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Immunology

Rearrangement of immunoglobulin light chain genes in the chicken occurs prior to colonization of the embryonic bursa of Fabricius

(chicken B-cell differentiation/gene conversion/prebursal stem cells)

ANTTI MANSIKKA*†, MINNA SANDBERG‡, OLLI LASSILA*, AND PAAVO TOIVANEN*

Departments of *Medical Microbiology and ‡Medical Biochemistry, Turku University, SF-20 520 Turku, Finland

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ABSTRACT **We have applied polymerase-chain-reaction-directed immunoglobulin gene analysis to study the embryonic differentiation of chicken B cells. Immunoglobulin light chain DNA segments in the rearranged configuration were amplified from cells of the intraembryonic mesenchyme as early as day 7 of incubation. We showed by sequencing that the rearranged variable region genes in these early B-cell progenitors were not different from the germ-line Vλ1 gene (the single functional light chain variable region gene in chickens). In the bursal B lymphocytes, on the other hand, clear gene conversion events were first observed at day 15 of embryonic development. The present data indicate that rearrangement of light chain genes in the chicken occurs independently of the bursa of Fabricius and that diversification of the variable region begins only later, when the surface immunoglobulin-positive B cells are proliferating in the bursal follicles.**

The bursa of Fabricius of the chicken is colonized by blood-borne B-cell precursors (1) between days 8 and 14 of embryonic development (2). These prebursal stem cells are first detected using reconstitution assays at day 7 in the hemopoietic foci of intraembryonic mesenchymal tissue (3); whereas the first IgM-positive B cells are observed in the bursa at day 12 of incubation (4).

The chicken has a unique way of generating immunoglobulin light (L) and heavy chain diversity. The L chain locus has only one functional variable (V) region gene (Vλ1) that is expressed. During B-cell development, the Vλ1 gene is diversified by gene conversion, using a pool of pseudogenes as donors of DNA segments (5, 6). A similar strategy is used for diversification of the heavy chain repertoire (7).

According to histological analysis, the prebursal stem cells appear to give rise to both B cells and granulocytes in the bursal anlage (2); however, several recent data suggest that the stem cells may become committed to B-cell lineage prior to bursal colonization (8–11). In this paper, we analyzed immunoglobulin L chain gene rearrangement during the early embryogenesis. By using polymerase chain reaction (PCR) amplification, we showed that early B cells with rearranged L chain genes exist in the intraembryonic mesenchyme at day 7 of embryonic development.

MATERIALS AND METHODS

Chickens. In these experiments, chickens of the H.B15 strain were used.

Nucleic Acid Hybridization Methods. Cellular RNA was prepared according to Schibler *et al.* (12) and enriched for poly(A)$^+$ RNA (13). Fractionation of the RNA samples was carried out as described by Lehrach *et al.* (14). Transfer onto GeneScreen membranes (New England Nuclear) and hybridization conditions were according to instructions from the manufacturer. *In situ* hybridization to embryonic bursal tissue sections was done as described (15, 16), with the modification of Maples (17). In all hybridization experiments, the V+C L chain probe (where C is constant) (18) was labeled according to Feinberg and Vogelstein (19). The radioactive isotopes were supplied by Amersham.

PCR. Cells were prepared from the intraembryonic mesenchyme and the bursal tissue as described (3, 16) and enriched for mononuclear cells on a Ficoll/Isopaque gradient (Pharmacia). The genomic DNA was prepared using standard methods (20). The thermal cycler and reagents for PCR (21) were supplied by Perkin–Elmer/Cetus. To obtain high sensitivity, the amplification was done using four nested primers (three pairs) in three successive rounds of amplification (20 cycles per round). The primers were selected according to published sequence data of the chicken L chain locus (5, 8). Primer: L1, 5′-CG GAA TTC AGC CTG CCG CCA AGT CC-3′; L2, 5′-CC GAA TTC TCA GGT TCC CTG GTG CAG GCA-3′; L3, 5′-CC TCT AGA GGA AGA AAG ACC GAG ACG-3′; L4, 5′-CC TCT AGA CGA CAA AAT GTC ACA ATT TCA CG-3′. In the rearranged L chain locus, use of these primers results in amplification of a 0.4-kilobase (kb) DNA fragment that contains the whole L chain V region and the V–J joint area (where J is joining). For subsequent sequence analysis, the specific amplification product was subcloned into M13mp19 vector. Twenty random recombinants from each ligation were sequenced (22). Reagents for sequencing were supplied by United States Biochemical (Sequenase, version 2.0).

RESULTS

Rearrangement of the Immunoglobulin L Chain Genes in the Intraembryonic Mesenchyme. We carried out DNA amplification (PCR) to establish when the B-cell progenitors start to rearrange their L chain genes. Lymphoid cells were prepared from the hematopoietic foci of intraembryonic mesenchyme at days 3, 5, 7, and 9 of incubation, and their DNA was subjected to PCR amplification. The expected 0.4-kb amplification product indicating L chain rearrangement was consistently observed at days 7 and 9 of incubation, whereas repeated attempts using day 3 or 5 mesenchymal cells were negative (Fig. 1).

Expression of L Chain Genes in the Embryonic Bursa of Fabricius. To study the emergence and anatomical distribution of the developing B cells, we analyzed the expression of L chain mRNA in the embryonic bursa of Fabricius. For this purpose, bursal mRNA was prepared at days 9, 11, 12, 13, 15, 17, 19, and 21 of embryonic development and subjected to

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Abbreviations: PCR, polymerase chain reaction; V, J, C, variable, joining, and constant, respectively; L, light.

†To whom reprint requests should be addressed at: Department of Medical Microbiology, Turku University, Kiinamyllynkatu 13, SF-20520 Turku, Finland.

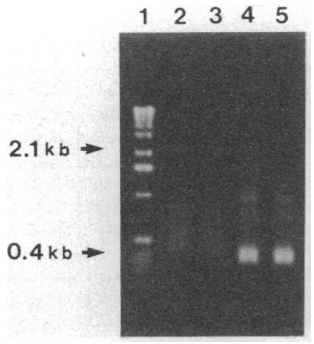

FIG. 1. Immunoglobulin L chain rearrangement in intraembryonic cells. Genomic DNA was prepared from mononuclear cells of intraembryonic mesenchyme at days 3, 5, 7, and 11 of incubation. After PCR amplification, 5 μl of the reaction mixture was fractionated on a 1% agarose gel. As a result, the 0.4-kb amplification product identified L chain rearrangement in day 7 and 9 intraembryonic cells (lanes 4 and 5, respectively), whereas repeated attempts using day 3 and 5 mesenchymal cells (lane 2 and 3) were negative. Molecular size markers (1-kb ladder) were included in lane 1.

Northern blot analysis. In addition, embryonic bursal tissue sections were hybridized *in situ* using the V+C L chain probe.

Fig. 2 shows a typical Northern blot hybridized with the V+C probe. The first clear hybridization signal to the 1.2-kb L chain mRNA was observed at day 12 of incubation. Between days 13 and 17 there was a vigorous increase of L chain mRNA concentration in the bursal tissue. During the late embryonic period until hatching (days 17, 19, and 21), the mRNA level in the bursal tissue remained relatively constant.

The increase of L chain mRNA expression in the bursal tissue was certainly due to the proliferation of B cells in the bursal follicles, known to start around day 12 of embryonic development (4). However, our *in situ* hybridization experiment showed that there is also an increase of cellular L chain mRNA concentration in the follicular B cells after day 13. In this experiment (Fig. 3), no cells with L chain expression were observed in the bursal follicles at day 13 of incubation. At day 15 most follicles were still negative, whereas at day 17 strong L chain mRNA expression was observed in most follicles. Interestingly, the increase in cellular L chain mRNA expression appeared to take place in multiple clusters of a few adjacent cells and then rapidly spread to practically all B cells of the individual follicle.

L Chain Diversification in the Prebursal and Bursal B Cells. To compare L chain diversification in the prebursal and bursal B cells, we also amplified L chains from embryonic bursal cells at days 11, 13, 15, 17, and 19 of incubation (data not shown). Sequence analysis of the L chains derived from the mesenchymal and bursal cells is shown in Fig. 4. L chain

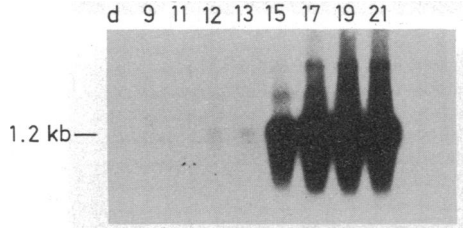

FIG. 2. Expression of L chain mRNA in the embryonic bursa of Fabricius. Bursal RNA was prepared from several H.B15 chicken embryos at various stages of embryonic development. Poly(A)$^+$ RNA (2 μg per lane) was fractionated and transferred onto hybridization membrane. The Northern blot was hybridized with the V+C probe. A faint hybridization to the 1.2-kb L chain mRNA was observed at days 12 and 13 of incubation. Between days 13 and 17, a clear increase in the bursal L chain mRNA content occurred.

sequences from day 7 and day 9 mesenchymal B cells, as well as those from the bursal cells up to day 13 of incubation, were of the germ-line Vλ1 type. The first clear gene conversion events were observed in bursal L chains at day 15 of incubation.

When we examined the sequence of the V–J joints, about one-third of the L chains derived from the day 7 and 9 mesenchymal B cells and from the bursal B cells up to day 13 were in-frame and two-thirds were out-of-frame. This indicates inaccuracy of the V–J joining event (8). Between days 13 and 15 a selective amplification of B cells having a productively rearranged L chain locus had occurred, since there was a clear increase in the proportion of L chains with in-frame V–J joints; among the 20 sequences from the day 15 embryonic bursa of Fabricius 16 (80%) were in-frame and 4 (20%) were out-of-frame. During the later embryonic period this finding was even more pronounced, since all 20 L chains from day 17 and 19 bursae were in-frame.

DISCUSSION

The most important finding of the present work is that B-cell progenitors in the intraembryonic mesenchymal tissue have started to rearrange their L chain genes already at day 7 of incubation, at a time when the bursal colonization by the hemopoietic B-cell precursors is not yet initiated. Since no specific cellular markers have been described that differentiate the prebursal stem cells from other mesenchymal cells, we cannot rule out that the day 7 intraembryonic cells with immunoglobulin gene rearrangement could be different from the prebursal stem cells migrating to the bursal anlage. Thus, these precursors could give rise to a bursa-independent lineage of B cells, analogously to the omentum-derived CD5$^+$ and bone marrow-derived CD5$^-$ B cells in mammals (24). It is also possible that only a fraction of stem cells would rearrange the immunoglobulin genes in the intraembryonic mesenchyme, whereas most would rearrange their genes during or after the migration. With these reservations, an obvious conclusion of the present finding is, however, that the embryonic bursa of Fabricius may be colonized by precommitted B-cell precursors that have started to rearrange their immunoglobulin genes before the bursal colonization.

The bursa of Fabricius appears to be a site for specific amplification of B cells with productively rearranged immunoglobulin genes, as shown by McCormack *et al.* (8) and confirmed in this paper. Since the V–J joining event is a random process, B cells with nonproductive L chain gene rearrangements are in the majority in the intraembryonic mesenchyme and also in the embryonic bursa itself until the follicular proliferation of B cells has started. Between days 13 and 15, the frequency of B cells with productive L chain rearrangements had increased up to 80% and, at day 17 and thereafter, L chain sequences with nonproductive V–J joining events were not observed. Thus, colonization of the bursal anlage appears not to be affected by a successful completion of the L chain gene rearrangement process in the stem cells. However, only those B cells with in-frame V–J joints and probably with surface immunoglobulin expression may proliferate in the bursal follicles.

The present sequence data also suggests that activation of the L chain diversification and induction of the follicular B-cell proliferation may be parallel events. As already pointed out, there is a rapid increase in the frequency of L chain sequences with in-frame V–J joints after day 13 of incubation. At day 13, the L chain sequences were still of the germ-line Vλ1 type. Importantly, at day 15 all those sequences with gene conversion events had an in-frame V–J joint (16/20 sequences), whereas among those with germ-line V regions (4/20 sequences), two had an out-of-frame V–J

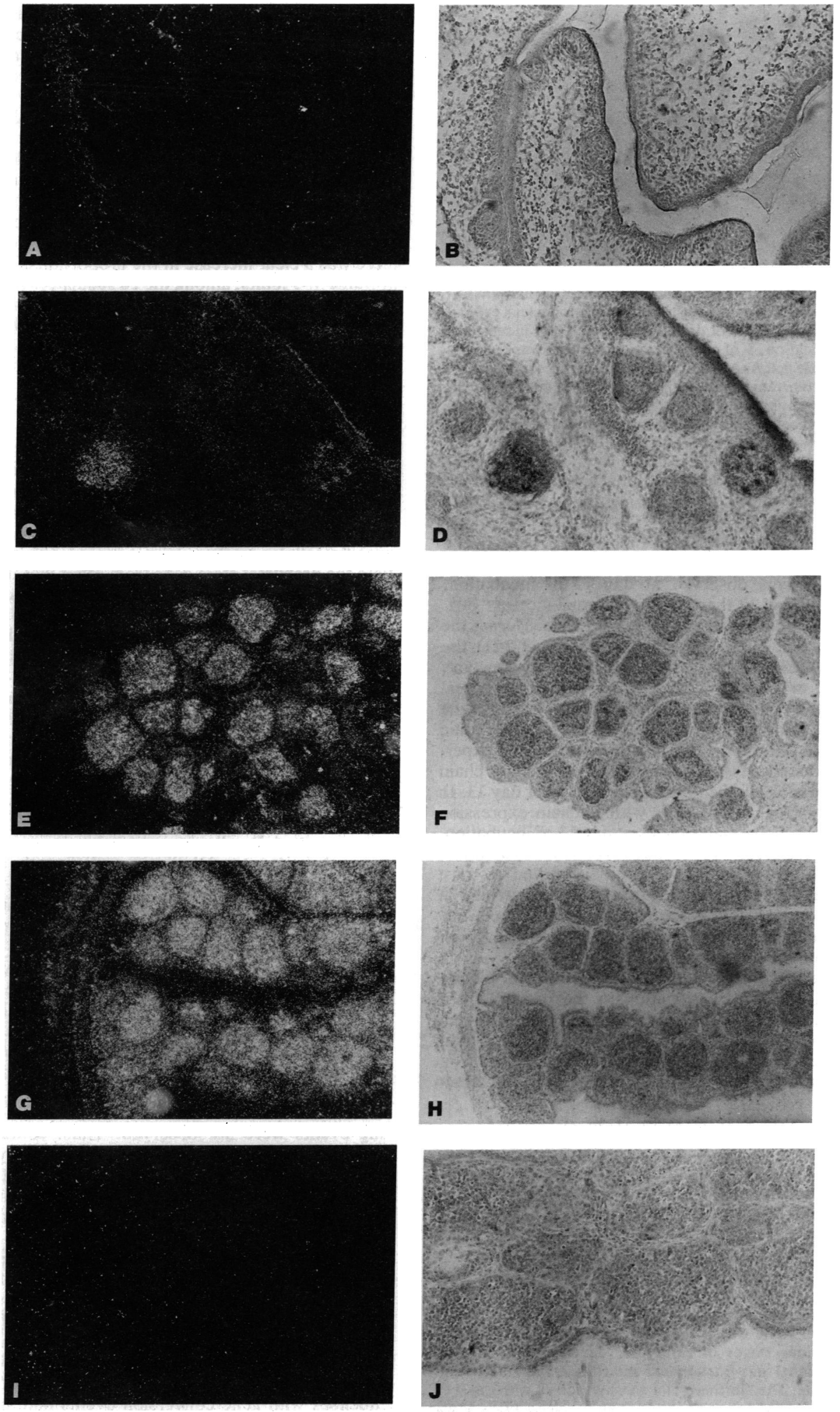

FIG. 3. *(Legend appears at the bottom of the opposite page.)*

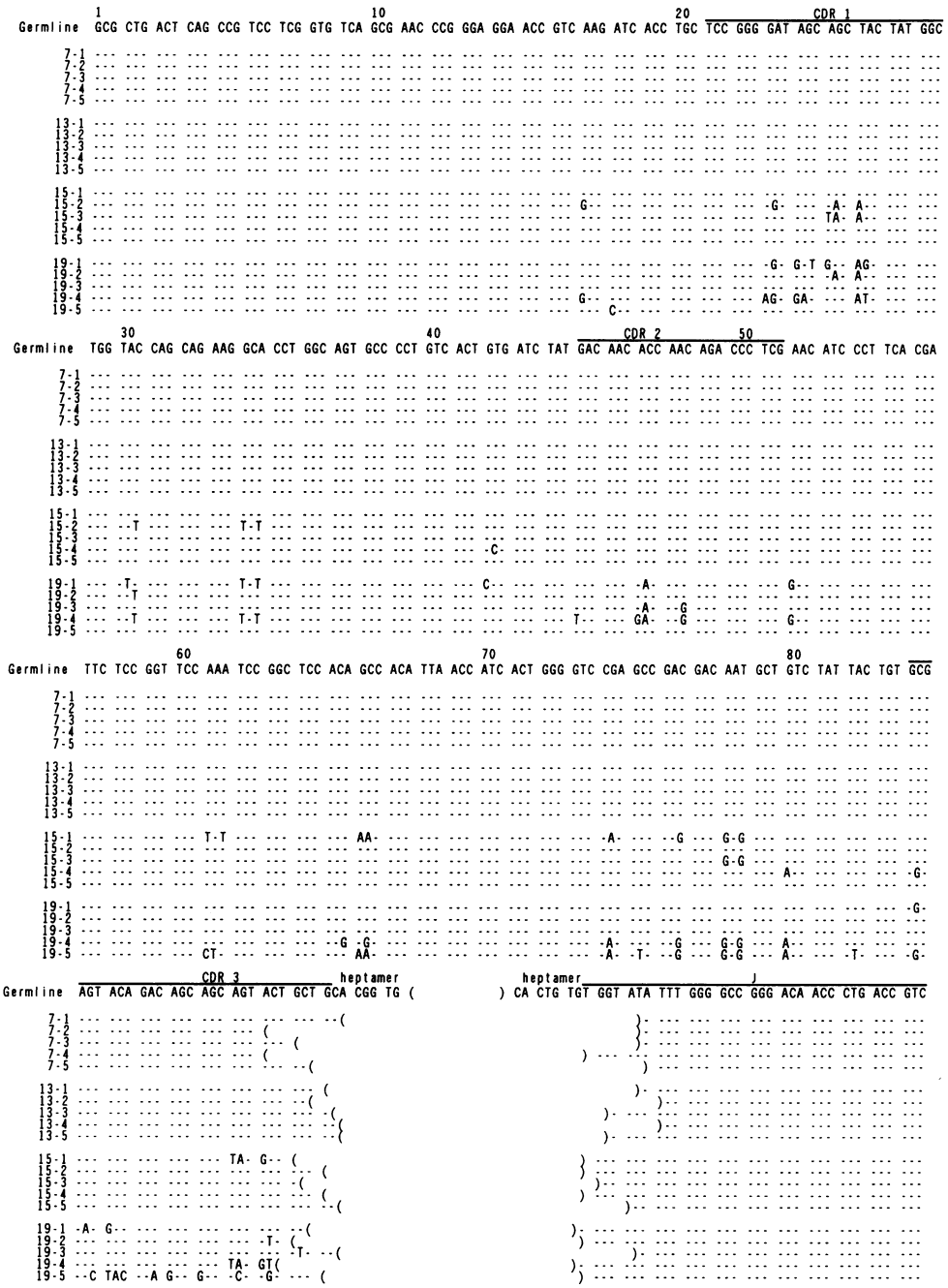

FIG. 4. L chain sequences from chicken embryos. L chain sequences in the rearranged configuration were amplified with PCR from day 7 intraembryonic cells and from bursal cells at days 13, 15, and 19 of incubation. The germ-line Vλ1 and J sequences of the H.B15 line have been determined (23). Dashes indicate nucleotide identity to the germ-line Vλ1 sequence. Positions of the three complementary determining regions (CDRs) are indicated. Numbering of codons starts at the beginning of V region. Positions of the putative heptamer and nonamer recombination signals are marked with bars.

joint. Thus, the follicular B cells had not divided extensively before the activation of the gene conversion machinery occurred. These results do not exclude that the follicular stem cells with germ-line V regions are maintained, while producing a progeny of B cells with mutated immunoglobulin loci during growth of the clones. This kind of clonal diversification by gene conversion is known to occur in the mating type switching of yeast cells (25).

Increase of the cellular L chain mRNA concentration in the follicular B cells after day 13 of embryonic development may

FIG. 3 (*on opposite page*). *In situ* hybridization to embryonic bursal tissue sections. Bursal tissue sections were hybridized *in situ* with the V+C probe to study the presence of L chain mRNA expressing B lymphocytes. (*A*, *C*, *E*, *G*, and *I*) Dark-field image. (*B*, *D*, *F*, *H*, and *J*) Bright-field images corresponding to *A*, *C*, *E*, *G*, and *I*, respectively. (*A* and *B*) Typical hybridization pattern of the V+C probe to day 13 bursal tissue sections. At this developmental stage, no clear hybridization signal was observed. At day 15 a few follicles gave a positive hybridization signal; interestingly, an increase in L chain mRNA expression first occurred in multiple clusters of a few adjacent B cells (compare the two positive follicles in *C* and *D*). At day 17 (*E* and *F*) and day 19 (*G* and *H*), most follicles were strongly positive. (*I* and *J*) The level of unspecific background was demonstrated by hybridizing serial bursal tissue (day 19 bursa of Fabricius) sections with similarly labeled λ phage restriction fragments. (×45–90.)

occur concomitantly with the activation of L chain diversification. Only L chains with germ-line V regions were derived from bursal cells at day 13 of incubation, whereas the first clear gene conversion events in the L chain V regions were observed at day 15. On the other hand, according to *in situ* hybridization, the increase of cellular L chain mRNA expression first occurred at isolated foci of a few adjacent B cells. Moreover, earlier studies have revealed that, after day 14 of incubation, B cells with certain antigen-specific reactivities may arise in a sequential manner at isolated foci in single bursal follicles (4).

The present data suggest that the early B cells may express the germ-line immunoglobulin molecule until the first gene conversion events appear in the L chain V regions between days 13 and 15 of incubation. What function might the germ-line immunoglobulin receptor have on the cell surface? Interestingly, reconstitution of the bursal follicles of chemically B-cell-ablated newly hatched chicks by B-cell precursors is inhibited by anti-IgM allotype antiserum (26). Also, administration of antibodies specific for μ heavy chains into very early chicken embryos leads into suppression of B-cell formation (27). Thus it is feasible to speculate that the germ-line immunoglobulin molecule could function in arresting functional B cells into the follicles and/or mediate the differentiation and growth signal(s) from the follicular microenvironment to the stem cells. This is actually what was first suggested by Jerne (28), since in a hypothesis about the bursa of Fabricius as a mutant-generating organ he proposed that lymphocytes with germ-line molecules would stick to self-antigens and be stimulated to divide. Thereafter, somatic diversification of immunoglobulin receptors would result in mutants that retain the self-reactivity and are subsequently suppressed (leading to self-tolerance) or that acquire new antigen-binding properties, escape the primary organ, and generate the peripheral, antigen-sensitive B-cell compartment. The bursal role as a mutant-generating organ is conceivable, since the chicken B-cell repertoire is mostly generated in an antigen-independent fashion (4). Moreover, according to our studies (23), gene conversion is going on quite slowly in chickens that have been surgically bursectomized at 60 hr of incubation.

In summary, the present data are in line with the hypothesis that induction of the chicken B-cell differentiation is a bursa-independent process and may take place in the intraembryonic hemopoietic foci, as in mammals (24, 29). Thereafter, the differentiation pathway in these species diverges. In the mammals, lymphoid stem cells are produced from multipotent hemopoietic precursor cells throughout life, first in the embryonic liver and spleen and later in the bone marrow; and immunoglobulin diversity is generated mostly by fresh recombination events using a large pool of functional V genes (30, 31). In the chicken, the generation of prebursal stem cells occurs only during a limited time period and the stem cells seed to various lymphoid organs through the circulation. A proportion of the stem cells appear to migrate into the bursal follicles, where those cells having productively rearranged immunoglobulin genes and surface immunoglubulin are trapped. In the follicular microenvironment, they are induced to divide and immunoglobulin diversification by gene conversion is activated. Thus, the early differentiation of the B-cell lineage appears similar in the avian and mammalian species; however, a special mutant-generating organ is utilized for diversification of the B-cell repertoire in the birds, due to the unique structure of immunoglobulin gene loci.

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