4). Analysis of the 5’- and 3’- truncation mutants showed that the sequences 322–451 (compare C6 with C2) and 1133–1400 (compare C13 with C2) do not make a significant contribution to the overall enhancement of transcription, as mediated by VH1 or ECS-I’y3 promoter. This was confirmed by the analysis of the 5’ and 3’ truncated construct C17, which displayed 97% of the activity of C2. Analysis of sequential 5’- deletion mutants showed that each of the four GRRs critically contributes to the overall enhancing activity of region 1 (C6–C11), whereas 3’ deletion mutants suggested a significant role for GATA, E-Box, Ikaros, AP-2, AP-4, and/or Myb in the overall enhancing activity of region 3 (C14 and C15). Furthermore, deletion of region 1 (Δ452–669, C3), 2 (Δ684–818, C4), or 3 (Δ819–1133, C5) DNA resulted in 84, 96, and 93% loss, respectively, of such an activity, regardless of the promoter used. Conversely, when alone, region 1, 2, or 3 failed to significantly enhance transcription, as driven by the VH1 or ECS-I’y3 promoter (C12, C16, C20, and C21). Regions 1 and 2 together displayed only partial enhancing activity when disjoined from region 3 (C18), whereas regions 2 and 3 together displayed only partial activity when disjoined from region 1 (C19). Thus, regions 1, 2, and 3 are all necessary, but none is sufficient per se to effect full Co2 hs1,2 activation.

The HoxC4 and HoxC4/Oct Motifs of Region 2 Critically Mediate hs1,2 Region 2-enhancing Activity—The absolute conservation of the HoxC4 and HoxC4/Oct sites prompted us to test the hypothesis that these two cis-elements mediate the hs1,2 activity. We transfected 4B6 cells with a luc-reporter gene vector containing the Co2 hs1,2 DNA sequence spanning regions 1, 2, and 3 (residues 452–1133), which were variously mutated in the region 2 HoxC4 and HoxC4/Oct sites to potentially hamper recruitment of the relevant trans-factors. Mutation of the HoxC4 element (mt1) reduced hs1,2-enhancing activity by 80% (VH1 promoter), 55% (ECS-I’y3 promoter), and 58% (β-globin promoter) (Fig. 5). Mutation of the HoxC4/Oct element (mt2) reduced hs1,2-mediated transcription by 75% (VH1), 43% (ECS-I’y3), and 58% (β-globin promoter). Finally, mutation of both the HoxC4 and the HoxC4/Oct sites (mt3) resulted in virtual ablation of the hs1,2-enhancing activity, regardless of the promoter used, thereby confirming that these cis-elements critically mediate the Co2 hs1,2-enhancing activity.

The HoxC4 and Oct-1/Oct-2 Homeodomain Proteins Are Specifically Recruited to Co2 hs1,2—To identify the trans-factors that are recruited to the HoxC4 and HoxC4/Oct sites of hs1,2 region 2, we used wild-type (wt) and mutated (mt) oligonucleotide probes encompassing residues 704–729 (HoxC4 site) and 764–789 (HoxC4/Oct site) (Fig. 6) in EMSAs involving 4B6 nuclear cell extracts. These gave rise to a distinct nucleoprotein complex when incubated with the HoxC4 probe (Fig. 7A, top panel). Such a complex was specific, as its formation was in-
The HoxC4 and HoxC4/Oct cis-elements are required for full hs1,2-enhancing activity. The hs1,2 HoxC4 and HoxC4/Oct-binding sites were mutagenized by site-directed PCR, resulting in single and double mutant enhancer pGL3 luc-reporter gene constructs driven by the V_{H1}, ECS-γ3, or β-globin promoter. Each construct was used to transfect 4B6 cells to measure Luc activity. Data are represented as percentage of wild-type hs1,2 (regions 1–3)-enhancing activity and are the mean values of three independent experiments plus standard deviations.

Fig. 5. The HoxC4 and HoxC4/Oct cis-elements are required for full hs1,2-enhancing activity. The hs1,2 HoxC4 and HoxC4/Oct-binding sites were mutagenized by site-directed PCR, resulting in single and double mutant enhancer pGL3 luc-reporter gene constructs driven by the V_{H1}, ECS-γ3, or β-globin promoter. Each construct was used to transfect 4B6 cells to measure Luc activity. Data are represented as percentage of wild-type hs1,2 (regions 1–3)-enhancing activity and are the mean values of three independent experiments plus standard deviations.

Fig. 6. hs1,2 HoxC4 and HoxC4/Oct oligonucleotides. HoxC4- and HoxC4/Oct-binding site oligonucleotides used as radiolabeled probes in EMSA. For the HoxC4 site, the ATT TT motif was mutated to cggg (HoxC4 mt4). For the HoxC4/Oct site, the ATT TTG CAT motif was mutated as follows: HoxC4/Oct mt5, complete element replaced with a KpnI sequence; HoxC4/Oct mt6, 5′ HoxC4 portion (ATT T) mutated to cggg; HoxC4/Oct mt7, AT of 5′ HoxC4 portion mutated to gg; and HoxC4/Oct mt8, AT of 3′ Oct portion mutated to cg.

Incubation of nuclear extracts from 4B6 cells with the HoxC4/Oct probe gave rise to the two major nucleoprotein complexes, A and B (Fig. 7B). These were specific, as shown by the failure of mutated HoxC4/Oct oligonucleotides (HoxC4/Oct mt1–4) or Igκ NF-κB and BSAP oligonucleotides to inhibit the formation of both complexes. They included the HoxC4 and Oct-1 or Oct-2 homeodomain proteins as well as the Oca-B coactivator, as inferred from their supershifting or inhibition by specific antibodies to HoxC4, Oct-1, Oct-2, and Oca-B (Fig. 7C) and the binding of GST-HoxC4, GST-Oct-1, and/or GST-Oct-2 to the HoxC4/Oct oligonucleotide probe (Fig. 7D, lanes 1–6). Such a binding was specific, as mutation of the whole HoxC4/Oct-binding site (mt5) or its 5′-end containing the HoxC4-binding motif (mt6 and mt7) abolished binding of HoxC4, Oct-1, and Oct-2 (Fig. 7D, lanes 7–24), whereas mutation of the HoxC4/Oct 3′ portion (mt8) abrogated the binding of Oct-1 and Oct-2 but not HoxC4 (lanes 25–30). Finally, the Hox/Oct nucleoprotein complexes did not include homeodomain-interacting Ku70/Ku86 proteins, as Ku-specific antibodies did not supershift either complex A or B (Fig. 7C, lanes 6–8).

That HoxC4, Oct-1, Oct-2, and Oca-B are bound in vivo to hs1,2 region 2 was demonstrated by ChIP experiments in which the hs1,2 HoxC4-HoxC4/Oct sequence was specified in DNA precipitated from HS Sultan B cells using antibodies to HoxC4, Oct-1, Oct-2, or Oca-B but not Ku70/Ku86 or Stat-1 (Fig. 7E). In addition, the binding of in vitro translated 35S-Oct-1, 35S-Oct-2, and 35S-Oca-B to immobilized GST-HoxC4 indicated that Oct-1, Oct-2, and Oca-B can be recruited to hs1,2 by DNA-bound HoxC4 through direct protein-protein interaction (Fig. 7F). Thus, the hs1,2 5′ HoxC4 and 3′ HoxC4/Oct cis-elements effectively recruit HoxC4, Oct-1/Oct-2 and Oca-B as
FIG. 7. Recruitment of HoxC4, Oct-1/Oct-2, and Oca-B to the 3′E<sub>1</sub> hs1,2. A, nuclear proteins from spontaneously switching 4B6 cells specifically bind an oligonucleotide probe containing the HoxC4-binding site of hs1,2 region 2 (top panel). Efficient competition was achieved by 50-fold molar excess of wild-type (HoxC4 wt) but not mutant (HoxC4 mt4), IgG, or BSAP cold oligonucleotides. The formation of the DNA-binding complex was inhibited by a specific mAb to HoxC4. Mouse IgG served as a negative control. The specificity of the HoxC4 oligonucleotide probe was further verified by binding of recombinant GST-HoxC4 protein (250 ng) to wild-type HoxC4 but not to mutated HoxC4 (mt4) oligonucleotide probe (bottom panel). NE, nuclear extract.

B, nuclear proteins from 4B6 cells bind specifically to radiolabeled hs1,2 HoxC4/Oct wt probe. Efficient competition was achieved by 50-fold molar excess of wt (HoxC4/Oct wt) but not mt (HoxC4/Oct mt5–8) or nonspecific (IgG) cold oligonucleotides.

C, identity of the nuclear protein complexes as assessed by supershift EMSA. 4B6 nuclear extracts were preincubated with the indicated antibodies prior to the addition of the HoxC4/Oct oligonucleotide probe and EMSA. Arrowheads indicate complexes A and B as well as the supershifted complex A′.

D, direct binding of HoxC4, Oct-1, and Oct-2 to hs1,2 DNA. Recombinant GST fusion HoxC4, Oct-1, Oct-2, HoxC4, and Oct-1 or HoxC4 and Oct-2 protein (250 ng) were analyzed for direct binding to HoxC4/Oct probes with GST serving as a negative control. Sequences of HoxC4/Oct mt5, mt6, mt7, and mt8 were as described in the legend to Fig. 6. Note that mutation of the 3′-end of HoxC4/Oct motif (mt8) allowed for binding of HoxC4 only, as predicted.

E, in vivo binding of HoxC4, Oct-1, and Oct-2 proteins to hs1,2 region 2 as determined by ChIP. Cross-linked chromatin from HS Sultan B cells was precipitated by mAb to human HoxC4 or antibodies specific for human Oct-1, Oct-2, or Oca-B. The precipitated DNA was sequenced by PCR using primers (black arrows) and detected by Southern blotting using a specific probe (gray bar) as listed under “Experimental Procedures.” mAbs that detect the interface between Ku70 and Ku86 and a rabbit antibody to Stat1 were used as controls, along with mouse and rabbit IgG. F, GST fusion protein pull-down assays. In vitro 35S-labeled translated HoxC4, Oct-1, Oct-2, or Oca-B proteins were mixed with GST or GST-HoxC4 immobilized on glutathione-agarose resin, subjected to 12% SDS-PAGE, and exposed for autoradiography.
Activation of IgH hs1,2 by HoxC4

Fig. 8. Enforced expression of HoxC4, Oct-1, Oct-2, and/or Oca-B activate the hs1,2 3′ E\textsubscript{H} promoter. Mammalian expression constructs of full-length HoxC4, Oct-1, Oct-2, and/or Oca-B proteins together with the luc-reporter vector containing the β-globin promoter and hs1,2 (regions 1, 2, and 3) were used to transfect 4B6 cells. A mutant HoxC4 (HD mt) was coexpressed in the presence or absence of Oct-1, Oct-2, or Oca-B expression vector. Results represent the fold induction of Luc activity expressed by the reporter gene vector containing the promoter only. Data are the mean values of three experiments plus standard deviations.

Activation of Co2 hs1,2 Is Maximal in Germinial Center B Cells and Plasma Cells—To confirm the relevance of our findings to B cell ontogeny, we transfected human cell lines corresponding to sequential stages of B cell differentiation with the Co2 hs1,2 (452–1133) enhancer-luc reporter gene vector, as driven by the β-globin promoter. We then measured Luc activity, the levels of endogenous germ-line I\textsubscript{H}-CH and mature V(H)DJ(H)-CH transcripts, and the expression of HoxC4, Oct-1/Oct-2, and Oca-B, and we monitored the formation of HoxC4-Oct-1/Oct-2.

Oca-B nucleoprotein complexes. Co2 hs1,2 was not activated in pro-B cells (RS4;11), was moderately activated (4-fold) in pre-B cells (Nalm-6), and was significantly activated in early (4B6) and late (HS Sultan) germinal center B cells (12–50-fold) and plasma cells (U266) (10-fold) (Fig. 9A). Increased hs1,2-enhancing activity was associated with the appearance of germ-line I\textsubscript{H}-C\textsubscript{H} and/or mature V(D)J(CH) transcripts, expression of HoxC4, Oct-1/Oct-2, and Oca-B transcripts and proteins, and the formation of related nucleoprotein complexes involving the ATTT and ATTTGCAT DNA motifs (Fig. 9, B–D). Thus, hs1,2 activation and consequent enhancement of IgH locus transcription is B cell stage-specific and occurs concomitantly with the formation of HoxC4, HoxC4/Oct-1, and HoxC4/Oct-2 nucleoprotein complexes.

**DISCUSSION**

Together with our previous studies on the human Iγ and Iε promoters (25, 28), these findings point to HoxC4 as an important regulator of transcription in the human IgH locus and outline a critical role for this homeodomain protein in B cell differentiation. We show here that HoxC4 mediates activation of the 3′E\textsubscript{H} hs1,2 enhancer in human B cells by binding to newly identified conserved ATTT and ATTTGCAT motifs and through synergy with two other homeodomain proteins, Oct-1/Oct-2, and the Oca-B coactivator. By showing that hs1,2 is dominant over hs4 and hs3 in enhancing transcription, as driven by a human V\textsubscript{H}, ECS-I\textsubscript{H} or the β-globin promoter, our experiments extend previous findings (18–21). Further, they show that hs1,2 can be segregated into three regions (1, 2, and 3), which are all necessary to enhance germ-line I\textsubscript{H}-C\textsubscript{H} and mature V\textsubscript{D}J\textsubscript{H}-C\textsubscript{H} transcription. Finally, they define the minimal requirements for human hs1,2-mediated transcriptional enhancement and determine a B cell stage specificity in HoxC4-dependent activation of hs1,2 (25, 28).

The enhancement of V\textsubscript{H}1 or ECS-I\textsubscript{H} promoter-driven transcription suggests a primary role for hs1,2 in the overall function of the IgH 3′ regulatory region, as V\textsubscript{H}1 and ECS-I\textsubscript{H} are IgH locus promoters. The V\textsubscript{H}1 promoter is required for steady-state V\textsubscript{H} gene transcription, V\textsubscript{D}J\textsubscript{H} gene rearrangement, and IgH SHM; the ECS-I\textsubscript{H} promoter is required for germ-line I\textsubscript{H}-C\textsubscript{H} transcription and CSR. The enhancing activity displayed by hs1,2 in conjunction with the Igκ-unrelated β-globin promoter emphasizes the strength of hs1,2 as a bona fide enhancer (1, 5, 6, 38, 39). This dominant transcription-enhancing activity of human hs1,2 would be reflected in the SHM-enhancing activity displayed by this element, but not hs4 or hs3, when inserted downstream of a SHM “inducible” human DNA V\textsubscript{H}DJ\textsubscript{H}-iEts-\textsubscript{Mu-Su}-Sy-C\textsubscript{H}1 construct (2) (40).

Our analysis of the human Co2 hs1,2 identified two HoxC4-binding sites, which are both critical for full hs1,2 activation. The 3′ cis-element is a HoxC4-Oct-binding site and was previously recognized as a mere Oct-binding site in the reports originally detailing the structure of the human IgH 3′ regulatory region (18–21). That Oct site was included together with I\textsubscript{H}κ-binding sites downstream of the ECS were not recognized, nor was a cis-acting region 3 defined and shown to be necessary for full hs1,2 activation (19). In another study (18), a sequence corresponding to our region 2, but also containing one copy of a −53-bp motif (equal to region 1 GR4), was identified as an ECS lacking CTGCACGCCTGAGCT, which includes the E-B box site, and resulting in an overall structure/function of hs1,2 significantly different from that reported here. In that study, the cis-binding sites downstream of the ECS were identified, not as a “region 3” defined and shown to be required for full hs1,2 activation. An analysis of the rat hs1,2 element resulted in the dissection of the enhancer into three domains, designated as A, B, and C, which were shown to be necessary for transcriptional enhancer activity (41). As in the human region 2, such enhancer activity was contributed mainly by the second hs1,2 DNA region, domain B. But unlike our human region 2, the main rat hs1,2 domain B cis-elements are a group of Ets-like binding sites.

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were normalized by calibrating Sp1 complex content using the Sp1 probe. 3 down-regulation is likely central to the full activation of the IgH evade BSAP-mediated repression. This and the demonstration in transcripts were used to normalize the amount of cDNA in each cell type.

ous (28) findings point to HoxC4 as an important element in the regulation of genes controlling lymphocyte activation and nuclear localization suggest an involvement of HoxC4 in HoxC4 function in the lymphoid system. Its early expression targeted deletions in the mouse (50, 51), have been available on and/or proliferating T, B, and NK cells. No data, even from committed lymphoid lineages. HoxC4 is expressed in activated cluster are preferentially expressed in developing and differentiated lymphoid lineages.

The maximal activation of hs1,2 in human germinal center B cells originally went unrecognized, as IgG+ HS Sultan B cells were classified as a myeloma rather than a germinal center B cell line (18, 19, 34). It has been further confirmed here by the demonstration that hs1,2 is highly activated in 4B6 cells, which effectively express Iγ-Cγ transcripts and undergo CSR to IgG, IgA and IgE (28). The maximal activation of hs4 in Ramos B cells (42) further indicates that the 3 regulatory region plays an important role in the IgH locus transcriptional regulation in germinal center B cells. However, significant hs1,2 activation was seen in murine Ig-secreting plasma cells but not in germinal center B cells (8–10, 43, 44), perhaps reflecting the presence of BSAP-binding motifs in this murine IgH enhancer element (5, 6, 39). By binding to hs1,2 and hs4, BSAP represses the 3 EγH activity in murine germinal center B cells, and BSAP down-regulation is likely central to the full activation of the IgH 3 regulatory region observed in plasma cells (45, 46). Because of the putative lack of BSAP-binding sites in the hs1,2 and hs4 sequences (2, 6, 19), human germinal center B cells would likely evade BSAP-mediated repression. This and the demonstration that, like hs3a, the murine hs1,2 is dispensable for germ-line Iγ-Cγ transcription and CSR (14) underscore significant differences between the mouse and human IgH 3 regulatory regions.

Hox proteins are phylogenetically conserved helix-loop-helix homeodomain proteins that recognize the ATTTA consensus (47, 48). They regulate embryonic pattern formation, axis specification and organogenesis, selective hematopoietic differentiation, and stem cell renewal (49). Genes belonging to the C cluster are preferentially expressed in developing and differentiated lymphoid lineages. HoxC4 is expressed in activated and/or proliferating T, B, and NK cells. No data, even from targeted deletions in the mouse (50, 51), have been available on HoxC4 function in the lymphoid system. Its early expression and nuclear localization suggest an involvement of HoxC4 in the regulation of genes controlling lymphocyte activation and/or proliferation (52). By defining critical new roles in the regulation of the IgH locus expression, our present and previous (28) findings point to HoxC4 as an important element in human lymphocyte differentiation.

HoxC4 synergizes with Oct-1/Oct-2 and the Oca-B coactivator, which it recruits to induce the human hs1,2 enhancer. Indeed, Oct-1/Oct-2 and Oca-B are components of the newly identified nucleoprotein complexes A and B, which assemble on the hs1,2 ATTTGCAT cis-element through recruitment of HoxC4 to the 5′-ATTT end (Fig. 7C). Although HoxC4/Oct-1 heterodimers can form and bind hs1,2 we propose that it is HoxC4/Oct-2 that is recruited preferentially, as suggested by the ChIP assays and the specific DNA binding by increased HoxC4 and Oct-2 proteins in germinal B cells (Figs. 7E and 9, C and D). This heterodimer assembly and recruitment further involves Oca-B. The ATTT cis-element would not recruit Oct-1/Oct-2 and Oca-B directly but through DNA-bound HoxC4, as suggested by the protein-protein interaction experiments (Fig. 7F). The trans-factors recruited at the HoxC4 site would synergize with the HoxC4-Oct-1/Oct-2-Oca-B complex recruited at the HoxC4-Oct site to potentiate hs1,2 activation. This paradigm of transcriptional regulation through protein-protein cooperation has been effectively shown for other Hox homeodomain proteins in which the transcriptional activation function appears to be dependent on the nature of the target DNA sequence, implicating the importance of partner(s) or cofactor(s) and the relative properties of this interaction in mediating specific transcriptional regulation.

The HoxC4 homeodomain is important for hs1,2 activation, as expression of a mutant HoxC4 lacking this domain abolished hs1,2 activity. Overexpression of Oct-1, Oct-2, or Oca-B could not overcome this inactivation, presumably because the HoxC4 homeodomain mutant behaved as a potent “dominant negative” regulator of Oct-mediated hs1,2 activation. Oct-1 and Oct-2 are members of the POU family, a group of homeodomain-containing trans-factors that contain the DNA-binding POU domain. This comprises the “POU-homeodomain” and “POU-specific” subdomains (53). Oct-1 and Oct-2 regulate both general and cell type-specific genes (54), including VH and CH (53). Although Oct-1 is ubiquitous, Oct-2 is preferentially expressed in B cells. In the human IgH 3′ regulatory region, Oct-2

**Fig. 9.** IgH hs1,2 activation is dependent on HoxC4, Oct-2, and Oca-B, and is B cell stage-specific. A, human B cell lines representing different stages of B cell ontogeny were transfected with the luc-reporter vector containing the β-globin promoter and hs1,2 (containing regions 1, 2, and 3). Results represent the fold induction of Luc activity expressed by the reporter gene vector containing the promoter only. Data are the mean values of three experiments plus standard deviations. B, expression of germ-line Iγ-Cγ and mature VγDJ-γCγ transcripts by pro-B cell (RS4;11), pre-B cell (Nalm-6), early germinal center B cells (4B6), late germinal center B cells (HS Sultan), and plasmacytoma cells (U266). β-Actin transcripts were used to normalize the amount of cDNA in each cell type. C, expression of HoxC4, Oct-1, Oct-2, and Oca-B in the same B cells as in A. Transcripts were detected by specific reverse transcriptase PCR analysis (RT-PCR, top panel) and proteins by immunoblotting (bottom panel). β-Actin transcripts and proteins were used as normalizing loading controls. D, EMSA involving hs1,2 region 2 HoxC4 and HoxC4/Oct DNA probes with nuclear extracts from the same B cells as in B. Arrowheads indicate each specified nucleoprotein complex(s). The amounts of proteins analyzed were normalized by calibrating Sp1 complex content using the Sp1 probe.
is required for not only hs1,2 (our data), but also hs4 activation (42). Accordingly, in Oct-2-deficient mice, B cell development to surface IgM expression is normal, but germinal center formation is impaired, and IgG1 and IgG3 levels are severely decreased (55, 56), indicating that Oct-2 is required for germinal center formation, CSR to secondary isotypes, and a high level of Ig transcription.

Coexpression of Oca-B and HoxC4 yielded the highest level of hs1,2 activation, presumably through interactions with the endogenous pool of Oct-1/Oct-2 proteins (Fig. 8). Oca-B (Oct coactivator from B cells, or Oct-binding factor-1 (OBF-1)) functions as an important transcriptional coactivator in B cells. It increases the binding affinity of Oct-1 and Oct-2 for DNA by clamping the POUH and POUS subdomains, which can further stimulate Oct-dependent gene transcription (57). Our demonstration that Oca-B plays an important role in HoxC4/Oct-2-mediated activation of human hs1,2 further emphasizes the critical role of this coactivator in B cell differentiation. Accordingly, Oca-B interacts with Oct-2 in modulating the activity of the 3′E\textsubscript{H} and IgH transcription in murine B cells (58, 59), and Oca-B−/− B cells stimulated with anti-CD40 and interleukin-4 fail to activate a luc-reporter gene construct bearing the regulatory hs3a-hs1,2-hs5b-hs4 cluster (44). Further, mice lacking Oca-B are viable and have normal serum IgM levels but lack GCs and show a significant impairment in CSR to IgG and serum IgG levels (60–62). Finally, Oct-2/Oca-B double deficient mice display a similar but more pronounced phenotype with impairment of T cell-dependent antibody responses (59).

The transcription-enhancing activity induced by the binding of HoxC4 to the human hs1,2 ATTT and ATTTGCAT motifs contrasts with the repression of germ-line I\textsubscript{H}-C\textsubscript{H} and I\textsubscript{H}-C\textsubscript{L} transcription that, as we showed (28), is dependent on recruitment of HoxC4 to ATTT sites embedded in the human ECS-I\textsubscript{H} and ECS-I\textsubscript{L} promoters. ATTT motifs exist as multiple copies in the human ECS-I\textsubscript{H} and ECS-I\textsubscript{L} promoters (28). Because of the lack of ATTT sites in the ECS-I\textsubscript{H} and ECS-I\textsubscript{L} promoters, the C\textsubscript{H}1/C\textsubscript{H}2 loci can undergo CSR to IgA even when HoxC4 expression is up-regulated (28). The repression exerted by HoxC4 on the ECS-I\textsubscript{H} and ECS-I\textsubscript{L} promoters, germ-line I\textsubscript{H}-C\textsubscript{H} and I\textsubscript{H}-C\textsubscript{L} transcription, and CSR to IgG and IgE is dependent on the recruitment of the Ku70/Ku86 heterodimer, as a mutant Ku70 lacking the homeodomain interaction motif relieved all HoxC4-mediated inhibition (28). As we show here (Fig. 7), instead of recruiting Ku70/Ku86, HoxC4 bound to human hs1,2 recruits Oct-1/Oct-2 and Oca-B, consistent with the notion that Hox proteins are multifunctional transcriptional regulators that interact with different cofactors and/or components of the transcriptional machinery depending on the broader structure of their target regulatory elements (63).

The combined recruitment of HoxC4 and Oct-2 to the HoxC4 and HoxC4/Oct sites, as complemented by Oca-B, would represent a paradigm of gene regulation by homeodomain transcription factors (Fig. 10). Once the HoxC4-Oct-1-Oct-2/Oca-B complex is bound to hs1,2, long-range interactions with V\textsubscript{H} and
ECS-I H promoters, presumably by looping of the 3’ regulatory region, would confer greater IgH locus accessibility. This would result, perhaps in the context of a promoter competition mechanism as proposed in the mouse (12, 13, 64), in markedly differential enhancement levels of transcription, as seen with human ECS-Iα/Iα2 and ECS-Iγ3 promoters (21). Further studies are needed to address such possible mechanisms and the role of hs1,2, hs3 and hs4 in the human IgH 3’ regulatory region as a LCR. Such studies would require the generation of constructs containing hs1,2, hs3 and/or hs4 together with the appropriate promoters and rearrangeable, switchable, or hy-

permutable Ig DNA for in vitro and in vivo expression, CSR, and SHM.

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permutable Ig DNA for in vitro and in vivo expression, CSR, and SHM.

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The HoxC4 Homeodomain Protein Mediates Activation of the Immunoglobulin Heavy Chain 3' hs1,2 Enhancer in Human B Cells: RELEVANCE TO CLASS SWITCH DNA RECOMBINATION
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