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Immunoglobulin Gene Conversion: Insights From Bursal B Cells and the DT40 Cell Line

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Chicken B cells diversify their immunoglobulin (Ig) light and heavy chain genes by pseudogene templated gene conversion within the bursa of Fabricius. Although Ig gene conversion was initially believed to occur only in birds, it is now clear that most farm animals also use this elegant mechanism to develop an immunoglobulin gene repertoire. The best model to study Ig gene conversion remains the chicken Ig light chain locus due to its compact size and the fact that all the pseudogene donors are sequenced. Furthermore, gene conversion continues in the bursa-derived DT40 cell line whose genome can be easily modified by targeted integration of transfected constructs. Disruption of the AID gene, which had been shown to control somatic hypermutation and switch recombination in mammals leads to a complete block of gene conversion in DT40 indicating that all B-cell specific repertoire formation is controlled by the same gene. Here, we review the genetics and the molecular mechanism of Ig gene conversion based on sequence analysis of bursal B cells and gene disruption studies in the DT40 cell line. *Developmental Dynamics 229:458–464, 2004.* © 2004 Wiley-Liss, Inc.

Key words: gene conversion; somatic hypermutation; chicken; the bursa of Fabricius; DT40; AID

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INTRODUCTION

Studies in the chicken have made important contributions to our understanding of the immune system. Most notable is the serendipitous discovery that antibody production depends on the bursa of Fabricius (Glick et al., 1956), leading to the general name of B (bursal) cells for antibody-producing cells. Equally important in the context of this review is the finding that chicken B cells create their immunoglobin (Ig) repertoire by gene conversion, rehabilitating an old theory (Smithies, 1967) that was previously rejected for mice and men.

Ig genes are not encoded in a functional form in the germline of all vertebrate species but are assembled from gene segments by site-specific recombination. In humans and mice, functional V, D, and J gene segments exist in large clusters and V(D)J recombination generates combinatorial diversity by the random assortment of individual V, D, and J segments (Hozumi and Tonegawa, 1976). After antigen stimulation, the functionally rearranged V segments are further modified by somatic hypermutation (Jacob et al., 1991) and the Ig isotope can be changed by class switch recombination (Honjo and Kataoka, 1978).

However, most species other than mouse and human use V(D)J recombination only to ensure the assembly and expression of a single functional gene. This was first demonstrated for the chicken where only single functional V and J segments are present in the light and heavy chain gene loci. Diversity is introduced into the rearranged V(D)J segments by gene conversion using pseudo V genes as donors (Reynaud et al., 1987). Not only avian species, but also rabbits, cattle, swine, and horses use gene conversion for B-cell repertoire formation (Butler, 1998).

The bursal B-cell line DT40 continues Ig gene conversion during in vitro culture (Buerstedde et al., 1990). Another unique feature of DT40 is that it integrates transfected gene constructs at high ratios into the endogenous loci (Buerstedde and Takeda, 1991). This high frequency of targeted integration in DT40 is a powerful tool to test the function of candidate genes by gene disruption (Takeda et al., 1992).

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	Ig gene remodelling	organ	ontogeny
pre-bursal stage			
	DH-JH recombination	yolk sac	embryonic day 5-6
	completion of V(D)J recombination	spleen, thymus, blood etc.	embryonic day 7-15
bursal stage			
	gene conversion (high) somatic hypermutation (low)	the bursa of Fabricius	embryonic day 15 - after hatching (until involution of the bursa at 4-6 months after hatching)
post-bursal stage			
	gene conversion (high in early GC stage low in late GC stage) somatic hypermutation (high)	splenic germinal centers (GCs)	after 3 month old

Fig. 1. B-cell repertoire formation in the chicken. Commitment to the B-cell lineage and V(D)J recombination takes place in extra-bursal sites. Prebursal stem cells migrate to the bursa of Fabricius, where they rapidly proliferate and diversify the rearranged immunoglobulin (Ig) genes. Diversity is mainly achieved by gene conversion in the bursa, although a low frequency of somatic hypermutation is also observed. In the adult stage, gene conversion as well as somatic hypermutation contribute to antigen-dependent affinity maturation of Ig within splenic germinal centers.

CHICKEN B-CELL DEVELOPMENT AND REPERTOIRE FORMATION

The bursa of Fabricius plays a central role for chicken B-cell development, which can be classified into prebursal, bursal, and postbursal stages (Fig. 1).

Prebursal Stage

Prebursal stem cells are derived from hematopoietic precursors in the dorsal aorta. The first DH–JH joints in the heavy chain locus can be detected in the yolk sac on embryonic day 5 or 6 (Reynaud et al., 1992). Rearrangement of V(D)J segments is stochastic in the chicken, and frequently VJ recombination in the light chain locus precedes VDJ recombination at the heavy chain locus (Benatar et al., 1992). V(D)J recombination at either locus is virtually complete by embryonic day 15 (Reynaud et al., 1992). Cells that have already completed V(D)J recombination at the heavy and light chain loci, can be detected in the blood, spleen, thymus, and even in nonlymphoid organs. These progenitor populations only expand in the bursa and decline with time at the other sites.

Bursal Stage

The mature bursa of Fabricius is composed of approximately 10,000 lymphoid follicles and presents the major site for B-cell proliferation and repertoire formation. Between embryonic days 10 and 15, each follicle is seeded by oligoclonal B stem cells (Pink et al., 1985). Gene conversion in bursal B cells is initiated around embryonic day 15 even in the absence of environmental antigen (Mansikka et al., 1990). Gene conversion most likely continues until the bursa involutes 4–6 months after hatching. Somatic hypermutation at low frequency also contributes to bursal Ig gene diversification (Arakawa et al., 2002a). Despite its oligoclonal colonization, each bursal follicle develops a highly diverse repertoire due to repeated rounds of gene conversion (Arakawa et al., 2002a).

Postbursal Stage

It had been believed for a long time that the peripheral lymphocytes proliferate by self-renewing amplification of the preimmune repertoire generated in bursa. However, adult chicken generate germinal centers in the spleen in response to antigen challenge. Germinal center formation is clearly detectable by day 7 after antigen stimulation and begins to wane 14 days after immunization. The Ig genes of antigen-activated B cells are diversified both by gene conversion and somatic hypermutation in the very early phase of the germinal center reaction (Arakawa et al., 1996). In the later stage, gene conversion is down-regulated, and most modifications are somatic hypermutations (Arakawa et al., 1998).

GENE CONVERSION IN BURSAL B CELLS

A cluster of pseudo V genes is located upstream of the functional V gene segment in both the light and heavy chain loci. The light chain locus includes 25 pseudo V genes (Reynaud et al., 1987), whereas the heavy chain locus encompasses around 80 pseudogenes similar to V-D joints (Reynaud et al., 1989). All pseudo V genes lack promoter, leader exon, or V(D)J recombination signal sequences. Only a few of the pseudogenes contain stop codons or frameshift mutations, but quite a

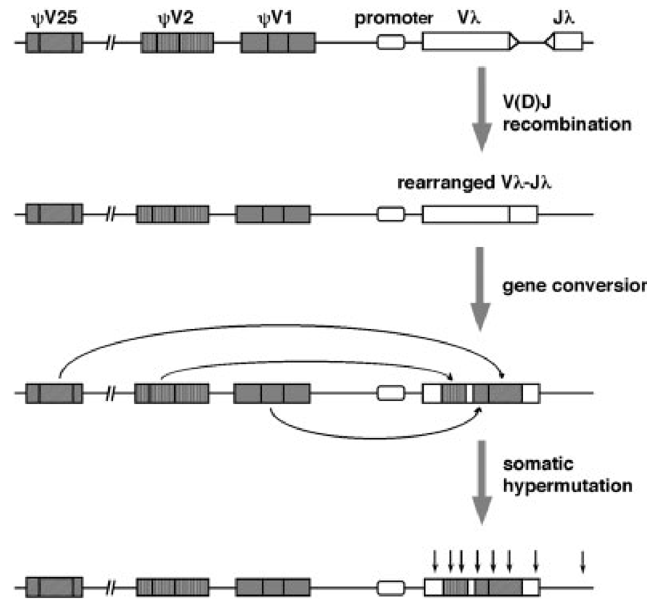


Fig. 2. Scheme of V(D)J recombination, gene conversion, and somatic hypermutation of chicken immunoglobulin light chain gene locus. Chicken has only a single V and J segment in the light chain locus, which can be functionally rearranged by V(D)J recombination. The rearranged VJ segment is diversified by segmental gene conversion using pseudo V genes as donors. Rearranged VJ segments also undergo somatic hypermutation in the bursa and spleen.

few are truncated in their 5′ or 3′ ends.

During B-cell development in the bursa, segments of pseudogene sequences appear in the rearranged V gene segments, but the pseudogene donor sequences do not change (Reynaud et al., 1987; Carlson et al., 1990). The phenomenon, schematically depicted in Figure 2, is called gene conversion in analogy to similar processes in yeast. Only the pseudogenes on the same chromosome are used as donors (Carlson et al., 1990) and pseudogenes that are either more homologous, closer or in the opposite orientation to the rearranged V segment are preferred (McCormack and Thompson, 1990; Sayegh et al., 1999). Conversion tracts range from 8 bp to around 200 bp (McCormack and Thompson, 1990). The 5′ ends of the gene conversion tracts always begin in regions of homology between the pseudogene donor and recipient V segment, whereas the 3′ ends can occur in regions of nonhomology and often encompass nucleotide insertions or deletions (McCormack and Thompson, 1990). These results suggest a 5′ to 3′ polarity in the gene conversion mechanism.

PHYLOGENETIC PREVALENCE OF GENE CONVERSION

The Ig light chain gene loci of quail, mallard duck, pigeon, turkey, cormorant, and hawk undergo a single major rearrangement event (McCormack et al., 1989). These species contain nearby families of V sequences that most likely serve as donors for gene conversions.

Based on the studies in mice, humans, and chicken it was assumed that mammals use V(D)J recombination, whereas birds use gene conversion for Ig repertoire formation (Table 1). It came as a surprise that rabbits, cattle, swine, and horses all develop their antibody repertoire by rearrangement of a relatively small number of V segments with limited diversity due to V(D)J recombination. It is now clear that these animals use gene conversion in combination with somatic hypermutation for repertoire development (Table 1; Butler, 1998).

Ig gene diversification occurs predominantly in the ileal Peyer's patches of cattle, swine, and horses and in the appendix of rabbits. These gut-associated lymphoid tissues—composed of thousands of lymphoid follicles until they involute some time after birth—can be considered the mammalian equivalent of the bursa of Fabricius. Ig gene conversion in birds, rabbit, and ruminants suggests that this strategy of repertoire development evolved a long time ago in a common ancestor of avians and mammals and was only recently abandoned in mice and men.

GENE CONVERSION IN THE BURSA-DERIVED B CELL LINE DT40

The ALV-induced lymphoma line DT40 seems to be arrested at the stage of bursal B cells and continues Ig light chain gene conversion during in vitro cell culture (Buerstedde et al., 1990; Kim et al., 1990). Although wild-type DT40 cells are dominantly surface immunoglobulin positive (sIg(+)), spontaneously arising sIg(−) subclones can be isolated, which contain frameshifts in the rearranged light chain V segment. Overlapping gene conversion events can repair these frameshifts leading to re-expression of sIg (Buerstedde et al., 1990). This reversion from sIg(−) status to sIg(+) status can be used to quantify gene conversion efficiency (Ig reversion assay).

DT40 possesses another homologous recombination activity as it integrates transfected gene constructs targeted into virtually any gene loci at high ratios (Buerstedde et al., 1991). Even transcriptional activity of a gene locus is not required for targeted integration. This high efficiency of targeted integration is present in other chicken B-cell lines, which do not continue Ig gene conversion (Buerstedde and Takeda, 1991). This finding suggests that the targeted integration activity is needed for Ig gene conversion, but in itself is not sufficient to support it. In contrast to chicken B-cell lines, high frequencies of targeted integration were not detected in chicken non–B-cell lines or B-cell lines of human or murine origin.

SUPPORTING ROLE OF THE RAD52 PATHWAY

Gene conversion and targeted integration in the yeast *S. cerevisiae* depends on the RAD52 pathway,

TABLE 1. Ig Gene Diversification System of Mammalian Species and Chicken

	Primary B cell repertoire formation	
	Mechanism	Organ
Human	V(D)J recombination	Bone marrow
Mouse	V(D)J recombination	Bone marrow
Rabbit	Gene conversion and somatic hypermutation	Appendix
Cattle	Gene conversion and somatic hypermutation	Illeal Peyer's partches
Swine	Gene conversion and somatic hypermutation	Illeal Peyer's partches
Horse	Gene conversion and somatic hypermutation	Illeal Peyer's partches
Chicken	Gene conversion and somatic hypermutation	The bursa of Fabricius

TABLE 2. DT40 Mutants Related to Gene Conversion and Somatic Hypermutation

	Enzyme activity	DT40 mutant phenotype	References
RAG-2	V(D)J recombinase	No defect in gene conversion	Takeda et al., 1992
RAD51	Homologous recombination	Lethal	Sonoda et al., 1998
RAD52	Homologous recombination	Slight reduction of targeted integration	Yamaguchi-Iwai et al., 1998
RAD54	Homologous recombination	Reduction of gene conversion Reduction of targeted integration Hypersensitivity to DNA damaging reagent	Bezzubova et al., 1997
NBS1	Homologous recombination	Reduction of gene conversion Reduction of targeted integration	Tauchi et al., 2002
AID	Cytidine deaminase	No gene conversion activity	Arakawa et al., 2002
RAD51-paralogues (XRCC2, XRCC3, RAD51B)	Homologous recombination	Shift from gene conversion to somatic hypermutation	Sale et al., 2001
UNG	Uracil DNA glycosilase (base excision repair)	Shift of hypermutation pattern from transversion to transition	Di Noia and Neuberger, 2002
REV1	Deoxycytidyl transferase	Reduction of somatic hypermutation	Simpson and Sale, 2003

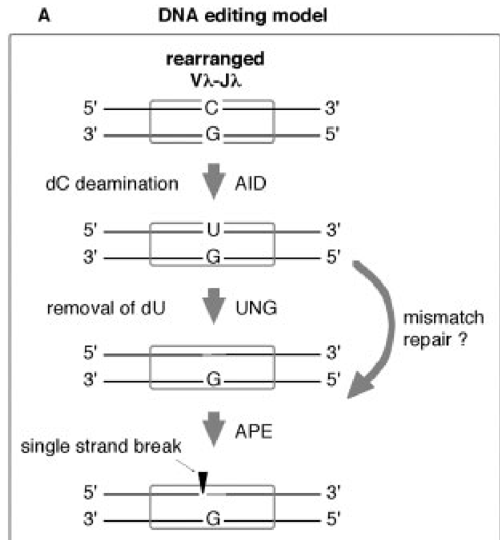
which mediates double-strand break (DSB) repair by homologous recombination. Genes belonging to the RAD52 pathway encode proteins that function either in the recognition of the double-strand break (RAD50, MRE11, and XRS2) or in the promotion of homology search and strand invasion (RAD51, RAD52, RAD54, RAD55, RAD57). The Rad51, Rad55, and Rad57 proteins are structural homologues of the bacterial recA protein.

Homologues of the RAD51, RAD52, and RAD54 genes were first cloned from chicken bursal cells by reverse PCR using degenerate primers derived for conservative sequence motifs of the yeast proteins. Gene disruptions in DT40 revealed that RAD51 deficient cells do not survive, most likely because they cannot repair replication-induced DSBs (Table 2; Sonoda et al., 1998). In contrast to the severe recombination and repair defect of the *S. cereviseae* RAD52 mutants, the DT40 RAD52-deficient cells have only modestly reduced targeted integration frequencies and are not hypersensitive to DNA damage (Yamaguchi-Iwai et al., 1998). However, RAD54-deficient DT40 cells are highly X-ray sensitive compared with wild-type cells, have 100-fold decreased targeted integration ratios, and also show reduced Ig light chain gene conversion activity (Bezzubova et al., 1997). The disruption of the NBS1 gene, which is the counterpart of the yeast XRS2 gene, produces a phenotype very similar to the RAD54 phenotype in DT40 (Tauchi et al., 2002). Vertebrates have five RAD51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) and DT40 mutants of each of these genes have reduced targeted integration frequencies and DSB repair deficiencies (Takata et al., 2001). Of interest, some of the functions of the XRCC3 may be compensated by RAD52, because inactivation of XRCC3 in cells lacking RAD52 results in chromosomal instability and cell death (Fujimori et al., 2001).

The studies of the RAD52 pathway in DT40 prove that DSB repair is well conserved during eukaryotic evolution and that targeted integration in vertebrate cells is a side effect of DSB repair (Table 2). The high ratios of targeted integration in DT40 most likely reflects the up-regulation of the RAD52 pathway in bursal B cells compared with other chicken and mammalian cells. This increased general homologous recombination activation is required for efficient Ig gene conversion, but it cannot explain how gene conversion events are specifically initiated in the Ig loci.

THE LEAD ACTOR AID

AID was identified as a novel gene that is specifically expressed in class



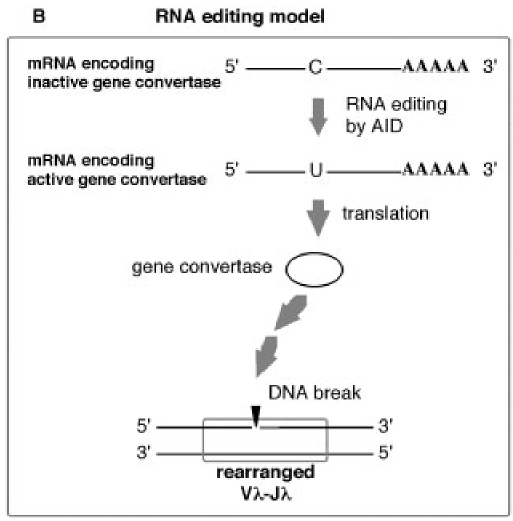


Fig. 3. DNA editing and RNA editing models of AID action. AID most likely initiates Ig gene conversion by introducing a DNA alteration in the rearranged V segment. In the DNA editing model, AID changes C to U by DNA deamination. A single-strand break is then generated after uracil base excision by uracil DNA glycosylase (UNG) and APE endonuclease or through a mismatch repair reaction. In the RNA editing model, AID edits a codon in a mRNA, which encodes a DNA-modifying enzyme. This change leads to the translation of an active protein that then introduces a DNA alteration in the rearranged V segment.

switch recombination active cells and shares homology with RNA editing enzyme APOBEC (Muramatsu et al., 1999). AID was later shown to be essential for somatic hypermutation and class switch recombination in the mouse (Muramatsu et al., 2000) and the human (Revy et al., 2000). Disruption of the chicken AID in DT40 completely blocks Ig gene conversion, and this block can be complemented by AID over expression (Arakawa et al., 2002b). This finding indicates that the AID master gene controls all B-cell–specific modifications of vertebrate Ig genes.

It is likely that AID induces a change in the DNA structure within the Ig locus action, which leads to Ig gene conversion in bursal B cells. AID-deficient DT40 cells still maintain high targeted integration activity, and there is no increased sensitivity to DNA-damaging reagents (unpublished results). It is, therefore, likely that the action of AID is specifically targeted to the Ig loci.

At the moment, two alternative models are proposed to explain the induction of recombination and hypermutation by AID. According to the first model AID catalyzes cytosine deamination leading to guanine/uracil (G/U) mismatches (DNA editing model, Fig. 3A), whereas in the second model AID edits an mRNA that encodes the DNA-modifying activity (RNA editing model, Fig. 3B). In both models, AID changes the DNA conformation at the Ig loci and it remains an open question how further processing leads to gene conversion, class switch or somatic hypermutation.

RELATIONSHIP TO SOMATIC HYPERMUTATION

DT40 clones deficient in either one of the RAD51 paralogues, XRCC2, XRCC3, or RAD51B accumulate single-point mutations in the rearranged V segment, which cannot be accounted for by known pseudogenes. Like in mammalian cell lines with ongoing somatic hypermutation activity (Bachl and Wabl, 1996; Faili et al., 2002; Martin et al., 2002), these mutations occur mainly at G/C base pairs and have a preference for known in vivo hotspots of somatic hypermutation (Sale et al., 2001). Although Ig gene conversion is only reduced in the mutants, the results indicate that impairment of homologous recombination can activate somatic hypermutation in Ig gene conversion active cells. Because somatic hypermutation in activated germinal center B cells occurs at A/T and G/C base pairs with comparable frequencies, hypermutating B-cell lines seem to miss the mechanism that mutates A/T base.

The exciting discovery of somatic hypermutation in mutants of the RAD51 paralogues triggered further studies about the hypermutation mechanism in DT40. It could be demonstrated that transfection of a dominant uracil DNA glycosylase inhibitor changes the balance of V gene mutations from transversions to transitions (Di Noia and Neuberger, 2002). This finding is strong support of the cytosine deamination model of AID which predicts cytosine (C) to thymine (T) transition mutations, if the excision of the uracil in the AID-induced G/U mismatch is blocked. It was also recently shown that disruption of the deoxycytidyl transferase Rev1p, which forms a complex with the subunits of DNA polymerase zeta, is reduces hypermutation in DT40 (Simpson and Sale, 2003), suggesting that polymerase zeta participates as an error-prone polymerase.

MODEL OF THE Ig GENE CONVERSION MECHANISM

A current model for Ig gene conversion needs to combine the early sequencing data from bursal B cells,

the knock-out studies in DT40, and the mechanism of AID action. The genes that affect Ig gene conversion can be grouped into three classes based on their disruption phenotype (Table 2): (1) AID, which is essential for Ig gene conversion; (2) the RAD54 and NBS1 genes, which facilitate Ig gene conversion; and (3) the RAD51 paralogues, which facilitate Ig gene conversion and prevent somatic hypermutation.

Because AID disruption blocks all conversion activity but does not produce a general DNA repair defect, the first step for Ig gene conversion is most likely an AID-dependent DNA modification within the rearranged V-segments. If the AID direct target is RNA (Fig. 3B), then AID would activate the mRNA encoding a DNA-modifying enzyme, which then interacts with the Ig locus.

In the DNA editing model (Fig. 3A), AID scans the rearranged V segments and deaminates C to U. The U in the G/U mismatch is then removed by UNG, leading to an abasic site that is recognized and cut by an APE endonuclease. The resulting single-stranded break (SSB) could be the starting point for a recombination reaction involving a homologous pseudogene. Alternatively the G/U mismatch may be processed by a mismatch repair pathway and give rise to other types of DNA repair intermediates. It has not been reported whether UNG inhibition affects Ig gene conversion. However, the predictions of this model can be easily tested by targeted gene disruption of UNG, APE, or mismatch repair genes in DT40.

G/U mismatches are one of the most frequently occurring natural mismatches, which are generated by random deamination of C. Because such mismatches are normally repaired correctly with high efficiency by base-excision or mismatch repair pathway, we have to postulate the presence of a mechanism that dissects the function of DNA repair machinery between Ig gene locus and other loci.

Whatever the initial DNA change will be, further processing toward a homologous recombination intermediate and interaction with a pseudogene sequence donor are required. The need for homologous recombination factors can explain the reduction of the Ig gene conversion activity in the RAD54 and NBS1 mutants. Models of how either SSBs or DSBs lead to conversion events in the rearranged V segments have been proposed some time ago and remain relevant today (McCormack and Thompson, 1990). An advantage of the SSB model is that it can be easily combined with the DNA editing model of AID, because this model predicts the generation of SSBs after uracil removal.

FUTURE PERSPECTIVE

The high frequency of targeted integration makes DT40 an excellent cell model to dissect the genetics of Ig gene conversion and AID action. In addition, somatic hypermutation and its relation to gene conversion can be investigated by using the DT40 mutants of the RAD51 paralogues.

Which DNA alteration initiates gene conversion and why disruption of homologous recombination pathways switch gene conversion to somatic hypermutation are still open questions. The specificity of Ig gene conversion and somatic hypermutation also needs to be clarified. Although AID expression in fibroblasts can induce hypermutation of non-Ig indicator genes, there is ample evidence that somatic hypermutation is specifically targeted to the rearranged V(D)J segments. This specificity is probably mediated by an unknown protein or a protein complex that binds to *cis*-acting sequences in the Ig loci and either to AID or a downstream mediator of AID action.

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