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Isolation of chicken major histocompatibility complex class II (B-L) β chain sequences: comparison with mammalian β chains and expression in lymphoid organs

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By cross-hybridization in low stringency conditions, using a probe derived from an HLA-DQβ cDNA clone, we have isolated several chicken genomic DNA clones. These clones were mapped to the major histocompatibility complex (MHC) of the chick (B complex) by virtue of their ability to detect restriction enzyme length polymorphisms between congenic lines of chicken. Evidence was obtained for the presence of at least three B-Lβ genes in the chicken genome. The B-Lβ genes are transcribed specifically in tissues containing cells of the B lymphocyte and myeloid lineages and expressing the B-L antigens. Exons encoding the β1, β2 and transmembrane domains of a B-Lβ chain have been identified with 63, 66 and 62% similarity with the HLA-DQβ sequence. This first isolation of an MHC class II gene outside of the mammalian class provides insight into the evolution of MHC genes based on the comparison of avian and mammalian class II β chain amino acid and nucleotide sequences.

Key words: interspecies cross-hybridization/DNA sequencing/evolution/major histocompatibility complex/Northern blot

Introduction

The major histocompatibility complex (MHC) was first discovered in mice as a genetic region governing the ability of transplants of neoplastic or normal tissue to succeed (reviewed in Klein *et al.*, 1983). The second animal species in which an MHC was subsequently described is the chicken (Briles *et al.*, 1950; reviewed in Longenecker and Mosmann, 1981) in which the B locus was first detected as a blood group system. An equivalent locus was discovered later in man, the HLA complex (reviewed in Dausset, 1981). Similar loci have now been described in all mammalian species examined as well as in amphibians, and evidence points to its existence in all vertebrate classes. Most of the immunologic phenomena linked to the murine H-2 and human HLA complexes are also associated with the chicken B complex, e.g. resistance to neoplastic diseases (Pazderka *et al.*, 1975; Collins *et al.*, 1977), control of T cell–B cell interactions (Toivanen and Toivanen, 1977) and susceptibilty to autoimmune diseases (Bacon and Rose, 1979).

Genes of MHC complexes encode polymorphic cell surface glycoproteins involved in the control of various aspects of immune responses. The class I antigens (HLA-A, B, C in man, H-2K, D, L in mice and B-F in chicken) are the classical transplantation antigens expressed by virtually all nucleated cells that function as restricting elements for cytotoxic T lymphocytes. The class II or Ia antigens (HLA-DR, DP, DQ in man, H-2, I-E and I-A in mice, B-L in chicken) are defined as the products of immune response genes which restrict antigen presentation to T helper lymphocytes. They are composed of two glycosylated chains, α (33-34K) and β (28-29K), noncovalently associated at the surface of B lymphocytes and cells of the myeloid lineage (macrophages and dendritic cells). In mouse and man, the MHC class II antigens have been extensively studied by serological, cellular and biochemical techniques, and the corresponding class II genes have been isolated and characterized in detail (reviewed in Hood *et al.*, 1983; Auffray and Strominger, 1986).

It is only recently that polyclonal and monoclonal antibodies have permitted the study of the structure, biosynthesis and polymorphism of the chicken B-L antigens (Crone *et al.*, 1981; Ewert *et al.*, 1984; Guillemot *et al.*, 1986). These reagents have also been used to study expression of B-L antigens during ontogeny (Ewert and Cooper, 1978; Guillemot *et al.*, 1984). With the use of the quail–chicken nucleolar marker (Le Douarin, 1969) in thymic interspecies transplantation schemes, it was possible to show that in the thymus two cell types of different embryonic origin express the B-L antigens (Guillemot *et al.*, 1984). These are the thymic epithelial cells and the dendritic cells differentiating from hemopoietic precursors which migrate in the thymic primordium in successive waves during embryonic development (Jotereau *et al.*, 1980).

This model offers an unique opportunity to dissect the series of early events which determine the ability to bind a functional immune response in the context of self MHC class II antigens. Moreover, since birds and mammals have evolved separately for more than 250 million years, a detailed study at the molecular level of the chicken B-L antigens should enhance our knowledge of structure–function relationships of class II antigens.

We have started a molecular analysis of the B complex by attempting to isolate the immune response B-Lβ genes. In this paper, we describe a successful approach using an HLA-DQβ probe in interspecies cross-hybridization, and we provide insight into the evolution of the MHC class II genes from comparisons of mammalian and avian sequences both at the amino acid and nucleotide levels.

Results

Isolation of chicken genomic clones using an HLA-DQβ probe

As shown in Figure 1, we have chosen as probe a 496 bp *Sac*I–*Eco*RI fragment derived from an HLA-DQβ clone

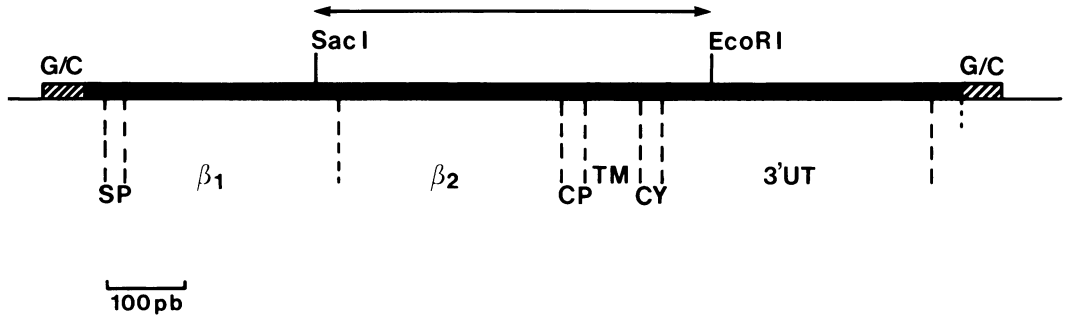

Fig. 1. The 496 bp *Sac*I–*Eco*RI DNA fragment was derived from the cDNA clone pIIβ-1 (Larhammar *et al.*, 1982). The GC tails generated during the cloning procedure are indicated by hatched boxes. The various parts of the DNA clone are delineated by a vertical dashed line; SP: signal peptides; β1, β2: first and second extracellular domains; CP, TM, CY: connecting peptide, transmembrane domain, cytoplasmic domain; 3'UT: 3' untranslated region.

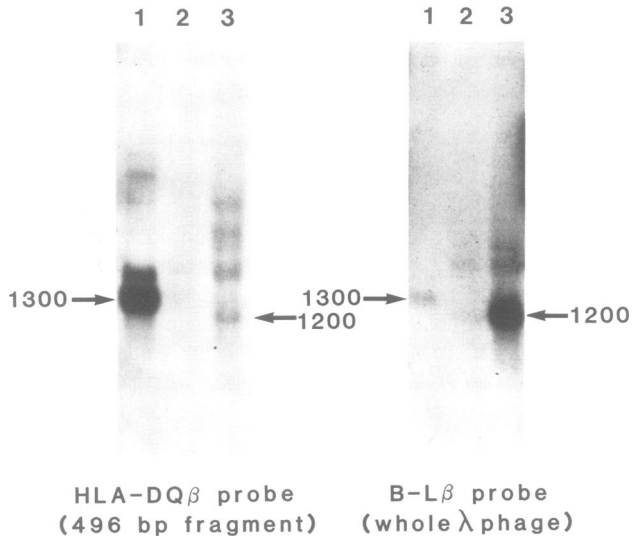

Fig. 2. Cross-hybridization between human and chicken class II β chain sequences. Northern blot analysis was performed as described in Materials and methods with the HLA-DQβ probe (left, see Figure 1) or the L6 phage clone (right) isolated with the human probe. **Lane 1**: mRNA from the human B cell line CA; **lane 2**: chicken liver mRNA; **lane 3**: chicken spleen mRNA. Size of hybridizing bands is indicated in nucleotides.

(Larhammar *et al.*, 1982). We searched for hybridization conditions in which this human probe would allow detection of an homologous chicken sequence. The DQβ probe labeled at high specific activity by nick-translation was hybridized to a Northern blot in low stringency conditions (see Materials and methods), and a series of bands were detected in chicken spleen mRNA. Among them, a band at 1200 nt, not detected in these conditions in chicken liver mRNA, which migrated slightly faster than the mature 1300 nt human DQβ mRNA (Figure 2), was a good candidate for a chicken class II transcript. It seems likely that the three other bands represent 18S and 28S ribosomal RNA and breakdown products of 28S RNA. Very similar results were obtained after washing in 0.2 × SSC at 35°C.

We then used the DQβ probe to screen chicken genomic DNA libraries in conditions similar to those described above. We screened 10^6 phage plaques from two independent libraries (Dodgson *et al.*, 1979; Ballivet *et al.*, 1983). We obtained 19 clones from the first library and 11 clones from the second which hybridized on duplicate filters and which were purified through successive rounds of screening. In order to verify that the isolated clones contained genes that had the expected characteristics of MHC class II genes, we used labeled whole phage to reprobe the same Northern blot that had been hybridized earlier with the DQβ probe. The 1200 nt band hybridized strongly with the chicken probe in chicken spleen mRNA, and faintly in chicken liver mRNA, whereas the 1300 nt HLA-DQβ mRNA was detected as a weak band (Figure 2).

Mapping to the chicken B complex

The sequence hybridizing to the HLA-DQβ probe in one clone from the λL47 library (L6) was isolated as a 3.2 kb *Hin*dIII DNA fragment. This fragment, referred to as the p14 probe (see Figure 4), was used to probe Southern blots of genomic DNA from homozygous inbred and congenic lines of chicken. The p14 probe detected multiple DNA fragments of various intensities, some of which were polymorphic in *Pvu*II and *Bam*HI digests (Figure 3). In contrast *Eco*RI and *Hin*dIII yielded only conserved patterns (not shown). Among the polymorphic bands, some were specific for the B4, B12 or B14 haplotypes and others for groups of two or three of them. The B4 and B12 haplotypes studied are those of the CC and CB congenic lines which have different B complexes on an identical genetic background. Thus the ability of the p14 probe to detect polymorphisms between these lines readily indicates that it does hybridize to sequences located within the B complex. Similar conclusions can be drawn from an extensive analysis of several individuals of different chicken lines using multiple restriction enzymes (A.M.Chaussé and C.Auffray, unpublished results).

In order to estimate the number of B-Lβ genes, we have used a shorter probe, p234, corresponding only to the β2 domain (see below and Figure 4). When hybridized to B4 and B12 genomic DNA digested with *Pvu*II, p234 reveals a subpattern of four of the bands detected with the p14 probe (Figure 3). Three of these bands common to the B4 and B12 haplotypes are also found in individual clones isolated from the genomic libraries of unknown haplotypes and thus represent three independent B-Lβ genes. The fourth *Pvu*II band detected with p234 is polymorphic and represents allelic forms of a fourth gene in the B4 and B12 haplotypes. Some of the isolated clones such as C6 (Figure 3) have a different *Pvu*II band, but whether it corresponds to a fourth gene or a truncated form of one of the three conserved fragments remains to be determined.

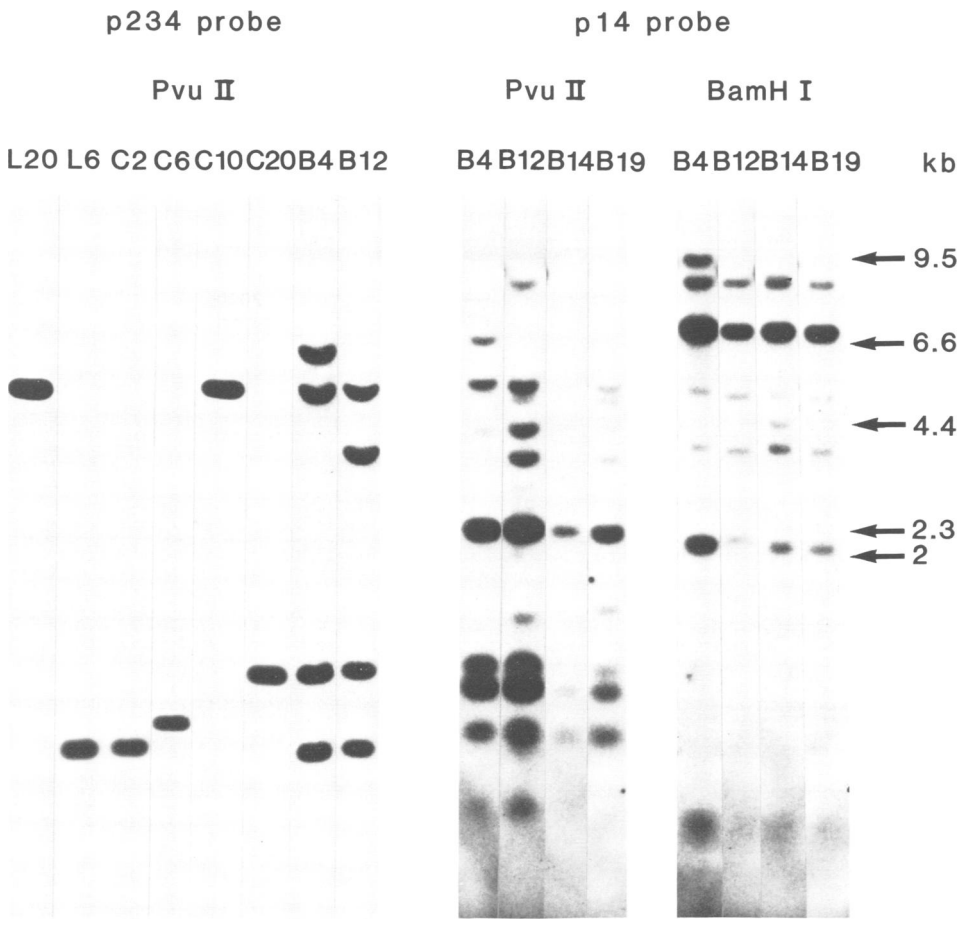

Fig. 3. Enumeration of the B-Lβ genes and mapping to the B complex. Southern blot analysis of chicken genomic DNA (right) and phage clones (left) was performed as described in Materials and methods. L20, L6: clones isolated from the λL47 library; C2, C6, C10, C20: clones isolated from the λCharon 4A library. The B haplotypes of the genomic digests are indicated for each lane.

Structure of a chicken B-Lβ gene

The 3.2 kb *Hin*dIII DNA fragment from the L6 clone was further subcloned and analysed in detail by DNA sequencing. The sequence obtained together with its direct comparison with the HLA-DQβ sequence is shown in Figure 4. Clearly regions of sequence similiarity where no gaps had to be introduced were distinguishable. They correspond to typical β1, β2 and transmembrane exons containing an open reading frame which can be connected through splicing using the typical signals which are found at their limits. It is worth noting that these intron/exon limits interrupt the coding frame between the first and second bases as is observed in all MHC genes and other members of the immunoglobulin supergene family.

Although we have sequenced 860 bp upstream of the β1 exon (Figure 4A and data not shown), it was not possible to detect the signal sequence exon which might be too divergent when compared to the HLA-DQβ or other class II β chain sequences. This is not too surprising because this region is very divergent between isotypes in a given species (Tonnelle *et al.*, 1985). Alternatively this exon may be located outside of the clone analyzed. Exons encoding the cytoplasmic domain and 3′ untranslated region were identified by comparison to a cDNA clone corresponding to a transcript of another B-Lβ gene in the B12 haplotype (Figure 4; R.Zoorob and C.Auffray, unpublished data). The four introns separating the five exons were found to be very short (108, 83, 86 and 86 nt) when compared to their homologues in human and murine class II β chain genes. For example, in the HLA-DQβ gene, they are 2889, 515, 484 and 610 nt long respectively (Boss and Strominger, 1984). The nucleotide sequence alignment between the B-Lβ gene and the HLA-DQβ sequence, part of which was used to clone it reveals that the extent of conservation is approximately the same in the three exons, ranging from 62−66% of shared residues (Figure 4 and Table I).

The B-Lβ chain

The predicted amino acid sequence of the chicken B-Lβ chain was compared to that of human and murine class II β chains (Figure 5). The chicken sequence has all the characteristics of a class II β chain. The β1 exon encodes amino acids 6−94 and contains conserved residues for N-glycosylation (NGT at positions 19−21) and intradomain disulfide bond formation (cysteines 15 and 79) as well as several clusters of conserved residues (Figures 4 and 5). The β2 exon (residues 96−188) is one codon shorter in the B-Lβ gene, probably due to a single mutation in the AG dinucleotide at the end of the polypyridine track in the preceeding intron followed by the use of a downstream AG allowing in-phase mRNA processing. The β2 domain is therefore 92 amino acids long with two conserved cysteines involved in the formation of the 55 amino acid intrachain disulfide loop that is a typical feature of members of the immunoglobulin superfamily.

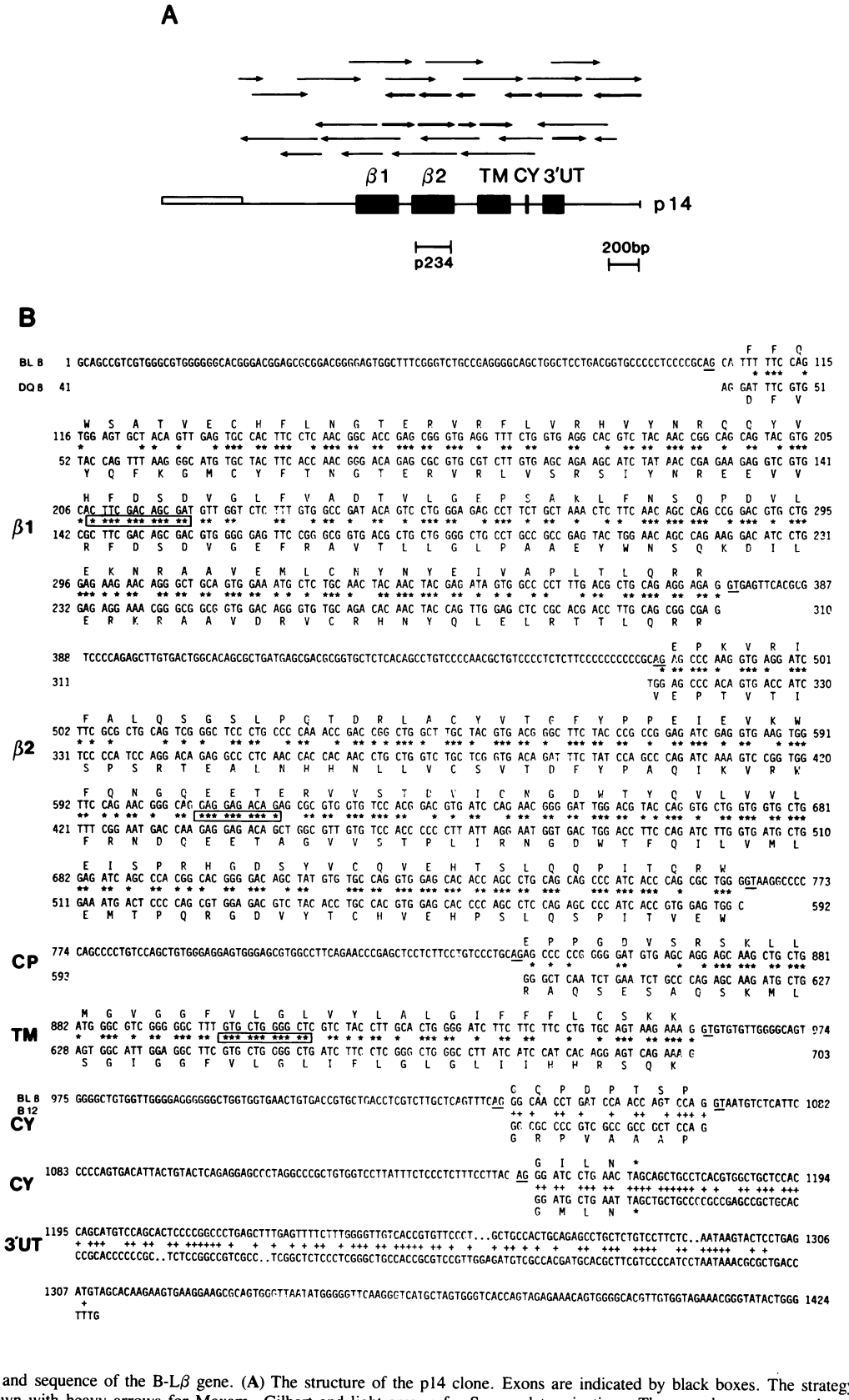

Fig. 4. Structure and sequence of the B-Lβ gene. (**A**) The structure of the p14 clone. Exons are indicated by black boxes. The strategy used for sequencing is shown with heavy arrows for Maxam−Gilbert and light arrows for Sanger determinations. The open box corresponds to the phage arm. (**B**) Partial sequence of p14 and comparison with HLA-DQβ. Only the three identified exons and their flanking sequences are shown (see text). The DNA sequences (B-Lβ on top, DQβ below) have been aligned and matching nucleotides are indicated by stars with the longest stretch in each exon boxed. The GT and AG dinucleotides at the intron/exon limits are underlined. The coding sequences have been translated using the single-letter amino acid code. Numbering starts at 1 in the B-Lβ sequence shown and follows that of the cDNA insert for the HLA-DQβ sequence (Larhammar *et al.*, 1982). β1, β2, CP, TM, CY: see legend to Figure 1. At the bottom, the B-Lβ sequence is aligned with that derived from a B-Lβ cDNA clone from the B12 haplotype, with matches indicated by '+' signs. CY, 3'UT: see legend to Figure 1. The stop codon is indicated by a star.

Similarity is clearly visible all along the domain except in the first 20 amino acids, another typical feature of β2 domains. The connecting peptide and the beginning of the cytoplasmic regions of class II β chains are encoded in the same exon as the transmembrane domain. The limits of the transmembrane region, defined by the presence of basic residues, is extremely conserved on the amino-terminal side, whereas it coincides with a region of high divergence on the carboxy-terminal side. Except for the last three residues, the connecting peptide bears no relationship to the corresponding sequence in other β chains. The transmembrane domain is 25 amino acids long with a majority of small side chain and hydrophobic residues, a length compatible with an almost exclusively α helical structure and contains one cysteine residue that could be a site for fatty acid attachment as is the case in HLA antigens having such a residue in the transmembrane domain of one of their polypeptide chains (Kaufman *et al.*, 1985).

The carboxyterminal cytoplasmic domain is 14 amino acids long, only two of which are encoded in the transmembrane exon. As in mammals, the following eight residues are encoded in a short exon and the last four residues in the same exon as the 3′ untranslated region.

Table I. Sequence similarities between MHC class II and B-Lβ chains

B-Lβ		DQβ	Aβ	DPβ	DOβ	Aβ2	DRβ	Eβ
β1	aa	50	45	47	49	48	51	48
	nt	63	54	60	61	64	65	63
β2	aa	56	54	56	47	48	53	49
	nt	66	68	66	61	60	62	61
TM	aa	43	46	51	35	30	43	41
	nt	62	62	65	57	50	63	60

Amino acid sequence similiarites (aa) were calculated from the alignment shown in Figure 5. Nucleotide sequence similarities (nt) were obtained after alignment of each sequence with the B-Lβ sequence using the computer program BESTFIT (see Materials and methods). Similarities are indicated as percentage of matching positions. β1, β2, TM: see legend to Figure 1.

Tissue-specific expression of avian B-Lβ genes

The β2 exon was used as a probe to study expression of the B-Lβ genes by Northern blot analysis of mRNA extracted from various tissues from chicken and quail (Figure 6). In conditions of relatively high stringency, no signal is detected in the control human B lymphocyte mRNA, whereas clear bands at 1200 nt are detected in the two avian species. The B-Lβ genes appear to be transcribed in all organs containing cells of the B lymphocyte and myeloid lineages, although to various extents. In order to better compare the level of expression, the same Northern blot was hybridized with an actin probe to estimate the amount of mRNA in each lane and a chicken immunoglobulin Cλ probe to estimate the presence of B cells in each organ (not shown). With these corrections, mRNA content as determined by the intensity of hybridization is maximum in spleen, intermediate in

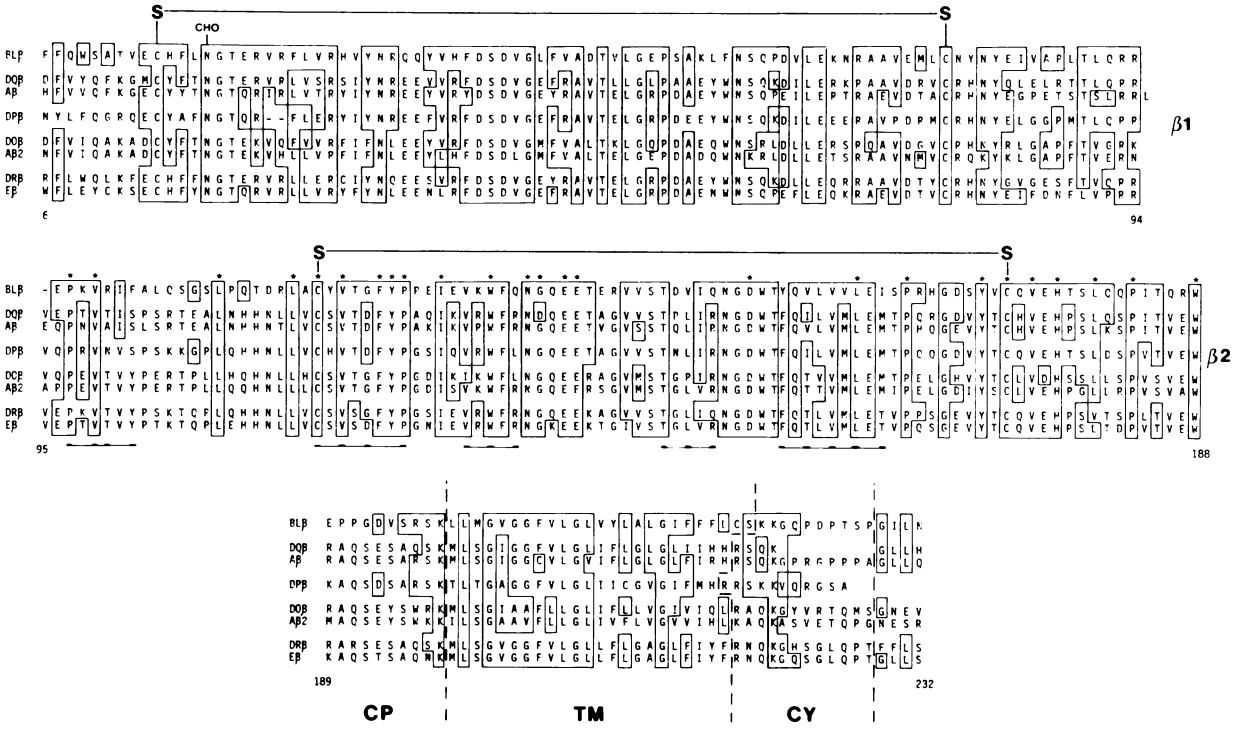

Fig. 5. Comparison of B-Lβ with human and murine class II β chains. The amino acid sequence derived from the B-Lβ gene was aligned and compared to the isotypic forms of human β chain, DQβ (Larhammar *et al.*, 1982), DPβ, DOβ and DRβ (Tonnelle *et al.*, 1985) and their murine equivalents Aβ (Malissen *et al.*, 1983), Aβ2 (Larhammar *et al.*, 1985) and Eβ (Mengle-Gaw and McDevitt, 1983). Residues identical to that of the B-Lβ sequence are boxed. Stars on top of the β2 domain indicate residues commonly found in the immunoglobulin light and heavy chain and MHC class I and class II α and β chains. The disulfide bridges are shown, as well as the carbohydrate attachment site (CHO). Residues involved in the formation of the β strands of the immunoglobulin-like domain are indicated below the β2 sequences. β1, β2, CP, TM, CY: see legend to Figure 1.

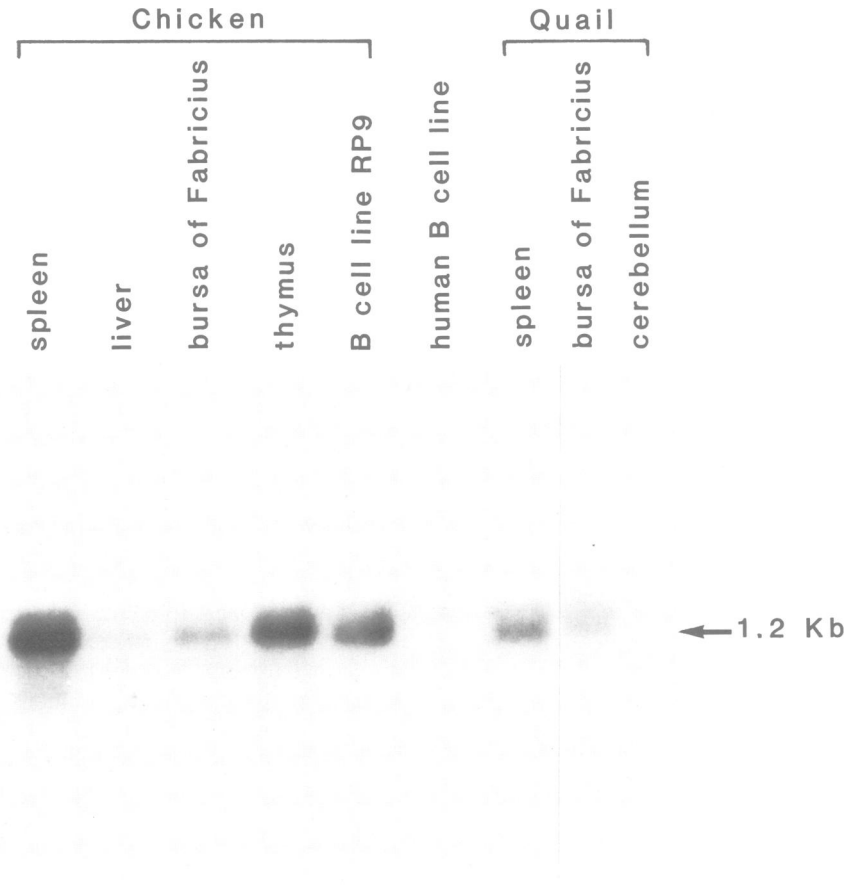

Fig. 6. Tissue specific transcription of the B-Lβ genes. Northern blot analysis was performed as described in Materials and methods using the B-L β2 domain probe p234, and 10 μg mRNA from the various sources indicated.

thymus and bursa of Fabricius, and low in liver, whereas no signal can be detected in quail cerebellum. This is also in good agreement with the number of cells and the intensity of immunofluorescence staining detected with the anti B-L monoclonal antibody TaPl in each organ or cell type (Figure 7). TaPl labels strongly myeloid (macrophage-like) cells in all tissues analyzed but these cells are found in greater numbers in the spleen around the blood vessels and in the thymic medulla than in liver or bursa of Fabricius. In the thymic cortex, epithelial cells are stained weakly whereas thymic lymphocytes are negative. In contrast, immature lymphocytes located in the medulla of bursal follicules are labeled weakly, whereas more mature cells of the B lineage found in the cortex of bursal follicules and in splenic primary follicules express higher amounts of B-L antigens. Thus B-Lβ genes are transcribed and expressed with the same tissue specificity as mammalian class II β genes.

Discussion

The successful cloning of the human and mouse MHC genes accomplished in the past years has provided new tools to study the structure and function of MHC antigens and has allowed a detailed study of the chromosomal region containing this complex. Thus genetic markers defined with great precision are now being used in association with those defined by biochemical or cellular assays in attempts to unravel the genetic basis for MHC-linked immune regulation. The ability of an organism to develop immune defenses in the context of its own MHC antigens is learned during the embryonic life, but we know very little at present on the succession of molecular and cellular interactions which are critical in this process. One possible approach to identify the structure–functional relationships of MHC antigens is to reconstruct the evolutionary history of the MHC by describing the MHC genes, their products and functions in classes of vertebrates which have evolved separately for long periods of time.

We have been interested in the role of MHC class II antigens during ontogeny in the chicken embryo and we have therefore investigated the possibility of using HLA class II gene probes to characterize their chicken equivalents, the B-L genes. With the accumulation of DNA and protein sequences, most often derived from that of cDNA or genomic clones, for various isotypes of α and β chains in mouse and man, it became clear that the class II β chains are somewhat more conserved between isotypes in a given species or between species than α chains. Thus we assumed that an HLA class II β chain sequence would represent a good candidate probe for detection of chicken sequences in interspecies cross-hybridization experiments. The precise design of the probe derived from an HLA-DQβ cDNA clone was directed by the following considerations: the probe should contain the segment encoding the parts of the β chain with the highest degree of sequence conservation, that is the β2 immunoglobulin-like domain and the core of the transmembrane domain. The AT-rich 3′ untranslated region and the GC-rich regions generated during the cloning procedure should be

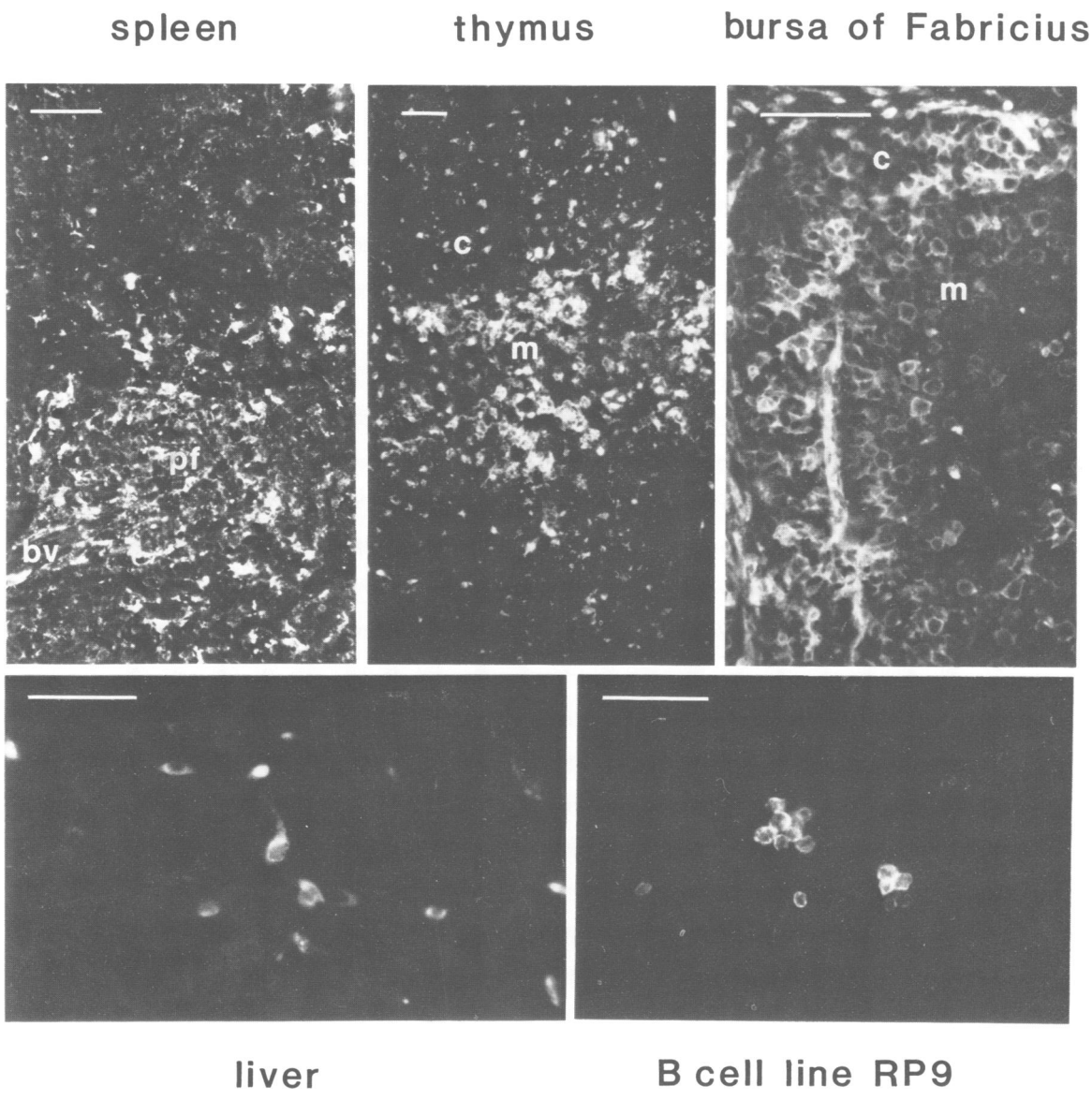

Fig. 7. Expression of B-L antigens in lymphoid organs. Immunofluorescence staining of tissue sections or cell culture was performed using the monoclonal antibody TaP1 as described in Materials and methods. c: cortex; m: medulla; bv: blood vessel; pf: primary follicule. The bars correspond to 10 μm.

eliminated in order to avoid non specific hybridization. Northern blot analysis rather than Southern blot analysis was chosen as a valuable assay for this type of probe because one can visualize sequences which are defined not only by their hybridization potential but also by their length and tissue specificity of expression. Thus in the case of MHC class II β chain genes, only signals which are in the correct size range (~1300 nt) and show the expected tissue specificity can be considered as significant.

Indeed, with the HLA-DQβ probe we were able to detect a 1200 nt band in chicken spleen mRNA which fulfills all the criteria for a class II β chain transcript. These conditions of rather low stringency should allow hybridization of sequences having around 70% similarity (Howley *et al.*, 1979). We have used similar hybridization conditions to isolate genomic clones containing sequences that hybridize strongly to the 1200 nt mRNA. These experiments provide strong evidence that we have indeed isolated class II β chain genes of the chicken MHC. This was confirmed by Southern blot analysis which detected restriction enzyme length polymorphisms between congenic lines of chicken and indicated the presence of at least three and probably four B-Lβ genes in the chicken genome.

Nucleotide sequence analysis established the presence in the chicken clone of two segments with 66 and 62% similarity with the probe, corresponding to the β2 and transmembrane exons of a chicken B-Lβ gene. In addition, we could identify a β1 exon which is almost as conserved as the β2 exon (63%). This observation came to us as a surprise since we had expected that the polymorphic β1 exon would be less conserved and we had not included the corresponding sequence in the HLA-DQβ probe (Figure 1). Moreover, the longest matching segment is 12 nt in β1, but only 10 nt in β2 and 11 nt in the transmembrane exon. These conserved segments appear to have been sufficient to serve as nucleation centers in the formation of stable mismatched hybrids in the conditions used. Following this procedure, all clones isolated contained B-Lβ genes and we obtained no false

positives. Thus the experimental protocol used in this study is both sensitive and specific. However, in similar conditions we were unable to obtain any positive clones using an HLA-DQα probe in several attempts. This might be due to the fact that MHC class II α chains have diverged more extensively than β chains (as pointed out above).

We have compared the B-Lβ chain sequence with all known mammalian class II chains both at the amino acid and nucleotide levels (Figure 5 and Table I). In the extracellular domains, the B-Lβ sequence has 46–56% amino acid sequence similarity and 55–68% nucleotide sequence similarity with its mammalian homologues. This suggests that the series of duplications that led to the present isotypes of class II β chains had already taken place at the time when birds and mammals diverged from a common ancestor around 250 million years ago. The transmembrane exon is overall more divergent, but this reflects the lack of conservation in the connecting peptide, whereas the hydrophobic core of the transmembrane segment is as conserved as the extracellular domains. Since the B-Lβ gene sequenced is equally similar to the various mammalian sequences, it can be anticipated that any of the human or murine probes would have been suitable for isolation of the B-Lβ genes. It is thus possible that the three genes isolated in this study represent a full complement of chicken class II β chain genes in the MHC haplotypes from which the genomic libraries were derived, with at least one member of different isotypic families. Analysis of these additional genes will provide a structural basis to answer these questions.

Although the overall homology between the chicken and mammalian sequences is smaller than that obtained in intra or interspecies comparison in the mammalian class, both the nature and the location of the conserved residues are similar. This is well illustrated by the fact that similarity reaches 71% when one takes into account the amino acids in the β2 domain of the B-Lβ sequence which have physical and chemical properties similar to the most common amino acids found in the other sequences (for example Ser/Thr at 106, Ala/Val at 116, Lys/Arg at 130). Among the 48 positions in the β2 domain, where most β chains share the same amino acids, 25 are also common with other members of the immunoglobulin gene superfamily (Figure 5). These include the majority of the hydrophobic residues found at alternate positions and involved in the formation of β strands which are the essential building blocks of the prototype three-dimensional immunoglobulin domain fold.

In addition to the distinctive features of a class II β chain, there are highly conserved segments in each domain. The longest of these segments in the polymorphic β1 domain is the VHFDSDVG sequence (38–45). It corresponds to the region where similarity with the fibronectin cell-attachment site has been noted previously (Auffray and Novotny, 1986), leading to the proposal that it represents the non-polymorphic binding site of the class II β chain interacting with the CD4 T-cell differentiation antigen (Auffray, 1986). The pentapeptide NGDWT (150–154) and a stretch of 9 amino acids, GGVGGFVLGL (202–210), appear as structural invariant signatures of the β2 and transmembrane domains, respectively.

We have investigated the pattern of transcription of B-Lβ genes in various tissues by Northern blot analysis using the β2 domain as probe. The faint signal detected in liver is related to the presence of macrophage-like cells expressing B-L antigens (Figure 7), probably analogous to the passenger macrophages and Kupffer cells of human liver (Lautenschlager *et al.*, 1984). The high level of expression seen in the thymus reflects the fact that this organ contains a large number of cortical epithelial cells expressing B-L antigens rather faintly and that most medullary dendritic cells express B-L antigens at a high level (Guillemot *et al.*, 1984 and Figure 7). In contrast, the signal detected in the bursa of Fabricius is surprisingly low considering the large number of bursal lymphocytes which stain with an anti-B-L monoclonal antibody when analyzed in suspension. However, this staining is restricted to the mature B lymphocytes and the medulla of the follicules is labeled only weakly. This result might also be explained by the fact that we have used 8-week-old animals in which involution of the bursa is in progress, or it could be related to a different turnover and level of B-Lβ mRNA in this organ. At this stage of the analysis, it is not possible to determine how many and which of the B-Lβ genes are transcribed and translated. The B-Lβ gene analyzed has an exon/intron structure similar to the mammalian class II β genes, except for the very small size of the introns. Because we have not identified the regions of this gene containing the signal peptide exon and the promoter sequences, further experiments will be necessary to establish that it is an expressed gene.

Since the B-Lβ probe also hybridizes to quail mRNA, it will be possible to isolate the corresponding genes and to determine the degree of similarity of class II antigens in two avian species in which interspecies intra-embryonic grafts are tolerated and are used extensively to study ontogeny. In addition, chicken class II gene probes will help defining new markers for the genetic analysis of the B complex using restriction enzyme polymorphisms and chromosome walking procedures. Hopefully it will be possible to manipulate these genes during the embryonic stages in order to better understand their role in the establishment of a functional immune system.

Materials and methods

The HLA-DQβ probe

A 496 bp *Sac*I–*Eco*RI DNA fragment from the HLA-DQβ cDNA clone pIIβ-1 (Larhammar *et al.*, 1982) was subcloned into pUC12 (Vieira and Messing, 1982). Plasmid DNA was prepared from an overnight saturated *Escherichia coli* culture by alkali/SDS extraction (Birnboim and Doly, 1979), followed by banding in two successive CsCl/ethidium bromide gradients, cut with *Eco*RI + *Sac*I to completion and the 496 bp fragment was isolated after electrophoresis on a 5% polyacrylamide gel by electroelution and DEAE–cellulose chromatography. The isolated fragment was labeled by nick-translation to a specific activity of $2-4 \times 10^8$ c.p.m./μg (Rigby *et al.*, 1977).

Screening of chicken genomic DNA libraries

A λ Charon 4A genomic library (Dodgson *et al.*, 1979) and a λL47 genomic library (Ballivet *et al.*, 1983) were screened with the HLA-DQβ probe on duplicate filters essentially as described (Maniatis *et al.*, 1982). Nitrocellulose filters (Millipore HAHY) were prewashed 2 h at 42°C in 50 mM Tris, pH 8.0, 1 mM EDTA, 0.1% SDS, prehybridized 4 h at 42°C in 50% formamide, 5 × SSPE, 0.1% SDS, 1 μg/ml poly(A), 1 μg/ml poly(C), 100 μg/ml denatured salmon sperm DNA. The nick-translated probe ($2-4 \times 10^8$ c.p.m./μg) was then added to the same buffer and hybridization carried out 24 h at 42°C and 24 h at 37°C. The filters were washed 2 × 1 h at 45°C and 2 × 1 h at 55°C in 2 × SSC, 0.1% SDS, and exposed to X-ray films (Kodak) for 3–7 days with two intensifying screens (Dupont Liplus). Positive clones were purified by rescreening under the same hybridization conditions.

Mapping, subcloning and DNA sequencing

Phage DNA was prepared as described (Maniatis *et al.*, 1982). A restriction map of the recombinant phage was determined using the oligo cos/partial

digest approach (Rachwitz *et al.*, 1984) and a 3.2 kb *Hin*dIII DNA fragment hybridizing to the HLA-DQβ probe was identified by Southern blot analysis using Genescreen (NEN) nylon membrane (Southern *et al.*, 1975). This DNA fragment was subcloned into pUC9 (Vieira and Messing, 1982), yielding the subclone p14. Restriction enzyme and Southern blot analysis indicated that the HLA-DQβ probe hybridized to two internal *Pst*I fragments from p14, 400 and 250 bp in length. The latter, corresponding to the β2 exon, was subcloned in pUC9, and is referred to as the p234 clone.

DNA sequencing of p14, and subclones thereof, was performed using the chemical degradation procedure (Maxam and Gilbert, 1980) by labeling 3′ ends with the Klenow fragment of DNA polymerase I and α-^{32}P-labeled deoxynucleotides, and by the dideoxy chain termination method (Sanger *et al.*, 1977) using [α-^{35}S]dATP and modified T7 DNA polymerase (Sequenase, US Biochemicals) after subcloning the p14 insert in M13mp18. 17mer oligonucleotides used as sequencing primers were synthesized on an Applied Biosystems DNA synthesizer. Nucleotide sequences were compiled on a micro computer using the PCGENE software package (Genofit). Alignments were performed using the program BESTFIT using the BISANCE facility (CITI2, Paris).

Southern blot analysis

High mol. wt DNA was extracted from red blood cells of B4, B12, B14, B19 chickens with proteinase K and SDS (Blin and Stafford, 1976), digested to completion with *Pvu*II or *Bam*HI, electrophoresed on 0.6% agarose gels and blotted on nylon membranes (Amersham) as described (Southern, 1975).

Northern blot analysis

Liver, spleen, thymus, bursa of Fabricius were dissected from 8-week-old White Leghorn chickens (*Gallus gallus*) and japanese quails (*Coturnix coturnix japonica*) and frozen in liquid nitrogen. The chicken RP9 cell line was maintained in culture in DMEM supplemented with 10% fetal calf serum and 1% chicken serum in a humidified atmosphere containing 5% CO_2. Cells were collected by centrifugation and washed in phosphate buffer saline. The final pellet was frozen in liquid nitrogen. RNA was extracted using the LiCl/urea procedure (Auffray and Rougeon, 1980). Poly(A)$^+$ RNA was purified by two cycles of oligo(dT)cellulose chromatography, 10 μg was denatured with glyoxal, electrophoresed on a 1.1% agarose gel (McMaster and Carmichael, 1977) and transferred to a nylon membrane (NEN Genescreen). The Northern blot was prehybridized for 4 h at 42°C in 50 mM Na phosphate, pH 6.5, 50% formamide, 5 × SSC, 1 × Denhardt, 250 μg/ml sonicated salmon sperm DNA. Hybridization was performed in the same buffer at 37°C for the HLA-DQβ probe, and at 50°C for the p234 or the whole L6 phage (see above). The probes were labeled by nick-translation at 2–4 × 10^8 c.p.m./μg. The final wash was in 2 × SSC, 0.1% SDS at 55°C (HLA-DQβ) or in 0.1 × SSC, 0.1% SDS at 50°C (p234, L6 phage).

Immunofluorescence analysis

Indirect immunofluorescent staining of 1-week-old chicken spleen, thymus, bursa of Fabricius and liver was performed on fixed tissue sections, whereas labeling of the RP9 cell line was done on living cells as previously described (Guillemot *et al.*, 1984). Reagents used were the anti-chicken B-L monoclonal antibody TaP1 (Guillemot *et al.*, 1986) and a fluoresceinated goat anti-mouse immunoglobulin serum (Nordic).

Materials

Enzymes and chemicals were obtained from Appligene, Amersham, Biolabs, Boehringer, BRL, Genofit, Pharmacia and Sigma. Radiochemicals were from Amersham.

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