Cell, Vol. 64, 201-208, January 11, 1991, Copyright © 1991 by Cell Press

Selective Expression of RAG-2 in Chicken B Cells Undergoing Immunoglobulin Gene Conversion

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Summary

Chickens create their immunoglobulin (Ig) repertoires during B cell development in the bursa of Fabricius by intrachromosomal gene conversion. Recent evidence has suggested that Ig gene conversion may involve *cis*-acting DNA elements related to those involved in V(D)J recombination. Therefore, we have examined the potential role of the V(D)J recombination activating genes, RAG-1 and RAG-2, in regulating chicken Ig gene conversion. In contrast to the coexpression of RAG-1 and RAG-2 observed in mammalian B cells that undergo V(D)J recombination, chicken B cells isolated from the bursa of Fabricius express high levels of the RAG-2 mRNA but do not express RAG-1 mRNA. The developmental and phenotypic characteristics of the bursal lymphocytes and chicken B cell lines that express RAG-2 mRNA demonstrate that selective RAG-2 expression occurs specifically in B cells undergoing Ig diversification by gene conversion. These data suggest that RAG-2 plays a fundamental role in Ig-specific gene conversion.

Introduction

The primary repertoire of mammalian immunoglobulin (Ig) and T cell receptor (TCR) genes is generated by a series of genomic rearrangements that occur during lymphoid development (reviewed by Alt et al., 1986; Hunkapiller and Hood, 1989). In both sets of genes, rearrangement is mediated by recombination signal sequences that flank variable (V), diversity (D), and joining (J) gene segments (Tonegawa, 1983). These signal sequences have been highly conserved in species that carry out V(D)J rearrangement and are functionally interchangeable (Yancopoulos et al., 1986; Bucchini et al., 1987; Goodhardt et al., 1987; Lieber et al., 1987). V(D)J recombination requires the expression of two unrelated but tightly linked genes, RAG-1 and RAG-2 (Schatz et al., 1989; Oettinger et al., 1990). Upon transfection, neither RAG-1 nor RAG-2 alone is sufficient to induce V(D)J rearrangement in fibroblasts. However, cotransfection of the RAG-1 and RAG-2 cDNAs induces a high frequency of V(D)J-specific recombination without inducing the expression of other pre-B or pre-T cell markers (Schatz et al., 1989; Oettinger et al., 1990). A survey of mammalian pre-B and pre-T cell lines that undergo Ig or TCR gene rearrangement demonstrated that all of these cell lines coexpress RAG-1 and RAG-2. Thus, the data suggest that together RAG-1 and RAG-2 encode the lymphoid-specific component of the V(D)J recombination system.

In contrast to mammals, Ig gene rearrangement in chickens does not lead to the creation of an immunoglobulin repertoire. Instead, chickens use rearrangement of the Ig heavy (H) and light (L) chain loci only to activate the expression of surface Ig. During chicken B cell development, B cell progenitors rearrange their IgH and IgL genes for only a brief period between days 10 and 15 of embryogenesis (Weill et al., 1986; McCormack et al., 1989a). The cells that undergo Ig gene rearrangement are present primarily in the spleen, which is a major site of hematopoeisis during this embryonic period (reviewed by Ratcliffe, 1985; Pink, 1986; McCormack and Thompson, 1990a). Following Ig gene rearrangement the B cell progenitors migrate to the bursa of Fabricius, where those progenitors that express surface Ig undergo rapid clonal expansion (McCormack et al., 1989a). Beginning between days 15 and 18 of embryogenesis, this proliferating Ig^+ population of cells undergoes repetitive gene conversion of the single rearranged V_{H1} and V_{L1} segments using V segment pseudogenes as sequence donors (Reynaud et al., 1987, 1989; McCormack et al., 1989b; Carlson et al., 1990). After accumulating four to ten gene conversion events within their V_{H1} and V_{L1} gene segments, individual bursal lymphocytes cease to proliferate and begin to migrate to the peripheral lymphoid organs (Thompson and Neiman, 1987). Ig gene conversion is an ongoing process in the proliferating population of bursal lymphocytes until the bursa involutes at 4–6 months of age.

Previous studies have demonstrated that the gene conversion process in bursal lymphocytes is restricted to the Ig loci. The lymphoid-specific Cα5-tubulin gene has a genomic organization similar to the IgL gene. It is composed of three exons, is actively transcribed in bursal lymphocytes, and is preceded by a 5′ pseudogene segment that has an extensive open reading frame (Pratt and Cleveland, 1988). Despite this, the Cα5-tubulin gene fails to undergo gene conversion in developing bursal lymphocytes (Thompson, 1989). Similarly, the histone H2b genes, which are organized in two tightly linked clusters, fail to display evidence of gene conversion in developing bursal lymphocytes (Thompson, 1989).

The Ig specificity of bursal-dependent gene conversion led us to investigate whether RAG-1 and/or RAG-2 might also play a role in the regulation of Ig diversification by

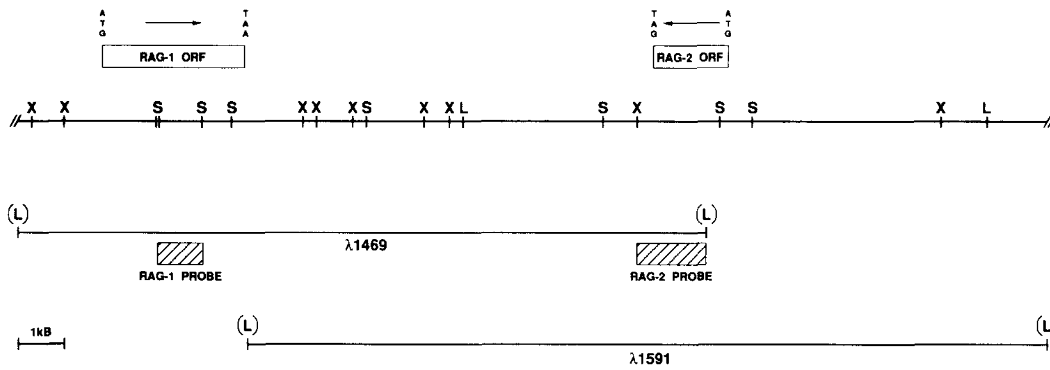
gene conversion. In this report, we demonstrate that RAG-2, but not RAG-1, is expressed during B cell development in the bursa of Fabricius. These results suggest that a specific mechanism has evolved for the differential regulation of RAG-1 and RAG-2 during B cell development. Bursal RAG-2 expression was first detected between days 15 and 18 of embryogenesis, the same time period during which developing bursal lymphocytes begin to diversify their Ig genes by gene conversion. Selective RAG-2 expression within the bursa continued until bursal involution at 4–6 months of age. Since gene conversion has been associated with cells undergoing proliferative expansion within the bursa of Fabricius, we examined RAG-2 gene expression with respect to the proliferative state of the bursal lymphocyte. Quiescent bursal lymphocytes destined to migrate into the periphery failed to express RAG-2, whereas B cells undergoing proliferative expansion in the bursa of Fabricius expressed high levels of RAG-2 mRNA. Finally, in contrast to other non-gene-converting avian B and T cell lines, the bursal lymphoma cell line DT40, which undergoes constitutive Ig-specific gene conversion in culture, was found to express RAG-2 but not RAG-1 mRNA. Together these data suggest that RAG-2 plays a role in the Ig-specific gene conversion process that occurs during B cell development in the bursa of Fabricius.

Results

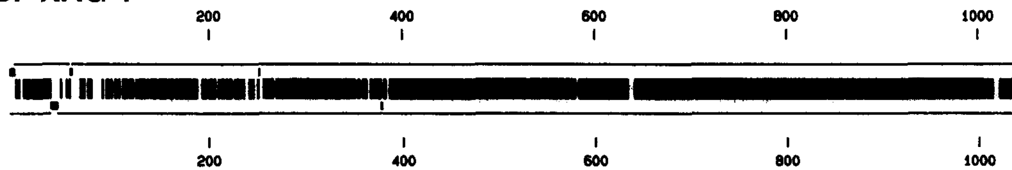
The Genomic Organization of RAG-1 and RAG-2 Has Been Highly Conserved during Vertebrate Evolution

To characterize the expression of RAG-1 and RAG-2 during avian B and T cell development, it was first necessary to clone the chicken RAG-1 and RAG-2 genes. Hybridization of chicken DNA with a human RAG-1-specific probe detected a single-copy gene in the chicken genome (data

A.



B. RAG-1



C. RAG-2

Figure 1. Genomic Organization of the Chicken Recombination Activating Locus

(A) The genomic organization of the chicken RAG-1 and RAG-2 genes has been deduced by characterizing two genomic clones (λ1469 and λ1591) containing overlapping fragments of chicken genomic DNA. The positions of the RAG-1 and RAG-2 open reading frames are indicated. Arrows indicate the transcriptional orientation (S, SacI; X, XbaI; L, SalI). Schematic comparisons of the amino acid sequences of chicken and mouse RAG-1 (B) and RAG-2 (C) were generated using the GAP and GAPSHOW programs of the Genetics Computer Group software package (University of Wisconsin). Amino acid sequences are shown as horizontal lines, with gaps denoted by openings in the horizontal lines and by small vertical lines. Identity between the chicken (top line) and mouse (bottom line) sequences is denoted at each position by a large vertical line.

not shown), and several genomic clones were isolated from a chicken genomic library. One genomic clone, λ1469, was found to hybridize at low stringency to a murine RAG-2 probe, and this clone was used in the initial characterization of the chicken RAG locus (Figure 1).

The organization of the RAG locus has been highly conserved during vertebrate evolution. The coding regions of both chicken and murine RAG-1 and RAG-2 are located on single large exons that are separated by approximately 9 kb of genomic DNA and are convergently transcribed. Sequence analysis of chicken RAG-1 demonstrated that the amino acid sequences of the chicken and mouse proteins are 75% identical. Conservation is most striking within the carboxy-terminal half of the molecule (86% amino acid identity), the same region in which homology was recently reported between RAG-1 and the yeast gene *HPR1* (Wang et al., 1990). The *HPR1* gene itself shares homologies with yeast DNA topoisomerase I, and mutations in *HPR1* increase intrachromosomal recombination and, to a lesser extent, gene conversion (Aguilera and Klein, 1988, 1990). Alignment of the chicken and murine RAG-2 amino acid sequences revealed 70% amino acid identity between the two sequences. Small blocks of higher conservation were identified throughout the RAG-2 sequence. These regions were used to search the GenBank and NBRF data bases, but no significant homologies to known proteins were identified. DNA fragments that were specific for the coding regions of chicken RAG-1 and RAG-2 were isolated from genomic clone λ1469. When hybridized to Southern blots containing chicken genomic DNA, each of these fragments hybridized only to single-copy genes (data not shown).

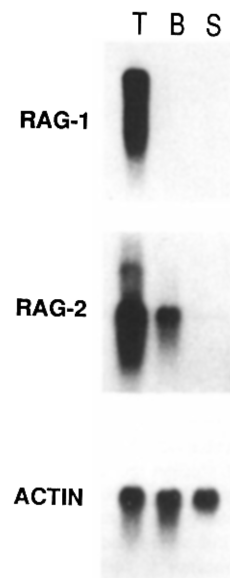


Figure 2. Northern Blot of Bursal, Thymic, and Splenic Lymphocyte RNA Isolated from a 4-Week-Old Chicken

A Northern blot was prepared using lymphocyte RNA isolated from the thymus (T), bursa (B), and spleen (S) of a 4-week-old chick, as described in Experimental Procedures. The Northern blot was hybridized sequentially with ^{32}P-labeled probes specific for RAG-1, RAG-2, and β-actin, and the resulting autoradiograms are shown.

RAG-2 but Not RAG-1 Is Expressed in Lymphocytes Isolated from the Bursa of Fabricius

Unlike mammals, chickens contain two organs for lymphoid development. As described above, the Ig repertoire of chicken B cells is generated by Ig-specific gene conversion events during B cell development in the bursa of Fabricius. The chicken T cell repertoire is generated by recombination of TCR V, D, and J elements during T cell development within the thymus (Tjoelker et al., 1990). TCR gene rearrangement is an ongoing process within the thymus during development, as T cell precursors continue to seed the thymus during the first several months of life (Coltey et al., 1989; W. T. M. and C. B. T., unpublished data). Lymphocytes isolated from the chicken thymus were found to express high levels of both RAG-1 and RAG-2 mRNA (Figure 2). In contrast, we found that bursal lymphocytes isolated from a 4-week-old chick expressed only RAG-2-specific mRNA. RNA isolated from bursal lymphocytes consistently failed to hybridize to RAG-1-specific probes. Lymphocytes isolated from the spleen failed to express either RAG-1 or RAG-2 mRNA.

Selective expression of RAG-2 mRNA has not been observed previously. It is unlikely to result from ongoing Ig gene rearrangement, because V(D)J recombination requires the expression of both RAG-1 and RAG-2 (Oettinger et al., 1990) and because we have been unable to detect the circular episome that results from IgL rearrangement during this period of development (McCormack et al., 1989a, 1989b). Furthermore, we found that at 4 weeks of age, bursal lymphocytes uniformly express surface Ig (Figure 3) and therefore no longer require Ig recombinase activity for V(D)J rearrangement.

These data argue that a specific mechanism has evolved in the regulation of the recombination activating locus that results in the selective expression of RAG-2. The bursal lymphocyte has been shown to be the site of a novel Ig gene recombination process that results in Ig diversification by intrachromosomal gene conversion (Reynaud et al., 1987, 1989; Carlson et al., 1990). To establish whether there is a correlation between selective RAG-2 mRNA expression and bursal-specific gene conversion, a series of experiments was carried out to correlate the known phenotypic characteristics of B cells undergoing gene conversion in the bursa of Fabricius with the expression of RAG-2.

Both Bursal Lymphocyte RAG-2 Expression and Ig Gene Conversion Begin between Days 15 and 18 of Embryogenesis

Rearranged V gene segments isolated from bursal lymphocytes on or before day 15 of embryogenesis fail to display evidence of Ig gene conversion (Thompson and Neiman, 1987; Reynaud et al., 1987; McCormack et al., 1989b). In contrast, bursal lymphocytes isolated from day 18 of embryogenesis display between one and four individual gene conversion events within their rearranged V gene segments (Reynaud et al., 1987; McCormack and Thompson, 1990b). These data suggest that the bursal-

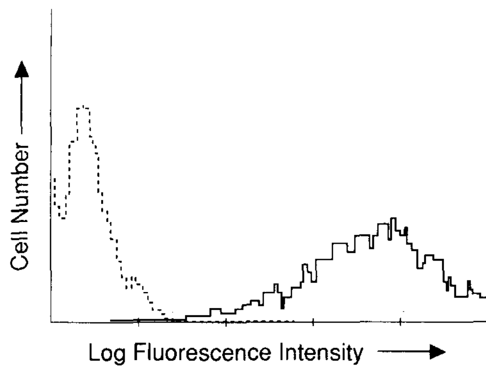


Figure 3. Expression of Surface Immunoglobulin in Bursal Lymphocytes Isolated from a 4-Week-Old Chicken

Chicken bursal lymphocytes isolated from a 4-week-old chick were incubated with rabbit anti-chicken IgG antibody. Binding was assayed by indirect immunofluorescence following staining with a FITC-conjugated donkey anti-rabbit IgG secondary antibody (solid line) using a fluorescence-activated cell sorter. Background staining was assayed by direct immunofluorescence of cells stained with the FITC-conjugated donkey anti-rabbit IgG antibody alone (dashed line).

specific process of Ig gene conversion begins in most bursal lymphocytes between days 15 and 18 of embryogenesis. After that time, B cells proliferating within the bursal environment continue to accumulate Ig gene conversion events until the bursa involutes at sexual maturity at 4–6 months of age.

To determine the temporal pattern of RAG-2 gene expression during B cell development in the bursa of Fabricius, RNA was isolated from the bursal anlage or from bursal lymphocytes at successive stages of B cell development, and analyzed for RAG-1 and RAG-2 expression by Northern blot analysis (Figure 4). We were unable to detect RAG-2 mRNA expression on or before day 15 of embryogenesis. However, RAG-2 gene expression was consistently observed at day 18 of embryogenesis and at all subsequent time points of bursal development.

In contrast to selective RAG-2 expression in the bursa of Fabricius, coexpression of RAG-1 and RAG-2 was observed in the developing thymus (data not shown). Expression of both RAG-1 and RAG-2 was detected in thymic lymphocytes as early as day 15 of embryogenesis. This coincides with the peak of the first major wave of TCR gene expression observed during avian thymic development (Coltey et al., 1989). No discordant expression of RAG-1 or RAG-2 was observed in thymic lymphocytes at any developmental time point.

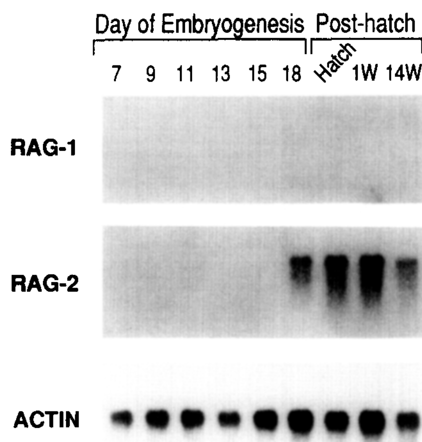


Figure 4. Analysis of RAG-1 and RAG-2 Expression during B Cell Development in the Bursa of Fabricius

RNA was isolated from the bursal anlage at embryonic days 7, 9, 11, and 13, and from bursal lymphocytes isolated by Ficoll-Hypaque separation on embryonic days 15 and 18, the day of hatching, and 1 week and 14 weeks after hatching. RNA was equalized from all samples, and Northern blots were prepared. Blots were hybridized sequentially with probes specific for RAG-1, RAG-2, and β-actin, and the resulting autoradiograms are shown. The integrity of the RAG-1 probe was confirmed by simultaneous hybridization to RNA isolated from the thymus (data not shown).

RAG-2 Is Selectively Expressed in Proliferating Bursal Lymphocytes

We have suggested previously that bursal lymphocytes that complete the gene conversion process enter a quiescent phase that renders them capable of migrating to the peripheral lymphoid organs (McCormack et al., 1989a). After hatching, an increasing proportion of bursal lymphocytes cease active clonal expansion and begin to migrate to the periphery. Therefore, if the RAG-2 gene product is involved in gene conversion, we would expect RAG-2 to be expressed in proliferating, but not quiescent, bursal lymphocytes. To test this hypothesis, proliferating and nonproliferating bursal populations were separated on the basis of the size differences between quiescent versus proliferating lymphocytes using counterflow centrifugation (elutriation) (Figure 5). RAG-2 mRNA expression was found to be confined to fractions of bursal lymphocytes containing a high proportion of cells that were progressing through the cell cycle, as measured by cell volume, nuclear volume, and nuclear DNA content. In contrast, subpopulations that were composed almost exclusively of quiescent lymphocytes (fractions 1 and 2) failed to express the RAG-2 gene product. Because it is possible that RAG-2 is selectively expressed only in proliferating B cells, we also examined proliferating B cells from the spleen. RNA isolated from proliferating splenic B cells failed to hybridize with either RAG-1- or RAG-2-specific probes (data not shown).

Selective RAG-2 Expression Is Observed in an Avian B Cell Line That Undergoes Gene Conversion In Vitro

If RAG-2 expression is required for gene conversion to occur, then a cell line that continues to undergo Ig-specific gene conversion in vitro would be expected to express RAG-2. Several years ago we screened a variety of avian B cell lines for evidence of ongoing gene conversion. One bursal lymphoma cell line, DT40, demonstrated diversity within its rearranged V_L gene segment (Thompson et al., 1987). Although the level of gene conversion within the DT40 cell line is at least 20-fold lower than the level observed during B cell development in the bursa of Fab-

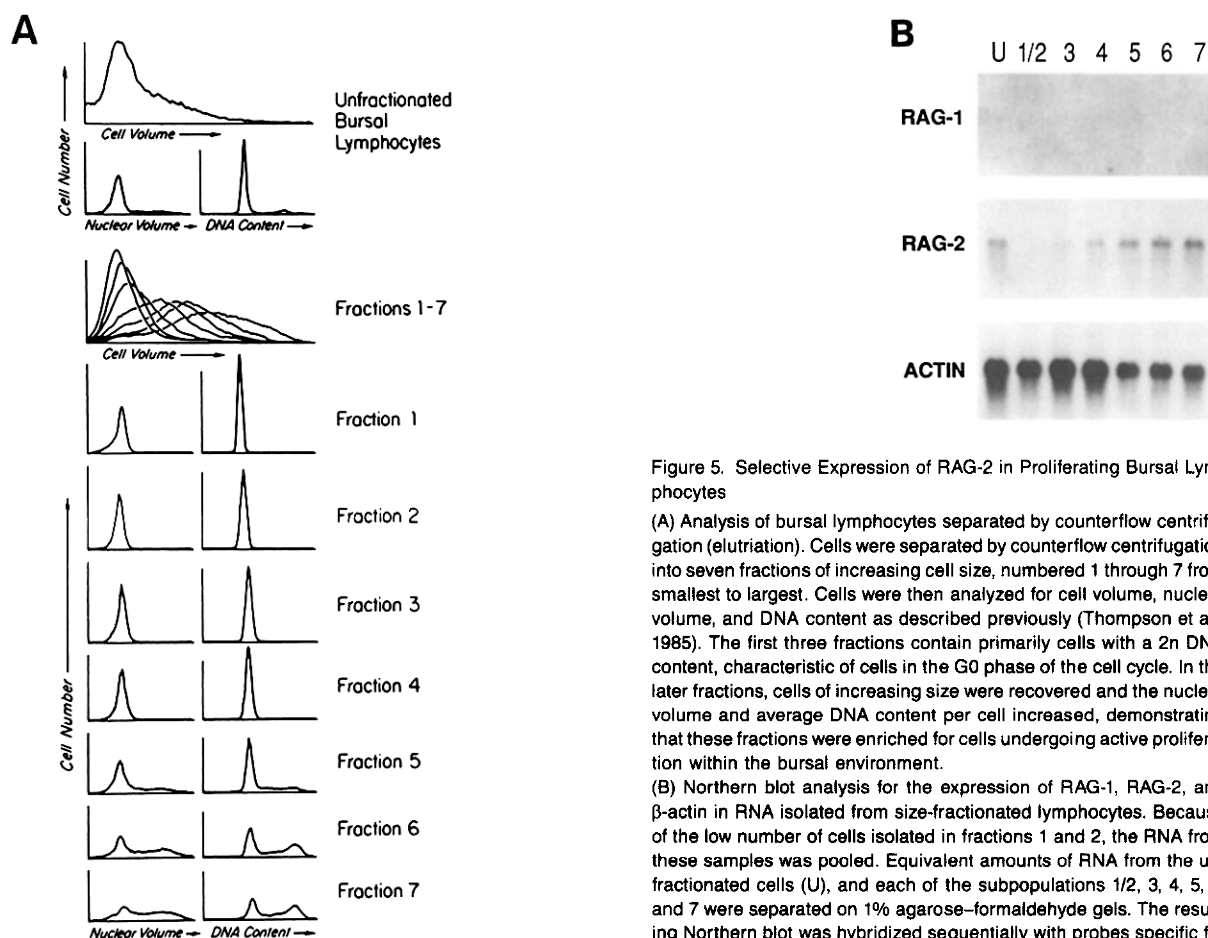


Figure 5. Selective Expression of RAG-2 in Proliferating Bursal Lymphocytes

(A) Analysis of bursal lymphocytes separated by counterflow centrifugation (elutriation). Cells were separated by counterflow centrifugation into seven fractions of increasing cell size, numbered 1 through 7 from smallest to largest. Cells were then analyzed for cell volume, nuclear volume, and DNA content as described previously (Thompson et al., 1985). The first three fractions contain primarily cells with a 2n DNA content, characteristic of cells in the G0 phase of the cell cycle. In the later fractions, cells of increasing size were recovered and the nuclear volume and average DNA content per cell increased, demonstrating that these fractions were enriched for cells undergoing active proliferation within the bursal environment.

(B) Northern blot analysis for the expression of RAG-1, RAG-2, and β-actin in RNA isolated from size-fractionated lymphocytes. Because of the low number of cells isolated in fractions 1 and 2, the RNA from these samples was pooled. Equivalent amounts of RNA from the unfractionated cells (U), and each of the subpopulations 1/2, 3, 4, 5, 6, and 7 were separated on 1% agarose–formaldehyde gels. The resulting Northern blot was hybridized sequentially with probes specific for RAG-1, RAG-2, and β-actin, and the resulting autoradiograms are shown. The integrity of the RAG-1 probe was confirmed by simultaneous hybridization to RNA isolated from the thymus (data not shown).

ricius, ongoing gene conversion has been documented during in vitro passage of this cell line (Buerstedde et al., 1990; Kim et al., 1990). In contrast, detailed analysis of several other bursal-derived cell lines failed to demonstrate diversity within their rearranged V gene segments (Carlson et al., 1990).

To test whether in vitro Ig gene conversion is associated with selective RAG-2 expression, chicken B and T cell lines were assayed for the expression of RAG-1 and/or RAG-2 during exponential growth in culture (Figure 6). The bursal lymphoma cell line DT40 was found to express a low but detectable level of RAG-2 mRNA. As with bursal lymphocytes, DT40 failed to express detectable levels of RAG-1 mRNA. In contrast, avian B cell lines that fail to undergo Ig diversification in vitro did not express either RAG-1 or RAG-2 mRNA. The TCR^+ chicken T cell lines we have examined also failed to express detectable levels of either RAG-1 or RAG-2 mRNA.

Discussion

In this report we have demonstrated that RAG-2 but not RAG-1 is expressed during B cell development in the bursa of Fabricius. The failure of bursal lymphocytes to coexpress RAG-1 and RAG-2 is consistent with previous observations that ongoing V(D)J recombination does not occur during B cell development in the bursa of Fabricius (Weill et al., 1986; McCormack et al., 1989a). The isolated expression of RAG-2 has not been observed previously. This selective RAG-2 expression does not appear to be due simply to differences between avian and mammalian species, because the overall organizations of the chicken and murine RAG loci are very similar, and ongoing V(D)J recombination in the chicken thymus is associated with coexpression of RAG-1 and RAG-2. Therefore, our results demonstrate that a specific mechanism(s) exists for differential expression of the RAG genes. The pattern of RAG-2 expression in bursal lymphocytes and avian B cell lines demonstrates that RAG-2 is selectively expressed in cells that undergo Ig-specific gene conversion. Chun et al. (1991; see accompanying article) have demonstrated that selective expression of RAG-1 occurs in the central nervous system. Thus, although RAG-1 and RAG-2 together are required for V(D)J recombination, our papers suggest that RAG-1 and RAG-2 each have an independent role(s) in other recombination processes.

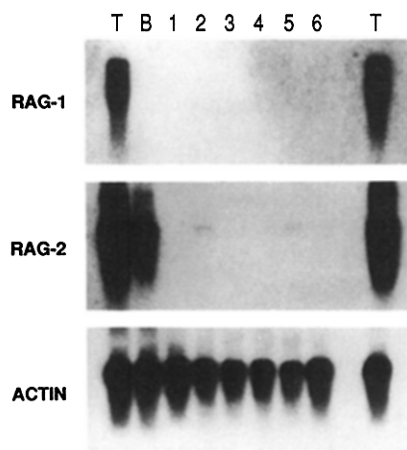


Figure 6. Expression of RAG-1 and RAG-2 in Bursal and Thymic-Derived Cell Lines

RNA samples (10 μg) from thymus (T), bursa (B), chicken bursal-derived cell lines 30LI (lanes 1 and 6) and DT40 (lanes 2 and 5), and chicken thymus-derived cell lines MSB-1 (lane 3) and UG-9 (lane 4) were separated on a 1% agarose–formaldehyde gel and transferred to nitrocellulose. The resulting Northern blot was hybridized sequentially with probes specific for RAG-1, RAG-2, and β-actin. The two bursal-derived cell lines have been extensively characterized, and DT40 (lanes 2 and 5) has been shown to undergo constitutive gene conversion in culture, whereas 30LI (lanes 1 and 6) does not display this ability (Buerstedde et al., 1990; Kim et al., 1990; Carlson et al., 1990). None of the cell lines expressed RNA that hybridized with the RAG-1-specific probe, but the DT40 cell line mRNA consistently hybridized at a low level with the RAG-2-specific probe (lane 2, passage 58; lane 5, passage 150). Analysis of four additional bursal lymphoma cell lines failed to demonstrate expression of either RAG-1 or RAG-2 (data not shown).

Our data suggest that the RAG gene products may play a wider role in the creation of Ig diversity than previously considered. Diversity within the primary immunologic repertoire has been shown to result from three processes: V(D)J recombination, V region replacement, and gene conversion. Based on our current and past results, it seems possible that the RAG locus is involved in the generation of Ig diversity by each of these processes. We consider each in turn.

V(D)J recombination is a complex process (for review see Lewis and Gellert, 1989) that normally requires the presence of two pairs of recombination signal sequences that are comprised of conserved heptamer and nonamer elements. A complete V(D)J recombination requires the activity of multiple gene products. It appears that RAG-1 and RAG-2 encode proteins that are the lymphoid-specific components of the V(D)J recombinase (Oettinger et al., 1990). In addition, other, ubiquitously expressed factors are involved. For example, the *scid* mutation, which results in the failure to form a coding joint during V(D)J recombination, also results in the failure to repair radiation-induced DNA damage (Fulop and Phillips, 1990). Together, the components of the V(D)J recombinase serve to create diversity as a result of combinatorial and junctional heterogeneity.

Another way in which B cells create Ig diversity is V region replacement by V(D)J recombination between an expressed Ig gene and a germline V gene segment (Reth et al., 1986; Kleinfield et al., 1986). Such recombination appears to result from the recognition of a conserved heptamer element present in the rearranged V gene segment immediately 5′ of CDR3. Thus the minimal sequence element required to target the V(D)J recombinase to a rearranged V segment is the presence of a heptamer sequence at the site of recombination.

Immunoglobulin diversification by gene conversion displays several features that suggest that gene conversion may also involve components of the Ig/TCR recombinase. Like V region replacement, gene conversion is highly restricted to an expressed V gene segment (Thompson and Neiman, 1987). Other genes that undergo equivalent rates of lymphoid-specific transcription and that are located in close proximity to homologous donor templates fail to undergo gene conversion in developing bursal lymphocytes (Thompson, 1989). We have found that three heptamer sequences similar to those present in Ig and TCR recombination signal sequences have been conserved in the avian V_L gene segment (McCormack et al., 1989c). One of these is immediately 5′ of CDR1, a second is at the FR2–CDR2 boundary, and the third is located immediately 5′ of the CDR3 domain. Similar heptamer sequences are located 5′ of CDR3 in the functional V_H element and within the coding region of the D elements (Reynaud et al., 1989).

Reynaud et al. (1987) have speculated that the heptamer sequence 5′ of CDR3 might play a role in the high frequency of gene conversion observed in that domain. In support of this hypothesis, recent data have shown that nucleotides that encompass the three conserved heptamer sites in the chicken V_L region are involved in gene conversion tracts at a statistically higher frequency than other sequences within the V region (McCormack and Thompson, 1990b). It has been postulated that heptamer-specific strand nicking or cleavage might be involved in the initiation of a gene conversion event, and a molecular model based on these observations has recently been proposed (McCormack and Thompson, 1990b). The observation that RAG-2 is selectively expressed in bursal lymphocytes suggests that RAG-2 may be the V(D)J recombinase component involved in the gene conversion process. Based on the existing data, it is tempting to speculate that RAG-2 may encode or regulate a heptamer-nicking activity.

As suggested above, the recombination events that serve to create primary immunologic repertoires in different species appear to share some common *cis*-regulatory elements and *trans*-acting mediators. In particular, the RAG locus seems to be involved not only in V(D)J recombination, but also in Ig gene conversion during avian B cell development. The conservation of the RAG locus during evolution suggests that the multiple roles of RAG genes in Ig diversification may have evolved early during vertebrate evolution. Consistent with this hypothesis, some avian species have been shown to use combinatorial diversity to generate a primary immunologic repertoire

(McCormack et al., 1989c), and rabbits appear to create an IgH repertoire primarily by gene conversion (Becker and Knight, 1990; Roux et al., 1991).

In summary, our data suggest that the ability of avian B cells to generate an Ig repertoire by gene conversion results from an alteration in the expression of genes involved in Ig gene rearrangement. A simple model can be proposed to account for the relationship of Ig gene rearrangement and gene conversion. The expression of RAG-2 in cells undergoing either V(D)J recombination or Ig gene conversion suggests that RAG-2 is involved in initiating the assembly of a recombination intermediate. It is possible that both Ig-specific recombination events involve a common intermediate. In the presence of RAG-1, such an intermediate would result in the production of a V(D)J rearrangement. In the absence of RAG-1, V(D)J rearrangement does not occur and the recombinational intermediate is resolved to leave a gene conversion tract.

Experimental Procedures

Cells and Cell Lines

Bursal and thymic lymphocytes were isolated as single-cell suspensions by gently teasing apart the bursa of Fabricius or thymus, and filtering the cells through nylon mesh to remove clumps. Contaminating nonlymphoid cells were removed by centrifugation of the cell suspension over Ficoll-Hypaque. Greater than 95% of the cells prepared from either organ in this manner stained positively with monoclonal antibodies specific for cells of the respective organ (data not shown). For embryonic samples isolated prior to day 15 of embryogenesis, it was not possible to efficiently separate the lymphoid component of the bursa; therefore, RNA was prepared from the total bursa or the posterior cloaca. To obtain size-dependent subpopulations of cells, single-cell suspensions were loaded into a counterflow centrifuge (elutriator), and cell subpopulations of increasing mean cell volume were sequentially isolated as previously described (Thompson et al., 1985). Cell volume, nuclear volume, and average DNA content of cells from each fraction were determined as previously described (Thompson et al., 1985). Avian B cell lines derived from the bursa of Fabricius following retroviral infection on the day of hatching were isolated and propagated as previously described (Thompson et al., 1987). The avian T cell lines were grown and passaged using standard conditions (Chan et al., 1988).

FACS Analysis

One million bursal lymphocytes isolated from a 4-week-old chicken were stained with 2 μl of rabbit anti-chicken IgG (Cappel-Worthington, 2 mg/ml) and incubated on ice for 30 min. After two washes with 1 ml of PBS, 0.1% azide, the cells were stained with 5 μl of FITC-donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., 1.4 mg/ml) for 30 min on ice. The cells were washed twice with 1 ml PBS, 0.1% azide, and analyzed immediately after staining using a Becton-Dickinson FACScan.

Avian RAG-1/RAG-2 Cloning

The 0.9 kb human RAG-1 fragment was labeled by nick translation and used to probe nitrocellulose lifts of a primary plating of a chicken λFIX genomic library (the generous gift of K. Conklin, University of Minnesota). Blots were prehybridized at 42°C in 50% (v/v) formamide and 5× SSC, 1× Denhardt's solution, 25 mM sodium phosphate (pH 6.5), 250 μg/ml torula RNA. Hybridizations were carried out for 16 to 20 hr under identical conditions except for the addition of 10% (w/v) dextran sulfate and labeled probe at 10^6 dpm/ml. After hybridization, blots were rinsed twice at room temperature in 2× SSC, 0.1% SDS and then washed for 20 min in 0.2× SSC, 0.1% SDS at 56°C. Blots were then air dried and exposed to X-ray film for 8 to 20 hr at −70°C with intensifying screens. Following initial isolation, RAG-1^+ genomic clones were rescreened with the 2.1 kb mouse RAG-2 cDNA probe. A resulting RAG-1^+/RAG-2^+ clone (λ1469) was subcloned in two SalI fragments into pBluescript SK(−), from which RAG-1- and RAG-2-specific probes were isolated.

Probes

The following probes were utilized: a 0.9 kb XhoI–HindIII human RAG-1 probe derived from the 3′ end of the coding region (Schatz et al., 1989), and a 2.1 kb murine RAG-2 cDNA probe containing the entire coding region and flanking 5′ and 3′ untranslated regions (Oettinger et al., 1990). Chicken-derived probes included: a 1.1 kb SacI fragment of RAG-1, isolated from the middle of the coding region; a 1.8 kb XbaI–SalI fragment, which contains two-thirds of the RAG-2 coding region at the 3′ end and the 3′ untranslated region; and a 0.59 kb HindIII fragment from the 3′ untranslated region of the β-actin gene (Cleveland et al., 1980).

Northern Blots

For analysis of gene-specific mRNA levels, RNA was extracted using guanidinium isothiocyanate (Chirgwin et al., 1979). Samples were equalized for ribosomal RNA and the equalization confirmed by ethidium bromide staining of RNA samples separated on a nondenaturing 1% agarose gel. Equalized RNA samples (2–10 μg) were separated on 1% agarose–formaldehyde gels and transferred to nitrocelluose. Filters were then hybridized sequentially with gene-specific probes for RAG-1, RAG-2 and β-actin. Resulting autoradiograms were exposed for 2 to 48 hr. The relative molecular masses of the mRNAs that hybridized to each of the probes agreed with previously published values (Schatz et al., 1989; Oettinger et al., 1990; Cleveland et al., 1980).

Acknowledgments

We thank our colleagues Jeff Leiden, Eric Humphries, and John Lowe for helpful discussions and review of the manuscript, and Beverly Burck for preparation of figures. This work was supported by Public Service Award CA48023 to C. B. T., and CA51466 to D. B. from the National Institutes of Health, and by fellowships from the Whitaker Health Sciences Fund (M. A. O.) and the Life and Health Insurance Medical Research Fund (D. G. S.).

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Received November 2, 1990; revised November 20, 1990.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are M58530 (chicken RAG-1) and M58531 (chicken RAG-2).