Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 4023–4027, May 1992
Immunology

RAG-2 expression is not essential for chicken immunoglobulin gene conversion

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Communicated by Martin Gellert, January 21, 1992

ABSTRACT **Chicken B cells diversify their immunoglobulin genes by gene conversion in the bursa of Fabricius. The avian leukosis virus-induced B-cell line DT40 continues to diversify its immunoglobulin light chain locus by gene conversion during *in vitro* passage. Since a variable(diversity)joining recombination-activating gene, RAG-2, is specifically expressed in chicken B cells undergoing immunoglobulin gene conversion, it has been suggested that RAG-2 may be involved in the immunoglobulin gene conversion process. We previously reported high ratios of targeted to random integration after transfection of genomic DNA constructs into DT40. This allows us to easily investigate the function of a gene product by gene disruption. We show here that subclones of DT40 maintain the ability to diversify their immunoglobulin light chain locus by gene conversion even after both copies of the RAG-2 coding regions are deleted. These results demonstrate that the RAG-2 product is not required for gene conversion activity in the immunoglobulin light chain locus.**

Chicken B-cell precursors rearrange their immunoglobulin genes before or during colonization of the bursa of Fabricius (1, 2). Subsequent B-cell proliferation in bursa follicles is accompanied by diversification of the rearranged light chain genes through segmental gene conversion with nearby pseudogenes serving as donors (3–6). Like most bursal B cells, the avian leukosis virus-transformed chicken B-cell line DT40 (7) contains only one rearranged light chain gene, whereas the other allele remains in germ-line configuration. DT40 continues to diversify the variable (V) segment of its rearranged light chain gene during passage *in vitro* (8, 9).

Transfection of the two recombination-activating genes RAG-1 and RAG-2 into NIH 3T3-fibroblast cells synergistically induced recombination in an artificial DNA substrate containing the heptamer- and nonamer-specific recombination signals shared by the immunoglobulin and T-cell receptor (TCR) genes (10, 11). Such transfection did not induce any other lymphoid-specific properties. In addition, all mammalian lymphoid cell lines undergoing immunoglobulin or TCR gene rearrangement coexpress RAG-1 and RAG-2. These data suggest that RAG-1 and RAG-2 encode components of the V(diversity)joining [V(D)J] recombination system. RAG-2, but not RAG-1, is expressed during B-cell development in the bursa of Fabricius (12). Whereas most avian lymphoid cell lines do not undergo gene conversion in their immunoglobulin light chain loci and express neither RAG-1 nor RAG-2 mRNA, DT40, which does undergo light chain gene conversion, expresses RAG-2 mRNA but not RAG-1 mRNA. This correlation between the selective expression of RAG-2 and the gene conversion activity suggested that RAG-2 is involved in the immunoglobulin gene conversion system.

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The approximate ratio of targeted to random integration is $1:10^2$ to $1:10^5$ after transfection of genomic DNA constructs into mammalian cells (13). Surprisingly, DT40 incorporates foreign DNA by targeted integration at frequencies similar to those seen for random integration (14). Since targeted integration occurred at all four different loci analyzed, we expected that the RAG-2 locus would also be efficiently targeted. We therefore decided to test the function of the RAG-2 gene by targeted disruption.

We have previously described subclones of DT40 that lack surface IgM (s-IgM) expression due to a frameshift mutation within the rearranged immunoglobulin light chain V segment (9). It was shown that repair of the frameshift occurs through overlapping gene conversion events, leading to reexpression of s-IgM. This makes it possible to measure immunoglobulin gene conversion activity in these cells by quantitating the appearance of s-IgM-positive revertants.

We generated two DT40 subclones in which both copies of the RAG-2 gene were completely deleted. Both DT40 subclones were found to maintain the ability to undergo immunoglobulin light chain gene conversion. We were unable to detect any difference in the nature of the gene conversion events in one of these subclones by DNA sequence analysis. These data demonstrate that RAG-2 is not essential for the generation of immunoglobulin gene conversion events in avian B-cell lines.

MATERIALS AND METHODS

Cells. Clone 18 (CL18), a s-IgM-negative variant derived from DT40, has been described (9). This clone and all other subclones were cultured in modified Dulbecco's medium containing 10% fetal calf serum, 1% chicken serum, 2 mM L-glutamine, 0.01 mM 2-mercaptoethanol, and penicillin/streptomycin at 40°C in a 5% CO_2 in air humidified incubator.

Gene Constructs. As a source of chicken genomic DNA of RAG-2 we used an 8.7-kilobase (kb) fragment from the phage clone π1591 (12). The fragment contains the RAG-2 coding sequence and the flanking sequence from the first upstream *Hin*dIII site to the first downstream *Sal* I site. The *Sac* I–*Xba* I fragment including the whole RAG-2 coding region was replaced by the neomycin-resistance gene (Neo^R) or Ecogpt gene under the control of the β-actin promoter (15), and the recombinant DNA fragment was inserted into the Bluescript (Stratagene) plasmid (Fig. 2). Before transfection of the construct into DT40, we linearized the construct by *Xba* I digestion at the *Xba* I site in the polylinker of the Bluescript plasmid. The RAG-2 coding probe was a 1.8-kb *Sac* I–*Xba* I fragment, the 3′ RAG-2 probe was a 0.9-kb *Hin*dIII fragment, the Neo^R probe was a 0.65-kb *Pst* I fragment, and the Ecogpt probe was the *Hin*dIII–*Bam*HI fragment from pSV2 Ecogpt (16). The immunoglobulin light chain histidinol-resistance gene (17) ($Ig\text{-}His^R$) construct consisted of a *Bgl* II–*Xba* I

Abbreviations: s-IgM, surface IgM; V, variable; D, diversity; J, joining; CL, clone; R, resistance; Neo, neomycin; His, histidinol.

fragment of the rearranged light chain locus (3) in which V–J segment was replaced by the His^R gene, including its own poly(A) signal.

Screening. Conditions for transfection and selection of DT40 with G418 have been described (14). Selection of transformants with the Ecogpt construct was done in medium containing 30 μg of mycophenolic acid per ml. After transfection of CL18 with the RAG-2 Neo construct, individual clones were analyzed by Southern blotting of *Xba* I-digested DNA using the 3′ RAG-2 probe for hybridization. We then transfected the RAG-2 Ecogpt construct into a clone in which one of the RAG-2 genes had already been disrupted. Targeted events were confirmed by Southern blot analysis of *Xba* I-digested genomic DNA from G418- and mycophenolic acid-resistant clones using the RAG-2 coding probe, the 3′ RAG-2 probe, the Neo^R probe, and the Ecogpt probe.

Quantification of Gene Conversion by Flow Cytometric Analysis. We analyzed six clones: CL18, which contains two intact RAG-2 genes; DT40(−), in which the rearranged light chain gene is irreversibly disrupted by targeted integration (14); CL18.1 and CL18.1.1, in which one of the two RAG-2 loci is disrupted; CL18.1.2 and CL18.1.3, in which both RAG-2 genes are disrupted. Two weeks after limiting dilution we randomly selected 16 subclones from DT40(−) and 24 subclones from each of the other five original clones and measured the frequency of s-IgM-positive cells of each subclone with a Becton Dickinson FACScan. The method of staining for s-IgM has been described (9). Propidium iodide was added just before analysis to gate out any dead cells.

Sequence Analysis. s-IgM-positive cells from CL18.1.3 and one subclone derived from CL18.1.3 were enriched by fluorescence-activated cell sorting. The populations are called CL18.1.3(+). The methods of isolating genomic DNA from the populations, PCR amplification, and nucleotide sequencing have been described (9).

RESULTS

An outline of the whole experimental procedure is shown in Fig. 1. As a first step we introduced the RAG-2 Neo construct into CL18, which does not express s-IgM due to a single base-pair insertion in the rearranged V segment of its light chain gene (Fig. 5); this clone carries two intact copies of the RAG-2 gene [RAG-2 (+/+)]. Southern blot analysis indicates that 6 of 15 independent G418-resistant clones had targeted integration (data not shown). We then chose one of the six clones, designated CL18.1, in which one of the RAG-2 genes was disrupted by targeted integration of the RAG-2 Neo construct [RAG-2 (+/−)], for transfection of the RAG-2 Ecogpt construct. The RAG-2 Ecogpt construct disrupted the other copy of the RAG-2 gene in two independent clones, CL18.1.2 and CL18.1.3, which therefore lost both RAG-2 genes. In another clone, CL18.1.1, the RAG-2 Ecogpt construct integrated randomly [RAG-2(+/−)]. We compared the activity of light chain gene conversion in these clones. From the CL18.1.3 RAG-2(−/−) clone, we purified a s-IgM-positive revertant population designated CL18.1.3(+) to determine whether the frameshift mutations had been repaired by gene conversion.

The structure of the targeting plasmids used for transfection into CL18, a map of the RAG-2 locus, and a map of the relevant region of a homologous recombinant after targeted integration of the RAG-2 Neo construct are illustrated in Fig. 2. Fig. 3 shows Southern blot analysis of *Xba* I-digested DNA using the 3′ RAG-2 probe (Fig. 3*A*) and the RAG-2 coding probe (Fig. 3*B*). The *Xba* I site downstream of the RAG-2 gene would be deleted during targeted integration of either the RAG-2 Neo construct or the RAG-2 Ecogpt construct (see Fig. 2); in clones with a targeted integration event, hybridization with the 3′ RAG-2 probe were expected to give

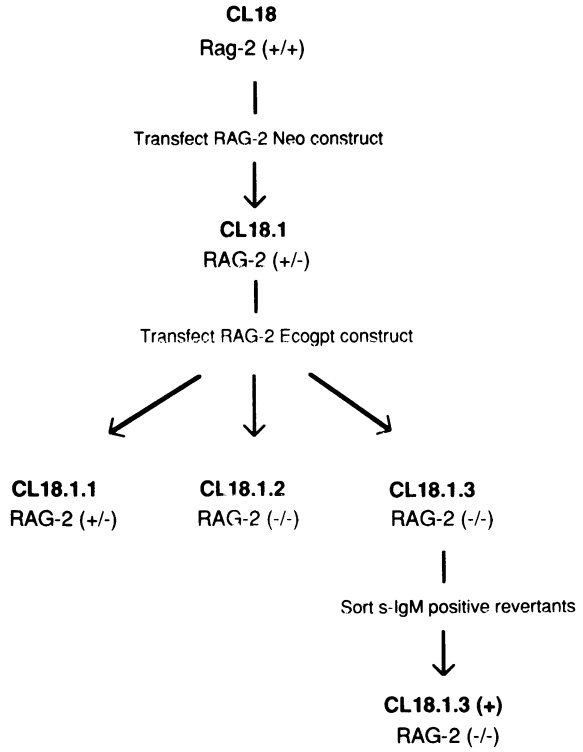

FIG. 1. Outline of two-step transfection procedure to isolate a homozygous RAG-2 mutant.

a new 11-kb band instead of the 4.2-kb band from the intact RAG-2 gene. The two equally intense bands of 4.2 kb and 11 kb in lanes 3 and 4 of Fig. 3*A* indicate that one copy of the RAG-2 gene in CL18.1 and CL18.1.1 has been disrupted [RAG-2(+/−)]. CL18.1.2, CL18.1.3, and CL18.1.3(+) gave only the 11-kb band (Fig. 3*A*, lanes 5–7), indicating that both RAG-2 genes of these cells are modified by homologous recombination. To confirm that the coding sequences of both RAG-2 genes were deleted in CL18.1.2, CL18.1.3, and the CL18.1.3-derived s-IgM-positive revertants, we analyzed the Southern blot hybridization pattern of their DNAs using the RAG-2 coding probe, the Neo^R probe, and the Ecogpt probe. Though no bands were detected with the RAG-2 coding probe, which includes most of the RAG-2 coding sequence (Fig. 3*B*, lanes 5–7), the Neo^R and Ecogpt probes hybridized with the 11-kb band (data not shown), suggesting that the coding sequence of the two RAG-2 genes had been replaced by the Neo^R and Ecogpt genes in these cells.

We then compared the immunoglobulin gene conversion activity in the light chain locus among the CL18, CL18.1, CL18.1.1, CL18.1.2, and CL18.1.3 clones. We have previously shown that spontaneous reexpression of s-IgM from s-IgM-negative DT40 subclones is caused by repair of the light chain gene frameshifts through gene conversion events using donor sequences from the pseudogene pool (9). We measured the frequency of s-IgM-positive revertants of 24 subclones from each of the 5 clones 2 weeks after subcloning to obtain an estimate of the gene conversion activity. As a negative control, we stained 16 subclones of DT40(−), a stable s-IgM-negative DT40 clone in which the rearranged immunoglobulin locus was disrupted by a targeted integration event (Fig. 4*A*) (14). Fig. 4 shows s-IgM expression patterns of DT40 (Fig. 4*B*) and of representative subclones of DT40(−) (Fig. 4*A*), CL18 (Fig. 4*C*), CL18.1 (Fig. 4*D*), CL18.1.2 (Fig. 4*E*), and CL18.1.3 (Fig. 4*F*). Table 1 shows the relative frequencies of the cells defined to be positive for s-IgM by falling into the rectangle in the dot plots shown in Fig. 4. Since reversion events may happen at different times during ex-

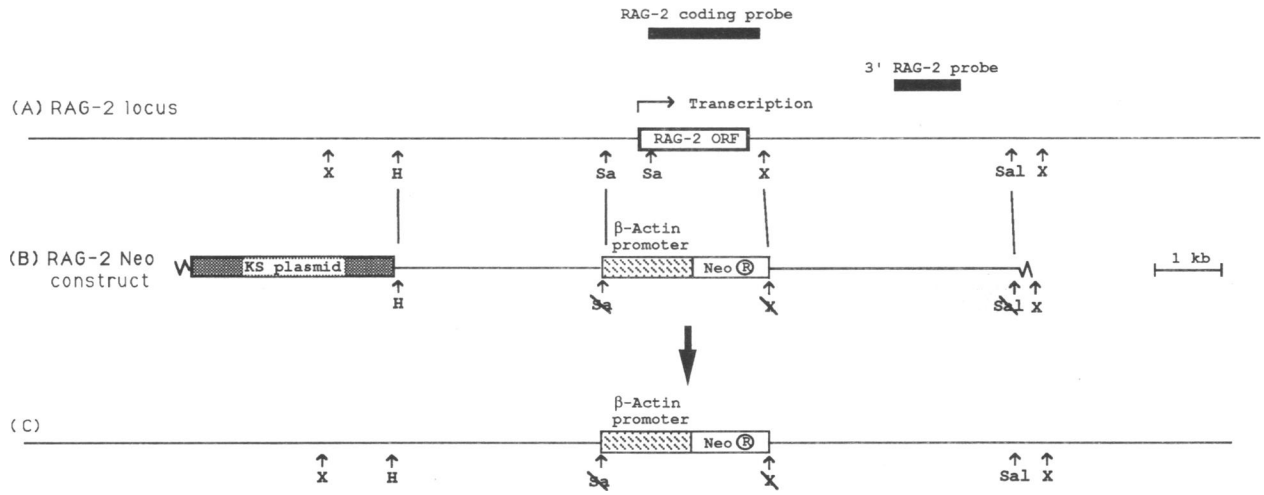

FIG. 2. Schematic diagram of the homologous recombination resulting in the deletion of the RAG-2 coding sequence. (*A*) RAG-2 locus in the genome of DT40 cells. An open box indicates the RAG-2 open reading frame (ORF). Locations of the two probes are indicated by bars. (*B*) Targeting construct, RAG-2 Neo. An open box indicates the Neo^R gene under the control of the chicken β-actin promoter represented by a hatched area. The RAG-2 Ecogpt construct is similar to the RAG-2 Neo construct and the Neo^R gene was replaced by a Ecogpt gene. The plasmids were linearized at the *Xba* I site in the polylinker of Bluescript plasmid. (*C*) RAG-2 locus in DT40 clones after targeted integration of the RAG-2 Neo construct. H, *Hin*dIII; Sal, *Sal* I; X, *Xba* I; Sa, *Sac* I. Only relevant restriction sites are indicated.

pansion of the subclones, the frequency of s-IgM-positive cells would accordingly fluctuate from one subclone to another (19). In addition, some of the subclones are predominantly s-IgM-positive and they may be derived from a cell that was already s-IgM-positive (CL18.1 subclone 18, CL18.1.2 subclone 15, CL18.1.3 subclone 5). Excluding these predominantly s-IgM-positive subclones, there are no significant differences in the fraction of s-IgM-positive cells among the five pools of measurements, suggesting that the homozygous mutant subclones maintain the ability to generate s-IgM-positive cells by immunoglobulin light chain gene conversion even in the absence of RAG-2 expression.

We determined the immunoglobulin light chain V segment sequence of s-IgM-positive revertant populations, CL18.1.3(+), which are derived from the original CL18.1.3 RAG-2(−/−) clone and from one of its subclones. In Fig. 5 these sequences are aligned with the germ-line V sequence and the sequence found in s-IgM-negative cells of CL18.1.3, which retains the frameshift mutation described for CL18 (9). The most likely pseudogene donors are shown above the sequences of s-IgM-positive revertants. Converted segments can be identified over various lengths from 5 to >40 base pairs. Most of these somatic modifications can be assigned to a pseudogene sequence. The sequence of CL18.1.3(+).1 was by far the most frequent sequence among the sequences obtained from both s-IgM-positive populations. Predominant usage of pseudogene V8 may be due to the high degree of homology of this pseudogene with the defective CL18 sequence. The 3-base-pair addition at the border of a conversion segment in the sequence CL18.1.3(+).4 is best explained by a shifted alignment of the pseudogene V2 donor and the target sequence. No potential pseudogene donors are known for the CL18.1.3(+).5 sequence, which is repaired by a single base-pair deletion. Together, these sequence data suggest that most sequence modifications were generated by pseudogene templated gene conversion events, which display the

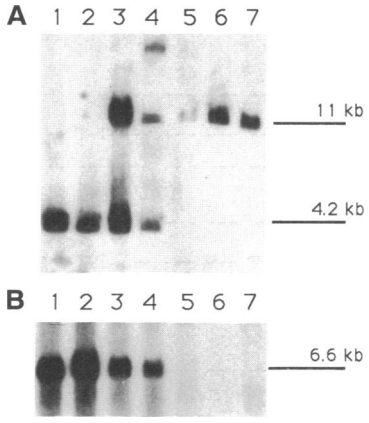

FIG. 3. Southern blot analysis of homologous recombination events. Genomic DNA was digested with *Xba* I, and the blots were hybridized with the 3′ RAG-2 probe (*A*) or the RAG-2 coding probe (*B*). Lanes: 1, DT40; 2, CL18; 3, CL18.1; 4, CL18.1.1; 5, CL18.1.2; 6, CL18.1.3; 7, CL181.3(+).

Table 1. Percentages of cells defined to be s-IgM positive

Subclone	DT40(−)	CL18	CL18.1	CL18.1.1	CL18.1.2	CL18.1.3
1	0.02	1.30	1.04	0.44	0.90	0.96
2	0.22	2.56	0.60	0.66	0.46	1.38
3	0.08	1.90	0.64	1.18	2.28	0.66
4	0.12	1.22	0.76	1.06	1.34	1.38
5	0.22	1.04	0.52	0.48	0.88	98.16*
6	0.06	0.48	1.06	0.90	0.46	0.70
7	0.28	2.12	0.64	0.14	0.08	0.68
8	0.10	1.40	0.62	1.02	0.54	0.72
9	0.16	2.00	1.48	0.88	1.20	0.78
10	0.12	1.62	0.84	1.00	0.64	1.04
11	0.14	1.76	0.94	1.60	1.10	0.86
12	0.06	0.18	0.98	1.30	0.84	1.84
13	0.04	0.64	0.86	0.34	0.92	1.14
14	0.02	0.48	1.80	0.92	0.88	0.62
15	0.12	1.40	1.28	1.14	98.44*	0.62
16	0.08	1.66	2.46	0.36	0.78	1.16
17		0.96	1.26	0.36	1.20	0.84
18		0.48	76.12*	0.84	0.54	0.90
19		0.26	1.38	0.88	1.00	0.64
20		0.20	1.34	1.06	0.44	1.22
21		1.20	1.30	1.18	1.10	0.84
22		0.84	1.52	0.88	0.90	1.30
23		0.84	0.28	1.22	0.74	1.28
24		1.70	1.96	1.10	0.26	0.86
Average	0.115	1.18	1.11	0.873	0.847	0.974

*Data excluded from the calculation.

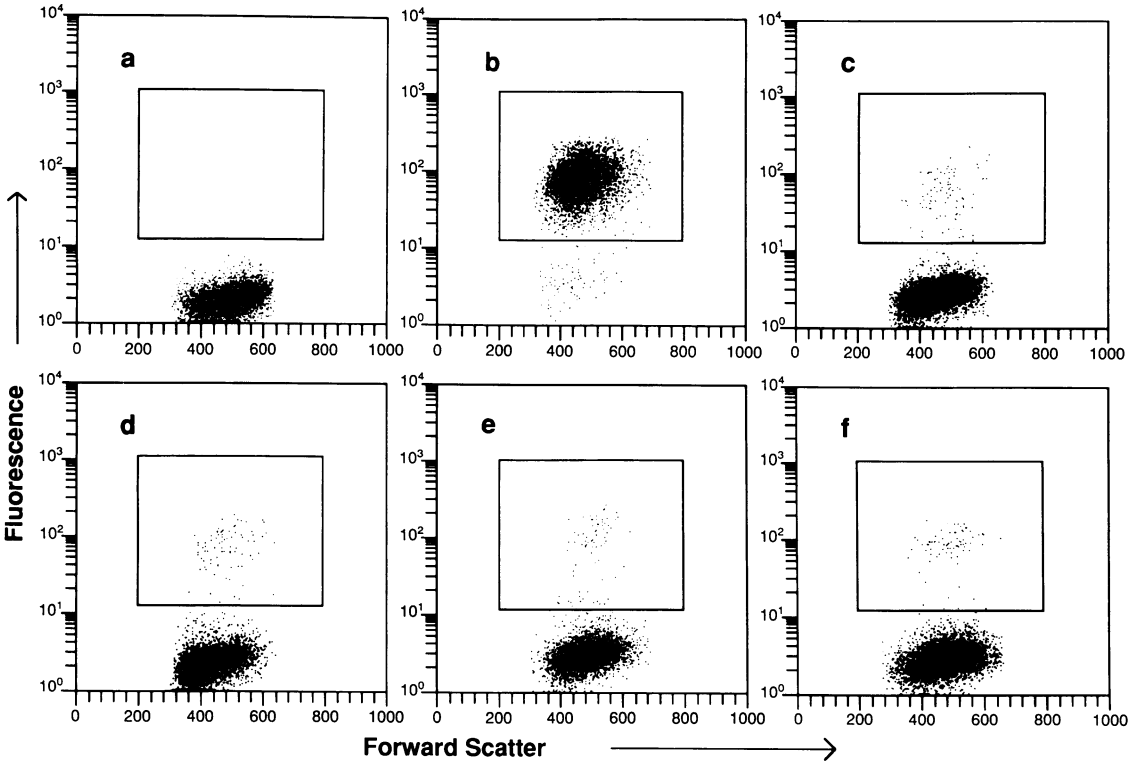

FIG. 4. s-IgM reexpression of subclones of CL18 and its RAG-2 defective mutants. The cells were analyzed for s-IgM expression by flow cytometry using the fluorescence-conjugated anti-IgM monoclonal antibody M-1 (18). Five thousand events were displayed by dot plots. (*a*) Subclone of DT40(−). (*b*) DT40. (*c*) Subclone of CL18. (*d*) Subclone of CL18.1. (*e*) Subclone of CL18.1.2. (*f*) Subclone of CL18.1.3. The cells in a rectangle were counted as s-IgM-positive cells.

same features as those of wild-type DT40 (9). This demonstrates that light chain gene conversion continues in the DT40 cell line independent of RAG-2 expression.

Finally, we compared targeted integration frequencies of RAG-2(−/−) cells and wild-type DT40. We previously reported that the frequency of s-IgM-negative cells after transfection of a rearranged immunoglobulin construct reflects the relative frequency of targeted integration (14). The Ig-His^R construct was introduced into CL18.1.3(+) or wild-type DT40, and the histidinol-resistant bulk population was analyzed by fluorescence-activated cell sorting to quantitate s-IgM-negative cells. There was no significant difference in either the efficiency of transfection or the fraction of s-IgM-negative cells (30–70%) (data not shown), indicating that the RAG-2 product is not required for the high frequency of targeted integration.

DISCUSSION

Identification of gene function has often relied on isolation of mutant cells in which expression of the gene was inactivated. Here we generated RAG-2-negative DT40 clones to examine the role of RAG-2 expression for immunoglobulin gene conversion activity.

Germline	TCC GGG GAT AGC AGC TAC TAT GGC TGG TAC CAG CAG AAG GCA CCT
CL18.1.3(-)	--- --- -G- G-- --T ---↓G GCT GGA AGT TAC TAT --- --- --- --- --- --- --- T-T ---
ψV8	--- --- -G- G-- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
CL18.1.3(+).1	--- --- -G- G-- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- T-T ---
CL18.1.3(+).2	--- --- -G- G-- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
ψV18	--- --- -G- --- --- G-T -A- --- --- --T --C --- A-- --- C-- --- --- --- T-T ---
CL18.1.3(+).3	--- --- -G- --- --- G-T -A- --- --- --T --C --- A-- --- --T --- --- --- T-T ---
ψV2	--- --- -G- TA- --- GG- TA- -C --
CL18.1.3(+).4	--- --- -G- TA- --- GG- TA- -CT↓GGA --- --- --- --- --- --- --- --- --- --- T-T ---
CL18.1.3(+).5	--- --- -TG GCA GTT ACG --- --- --- --- --- --- --- --- --- --- --- --- --- T-T ---

FIG. 5. The frameshift in the V segment of the RAG-2(−/−) cells is repaired by gene conversion. V segment sequences of immunoglobulin light chain genes were obtained from s-IgM-positive cells that originated either from CL18.1.3 or from a subclone derived from CL18.1.3. These sequences are compared with the sequence from s-IgM-negative CL18.1.3 cells [CL18.1.3(−)] and the most likely pseudo-V donor sequence. No likely pseudo-V donor was found for sequence CL18.1.3(+).5.

Cotransfection of the RAG-1 and RAG-2 genes into the NIH 3T3 fibroblast line induces a high frequency of V(D)J-specific recombination without inducing the expression of other pre-B- and pre-T-cell markers, suggesting a direct participation of the RAG-1 and RAG-2 gene products in the recombination reaction (10, 11). On the other hand, RAG-2 but not RAG-1 is selectively expressed in the cells that undergo immunoglobulin gene conversion, although both RAG genes are tightly linked. Strong correlation between selective RAG-2 expression and the gene conversion activity has suggested that RAG-2 is one of the necessary components for immunoglobulin gene conversion (12). It is, however, also possible that the RAG-2 gene products induce V(D)J recombination or immunoglobulin gene conversion by regulating the activity of the other genes or proteins.

In this report, we demonstrate that light chain gene conversion continues in DT40 subclones even after we have completely deleted the RAG-2 coding regions. The gene conversion activity was assayed by quantitating the appearance of s-IgM-positive revertants from cells containing a frameshift mutation in their rearranged light chain V segment due to single base-pair insertion. We believe that the appearance of s-IgM-positive cells from RAG-2(−/−) clones reflects gene conversion at the immunoglobulin light chain locus for the following reasons. (*i*) The cells that are s-IgM-positive have an in-frame light chain V sequence, indicating that these small fractions of RAG-2(−/−) cells indeed express the immunoglobulin light chain. (*ii*) The revertant population, CL18.1.3(+), consisting of >95% s-IgM-positive cells after sorting, do not contain the RAG-2 coding sequence (Fig. 3 *A* and *B*, lane 7). Hence, contamination of wild-type cells does not account for the appearance of s-IgM-positive cells. (*iii*) The sequence data from CL18.1.3(+) (Fig. 5) indicate that gene conversion events were responsible for the repair of the frameshift, since most of the modifications can be correlated with stretches of identical sequences from the pseudogene pool. There are no obvious differences in the nature of the gene conversions among s-IgM-positive revertants from wild-type DT40 (9) and its RAG-2(−/−) mutants. Furthermore, the gene conversion activity of the RAG-2(−/−) cells is quite stable, because the appearance of s-IgM is reproducible after serial subcloning. Since there appears to be no difference in the nature of gene conversion events of RAG-2(+/+) or RAG-2(−/−) clones, it is unlikely that continued expression of RAG-2 is required for immunoglobulin gene conversion.

Although chicken B-cell progenitors rearrange their immunoglobulin genes for only a brief period between day 10 and day 15 of embryogenesis (1, 2), selective RAG-2 expression is found from day 18 of embryogenesis to 14 weeks after hatching (12). Consequently, we could still speculate that RAG-2 plays a role during B-cell development in the bursa of Fabricius, rather than being left activated even after V(D)J-specific recombination has stopped. Since the DT40 cell line may not fully represent bursal lymphocytes with respect to their immunoglobulin gene conversion activity, it is still an open question whether expression of the RAG-2 gene is required for immunoglobulin gene conversion of B cells in the bursa of Fabricius. The RAG-2 product may be required, for example, to activate other genes that participate in the gene conversion reaction, even if RAG-2 expression is no longer necessary to maintain gene conversion activity in DT40.

The peculiar property of DT40 to integrate transfected constructs at high frequencies by targeted integration promises easy isolation of mutant cells, allowing us to test the function of a gene product as well as of a cis-acting sequence. We can use at least four reagents—G418, hygromycin, mycophenolic acid, and histidinol—to select DT40 stable transfectants (unpublished data). It is possible to thereby introduce four different specific mutations, suggesting that this cell line is useful as a genetic tool for the dissection of various biological processes.

We thank Drs. Alexandra Livingstone, Michael Wiles, and Klaus Karjalainen for critical comments and Gholan Reza Dastoornikoo and Francis Vilbois for excellent technical assistance. The Basel Institute for Immunology was founded and is supported by F. Hoffmann–La Roche, Basel, Switzerland.

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