Antibody diversification in B cells is an important component of immune responses against foreign antigens. Ig gene diversity is first created by V(D)J recombination, which is responsible for assembling the primary functional repertoire, and this is followed by gene conversion (GCV) and somatic hypermutation (SHM), processes that further increase sequence diversity at the variable regions of Ig genes.

GCV is achieved by copying patches of sequences from sequence donors into the variable regions, whereas SHM events are nontemplated and exist mainly as single point mutations. Both GCV and SHM are dependent on activation-induced cytidine deaminase (AID) (1–4), which is thought to initiate the reactions by deaminating deoxycytosine residues in Ig genes. The uracils thereby generated are then processed by uracil DNA glycosylase and mismatch repair proteins and channeled into homology-based repair for GCV or by error-prone repair for SHM (5–8).

GCV and SHM are thought to be restricted largely to Ig genes. The molecular basis of this restriction has been the subject of intensive investigation, with cis-acting DNA elements in Ig genes being the prime candidates for providing targeting specificity. Numerous studies indicate a tight link between transcription and SHM, which spans the variable region exon (9–11). Studies using knockout mice and transfected cell lines have shown that no SHM occurs in the absence of an active promoter (12, 13). Additional studies with transgenic mice demonstrated that promoter location dictates the region of SHM, as a newly inserted promoter creates a new window of mutation, and altered positioning of the promoter shifts the mutating region (10, 14). Nonetheless, the endogenous Ig promoters are thought not to be necessary because several non-Ig promoters in different model systems could support SHM when used to replace the Ig promoter (13, 15, 16). Similarly, studies of transgenes in mice and heterologous expression vectors in hypermutating B cell lines indicated that the Ig variable region exon itself is not required for SHM (17–19).

Importantly, experiments performed in cell lines have indicated a positive correlation...
between transcription levels and SHM frequencies (12, 19). These results have led to the conclusion that rather than acting as targeting elements, Ig promoters simply mediate the transcription essential for SHM. However, very few experiments have been performed to examine the role of the promoter in the context of endogenous Ig genes (13, 15).

Another focus of interest has been Ig gene enhancers. Studies using mouse Ig light chain κ (Igκ) transgenes showed that deletion of the intronic or 3′ Igκ enhancer strongly reduced SHM, suggesting an important role of enhancers in SHM (16, 20, 21). However, when the mouse Igκ intronic enhancer, 3′κ enhancer, or Ig heavy chain (IgH) intronic enhancer were deleted in their endogenous context, SHM was not substantially affected (22–24). The discrepancy could reflect redundant functions provided by other cis-acting elements in the endogenous loci or limitations of transgenic studies related to low mutation rates and position effects coming from different integration sites. For its part, deletion of endogenous enhancers from the germline has the potential limitation that epigenetic alterations or selection might take place to compensate for the lack of the enhancer during earlier stages of B cell development. Recently, several reports have indicated positive effects of E2A proteins on AID-dependent diversification processes (25–27). As E2A binding sites are found in Ig enhancers, it is possible that the enhancers play an important role in Ig gene sequence diversification. In investigating such a role, it is important to distinguish a putative targeting activity of enhancers from their ability to facilitate transcription, because stronger transcription can itself contribute to higher levels of SHM.

To investigate the molecular mechanisms of targeting in Ig sequence diversification, we analyzed two regions in the Ig light chain (IgL) locus of DT40 cells, the promoter and the enhancer. DT40 is a chicken B cell line that undergoes continuous GCV and SHM in its IgL and IgH loci, and it performs homologous recombination efficiently, thereby enabling manipulation of DNA elements in their endogenous context (28–30). Deletion of the single known IgL enhancer element while maintaining high levels of transcription showed that the enhancer was not essential for GCV/SHM. Analysis of the promoter region revealed that an active promoter is crucial for GCV/SHM and that the endogenous promoter could befunctionally replaced by a strong heterologous promoter. Surprisingly, we found that the human elongation factor 1-α (EF1-α) promoter supported GCV/SHM poorly, even though it drove transcription efficiently. This is the first time a strong non-Ig promoter has been shown to have a substantial defect in supporting Ig sequence diversification in an endogenous Ig locus, which argues that the function of the promoter in GCV/SHM must extend beyond simply driving transcription. Overall, our results indicate that cis-acting elements contribute to the targeting efficiency of Ig gene diversification and that high-level transcription in an Ig locus is not sufficient for efficient GCV/SHM.

RESULTS

Generation of IgL promoter-substituted clones

There is a well-established link between transcription and SHM in mammalian B cells, but this issue has not been investigated for GCV/SHM in chicken B cells. To address this, we replaced the endogenous IgL promoter in DT40 cells with three heterologous promoters: the bacteriophage T7 promoter, which should not be able to initiate transcription in eukaryotic cells, and two strong Pol II promoters, the chicken β-actin and the human EF1-α promoter. The IgL promoter region that was replaced extended from 206 bp upstream to 6 bp downstream of the transcription start site and contained the octamer binding motif and TATA-box essential for IgL promoter function (31, 32). The CL18 clone of DT40 cells was transfected with targeting constructs containing different promoters and seeded out at limiting dilution, and stable transfectants were identified after 6–8 d (see Materials and methods; Fig. 1 A) (30). Southern blots were performed to distinguish clones in which targeted integration of the constructs into the rearranged IgL allele had occurred (targeted integrants) from those in which random integration into other parts of the genome had taken place (random integrants). At least two independent targeted integrants were selected for subsequent analysis and treated with recombinant Cre protein to remove the puromycin selection cassette inserted upstream of the promoter as a result of targeted integration (Fig. 1 A). Cre-treated cells were single-cell seeded again, and clones that grew out were verified for successful Cre-mediated deletion by Southern blots (Fig. 1 B). Overall, the targeting efficiency of our promoter-swapping constructs was ~45%, and the efficiency of Cre-mediated deletion was ~90% (not depicted).

IgL expression driven by the new promoters was measured by Northern blots. As expected, no transcription was detected from the T7 promoter (T7 cells), although removal of the normal V promoter appeared to activate cryptic promoters that generated low-level transcription initiating at the 3′ end of the V segment (not depicted). Conversely, both β-actin and EF1-α promoter-substituted cells (βA and EF cells, respectively) expressed the IgL locus at levels higher than the endogenous promoter (Fig. 1 C and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20061835/DC1).

The β-actin promoter supports GCV/SHM

Two established assays were used to measure levels of GCV/SHM. First, direct sequencing was performed on cloned PCR products of the 500-bp region downstream of the IgL transcription start site from genomic DNA of cells cultured for 28 d. Second, IgM reversion assays were performed, taking advantage of a frameshift mutation in the IgL variable region of CL18-derivative clones that can be corrected by certain GCV events to generate IgM+ cells (30). As only a few pseudogenes are able to correct the frameshift mutation and each culture begins as a single cell, the percentages of IgM+ cells in different clones of the same genotype exhibited considerable fluctuation. Consequently, multiple
clones of a given genotype were analyzed, and the median of each group was used for comparison. In addition, successive rounds of subcloning were performed to ensure that GCV/SHM phenotypes were stable (cells that have undergone different numbers of subclonings will be referred to as belonging to different generations). Subcloning was performed when cells had been grown for 3 wk since they were last subcloned. Cells were maintained for a total of 4 wk for each round of subcloning, during which IgM reversion frequencies were assessed; at the end of this time, DNA was prepared for sequencing.

After 4 wk of culture, a significant percentage of βA cells had become surface IgM+ (sIgM+) compared with AID−/− controls (combined dataset from three generations of βA cells; P ≤ 0.0001; Fig. 2 A) (2). When the IgM reversion results were separated based on the number of subclonings the
cells had gone through, no difference was found between different generations, indicating that the GCV/SHM phenotype was stable over time (Fig. 2 B). Moreover, weekly examination of the cells showed that the percentages of IgM+ cells increased over time (Fig. 2 C shows median values; data for all clones are shown in Fig. S2 B, available at http://www.jem.org/cgi/content/full/jem.20061835/DC1), confirming the ability of the β-actin promoter to support GCV in the IgL locus. Although we noted a slowing of accumulation of sIgM+ cells in the βA cell cultures between days 21 and 28 as compared with between days 14 and 21, the data from successive subclonings of these cells indicate no loss of GCV/SHM activity with the age of the cells (Fig. 2 B).

Sequence analysis corroborated the findings from the IgM reversion assays. At day 28, we found 43 total events (long-track GCV, templated mutations, and nontemplated events combined) out of 274 sequences analyzed for the βA cells (combined dataset over three generations; Table I). In comparison, T7 cells generated no diversification events in the 138 sequences obtained (βA vs. T7 cells; P ≤ 0.0001). These results indicate that GCV/SHM in DT40 cells relies on a functional promoter and that the endogenous promoter is not essential.

Manipulation of the IgL locus around the promoter interferes with optimal GCV/SHM

Although the βA cells were able to perform GCV/SHM, we noticed that they did so only half as efficiently as the wild-type CL18 cells (WT cells; Fig. 2, A–C; and Table I). This was not an artifact resulting from transfection, subcloning, selection, or phenotypic fluctuation of the cells, as random-integrant clones (unaltered in the IgL locus) derived from transfections of different targeting constructs performed GCV/SHM in the endogenous IgL locus at levels comparable to those of untransfected CL18 cells (not depicted). Sequencing and IgM reversion results from random integrants and untransfected CL18 cells have thus been pooled together as the wild-type dataset.

We therefore considered the possibility that the promoter-exchange scheme altered the IgL locus in a manner that impaired GCV/SHM. To address this, a targeting vector similar to that used to generate βA and EF cells was created to replace the IgL promoter with an exact copy of itself. We expected that cells arising from targeted integration of this construct (L cells, derived from one parental clone) would be identical to WT cells, except that they would have undergone the same recombination events in the IgL locus as other promoter-substituted cells and retained a loxP site upstream of the promoter. Northern blots demonstrated that IgL expression was not affected by this synonymous substitution (compare WT with L; Fig. 1 C).

Interestingly, L cells exhibited a twofold reduction in IgM reversion relative to WT cells (P = 0.0016; Fig. 2 A and Fig. S2 C), indicating that the promoter replacement scheme reduced the efficiency of GCV/SHM. Furthermore, comparison of βA and L cells (Fig. 2 A) indicates that the β-actin promoter supports the same frequency of GCV/SHM as the normal IgL promoter, and that the reduction in GCV/SHM seen in βA cells relative to WT cells was most likely caused by locus manipulations (see Discussion).

The EF1-α promoter is defective in supporting GCV/SHM

To our surprise, the EF1-α promoter yielded results quite different from those of the β-actin promoter. EF cells generated very few sIgM+ cells over the course of 28 d (three- to fourfold less than L cells; Fig. 2 A). This was a stable phenotype, observed in three successive generations of EF cells (Fig. 2 B). When the medians of the percentages of IgM+ cells...
We next tested whether the IgL locus is silenced specifically for GCV/SHM. Though the defect in GCV/SHM of EF cells as assayed by IgM reversion analysis also confirmed the GCV/SHM frequencies in EF-L cells were similar to those seen in WT and L cells (Fig. 1 C). Deletion of the IgL enhancer construct into WT and BA cells were screened by Southern blots to identify targeted integrants (Fig. 3 B), and for each at least two independent clones were selected for further characterization. As a control, a cell line in which the enhancer was retargeted into the rearranged IgL allele of CL18 cells was also generated (E cells); the only genotypic difference between E cells and CL18 cells should be an 80-bp fragment (Fig. 2 A and Table I), consistent with observations in L cells and demonstrating again the effect of the targeting scheme on GCV/SHM.

### Deletion of the IgL enhancer

The only known transcriptional enhancer in the chicken IgL locus was identified in a transgenic mouse study as a 1.7-kb fragment 2 kb downstream of the constant region (33). Transient transfection assays were subsequently used to localize the enhancer to a ~500-bp region (32). To test the involvement of the enhancer in IgL GCV/SHM, this region was deleted in CL18 and BA cells (see Materials and methods; Fig. 3 A). Deletion of the IgL enhancer in the BA cells was of particular interest, because we anticipated that transcription driven by the BA–actin promoter would be minimally dependent on the enhancer, thereby allowing us to identify potential roles of the enhancer in GCV/SHM beyond facilitation of transcription. Stable transfectants of the enhancer deletion construct into WT and BA cells were screened by Southern blots to identify targeted integrants (Fig. 3 B), and for each at least two independent clones were selected for further characterization. As a control, a cell line in which the enhancer was retargeted into the rearranged IgL allele of CL18 cells was also generated (E cells); the only genotypic difference between E cells and CL18 cells should be an 80-bp fragment.
including the loxP site inserted immediately upstream of the enhancer. Targeted integration efficiency of the enhancer deletion construct was ~24% (not depicted). Clones were subsequently treated with Cre protein and seeded out as described in Generation of IgL promoter..., and deletion of the puromycin selection cassette was verified by Southern blotting (Fig. 3 B).

Northern blotting demonstrated that deletion of the enhancer in the context of the wild-type promoter (∆E cells) reduced transcription twofold compared with WT cells, whereas deletion in the context of the β-actin promoter (βA∆E cells) reduced transcription by only 15% compared with βA cells, with transcription levels remaining 2.6-fold higher than in WT cells (Fig. 1 C). These results indicate that the enhancer only contributes moderately to transcription in the DT40 IgL locus. In the E cells, IgL expression was unaltered compared with WT cells (Fig. 1 C), showing that synonymous replacement of the enhancer had no measurable effect on IgL transcription.

The IgL enhancer is dispensable for GCV/SHM
We analyzed the ∆E, βA∆E, and E cells for IgL GCV/SHM and included the same subcloning procedure as described in The β-actin promoter... to test the stability of GCV/SHM phenotypes. Sequencing results from two generations of ∆E cells indicated that GCV/SHM levels were reduced by almost fivefold compared with WT cells (Table I). IgM reversion assays showed reductions of about four- and twofold relative to WT and E cells, respectively (two generations of cells analyzed; Fig. 2 A, Fig. 3 C, and Fig. S2 E). Both assays indicated that some GCV/SHM activity remained in the absence of the enhancer. In addition, much of the decrease in GCV/SHM observed in ∆E cells compared with E cells was likely caused by the twofold decrease in IgL transcription caused by deletion of the enhancer. In the βA∆E cells, IgL transcription was substantially stronger than in WT or L cells (Fig. 1 C); thus, effects on GCV/SHM are unlikely to be results of reduced transcription. Analysis of three generations of βA∆E cells demonstrated that they were able to produce...
some sIgM+ cells, confirming the finding from ΔE cells that the enhancer is not essential for GCV/SHM. Nevertheless, the median was reduced by 30% compared with βA cells (P ≤ 0.0001; Fig. 2 A, Fig. 3 C, and Fig. S2 F). Interestingly, by IgM reversion assay, E cells also showed a ∼30% reduction in levels of GCV/SHM compared with WT cells (Fig. 2 A), which was similar in magnitude to the reduction observed in βAΔE cells compared with βA cells. This suggests that the IgL locus manipulations that accompanied enhancer deletion cause a measurable reduction in GCV/SHM and that the drop in GCV/SHM activity seen in βAΔE cells compared with βA cells was the result of the manipulations and not the absence of the enhancer sequences. This was supported by sequencing analyses of two generations of βAΔE cells, as the GCV/SHM levels were indistinguishable from that observed in βA cells (Table I). Collectively, our results indicate that the IgL enhancer is not required for GCV/SHM of the locus and that when transcription is driven at high levels, the contribution of the enhancer to GCV/SHM is quite modest.

**DISCUSSION**

In this study, the roles of two cis-acting elements in GCV/SHM of the chicken DT40 IgL locus were investigated: the promoter and the enhancer. Replacement of the endogenous IgL promoter with the inactive bacteriophage T7 promoter led to complete abrogation of GCV/SHM, indicating an essential role for an active promoter in chicken Ig gene diversification. Two other non-Ig promoters were also tested for their ability to substitute functionally for the IgL promoter. Unexpectedly, the EF1-α promoter supported GCV/SHM poorly despite driving IgL expression at levels 1.5-fold higher than that obtained with the endogenous IgL promoter. Given the proposal that a positive correlation exists between transcription levels and Ig gene diversification frequencies (12, 19), the GCV/SHM defect in EF cells could be as much as sixfold compared with L and EF-L cells. To our knowledge, this is the first time a strong promoter has been shown to have a significant defect in supporting SHM or GCV of an endogenous Ig gene. These results indicate, in contrast to currently prevailing models, that there are roles of the promoter in GCV/SHM beyond driving high levels of transcription.

Although the β-actin promoter was able to substitute for the endogenous IgL promoter to support GCV/SHM, it did so at levels similar to those supported by the endogenous IgL promoter (L and EF-L cells), despite driving more than threefold higher steady-state IgL transcript levels than the endogenous promoter. That a higher GCV/SHM activity was not observed in the βA cells could reflect saturating levels of transcription driven by the endogenous IgL promoter for GCV/SHM. Alternatively, the β-actin promoter, like the EF1-α promoter, could be suboptimal for recruitment of AID or other aspects of GCV/SHM, which in turn would suggest that the endogenous IgL promoter has features that render it particularly efficient in mediating GCV/SHM. A third possibility is that the positive correlation between transcription and diversification levels inferred from studies of transgenes and artificial SHM substrates does not apply to the endogenous Ig loci. Our experiments only assessed steady-state mRNA levels, but because the transcribed region is virtually identical in all of the cell lines examined in this paper, the results are likely to reflect relative transcription rates as well.

Most previous analyses of the role of promoters in SHM have been performed using transgenes in mice and cell lines, and the ability of non-Ig promoters to support SHM has led to the proposal that endogenous Ig promoters are entirely replaceable (12, 16, 18, 19, 34, 35). These results should be interpreted cautiously, because transgenes typically mutate at substantially lower frequencies than endogenous Ig genes and are subject to integration site effects. In the two previous studies in which an endogenous Ig promoter was manipulated (13, 15), mutation frequencies were reduced but so was transcription. In our study, targeted manipulation of the endogenous IgL promoter yielded loci with high-level transcription, which revealed previously unappreciated roles of the promoter region in the targeting of GCV/SHM.

Cell line–based studies, as well as bacterial and in vitro assays, have led to the model that transcription is important for generating single-stranded DNA suitable for AID deamination (12, 36, 37), implying that stronger promoter activity translates into more AID substrates being available, thereby leading to higher levels of mutation. There is also evidence to indicate that AID interacts directly with RNA polymerases, supporting the idea that AID could traverse the transcribed region with the transcription elongation complexes (14, 38, 39). Though our results do not contradict the role of transcription in such models, they demonstrate that an active promoter is important for GCV/SHM for reasons beyond driving high levels of transcription. One possibility is that the EF1-α promoter fails to recruit certain transcription factors to its transcription initiation or elongation complexes that are important for AID recruitment or for locus interactions involved in GCV/SHM.

Our experiments also revealed that the targeted integration scheme used interfered with GCV/SHM. After targeted integration of the designated DNA elements and Cre-mediated deletion of the selection cassette, 59- and 80-bp regions including the loxP site are left in the IgL locus immediately upstream of the promoter and in place of the enhancer with the respective targeting constructs (Fig. 1 A and Fig. 3 A). These insertions might interrupt DNA elements needed for optimal GCV/SHM. Alternatively, sequences within the insertions or the targeted integration process itself could have interfered with GCV/SHM.

A recent study reported that deletion of the enhancer in the DT40 IgL locus leads to a 1,000-fold reduction in IgL transcription (25). However, additional Southern blot analyses of the single enhancer-deleted clone generated by Conlon and Meyer indicated that Cre-mediated removal of the selection cassette integrated at the site of the deleted enhancer was unsuccessful (not depicted; Meyer, K.B., personal...
Although the reduction in GCV/SHM of ∆E cells could be due to the proposal that E2A proteins facilitate GCV/SHM gene substantially reduces GCV/SHM in DT40 (27), leading to the targeting specificity of GCV/SHM is established through interactions between multiple cis-acting elements, including the promoter, organized into higher order structures through interactions to the enhancer deletion construct contained homology arms 2.2 kb upstream and downstream of the enhancer flanking a Cre-removable puromycin selection cassette. The arms were PCR-amplified from DT40 genomic DNA (upstream arm: CLEUF2-5’-ggggeccataccacatcttg-3’; CLEUPR-3’-gctgttaattgcctgtctc-3’; downstream arm: CLEDFH-3’-ccacgataagcttggcgcac-3’; CELDXR-3’-gattacttcggagagac-3’). The targeting construct for reinsertion of the IgL enhancer contained a different downstream homology arm (CLE-HF-3’-cttcggcagataagcttggcgcac-3’; CLEDXR).
and downstream of it. All other changes were classified as nontemplated events. Sequence diversification in IgH was not subcategorized, because the IgH 4′V gene database is incomplete. Instead, the percentages of sequences that contained at least one nucleotide change relative to the germline sequence were calculated and used for comparisons.

**Statistics.** Two-sample t tests were used to determine the statistical significance of differences in IgM reversion levels and mutation frequencies by sequencing using Data Desk software (version 6.2; Data Description, Inc.). χ² analyses were performed to determine if the distribution of GCV/SHM events in different cell types were statistically significant.

**Online supplemental material.** Fig. S1 is a Northern blot analysis of IgL expression in promoter-substituted cells. Fig. S2 is a time course analysis of IgM expression in individual subclones of various promoter-substituted cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20061835/DC1.

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