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Cutting Edge: A cis-Acting DNA Element Targets AID-Mediated Sequence Diversification to the Chicken Ig Light Chain Gene Locus

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Somatic hypermutation and gene conversion are two closely related processes that increase the diversity of the primary Ig repertoire. Both processes are initiated by the activation-induced cytidine deaminase that converts cytosine residues to uracils in a transcription-dependent manner; these lesions are subsequently fixed in the genome by direct replication and error-prone DNA repair. Two alternative mechanisms were proposed to explain why this mutagenic activity is targeted almost exclusively to Ig loci: 1) specific cis-acting DNA sequences; or 2) very high levels of Ig gene transcription. In this study we now identify a novel 3’ regulatory region in the chicken Ig light chain gene containing not only a classical transcriptional enhancer but also cis-acting DNA elements essential for targeting activation-induced cytidine deaminase-mediated sequence diversification to this locus. The Journal of Immunology, 2008, 180: 2019–2023.

During B cell development, functional Ig genes are assembled from individual gene segments by V(D)J recombination. Subsequently, B cells use distinct cellular programs, in particular somatic hypermutation (SHM)1 and gene conversion (GCV), to actively mutate the DNA of their Ig genes to improve Ag recognition. Both processes, as well as the related class switch recombination, share an essential initiating factor, the activation-induced cytidine deaminase (AID) (1–4). This protein acts on individual cytosine residues within ssDNA that is transiently generated during transcription of the Ig genes (reviewed in Refs. 5 and 6). The resulting U:G mismatch gets resolved by direct replication and DNA repair mechanisms involving uracil DNA glycosylase, general mismatch repair factors, and error-prone DNA polymerases (reviewed in Refs. 6 and 7). While SHM creates individual “nontemplated” point mutations and occurs in all jawed vertebrates, GCV introduces single or multiple “templated” nucleotide changes and has only been observed in selected vertebrates, including chicken and rabbits. Although the end products differ, it is thought that the key molecular principles that govern their AID-dependent initiation phase are the same (reviewed in Refs. 5 and 6).

SHM and GCV are highly limited to Ig genes, but at very low frequencies non-Ig genes are also targeted and such events, as in the case of Bcl6, can contribute to B cell lymphoma formation (reviewed in Ref. 8). The molecular principles enforcing this tight restriction are unknown. Transgenic mice harboring murine Igκ mini loci suggested that the regulatory DNA elements controlling Igκ transcription, the κ intronic enhancer (iEκ) and the 3′κ enhancer (3′Eκ), serve a role in the targeting of SHM (9–11). However, neither of them was found to be essential for SHM when they were deleted individually from the endogenous Igκ locus (12). Hence, these and other approaches to identify cis-acting DNA elements (also referred to as targeting elements) recruiting AID-dependent mutation activities to Ig loci have yet to yield definitive results (reviewed in Ref. 13). Early studies in mouse models indicated that any active promoter is able to support SHM of Ig genes (9, 14, 15). The observation that AID was able to mutate highly transcribed non-Ig transgenes in AID-expressing cell lines contributed to the alternative hypothesis that the high levels of transcription themselves are the major determinants for rendering a gene to be subjected to SHM (reviewed in Ref. 13). Recent findings in chicken DT40 cells, however, indicated that not only the high activity of promoters driving Ig gene transcription but also the very nature of such promoters themselves contribute significantly to the rate of GCV/SHM (16). To resolve the controversy between the two models, we sought to identify cis-acting DNA elements that target GCV to the Ig light chain (IgL) locus in chicken DT40 cells. Using a systematic deletion strategy, we identified a novel regulatory region downstream of the only known Igκ enhancer. Importantly, this region not only promoted high levels of transcription but harbors a cis-acting DNA element that targets GCV and SHM to the IgL locus.

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3 Abbreviations used in this paper: SHM, somatic hypermutation; AID, activation induced cytidine deaminase; GCV, gene conversion; IgH, Ig heavy chain; IgL, Ig light chain; 3′RR, 3′ regulatory region; MAR, matrix attachment region.
genomic DNA, gel purified, and body labeled with microfarads, and infinite resistance. Stable transfectants were selected with 0.5 NLS is "nuclear localization signal”) as described previously (16) and

The IgL locus components of all targeting constructs were generated by PCR amplification of respective genomic DNA fragments using Phusion polymerase (New England Biolabs). Briefly, left arm (upstream) and right arm (downstream) fragments were cloned into the NotI and XhoI sites of pBluescriptSK+ (Stratagene) with a central BamHI site between the arms. The puromycin cassette of pLoxPuro (17) was inserted as a BamHI fragment to obtain the final MES construct (see Fig. 1 and legend), the SV40 enhancer was amplified from pECFP-1 and ligated as a BamHI/BamHI fragment into the central BamHI site of ΔMES before insertion of the puromycin selection cassette.

Materials and Methods

Oligonucleotides

All primer sequences will be provided upon request.

Targeting constructs

The IgL locus components of all targeting constructs were generated by PCR amplification of respective genomic DNA fragments using Phusion polymerase (New England Biolabs). Briefly, left arm (upstream) and right arm (downstream) fragments were cloned into the NotI and XhoI sites of pBluescriptSK+ (Stratagene) with a central BamHI site between the arms. The puromycin cassette of pLoxPuro (17) was inserted as a BamHI fragment to obtain the final targeting constructs. For the ΔMES construct (see Fig. 1 and legend), the SV40 enhancer was amplified from pECFP-1 and ligated as a BamHI/BamHI fragment into the central BamHI site of ΔMES before insertion of the puromycin selection cassette.

Tissue culture and cell lines

All DT40 CI18 clones were grown at 41°C and 5% CO2 in RPMI 1640 (Mediatech) supplemented with 10% FBS (Invitrogen Life Technologies), 1% chicken serum (Invitrogen Life Technologies), 10 mM HEPES, 2 mM L-glutamine, and penicillin/streptomycin. Transfections were performed using 30 μg of linearized plasmid DNA with a Bio-Rad Gene Pulser at 580 volts, 25 μF. The transfected cells were cultured continuously for 4 wk (corresponding to 84 generations). Surface IgM expression was determined by staining cell aliquots with PE-labeled anti-IgM Ab (Southern Biotechnology), and VJ region sequences were amplified and sequenced from the genomic DNA of unsorted cultures.

Gene conversion assays

Gene conversion and mutation analysis was performed as described previously (16). Briefly, single cell clones were obtained by limiting dilution and cultured continuously for 84 generations without media changes. Surface IgM expression was determined by staining cell aliquots with PE-labeled anti-IgM Ab (Southern Biotechnology), and VJ region sequences were amplified and sequenced from the genomic DNA of unsorted cultures.

Gene expression analysis

Total RNA was isolated using RNAzol (Tel-Test) according to the manufacturer’s protocol, and transcript levels were determined by Northern blotting using probes for the constant region of IgL and GAPDH. Signals were detected using a Storm Phosphorimage (Amersham Biosciences) and quantified using ImageQuant (Amersham Biosciences).

Results and Discussion

To identify and locate cis-acting elements that recruit AID-mediated sequence diversification to the IgL locus, we systematically removed noncoding DNA segments from this locus by gene targeting (Fig. 1). All targeted manipulations of the IgL locus, the initial gene targeting, and the subsequent Cre-mediated removal of the puromycin-resistance cassette were confirmed by Southern blotting (data not shown). The effect of each deletion on GCV was determined by tracking the appearance of IgM+ cells from individual IgM+ single cell clones over 8 wk of culture, and mutation event frequencies were determined by sequencing the IgL gene at the end of this period. To minimize experimental errors, we adhered to the following five precautions for each of our IgL deletion mutants. First, to rule out any effects caused by clone to clone variations, all experiments were performed using two clones that had undergone the desired gene targeting event obtained from independent transfections. Second, puromycin-resistant clones with a random integration of the targeting constructs (random integrants) were cultured in parallel to targeted clones with a random integration of the targeting construct. Third, puromycin-resistant clones with a random integration of the targeting construct were cultured in parallel to targeted clones with a random integration of the targeting construct. All DT40 Cl18 clones were grown at 41°C and 5% CO2 in RPMI 1640 (Mediatech) supplemented with 10% FBS (Invitrogen Life Technologies), 1% chicken serum (Invitrogen Life Technologies), 10 mM HEPES, 2 mM L-glutamine, and penicillin/streptomycin. Transfections were performed using 30 μg of linearized plasmid DNA with a Bio-Rad Gene Pulser at 580 volts, 25 μF. The transfected cells were cultured continuously for 4 wk (corresponding to 84 generations). Surface IgM rever-

FIGURE 1. The structure of the IgL locus in DT40 cells and in derivatives thereof. The leader (L), VJ, and constant (C) region exons are depicted as open boxes, the putative MAR (M) and enhancer (E) as red ovals, and the SV40 enhancer (SV40E) as a purple oval. The position and size of each of these elements are drawn to scale. The polyadenylation site (Poly(A)) is represented by a green circle, and the loxp sites that are retained in the genome after Cre-mediated deletion of the puromycin selection cassette are depicted as filled triangles. The areas that are deleted in the DT40 derivatives are shown in lighter colors (gray and pink, respectively). The location of the newly discovered 3’ regulatory region, 3’RR, is indicated with a set of brackets, and its sequence corresponds to nt113822–117952 in GenBank entry AC171016. WT, Wild type.

FIGURE 2. IgL transcription and mutation frequencies in mutant DT40 cell with deletions in the IgL gene locus. A, Two Northern blots with independently derived RNA samples from each cell clone were hybridized with probes for the IgL gene and GAPDH as a loading control. The signals were quantified, and all values were first normalized to the GAPDH signal in each lane and subsequently normalized to the IgL level in wild-type (WT) DT40 cells. The bars represents the average of four independent experiments (two per genotype) except for ΔMES1.1 and ΔMES34.2, where the average of two values per clone is shown. Sorted IgM− cells were used for the RNA preparation to avoid issues of differential RNA stability due to non-sense-mediated decay. B, The frequency of mutation events (nontemplated and templated combined) was determined by sequencing the VJ region of the IgL gene from two individual clones of each genotype after 4 wk of continuous culture. A minimum of 77 sequences was obtained for each genotype. The dashed line indicates the background level of 5 × 10−3 events/bp for our experimental design as determined by sequencing the IgL gene in AID-deficient DT40 clones.

using a Storm Phosphorimage (Amersham Biosciences) and quantified using ImageQuant (Amersham Biosciences).
The status is determined by FACS and the appearance of IgM surface IgM. Limiting dilution and continuously culturing for 4 wk. Each cell clone starts as mutants. Eight to 12 single cell subclones of each genotype were obtained by gene sequencing, to determine GCV and SHM activity in each clone. Fifth, to rule out the possibility that the Cre-mediated deletion would lead to a transient susceptibility to GCV/SHM of an otherwise protected IgL locus (18), all clones were grown for at least 10 days before the 4-wk culture experiments.

We first deleted the known enhancer (ΔE) and the VJ-C intron (ΔM) containing a predicted matrix attachment region (MAR) (Fig. 1). The absence of the entire VJ-C intron (ΔM), including the MAR, resulted in a 3-fold increase of the steady-state IgL transcripts levels (Fig. 2A), similar to the result of an earlier deletion of only the MAR (19). In contrast, deletion of the 467-bp core IgL enhancer showed a modest reduction in transcription (Fig. 2A). Although one earlier report concluded that the enhancer is essential for IgL transcription (19), our observation is in better agreement with a more recent report showing that this enhancer plays only a minor role in IgL transcription in DT40 cells (16). The frequency of GCV and SHM was not dramatically altered in the ΔM and ΔE cell lines, consistent with previous reports, and small differences in the magnitude of effects are likely to be caused by differences between the targeting construct used (16, 19) (Figs. 2B and 3A).

The robust levels of AID-mediated IgL diversification observed in the individual VJ-C intron and enhancer deletion mutants led us to wonder whether these DNA control elements might function redundantly. There is precedence for such redundancy between the κ intronic enhancer iEκ and the 3′κ enhancer 3′Eκ in mice with respect to their roles in the targeting of SHM to an Igκ transgene itself (10). We created a large deletion (ΔME) that lacks the VJ-C intron, the enhancer, and an additional 2-kb of noncoding DNA located between the polyadenylation signal and the enhancer (Fig. 1). Northern blot analysis revealed that the steady-state level of IgL transcripts was slightly elevated (1.35-fold) compared with that of wild-type DT40 cells (Fig. 2A). Interestingly, the surface IgM reversion assay and the sequencing analysis after 4 wk of continuous culture revealed a modest 2-fold decrease of GCV/SHM rates in both of the ΔME clones (Fig. 2, B and D), suggesting that the deleted sequences might play a minor role in targeting. We concluded that a putative essential targeting sequence did not reside in these gene proximal sequences and that neither the VJ-C intron nor the enhancer is essential for IgL transcription in DT40 cells.

To search for other distal control elements within the chicken IgL locus, we deleted additional noncoding DNA regions downstream of the enhancer. Deletion of an additional 4 kb downstream of the enhancer (ΔM6K; Fig. 1) resulted in the complete absence of IgL transcripts as determined by Northern blotting and RT-PCR (Fig. 2A and data not shown). Because GCV and SHM are strictly dependent on active transcription of the ΔME6K transgene itself (10). We created a large deletion (ΔME) that lacks the VJ-C intron, the enhancer, and an additional 2-kb of noncoding DNA located between the polyadenylation signal and the enhancer (Fig. 1). Northern blot analysis revealed that the steady-state level of IgL transcripts was slightly elevated (1.35-fold) compared with that of wild-type DT40 cells (Fig. 2A). Interestingly, the surface IgM reversion assay and the sequencing analysis after 4 wk of continuous culture revealed a modest 2-fold decrease of GCV/SHM rates in both of the ΔME clones (Fig. 2, B and D), suggesting that the deleted sequences might play a minor role in targeting. We concluded that a putative essential targeting sequence did not reside in these gene proximal sequences and that neither the VJ-C intron nor the enhancer is essential for IgL transcription in DT40 cells.

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the Ig genes, it was not surprising that AID-mediated sequence diversification of IgL was absent in the ΔMES6K clones (Figs. 2B and 3C). This was not caused by a loss of AID activity, as the mutation frequencies in the unaltered Ig heavy chain (IgH) locus of ΔM (6.22 × 10^{-4} events/bp) and ΔMES6K (4.79 × 10^{-4} events/bp) were found to be comparable. We conclude that this 4 kb region (hereafter referred to as the 3'-regulatory region, 3’RR) contains a transcriptional control element that is essential for high levels of IgL gene transcription in DT40 cells.

To determine whether the 3’RR also contained cis-acting DNA elements that target GCV/SHM to this locus, we decided to rescue IgL transcription by replacing the 3’RR with a single copy of the strong SV40 enhancer in the context of the ΔMES6K genotype (ΔMES; Fig. 1). Two such cell clones, ΔMES1.1 and ΔMES34.2, were chosen for further analysis as expected, Northern blot analysis showed that the SV40 enhancer strongly increased the level of steady-state IgL transcripts close to that found in wild-type, ΔE, and ΔME cells (Fig. 2A), all of which showed readily detectable GCV and SHM activity. Strikingly, none of the 20 subclones of ΔMES1.1 and ΔMES34.2 showed any significant increase in the percentage of IgM+ cells (Figs. 4 and 3D). This complete loss of GCV/SHM in the IgL gene was further confirmed by sequence analysis (Fig. 2B). The mutation frequency (0.977 × 10^{-5} events/bp) is dramatically lower (20-fold) than that of ΔME cells and is again below the background level of our experiments. Importantly, mutation frequencies in the unaltered IgH locus in ΔMES (4.26 × 10^{-4} events/bp) remained comparable to that of the parental ΔM lines (6.22 × 10^{-4} events/bp). These observations demonstrate that the 3’RR harbors DNA sequences that are essential for targeting AID-mediated sequence diversification to the chicken IgL locus.

The 3’RR constitutes one of the first cis-acting targeting element within an endogenous Ig locus. While previous studies suggested that high levels of transcription and the promoters themselves are important determinants in licensing Ig genes for AID-mediated sequence diversification (20, 21), our data show that distinct DNA sequences within the Ig loci mark the genes as targets for localized mutagenesis. Future analysis will reveal whether the same elements within the 3’RR are responsible for both enhancing transcription and GCV/SHM or whether these functions are conferred by different sets of sequences. One popular model of how targeting elements work invokes the binding of transcription factors to such DNA elements and the recruitment of AID via protein-protein interactions (reviewed in Ref. 13). Evidence is emerging that E2A could be one such factor, as the deletion/overexpression of E2A in DT40 cells leads to a corresponding decrease/increase of GCV and SHM without significantly affecting Ig transcription (19, 22). Similarly, additional E2A binding sites in murine Igκ transgenes also increase SHM frequencies (23). The 3’RR described here contains five predicted E2A binding sites (data not shown), and additional experiments will reveal whether any of these are important for targeting. It is worth noting, however, that none of the six putative E2A sites within the IgL enhancer is essential for GCV (16). To narrow our search to smaller regions within the 3’RR, we compared this DNA segment with IgL and IgH loci of other vertebrate species, but no highly conserved sequence stretches indicative of conserved elements were found (data not shown).

This is not surprising, as control elements in Ig loci are rapidly evolving at the sequence level while still maintaining their regulatory function (24). Because AID-mediated Ig gene diversification is an evolutionary conserved pathway to generate Ag receptor diversity, we predict that cis-acting targeting elements are present in the Ig loci of all vertebrates, including humans and mice, and that functional complementation assays represent a direct approach to reveal their nature and identity. The identification of the 3’RR now sets the stage toward understanding the molecular mechanism and the protein factors involved that restrict these unique mutagenic processes to distinct genomic loci. It is tempting to speculate that the 3’RR is the member of a novel category of cis-acting elements that are able to control genomic (in)stability on a local scale, e.g., in the context of antigenic variation of pathogens and recombination hot spots.

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Disclosures

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