Deletional analyses reveal an essential role for the hs3b/hs4 IgH 3’ enhancer pair in an Ig-secreting but not an earlier-stage B cell line

Xuerong Shi1 and Laurel A. Eckhardt

Hunter College and Graduate Center of The City University of New York, 695 Park Avenue, New York, NY 10021, USA

1Present address: Department of Pathology, Joan and Sanford I. Weill Medical College, Cornell University, 1300 York Avenue, New York, NY 10021, USA

Keywords: antibodies, B lymphocytes, gene regulation, molecular biology

Abstract

The Ig heavy chain (IgH) locus is controlled by multiple regulatory sequences mapping both within the IgH transcription unit (Eµ) and downstream (3’) of IgH coding sequences (hs3a, hs1,2, hs3b and hs4). Enhancer knockout studies in mice have implicated Eµ in the control of IgH variable region gene assembly, but single-enhancer knockouts involving the 3’ IgH enhancers have yet to shed light on their function. Transfection studies in mice and cell lines have suggested that the 3’ enhancers behave similarly to a locus control region as first identified in the β-globin locus. We have exploited this property to form mini-loci in a surface Ig/c9059 and an Ig-secreting cell line as a means for studying the functions of the 3’ IgH enhancers. Importantly, this experimental system allows for the analysis of enhancer function within the context of chromatin. The mini-loci consisted of an Igγ2b transcription unit linked to the four murine 3’ IgH enhancers. Using targeted deletions of enhancer pairs within these mini-loci, we have discovered a critical and apparently developmentally regulated role for the hs3b/hs4 enhancer pair in IgH transgene expression.

Introduction

The transcriptional enhancer Eµ lies within the intron separating JH (joining) gene segments and Cµ (Fig. 1), and has been implicated in regulation of IgH variable region gene assembly (V to DJ joining), as well as IgH gene transcription (1–5). Several years ago, we and others identified additional enhancers downstream of Cµ, the last in the tandem array of Cµ genes in the murine IgH locus (Fig. 1) (reviewed in 6). There are four murine enhancers (hs3a, hs1,2, hs3b and hs4), the last two of which are 97% identical in sequence and three of these have analogues in the human IgH loci (hs3, hs1,2 and hs4), lying downstream of each of the two human Cµ genes (7,8). Evolutionary conservation of these control elements attests to their probable importance to IgH gene expression, but previous studies have fallen short of clarifying their roles.

We and another group analyzed the function of hs1,2 by enhancer knockout studies in a cell line (9) and in mice (10). In both studies, hs1,2 was replaced by a marker gene encoding neomycin resistance (ned′) and a pronounced effect was seen: complete loss of IgH promoter activity in the cell line and a striking effect on heavy chain class switching in mice. Subsequent knockout studies of hs1,2 and of hs3a in mice, however, demonstrated that the effect on class-switch recombination (CSR) was not the result of enhancer deletion but, rather, was due to insertion of the ned′ gene into this region of the IgH locus (11). In fact, simple insertion of ned′ at two additional sites within the Cµ cluster led to an impairment of CSR to Cµ genes upstream but not downstream of the pgk-ned′ insertion (12). These findings suggest that the ned′ gene somehow disrupts necessary communication between the enhancers and the constant region genes during the process of heavy chain class switching, and likely was similarly responsible for disrupting communication between the enhancers and the IgH promoter in the Eµ-deficient cell line described above (9). These studies, therefore, provide...
Deletion analyses of 3′ IgH enhancer pairs

Fig. 1. Diagram of the murine IgH locus after assembly of an IgH variable region gene (P-VDJ) upstream of μ constant region coding sequences (Cμ). Black boxes denote coding sequences and open circles represent enhancer regions. Relative distances between elements are not indicated. For reference, the enhancer hs3a lies ~170 kb downstream of Eμ.

Strong support for a role for the 3′ IgH enhancers in both processes (IgH gene transcription and CSR), but do not identify the critical enhancer(s) involved in either process.

In the present study, we have identified the 3′ IgH enhancers critical to IgH gene transcription in Ig-secreting cells, using a stably integrated IgH transgene as a target for enhancer deletions. As in the previous knockout studies, we were able to analyze enhancer function in the context of chromatin, but this IgH mini-locus system allowed us to circumvent difficulties we and others encountered in targeting the 3′ IgH region for homologous recombination and, moreover, allowed us to directly examine 3′ IgH enhancer function in the absence of the possibly compensatory effects of Eμ. Transgenes consisting of an Igγ2b transcription unit linked to the 3′ IgH enhancers were introduced into both an Ig-secreting plasmacytoma cell line and a surface Ig cell line. We used B lymphoid cell lines representing two different functional stages since earlier transient transfection studies (where reporters were not integrated into chromosomes) had revealed a pronounced difference in the behavior of the 3′ IgH enhancers at these two stages (13). After stable expression of the integrated transgenes had been established, we deleted either hs1.2.hs3a or hs3b.hs4 from the transgenes of individual transformants and measured the effect on IgH expression levels.

As described below, we found that the hs3b.hs4 enhancer pair was essential for IgH gene expression from this IgH mini-locus when incorporated into the genome of an Ig-secreting cell line. In contrast, the function of this enhancer pair could be supplanted by the hs3a/hs1,2 enhancer pair in a surface cell line. In contrast, the function of this enhancer pair could.

The IgH transcription unit was assembled from:

Methods

Cell lines

Cell line 9921 is an IgG2a-producing class-switch variant that was derived, through an intermediate, from the IgG2b-producing plasmacytoma MPC11 (14). In the course of the heavy chain class switch, Eμ was deleted from the γ2a heavy chain transcription unit in 9921. MPC11 used in these studies is a tissue culture adapted subtype of the BALB/c mouse and was obtained from the ATCC (Rockville, MD; TIB-20816).

Cell lines 9921 and MPC11 were maintained in DMEM with 10% BCS. A20 was maintained in RPMI 1640 medium with 10% BCS.

Plasmid constructs

pBS185 is a cre recombinase gene expression vector (Gibco/ BRL, Grand Island, NY; cat. no. 10347-011) in which cre is expressed under control of the major immediate early promoter from human cytomegalovirus (hCMV). Cre recombinase mediates loxP site-specific DNA recombination.

pEGFP-C1 encodes a red-shifted variant of green fluorescence protein (Clontech, Palo Alto, CA; cat. no. 6081). It was co-transfected with pBS185 in order to isolate cells that had taken up DNA as made evident by their fluorescence.

The following enhancer fragments were used in the construction of the IgH mini-locus diagramed in Fig. 2:

- **Hs1.2**: a 3.6-kb XbaI–HindIII fragment isolated from λ phage clone M2 containing BALB/c genomic DNA (17).
- **Hs3a**: a 1.1-kb XbaI fragment, also isolated from λ phage clone M2.
- **Hs3b,4**: a 2.6-kb NcoI–HindIII fragment from plasmid pH53.4 which contains a fusion of hs3b and hs4 (18). hs3b and hs4 DNA were originally isolated from the 129 mouse strain.

The IgH transcription unit was assembled from:

- **V297**: a 2.2-kb XbaI fragment containing the MPC11 (and 9921) IgH variable region, isolated from plasmid p297γ2b (19).
- **Cγ2b**: a 5.2-kb XbaI genomic DNA fragment containing BALB/c mouse Cγ2b isolated from plasmid pγ2bR1.4 (19). This fragment does not contain the Cγ2b membrane exons and membrane-form polyadenylation site so that only secreted-form γ2b heavy chain is made from this gene.

The loxP sites inserted into the IgH mini-loci were derived from psk-2loxPneo (5.0 kb). This plasmid was constructed by inserting a loxP-flanked neomycin-resistance gene (neo) into pBS-SK + (Stratagene, La Jolla, CA; cat. no. 21120). The loxP-flanked neo was obtained from plox2neo, a gift of Dr. F. W. Alt (The Children’s Hospital, Center for Blood Research, Harvard Medical School, Boston, MA).

A complete description of the mini-locus constructions is available upon request.
Deletion analyses of 3' IgH enhancer pairs

**Fig. 2.** IgH Mini-loci before and after enhancer deletions. (A) γ2b-hs1-4 locus with loxP sites surrounding the enhancers hs3a and hs1.2 (γ2b-hs1-4/loxPs123a). LoxP sites are indicated with wide arrows. The γ2b gene in this mini-locus consists of V_H coding sequences (identical to that expressed in MPC11 and 9921, see Methods) and γ2b constant region sequences (C_γ2b). DNA probes in Southern analyses of transformants are indicated (probes A and B). Probe B is homologous to both hs3a and hs3b, as shown. The bacterial gene for ampicillin resistance is shown (amp). Although this gene is not expressed in eukaryotic cells, Plasmids were linearized with Pvu (P) before introduction into cells. This site was usually destroyed in the course of integration into the genome (slashes in P). Thick lines surrounding the mini-locus denote genomic DNA at the site of mini-locus integration. HindIII restriction sites (H) within the mini-locus were used to analyze transformants both for transgene copy number and for cre-mediated enhancer deletions (see text). Bracket above the HindIII fragment detected with probe A indicates that the size of this fragment will be $\geq$8.2 kb, depending upon integration site (see text). The predicted changes in mini-locus structure, subsequent to cre-mediated enhancer deletion, are shown below the intact mini-locus map (γ2b-hs1-4/hs123a). (B) γ2b-hs1-4 locus with loxP sites surrounding the enhancers hs3b and hs4 (γ2b-hs1-4/loxPhs3b4). Designations are as described (A).

**Stable transformations**

Linearized plasmid DNAs were introduced into both 9921 and A20 cells by electroporation. Twenty micrograms of Pvu-linearized γ2b-hs1-4/loxPhs123a, Pvu-linearized γ2b-hs1-4/loxPhs3b4 or NotI-linearized, enhancerless psk-γ2b were introduced into 9921 and A20 cells along with XhoI-linearized psk-2loxPneo (molar ratio of the γ2b constructs:neo drug selection plasmid was 1:1). DNAs were combined with a 1 ml suspension of $10^7$ 9921 or A20 cells and the mixture was dispensed into a 0.4 cm (width) electroporation cuvette (BioRad, Hercules, CA). An electric pulse was delivered at 960 µF and 250 V by a BioRad Gene Pulser electroporator and Capacitance Extender (BioRad). G418 was added to the medium (1.5 mg/ml) 48 h after transfection and neo' colonies were visible within ~2 weeks.

**Transient transfections and cell sorting**

Thirty micrograms of pBS185 (cre-expressing plasmid) and 2.5 µg pEGFP-C1 (green fluorescence protein-expressing plasmid), molar ratio 8:1, were simultaneously introduced, by electroporation, into 9921 and A20 clones that carried a single copy of one of the γ2b mini-loci. DNAs were combined with a 1 ml suspension of $5\times10^6$ 9921 or A20 cells for electroporation (960 µF, 290 V). Forty-eight hours after transfection, fluorescent cells (expressing the EGFP gene) were identified and bulk sorted with a FACS Vantage (Becton Dickinson, San Jose, CA) and then subcloned by limiting dilution into 96-well culture plates. Up to 15 subclones were analyzed from each of 24 single-copy transformants transfected with cre. The overall frequency of CRE-mediated enhancer deletion was 10%, with a range from 0 to 12 deletion subclones isolated per 15 subclones analyzed. Data shown are for all of the single-copy transformants that yielded one or more CRE-mediated enhancer deletion subclones.

**Southern blot analyses**

Agarose gel electrophoresis, transfers to membranes and DNA hybridizations were performed essentially as described previously (20). In all Southern analyses, ~25 µg of restriction enzyme-digested DNA was loaded into individual lanes of an 0.7% agarose gel. Probes included pJ11HE and Hs3b (probes A and B respectively, Fig. 2). pJ11HE is a 1.5-kb HindIII–EcoRI fragment isolated from plasmid containing the J_H gene region of BALB/cJ liver DNA (21). hs3b is a 1.2-kb XbaI fragment isolated from plasmid pH33S.4 (18).
Northern blot analyses

Total cellular RNA was isolated by Trizol reagent (Gibco/BRL; cat. no. 15596-026) according to the manufacturer’s instructions. Approximately 25 μg of total RNA were analyzed per sample. Northern blots were performed as previously described (20).

Probes were a 0.3-kb SacI fragment containing the Cγ3 domain of Cγ2b and which does not cross-hybridize to γ2a transcripts (22), and β-actin (Ambion, Austin, TX; cat. no. 7323) and GAPDH (Ambion; cat. no. 7330) probes for normalization.

ELISA

Microtiter plates were coated with purified rat anti-mouse γ2b (PharMingen, San Diego, CA; cat. no. 02041D). Coated wells were then incubated with 50 μl cell lysate, which was prepared by lysis of 10^6 cells in 0.5% Nonidet P-40 lysis buffer (19). Mouse γ2b heavy chains were then detected with alkaline phosphatase-conjugated rabbit anti-mouse γ2b (Zymed, South San Francisco, CA; cat. no. 61-0322), using p-nitrophenol phosphate as the enzyme substrate.

Results

The murine 3’ IgH enhancers mediate position-independent but not copy number-dependent expression of an IgH reporter gene in Ig-secreting and surface Igγ+ B-lineage cells

To test the ability of the 3’ IgH enhancers to activate IgH expression regardless of chromosomal context, we compared expression levels of a γ2b reporter gene lacking enhancers with two reporters carrying two different configurations of the 3’ IgH enhancers. The reporter genes carrying enhancers are diagrammed in Fig. 2(A and B) (γ2b-hs1-4loxPhs123a and γ2b-hs1-4loxPhs3b4). The enhancerless γ2b reporter was identical to the latter two constructs except that it lacked any enhancers (not shown).

Each linearized plasmid construct was co-transfected with a neo+ selectable marker gene (psk-2loxPneo, see Methods) into 9921, an IgG2a-secreting cell line, and into A20, a surface IgG+ cell line. Immediately following transfection, the cells were dispensed into 96-well plates. G418-resistant clones were recovered in 30–40% of the wells in each plate so that each growing well represented, on average, a single expression was always lower than that of a single-copy transformant.

In one of the mini-loci prepared for our experiments, hs3a and hs3b flank hs1,2 (γ2b-hs1-4loxPhs123a, Fig. 2); in the other, they flank hs4 (γ2b-hs1-4loxPhs3b4, Fig. 2). The former construct more closely resembles the endogenous locus where hs3a, hs1,2 and hs3b are part of a large palindromic domain (~25 kb) with hs1,2 at the axis of symmetry and hs4 outside the palindrome (25,26). There has been speculation that this palindromic arrangement is important to enhancer function. Since our two mini-loci differed with respect to

Transgene expression is unaffected by enhancer order

In one of the mini-loci prepared for our experiments, hs3a and hs3b flank hs1,2 (γ2b-hs1-4loxPhs123a, Fig. 2); in the other, they flank hs4 (γ2b-hs1-4loxPhs3b4, Fig. 2). The former construct more closely resembles the endogenous locus where hs3a, hs1,2 and hs3b are part of a large palindromic domain (~25 kb) with hs1,2 at the axis of symmetry and hs4 outside the palindrome (25,26). There has been speculation that this palindromic arrangement is important to enhancer function. Since our two mini-loci differed with respect to
Table 1. Frequency of transgene expression in A20 and 9921 transformants

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Constructs</th>
<th>No. of clones with γb transgene</th>
<th>No. of clones expressing γb transgene</th>
<th>Percent clones expressing γb transgene (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9921</td>
<td>γ2bhs1-4/loxPhs123a</td>
<td>26</td>
<td>25</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>γ2bhs1-4/loxPhs3b4</td>
<td>36</td>
<td>35</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>psk-γ2b</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A20</td>
<td>γ2bhs1-4/loxPhs123a</td>
<td>28</td>
<td>27</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>γ2bhs1-4/loxPhs3b4</td>
<td>28</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>psk-γ2b</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 3. Comparisons of transgene copy number with transgene expression level. (A) Representative data from eleven 9921 transformants carrying different numbers of γb transgenes (the transgene copy number is indicated above mRNA lanes). mRNA was isolated from the 11 clones and analyzed by Northern blot. The same blot was sequentially hybridized with a γb probe and a β-actin probe (for sample loading normalization). (B) Graph of γb mRNA level relative to transgene copy number. Multiple exposures of the Northern blot shown in (A) (including very light exposures for the more abundant mRNAs) were quantified by densitometry and data analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Transgene copy number was determined by Southern analysis as described in the text. The data for 11 individual clones are shown (dots). A line connects the average γb mRNA values for 1, 2, 4, 5 and 7 copy-number transformants.

enhancer configuration, we compared their expression levels to ask whether this difference in arrangement yielded a difference in function. As shown in Table 2, single-copy transformants expressed either transgene at similar levels, ranging from 10 to 50% endogenous locus expression levels. The average expression level of both of the γb transgenes in 9921 cells (Ig-secreting cells) was 26% of that of a natural γb locus in the IgG2b-secreting cell line MPC11. In A20 cells, the averages were 22% (γ2b-hs1-4/loxPhs123a) and 24% (γ2b-hs1-4/loxPhs3b4) of MPC11 γb expression levels. In the context of these reporter constructs, therefore, the order of the enhancers had little effect on overall enhancer activity.

hs3a/hσ1,2 and hs3b/hσ4 are functionally redundant in the γb mini-loci of surface Igσ cells

Having established that we could measure expression from a single copy of either γb-hs1-4 mini-locus in multiple transformants, we chose several single-copy transformants of the A20 cell line (surface Igσ+ cells) for further analysis of enhancer function. Since each transgene contained loxP sites flanking either the hs3a/hσ1,2 or the hs3b/hσ4 enhancer pair, we could induce deletion of these pairs without changing the chromosomal location of the transgene. In this way, we could test directly whether one or the other enhancer pair was critical to maintaining gene expression when in a chromosomal context.

A20 transformants carrying the γb-hs1-4/loxPhs123a transgene were transiently transfected with both a cre-expressing plasmid (pBS185) and a plasmid expressing green fluorescence protein (pEGFP-C1). Cells that had taken up the EGFP plasmid were identified and isolated by flow cytometry (see Methods, data not shown). We expected that most of these cells would have also incorporated the cre-expressing plasmid. The sorted cells were cloned by limiting dilution and growing clones were recovered 3 weeks later.

Four independent transformants yielded subclones with deletion of hs3a/hσ1.2, as determined by Southern blot. Genomic DNA from the A20-γb-hs1-4/loxPhs123a transformants was digested with HindIII and Southern blots hybridized with probe B (refer to maps, Fig. 2). Two HindIII fragments detected with this probe are derived from the endogenous IgH loci of A20 (Fig. 4). Just below the smaller of these two fragments is the 7.9-kb fragment derived from the transgene (migrates near the 8.0-kb marker, see legend to Fig. 4). This fragment was detected in each transformant before enhancer deletion (Fig. 4, ‘B’ lanes = before deletion; right panel, Δhs123a). After loxP-mediated deletion of hs3a/hσ1.2, the 7.9 kb transgene fragment should be lost and a new HindIII fragment formed, its size dependent upon the site of transgene integration (see maps, Fig. 2). As expected, therefore, in the transformant subclones that had undergone loxP-mediated enhancer deletion, the 7.9-kb HindIII fragment was missing and a new HindIII fragment was detected (Fig. 4, ‘A’ lanes = after deletion; right panel, Δhs123a). In two of the deletion clones (P19 and P71), the new HindIII fragment co-migrated with one of the endogenous HindIII fragments. This was obvious from the difference in relative signal intensity of these two endogenous fragments in A20 and in the transformants.
Deletion analyses of 3' IgH enhancer pairs

Table 2. γ2b mRNA levels in single-copy transformants

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Cell lines</th>
<th>γ2b mRNA levels in individual clones (% endogenous locus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ2bhs1-4loxPhs123a</td>
<td>9921</td>
<td>P1, P7, P11, P13, P18, P37, P42, P62, mean = 26</td>
</tr>
<tr>
<td></td>
<td>A20</td>
<td>P17, P18, P19, P32, P34, P37, P69, P71, mean = 22</td>
</tr>
<tr>
<td>γ2bhs1-4loxPhs3b4</td>
<td>9921</td>
<td>P15, P25, P27, P29, P42, P48, P50, P66, mean = 26</td>
</tr>
<tr>
<td></td>
<td>A20</td>
<td>P29, P31, P34, P35, P38, P39, P68, P69, mean = 24</td>
</tr>
</tbody>
</table>

γ2b mRNA levels produced by transgenes were compared, by Northern blot, to γ2b mRNA levels in a γ2b-producing plasmacytoma (MPC11). GAPDH was used to normalize sample loading. As described in Fig. 3(b), multiple exposures of Northern blots were quantified by densitometry.

Individual transformants are given unique designations: P1, P7, etc.

Fig. 4. Genomic Southern blot analyses of A20 transformants before and after enhancer deletion. Right panel shows data from transformants before (B) and after (A) deletion of hs1,2/hs3a (ΔHs123a). Individual transformant pairs are given unique designations (P71, P69, etc.). Left panel shows data from transformants before (B) and after (A) deletion of hs3b/hs4 (ΔHs3b4). Transformants P34 and P34.4 are two clones recovered from one culture well in the initial transfections with the γ2b-hs1-4loxPhs3b4 mini-locus. As is evident with probe A, however, these subclones represent independent integration events (compare size of fragment detected in the P34 transformant pair with that detected in the P34.4 transformant pair). Upper blot: hybridized with Probe B. Fragments derived from the endogenous IgH loci of A20 are indicated with arrows. Because of faster migration of the outer-most lanes of the gel, the 7.9 kb transgene fragment detected by probe B appears slightly larger than its actual size. In clones P19, P71, P29 and P34.4, the 7.9 kb transgene fragment present before deletion (B lanes) was replaced by a new fragment that co-migrated with one or the other of the fragments derived from the endogenous A20 IgH loci (A lanes, see text). Lower blot: the same blot shown in the upper panel was ‘erased’ and then re-hybridized with probe A. Fragments derived from the endogenous IgH loci of A20 migrate below the region shown on this blot.

Prior to enhancer deletion when compared to the deletion subclones. P19 deletion subclone ('B' lane in Fig. 4) has a new HindIII fragment that co-migrates with the larger endogenous fragment; P71 subclone has a new HindIII fragment that co-migrates with the smaller endogenous fragment.

These Southern blots were also hybridized with probe A (see map, Fig. 2) to confirm that the enhancer deletion left the rest of the transgene intact. This probe hybridizes to a HindIII fragment that spans most of the γ2b transcription unit and extends into adjacent DNA at the site of transgene integration. As shown in Fig. 2, this fragment is predicted to be at least 8.2 kb in size and to differ among transformants (because of differences in integration site). As shown in Fig. 4, a fragment >8.2 kb was detected in each single-copy transformant before enhancer deletion (Fig. 4, B' lanes). Moreover, the same size fragment was detected in each enhancer-deletion subclone, demonstrating that enhancer deletion had not disturbed the region of the transgene containing the γ2b transcription unit (Fig. 4, A' lanes).

Fig. 5. γ2b transgene expression in A20 clones before and after enhancer deletion. Representative northern blot of mRNA isolated from A20 clones before (B) and after (A) deletion of enhancers. The same blot was sequentially hybridized with a γ2b probe and a GAPDH probe (for sample loading normalization). The left panel shows transformant pairs with the mini-locus that allows cre-mediated deletion of hs3b/hs4 (ΔHs3b4) while the right panel shows pairs that carry the mini-locus that yields hs1,2/hs3a deletions (ΔHs123a).

Having achieved the desired enhancer deletions at four different chromosomal sites in four independent, single-copy transformant lines, we measured γ2b mRNA levels to determine the impact of these deletions on γ2b transgene expression. γ2b mRNA from the initial transformants and their enhancer-deletion subclones was quantified by Northern blot. As shown in Fig. 5, γ2b mRNA levels changed very little after hs3a/hs1,2 enhancer deletion (Fig. 5, cf. B and A lanes; right panel, ΔHs123a). In two transformant pairs (P17 and P19), transgene expression increased slightly after enhancer deletion.
dilution, and individual enhancer-deletion clones recovered. on a critical role in maintaining IgH transgene function.

fl expressing and EGFP-expressing plasmids. Cells expressing carrying all four enhancers (Figs 7 and 8B). Hence, in this

cre γ increase in ∆ this enhancer pair (Figs 5 and 8). As seen with the hs3a is present.

γ copy A20 transformants carrying the myc P71), transgene expression decreased slightly (a graph of oncogene c-

hs3b4). Northern blots again revealed hs3a and hs3b are present and homologous to this probe

γ transcription unit remained unchanged after enhancer

deletion while in the other two transformant pairs (P69 and P71), transgene expression decreased slightly (a graph of quantified data obtained from blot in Fig. 5 is provided in Fig. 8A).

The same kinds of analyses were done for five single-copy A20 transformants carrying the γ2b-hs1-4loxPhs3b4 transgene and their deletion subclones which had deleted the hs3b/hS4 enhancer partner. The Southern blots confirmed enhancer deletion without disruption of the γ2a transcription unit (Fig. 4, left panel, Ahs3b4). Northern blots again revealed no dramatic effect on transgene expression upon deletion of this enhancer pair (Figs 5 and 8). As seen with the hs3a/ hs1,2 deletion subclones, the hs3b/hS4 deletion resulted in a modest reduction of γ2b transgene expression in some cases (Fig. 5, cf. B and A lanes for clones P34 and P34.4) and an increase in γ2b expression in others (P29). Overall, the enhancer-deletion results in A20 transformants suggested that neither pair was essential to transgene expression after its integration into the chromosome. The fact that a gene lacking all enhancers was never expressed in these cells (Table 1), leads us to conclude that gene expression is enhancer-dependent but that both pairs of enhancers have comparable function in surface Ig-positive cells.

A dramatic effect is seen upon deletion of hs3b/hS4 in Ig-secreting cells

In total, 9921 single-copy transformants carrying either the γ2b-hs1-4loxPhs123a mini-locus or the γ2b-hs1-4loxPhs3b4 mini-locus were similarly co-transfected with the cre-expressing and EGFP-expressing plasmids. Cells expressing EGFP were sorted by flow-cytometry, cloned by limiting dilution, and individual enhancer-deletion clones recovered. As described for the A20 transformants, both types of 9921 transformant contained a 7.9-kb HindIII fragment that hybridized with probe B. This fragment migrated just below a HindIII fragment derived from 9921’s endogenous IgH loci (Fig. 6, ‘B’ lanes; 7.9-kb fragment migrates above 8-kb marker, see legend). The difference in signal intensity for the endogenous and the 7.9-kb transgene fragment is explained by the presence of four copies of the 3’ IgH enhancer region within endogenous IgH loci: one is associated with the expressed γ2a locus and the other three are associated with translocated copies of the IgH locus that juxtapose this region with the oncogene c-myc (27).

The 7.9-kb transgene fragment was lost after enhancer deletion in each of the enhancer-deletion clones and was replaced by a new HindIII fragment, its size dependent upon integration site (Fig. 6, ‘A’ lanes). The enhancer deletion itself explains the difference in signal intensity for the 7.9-kb fragment (before deletion) versus the new HindIII fragment that replaces it (after deletion): in the 7.9-kb fragment, both hs3a and hs3b are present and homologous to this probe while in the new fragment, only one of these (hs3a or hs3b) is present.

Probe A was used on HindIII-digested DNA to confirm that the γ2b transcription unit remained unchanged after enhancer deletion (lower panel, Fig. 6). Consistent with that expectation, DNA from individual transformants yielded unique HindIII fragments that did not change after enhancer deletion (Fig. 6, lower blot, cf. A and B lanes).

As shown in Fig. 7, the effect of hs3b/hS4 deletion on γ2b transgene expression differed dramatically from that of hs3a/ hs1,2 deletion. In all three independent, single-copy transformants carrying γ2b-hs1-4loxPhs3b4, cre-mediated deletion of hs3b/hS4 resulted in a precipitous drop in γ2b mRNA expression. In two of the clones, no γ2b mRNA was detectable. In the third, it was reduced to ~8% initial levels (Fig. 8). In contrast, deletion of hs3a/hS1,2 from γ2b-hs1-4loxPhs123a transformants had no such effect. Enhancer-deletion subclones lacking hs3a/hS1,2 expressed the γ2b transgene at levels that differed very little from those of the parental lines carrying all four enhancers (Figs 7 and 8B). Hence, in this Ig-secreting cell line, the hs3b/hS4 enhancer pair has taken on a critical role in maintaining IgH transgene function.

Fig. 6. Genomic Southern blot analyses of 9921 transformants before and after enhancer deletion. Right panel is of transformants before (B) and after (A) deletion of hs3b/hS4 (ΔHs3b4). Individual transformant pairs are given unique designations (P50, P42, etc.). Left panel is of transformants before (B) and after (A) deletion of hs1,2/hs3a (ΔHs123a). Upper blot: hybridized with Probe B (see Fig. 2). Fragments derived from the endogenous IgH loci of 9921 are indicated with an arrow. Because of faster migration of the outermost lanes of the gel, the 7.9-kb transgene fragment detected by probe B appears larger than its actual size. Lower blot: The same blot shown in the upper panel was “erased” and then re-hybridized with Probe A. The fragment derived from the endogenous IgH locus of 9921 migrates below the region shown on this blot.

Fig. 7. γ2b transgene expression in 9921 transformants before (B) and after (A) deletion of enhancers. The same blot was sequentially hybridized with a γ2b probe and a GAPDH probe (for sample loading normalization). The left panel shows transformant pairs with the mini-locus that allows cre-mediated deletion of hs3b/hS4 (ΔHs3b4) while the right panel is of pairs that carry the mini-locus that yields hs1,2/ hs3a deletions (ΔHs123a).
Discussion

Previous studies have suggested roles for the 3' IgH enhancer region in IgH gene transcription, in heavy chain CSR and in malignant transformation of B lineage cells (through de-regulation of the oncogene c-myc). Our understanding of how the enhancers mapping within this region function within the IgH chromosome remains cursory, however. As a method for directly assessing these enhancers' functions within the context of chromatin, we created IgH mini-loci amenable to manipulation within chromosomes. Individual deletions of hs1.2 and of hs3a in mice resulted in no apparent change in IgH locus activity, but Efi remained in the locus, as did the other 3' IgH enhancers (11). In the present study, therefore, we deleted enhancer pairs within an IgH locus that lacked Efi. Elimination of Efi serves not only to reduce the likelihood that 3' IgH enhancer activities will be masked by functional redundancy with Efi, it also mimics events frequently encountered in B-lineage cell lines. Efi-deficient loci have been described in numerous Ig-secreting cell lines (14, 19, 28–30), and the IgH/c-myc chromosome translocations characteristic of Burkitt's lymphoma and mouse plasmacytoma often result in a de-regulated c-myc locus lying in cis with the 3' IgH enhancers, but not with Efi (reviewed in 31, 32). By deleting enhancer pairs in our mini-loci, we also limit the effects of functional redundancy among the 3' IgH enhancers themselves.

Two mini-loci consisting of a y2b transcription unit and two different arrangements of the 3' IgH enhancers (hs1–4) were expressed in 115 out of 118 transformants analyzed from both a surface Ig+ cell line (A20) and an Ig-secreting cell line (9921). Without the 3' IgH enhancers, the same y2b transcription unit was inactive in all of 25 independent transformants isolated. Early studies of the individual 3' IgH enhancers showed that only hs4 had detectable activity in pre-B cells and surface Ig+ cells (17, 18, 33–35). The remainder of the enhancers were believed to achieve activity only at the Ig-secreting stage of B cell development since all were active in plasmacytoma cell lines. Subsequently, transient transfection studies with the group of 3' IgH enhancers revealed pronounced synergistic activity in both surface Ig+ cell lines and primary B cells from mouse spleen (13, 36). Consistent with the earlier studies, the 3' IgH enhancers (with the exception of hs4) had little or no detectable activity at this stage in development when assayed individually (13, 36).

In the present study, these findings are supported and extended by the demonstration that an IgH gene lacking the intronic enhancer Efi and carrying only the 3' IgH enhancers is very efficiently expressed when integrated into the genome of a surface Ig+ cell line. The 3' IgH enhancer region, therefore, begins to play a role in IgH gene expression well before B cells differentiate into Ig-secreting plasmacytes.

The murine 3' IgH enhancers have been described as a LCR or, more recently, as having some of the attributes of an LCR (18, 24). In the present study, site-independence was clearly demonstrated by active transgenes in all but three of 118 different chromosomal positions in two different B-lineage cell lines. There was no strict, copy number-dependence, however, and single-copy transgenes were expressed at varying levels that approached, but did not reach, that of an endogenous IgH locus. These findings are consistent with those of a transgenic mouse study (24). It is also worthy of note that in the latter study, the 3' IgH enhancers lay downstream of the reporter gene, rather than upstream as in our mini-loci, arguing against the notion that enhancer orientation relative to the promoter influenced LCR activity in our transgenes.

Unlike previous studies, the reporter in the present study was an IgH gene, presumably the gene that the 3' IgH enhancers are meant to regulate. We conclude that the cloned 3' IgH enhancers, when fused into a single unit, are not capable of establishing a fully insulated and independently acting IgH locus. We have yet to identify, therefore, the minimal elements required for such locus autonomy. Possibly, the natural spacing among enhancers is required or, perhaps more likely, there are additional control elements that await discovery.

Another feature of the 3' IgH enhancers that has attracted attention is the palindrome that extends from hs3a to hs3b with hs1.2 at the axis of symmetry (25, 26). While none of the assays of 3' IgH enhancer function published to date have re-created this palindrome in its entirety, our finding that two very different configurations of the 3' IgH enhancers yield equivalent and substantial activity argues against a strong dependence upon this palindromic arrangement for enhancer function. Notably, the palindrome found in the murine 3' IgH enhancer region is not conserved in the comparable region of human IgH loci (8).

The primary goal of the present study was to assess IgH 3' enhancer function in the context of chromatin, comparing IgH gene activity at a single chromosomal site before and after deletion of 3' IgH enhancer pairs. The experimental system allowed for the analysis of gene expression among multiple, independent clones, all of which were expressing the transgenes at appreciable levels before enhancer deletion. Enhancer deletion, therefore, provided a means for identifying the control elements required to sustain locus activity. Our results establish a critical and probably stage-restricted role for the hs3b/hs4 pair in IgH gene expression. In the Ig-secreting cell line, 9921, loss of this pair led to a dramatic

Fig. 8. Quantitation of Northern blot data presented in Figs 5 and 7. (A) Data generated from A20 transformant pairs. (B) Data generated from 9921 transformant pairs. Black bars are values for transformants before enhancer deletion and shaded bars are for their subclones, after deletion. Several different exposures of the autoradiographs shown in Figs 5 and 7 were quantified by densitometry (Molecular Dynamic densitometer SI; scanned images analyzed with ImageQuant). Values for y2b mRNA were normalized with GAPDH mRNA values. The normalized y2b mRNA levels were then compared for each transformant pair, with the value for the transformants before enhancer deletion (black bars) set to 100%.
decrease in (or loss of) IgH reporter gene expression. In contrast, in the surface Igα cell line A20, hs3a/hs1,2 and hs3b/hs4 were redundant with respect to their ability to enhance IgH gene transcription. Each pair sustained high levels of transgene expression in the absence of the other. The dramatic effect of hs3b/hs4 deletion in all three, independent transformants of 9921, each carrying a single copy of the γ2b-hs1-4loxP/neo mini-locus, and the lack of effect in all four, single-copy A20 transformants carrying the same mini-locus argues strongly for a cell-specific and against a chromosomal site-dependent mechanism. Prior work by us and others has shown 9921 and A20 to mirror the behavior of other cell lines at their respective stages, making these appropriate representatives of Ig-secreting and surface Ig+ cells respectively (13,17,35). We think it most likely, therefore, that the dramatically different effect of the hs3b/hs4 deletion in 9921 as compared to A20 cells reflects a stage-specific shift in the function of the 3’ IgH enhancers. Experiments are underway to extend these findings to other cell lines and to determine whether the effect of the hs3b/hs4 paired deletion can be mimicked by a single-enhancer deletion (of hs3b or of hs4), further delineating the unique functions of these 3’ IgH control elements.

The fact that the hs3b/hs4 deletion can be tolerated in the A20 transformants but not in 9921 transformants also suggests that hs3a/hs1,2 lose some aspect of their activity by the time cells reach the Ig-secreting stage. This was not apparent from transient transfection experiments in which hs3a and hs1,2 were active as an enhancer pair in Ig-secreting cell lines as well as in surface Ig+ cell lines and splenic B cells (J. Ong et al., unpublished data and 13,36). In these and other studies, hs3a and hs1,2 had low, but significant activity, even when assayed individually in Ig-secreting cell lines (13,17,33,37). These differences underscore the critical difference between assaying enhancer function within extra-chromosomal plasmids, as in transient transfections, and assaying enhancer function within a chromosome. Proteins that can bind enhancers on plasmids are not necessarily capable of penetrating a locus embedded in chromatin. We hypothesize that while there are transcription factors available in Ig-secreting cells that are capable of binding to and therefore mediating hs3a/hs1,2 enhancer function, this enhancer pair is unable, alone, to recruit the necessary chromatin-remodeling co-activators to allow transcription factor access to the transgene. Presumably, such remodeling factors that operate through interactions with hs3a and/or hs1,2 are available in surface Ig+ cells. This hypothesis will be explored in future experiments.

The mini-locus results presented here also shed light on our previous experiments in which hs1,2 was replaced with pgk-ned in 9921 cells (9). Given that deletion of hs3a/hs1,2 had no effect on mini-locus activity in 9921 cells while the hs3b/hs4 deletion had a pronounced effect, we conclude that the earlier hs1,2-deletion/ned-replacement experiments in 9921 constituted a technical knockout of all of the 3’ IgH enhancers. Promoter competition led to re-direction of hs3b/hs4 activity away from the IgH promoter and toward that of pgk-ned, depriving the endogenous IgY2α gene of its essential, positive control elements (hs3b/hs4). It remains to be determined whether diversion of this enhancer pair was responsible, as well, for the aberrant class-switching phenotype seen in mice in which hs1,2 (or hs3a) was replaced with pgk-ned (11).

Acknowledgements

The authors thank Drs Amy Kenter, Michael Young and Carol Nottenburg for critical readings of the manuscript. The plox2neo plasmid used for construction of psk-loxP/neo and used to introduce loxP sites into the IgH mini-loci was kindly provided by Dr Fred Alt (The Children's Hospital, Center for Blood Research, Harvard Medical School, Boston, MA). We thank both Mr Ryszard Sławowy and Mr David Miller for expert technical assistance. This work was supported by National Institutes of Health (NIH) grant AI-30653 to L. A. E. ‘Research Centers in Minority Institutions’ award RR-03037 from the NCRR of the NIH is also gratefully acknowledged for its support of infrastructure and instrumentation in the Department of Biological Sciences at Hunter College.

Abbreviations

LCCR locus control region
CSR class switch recombination

References

7 Chen, C. and Birshtein, B. K. 1997. Virtually identical enhancers containing a segment of homology to murine 3’ IgH-E(hs1,2) lie downstream of human Ig Calpha1 and Calpha2 genes. J. Immunol. 159:1310.
23 Lang, R. B., Stanton, L. W. and Marcu, K. B. 1982. On immunoglobulin heavy chain gene switching: two \( \gamma_{2b} \) genes are rearranged via switch sequences in MPC-11 cells but only one is expressed. Nucleic Acids Res. 10:611.