Class Switching in B Cells Lacking 3′ Immunoglobulin Heavy Chain Enhancers

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Summary

The 40-kb region downstream of the most 3′ immunoglobulin (Ig) heavy chain constant region gene (Cα) contains a series of transcriptional enhancers speculated to play a role in Ig heavy chain class switch recombination (CSR). To elucidate the function of this putative CSR regulatory region, we generated mice with germline mutations in which one or the other of the two most 5′ enhancers in this cluster (respectively referred to as HS3a and HS1,2) were replaced either with a pgk-*neo*^{r} cassette (referred to as HS3aN and HS1,2N mutations) or with a *loxP* sequence (referred to as HS3aΔ and HS1,2Δ, respectively). B cells homozygous for the HS3aN or HS1,2N mutations had severe defects in CSR to several isotypes. The phenotypic similarity of the two insertion mutations, both of which were *cis*-acting, suggested that inhibition might result from pgk-*neo*^{r} cassette gene insertion rather than enhancer deletion. Accordingly, CSR returned to normal in B cells homozygous for the HS3aΔ or HS1,2Δ mutations. In addition, induced expression of the specifically targeted pgk-*neo*^{r} genes was regulated similarly to that of germline C_H genes. Our findings implicate a 3′ CSR regulatory locus that appears remarkably similar in organization and function to the β-globin gene 5′ LCR and which we propose may regulate differential CSR via a promoter competition mechanism.

Key words: immunoglobulin genes • class switching • enhancers • gene-targeted mutation • transcription

Immunoglobulin (Ig) variable regions are encoded by germ-line V, (D), and J gene segments that are assembled during early B cell differentiation by V(D)J recombination (for review see reference 1). The Ig heavy (H)[1] chain locus contains eight different constant region (C_H) genes with the organization: 5′V(D)J-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3′. Differentiating B lymphocytes first produce H chains in the context of an IgM surface receptor. During antigen-driven B cell maturation, B cells can secrete specific antibody with a different C_H region effector function by juxtaposing the antigen-specific V(D)J gene to a different downstream C_H gene via a recombination/deletion process termed class switch recombination (CSR; for review see reference 2). CSR occurs between tandem repetitive switch (S) region sequences present 5′ of individual germline C_H genes.

CSR to particular C_H genes is directed by different combinations of activators and lymphokines (3–6). For example, stimulation of B cells with bacterial LPS induces CSR to the Cγ2b and Cγ3; whereas LPS plus IL-4 induces CSR to Cγ1 and Cε. Factors that modulate switching to particular C_H genes correspondingly modulate germline transcription of those genes before CSR (7–13). Thus, LPS treatment of splenic B cells induces germline Cγ2b and Cγ3 transcription; whereas LPS plus IL-4 treatment suppresses germline Cγ2b and Cγ3 expression, and induces germline Cγ1 and Cε gene transcription. All C_H gene transcripts initiate at an exon termed I that lies 5′ to each S region, proceed through the S region and C_H gene, and are processed to yield noncoding transcripts with the I exon spliced to the C_H exon. Gene-targeted mutational analyses of I region exons/promoters have confirmed that germline C_H transcription and/or transcripts play a direct role in CSR (14–19). Therefore, control of CSR is directly related to ability to control transcription of the various C_H genes.

Understanding of the mechanisms that regulate CSR will require elucidation of both *trans*- and *cis*-acting ele-

[1]*Abbreviations used in this paper:* CSR, class switch recombination; ES, embryonic stem; H, Ig heavy; LCR, locus control region.

The first three authors contributed equally to this work.

ments that modulate germline C_H transcription. In this context, C_H gene promoters contain consensus sequences that are responsive to specific lymphokines (reviewed by Coffman et al. [20]). Yet, properly regulated expression of germline C_H genes requires sequences beyond those of their proximal promoters (21). In this regard, efficient CSR from Cμ to a downstream C_H gene requires the transcriptional enhancer element (iEμ) found in the J_H/Cμ intron (22). However, the expression of switched transcripts or translocated oncogenes on alleles in which iEμ is absent suggested the presence of additional downstream positive regulatory sequences (23–25).

The first candidate for such a downstream transcriptional enhancer was the so-called 3′CαE or 3′EH (Fig. 1, *A* and *B*), which was identified ~15 kb 3′ of Cα based on ability to enhance transcription specifically in B lineage cells (26–28). Homozygous replacement of this element (also referred to as HS1,2, see below) with a pgk-*neo*^{r} gene disrupted CSR and germline transcription of a series of C_H genes, including Cγ3 which lies 120 kb upstream (29). Assuming that these effects were *cis*-acting, one hypothesis to explain this phenotype was that HS1,2 is critical for induction of germline C_H gene transcription and that its deletion was the primary cause of the CSR phenotype (29, 30). A second possibility is that insertion of the pgk-*neo*^{r} gene cassette in the 3′ IgH locus disrupts the normal regulation of germline transcription/CSR by interfering with the activities of additional required regulatory elements (29).

The 40-kb region just downstream from Cα contains four enhancer elements (that correspond to DNAase1 hypersensitive or HS sites): HS3a (or Cα3′E), which lies 4 kb 3′ to the Cα; HS1,2 (or 3′CαE), which lies 15 kb 3′ to Cα; HS3b, which lies 25 kb 3′ to Cα; and HS4, which lies ~30 kb 3′ to Cα (26–28, 31, 32). Like the HS1,2 sequence, HS3a (31) and HS3b (32) enhance reporter gene expression in activated B cells and plasma cells. On the other hand, HS4 is active throughout B cell development (32, 33). HS3a, HS1,2, and HS3b appear to represent a unit with HS3a and HS3b sharing high sequence homology, but lying in inverted orientation in the chromosome (34, 35). Recent studies have shown these enhancer sequences are conserved in man, consistent with an important regulatory function (36, 37).

Combinations of HS3b, HS1,2, and HS4 had synergistic transcriptional enhancing effects when assayed in a transgenic mouse model, and were able to induce copy number and position-independent reporter gene expression, suggesting that these sequences have locus control region (LCR) properties (32). In this regard, a similar set of HS sites that lie upstream of the β-globin gene locus constitute a β-globin LCR, that apparently is responsible for coordinating expression of the various β-globin genes during development (reviewed by Martin et al. [38]). Individual HS sites within the β-globin LCR also have transcriptional enhancer activity and replacement of 2 of these individually with an expressed selectable marker gene cassette resulted in decreased β-globin expression across the locus (39–41), an effect reminiscent of what was observed when HS1,2 was replaced with a *neo*^{r} gene (29). Yet "clean" deletion of the β-globin HS sites had no major effect, indicating that the effects of the pgk-*neo*^{r} gene insertion resulted from interference with additional regulatory sequences (40, 41).

To elucidate the function of the putative 3′ IgH regulatory region and assess the roles of specific enhancers, we have generated mice with germline mutations in which a pgk-*neo*^{r} cassette or a *loxP* site replaced either the HS3a or the HS1,2 elements and then assayed the effects of these mutations on the CSR process.

Materials and Methods

Vector Construction, Transfection, and Embryonic Stem Cell Screening. For the generation of HS1,2 mutant mice, a previously described construct (29) was modified by deleting its existing pgk-*neo*^{r} cassette and substituting a *loxP* flanked pgk-*neo*^{r} cassette in the central Not1 site, by blunt end ligation (Fig. 1 *D*). The deletion spanned from a Pst-1 site 1.8 kb 5′ of the HS1,2 core element (42) to an EcoR1 site 1.7 kb 3′ of the element. This construct was linearized using Pvu-1. A 600-bp EcoR1-Xba fragment 5′ of HS1,2 was used as a probe in Southern blotting.

The HS3a mutant embryonic stem (ES) cells were derived using pLNTK vector P3 (43), cloning the 3-kb H3 fragment containing the alpha membrane exon in the Sal1 site and a 3.4-kb Hind3-Xba fragment into the Xho site, and deleting the 2-kb fragment that contains the previously described 900-bp enhancer (31) HS3a (Fig. 1 *C*). This construct was linearized using Sal1. A 500-bp EcoR1-Hind3 fragment was used as a probe in Southern blotting.

Approximately 20 μg of linearized construct DNA was electroporated into 2×10^7 J1 or E14 ES cells (HS1,2 and HS3a targetings, respectively) and homologous recombinants were selected as previously described (44). One ES clone was injected into C57Bl/6 blastocysts to generate germline chimeric mice, each where a pgk-*neo*^{r} cassette inserted correctly into one allele of either HS3a or HS1,2. Germline transmitting mice were then interbred to produce either mice homozygous for the HS3aN or HS1,2N mutation. Females homozygous for either mutation were bred with males containing an EIIa-Cre transgene (43, 45) to produce mice heterozygous for HS3aΔ or HS1,2Δ. Subsequent breeding of these mice yielded animals homozygous for HS3aΔ or HS1,2Δ. To keep the strains in all mutant mice similar, we bred mice with the HS3aN or HS1,2N (which were in a 129 × C57BL/6 background) with FVB mice (the strain of the EIIa-Cre Tg mice). Again mice were interbred to produce homozygous mutants for HS3aN or HS1,2N.

Spleen Cell Cultures. Single-cell suspensions of spleen cells were cultured at 5×10^5 cells/ml in RPMI medium supplemented with 10% FCS and 20 μg/ml LPS with or without 50 ng/ml of mouse recombinant IL-4 as previously described (18). Cultures for IgG2a and IgA production were prepared as previously described (29). Cells were harvested for FACs, and supernatants assayed on days 4–5.

Flow Cytometry Analysis. Single-cell suspensions from spleens were prepared as previously described (18). Cells from day 4 or 5 cultures were washed in PBS, 2% FCS and stained with various antibodies conjugated with fluorescein (IgG1), phycoerythrin (IgM), biotin (IgG2b, IgG3, IgE, IgG2b^a/IgG2a^a, IgG2a^a), or Cychrome (B220; PharMingen, San Diego, CA). Biotin conjugates were revealed by phycoerythrin-streptavidin (PharMingen). The cells were analyzed on a FACScalibur® (Becton Dickinson &

Co., Sparks, MD) and analyzed using Cellquest software, and are presented as dot plots after gating for live cells.

ELISA Assays. Supernatants from spleen cell cultures and sera from normal B6/CBA mice or germline mutant mice were analyzed for the presence of different immunoglobulin isotypes by ELISA as described (18). Cultures were established in triplicate for each assay. In total, three independent culture experiments were performed for wild-type and the four different 3′ IgH enhancer mutant mice. Mice ranged in age from 6 wk to 3 mo, and culture supernatants were assayed after 5 d of stimulation.

Southern and Northern Blot Analyses. Genomic DNA was prepared as previously described (46). RNA was prepared using the TRIZOL reagent (GIBCO BRL, Gaithersburg, MD) as per the instructions of the manufacturer. Southern and Northern blot analyses were performed as described elsewhere (18). The mb-1 probe was generated from a full-length cDNA. The Pgk probe is a 300-bp R1-H3 fragment, and the neo probe is a 500-bp R1-Pst fragment. The Iγ2b probe is a 500-bp H3-Xho fragment. The Cε probe is a 2.5-kb Sac fragment.

PCR Amplification of Germline Transcripts. Total RNA was isolated from 1×10^6 day 3 LPS/IL-4–stimulated splenocytes, using the TRIZOL reagent (GIBCO BRL) as per the manufacturer's instructions. cDNA was generated using Superscript (GIBCO BRL), again according to manufacturer's instructions. For Cε, 1/10 the total cDNA was amplified using a forward PCR primer (these data are available from GenBank/EMBL/DDBJ under accession numbers 194466 and 194457) of 5′-acggggtgtgattacctacctgat-3′, and a reverse primer of 5′-gatattgttttcagtttcctg-3′. 30 cycles of 94°C × 1 min, 55°C × 30 s, and 72°C × 1 min were performed in a Perkin Elmer 9600 thermal cycler (Perkin-Elmer Corp., Norwalk, CT). The reaction was then directly digested with 10 U of Sty 1 for 1 h, electrophoresed on a 3% agarose gel, and directly visualized with EtBr staining.

The PCR amplification of germline transcripts was performed as previously described, using 5′-caagtggatctgaacaca-3′ and 5′-ggctccatagttccatt-3′, for γ3 forward and reverse, respectively. For germline ε the primers used are 5′-actagagattcacaacg-3′ and 5′-agcgatgaatggagtagc-3′, for forward and reverse, respectively. Both reactions were carried out as in Zelazowski et al. (47) for 28 cycles. mb-1 primers as described in Li et al. (48) were used in a reaction for 18 cycles. The products were run on a 2% agarose gel, transferred to a nylon membrane, and hybridized with the probes described above.

Generation of Chimeric Mutant Mice of a Randomly Integrated HS1,2 Targeting Construct. Four independent ES cell lines that contained randomly inserted HS1,2 targeting constructs (HS1,2RAN) and two HS1,2N/+ ES subclones were injected into blastocysts from the RAG-2–deficient mice and transplanted into foster mothers (B6/CBA) as described (49). Chimeric mice with lymphocytes derived from either the randomly or specifically targeted HS1,2N constructs were analyzed in splenic cultures as described above.

Results

Generation of Mice with Targeted Replacements or Deletions of HS3a or HS1,2. ES cells were transfected separately with constructs that replace either the entire HS3a or HS1,2 with a *loxP*-flanked pgk-*neo*^{r} gene cassette (Fig. 1, *B–D*; mutations termed HS3aN and HS1,2N, respectively). ES clones with appropriate targetings of the endogenous loci were used to introduce each of the mutations into the murine germline. Mice homozygous for either replacement mutation appeared normal and had B cell numbers in peripheral lymphoid organs that were similar to those of wild-type controls (data not shown). To generate mice with a "clean" deletion of each of the respective enhancer elements, the introduced *loxP*-flanked pgk-*neo*^{r} gene was deleted by breeding the mice containing the HS3aN or HS1,2N mutations with a mouse that expresses an EIIa-Cre recombinase transgene at a very early developmental stage, and which permits generation of progeny that have deleted the *neo*^{r} gene in the germline (45). From the progeny of this cross, we identified individuals in which the pgk-*neo*^{r} gene was deleted, resulting in replacement of the HS3a or HS1,2 sites with only a *loxP* sequence. Mice homozygous for these deletion mutations (termed HS3aΔ and HS1,2Δ, respectively; Fig. 1) again appeared normal, and contained B cell numbers comparable to those of wild-type mice (data not shown).

Deficient Serum Isotype Levels in Mice Homozygous for the HS3aN or HS1,2N Mutations. Previously, homozygous replacement of HS1,2 with a pgk-*neo*^{r} cassette in ES cells followed by assay via the RAG-2–deficient blastocyst complementation system showed that this mutation led to chimeric mice with markedly decreased serum levels of IgG3 and IgG2a, but relatively normal levels of other isotypes including IgM (29). To assay the effects of the germline replacement mutations, we quantified serum Ig levels in mice homozygous for the HS1,2N and HS3aN mutations (referred to as HS1,2N/N and HS3aN/N mice, respectively). The serum IgM levels in both mutant lines were similar to those of wild-type control mice (Fig. 2). Levels of IgG1, IgG2b, and IgA also were substantial but there was no detectable IgG3 or IgG2a (Fig. 2). IgE levels were not determined as they were below the detection level of our assay even in normal mice (not shown). Thus, replacement of either HS1,2 or HS3a with a pgk-*neo*^{r} cassette resulted in essentially identical defects in serum Ig expression, even though the sites of the replacement mutations were separated by 12 kb within the downstream IgH region. This finding indicates that either HS3a and HS1,2 are independently essential for promoting CSR to these isotypes or that the *neo*^{r} gene interferes with additional elements when placed at either site.

Severe Class-switch Defects In Vitro in HS3aN/N and HS1,2N/N Splenic B Cells. Our previous studies showed that splenic B cells generated from ES cells homozygous for a HS1,2-pgk-*neo*^{r} gene via replacement mutation by RAG-2–deficient blastocyst complementation had severe defects in CSR and in transcription of the Cγ3, Cγ2b, Cγ2a, and Cε genes (29). To determine whether HS1,2N/N or HS3aN/N splenic B cells were similarly affected, we cultured both types of mutant B cells for 4 or 5 d in the presence of either LPS, LPS + IL-4, LPS + IFN-γ, or LPS + TGF-β, agents that collectively are known to induce class switching to Cγ3 and Cγ2b, Cγ1 and Cε, or Cγ2a and Cα, respectively. Both mutant cell types proliferated similarly to normal cells after the various treatments (data not shown). Supernatant antibody levels in the cultures after 5

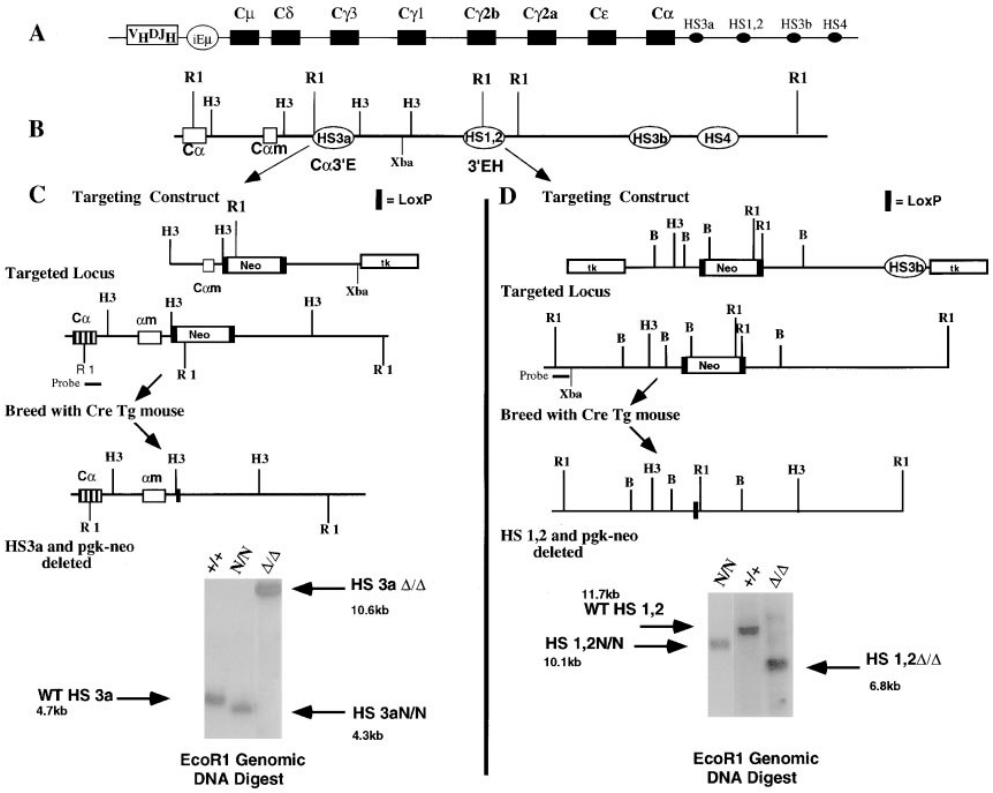

Figure 1. Targeted mutations of the HS3a and HS1,2 enhancers. (*A*) Partial map of the 3′ IgH locus representing the arrangement of the heavy chain constant region genes and enhancers depicted by filled boxes or ovals. The open circle labeled iEμ represents the intronic heavy chain enhancer (map not drawn to scale). (*B*) The open box represents the Cα membrane coding region. The open ellipses represent various enhancer elements, HS3a (*CαEH*), HS1,2 (*3′EH*), HS3b, and HS4. (*C*) The targeting for the HS3a was detected using the depicted H3-R1 500-bp probe, yielding a genomic band of 4.7 kb by EcoR1 digest, and a 4.3-kb band after homologous recombination with a lox flanked pgk-*neo*^{r} gene. Upon Cre-mediated deletion of the pgk-*neo*^{r} cassette a 10.6-kb band is detected. (*D*) HS1,2 was targeted using the depicted construct, where a 600-bp EcoR1-Xba fragment detects a 11.7-kb germline band and a 10.1-kb targeted band. Upon Cre-mediated deletion of the selectable marker, the mutated band is detected at 6.8 kb. Restriction enzymes: *B*, BamH1; *R1*, EcoR1; *H3*, Hind3; *Xba*, Xba 1.

days of treatment were measured by ELISA using isotype specific antibodies. After appropriate stimulation, secreted levels of IgM were similar in cultures of HS1,2N/N, HS3aN/N and wild-type B cells, while the levels of IgG1 and IgA secreted by the mutant cells were significant but generally lower than those of normal control B cells (Fig. 3). However, IgG3, IgG2b, IgG2a, or IgE were not detectable in the supernatants of the HS1,2N/N and HS3aN/N B cells, which indicated a reduction of 100-fold or more from wild-type levels (Fig. 3). Surface stains of day 4 stimulated cells confirmed a substantial inhibition of switching to IgG3, IgG2b, IgG2a, and IgE, accompanied by an apparently slight inhibition of switching to IgG1 and relatively normal switching to IgA (data not shown).

To analyze the consequences of replacing HS3a with pgk-*neo*^{r} cassette on expression of germline C_H transcripts, we assayed total RNA from LPS and LPS plus IL-4–stimulated HS1,2N/N and HS3aN/N B cells for hybridization to an Iγ2b-region specific probe (Fig. 4 *A*). These studies demonstrated major inhibition in the expression of germline Iγ2b transcripts in LPS cultures of the N/N cells (Fig. 4 *A*). Additional assays revealed a similar lack of germline

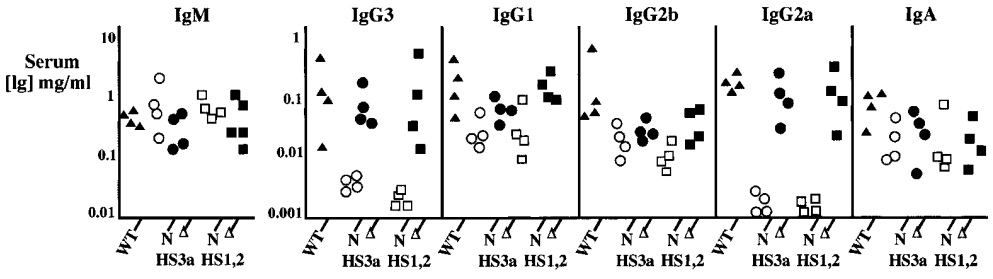

Figure 2. Selective serum immunodeficiency in HS1,2N/N and HS3aN/N mice but not in HS3aΔ/Δ or HS1,2Δ/Δ mice. Concentrations of specific immunoglobulin isotypes in sera from HS1,2N/N (*open squares*), HS3aN/N (*open circles*), HS3aΔ/Δ (*closed circles*), HS1,2Δ/Δ (*closed squares*) mice with their wt (*closed triangles*) littermates are shown. N represents the N/N mice, Δ represents the Δ/Δ mice, and WT indicates wild-type littermate controls. All isotypes except IgE were quantified by ELISA.

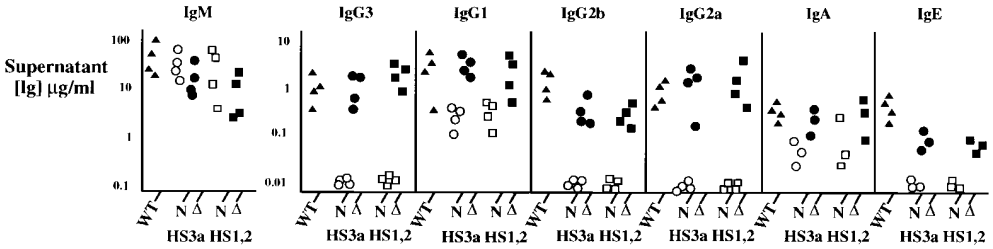

Figure 3. In vitro defect of class switching in HS1,2N/N and HS3aN/N mice but not in HS3aΔ/Δ or HS1,2Δ/Δ mice. Splenic B cells from the indicated mice were cultured for 5 d in the presence of LPS (*IgM*, *IgG3*, and *IgG2b*), or LPS + IL4 (*IgE* and *IgG1*), or LPS + IFN-γ for IgG2a, or LPS + TGF-β (for IgA). Culture supernatants were analyzed by ELISA to quantify secreted immunoglobulin levels. N represents the N/N mice, Δ represents the Δ/Δ mice, and WT indicates wild-type littermate controls.

transcripts from other C_H genes (e.g., Cγ3 and Cε) after appropriate in vitro stimulation of HS1,2N/N and HS3aN/N B cells (Fig. 4 *C*). Together, these findings show that homozygous replacement of either HS3a or HS1,2 with a pgk-*neo*^{r} gene results in an essentially identical defect of CSR to IgG3, IgG2b, IgG2a, and IgE after in vitro stimulation, and that this defect corresponds in turn to a block in the induction of the respective germline C_H transcripts. Again, these findings could be consistent with either a required function for both HS3a and HS1,2 in the CSR process or an effect of the inserted pgk-*neo*^{r} gene on an additional regulatory element.

Inhibition of CSR by the pgk-neo^r *Replacement Mutations Occurs in a Cis-acting Fashion.* The defect in CSR observed in either the HS3aN/N or HS1,2N/N B cells could occur via disruption of a critical cis-acting control element for CSR (29). To directly assay for this, we generated mice harboring one allele with a pgk-*neo*^{r} replaced enhancer element, and one wild-type allele. The ES cells used in our targetings were derived from the 129 mouse strain which carries the IgH^a haplotype. The mutant mice were bred with C57Bl/6 mice that carry the IgH^b haplotype, allowing the generation of heterozygous mutant progeny that harbor an IgH^a allele with the *neo*^{r} mutation and a wild-type IgH^b allele. The haplotypic differences between the IgH^a and the IgH^b alleles can be distinguished at the DNA level (based on nucleotide sequence differences manifested as unique restriction endonuclease sites), as well as at the protein level by antibodies specific for the IgH^a and IgH^b polymorphisms.

Splenocytes from the wild-type, HS3aN/+, and HS1,2N/+ mutant mice were stimulated in vitro with either LPS or LPS+IL-4, and activated B cells were collected for analysis after 4 d of stimulation. LPS-stimulated cells were doubly stained with a biotin-conjugated antibody that recognizes the IgH^a type allele of IgG2b and IgG2a isotypes (IgG2b^a and IgG2a^a, respectively), and one that recognizes total IgG2b (both IgH^a and IgH^b allotypes). Wild-type B cells heterozygous for the normal IgH^a and IgH^b alleles had similar numbers of B cells that stained for either surface IgG2b^a or IgG2b^b positive cells (Fig. 5 *A*). However, mice heterozygous for either mutant IgH^a allele and the wild-type IgH^b allele expressed only surface IgG2b^b positive B cells indicating a *cis*-acting defect of the mutations on class switching to IgG2b (Fig. 5 *A*, labeled HS3aN^a/+^{b} or HS1,2N^a/+^{b}).

To assay for allele-specific transcripts of the Cε region, we employed PCR for specific amplification of all transcripts through the Cε constant region. The IgE^b haplotype contains an additional Sty-1 restriction site in the 453 base pairs spanning the first 2 Cε constant region exons, as compared with the IgE^a haplotype. Therefore, RT-PCR products of transcripts arising from the two alleles can be differentiated based on the size of fragments generated after digestion with Sty-1. LPS plus IL-4–stimulated B cells from mice heterozygous for the wild-type IgH^a and IgH^b alleles generated an approximately equal ratio of Cε-containing transcripts from the two alleles (Fig. 5 *B*). In contrast, almost all detectable Cε-transcripts in LPS plus IL-4–stimulated B cells from mice heterozygous for either the HS1,2N or HS3aN IgH^a allele and the wild-type IgH^b allele were generated from the wild-type (IgH^b) allele (Fig. 5 *B*).

In summary, both pgk-*neo*^{r} replacement of either HS3a or HS1,2 regions resulted in a *cis*-acting defect in CSR, as measured both by transcripts and surface protein expression, indicating that the effects of the *neo*^{r} gene replacement mutations disrupted a *cis*-acting control element.

Normal Class Switch Recombination and Germline C_H Transcription in HS3aΔ/Δ or HS1,2Δ/Δ B Cells. To further elucidate the mechanism by which the HS3aN and HS1,2N insertion mutations inhibited CSR, we assayed for class switching by B cells of HS3aΔ/Δ and HS1,2Δ/Δ mice. Splenic B cells from both homozygous deletion mutant mice expressed a wild-type distribution of surface IgM and IgD (data not shown). Likewise, the levels of serum IgM and all other assayed downstream isotypes were similar to those of the wild-type control animals (Fig. 2). Thus, specific deletion of either the HS3a or HS1,2 enhancers had no readily measurable effect on serum Ig levels. Moreover, after LPS or LPS plus lymphokine stimulation of HS3aΔ/Δ and HS1,2Δ/Δ splenic B cells, we observed induction of surface and secreted Ig isotypes that were generally similar

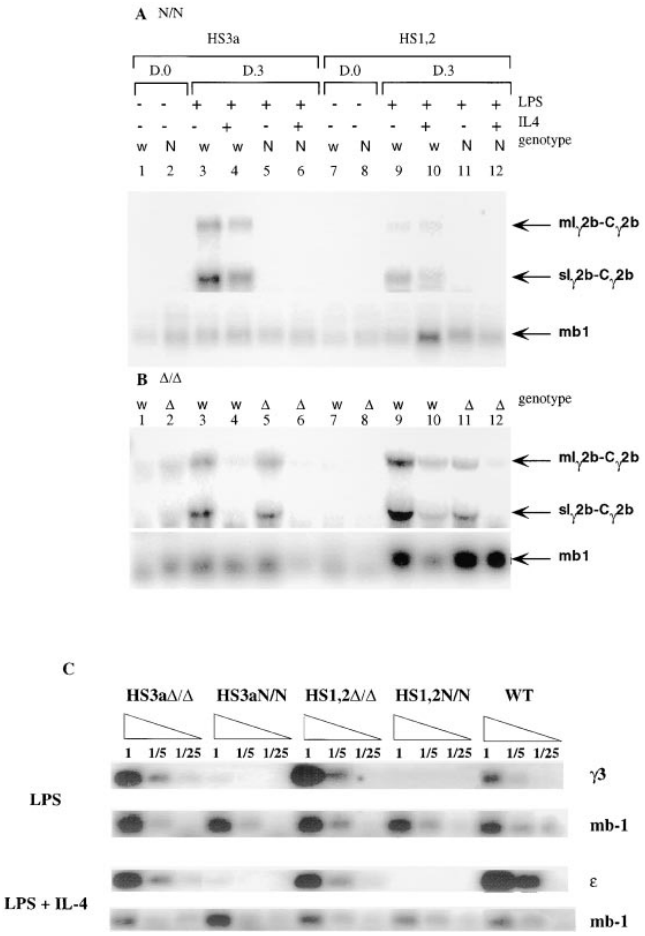

Figure 4. Marked inhibition of inducible germline Cγ2b transcription in HS12N/N and HS3aN/N but not HS3aΔ/Δ or HS1,2Δ/Δ B cells. Splenic B cells from wt, HS3aN/N and HS12N/N mice (*A*), or HS3aΔ/Δ, and HS1,2Δ/Δ (*B*). B Cells were cultured for 5 d with LPS or LPS plus IL-4. Total RNA was isolated on days 0 (*D.0*) and 3 (*D.3*) and separated on a denaturing 1% agarose gel and assayed for expression of Iγ2b and mb-1 hybridizing transcripts. The Iγ2b probe detects both the membrane (*m-Iγ2b*) and secreted (*s-Iγ2b*) forms of transcript as indicated. RNA from wild-type controls was obtained from cultures derived from littermates of the mutant mice. (*C*) RT-PCR for γ3 or ϵ germline transcripts was performed on the above samples, as outlined in the methods. Fivefold dilutions of cDNA were diluted into mouse genomic DNA to keep a constant amount of 100 ng total DNA per reaction. Probes internal to the PCR primers for Iγ3 or Iϵ were used for hybridization. Wild-type controls are indicated with w and mutant N/N and Δ/Δ mice are indicated, respectively, with N and Δ.

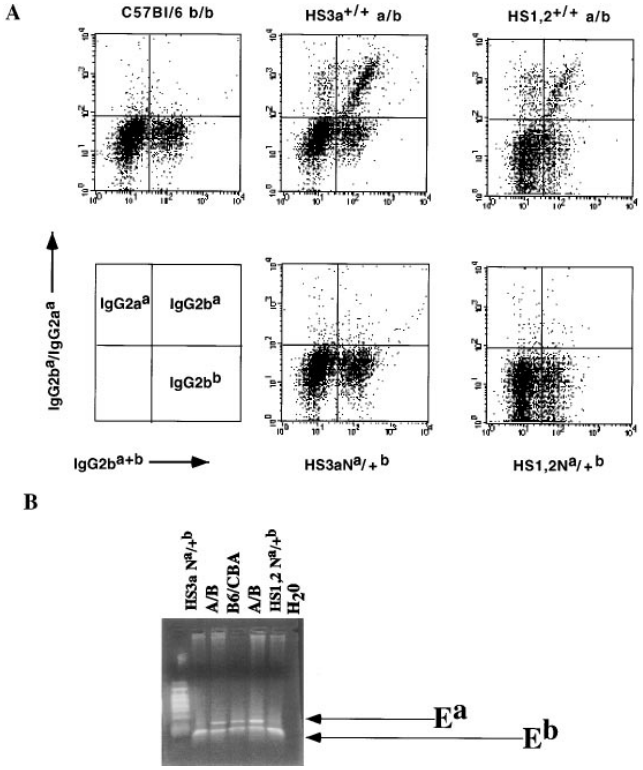

Figure 5. The HS1,2N and HS3aN mutations disrupt class switching in *cis*. Splenocytes from mice with either the HS3a or the HS1,2 elements replaced by a pgk-*neo*^{r} cassette on their IgH^a haplotype allele, and a wild-type IgH^b haplotype allele, were analyzed. (*A*) Day 4 splenocyte cultures were stained with an antibody detecting both IgG2b^a or IgG2b^b, and one which detects IgG2b^a and IgG2a^a. LPS-stimulated B cells from mutant mice reveal cells staining only for IgG2b^b. (*B*) RT-PCR of transcripts through Cϵ, digested with Sty 1 reveal a 200-bp fragment generated from the IgH^a allele, and a 150-bp fragment from the IgH^b allele.

in level to those of wild-type control mice (Fig. 3 and data not shown). Although there were potentially small effects on switching to certain isotypes (e.g., IgG2b and IgE; Fig. 3), these are difficult to assess given the inherent variations in these population assays. Overall, our results indicate that neither the HS1,2 nor the HS3a enhancers are required for LPS and lymphokine-stimulated CSR and expression of IgH isotypes.

To test for the effects of specific deletions of HS3a or HS1,2 on expression of germline C_H transcripts, we assayed RNA prepared from the LPS or LPS plus IL-4–treated cultures of HS3aΔ/Δ and HS1,2Δ/Δ B cells for hybridization to an Iγ2b-specific probe (Fig. 4 *B*). We readily observed the induction of Iγ2b-containing germline transcripts upon LPS activation of these cells, in striking contrast to the lack of induction observed in LPS-activated cultures of HS3aN/N and HS1,2N/N B cells. (compare Fig. 4, *A* with *B*). PCR amplification of germline transcripts containing Iγ3 or Iϵ yielded similar findings (Fig. 4 *C*). We do note that the extent of induction appeared to be less in the HS1,2 Δ/Δ B cells than in wt cells (Fig. 4 *B* and data not shown). Although the potential for more modest effects on expression will need to be examined in further detail, we conclude from these studies that neither the HS3a nor the HS1,2 enhancers are absolutely needed for substantial induction of germline C_H gene transcription or the CSR process that follows.

The pgk-neo^r Gene Is LPS Inducible When Inserted into the 3′ IgH Locus. Insertion of the pgk-*neo*^{r} cassette into either the HS3a or HS1,2 location in the 3′ IgH locus inhibits germline transcription of C_H genes up to 120 kb away (Fig. 4). To further analyze the potential mechanisms involved, we assayed for expression of the pgk-*neo*^{r} cassette when it

was inserted in place of the HS1,2 sequences. For this purpose, we assayed total splenocyte RNA from HS1,2N/+ mice for hybridization to a *neo*^{r} gene-specific probe. Expression of endogenous pgk sequences (which are ubiquitously expressed) and the Mb-1 sequence (which is B cell specific) was measured as a control. RNA from thymus and from nonlymphoid tissues had very low levels of pgk-*neo*^{r} transcripts, from the specifically inserted pgk-*neo*^{r} gene (data not shown). However, HS1,2N spleen RNA contained significant levels of *neo*^{r} transcripts (Fig. 6, lanes *3* and *5*). To determine if these *neo*^{r} transcripts were expressed and inducible in B lymphocytes, we assayed RNA from cultures of HS1,2N/+ spleen cells treated for 4 or 5 d with LPS. These RNA preparations showed a significantly higher level of *neo*^{r} gene transcripts (representing as much as a fivefold induction; Fig. 6; compare lanes *3* and *5* with lanes *9*, *11*, *14*, and *16*). Furthermore, we also observed similarly elevated expression levels of the pgk-*neo*^{r} gene in LPS-treated HS3aN/N splenic B cells (data not shown), indicating that such induction occurs with respect to *neo*^{r} genes targeted at two independent sites in the 3′ IgH locus.

These findings suggested that the pgk-*neo*^{r} sequences inserted into the 3′ IgH region are specifically expressed and LPS-inducible in B cells. As a control for this experiment, we used ES cell lines that contained randomly inserted HS1,2 targeting constructs to generate splenic B cell populations by the RAG-2–deficient blastocyst complementation method (49). Spleen cells from these chimeric mice were then assayed for *neo*^{r} and control gene transcription before and after 5 d of culture in LPS. These cells proliferated and underwent class switching to IgG3 and IgG2b in a manner similar to that of normal splenic B cells. However, none of four independent populations of spleen cells with random integrations of the HS1,2 targeting construct expressed levels of the pgk-*neo*^{r} gene above the very low levels found in thymus or non-lymphoid tissues, either before or after LPS treatment (Fig. 6, compare lanes *1*, *2*, and *4* with lanes *7*, *8*, *10* and *12*, *13*, *15*).

Together, these data indicate that specific targeting of the pgk-*neo*^{r} gene into the 3′ IgH locus leads to its upregulated expression in total splenic B cells and that this expression level is further augmented in LPS-stimulated B cell populations.

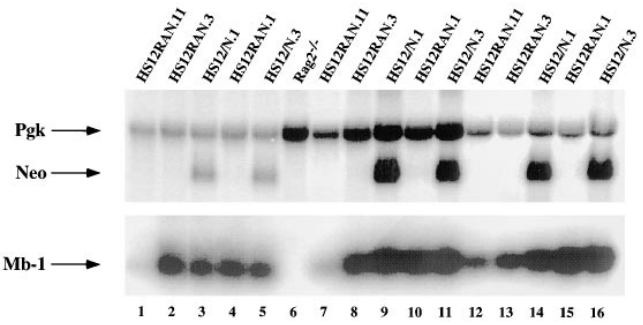

Figure 6. LPS-inducible *neo*^{r} gene expression from HS1,2N. Total RNA samples isolated from spleen cells of either unstimulated or day 4 or 5 of LPS activation, and were separated on a denaturing formaldehyde containing 1% agarose gel. Respectively, *neo*^{r}-, pgk-, and mb-1-RNA transcripts were detected with specific probes as mentioned in experimental procedures and are indicated with an arrow. Lanes *1*, *2*, and *4* contain RNA from unstimulated splenocytes that harbor a randomly integrated HS12 pgk-*neo*^{r} targeting construct (*HS12RAN*) and are derived from ES cell clones R12.3, R12.11 and R12.1, respectively. Lanes *7*, *8*, and *10*, contain RNA samples of ES-derived splenocytes (again clones R12.3, R12.11 and R12.1) after 4 d of LPS stimulation, and lanes *12*, *13*, and *15* contain RNA samples of the same spleen cells after 5 d of LPS stimulation. Lanes *3* and *5* contain RNA samples derived from unstimulated spleen cells that harbor a targeted replacement of the HS12 pgk-*neo*^{r} targeting construct. Lanes *9* and *11* contain RNA samples of the same spleen cells after 4 d of LPS stimulation and lanes *14* and *16* contain RNA samples of the same spleen cells after 5 d of LPS stimulation. Lane *6* contains RNA from unstimulated RAG-2–deficient spleen cells.

Discussion

The 3′ IgH Regulatory Region. The 40-kb region directly 3′ of the IgH locus contains four known transcriptional enhancer sequences, including HS3a, HS1,2, HS3b, and HS4 (reviewed by Birshtein[42]) and is referred to as the 3′ IgH regulatory region. Cell transfection and transgenic studies indicated that the individual enhancers are active in stimulated or terminally differentiated B cells, suggesting they are involved in controlling late B cell differentiation events such as CSR and high level IgH gene expression (26, 30, 32, 50). However, until now, there has been no direct examination of the potential role of any of these elements during normal B cell development. Although we previously demonstrated that replacement of the HS1,2 enhancer with a pgk-*neo*^{r} cassette disrupted CSR to multiple C_H genes, the mechanism was not clear. Thus, the block in CSR could have resulted from the deletion of the HS1,2 enhancer, an inhibitory effect of the inserted pgk-*neo*^{r} cassette on other elements, or both (29). Furthermore, the effects of the previous mutation could have resulted from disrupting expression of a gene encoding a *trans*-acting factor necessary for CSR (29). Our current work clearly resolves these possibilities by showing that neither HS3a nor HS1,2 are necessary for CSR or IgH gene expression; whereas replacement of either with a pgk-*neo*^{r} cassette interferes with CSR and germline transcription of distant, *cis*-linked C_H genes.

HS3a and HS1,2 Are Not Required for CSR or IgH Expression. HS1,2 was identified as a strong transcriptional enhancer that is active specifically in stimulated B cells and plasma cells (26, 28). Previous studies demonstrated that HS1,2 increased expression of linked transgenes and rendered them LPS-inducible in B lineage cells (50, 51). HS1,2 also contains numerous transcription factor binding sites (52–55), further implicating it in transcriptional regulation or related processes. HS3a, which lies 12 kb upstream of HS1,2 and immediately downstream of the Cα gene, was similarly defined based on ability to enhance reporter gene expression specifically in late-stage B lineage cell lines (31). This activity, combined with the finding that HS3a also contains numerous factor-binding motifs, again led to speculation about its potential roles in Ig HC gene

expression or CSR (31). Our current studies are consistent with the possibility that these enhancers, in particular HS1,2, may have some role in influencing expression (Fig. 4). However, our finding that neither HS1,2 nor HS3a is essential for V(D)J recombination, substantial expression of germline C_H transcripts, or IgH CSR and IgH expression is surprising and necessitates consideration of other potential functions for these elements. One possibility is in the somatic hypermutation process as 3′ IgH region sequences have been implicated by the finding of an increased occurrence of mutations in V_HDJ_H transgenes recombined into the IgH locus (56). The availability of mice containing germline deletions of the individual 3′ IgH enhancers will facilitate searches for more specific functions.

The lack of major effect of the HS3a or HS1,2 deletions on measured processes might result from redundancy. HS3a and/or HS1,2 share potential transcription factor binding sites with HS3b and/or HS4 (36). Therefore, these enhancers may have overlapping or redundant functions, which could obviate major effects of the deletion of a single element. In this context, disruption of the NF-κB p50 gene led to a phenotype reminiscent of the homozygous HS1,2N/N or HS3aN/N phenotype, with impaired CSR to an overlapping set of C_H genes (57). As there are NF-κB–binding sites in both the HS1,2 and HS4 enhancers (58), it is possible that the NF-κB KO phenotype could result, at least in part, by interfering with the function of both enhancers. Enhancer redundancy is found in the Igκ locus, where the intronic and 3′ enhancers appear to have overlapping functions with respect to Igκ gene rearrangement and expression (43, 59). For some processes, the intronic Eμ also may work in conjunction with 3′ IgH sequences, as expression of rearranged V(D)J sequences in iEμ deleted cell lines was suppressed upon replacement of HS1,2 with a pgk-*neo*^{r} gene (30).

Our studies also demonstrate that expression of the Cγ1 and Cα genes are less affected in vitro or in vivo even with respect to the HS1,2N or HS3aN mutations, suggesting that expression of these genes may be positively influenced by control elements not influenced by the 3′ IgH regulatory locus (29). Likewise, the finding that IgG2b levels are suppressed with respect to in vitro LPS stimulation of HS3aN/N and HS1,2N/N B cells but are relatively normal in vivo in the corresponding mice, supports the notion that Cγ2b can be activated in vivo by a novel pathway which is independent of the 3′ IgH regulatory region. The availability of the germline mutant mice will facilitate the search for such novel activating pathways and elements.

Similarities between the 3′ IgH Regulatory Region and the β-Globin 5′LCR. The 3′ IgH regulatory region is quite reminiscent in organization to the LCR region 5′ of the β globin locus which similarly contains four erythroid-specific HS sites that individually harbor distinct transcriptional enhancer activity (60). The β-globin LCR confers tissue-specific, high level, position-independent expression to *cis*-linked genes (61, 62), enhances transcription as far as 70 kb away, and influences chromatin structure and timing of replication over >200 kb (63, 64). Similarly, the combined HS1,2, HS3b, and HS4 sequences induced copy number and position independence to transfected constructs, suggesting the 3′ IgH region also may function as an LCR (32). In another striking parallel to our current findings, replacement of either the HS3 or HS2 enhancers of the β-globin LCR with an expressed selectable marker gene resulted in a severe block in the expression of the linked β-globin locus; but, in both cases, β-globin locus gene expression was substantially restored upon removal of the selectable marker gene (40, 41). Heterozygous deletion of the complete β-globin LCR in human cells of a thallassemia carrier eliminated β-globin gene expression in *cis*, implicating its essential role in β-globin expression and suggesting that the individual enhancer elements are redundant (65). By analogy, more significant effects on CSR or expression may occur upon simultaneous deletion of multiple 3′ enhancer elements (66). As insertion of the pgk-*neo*^{r} cassette into both the 3′ IgH HS3a and HS1,2 locations blocks CSR to the same upstream genes and, in both cases renders the inserted pgk-*neo*^{r} cassette LPS inducible, it seems likely that major distal elements necessary for LPS induction of germline C_H transcription still lie downstream of HS1,2, with HS3b and HS4 being prime candidates.

In addition to the 3′ IgH locus and the β-globin locus, inhibition of expression due to an inserted pgk-*neo*^{r} cassette has been observed in several other loci (41, 43, 59, 67–70). Although the effects of the inserted pgk-*neo*^{r} gene in different loci may not necessarily occur via a single mechanism, a common theme is the potential of complex, long-range mechanisms that have evolved to control expression of developmentally regulated multi-gene loci. The effect of the pgk-*neo*^{r} gene insertion in the β-globin LCR or the 3′ IgH region could be via the inhibition of the neighboring enhancer elements (38, 40, 41); for example, the HS1,2 replacement might inhibit transcription factors binding to the HS3a, HS3b, or HS4 elements, and thereby inhibit all three enhancer elements. However, a more likely scenario is that the pgk-*neo*^{r} gene insertion results in interference with long-range transcriptional control elements involved directly with promoter–LCR interactions (71, 72) or long-range effects on chromatin structure that modulate distal promoter function (73). Recent studies of a transgenic β-globin locus showed that a second β-globin gene competed more efficiently with other genes to which it was LCR-proximal (74), supporting the looping model of LCR function in which one gene interacts with the LCR at a time (75).

Additional insight into the potential mechanisms by which the 3′ IgH regulatory locus may modulate germline CH transcription and/or CSR comes from recent studies of lymphocytes in which the Cε or the Iγ2b exons were replaced with a pgk-*neo*^{r} gene; class switching to CH genes upstream, but not downstream of the pgk-*neo*^{r} insertion was inhibited, again with the exception of switching to IgG1 (Seidl, K., H. Oettgen, and F. Alt, manuscript in preparation). On the other hand, other recent studies of B cells in which the intronic Eμ element was replaced with a pgk-*neo*^{r} cassette showed that the inserted pgk-*neo*^{r} cassette

maintained CSR to downstream CH genes at relatively normal levels even in the absence of Eμ (76). In this case, the expressed pgk-*neo*^{r} cassette may have provided the necessary transcriptional functions to promote CSR at the Sμ region whereas downstream germline transcription units were unaffected by this 5′ insertion. Together, all of these studies are consistent with the notion that the pgk-*neo*^{r} insertions inhibit CSR in a polarized fashion, primarily affecting germline transcription units 5′ to the insertion site. In this regard, the β-globin LCR has been speculated to regulate differential β-globin locus gene expression via a promoter competition mechanism (71, 77). The 3′ IgH regulatory region might employ a similar mechanism to regulate differential expression of C_H genes dependent on this region (29). Whatever the absolute mechanism, the many similarities between the overall organization of the β-globin LCR and the 3′ IgH region suggest that these two loci have evolved similar strategies to regulate differential gene expression.

We thank W. Forrester for critical reading of the manuscript.

This work was supported by the Howard Hughes Medical Institute and National Institutes of Health Grants AI-240047 and AI-31541 (to F.W. Alt) and AI-01285 to (J.P. Manis). N. van der Stoep was supported in part by European Molecular Biology Organization fellowship AltF-300-1994, and M. Tian was supported in part by an Irvington Institute fellowship.

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Received for publication 24 April 1998 and in revised form 28 July 1998.

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