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# Gene conversion in the chicken immunoglobulin locus: A paradigm of homologous recombination in higher eukaryotes

O. Y. Bezzubova and J.-M. Buerstedde

*Basel Institute for Immunology, Grenzacherstr. 487, CH-4005 Basel (Switzerland)*

*Abstract.* Gene conversion was first defined in yeast as a type of homologous recombination in which the donor sequence does not change. In chicken B cells, gene conversion builds the antigen receptor repertoire by introducing sequence diversity into the immunoglobulin genes. Immunoglobulin gene conversion continues at high frequency in an avian leukosis virus induced chicken B cell line. This cell line can be modified by homologous integration of transfected DNA constructs offering a model system for studying gene conversion in higher eukaryotes. In search for genes which might participate in chicken immunoglobulin gene conversion, we have identified chicken counterparts of the yeast *RAD51*, *RAD52*, and *RAD54* genes. Disruption and overexpression of these genes in the chicken B cell line may clarify their role in gene conversion and gene targeting.
*Key words.* Immunoglobulin; V(D)J recombination; gene conversion; targeted integration; yeast; *RAD52* epistasis group.

## Recombination in the immune system

In vertebrates, B cells can produce millions of different immunoglobulin molecules, which are able to bind to an essentially unlimited number of antigens. It is recombination that serves the special task of creating a repertoire of genes able to encode these immunoglobulin variants. Not all vertebrate species use, however, the same strategy to establish their immunoglobulin diversity as will be discussed in more detail for mice and chicken.

## Immunoglobulin gene diversification in mice

Murine B lymphocytes assemble their immunoglobulin light and heavy chain genes from families of V (variable), D (diversity), J (joining) gene segments[19, 57] (fig. 1). The mechanism of the V(D)J joining involves site-specific recognition of conserved 'heptamer-spacer-nonamer' signal recognition (RS) sequences that flank the germline V, D, and J segments[57]. Recombination takes place at the border between the signal heptamer and the coding sequence, and generates two products: a fusion of the coding segments (coding joint) and a heptamer to heptamer junction of the signals (signal joint). It has been proven that DNA double-strand breaks (DSBs) occur indeed near the RS sequences[46]. Following antigen stimulation, point mutations are introduced at high frequency into the rearranged VJ segment by a process named somatic hypermutation[4] (fig. 1).

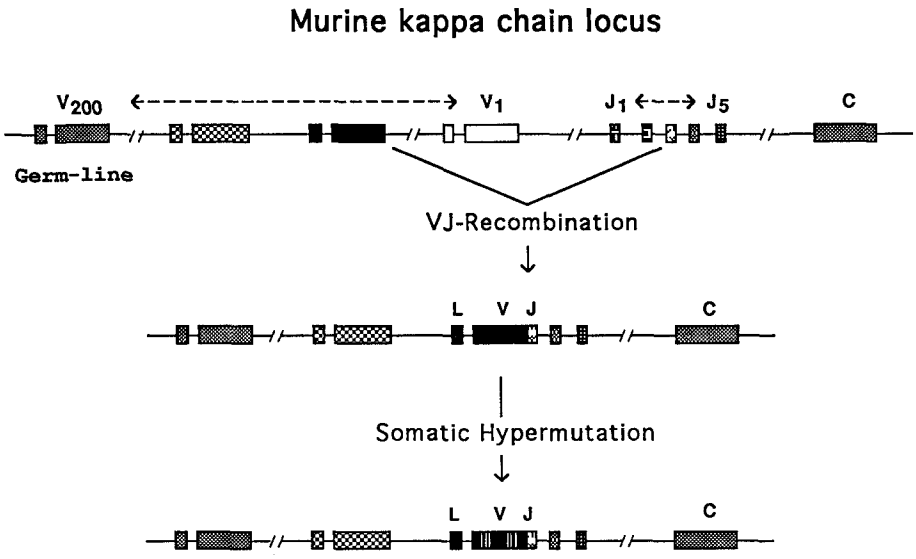


Figure 1. Diversification of the mouse kappa chain locus. The locus consists of approximately 200 V segments spanning a region of about 3000 kbp, 5 J segments 20 kbp downstream of the nearest V segment, and a C gene segment. V(D)J recombination joins one of the V gene segments to one of the J gene segments. Following antigen stimulation somatic mutations are introduced into the rearranged V gene.

V(D)J recombinase.

| Factor | Gene cloned | Expression | Putative function |
|---|---|---|---|
| RAG-1 | Yes | Early lymphocytes Brain | Activate V(D)J recombination; |
| RAG-2 | Yes | Early lymphocytes | May recognise RS sequences |
| TdT | Yes | Early lymphocytes | Adds nontemplated sequences to coding joints |
| SCID | No | Early lymphocytes | Repairs DSBs; May ligate coding joints |
| XR-1 | No | Fibroblasts (Ubiquitous?) | Repair DSBs; May ligate RS and coding joints |
| xrs-6 | No | | |

Little is known about the biochemistry of the V(D)J recombination, but genetic studies indicate that at least five genes influence the activity of the putative V(D)J recombinase (table). Two of these genes, *RAG-1* and *RAG-2*, are expressed at high levels only in lymphocytes and were cloned by their ability to activate rearrangement of test substrates in mouse fibroblasts[39,49]. Another factor involved in V(D)J recombination is defined by a mutation causing severe combined immune deficiency (*scid*) in mice[9], which preferentially affects the formation of coding joints between V, D, and J gene segments[32]. It seems that the putative SCID factor is not lymphoid-cell specific, since fibroblast cell lines derived from *scid* mice are significantly more X-ray sensitive than wild-type cells[7] and deficient in the repair of DNA DSBs[16,26]. Thus, the SCID protein may have a general DNA repair activity also engaged for V(D)J recombination. It was recently discovered that two radiation sensitive Chinese hamster ovary cell lines, *xrs-6* and *XR-1*, were unable to rearrange V(D)J recombination substrates after transfection of the *RAG-1* and the *RAG-2* genes[51]. Since the defective genes of these cell lines map to chromosomal positions different from the *scid* locus, they appear to encode two additional components of the V(D)J recombinase (table).

**Generation of immunoglobulin diversity in chicken**

Chickens develop their immunoglobulin repertoire by a different strategy already reflected in the particular genomic organisation of their immunoglobulin loci[37,54,60]. The chicken light chain locus contains only a single functional V- and J-gene segment[44]. However, 25 pseudo-V gene ($\psi$V) segments have been found upstream of the functional V gene segment spanning a region of about 22 kbp[45] (fig. 2). These were called pseudo-V genes because they are homologous to the V gene, but lack the common transcription regulatory and signal recognition sequences.

The single V and J gene segments are rearranged by V(D)J recombination (fig. 2) during a brief period of early chicken B cell development[34,58] creating only limited diversity at the junction of the V and J gene segment[36]. Further diversity of the rearranged V gene is acquired during B cell proliferation in the bursa of Fabricius, a critical organ for avian B cell development. There, blocks of pseudogene sequences appear in the rearranged V gene (fig. 2), whereas the sequences of the pseudogenes and the unrearranged V gene segment do not change[45,55]. This nonreciprocal transfer of sequence information from the pseudogenes into the rearranged V gene was named gene conversion in analogy to similar processes in yeast[24].

The conversion tracts comprise from 10 to more than 120 bp, and a single V gene can receive segmental exchange from up to six different pseudogenes[45]. The

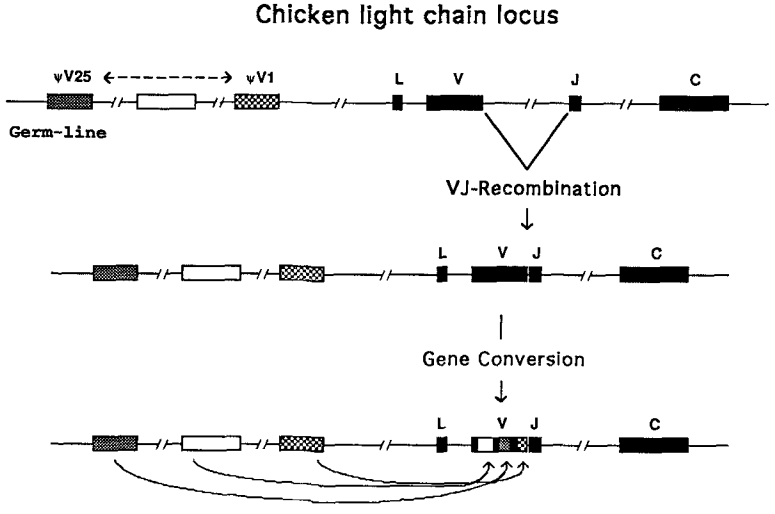


Figure 2. Diversification of the chicken immunoglobulin locus. The locus extends only approximately 30 kbp containing a single V, J and C gene segment downstream of an adjacent pool of 25 pseudogenes ($\psi$V1–$\psi$V25). After V(D)J rearrangement joins the V and J segments, gene conversion within the bursa of Fabricius introduces sequence substitutions in the functionally rearranged V gene.

number of events increases with the time that the B cells spend in the bursal environment, consistent with the idea that gene conversion occurs in a stochastic manner, with more events accumulating as the number of cell divisions increases. It was estimated that one successful conversion event occurs every 10 to 15 cell divisions[60].
The frequency of usage of the $\psi$V segments for conversion events appears to depend on a number of variables[34,45]. First, pseudogene segments proximal to the V gene are used more frequently than distal ones. Second, $\psi$V segments in the antisense orientation are used preferentially over segments in the sense orientation. This happens probably due to simpler DNA folding required for the alignment of donor and recipient sequences. Finally, sequence homology seems to be important for the reaction, since pseudogenes with greater sequence similarity to the V gene serve more often as donors.
Analysis of the sequence exchange between V and $\psi$V segments has shown that it occurs intrachromosomally[13] and proceeds in a directional manner[34]. Starting in the regions of sequence identity at the 5′ end, gene conversion tracts extend in the 3′ direction and end at positions where the donor and acceptor gene segments are not homologous any more.
The fact that only the rearranged V gene segment undergoes gene conversion, but not the V gene segment of the unrearranged allele[55] or the pseudo-V genes[13] suggests the possibility that the recombination process is associated with gene expression, see Gangloff, Lieber, Rotstein in this issue. This particular feature of immunoglobulin gene conversion is reminiscent of somatic hypermutation in mammals, also targeted to the rearranged, transcribed genes.
Quite surprisingly, immunoglobulin gene conversion is not limited to the avian line of evolution, but is also used by rabbits, where it diversifies a preferentially rearranging heavy chain V gene segment[5,31].

## Homologous recombination in chicken B cell lines

### Gene conversion in ALV-induced tumors and B cell lines

Chicken immunoglobulin gene conversion is difficult to study using primary bursal B cells, as these cells survive only a short time during in vitro culture. It was, however, found that the avian leukosis virus (ALV) induces B cell tumors in the bursa, which seem to be arrested at the development stage of the bursal B cells. Light chain gene diversification of the rearranged VJ segment continues indeed during tumor development and in a cell line (DT40) derived therefrom[55,56]. Ongoing gene conversion within tumor cells is not dependent upon the bursal environment, since it happened in a thymic metastasis and during the in vitro passage of the cell line DT40[11]. The estimated average gene conversion rate for this cell line was one event per 40 cell divisions, which is lower than in bursal B cells, but still high enough to serve as a model for in vitro studies.
Some subclones of DT40 lack surface immunoglobulin expression due to nucleotide losses or additions in the light chain gene coding region[11]. Repair of this frameshift by pseudogene templated conversions restores surface immunoglobulin expression and therefore allows a rough estimation of the rate of gene conversion by flow cytometry.

### Targeted integration in DT*40*

It was noticed that transfected constructs of the light chain locus integrated at high frequency into the endogenous light chain loci of DT40[12]. This was surprising, since transfection of mammalian cell lines leads to integration of the gene constructs predominantly at random chromosomal positions and only infrequently into the homologous gene loci[2,50,53]. The high ratio of targeted to random integration was not specific for light chain gene constructs, but was also observed after transfection of $\beta$-actin and ovalbumin gene constructs[12]. Targeted integration of the $\beta$-actin construct occurred at highest frequency in DT40, at somewhat lower frequency in two other chicken B cell lines with no conversion activity and was undetectable in a chicken T cell line and other chicken hematopoietic cell lines. It is currently unresolved whether the increased ratio of gene targeting in chicken B cell lines is related to the immunoglobulin gene conversion activity of bursal B cells. Both processes require sequence homology and a common factor may enhance the search or the integration of the recombining DNA substrates. If this is the case, it should be highly attractive to characterize this factor in more detail because it may facilitate homologous recombination in other organisms. At the moment the increased frequency of targeted integration in DT40 proves advantageous for the study of gene conversion and targeted integration since it allows the function of genes to be tested by gene disruption. At this point the question arises: Are there good candidates for genes which might be involved in these recombination processes?

## Putative enzymes involved in homologous recombination in chicken B cells

Akin to the V(D)J recombination system in the mouse, we suspect that a complex of multiple factors mediates chicken immunoglobulin gene conversion. Some of these factors may be lymphoid specific, but others may play a more universal role for DNA recombination and repair.

### The *RAG-2* gene

Pseudogene sequences are selectively transferred into the rearranged V segment indicating that this segment is distinguished as the conversion target. One possibility is that there are *cis*-acting regulatory sequences which are recognised by factors initiating the conversion event.

Selective expression of the *RAG-2* gene in the bursal B cells and in DT40, but not in other B cell lines which do not undergo light chain gene conversion, suggested that *RAG-2* may play a role in the gene conversion process[14]. However, subclones of DT40 maintained the ability to undergo light chain gene conversion even after both copies of the *RAG-2* coding region were deleted[52], demonstrating that at least in this cell line *RAG-2* expression is not essential for gene conversion.

### The yeast *RAD52* epistasis group

Double mutant analysis of radiation sensitive mutations in *S. cerevisiae* had led to the definition of the three epistasis groups which may represent different DNA repair pathways[17,18,25]. Mutations of genes belonging to the *RAD52* epistasis group influence mitotic and meiotic recombination[41,42] leading to the hypothesis that the *RAD52* pathway repairs DNA damage through recombination intermediates. A common feature of the recombination deficient mutants is their inability to repair double-strand breaks (DSBs)[10,27,30,43]. Three genes of the *RAD52* group, *RAD51*, *RAD52*, and *RAD54* are also required for mating-type switching (refs 1, 33; Game, unpubl. data cited in 17), a gene conversion event initiated by the cut of a site-specific endonuclease[22]. The enzymatic activities of the different members in the *RAD52* pathway still need to be explored. It has recently been shown that the *RAD51* gene product shares significant homology with the bacterial RecA protein[47] and forms nucleoprotein filaments with double-stranded DNA[38]. The primary structure of the Rad52 protein offers no clues to its function, but Rad54[15] possesses sequence motifs common to DNA and RNA helicases[20].

### Isolation of the chicken *RAD51*, *RAD52* and *RAD54* homologues

Encouraged by the finding that the *RAD3* excision repair pathway is well conserved throughout eukaryotic evolution[28,29], we decided to search for chicken homologues of the Rad51, Rad52, and Rad54 proteins (fig. 3). In our attempt to clone the chicken genes we assumed that at least part of the primary amino acid sequence would be conserved during evolution. In addition, we reasoned that comparison of homologues from different species and comparison with other structurally related proteins would reveal those regions in the protein sequence which are under strongest selective pressure.

To clone a chicken *RAD51* counterpart, we compared the Rad51[47] and Dmc1[8] of *S. cerevisiae* with Rad51 of *S. pombe*[48]. All three proteins are homologues of bacterial RecA protein, but are more similar to each other than to RecA. Degenerate oligonucleotides encoding two highly conserved regions were used for polymerase chain reaction (PCR) starting with cDNA of the chicken B cell line DT40. The primers amplified an open reading frame of a chicken RecA-like gene, and the full length cDNA was isolated from a bursal cDNA library. The encoded protein shares 68% and 49% of identical amino acids with the Rad51 and Dmc1 proteins of *S. cerevisiae* respectively (see Heyer in this issue), indicating that the chicken gene is a *RAD51*, not a *DMC1* counterpart[6]. The highest degree of homology is found in the part corresponding to the so-called core region of the Rad51 protein believed to be involved in ATP hydrolysis, DNA binding[47], and the formation of nucleoprotein filaments[38].

A similar strategy was employed to isolate the chicken *RAD52* gene. Comparison of the Rad52 protein of *S. cerevisiae*[3] and its *S. pombe* homologue, Rad22[40], had revealed a conserved region near the N-terminus. Using degenerate oligonucleotides for PCR, we cloned a chicken gene encoding a protein with strong homology in the N-terminal region with the yeast Rad52 and Rad22 proteins. Outside this conserved region of about 160 aa the homology among the three proteins is low and probably insignificant[61].

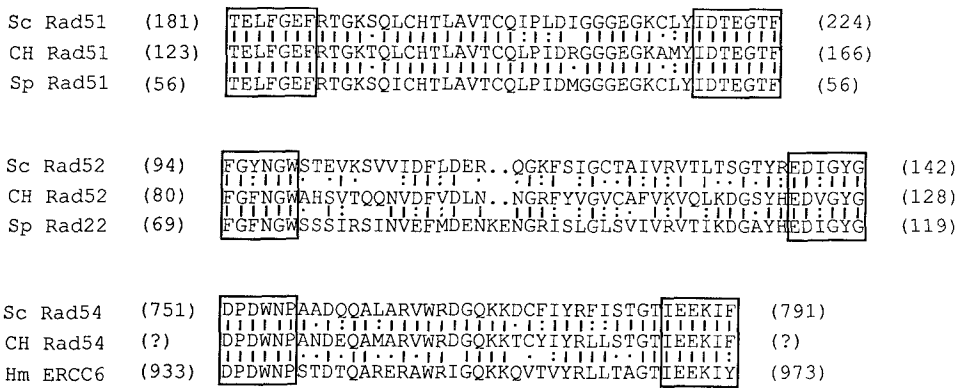


Figure 3. Sequence comparison of the chicken Rad51, Rad52, and Rad54 proteins with their yeast homologues and the human ERCC6 protein within the region encoded by the PCR amplified fragments. The peptides encoded by the degenerate oligonucleotides are boxed. Sequences for the Sc Rad51, Sp Rad51, Sc Rad52, Sp Rad22, Sc Rad54 and Hm ERCC6 are taken from references 47, 48, 3, 40, 15 and 58 respectively.

The *S. cerevisiae RAD54* gene product is a member of a subfamily of helicases which includes several proteins implicated in transcriptional regulation and DNA repair[58]. Among them, the Rad54 is most similar to the recently cloned excision repair protein ERCC6[58]. Primers corresponding to the conserved sequence motifs were designed in such a way, that they would amplify the *RAD54* and the *ERCC6* gene, but not the genes of the other members of the helicase family. The gene cloned from a bursal cDNA library using the PCR amplified fragment as a probe encodes a protein with high homology to the Rad54 protein (unpubl. results). As the similarity to Rad54 is preserved even outside the helicase motifs, we consider the chicken gene a bona fide *RAD54* counterpart.

The expression pattern of the three cloned chicken genes are rather similar. High abundance of mRNAs is found in the sites of lymphoid and germ cell development and a low level of expression is detected in all other organs examined[6, 61].

## What is the function of the putative chicken *RAD52* pathway

The structural homology to the yeast proteins and the expression pattern of the chicken *RAD51*, *RAD52* and *RAD54* genes suggest that they fulfil a similar role as their yeast counterparts. Nevertheless, functional studies are necessary to ascertain a place for these genes in the abstract picture of homologous recombination. The most straightforward approach would be to generate null mutations of DT40 by gene disruption and analyze these with respect to immunoglobulin gene conversion, targeting integration and X-ray sensitivity. It may be that the genes in question are essential for the proliferation of DT40 cells, since even some of the yeast mutants grow slowly, perhaps due to a defect in chromosome segregation[23]. In this case recovery of cell clone with the disruption of both gene copies will prove impossible. This problem may, however, be circumvented by using the recently developed tetracycline responsive gene expression system[21], which works well in DT40 (unpubl. results).

Genes belonging to the *RAD52* epistasis group may be involved in the repair of double-strand breaks which accompany V(D)J recombination[46]. The DT40 cell line does not undergo V(D)J recombination at its immunoglobulin loci, but its *rad51*, *rad52* or *rad54* mutants could be cotransfected by the *RAG-1* gene and a recombination substrate and then be tested for V(D)J recombination activity.

## Chicken B cells as a model system for homologous recombination

The conservation of the *RAD52* pathway during eukaryotic evolution proves that lower eukaryotes are excellent models for the study of recombination. Nevertheless, certain types of recombination, like lymphoid-specific recombination, cannot be investigated in yeast. Yeast and mammalian cells differ in another important aspect: gene constructs transfected into yeast cells integrate predominantly by homologous recombination and only rarely at random positions, whereas the opposite holds true for mammalian cells. The study of recombination in chicken B cells should yield new insight into the processes of gene conversion and targeted integration, thus serving as a bridge between the two systems.

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