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Identification of 3′α-hs4, a novel Ig heavy chain enhancer element regulated at multiple stages of B cell differentiation

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ABSTRACT

In addition to Eμ, several elements downstream of the IgH cluster, i.e. 3′ of the Cα gene, are involved in regulating IgH gene rearrangement and expression. This entire downstream regulatory region was shown to be deleted in the mutant myeloma cell line, LP1.2. The deletion encompasses ~34 kb and is presumably responsible for the reduced levels of IgH expression in this cell line. An additional regulatory element, included in the LP1.2 deletion, was identified by investigation of a DNase I hypersensitivity site located ~33 kb downstream of the α gene and present in pre-B and plasma cells. This novel IgH gene enhancer element, termed 3′α-hs4, is capable of activity throughout B cell development. Transient transfection of 3′α-hs4 in a CAT reporter gene construct shows transcriptional enhancement activity approximating that of Eμ in S194 plasmacytoma and M12.4.1 and A-20 B cell lines; while in a pre-B cell line, 18-81, the average activity is 25% that of Eμ. Enhancer activity was localized to an 800 bp fragment. The activity of 3′α-hs4 is orientation independent and appears to be B cell specific. Tight regulation of 3′α-hs4 is inferred from its variable activity in different plasmacytoma cell lines and within the pre B cell line, 18-81.

INTRODUCTION

Immunoglobulin heavy chain genes undergo a number of processes, including V(D)J recombination, class switching, somatic mutation and high levels of expression [reviewed in (1)]. These events are regulated by multiple *cis*-acting elements [reviewed in (1)]. One such element is the intron enhancer, Eμ, located upstream of the μ constant region gene. Initial evidence supporting the notion that regulatory elements other than Eμ might exist came from several myeloma mutants which produced normal levels of heavy chain mRNA protein despite deletion of Eμ (2–5). Moreover, a mutant myeloma cell line, LP1.2, which has a very low rate of alpha transcription, was shown to have a deletion of at least 3.8 kb in the region 3′ of the alpha gene (6). This finding suggested that sequences downstream of Cα, i.e. downstream of the immunoglobulin heavy chain gene cluster, that were missing from LP1.2 might be responsible for regulating transcription from the α gene. We have now mapped the deletion encompassing the region 3′ of Cα in the low expressing cell line LP1.2. This deletion encompasses ~34 kb and includes a number of *cis*-acting regulatory elements, which have been mapped to the region 3′ of the alpha gene (Fig. 1).

The first of these, 3′αE, was identified in rat (26 kb downstream of Cα) (7) and in mouse (12.5 kb downstream of Cα membrane exon; Fig. 1) (8,9). 3′αE is a B cell specific element, which exhibits activity ranging from 25 to 100% that of Eμ when transiently transfected into plasmacytoma lines (MPC11, S194, HOPC1 and J558L), but is generally inactive upon transfection into B cell lines (A-20 and W231) (8–10). Data from a transgenic mouse model have confirmed that the activity of 3′αE is, in fact, restricted to late stage immunocompetent B cells (11). This stage specific activity is correlated with the identification of BSAP, a B cell specific transcription factor, as a negative *trans*-acting regulator of 3′αE (10,12). BSAP is a B cell specific transcription factor expressed in pro-B, pre-B and B-cell lines; thus, in plasmacytoma cells, where BSAP is no longer expressed, 3′αE is fully active. Recently, a role for 3′αE in class switching was demonstrated when targeted deletion of 3′αE in mouse resulted in isotype switch deficiencies (13). Mice whose B cells lacked 3′αE were deficient in serum IgG2a and IgG3, while cultured B cells from these mice failed to undergo switching to IgG2a, IgG2b, IgG3 and IgE.

A second and weaker positive regulatory element in the 3′ α region was described by Matthias *et al.* (14). Located in close proximity to the Cα gene (Fig. 1), Cα3′ enhancer is only ~5–15% as active as Eμ when transiently transfected into either B cell or plasmacytoma cell lines.

A general approach to identifying potential control elements in the DNA sequence, such as enhancers, is to characterize sites that are hypersensitive to digestion with DNase I. Accordingly, our previous studies showed that a pair of DNase I hypersensitivity sites, present at the plasma cell stage, was associated with 3′αE (15). In addition, a site exhibiting DNase I hypersensitivity in pre-B cells (18-81 and 3-1 cell lines) as well as in plasma cells (MPC11 and F5.5 lines), was identified ~17 kb downstream of 3′αE, i.e. 33 kb 3′ of Cα (15). We speculated that this

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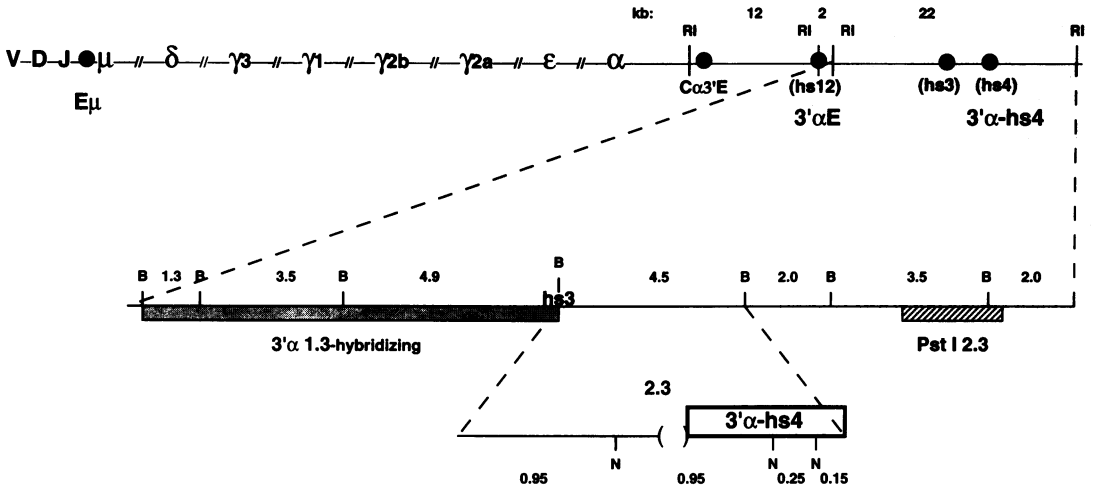

Figure 1. Map of mouse Ig heavy chain locus. Filled circles indicate regulatory elements (8,9,14,28). Shaded regions denote sequences which hybridize to the probe 3'α-1.3 (15); striped region marks location of *Pst*I-2.3 probe. Parentheses indicate an internal deletion within the 2.3 kb *Bam*HI fragment (as cloned from clone D) which derives from the 4.5 kb *Bam*HI fragment. The sequence at the extreme 5' end of the 2.3 kb *Bam*HI fragment matches that of the 3' portion of hs3, commencing at base pair 739 (*Bam*HI site) of hs3 (28). Numbers refer to kb between adjacent restriction enzyme sites. Restriction enzyme sites shown are *Eco*RI (RI), *Bam*HI (B) and *Nco*I (N). The size of the 3'-most *Eco*RI fragment in BALB/c genomic DNA, based on genomic Southern blot analysis, was originally reported to be 26 kb (15). Our cloning data indicate that the actual size is ~22 kb. Note that the size of the corresponding fragment in W3129 is larger (6).

hypersensitivity site might be indicative of an additional control element.

In this study, we have cloned and mapped a segment of DNA in the vicinity of the distal 3' hypersensitivity site and have shown that it houses transcriptional enhancement activity comparable to that of Eμ. This enhancer is active not only in plasma cells, as is 3'αE, but in the pre-B and B-cell stages of differentiation as well. We have termed this novel enhancer element 3'α-hs4. The activity of 3'α-hs4 appears to be restricted to cells of the B lineage. The deletion in LP1.2 includes all of the previously identified regulatory elements in the region 3' of Cα, as well as 3'α-hs4.

MATERIAL AND METHODS

Cell lines

The following mouse cell lines representing different stages of B cell differentiation were used: 18-81 (pre-B), M12.4.1 and A-20 (B-cell lymphoma), and S194 and J558L (plasmacytoma). Mouse T cell lines SL3B and L691-6 (16), and a human T cell line, Jurkat, were used as representative non-B lymphoid lines. All cell lines were grown in RPMI (BioWhittaker) and supplemented with heat-inactivated 10% fetal bovine serum (Gibco-BRL), 50 μM β-mercaptoethanol, 1% penicillin–streptomycin and 2 mM L-glutamine; except for J558L, which was grown in DMEM (JRH Biosciences), supplemented with 20% heat-inactivated donor horse serum (Mediatech) and 1% pen-strep (Gibco-BRL), and L691-6, which was grown in DMEM with 10% fetal bovine serum and 1% pen-strep. All cells were maintained in suspension at 37°C in an atmosphere of 7–8% CO_2. 18-81 subclones 316.10 and Vγ2a-8 were a kind gift of N. Green and M. Scharff (Albert Einstein); L691-6, SL3B and Jurkat cells were kindly given by J. Lenz (Albert Einstein); A-20 cells were generously given by S. Desiderio (Johns Hopkins).

Phage and plasmid DNA cloning

A phage library was constructed in Charon 40 phage by complete digestion of F5.5 [a mouse myeloma cell line derived from MPC11 (17)] DNA with *Bgl*II. The library was screened with a probe from the region 3' of Cα called 3'α-540 [a derivative of 3'α-1.3 (15)]. Clone D, containing an 11.5 kb insert commencing 6 kb downstream of the 5' end of the 22 kb *Eco*RI fragment, was chosen for further study. Fragments from clone D were subcloned into pWSK129 (18), a kind gift of S. Kushner, by transformation into the MB408 cell line (19), a gift from P. Stanley of Albert Einstein.

An S107 partial *Mbo*I library, cloned into Charon 35, was obtained from R. DePinho (Albert Einstein). *Pst*I-2.3 (6) was used to screen the library, and clone 4A was isolated. The 11 kb insert in clone 4A is derived from the 3' portion of the 22 kb *Eco*RI fragment.

PCR and sequencing

The 800 bp enhancer fragment was amplified by PCR using *Taq* DNA Polymerase (Boehringer Mannheim) with an annealing temperature of 55°C for 35 cycles. This fragment was sequenced by the chain termination method (20), using a Sequenase kit (US Biochemicals), and by automated sequencing on a 373A ABI DNA sequencer.

Transient transfections and CAT assays

All cells were grown to log phase and transiently transfected by the DEAE–dextran method, primarily as previously described (10,16). 2×10^7 cells were used per transfection for S194, 18-81 and J558L; for M12.4.1 and Jurkat, 10^7 cells were transfected; for L691-6 and SL3B, 5×10^6 cells were transfected. Transfection of S194 and A-20 was followed by chloroquine treatment (21,22), while transfection of M12.4.1 was followed by brief trypsinization (23). 18-81, M12.4.1, A20, S194 and J558L were transfected with 10 μg of reporter plasmid. M12.4.1, A-20 and

S194 were co-transfected with 5 μg of Rous sarcoma virus-β-galactosidase (RSV-β-gal) expression vector. For L691-6, SL3B and Jurkat, 5 μg of reporter plasmid were transfected; L691-6 was co-transfected with 1 μg of RSV-β-gal. Following 44 h of incubation at 37°C, cells were harvested, washed, resuspended in 100 μl Tris–HCl pH 8.0 and lysed by the freeze–thaw method (21). Protein concentrations were determined by the BioRad Protein Assay. Thirty μl of extract was used for the β-galactosidase assay with chlorophenol red β-D-galactopyranoside (CPRG) as the substrate (24).

CAT assays were performed as described (10). Briefly, following heat inactivation at 65°C for 10 min, equal amounts of β-galactosidase units (for M12.4.1, A-20, S194 and L691-6) or protein (for 18-81, J558L, SL3B and Jurkat, since β-galactosidase activity was low in these cell lines), were taken for use in the CAT assay. CAT activity was quantitated by the solvent extraction method (21) following 2 h (2.5 h for A-20; 4 h for 18-81, SL3B and Jurkat) of incubation at 37°C.

QM293 was the CAT expression vector used in these experiments. Constructs were prepared by inserting test DNA into the *Sma*I site 5′ to the Ig light chain λ_1 promoter of this vector. QM293 and QM351 (containing Eμ as an insert) were prepared by M. Anderson and S. Morrison of UCLA and were a gift of L. Eckhardt of Hunter College. SL3-LTR (21), used as a positive control for CAT activity in the T cell lines, was a gift from J. Lenz (Albert Einstein).

RESULTS

Deletion of ~34 kb from 3′ regulatory region in LP1.2

Analysis of a mutant myeloma cell line, LP1.2, provided evidence for the role of elements from the region downstream of Cα in regulating Ig heavy chain gene expression. Gregor and Morrison noted that LP1.2 had a unique phenotype of low Cα gene transcription relative to other members of its lineage (W3129 → R15 → Δ15.8 → LP1.2) (6), including its immediate parent, Δ15.8. Correlated with this phenotype was their estimation of a DNA deletion of at least 3.8 kb commencing 11 bp downstream of the polyadenylation site associated with the secreted Cα gene (6). A 6.2 kb *Eco*RI fragment containing the rearranged Cα gene segment from LP1.2 was isolated and was the source of a unique DNA probe, *Pst*I-2.3 (Fig. 2A). This probe was shown to hybridize to a single ~30 kb *Eco*RI fragment in BALB/c genomic DNA, to two fragments in W3129 and Δ15.8, of 30 and 20 kb, and to two fragments in LP1.2 of 30 and 6.2 kb (6).

We sought to define the extent of the lesion in LP1.2 by locating *Pst*I-2.3 in its original genomic context. Because the size of the largest *Eco*RI fragment was similar to that identified in several mouse strains by the probe 3′α-1.3 (~22 kb) (15) (Fig. 1), we hypothesized that 3′α-1.3 and *Pst*I-2.3 hybridized to the same fragment. Support for this hypothesis came from a study of a panel of wild mouse DNA, which revealed one mouse strain, *Papaeus cervicolor*, with a smaller 3′α-1.3 hybridizing fragment, of 11.5 kb, that also hybridized uniquely with *Pst*I-2.3 (25).

Further documentation linking 3′α-1.3 sequences with *Pst*I-2.3 sequences in BALB/c mice came from two overlapping phage clones which we isolated from the region. Clone D, initially isolated from a phage library by hybridization with a probe derived from 3′α-1.3 (see Materials and Methods), contained sequences at its 3′ end that hybridized to *Pst*I-2.3. Figure 2B demonstrates hybridization of both probes to clone D digested DNA. Similarly, a second phage clone, clone 4A (see Materials and Methods), isolated by hybridization to *Pst*I-2.3, hybridized to two probes (2.0 and 2.3 kb *Bam*HI fragments isolated from clone D) derived from the 22 kb *Eco*RI fragment (data not shown). Moreover, the DNA sequence at the extreme 3′ end of the 22 kb *Eco*RI fragment, as sequenced from clone 4A, directly matched that of the 3′ end of the 6.2 kb *Eco*RI fragment from LP1.2. The regions sequenced are denoted by arrows in Figure 2A. These data confirmed that the 3′ portion of the 6.2 kb fragment was derived from the 3′ end of the 22 kb *Eco*RI fragment.

Because the 6.2 kb *Eco*RI fragment inclusive of the rearranged Cα gene in LP1.2 contained sequences from the 3′ end of the 22 kb *Eco*RI fragment, we concluded that the deletion in LP1.2

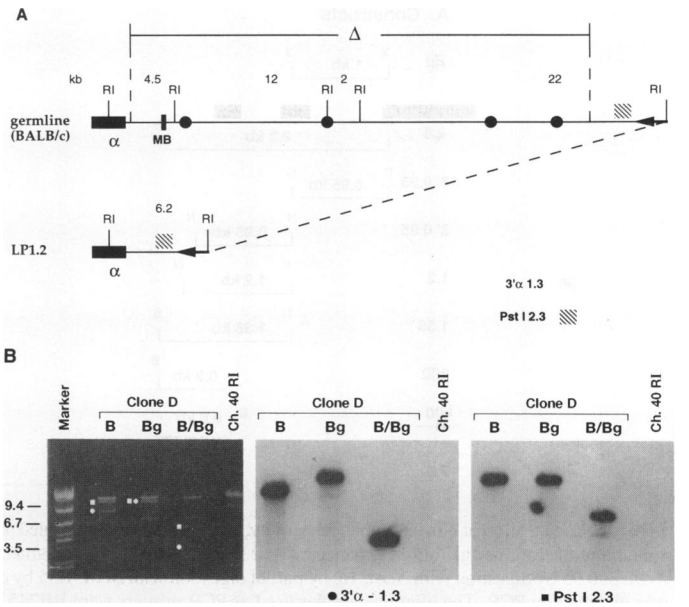

Figure 2. Deletion in LP1.2 encompasses ~34 kb from region 3′ of Cα. (**A**) The BALB/c germline map of the region 3′ of Cα is compared to that of LP1.2 (6). Δ marks the region deleted in LP1.2. The 5′ end of the deletion commences 11 bp downstream of the secreted polyadenylation site (6). Numbers refer to kb between adjacent *Eco*RI sites. MB indicates the membrane exon associated with Cα. Filled circles indicate regulatory elements in the region. Shaded regions denote sequences which hybridize to the probe 3′α-1.3 (15); striped region marks location of *Pst*I-2.3 probe. Segments denoted by arrows were sequenced. Germline sequences were obtained from the extreme 3′ end of clone 4A; for LP1.2, the extreme 3′ end of the 6.2 kb RI fragment was sequenced [dideoxy method; (20)]. The sequences were 100% identical over the 132 bp region sequenced. RI = *Eco*RI. (**B**) The left panel represents an ethidium bromide stained 0.8% agarose gel. The marker lane contains λDNA digested with *Hin*dIII and *Hin*dIII/*Eco*RI and φX174 *Hae*III digested DNA. Clone D was digested with B (*Bam*HI), Bg (*Bgl*II) or B/Bg (*Bam*HI and *Bgl*II). Charon 40, the cloning vector from clone D, was digested with RI (*Eco*RI) to release the phage arms. The gel was transferred to Nytran by Southern blotting and hybridized with ^{32}P-labelled 3′α-1.3, as indicated by circles (middle panel), and subsequently erased and rehybridized with ^{32}P-labelled *Pst*I-2.3, as marked by squares (right panel). 3′α-1.3 hybridized to a 9.1 kb *Bam*HI fragment which derives from the 5′ end of the clone D and includes a stuffer fragment; a 12.5 kb *Bgl*II fragment representing the *Bgl*II insert and including 1.8 kb of the left phage arm; and a 3.2 kb *Bam*HI–*Bgl*II fragment from the 5′ end of clone D. *Pst*I 2.3 hybridized to a 11.5 kb *Bam*HI fragment from the 3′ end of clone D which includes 1.8 kb of the left phage arm; to the 12.5 kb *Bgl*II fragment (also hybridizing to 3′α-1.3); and to a 5.3 kb *Bam*HI–*Bgl*II fragment which derives from the 3′ end of clone D and includes 1.8 kb of the left phage arm. Neither probe hybridized with Charon 40 phage arms.

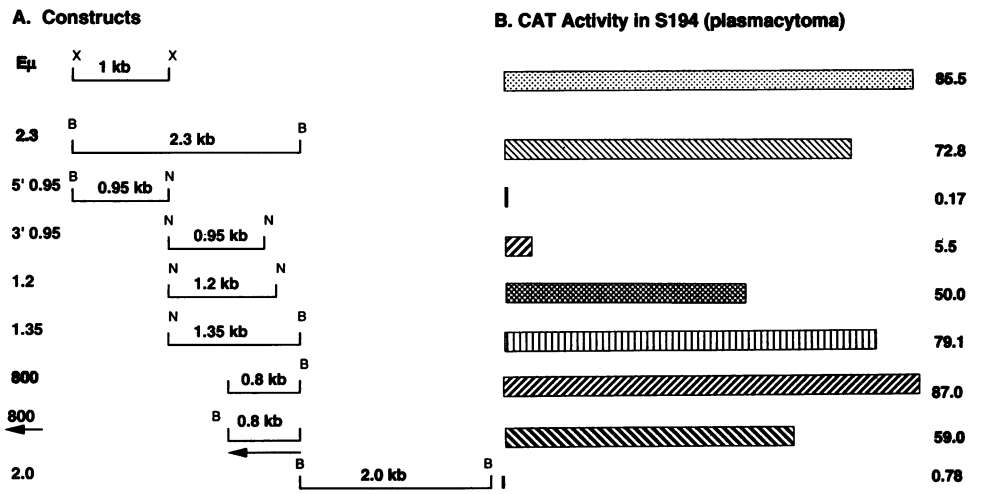

Figure 3. Localization of 3′α-hs4 enhancer activity. (**A**) Constructs were cloned into the *Sma*I site of QM293, a CAT expression vector. Eμ refers to a 1 kb *Xba*I fragment containing the murine IgH intron enhancer. The 2.3 and 2.0 kb *Bam*HI fragments were subcloned from clone D. 5′ 0.95 was derived by digestion with *Bam*HI and *Nco*I, 3′ 0.95 by digestion with *Nco*I, 1.2 by partial digestion with *Nco*I, 1.35 by complete digestion with *Bam*HI and partial digestion with *Nco*I. The 800 bp fragment was obtained by PCR. The oligonucleotides used as PCR primers were BB245 and BB246. The sequence of BB245 is aaggatccCACGTTGAAAGTCTTCAG; the sequence of BB246 is aaGATCCTGAGGCTGGGGATTGC. The PCR product was digested with *Bam*HI and blunt-end ligated into the vector. The arrow denotes reverse orientation. X = *Xba*I; B = *Bam*HI; N = *Nco*I. (**B**) CAT activity following transient transfection of these constructs into a plasmacytoma cell line, S194. Values represent fold induction, which is determined by comparing CAT assay values for each of the constructs with that of the enhancerless CAT vector, QM293. Values represent averages of 3–6 independent transfection experiments, using two different preparations of DNA in most cases. The average experimental CAT value for Eμ in S194 is 133 000 c.p.m.

encompasses a region of ~34 kb including all of the regulatory elements in the region 3′ of Cα. Presumably, the deletion of this regulatory region accounts for the greatly diminished levels of Cα transcription in this mutant cell line.

Cloning of distal 3′ hypersensitivity site

Analysis of the deletion in the mutant cell line LP1.2 resulted in a map of the 3′ regulatory region (8,15,26). DNase I hypersensitivity studies had indicated a distal hypersensitivity site located ~17 kb downstream of 3′αE. Mapping studies indicated that phage clone D might contain this site, since its 11.5 kb insert, deriving from the 22 kb *Eco*RI fragment, commenced ~8 kb downstream of 3′αE (Fig. 1). Portions of clone D were subcloned into pWSK129 (18), a low copy number plasmid, by transformation into MB408 (19). A 2.3 kb internal *Bam*HI fragment, which hybridized to a larger genomic *Bam*HI fragment of 4.5 kb (Fig. 1), was chosen for further analysis, since it was mapped to the vicinity of the distal 3′ DNase I hypersensitivity site.

Identification of enhancer activity

The 2.3 kb *Bam*HI fragment was cloned into QM293, a CAT expression vector, and assayed for CAT activity following transient transfection (Figs 3 and 4). In a plasmacytoma line, S194, the enhancement activity of the 2.3 kb fragment was nearly equivalent to that of Eμ, showing 73-fold induction over basal CAT activity. As schematized in Figure 3A, the enhancer activity was further localized by subsequent trimming of the 2.3 kb fragment. A 0.95 kb *Bam*HI–*Nco*I fragment showed basal activity, while the remaining 1.35 kb *Nco*I–*Bam*HI fragment retained full activity (Fig. 3B). When the 1.35 kb fragment was trimmed successively from the 3′ end, the activity decreased; a 1.2 kb *Nco*I fragment, derived by partial *Nco*I digestion, housed 68% activity while a 0.95 kb *Nco*I fragment retained only 7% of the activity. When the 1.35 was trimmed from the 5′ end, an 800 bp fragment, generated by PCR, retained full activity. A 2.0 kb *Bam*HI fragment immediately downstream of the 2.3 kb fragment had only basal CAT activity.

Since enhancer elements are known to function in an orientation independent manner, we tested the 800 bp fragment in the opposite orientation. Figure 3B demonstrates that this fragment was able to enhance transcriptional activity when present in the reverse orientation as well.

The sequence of this 800 bp fragment housing transcriptional enhancement activity is shown in Figure 5. We have termed this novel enhancer element, 3′α-hs4.

Enhancement activity of 3′α-hs4 at early stages of B cell development

Having demonstrated activity in a plasmacytoma line, we sought to determine whether 3′α-hs4 was active at earlier stages of B cell differentiation as well. The presence of the distal DNase I hypersensitivity site indicative of this enhancer at the pre-B cell stage suggested that its associated enhancer, 3′α-hs4, might be active at that time in development, in contrast to 3′αE, which is active only at the plasma cell stage. Thus cell lines representing earlier stages of B cell differentiation were transiently transfected to test whether the activity of 3′α-hs4 correlated with the early appearance of the hypersensitivity site.

Transient transfections into two B cell lines, M12.4.1 and A-20, were performed. In M12.4.1 the 2.3 kb fragment showed 17-fold enhancement, approximating that of Eμ in this cell line (Fig. 4A). The 800 bp segment retained essentially all of the activity. Similarly, in a second B cell line, A-20, the 800 bp segment showed 5.7-fold induction as compared to 4.5-fold induction by Eμ (Fig. 4A).

Transient transfection into a pre B cell line, 18-81, resulted in an average enhancement activity ~25% that of Eμ. The 2.3 kb and the 800 bp fragments resulted in an average of a 3.1-fold induction, compared to 12.9-fold for Eμ (Fig. 4B). Interestingly,

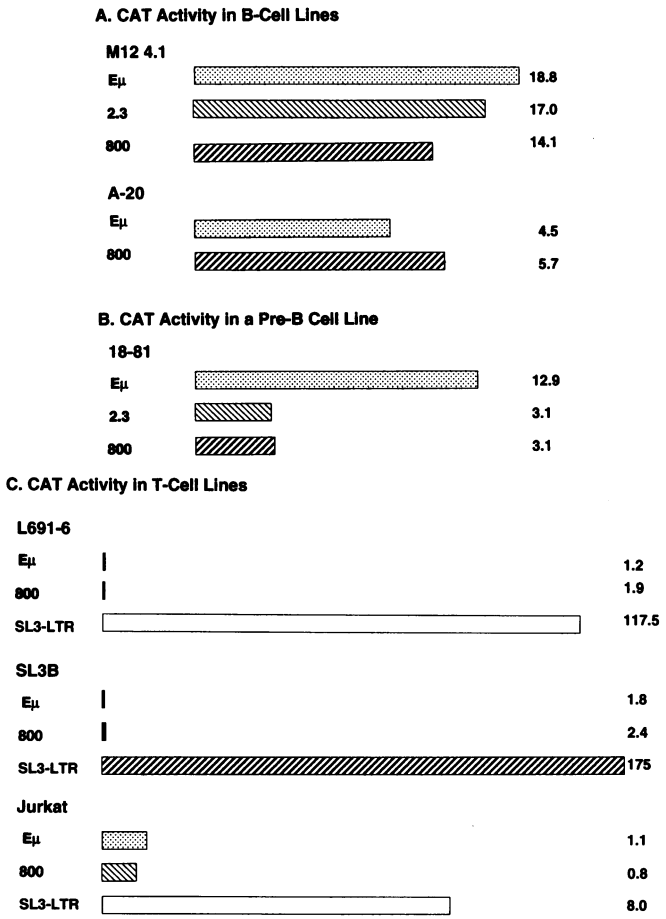

Figure 4. Activity of 3′α-hs4 in B cell, pre-B cell and T cell lines. (**A**) CAT activity following transfection into B-cell lines, M12.4.1 and A-20; (**B**) a pre-B cell line, 18-81; and (**C**) T cell lines, L691-6, SL3B and Jurkat. SL3-LTR, which refers to the long terminal repeat derived from SL3 retrovirus (21), was used as a positive control for CAT activity in the T cell lines. When linked to a non-B cell specific enhancer, the Ig light chain λ_1 promoter can drive transcription in non-B cells (36,37). Values represent fold induction, which is determined by comparing CAT assay values for each of the constructs with that of the enhancerless CAT vector, QM293. Values represent averages of 3–6 independent transfection experiments, using two different preparations of DNA in most cases. Average experimental CAT values for Eμ are as follows: 104 000 c.p.m. (M12.4.1); 1800 (A-20); 2800 c.p.m. (18-81); 150 (L691-6); 200 (SL3B); 150 (Jurkat).

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  1  CACGTTGAAA GTCTTCAGTA GACTGGTATC ATAGCCCTGA TATGGGCAAC
 51  ACAGCAAGCC TGGGTCACAC TCAAGCTGAG TATCAGGGTA GCCAGGGCCT
101  TCTAACCAAG GGTAGATGCA GCCTGTGTTC CGTTTACTGA CCAGTGAGAA
151  GCCATGAGCT GAACCAGACC AGAAGACCCT TACTGTTCCC ACCCAACCCC
201  CACCCAGTTT AGTCTCAGCA AGACCCTGTA CTGTGGGCCA CAGCTCTCCC
251  CCACACCCCA CCTGTAGCAC AAACACTATT TGCAAACATT TCTAAAAATG
301  ATGAGAACAG GAACCACAGA GCAGAGGGGG GGACTGGCGT GGAAAGCCCC
351  ATTCACCCAT GGGACTGAAA CTCAGGGAAC CAGAACCGTA AGGAGATTTG
401  CATGGTGCTG GGGGAGGTTG GCCCTGGATC AGTGAGCCCA GAGAGTTACT
451  GGTTTCTCAC TTCCATCATG TCAACCTCCT CAACCCCCAA AAATGGCCAG
501  GCCTAGGCTA TGGATGAGTT TCAATGACCA GGCCCTAAGG ACGAGTCACA
551  GAGGACTTCC TGGTGGGCTC AGGCAGCAGA CCTGCTCAGA TGGATTGCAG
601  AGCCAGAGGG AGCCATGGCC AGGAAGGCCA GACGCCTTAG GGGTGTGCTG
651  TCTCTGCATC CTTTGCCCTC TCTGCTCCTC ACAGTCCATC TGCCATCTCA
701  CAATCCCTGC TGTCGCTCTG GGGCCCAGAC CTGGCCAGTC TGGGTACCTG
751  TGGAATACAC CCAGAGAAGC AATCCCCAGC CTCAGGATC
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Figure 5. Sequence of 3′α-hs4. The 789 bp sequence of 3′α-hs4 is shown. The sequence was obtained by an amalgamation of machine sequencing on a 373A ABI DNA sequencer and dideoxy sequencing (20). Underlined nucleotides represent putative octamer motifs. The sequence corresponds to base pairs 320–1109 of hs4 (28). There is a 99% sequence similarity and identity between the BALB/c sequence shown here and the sequence of hs4 as derived from 129 ES; disparities occur at base pairs 195, 250, 252, 257, 299, 300, 302 and 303 of 3′α-hs4 sequence.

both the 2.3 kb and the 800 bp fragments demonstrated a reproducible variability in each of three different subclones of 18-81 tested, whereby depending on the experiment, the values ranged from levels equivalent to Eμ activity to levels close to basal activity (see Discussion). In all transfection experiments, values for Eμ were consistently high.

Basal activity of 3′α-hs4 in T-cell lines

To determine whether the enhancer activity of 3′α-hs4 is restricted to cells of the B lymphocyte lineage, the 800 bp fragment was transiently transfected into a panel of T cell lines. The activity of the 800 bp fragment appeared to be B cell specific, as transfection into L691-6, a T-lymphoma cell line, resulted in only basal levels of CAT activity (Fig. 4C). Likewise Eμ, a known B-cell specific element, did not enhance transcriptional activity in this T cell line. Similar results were obtained upon transfection into two other T cell lines, SL3B and Jurkat (Fig. 4C). SL3-LTR, derived from the long terminal repeat of SL3 retrovirus (27), which was used as a positive control in these experiments, manifested high levels of enhancement activity in all the T cell lines.

The activity of 3′a-hs4 thus appears to be specific to cells of the B lineage, since it was shown to be active at multiple stages of B cell development but inactive in T cell lines.

DISCUSSION

The deletion of the 3′ regulatory region in the cell line LP1.2 provides an *in vivo* approach to study the elements that control Ig heavy chain gene expression. We have shown that the deletion, which is presumably associated with the low levels of α transcription in this cell line (6), encompasses ~34 kb of DNA in the region downstream of the α gene. The deletion commences 11 bp downstream of the polyadenylation site for the secreted α chain (6) and extends to ~2 kb downstream of 3′α-hs4. It includes all of the 3′ regulatory elements that have been identified: Cα3′ enhancer (14), 3′αE (8,9), hs3 (28) and 3′α-hs4 as described here and by Madisen and Groudine (28). Low levels of Cα transcription in this cell line presumably reflect retention of the intron enhancer, Eμ. Analysis of LP1.2, together with the documentation that deletion of Eμ has little detectable effect on C_H expression in plasmacytoma cell lines (2–5), implies that in plasmacytes the high level of secreted immunoglobulins is dependent on elements within the region 3′ of Cα.

We have identified a novel regulatory element, located ~33 kb downstream of the α gene of the IgH gene cluster and reflecting a previously identified DNase I hypersensitivity site (15). We have termed this element 3′α-hs4. 3′α-hs4 acts as a potent transcriptional enhancer when transiently transfected into a variety of cell lines representing different stages of B cell differentiation. Enhancer activity of 3′α-hs4 was localized to an 800 bp fragment.

The distal DNase I hypersensitivity site associated with 3′α-hs4 is present at the plasma cell stage as well as the pre-B cell stage of development. We have shown that the transcriptional enhancement activity of 3′α-hs4 is equivalent to that of Eμ in the plasmacytoma line, S194. Furthermore, we tested the activity of 3′α-hs4 as an enhancer in B cell and pre-B cell lines in an attempt to correlate enhancer activity with the early appearance of the hypersensitivity site. Indeed we have observed that 3′α-hs4 is fully active in two B-cell lines, M12.4.1 and A-20; in a pre-B cell line, 18-81, the average activity is ~25% that of Eμ.

The activity of 3′α-hs4 appears to be specific to cells of the B-lymphocyte lineage. Transfection into a panel of T cell lines resulted in basal levels of transcriptional activity. Eμ, a known B-cell specific element, also showed no enhancement activity in T cells. The fact that 3′α-hs4 is inactive in T cells is consistent with the absence of the associated DNase I hypersensitivity site in T cell lines (15,28). Further evidence for the B cell specificity of 3′α-hs4 comes from the recent observation by Madisen and Groudine that hs4, a 1.35 kb fragment which includes 3′α-hs4, is inactive in L-cell fibroblasts (28).

Interestingly, although transfection into the plasmacytoma line S194 resulted in transcriptional activation approximating that of Eμ, the same was not observed upon transfection into other cell lines representing the plasma cell stage of differentiation. Transfection into the plasmacytoma line J558L resulted in only minimal induction over basal transcriptional levels (data not shown). Similarly, Madisen and Groudine (28) recently observed that hs4 only weakly enhanced transcription upon transient transfection into 1165, another plasmacytoma line. It is interesting to note that, like 3′α-hs4, 3′αE alone is also relatively inactive when transiently transfected into 1165 (28). Likewise in J558L, 3′αE activity is weak relative to its activity in S194 (8,10). Thus in S194, both 3′α-E and 3′α-hs4 are highly active, while in J558L and 1165 both elements show weak activity. Possibly, S194 represents a slightly different stage of plasmacytoma development or activation compared to J558L and 1165, characterized by a distinct set of *trans*-acting factors and/or a shift in accessibility to both enhancer elements.

The fact that both 3′αE and 3′α-hs4 exhibit similar relative levels of activity in the different plasmacytoma lines suggests that related factors may be responsible for regulating these two enhancer elements. In the case of 3′αE, octamer binding proteins appear to be important regulatory factors. Grant *et al.* (29) showed that deletion of a putative octanucleotide motif resulted in a 30% decrease in transcriptional activity of 3′αE in MPC11. Octamer binding proteins may also be involved in the regulation of 3′α-hs4, since the sequence of 3′α-hs4 shows two putative octanucleotide elements (Fig. 5). However, while the two enhancer elements may bind certain factors in common, it is clear that 3′αE and 3′α-hs4 are not entirely governed by the same set of regulatory conditions. Unlike 3′α-hs4, 3′αE is only active in the plasma cell stage of B cell development; at the B cell stage, the activity of 3′αE is repressed by BSAP (10,12). Since 3′α-hs4 is active at earlier stages of B cell development (see Discussion below), at times when BSAP is expressed, 3′α-hs4 is likely not to be regulated by BSAP. Nevertheless, it is possible that BSAP could regulate 3′α-hs4 in a different manner, i.e. as an activator, at earlier stages of development. We have in fact demonstrated that BSAP can function as a positive *trans*-acting factor in promoting the production of epsilon germ-line transcripts (30).

Interestingly, not only was there a difference in 3′α-hs4 activity among cell lines representing a given stage of development, i.e. plasmacytoma (S194 compared to J558 and 1165), but within the pre-B cell line 18-81, the results were variable. This is in contrast to the results obtained in S194 and M12.4.1 which showed reproducibly high activity for both the 2.3 kb and 800 bp fragments. In 18-81, the values for the 2.3 kb and 800 bp fragments ranged from being nearly basal to being comparable to Eμ, while the values for Eμ were consistently high and uniform. Perhaps this is indicative of an on/off switch, whereby 3′α-hs4 undergoes tight regulation dependent upon the strict titration of cellular factors which affect the activity and/or accessibility of the enhancer. It is intriguing to consider whether such a regulatory switch might be associated with the differential response of a B cell to activating versus tolerogenic signals.

What is the role of 3′α-hs4? If individual enhancers are responsible for specific and identifiable functional processes, then potential functions for this enhancer include those not associated with previously identified enhancers. Eμ appears to be responsible for μ transcription and high levels of V(D)J recombination, as documented by targeted deletion of this element in mouse (31,32). 3′αE apparently plays a role in class switching, since mice lacking this element were deficient in serum IgG2a and IgG3 (13). In addition, *in vitro* activated B cells from these mice were deficient in IgG2a, IgG2b, IgG3 and IgE; levels of IgG1 were unaffected (13). Thus candidate activities for the newly identified enhancer, 3′α-hs4, include high levels of DJ joining, basal levels of V(D)J joining, and switching to certain isotypes, such as IgG1.

3′α-hs4 may also play a role in regulating IgH expression. In the cell line LP1.2, where 3′α-hs4 and the other 3′ control elements have been deleted, α expression is significantly diminished. Yet, α production does not appear to be regulated by 3′αE, as documented by the only slightly lower than normal levels of serum IgA in the 3′αE$^{-/-}$ mice and the only minimally reduced IgA secretion from *in vitro* activated B cells from these animals (13). Similarly, Cα3′ enhancer did not significantly differ in its effect on activity in an α-expressing cell line as compared to a μ-expressing line (14). A candidate for the element the deletion of which is responsible for low level α transcription in LP1.2 is thus 3′α-hs4.

Another possible functional role for 3′α-hs4 is in somatic hypermutation. In a transgenic model, it was shown that a 3′ κ enhancer is necessary for somatic mutation of the V gene (33,34). The potential involvement of 3′α-hs4 in somatic mutation or V(D)J joining would necessitate interaction with the V region, located >150 kb upstream of this element. Indeed we have documented an example of a physical interaction between the V region and the region 3′ of α (26,35).

It is likely that 3′α-hs4 functions in concert with the other regulatory elements in the region. In fact, Madisen and Groudine (28) have demonstrated that hs4 can synergize with other elements in transcriptional assays and in effects on c-*myc* regulation. When present in combination with other hypersensitive

sites such as hs1,2 (3′αE) or hs3 (located ~12 kb downstream of 3′αE), hs4 dramatically increased the levels of enhancement to that of Eμ. Moreover, hs12 (3′αE), hs3 and hs4 (3′α-hs4) together formed a complex regulatory unit, which they term a locus control region, which was sufficient to impact on c-*myc* expression in plasmacytomas (28). The complex interplay among the various elements along with the available *trans*-acting factors perhaps determines the functional role this region may play in any given situation. For example, the ability of a B cell to switch to a particular isotype may be dictated not only by exposure to particular T cell lymphokines, but also by the constellation of 3′ regulatory elements present in distinct combinations of on/off states.

Future studies, including targeted deletion of 3′α-hs4, will help to reveal the role of this novel enhancer element throughout B cell development.

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