Enhancer Complexes Located Downstream of Both Human Immunoglobulin Cα Genes

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Summary

To investigate regulation of human immunoglobulin heavy chain expression, we have cloned DNA downstream from the two human Cα genes, corresponding to the position in the mouse IgH cluster of a locus control region (LCR) that includes an enhancer which regulates isotype switching. Within 25 kb downstream of both the human immunoglobulin Cα1 and Cα2 genes we identified several segments of DNA which display B lymphoid–specific DNase I hypersensitivity as well as enhancer activity in transient transfections. The corresponding sequences downstream from each of the two human Cα genes are nearly identical to each other. These enhancers are also homologous to three regions which lie in similar positions downstream from the murine Cα gene and form the murine LCR. The strongest enhancers in both mouse and human have been designated H S12. Within a 135-bp core homology region, the human HS12 enhancers are ~90% identical to the murine homolog and include several motifs previously demonstrated to be important for function of the murine enhancer; additional segments of high sequence conservation suggest the possibility of previously unrecognized functional motifs. On the other hand, certain functional elements in the murine enhancer, including a B cell–specific activator protein site, do not appear to be conserved in human HS12. The human homologs of the murine enhancers designated H S3 and H S4 show lower overall sequence conservation, but for at least two of the functional motifs in the murine H S4 (a κB site and an octamer motif) the human H S4 homologs are exactly conserved. An additional hypersensitivity site between human HS3 and HS12 in each human locus displays no enhancer activity on its own, but includes a region of high sequence conservation with mouse, suggesting the possibility of another novel functional element.

The regulation of human immunoglobulin heavy chain gene expression is incompletely understood, despite clinically significant conditions in which specific isotypes are inappropriately up- or downregulated, e.g., allergies due to inappropriate IgE response, and various forms of immunodeficiency associated with low IgA expression. Clearly, cytokines and interactions between B and T cells play a role in regulating isotype switching, and cis elements in the IgH gene locus which mediate these effects have been documented in the murine and human promoters of the sterile transcripts associated with each heavy chain constant region gene (1, 2). However, in the mouse an additional control region which contributes to regulation of isotype switching has been reported to lie downstream from Cα, and the corresponding region of the human heavy chain locus has not yet been investigated.

The existence of a regulatory region downstream from murine Cα was originally inferred when it was found that plasmacytomas which had undergone spontaneous deletions of the only heavy chain enhancer then known, which lies in the intron between JH and Cμ, nevertheless remained capable of high level immunoglobulin secretion (3–6). Conversely, a myeloma subclone which retained the intronic enhancer but lost a segment of DNA downstream from the murine Cα gene was found to have markedly reduced its heavy chain gene expression (7). A systematic search in the homologous region of the rat heavy chain locus revealed an enhancer (8), and a homologous mouse enhancer designated 3′αE was found soon after (9, 10) positioned ~16 kb downstream from Cα. The mouse and rat 3′αE segments lie in opposite orientations and are flanked by inverted repeats (9). In addition to the 3′αE, Matthias and Baltimore also reported a weak enhancer in mouse lying only 4 kb downstream from Cα (Fig. 1 and reference 11).
More recently, Madisen and Groudine (12) analyzed B cell–specific DNase I hypersensitivity downstream from Cα and identified four hypersensitive sites. H51 and H52 fall in the previously described 3′αE, whereas H53 and H54 lie further downstream and identify two new regions with somewhat weaker enhancer activity in transient transfection assays. The H53 sequence is almost identical to that of the enhancer described by Matthias and Baltimore but has an inverted orientation. This reflects the fact that the sequence surrounding the H512-3′αE is present in the mouse in a long inverted repeat which includes H53 sequences at both ends (Fig. 1 and reference 13). When constructs containing H53, H512, and H54 linked to a reporter gene were transfected into a B cell line, subsequently isolated stable transfectants were found to express the reporter gene in a position-independent manner. This suggested that the three enhancer sequences (H512, H53, and H54) acted together as a locus control region (LCR). LCRs, first defined in the globin locus (14), activate large domains of chromatin in vivo (100 kb in the human β globin locus), and, as components of DNA constructs in transgenic mice, support gene expression proportional to the number of integrated copies. In contrast, integrated gene constructs lacking LCR sequences are variably expressed, depending on the positions of integration. LCRs typically contain several DNase I hypersensitive sites, which often represent DNA with enhancer activity. In addition to the LCRs found in the β globin and mouse IgH loci, LCRs have also been described as associated with macrophage–specific lysozyme, CD2, and α/β TCR loci (15).

Analyses of the regulatory regions downstream from murine Cα have identified several motifs which bind specific transcription factors to mediate different aspects of regulation of enhancer function. The 3′αE has been found to activate transcription strongly in plasmacytomas, but only weakly in earlier B lymphoid cells. Part of this developmental change is attributable to a motif known as E5, which matches the “E-box” consensus binding site (CANNTG) characteristic for members of the basic helix-loop-helix family of transcription factors. The contribution of the E5 site to enhancer activity is inhibited in early stages of development by the dominant negative nuclear regulator Id3, which is expressed in early B lineage cells but downregulated in plasma cells (16). At least four other motifs in the 3′αE have been reported to contribute to enhancer activity specifically in plasmacytomas, motifs whose contribution in B cells is inhibited by BSAP (the B cell–specific activator protein), which disappears as B cells mature to plasma cells. These sites include αP (17), the octamer motif (ATG-CATAAT; reference 18), a κB-like site (16), and a G-rich sequence (19). In B cells, BSAP prevents the binding of the transcriptional activator NF-κB to the αP site, and causes the octamer, G-rich, and κB-like motifs to exert an active repressive influence on transcription (17, 19–21).

Apart from the motifs mediating upregulation of the 3′αE during maturation to plasma cells, a response element in the enhancer for activation induced by B cell receptor cross-linking has been traced to partially overlapping sites for the ETS family member Elf-1 and for members of the AP-1 transcription factor family (22). Two other motifs in the enhancer have been proposed to contribute to its regulation, but are less well documented: the μE1 and the μB motifs, which were first noted in the rat 3′αE and which are partially conserved in mouse. The H53 and H54 enhancer regions of mouse have been studied in less detail, but the H54 enhancer apparently contains functional O ct-1 and BSAP binding sites (23).

A role for the 3′αE in isotype switching was revealed by experiments in which this region was replaced by a neomycin resistance gene through homologous recombination in embryonic stem cells which were then used to reconstitute the B cell population in RAG-2 knockout mice. The re-
sulting B cells showed normal V(D)J recombination but marked deficiencies in switching to IgG2a, IgG2b, IgG3, and IgE in vitro, whereas expression of IgM and IgG1 was normal (24). This observation suggests that the enhancer exerts isotype-specific effects on switch recombination, possibly through its regulation of germline transcription of the different isotypes before switch recombination.

Because the enhancer regions lying downstream from the mouse Cα gene have been found to be important for heavy chain gene expression and isotype switching, there has been considerable interest in determining how homologous regions might regulate immunoglobulin gene expression in humans. The human heavy chain locus includes two γγ-γ-ε-α segments (25, 26), apparently the product of a large duplication in the primate lineage (27). Isotypes from the upstream duplication, comprising the γ3-γ1-ε-α1 constant region genes, are generally expressed at a much higher level than those of the downstream γ2-γ4-ε-α2 duplication. The existence of two Cα genes in humans suggests the possibility that two Cα enhancer complexes may regulate the locus, one downstream from each Cα gene; differences in these complexes could contribute to the differential regulation of the two duplications. Moreover, individuals who have a ψε-α1-εγ deletion on one chromosome show reduced expression of the downstream γ2 and γ4 genes on that chromosome, indicating that this deletion may have removed a region which exerts distal control over at least some of the human heavy chain genes (28). Finally, the possibility that there are two Cα enhancer complexes makes the human IgH locus an attractive candidate for study because there could be interactions between two adjacent LCRs, a situation which has not been described in any other system.

Several laboratories have attempted to characterize sequences lying downstream from the two human Cα genes, but technical difficulties have impeded this work. Gene walking downstream from the Cα gene has been difficult, apparently because of a segment of 20-bp tandem repeats which lies almost immediately downstream of the most 3′ exon of both Cα genes. These tandem repeats, described independently by three laboratories (29–31), include the sequence GATC recognized by the isochizomer restriction enzymes Sau3A and MboI. Since commercial human DNA libraries in λ phage have been constructed using genomic DNA fragments generated by partial MboI/Sau3A digestion, the repeated Sau3A sites downstream of the Cα genes make it unlikely that library clones isolated by hybridization to Cα probes will contain DNA downstream from the repeats.

As an initial step toward defining the role of global control regions in the activation of the human IgH genes, our laboratory has sought to determine: (a) are enhancer complexes located downstream from the human Cα gene and (b) how do any such human enhancers correspond to the regulatory sequences described downstream from the murine Cα gene? Towards this end we have successfully applied several strategies other than gene walking from Cα to obtain DNA clones extending downstream from the human Cα genes, enabling us, by functional analysis and sequencing, to characterize enhancer regions 3′ of each Cα gene homologous to those of the mouse Hs12, Hs3, and Hs5.

Materials and Methods

Cloning of 3′ α regions from human genomic DNA. To obtain DNA between Cα1 and the previously reported ψγ pseudogene which lacks associated SY sequences, we initially sought clones containing the pseudogene, which should hybridize to a Cγ probe but not to an SY probe. We screened a commercial library of partial MboI-digested human placental DNA in the λFixII phage vector (Stratagene, La Jolla, CA) with a Cγ probe: probe f (Fig. 2), a 7-kb HindIII fragment, was isolated from a pBR322 plasmid clone originally derived from λ phage CH-lg-H·g-11 (32) (Health Science Research Resources Bank, Osaka, Japan; e-mail: hsrb@nih.go.jp). Cγ plaques were replated and duplicate plaque-lift filters were hybridized with the Cγ probe and an SY probe (1,100-bp KpnI to PstI fragment containing human SYγ2; reference 33). Southern blots of BamHI-digested DNA from eight candidate clones (Cγ+, SYγ−) were hybridized with a 32P-labeled oligonucleotide Pseudogam-1 (see Table 1 for sequences of oligonucleotides) specific for the ψγ hinge region at 48°C in hybridization buffer (1 M N aC l, 0.1 M sodium phosphate, pH 7.0, 10% dextran sulfate, 10 mM EDTA, 1% SDS), followed by washing at 48°C in 1× SSC, 10 mM EDTA, 0.1% SDS. This analysis identified three overlapping ψγ+ clones (ψγ-25, ψγ-2, and ψγ-38) extending over an ~30-kb region containing the ψγ gene (Fig. 2).

To obtain clones for the expected homologous region downstream of the Cα2 region, two probes derived from the farthest upstream ψγ probe (ψγ-25) were used to rescreen two duplicate plaque lifts of the λFixII library. Probe b (Fig. 2) was a 4.5-kb fragment extending from the 5′ end of ψγ-25 to the first internal EcoRI site. Probe e was a 1.3-kb PCR fragment which lies ~2.5 kb upstream from ψγ and which was obtained by amplification from a ψγ-25 subclone containing a 10.5-kb EcoRI-Xbal fragment which extends from the farthest downstream EcoRI site in ψγ-25 to an artificial XbaI site at the 3′ end of the phage insert. Primers used for this amplification were a reverse sequencing primer (Table 1) and elkb2, a primer based on sequence data from a ψγ-25 subclone. This probe contains a sequence homologous to a gene encoding the transcription factor elk-1, which is known to lie on the X chromosome (34). Clones EG3-6 and EG3-5 were selected for the following properties: they hybridized to probe e but not to a Cγ probe (as expected for DNA downstream of Cα2), and they had restriction maps distinct from that of the α1 locus and that of the elk-1 gene on the X chromosome, as established by genomic Southern blots of DNA from a mouse human somatic hybrid cell line (NIGMS repository No. GM 063188; Coriell Institute for Medical Research, Camden, NJ) carrying the human X chromosome as its only human DNA. Clone H3E-1 was isolated on the basis of hybridization with probe e but not with a Cγ probe. By restriction maps and Southern blots, clones H3E-1, EG3-6, and EG3-5 were found to overlap with each other, forming a contig of ~30 kb.

From genomic Southern blots, the phage contigs downstream of the two Cα genes were found to lie ~20 kb away from clones containing the respective Cα genes. To bridge these gaps, a bacterial artificial chromosome (BAC) library of human genomic DNA (Genome Systems Inc., St. Louis, MO) was screened using both a Cα membrane exon probe (Fig. 2, probe a) made by amplification from the Cα1 plasmid GMAS using primer 5’T M-A1
In combination with primer 3'TM-B (35) and a probe extending from the most 5' HindIII site in ψγ-25 to a position ~1,300 bp upstream (Fig. 2, probe b), a single BAC clone was obtained, spanning ~120 kb, from ~20 kb upstream of Cγ2 to 35 kb downstream from the Cα3 membrane exon. From this clone, designated BAC 11771, two subclones were prepared: a 10.5-kb HindIII fragment (A2H10.5) and an overlapping 14-kb EcoRI fragment (A2E14). Fragments of these subclones were found to have sequence homology to murine Hα12, H3, and H4, and enhancer activity in a transient transfection assay (see Results). With the same primer pair, the corresponding regions near Hα12 in line C indicate that the Hα12 regions near Hα1 and Hα2 lie in opposite orientation. This inversion made it possible to selectively amplify α2-derived sequence from genomic DNA using two primers which both corresponded to the sense strand of the α2 locus. Amplification with the upstream primer SA2.5-A2 and the downstream primer SA2.1-A2 was performed using Taq polymerase XL (PE Applied Biosystems, Foster City, CA) for 32 cycles (94°C for 1 min, 61°C for 2 min, and 72°C for 10 min) and yielded the 5.5-kb A1-HS3-12 PCR fragment (Fig. 2). From this fragment a 982-bp α2 H5-3 product was amplified using primers SA2.5A and SA2.6A; and 964 bp α1H512T and 892 bp α1HS12B fragments were amplified using primers SA2.1A and SA2.2B. For comparisons between enhancer activities of corresponding α1 and α2 fragments, the homologous 1070-bp A2H12 PCR product was generated with the same primer pair but using plasmid pEHi.3 as template. In experiments seeking the α1 homolog of H54, amplifications exploiting the inverse were unsuccessful. As an alternative strategy, we prepared an α1-specific genomic template for PCR: a 23-kb HindIII fragment from the human myeloma HS Sultan (American Type Culture Collection, Rockville, MD) which Southern blots indicated extended from the α1 membrane exon to a HindIII site in our ψγ-25 clone, including DNA homologous to H54. The corresponding regions from α2 fall in 12-kb and 14-kb HindIII fragments. A 23-kb preparative electrophoresis fraction of HindIII-digested DNA from HS Sultan was used as template for amplification with primers SA8.A and SA11.B based on sequence from the α2 locus. The resulting 4.2-kb fragment (A1-H54-3’PCR; Fig. 2) was cloned into a λZAP Express phage vector (Stratagene), yielding the clone α1HS54-4.5; the cloned insert showed a restriction map consistent with the α1 locus and distinct from the α2 locus. From the 4.2 A1-H54-3’PCR fragment a 468-bp segment containing the α1 H54 region was amplified using primers SA8.A and SA9.B; this segment was used for sequence analysis and enhancer studies. With the same primer pair, the corresponding α2 fragment A2-H54 was generated using plasmid pA2E14 as template in order to enable meaningful α1 versus α2 comparisons of H54 enhancer activity.

Figure 2. Regulatory loci downstream of human Cα1 and Cα2. Lines A and C, based on this study, show an expanded map of the region downstream of Cα1 and Cα2, respectively, as well as available DNA clones, which are shown above (α1) or below (α2) the line. Phase clones are marked with diagrammatic phase heads, while the subclones of PCR-amplified segments A1-H53-12 and A1-H54-3’ are drawn with hatched lines, and a BAC clone is drawn as a double line, containing a deletion (dashed box). Vertical ovals mark DNA1 membrane exon to a HindIII site in our ψγ-25 clone, including DNA homologous to H54. The corresponding regions from α2 fall in 12-kb and 14-kb HindIII fragments. A 23-kb preparative electrophoresis fraction of HindIII-digested DNA from HS Sultan was used as template for amplification with primers SA8.A and SA11.B based on sequence from the α2 locus. The resulting 4.2-kb fragment (A1-H54-3’PCR; Fig. 2) was cloned into a λZAP Express phage vector (Stratagene), yielding the clone α1HS54-4.5; the cloned insert showed a restriction map consistent with the α1 locus and distinct from the α2 locus. From the 4.2 A1-H54-3’PCR fragment a 468-bp segment containing the α1 H54 region was amplified using primers SA8.A and SA9.B; this segment was used for sequence analysis and enhancer studies. With the same primer pair, the corresponding α2 fragment A2-H54 was generated using plasmid pA2E14 as template in order to enable meaningful α1 versus α2 comparisons of H54 enhancer activity.
**Table 1.** Sequences of Oligonucleotides Used in This Study

| Oligonucleotide name | Sequence |
|---------------------|----------|
| Pseudogam-1 reverse sequencing primer | AGATGCCCACCACATGTCAGT |
| elkb2 | AACAGCTATGACCATG |
| 5'TM-A1 | TAAAGCTCTTGAGAAAGGGTTGGGGAGG |
| 3'TM-B | CTTTCTACGAGTCTGGGCGGGCTG |
| SA2.5-A2 | TCCAAGAGGTCTCCACACCTTCC |
| SA2.1-A2 | ggcggctccCTTCTGCCAACCTGGGGCTG |
| SA2.5A | ggcggctcGAGATCCCGGTTTCTCCATGACCTG |
| SA2.6A | ggcggctccCTTCCACACCTTCCAGGAGG |
| SA2.1A | ggcggcttcCTTCTGCCAACCTGGGGCTG |
| SA2.2B | ggcggctccCGCTCGCTGCCCCACTCAGGAGG |
| SA11.B | CAGTGCCCCAACCCAGGACGCCAGCC |
| SA2.2A | ggcggctccCTTCTGCCAACCTGGGGCTG |
| SA2.6A | ggcggctccCTTCTGCCAACCTGGGGCTG |
| SA2.1-A2 | ggcggctccCTTCTGCCAACCTGGGGCTG |
| β3PR-A | GGCACAATCCAGATGCTCAAGGCCC |
| β3PR-B | TM-A1 CTGTTCACACGAGTCTGGGCCTGG |
| | TM-B TCCAAGAGGTTCCTCCACACTTCC |
| | 5PR-A GAGGAGAAGTCTGCCGTTACTGCCC |
| | 3PR-B GGCACAATCCAGATGCTCAAGGCCC |
| | 9PR-A GAGGAGAAGTCTGCCGTTACTGCCC |
| | 3PR-B GGCACAATCCAGATGCTCAAGGCCC |

All primer sequences are presented 5' to 3'. For certain primers derived from human genomic DNA sequence, additional nucleotides were added at the 5' end to provide restriction sites; these nongenomic nucleotides are written in lowercase letters, with the restriction site underlined. All oligonucleotides were prepared in the Facility for Biotechnology Research (Center for Biologics Evaluation and Research, Bethesda, MD).

**Results**

Cloning DNA downstream of Cα1 and Cα2. Because attempts to clone the human 3' Cα regions using strategies based on gene walking from Cα or cross-species hybridization had failed in other laboratories, we used an alternative strategy based on the fact that a Cγ-like pseudogene (ψγ) has been described downstream of human Cα1 (32, 38). We reasoned that we could clone DNA downstream of Cα1 by walking upstream from ψγ. The similarity in the restriction maps reported downstream of the two Cα genes...
based on Southern blotting (26) furthermore suggested that these regions would be highly similar to each other. Therefore, probes derived by walking upstream from \( \psi \gamma \) should also hybridize to clones containing DNA from downstream of C\( \alpha \)2.

By screening a phage library for clones that hybridized to C\( \gamma \) but not S\( \gamma \), and then screening those clones with a \( \psi \gamma \)-specific oligonucleotide (see Materials and Methods), we obtained three overlapping clones spanning \(~30\) kb (Fig. 2). To obtain clones covering the corresponding region from the \( \alpha \)2 locus, the library was then rescreened with probes b and e (Fig. 2). Clones deriving from downstream of C\( \alpha \)2 were selected on the basis of restriction site differences between the \( \alpha \)1 and \( \alpha \)2 which had been established by analysis of genomic Southern blots and comparisons with our cloned DNA from the \( \alpha \)1 duplication. Three clones deriving from the \( \alpha \)2 locus and spanning \(~30\) kb were obtained (Fig. 2).

To estimate the distance between our two phage contigs and the corresponding C\( \alpha \) genes, we performed genomic Southern blot experiments using a panel of restriction enzymes with known sites in the C\( \alpha \) loci and in our contigs. For example, co-migrating BglII bands of \(~35\) kb were found to hybridize to both probe a (from the C\( \alpha \) membrane exon \( \alpha \)m) and probe b (Fig. 2); these experiments suggested a gap of \(~20\) kb between each contig and the corresponding membrane exon of C\( \alpha \) (data not shown). For the \( \alpha \)2 locus, the gap was bridged by a BAC clone (see Materials and Methods). The corresponding regions from the \( \alpha \)1 locus have resisted direct cloning and have been obtained by PCR using primers designed from the sequence of the \( \alpha \)2 locus, as described in Materials and Methods.

DNase I Hypersensitive Site Analysis. To map potential regulatory sequences downstream of the C\( \alpha \) genes, we used fragments from the cloned DNA downstream of the C\( \alpha \)2 gene to search for DNase I hypersensitivity sites in cells where such enhancers or promoters are active, they generally are hypersensitive to endonucleases, apparently because binding of transcription factors disrupts the nucleosome protection afforded by nucleosomes. Intact nuclei from the human myeloma H S Sultan were incubated briefly with various concentrations of DNase I; DNA samples purified from these treated nuclei were then analyzed using several Southern blot strategies in order to localize the positions of DNase I cleavage. The analyses were complicated by the fact that all of the probes used hybridized to both the \( \alpha \)1 and \( \alpha \)2 loci, but various restriction map differences between the two loci allowed us to position all the hypersensitivity sites with respect to restriction sites mapped from our clones.

Fig. 3 A demonstrates analyses of DNA from the promyelocytic K562 line, representative of a cell not expressing immunoglobulin genes, and the myeloma H S Sultan line. The DNase I-treated DNA was digested with BglII, which cuts \(~1\) kb upstream of the membrane exon of both C\( \alpha \) genes; the blasts were hybridized with a probe corresponding to this exon. Since the next downstream BglII site is \(~20\) kb away, this strategy can display hypersensitivity sites over this wide distance from the \( \alpha \) membrane exon. The blot demonstrates at least seven hypersensitivity sites, which were subsequently assigned to the \( \alpha \)1 or \( \alpha \)2 locus by other blotting experiments and were named according to sequence similarity to the homologous murine regions as described below. The sites we have tentatively designated \( \alpha \)1X and \( \alpha \)2X do not correspond to any reported murine enhancer sequence. None of these sites were visible in the DNA from K562, in which the enhancer region is expected to be inactive.

The BglII blot of H S Sultan DNA fails to resolve the two H S4 sites because they are too far away from the BglII sites. The two H S4 sites were resolved by an alternative Southern blot strategy employing EcoRI digests of the DNA from DNase I-digested nuclei (Fig. 3 b). To determine which of the two resulting bands represented H S4 from the \( \alpha \)1 versus \( \alpha \)2 loci, we exploited the observation from our sequence analysis that recognition sites for the restriction enzyme SspI lie in each H S4 site. Since many regulatory regions accessible to DNase I are also accessible to restriction endonucleases, we digested H S Sultan nuclei with SspI and localized the cleavage sites by isolating the DNA, digesting with EcoRI, and hybridizing Southern blots with probe b' as shown in Fig. 3 b. From the map positions of the \( \alpha \)1 and \( \alpha \)2 SspI sites we assigned the SspI hypersensitivity bands as shown in Fig. 3. A control experiment on the same DNA isolated from SspI-digested H S Sultan nuclei demonstrated that SspI recognition sites in the \( \beta \) globin locus were not cut, indicating that the SspI sites associated with H S4 were indeed hypersensitive in H S Sultan cells (data not shown; see Materials and Methods).

Fig. 3 C illustrates an experiment which allowed assignment of several HS sites to the \( \alpha \)2 locus rather than \( \alpha \)1. DNA samples from DNase I-treated H S Sultan nuclei were digested with HindIII and hybridized with a probe for the HS12 site derived from the \( \alpha \)2 locus. Although this probe hybridizes to both the \( \alpha \)1 and \( \alpha \)2 loci, in this DNA the \( \alpha \)1 band is \(~23\) kb, so large that any fragments from this locus that were generated by HindIII and DNase I and which hybridized to the probe would be larger than the 12-kb band representing the \( \alpha \)2 locus. Thus all HS bands <12 kb derive from \( \alpha \)2. This blot therefore defines the position of the HS12, X, and HS3 sites in the \( \alpha \)2 locus. By implication, the other HS sites in the BglII blot of Fig. 3 A must derive from \( \alpha \)1. (It should be noted that a common allele in other DNA samples shows an additional polymorphic HindIII site which cuts within the 23 kb corresponding to the H S Sultan band; this allele would have confused the above strategy, but was absent in H S Sultan.)

Enhancer Function. To analyze enhancer activity in the cloned DNA downstream of the C\( \alpha \)2 locus, fragments containing one or more DNase I hypersensitive sites were subcloned into a luciferase reporter gene driven by a \( \beta \)k promoter as described in Materials and Methods. Luciferase activity in each sample was normalized to the \( \beta \)-galactosidase activity of the promoter-only control plasmid, and expressed as fold-increase over luciferase activity of that control plasmid.
The luciferase assays (Fig. 4) revealed strong enhancer activity in the 5-kb SmaI-HindIII fragment (SM5) which was found to contain sequence homologous to murine HS12 (see below). Within this fragment, enhancer activity seemed to be confined to the 1.3-kb EcoRI-HindIII fragment (EH1.3) containing the HS12 site. This segment was further cut into an upstream 0.3-kb EcoRI-PstI fragment (EP300), a 0.3-kb PstI-PstI fragment (P300), and a 0.6-kb PstI-HindIII fragment (PH600). Of these, only the P300 fragment, which contained HS12, showed enhancer activity. However, it should be noted that the enhancer activity of P300 was less than that measured for a larger PCR-generated fragment A2H S12. Thus it is possible that additional elements that do not show intrinsic enhancer activity when
isolated in constructs can nevertheless augment the activity of the core H S12 enhancer lying in the P300 fragment. Furthermore, the EH1.3 fragment, but not the slightly shorter A2HS12 fragment, showed significantly less enhancer activity in the A orientation, suggesting the possibility of inhibitory sequences located near one of the ends of EH1.3.

Two fragments containing the a2 HS3 site showed no significant enhancer activity in the luciferase construct, but a plasmid in which the H S3-containing 0.7-kb BamHI-Smal fragment was dimerized showed consistent low activity (Fig. 4, SM 0.7-X2). The homologous murine enhancer was reported to show greater activity with a c-myc promoter than with an immunoglobulin Vκ promoter (12), but we found no significant increase in enhancer activity when the Vκ promoter was replaced by a human c-myc promoter (data not shown; construct described in Materials and Methods).

Two restriction fragments, B5.5 and SpB0.9, containing the a2 H S4 site demonstrated significant stimulation of luciferase activity, but only when cloned in the B (inverted) orientation (Fig. 4 A). When these fragments were oriented so that the DNA strand continuous with the transcribed strand in the luciferase gene corresponded to the transcribed strand of the immunoglobulin gene in genomic DNA (the A orientation), luciferase activity was no greater than that from the promoter-only plasmid. Unexpectedly, a smaller fragment (the 468-bp PCR-generated fragment designated A2-HS4, designed to span the sequence showing the strongest homology to the murine H S4 site) was found to give a strong activation of luciferase activity. The stronger activation of luciferase conferred by the short A2-H S4 PCR fragment compared with that of the longer SpB0.9 fragment (in the same A orientation) suggests that the latter may contain an inhibitory sequence that is either position- or orientation-dependent. This possibility is currently being explored.

Because of the lack of genomic clones spanning the H S3, H S12, and H S4 regions of a1 locus, fragments corresponding to each of these sites were obtained by PCR and analyzed; and, to facilitate α1 versus α2 locus comparisons, the same primer pairs were used to generate corresponding fragments from the a2 locus. As shown in Fig. 4, the activity of each H S site fragment from α1 was similar to that from the a2 locus (compare SM 0.7-X2 a2 versus H S3-X2 a1; A2HS12 a2 versus A1HS12 a1; and A2-HS4 a2 versus A1-HS4 a1). The PCR amplification of the H S12 site yielded two fragments, designated A1HS12T (top) and A1HS12B (bottom), which differ in size by <0.1 kb and may represent alleles (see below). Both fragments showed substantial enhancer activity (Fig. 4 B).

Since the mouse 3'α enhancers show different activation patterns during B cell differentiation, with H S4 being activated at the pre-B cell stage, while H S12 and H S3 are only active in mature B cells, we examined the activity of the human 3'α enhancers in a range of human and mouse B cell lines (Table 2). The pattern of activation for the human H S12 enhancer is similar to that of mouse H S12; i.e., human H S12 is inactive in the human pro-B cell line FLEB14 and the mouse pre-B cell line 18-81, but functions in the human mature B cell line Raji, as well as three plasmacytomas (HS Sultan, human; S194, mouse; and MOPC 315, mouse). H S3 is also inactive in mouse 18-81 pre-B cell line, but shows modest activity in most of the more mature lines tested. H S3 shows surprisingly strong activity in the mouse S194 myeloma, indicating that unknown factors varying between cell lines at similar stages of differentiation can modulate the activity of this enhancer. Finally, the H S4 enhancer shows strong activity in the human pro-B cell line FLEB14, and is also variably active in all of the more mature cell lines (except 18-81) in which this enhancer was assayed in the B orientation.

Figure 4. Enhancer activity of selected regions downstream of human Cα1 and Cα2 genes. (A) Analysis of the locus downstream of α2, which was studied in detail. The map shows the position of DNase I sites below this are diagrammed the restriction sites defining the boundaries of each fragment tested for enhancer activity by insertion into pGL3-Vκ, transfection into the human myeloma HS Sultan, and assay of resulting luciferase activity, as described in the text. The enhancer activities are given for constructs in the A orientation (the same orientation with respect to transcribed strands of immunoglobulin and luciferase) or the opposite B orientation, where examined. The luciferase activities were normalized to β-galactosidase activity encoded by a cotransfected plasmid, and expressed as fold-increase over the activity of an enhancerless control plasmid. For fragments showing enhancer activity, assays were performed at least in triplicate, and standard deviations are given. (B) Comparable analysis of selected fragments amplified from the homologous locus downstream from Cα1.
Table 2. Cell Specificity of Human α2 Enhancer Elements

| Cell line         | H S12 (EH 1.3) | H S3 (SM 0.7) | H S4 (SpB 0.9) |
|-------------------|----------------|---------------|----------------|
|                    | A    | B    | A    | B    | X2   | A    | B    |
| Human             |      |      |      |      |      |      |      |
| FLEB 14 (pro B)   | 1.9 ± 0.1 | 1.9 ± 0.1 | N D  | N D  | N D  | 2.8 ± 0.2 | 18.4 ± 2.4 |
| Raji (Mature B)   | 3.1 ± 2.2 | 3.2 ± 1.8 | 0.7 ± 0.2 | 1.0 ± 0.3 | 2.8 ± 0.5 | 1.2 ± 0.1 | 2.7 ± 0.5 |
| HS Sultan (Plasmacytoma) | 5.3 ± 1.4 | 22 ± 10 | 1.54 | 0.59 | 2.3 ± 0.5 | 1.1 ± 0.2 | 5.4 ± 0.5 |
| Mouse             |      |      |      |      |      |      |      |
| 18-81 (Pre-B)     | 0.9 ± 0.2 | 1.1 ± 0.3 | 1.2 ± 0.1 | 0.8 ± 0.2 | 1.6 ± 0.5 | 0.5 ± 0.1 | 0.9 ± 0.2 |
| S 194 (Plasmacytoma) | 5.3 ± 0.2 | 14.4 ± 1.0 | 8.5 ± 0.8 | 2.6 ± 0.4 | 16.3 ± 0.3 | 7.2 ± 1.4 | 28.4 ± 2.3 |
| MOPC 315 (Plasmacytoma) | 5.9 ± 1.1 | 16.9 ± 1.9 | 1.4 ± 0.2 | 0.7 ± 0.2 | 2.9 ± 1.9 | 1.3 ± 0.6 | 5.8 ± 0.9 |

The numbers given represent the fold-increase of luciferase activity seen with a promoter-only control plasmid, with standard deviation. Numbers in bold were judged to represent significant enhancer activity.

The numbers given represent the fold-increase of luciferase activity seen with a promoter-only control plasmid, with standard deviation. Numbers in bold were judged to represent significant enhancer activity.

sequence identity between the human α1 and α2 elements, and similarity between the human and mouse enhancers ranging from 74 to 90%.

H S12, the strongest enhancer, showed 90% sequence identity to the homologous murine enhancer over a 135-bp core homology (Fig. 5 A). In the α2 locus, four tandem repeats with a 59-bp consensus sequence lie immediately upstream of the HS12 core. However, this sequence has been inverted in Fig. 5 A, to facilitate comparison with the homologous α1 sequence in opposite orientation. In the corresponding region of α1 (which, due to the inversion, lies downstream of the core homology region) a 115-bp deletion removes the second and third repeats; the α1H S12B region shows an additional deletion of 70 bp. The core homology region includes several of the functional motifs identified in the murine enhancer: the AP1-ETS site reported to confer responsiveness to B cell receptor cross-linking (22, 39); an exact octamer sequence (ATGCAAAT); and a μE5 site (except in the α1H S12B sequence, in which the μE5 is missing owing to the 70-bp deletion). The sequences of these three motifs from the human H S12 are identical to their murine homologs except for a single base change in the AP1 site which causes the human sequence to exactly match the consensus AP1 site, where the murine motif has one mismatch. The murine element designated μE1 (39), which has never been thoroughly documented even in the murine enhancer, is poorly conserved in the human homologs. Although the murine binding site for NF-κB lies outside the 135-bp region of strongest sequence similarity, a reasonable match to consensus for this element is found in a position roughly homologous to the murine κB site in the α1H S12T and α2H S12 sequences, but is part of the 70 bp deleted in α1H S12B. One of the mouse BSAP sites (BSAP2) is not conserved, but most residues in a second mouse BSAP binding site (BSAP1) are maintained in the human α2 H S12 enhancer. The murine αP site, which binds to an ETS-related transcription factor which augments enhancer activity (17), is not conserved in the human sequences.

The DNase I hypersensitive sites lying <3 kb downstream from the membrane exons of Cα1 and Cα2, roughly in the position of the weak enhancer reported by Matthias and Baltimore (11) and here designated H S3A, were found to contain sequences which are 74% identical to the murine H S3 over a 200 bp core segment. The two human H S3 segments are identical in the 326 bp shown (Fig. 5 B), and lie in the same orientation as H S3A. We have assumed that the correct orientation of the murine H S3A sequence is that described by Chauveau and Cogné (13). This orientation is opposite to that of the murine H S3B, which lies downstream from H S12 in the mouse, as described by Madisen and Groudine (12). Of the enhancer/HS sites downstream of murine Cα, H S3 is the least well investigated for functional motifs, in part because of its weak enhancer activity. Independent sequence analysis of the murine H S3A and H S3B regions detected several similarities to octamer motifs, AP1 sites, and consensus E box motifs (CANNTG). The AP1 site identified in Fig. 4 is a precise match to the consensus AP1 binding motif TGANTCA (40) in the human and two mouse H S3 sequences, and the murine sequence has been shown to bind to c-jun and c-fos in vitro (N eurath, M., personal communication). Similarly, several of the E box consensus motifs in the murine sequence have been shown to bind in vitro to proteins of the HLH family (N eurath, M., personal communication); some of these motifs are conserved in the two human H S3 sequences. The significance of the conserved motifs remains uncertain in the absence of a functional analysis of H S3 sequences.

The DNase I hypersensitivity sites furthest downstream
from human Cα1 and Cα2 in Fig. 3, which we have designated H S4, are 76% similar to the murine H S4 site over a core 145-bp sequence which spans the three functional motifs demonstrated in murine H S4 (23); see Fig. 5 C. The NF-κB motif and the downstream octamer motif in Fig. 5 C are both precisely conserved; in the murine H S4 these motifs both contribute to functional enhancer activity (23). In contrast, the BSAP site which upregulates murine H S4 enhancer activity in B cells but downregulates it in pre-B cells is completely absent from the human H S4 sequences. Lying between H S3 and H S12 in both the α1 and α2 loci are D N A ω I hypersensitive sites which are not associated with any of the known enhancer elements, but that do not map to the position of a 61-bp segment of 70% mouse-human homology. These conserved regions are provisionally designated X sites in part because of their unknown function, and in part because both the human and mouse segments contain XbaI restriction enzyme sites. In mouse this sequence is duplicated as part of the large inverted repeat centered on H S12, so that one copy lies between H S3A and H S12 while a second copy lies between H S12 and H S3B. A segment of (G A) n repeats is found near the X site in the direction of H S12 at an interval of 80 bp for both mouse X sites (13) and an interval of ~70 bp for the human (data not shown). Within the 61-bp conserved segment, the most highly conserved sequence is a consensus heat shock element (H S E; 41, 42). An H S E could potentially bind heat shock transcription factors (H S T F s), which are known to activate several heat shock response genes (H S P 70, H S P 90) in response to cellular stress such as heating (43–45). Fragments containing an X site do not appear to dramatically affect enhancer activity in H S Sultan, but may contribute to regulation through mechanisms not captured in our transient transfection assays.

Discussion

Structure of Human 3′ α Regions: Evolutionary Implications. The enhancers clustered 3′ of Cα in the mouse I g H locus can activate the upstream genes, functioning as an L C R. In human I g H locus, arrays of enhancers homologous to those 3′ of mouse Cα are located at two positions within the human I g H locus, 3′ of each Cα gene. On the basis of sequence homology and conservation of restriction
sites between the two human enhancer arrays, it is apparent that these enhancers lie near the 3' ends of the two duplication units which encompass the γ3-γ1-γe-α1 and γ2-γe-α2 gene clusters (25–27), indicating that the 3' α enhancer arrays were present in an approximation of the human arrangement preceding the duplication event that gave rise to the present human IgH locus structure. Moreover, the arrangement downstream of both human Cα genes is 5'-H S3-H S12-H S4-3', in contrast to the large palindromic structure downstream from mouse Cα that contains a 5'-H S3A-H S12-H S3B-H S4-3' arrangement (13). Therefore, although an arrangement containing an H S3 enhancer proximal to the Cα membrane exon and an H S4 enhancer farther downstream would seem to have been present in the common ancestor of rodents and primates, the mouse H S3A-H S12-H S3B palindrome probably arose after the primate–rodent divergence. Finally, there is a major structural difference between the 3'α1 and 3'α2 enhancer arrays; namely, that a DNA segment containing H S12 is inverted between the two loci. Using probes containing the 135-bp human H S12 core, it should now be possible to examine DNA from a number of primates for inversion of 3'α2 H S12 relative to 3'α1 H S12; such data may indicate when in evolution the inversion event occurred, and which orientation was present initially in the locus. What caused this inversion? Interestingly, the single mouse H S12 lies in opposite orientation from the rat H S12, and both are flanked by inverted repeats (13) which are known to mediate inversions in other genomic contexts, e.g., in the iduronate-2-sulfatase gene causing Hunter syndrome (46) and iduronic acid 2-sulfatase gene causing Maroteaux–Lamy (47). Limited Southern blot experiments have not provided evidence for inverted repeats flanking the human H S12 sequences (data not shown). Some hints about the mechanism of the inversion may be found when the inversion breakpoints are identified and sequenced, work currently in progress in our laboratory.

H S12 Structure and Function. The 135-bp H S12 core homology sequence is likely to contain essential motifs important for the strong, late, B cell–specific enhancer activity characteristic of H S12 in mice and humans. Although the function of transcription factor binding sites within the human H S12 core has not yet been demonstrated experimentally, this segment contains sequences nearly identical to the murine AP1, ETS, Oct, and, in α1H S12T and α2H S12, μ E5 motifs, all of which are functional in the mouse H S12 enhancer. However, the high degree of sequence conservation in the H S12 core homology extends beyond the transcription factor–binding sites identified in the mouse enhancer, indicating that there may be additional conserved motifs that have not been characterized in either mice or humans.

Despite the fact that a number of other transcription factor motifs whose function has been demonstrated in the mouse H S12 lie outside the H S12 135-bp core homology and are absent in one or more of the human α1H S12T, α1H S12B, and α2H S12 enhancers that we have studied, these enhancers all show roughly equivalent activities. This result suggests that elements missing from these enhancers are not essential for enhancer function in H S Sultan. These inconsistently conserved elements include human sequences corresponding to μ E5 and N F-κ B sites (absent from α1H S12B) and the BSAP2 site (absent from both α1 alleles). On the other hand, a 1.3-kb α2 EcoRI–HindIII fragment containing the α2 H S12 core plus considerable flanking sequence shows a dependence of enhancer activity on orientation of the fragment (Table 2), suggesting that uncharacterized elements beyond the H S12 core may have some inhibitory function.

Outside the human H S12 core are GC-rich 59-bp repeat units which by themselves do not have enhancer activity in the H S Sultan myeloma (EP300, Fig. 4 A), and are not conserved between mice and humans (Fig. 5 A). However, it is possible that these repeats contribute to enhancer activity because they are present in the A2H S12 PCR–generated fragment, which gives significantly higher enhancer activity than we observe in the p300 fragment containing the core homology. Deletions of the 59-bp repeats have given rise to apparent allelic polymorphisms, as evidenced by α1H S12T (deletion of the second and third repeats found in α2H S12), α1H S12 (deletion extending from 28 bp 5' of the first repeat through the third repeat), and other alleles (H arin anath, N., unpublished results).

H S3 and H S4. The other two enhancer components of the mouse 3'α LCR, H S3 and H S4, are weaker enhancers than H S12, but nonetheless are essential for locus control activity (12). Although these elements are less well characterized than the H S12 enhancer, the existing data indicate general human–mouse similarity of the H S3 and H S4 elements, with some notable differences.

In the mouse system, the H S3A element assayed in CAT reporter gene constructs driven by c-fos or thymidine kinase promoters (11) showed weak enhancer activity, although the nearly identical H S3B enhancer showed substantial activity in certain constructs with other promoters tested by another laboratory (12). These disparate results resemble our data on the human α1 and α2 H S3 elements in that single copies and dimers of human H S3 generally gave very low enhancer activity, except in the mouse S194 myeloma in which the same constructs gave substantial enhancer activity comparable to that of H S12 (Table 1). Taken together, these data suggest that H S3, though typically the weakest of the 3' α enhancers, contains uncharacterized motifs that in some cells and/or in combination with certain promoters, can mediate a strong enhancer function.

H S4 is the most downstream 3' α enhancer in both mice and humans, and shows activity intermediate between that of H S3 and H S12. The H S4 enhancer data in the mouse (12, 48), as well as our data on the human α1 and α2 H S4 elements, demonstrate that H S4 is active from the early stages of the B cell lineage onward (Table 2), and thus is qualitatively different from H S3 and H S12. In mouse H S4, there is a binding site for the BSAP, which is expressed in the early B cell lineage. However, in the human α1 and α2 H S4 enhancers, the BSAP site is deleted, indicating that BSAP binding is not an essential feature for H S4 activation.
in human pre-B cells. The human HS4 is inactive in the 18-81 mouse pre-B cell line, which was reported to support the activity of mouse HS4 (12). The significance of this difference is not clear; it could be related to the BSAP site deletion in the human HS4, or perhaps to other differences between the mouse and human HS4 sequences.

X Site. The DNase I X sites may represent novel control elements that function together with the H3S, H3S12, and H3S4 enhancers to activate the IgH locus. Although the significance of the conserved HSE motif is unclear, binding of HSTF protein to an HSE has been shown to be critical for maintaining the DNase I hypersensitivity of the yeast HSC82 gene promoter (49). Heat shock activation of the Drosophila HSP70 gene promoter results from binding of HSTF to HSE sites after accessibility of the HSEs has been established by binding of the GAGA protein to adjacent (GA)ₙ repeats (50). This demonstrated interaction between HSEs and (GA)ₙ motifs suggests that the location of a (GA)ₙ repeat region 70–80 bp away from the X site HSE may be of some significance. Furthermore, in the context of IgH gene regulation, it is of interest to note that HSE motifs have been shown to respond to IL-2 and IL-4 (51).

Potential LCR Control Region. In the mouse, it has been demonstrated that when H3S, H3S12, and H3S4 are linked together in a construct containing the c-myc gene and stably transfected into the Raji human B cell line, the c-myc gene is transcribed independent of integration site (12). This observation suggests that the HS1234 combination confers LCR activity, although LCR's have more typically been described based on position-independent transcription of mouse transgenes rather than genes transfected into a cell line. Because the regions 3' of the human Cα1 and Cα2 genes contain similar H3S, H3S12, and H3S4 elements that function as enhancers, it is reasonable to hypothesize that these elements also function together in the human system as LCR's. The different arrangement of 3' α enhancers in mice and humans (H3S3A-H3S12-H3S3B-H3S4 versus H3S3-H3S12-H3S4) may cause some functional differences in these control regions. Moreover the distance between 3' α enhancers also differs between mice and humans, with the mouse enhancer complex spanning a 30-kb region (13), whereas both the human 3'α1 and 3'α2 enhancers span ~15 kb.

Our finding that arrays of enhancers homologous to those in the mouse 3'α LCR lie downstream of both human Cα genes raises the possibility that differences in the activation of each human γ-γ-α-α duplication unit result from differences between the putative 3'α1 and 3'α2 LCR's. Even though sequence comparison shows that there is near identity between homologous enhancer elements in the α1 versus α2 locus (Fig. 5), transcription and expression of the upstream heavy chain duplication unit (γ3-γ1-ι(e-α1)) is greatly elevated relative to the downstream unit (γ2-γ4-ε-α2; reference 2). This difference could result from the fact that the 3'α2 H3S12 element is inverted relative to the 3'α1 H3S12, and is also at a greater distance from H3S3 than in the α1 locus, possibly reducing synergistic interactions between H3S3 and H3S12. Alternatively, it may be that only the 3'α1 enhancers are activated early in B cell development, possibly falling under the influence of the upstream Eμ enhancer, which itself can function as an LCR (52, 53). In this model, the Eμ and 3'α1 enhancers together would activate a large domain encompassing the first duplication unit, whereas the second duplication unit and the 3'α2 enhancers would fall outside this combined domain. Thus activation of the second duplication unit would depend solely on the 3'α2 enhancers, and expression of genes in this unit might therefore be reduced. Clarification of the basis for this difference will have to await experiments that involve specific deletion of either the 3'α1 or 3'α2 enhancers, as well as studies identifying matrix attachment sites and chromatin insulator elements that define domains within the human IgH region (52–54).

In the work presented here, we have laid the foundation for experimental studies on the activation of the human IgH gene transcription, as well as the regulation of isotype switching. In addition, knowledge of the action of these enhancers on distant constant region genes should contribute to a general understanding of the mechanisms underlying activation of large gene clusters.
References

1. Coffman, R.L., D.A. Lebman, and P. Rothman. 1993. The mechanism and regulation of immunoglobulin isotype switching. A dv. Immunol. 54:229–270.

2. Sideras, P., L. Nilsson, K.B. Islam, I.Z. Quintana, L. Freihof, G.J. Rosén, L. Hammarström, and C.I. Smith. 1992. Transcription of unrearranged Ig H chain genes in human B cell malignancies. Biased expression of genes encoded within the first duplication unit of the Ig H chain locus. J. Immunol. 149: 244.

3. Klein, S., F. Sabilitzky, and A. Radbruch. 1984. Deletion of the IgH enhancer does not reduce immunoglobulin production of a hybridoma IgD class switch variant. EMBO (Eur. Mol. Biol. Organ.) J. 3:2473–2476.

4. Eckhardt, L.A., and B.K. Birshtein. 1985. Independent immunoglobulin class-switch events occurring in a single myeloma cell line. Mol. Cél. Biol. 5:856–868.

5. Wabl, M., and P.D. Burrows. 1984. Expression of immunoglobulin heavy chain at a high level in the absence of a proposed immunoglobulin enhancer in ds. Proc. Natl. Acad. Sci. USA. 81:2452–2456.

6. Aguilera, R.J., T.J. Hope, and H. Sakano. 1985. Characterization of immunoglobulin enhancer deletions in murine plasmacytomas. EMBO (Eur. Mol. Biol. Organ.) J. 4:3689–3693.

7. Gregor, P.D., and S.L. Morrison. 1986. Myeloma mutant with a novel 3’ flanking region: loss of normal sequence and insertion of repetitive elements leads to decreased transcription but normal processing of alpha heavy-chain gene products. Mol. Cél. Biol. 6:1903.

8. Pettersson, S., G.P. Cook, M. Bruggemann, G.T. Williams, and M.S. Neuberger. 1990. A second B cell-specific enhancer of the immunoglobulin heavy-chain locus. Nature (Lond.). 344:165–168.

9. Dariavach, P., G.T. Williams, K. Campbell, S. Pettersson, and M.S. Neuberger. 1991. The mouse IgH 3’-enhancer. Eur. J. Immunol. 21:1499–1504.

10. Lieberson, R., S.L. Giannini, B.K. Birshtein, and L.A. Eckhart. 1991. An enhancer at the 3’ end of the mouse immunoglobulin heavy chain locus. Nucleic Acids Res. 19:933–937.

11. Matthias, P., and D. Baltimore. 1993. The immunoglobulin heavy chain locus contains another B-cell-specific 3’ enhancer close to the constant region. Mol. Cél. Biol. 13:1547–1553.

12. Madisen, L., and M. Groudine. 1994. Identification of a locus control region in the immunoglobulin heavy-chain locus that contains another B-cell-specific 3’ enhancer close to the constant region. Mol. Cél. Biol. 13:1547–1553.

13. Chauve, C., and C. Cogné. 1996. Palindromic structure of the IgH 3’ locus control region. Nat. Genet. 14:15–16.

14. Tabot, D., P. Collis, M. Antoniou, M. Vidal, F. Grosfeld, and D.R. Greaves. 1989. A dominant control region from the human β-globin locus conferring integration site-independent gene expression. Nature (Lond.). 338:352–355.

15. Ernst, P., and S.T. Smale. 1995. Combinatorial regulation of transcription. J. General aspects of transcriptional control. Immunity. 2:311–319.

16. Meyer, K.B., M. Skoberg, C. Morgenfeld, J. Ireland, and S. Pettersson. 1995. Repression of the immunoglobulin heavy chain 3’ enhancer by helix-loop helix protein Id3 via a functionally important E47/E12 binding site: implications for developmental control of enhancer function. Eur. J. Immunol. 25:1770–1777.

17. Neurath, M.F., E.E. Max, and W. Strober. 1995. Pax5 (BSAP) regulates the murine immunoglobulin 3’α enhancer by suppressing binding of NF-κB, a protein that controls heavy chain transcription. Proc. Natl. Acad. Sci. USA. 92: 5336–5340.

18. Ernst, P., and S.T. Smale. 1995. Combinatorial regulation of transcription II: the immunoglobulin μ heavy chain gene. Immunity. 2:427–438.

19. Singh, M., and B.K. Birshtein. 1993. NF-κB (BSAP) is a repressor of the murine immunoglobulin heavy-chain 3’α enhancer at early stages of B cell differentiation. Mol. Cél. Biol. 16:3611–3622.

20. Neurath, M.F., W. Strober, and Y. Wakatsuki. 1994. The murine Ig3’α enhancer is a target site with repressor function for the B cell lineage-specific transcription factor BSAP (NF-kB, S6-5P). J. Immunol. 153:730–742.

21. Singh, M., and B.K. Birshtein. 1996. Concerted repression of an immunoglobulin heavy chain enhancer, 3’α-E(12). Proc Natl. Acad. Sci. USA. 93:4392–4397.

22. Grant, P.A., C.B. Thompson, and S. Pettersson. 1995. IgM receptor-mediated transactivation of the IgH 3’ enhancer couples a novel Elf-1-AP-1 protein complex to the development control of enhancer function. EMBO (Eur. Mol. Biol. Organ.) J. 14:4501–4513.

23. Michaelson, J.S., M. Singh, C.M. Snapper, W.C. Sha, D. Baltimore, and B.K. Birshtein. 1996. Regulation of 3’ IgH enhancers by a common set of factors, including κB-binding proteins. J. Immunol. 156:2828–2839.

24. Cogné, M., R. Lansford, A. Bottaro, J. Zhang, J. Gorman, F. Young, H.-L. Cheng, and F.W. Alt. 1994. A class switch control region at the 3’ end of the immunoglobulin heavy chain locus. Cell. 77:737–747.

25. Flanagan, J.G., and T.H. Rabbits. 1982. Arrangement of human immunoglobulin heavy chain constant region genes implies evolutionary duplication of a segment containing γ, ε and α genes. Nature (Lond.). 300:709–713.

26. Holker, M.H., M.A. Walter, and D.W. Cox. 1989. Complete physical map of the human immunoglobulin heavy chain constant region gene complex. Proc. Natl. Acad. Sci. USA. 86:5567–5571.

27. Kawamura, S., and S. Ueda. 1992. Immunoglobulin Cγ gene family in hominoids and its evolutionary history. G enomics. 13:194–200.

28. Hammarström, L., A.O. Carbonara, M. DeM archi, G. LeFranc, M.-P. LeFranc, and C.I.E. Smith. 1987. Generation of the antibody repertoire in individuals with multiple immunoglobulin heavy-chain constant region gene deletions. Scand. J. Immunol. 25:189–194.

29. Gualandi, G., D. Frezza, A. Scotto d’Abusco, E. Bianchi, S. Gargano, S. Giorgi, A. Fruscalzo, and E. Calef. 1995. Integration of an Epstein-Barr virus episome 3’ into the gene encoding immunoglobulin heavy-chain α1 in a lymphoblastoid line. Gene. 166:221–226.

30. Chen, C., and B.K. Birshtein. 1995. A region of 20-bp repeats lies 3’ of human Ig Ca1 and Ca2 genes. Int. Immunol. 8: 115–122.

31. Kang, H.K., and D.W. Cox. 1996. Tandem repeats 3’ of the IGHα genes in the human immunoglobulin heavy chain cluster. Genomics. 35:189–195.

32. Takahashi, N., S. Ueda, M. O bata, T. N ikaido, S. Nakai, and
T. Honjo. 1982. Structure of human immunoglobulin gamma genes: implications for evolution of a gene family. Cell. 29: 671–679.

33. Mills, F.C., M.P. Mitchell, N. Harindranath, and E.E. M. 1995. Human IgS regions and their participation in sequential switching to IgE. J. Immunol. 155:3021–3036.

34. Rao, V.N., K. Huebner, M. Isobe, A. Ar-Rushdi, C.M. Croce, and E.S.P. Reddy. 1989. elk, tissue-specific ets-related genes on chromosomes X and 14 near translocation breakpoints. Science (Wash. D.C.). 244:66–70.

35. Bensmana, M., and M.-P. Lefranc. 1990. Gene segments encoding membrane domains of the human immunoglobulin gamma 3 and alpha chains. Immunogenetics. 32:321–330.

36. Siebenlist, U., L. Hennighausen, J. Battey, and P. Leder. 1984. Chromatin structure and protein binding in the putative regulatory region of the c-myc gene in Burkitt lymphoma. Cell. 37:381–391.

37. Bergman, Y., D. Rice, R. Grosschedl, and D. Baltimore. 1984. Two regulatory elements for immunoglobulin k light chain gene expression. Proc. Natl. Acad. Sci. USA. 81:7041–7045.

38. Bensmana, M., H.S. Huck, G. Lefranc, and M.-P. Lefranc. 1988. The human immunoglobulin pseudo-gamma IGHGP gene shows no major structural defect. Nucleic Acids Res. 16: 3108.

39. Grant, P.A., V. Arulampalam, L. Arhlumd-Richter, and S. Pettersson. 1992. Identification of Ets-like lymphoid specific elements within the immunoglobulin heavy chain enhancer. Nucleic Acids Res. 20:4401–4408.

40. Janknecht, R., and A. Nordheim. 1993. Gene regulation by Ets proteins. Biochim. Biophys. 23: 155:346–356.

41. Amin, J., J. Ananthan, and R. Voellmy. 1988. Key features of heat shock regulatory elements Mol. Cell. Biol. 8: 3761–3769.

42. Xio, H., and J. T. Lis 1988. Germline transformation used to define key features of heat-shock response elements. Science (Wash. D.C). 239:1139–1142.

43. Zmarino, V., C. Tsa, and C. Wu. 1990. Complex modes of heat shock factor activation. Mol. Cell. Biol. 10:752–759.

44. Schuetz, T.J., G. Gallo, J.L. Shelton, P. Tempst, and R.E. Kingston. 1991. Isolation of dCDNA for HS F2: evidence for two heat shock factor genes in humans. Proc. Natl. Acad. Sci. USA. 88:6911–6915.

45. Rabindran, S.K., G. Giorgi, J. Clos, and C. Wu. 1991. Molecular cloning and expression of a human heat shock factor, HS F1. Proc. Natl. Acad. Sci. USA. 88:6906–6910.

46. Bondeson, M.L., N. Dahl, H. Malmgren, W.J. Kleijer, T. Tonnisen, B.M. Carlborg, and U. Pettersson. 1995. Inversion of the IDS gene resulting from recombination with IDS-related sequences is a common cause of the Hunter syndrome. Hum. Mol. Genet. 4:615–621.

47. Naylor, J.A., D. Buck, P. Green, H. Williamson, D. Bentley, and F. Giannelli. 1995. Investigation of the factor VIII intron 22 repeated region (int22h) and the associated inversion junctions. Hum. Mol. Genet. 4:1217–1224.

48. Michaelson, J.S., D.L. Lefranc, and B.K. Birshtein. 1995. Identification of 3α-hs4, a novel Ig heavy chain enhancer element regulated at multiple states of B cell differentiation. Nucleic Acids Res. 23:975–981.

49. Ergne, A.M., C.C. Adams, T. Dikaen, and D.S. Gross 1996. Heat shock factor gains access to the yeast HSC82 promoter independently of other sequence-specific factors and antagonizes nucleosomal repression of basal and induced transcription. Mol. Cell. Biol. 16:7004–7017.

50. Sukiya, T., P.B. Becker, and C. Wu. 1994. ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. Nature (Lond.). 367:525–532.

51. Metz, K., J. Ezerniesks, W. Sebald, and A. Duschl. 1996. Interleukin-4 upregulates the heat shock protein Hsp90a and enhances transcription of a reporter gene coupled to a single heat shock element. FEBS (Fed. Eur. Biochem. Soc.) Lett. 385: 25–28.

52. Jenuwein, T., W.C. Forrester, R.G. Qiu, and R. Grosschedl. 1993. The immunoglobulin mu enhancer core establishes local factor access in nuclear chromatin independent of transcriptional stimulation. Genes Dev. 7:2016–2032.

53. Forrester, W.C., C. van Genderen, T. Jenuwein, and R. Grosschedl. 1994. Dependence of enhancer-mediated transcription of the immunoglobulin mu gene on nuclear matrix attachment regions. Science (Wash. D.C). 265:1221–1225.

54. Chung, J.H., M. Wittenly, and G. Felsenfeld. 1993. A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in drosophila. Cell. 74:505–514.