T-cell specific avian TdT: characterization of the cDNA and recombinant enzyme

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ABSTRACT

A cDNA clone coding for avian terminal deoxynucleotidyl transferase (TdT) has been isolated and sequenced. The size of this cDNA was 2545 bp with an open reading frame of 1521 bp and a predicted translation product of 58 kDa. Comparison of this TdT sequence with other known TdT sequences has revealed a very high degree of homology at both the DNA and predicted amino acid levels. The chicken TdT cDNA was expressed in a bacterial system and the protein was purified by affinity chromatography. The purified recombinant enzyme, with a specific activity of ~1700 U/mg protein, was significantly less active than TdTs from mammalian species. This finding correlates with the observation that TdT isolated from avian thymus has lower activity than that isolated from any mammalian thymus source. Northern blot hybridization analyses and reverse transcription PCR of RNA preparations were carried out with the chicken cDNA. The data generated from these experiments revealed that the TdT RNA was only expressed in the thymus and not in the bone marrow or the bursa of Fabricius during pre- and post hatching chicken development. These data suggest that while TdT is probably involved in N region addition in chicken T-cell receptor genes, it is unlikely to play a role in diversification of immunoglobulin genes.

INTRODUCTION

Terminal deoxynucleotidyl transferase (EC 2.7.7.31) catalyzes the random polymerization of deoxynucleoside 5'-triphosphates to the 3'-OH of a DNA initiator (1,2). In mammals TdT is only expressed in primitive lymphocytes present in thymus and bone marrow (3,4) and has been identified as a component of the mammalian V(D)J recombinase. TdT adds nucleotides (N regions) at the junctions of rearranged immunoglobulin (Ig) heavy chain and T-cell receptor (TCR) gene segments during B- and T-cell maturation to increase immunological diversity (5,6).

The biological distribution of TdT supports its postulated role in the diversification of the immunoglobulin and T-cell receptor genes. Activity of the enzyme correlates with the level of complexity of the evolving immune system (7). In mammalian B- and T-cell precursors, complex immunoglobulin gene rearrangements involving multiple gene segments occur throughout life, and TdT enzyme activity is continuously detected in these precursor cells (8). In contrast, in chickens, immunoglobulin gene rearrangements are much less complex, involve a single gene segment and occur at a limited period at the beginning of colonization of the bursa (9,10). TdT containing cells begin to appear in the embryonic thymus between 11 and 12 days of gestation (11). The proportion of TdT-positive cells increases throughout embryonic development and reaches a plateau at 20 days (12). In marked contrast to the mammals studied, no stable population of TdT-positive cells outside the thymus has been found in avian species. In fact, no other measurable transient population of TdT-positive cells has been found in birds when many embryonic tissues were tested (11).

The role of TdT in other genera is still the subject of debate. While the amphibian *Xenopus* possesses distinct B- and T-cells and an immunoglobulin gene structure reminiscent of mammals, the expressed antibody diversity is much lower than that of mammals and avians (13). Gene rearrangement does occur, but TdT activity has not been detected in any amphibian tissue. Recently, a TdT homolog cDNA has been isolated and sequenced in *Xenopus laevis* (14), although the gene product has not been expressed or analyzed for enzyme activity.

The pattern of biological occurrence of TdT, and its limitation to species that have a bifurcated immune system and gene rearrangement coupled with sequence diversification mechanisms, generates interest about the origins of this DNA polymerase. Earlier studies revealed a high degree of sequence conservation among all known mammalian TdTs. In addition, polyclonal antibody directed against homogeneous bovine TdT showed cross-reactivity of TdT in species ranging from chicken to human (15). Along with the conservation of immunological determinants, the size of the TdT molecules from these diverse species was also found to be similar (16). The amino acid sequences of human, bovine and murine TdTs are 86% identical

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and the enzymatic properties of all of these forms of the enzyme are very similar (17,18). The goal of the present work was to isolate a TdT cDNA from a non-mammalian species in order to expand our understanding of the evolution of this protein and its enzymatic properties.

MATERIALS AND METHODS

Screening of the chicken thymus cDNA library

A chicken thymus cDNA library was kindly provided by Dr Craig Thompson (University of Chicago). Screening of this cDNA library was carried out according to the standard protocol of Sambrook *et al.* (19) with modifications. An oligonucleotide mixture that contained eight oligonucleotides for both human and mouse cDNAs as shown in Table 1 was used as probe. Phage DNA isolated from positive clones was mapped with restriction endonucleases. The cDNA insert was subcloned into the single *EcoRI* site of M13 mp19 and sequenced.

RNA isolation

Total RNA from chicken tissues was isolated by a modified protocol of Chomczynski and Sacchi (20) using TRI REAGENT from Molecular Research Center, Inc. (Cincinnati, OH). Between 1 and 5 g of tissue were removed from different organs including bone marrow, bursa of Fabricius, liver, muscle, spleen and thymus from 1 and 6 week old chickens. RNA pellets were resuspended in FORMAZOL (Molecular Research Center, Inc.). RNA concentration was determined by the OD₂₆₀.

5' RACE of the chicken TdT mRNA

The 5' end of chicken TdT cDNA was amplified by the method of 5' RACE (rapid amplification of complementary DNA ends;

21) with reagent kits from Life Technologies, Inc. The sequences of the chicken TdT specific primers (CKTdT1 and CKTdT2) are listed in Table 1. The PCR amplified product was subcloned into M13 and sequenced.

Nucleotide sequencing

DNA sequencing was carried out using the dideoxy chain termination method of Sanger *et al.* (22). Reagents used for sequencing were from USB. The cDNA was sequenced employing oligonucleotide primers for specific sites. The full length cDNA was sequenced in both directions. The sequence data were analyzed by Clone 3 and Alignment, computer clone, sequence analysis and alignment programs from Scientific and Educational Software (State Line, PA).

Northern blot of total RNA from chicken tissues

Northern blot hybridization analyses of chicken total RNA were carried out according to a standard protocol (19). Full length chicken TdT coding sequence (1.5 kb) was used as the probe. DNA was labeled with [α -³²P]dATP and a megaprimer DNA labeling system from Amersham Corp.

For high stringency hybridization, the membrane was first pre-hybridized for 15–30 min at 65°C in the rapid hybridization solution (Amersham Corp.) and hybridized for 5–8 h at the same temperature in this solution. After hybridization, the membrane was first rinsed twice with 2 × SSC/0.5% SDS at room temperature and then washed with 2 × SSC/0.5% SDS for 30 min at 65°C. For low stringency hybridization, the temperature used for hybridization and washing was 55°C instead of 65°C and the washing solution was started with 5 × SSC/0.1% SDS. Washing stringency was increased gradually if necessary to remove excessive background.

Table 1. Sequences of oligonucleotides used in this study

Number	Name	Species	Sequences	Strand
1	RACE-3	Human	(245)CATTTTCAACCCTGAACCCTTTCC(222)	C
2	RACE-4	Human	(296)CCGAACCCGAGTTGTTCTCTGCTAC(272)	C
3	RACE-5	Human	(343)TTGTGAGCTGACTTGTACTTTCTGTG(318)	C
4	mTdT-21	Mouse	(896)GCCTGAGGTTACAAAAATGC(916)	
5	mTdT-22	Mouse	(956)CAGCTAACGAGGTCTTCG(928)	C
6	mTdT-23	Mouse	(766)GCTGTGTTAAATGATGAGCG(785)	
7	mTdT-24	Mouse	(828)TCCCACACCAAACACAGAGG(809)	C
8	TdT JR4	Mouse	(256)GATTCGGTCACCCACATT(273)	C
9	CKTdT1	Chicken	(819)CTGAACCATTTCTCTGATGTC(799)	C
10	CKTdT2	Chicken	(863)GGGTCTTGTCAGCTTTTACCTC(842)	C
11	5' CKTdT	Chicken	(1)ACTTCTGCCTTGCCATATGGAGAGGATCAGACCTCC(36)	
12	3' CKTdT	Chicken	(2323)GCCTGGGGAAGTGTTGGATCCTGCATCCCTGAAGG(2288)	C

Oligonucleotides are numbered from 1 to 12 for easy identification. All oligonucleotide sequences are listed in the 5'→3' direction. Numbers at the beginning and end of each oligonucleotide are distance in nucleotides from the beginning of each cDNA. Oligonucleotides 1–8 are used for the screening of chicken thymus cDNA library; 9 and 10 are used for the 5' RACE of the 5' half of chicken TdT cDNA; 11 and 12 are used for the subcloning of chicken TdT cDNA into the pET16b expression vector for the expression of TdT in a bacterial system. Non-TdT sequences included for generating restriction site were underlined. C: complementary to the coding strand.

Figure 1. Full length chicken TdT cDNA and subcloning of chicken TdT cDNA for the expression. (a) A positive clone from the chicken thymus cDNA library; (b) PCR product from the 5' RACE reaction; (c) scale drawing of the full length chicken TdT cDNA. Selective restriction enzyme sites and functional sites, including the ATG, TAA and AATAAA, are shown. The sizes and locations of primers used for 5' RACE and for subcloning the full length cDNA are also shown. Enzymes in parenthesis are introduced enzyme sites to facilitate the subcloning. Full length chicken TdT cDNA was subcloned into the expression vector pET16b for the expression in *E. coli* cells. Both forms of chicken TdT cDNA used for the expression, with and without the 3'-untranslated region, are shown.

Construction of the full length chicken TdT cDNA

The 5' and 3' segments of the chicken cDNA were cloned in bacteriophage M13 mp19 sequencing vector and subcloned into plasmid pUC19 to form the full length chicken TdT cDNA as shown in Figure 1. A *Nde*I site just 5' to the ATG start codon and a *Bam*HI site just 3' to the polyadenylation site of chicken TdT at position 2250 (relative to the ATG site) were introduced by PCR with primers 5' CKTdT and 3' CKTdT (Table 1). An expression plasmid pETckTdT (8.0 kb), which is derived from pET16b (Novagen Inc., Madison, WI) and contains the full length chicken TdT cDNA, was generated by a two step subcloning procedure as shown in Figure 1. This expression plasmid was used for the production of the chicken TdT in a bacterial expression system.

A short form of chicken TdT cDNA without the 3'-untranslated region was also subcloned into pET16b to generate the pETckTdT'. This was accomplished by a complete digestion of pETckTdT with *Bam*HI and a partial digestion of the linearized plasmid with *Hinc*II, as shown in Figure 1. The resulting 7.3 kb fragment was isolated from an agarose gel, treated with polymerase and self-ligated.

Expression of the chicken TdT

Chicken TdT was expressed in a bacterial system according to Studier and Moffatt (23) using reagents from Novagen Inc. After the construction of pETckTdT and pETckTdT', the plasmids were transformed into *E. coli* cell lines, including BL21(DE3) and

Table 2. Effect of metal ions on TdT activity

Protein	Substrate Concentration		Relative Enzyme Activity (Units per mg of Protein)				
	dGTP	dA ₅₀	Mg ²⁺	Mn ²⁺ (mM)		Co ²⁺ (mM)	
	(mM)	(μM)	(mM)	(mM)	(mM)	(mM)	(mM)
Chicken TdT (chicken thymus)	1.00	10.0	4,000	1,000	1,300	1,120	800
Recombinant Chicken TdT (bacterial system)	1.00	10.0	1,700	680	340	680	340

Standard TdT assay was carried out as described in Materials and Methods with the substrate concentrations as indicated in the table. Mg²⁺, Mn²⁺ or Co²⁺ was used individually in each assay with concentrations as indicated in the table.

BL21(DE3)pLysS (Novagen Inc.). Cells were cultured in LB broth and the expression of TdT was induced by adding IPTG to the culture medium to a final concentration of 1 mM. The cell culture was incubated for an additional 3–24 h either at 37°C with shaking or at room temperature while stirring at high speed (an additional aliquot of carbenicillin was added). Cells were collected by centrifugation at the end of incubation.

Protein purification with affinity chromatography

The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4 and 0.5 M KCl) or His-binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl and 5 mM imidazole) in the presence of lysozyme and NP-40 or Triton-X and sonicated on a salt-ice bath. Recombinant TdT was purified from cell lysates by monoclonal antibody affinity chromatography (24) and His-Tag affinity chromatography (Novagen Inc.). The protein was eluted from a monoclonal antibody column with 2 M MgCl₂ as described previously (24). For the metal chelating column, there was a stretch of 10 consecutive histidine residues (His-Tag) at the N-terminal end of the chicken TdT expressed from the pETckTdT and pETckTdT' expression vectors. The His-Tag sequence binds to divalent cations (Ni²⁺) immobilized on the His-Bind metal chelation resin (Novagen Inc.). After unbound proteins are washed away, the target protein is recovered by elution with 1 M imidazole. Protein concentrations were determined by the method of Bradford (25) with bovine serum albumin as a standard and reagents from BioRad.

TdT enzymatic assay

Terminal transferase activity was measured by incorporation of [³H]dGTP or [³H]dATP into single-stranded polymeric DNA using dA₅₀ as initiator as previously described (26,27). For divalent cation effect assays, MgCl₂, MnSO₄ or CoCl₂ was added individually to the reaction mix at the concentration indicated in Table 2. One unit of enzyme activity is defined as one nanomole of radioactive nucleotide polymerized per hour.

RESULTS

In this study, we have isolated and sequenced the chicken TdT cDNA, and have compared this sequence with corresponding sequences from three mammalian species and with a recently discovered amphibian homolog. This information was useful in identifying targets for site-directed mutagenesis to probe functions of specific amino acids (24) in the protein. In addition, the

avian TdT sequence will permit us to begin to answer questions about the evolution of this enzyme and also the evolution of the immune system.

Isolation of the chicken TdT cDNA

A partial chicken TdT cDNA was obtained from screening a chicken thymus cDNA library. Initially, this library was probed with 0.9 kb TdT human or mouse cDNA fragments. No positive clones were isolated from these screening experiments. However, when a mixture of oligonucleotides was used as probe for the screening, three positive clones were obtained. This mixture contained eight synthetic oligonucleotides from both human and mouse TdT cDNAs (Table 1, oligonucleotide numbers 1–8). Restriction enzyme mapping showed that the insert size ranged from 1.2 to 1.8 kb. The 1.8 kb insert was subcloned into M13 mp19 and sequenced.

Sequence alignment between the 1.8 kb chicken sequence and human TdT positioned the 5' boundary of the chicken TdT cDNA clone ~750 nt downstream from the ATG of human TdT. In order to construct a full length cDNA, a 5' RACE method was used. Oligonucleotides employed are listed in Table 1 (oligonucleotides numbers 9 and 10). The relative positions of primers used in the 5' RACE reaction are shown in Figure 1. An 800 bp fragment was amplified by PCR using anchor primers from the RACE kit and primer CKTDT1 as described in Materials and Methods. An ATG site was found in this fragment with a 16 bp 5'-untranslated sequence (Fig. 1). The total size of this TdT cDNA was 2545 bp in length with an open reading frame from position 17 to position 1537. The open reading frame was 1521 bp, which when translated predicted a protein of 507 amino acids with a molecular mass of 58 kDa. A polyadenylation site (AAUAAA sequence) was found at position 2190.

Sequence comparison of predicted amino acids among known TdTs

A sequence comparison was made among the TdT amino acid sequences, as predicted from the corresponding cDNA sequences of human, bovine, mouse, chicken and *X.laevis* (14). The predicted proteins from all of these species were nearly equal in length (507–520 amino acids). There are extensive amino acid homologies across the wide evolutionary range and numerous amino acids are conserved among all five genre. This suggests all variants are descended from a common ancestral gene. The invariant amino acid residues tend to occur in clusters; there are >10 regions with identical amino acid residues in clusters ranging from five to 15. Overall, the five species share 58% identical amino acids and 71% of all amino acids are substantially conserved among them. The percentages of identical and conserved amino acids are up to 75 and 83%, respectively, when only the mammalian sequences were compared (28). In chicken TdT, compared with the other four species, there were deletions of two and eight amino acids at positions 358 and 364, respectively, and there was also an insertion of six amino acid residues at position 425. Comparable levels of homology were also present at the DNA level among these five species.

The conservation of amino acid sequences reflects the importance and conservation of functions through evolution. The nucleotide and DNA binding domains identified by Evans *et al.* (29) and Farrar *et al.* (30) in bovine TdT were conserved in chicken TdT. Amino acids (Arg-336, Asp-343 and Asp-345)

proposed to be involved in the nucleotide binding and catalysis of the human TdT (24) were identical in the chicken protein. The conserved sequences, His-X₃-Cys-X₆₁-His-X₈-His, proposed by Chang and Bollum (31) for Zn²⁺ binding in mammalian TdTs were also present in chicken TdT. It is likely that this region plays an important role for the Zn²⁺ binding at the DNA initiator site. The putative nuclear-localization-sequence (PRKKRPR) was identical among mammalian proteins and was highly conserved in the *Xenopus* homolog as PLRKKAK (14). This sequence was not present in chicken TdT. Instead, a stretch of three basic amino acid residues (RKR) was located in the N-terminal region. Earlier studies have shown that chicken TdT is a nuclear protein (11) as is TdT from other species. Therefore, although the canonical nuclear localization sequence in chicken TdT is not present at the N-terminal, it is possible that this function is mediated by the similarly localized RKR sequence. Confirmation must await transfection of this cDNA into mammalian cells and immunolocalization of the gene product.

Analysis of the chicken TdT mRNA

Access to an avian TdT cDNA clone allowed studies of the mRNA distribution. These experiments have not been previously feasible since there is no cross-hybridization between avian and mammalian TdT mRNAs. The size of chicken TdT mRNA was determined by Northern blot hybridization analyses using a random primed TdT cDNA fragment from pUCckTdT as the probe. A band of ~2.2 kb was clearly detected in chicken thymus RNA (data not shown), but was not detected in the human thymus RNA preparation. The size of the endogenous RNA was consistent with an RNA species containing a shortened polyA tail. A slower moving band of ~4.5 kb, was also seen in chicken thymus RNA. This might have been the intermediate RNA splicing product of the primary transcript of the TdT gene. A similar second band was also seen in an earlier study of human TdT mRNA by Northern blot analysis (32). The human RNA species was estimated at ~3.5 kb in size, which is smaller than that of chicken TdT mRNA. The Northern blot hybridization analyses were carried out at both high and low stringencies to investigate the existence of cross-reaction between the human and chicken mRNA. Even at the low stringency condition, there was no TdT-specific RNA species of 2.2 kb present in the human RNA lane (data not shown). The apparent lack of cross-reaction between chicken and human TdT at the nucleotide level is intriguing since a high degree of sequence conservation exists.

The tissue-specific expression of chicken TdT RNA during development was also investigated. Northern blot hybridization analyses and RT-PCR were carried out on total RNA isolated from bone marrow, bursa, muscle, liver, spleen and thymus of 1 and 6 week-old chickens (Fig. 2a). The probe used in the Northern blot analyses was a full length chicken TdT cDNA (Fig. 2b). TdT specific mRNA was only seen in RNA from chicken thymus, not from bursa, bone marrow or any other tissues tested. The size of this band was ~2.2 kb long, as determined by its position relative to 18 and 28S RNAs. There was no apparent difference in band intensity between total RNA isolated from 1 or 6 week-old chickens with equal amounts of RNA loaded in each lane (compare lanes 2 and 8).

RT-PCR was also carried out on total RNA isolated from the bursa, bone marrow, liver and thymus of 7 and 14 days chicken embryos, and 1 and 6 week old chickens. No visible bands were

Figure 2. Northern blot hybridization analysis of total RNA isolated from different tissues of the chicken. Northern blot hybridization analysis was carried out as described in the Materials and Methods section. (a) Ethidium bromide stained agarose gel, (b) autoradiograph of the same gel transferred onto a nylon membrane. Thirty μ g of total RNA from different tissues of the chicken was applied in each lane. Lanes 1–6 and 7–12 are samples from bone marrow, bursa, liver, muscle, spleen and thymus, respectively. Arrow heads indicate the positions of the 18 and 28 S RNAs, while the hollow arrow indicates the position of the TdT mRNA.

seen after ethidium bromide staining in bursa, bone marrow and liver lanes (data not shown). Southern blot hybridization analysis of the RT-PCR gel with the same probe used for the Northern blot hybridization likewise showed no bands (data not shown).

Expression and purification of the chicken TdT

Chicken TdT was expressed from the cDNA in a bacterial system using vectors derived from pET16b. Two different forms of chicken TdT cDNA were used for the expression studies, one with the 3'-untranslated region (total length of cDNA was 2.3 kb)

Figure 3. Induction of the recombinant chicken TdT. Western blot analysis of crude extracts of bacterial cells at different times following induction. Cells taken at 0 and 1, 2 and 3 h after the IPTG induction were pelleted and resuspended in lysis buffer and loaded onto 12% SDS-PAGE. Two cell lines were used, BL21(DE3) and BL21(DE3)pLysS. The position of recombinant TdT was indicated by an arrow.

and the other one without this region (total length of cDNA was 1.6 kb).

Chicken TdT expressed in bacterial cells from the pETckTdT vector was detected on a Western blot (Fig. 3). Because of the inclusion of the His-Tag with the TdT open reading frame (see Methods), the final translated product was 60 kDa instead of 58 kDa predicted from the cDNA sequence. The gel illustrated in this figure included samples of bacterial extracts obtained at different times after IPTG induction of the recombinant protein. Lanes 1 and 5 are before induction, while lanes 2–4 and 6–8 are 1–3 h after induction. Lanes 1 and 5 showed that TdT was not present in cell extract from uninduced cells; while a band of 60 kDa was present in all cell extracts from cells after IPTG induction. The origin of a cross-reacting non-TdT band that was between 25 and 30 kDa and was consistently present in the Western blot regardless of induction of cells is unknown.

The recombinant chicken TdT was purified by both monoclonal antibody affinity chromatography (Fig. 4) and a metal chelating column using the His-Tag stretch of 10 histidine residues attached to the N-terminal of the protein. Comparable results were obtained with both methods. Panel A is the Coomassie brilliant blue staining of one gel while panel B is the Western blot of an equivalent gel. The specific activity of recombinant enzyme was 1700 U/mg protein, which is 2-fold lower than that of native protein isolated from chicken thymus (Table 2). This moderate difference in specific activity might be due to the lack of post-translational modification in the bacterial system or to the presence of the his-tag.

The expression of chicken TdT from pETckTdT', a chicken TdT cDNA construct without the 3' untranslated region, was 10 times higher than that from pETckTdT, a construct containing the 3' untranslated region. It is possible that an mRNA destabilizing sequence was present in this region, although the involvement of this sequence in bacterial cells has not been reported. Such a mammalian destabilizing sequence was reported in a yeast expression system (33).

Influence of divalent cations on recombinant chicken TdT activity

The purified recombinant chicken TdT exhibited similar activity in the presence of divalent cations (Mg^{2+} , Mn^{2+} and Co^{2+}) as does

Figure 4. Purification of the recombinant chicken TdT. Aliquots were taken at each step along the purification procedure and electrophoresed on two 20% SDS-PAGE gels. (a) Coomassie brilliant blue staining of one gel, while (b) is the Western blot of an equivalent gel. (a and b) Lane 1, crude extract; lane 2, column run through; lane 3 and 4, first and second wash of the column; lanes 5 and 6, 20 and 40 μ l of purified recombinant chicken TdT protein; lane 7, recombinant human TdT isolated from *E. coli* (48); lane 8, recombinant human TdT isolated from *Trichoplusia Ni* (24). Lane 9 in (a) is protein marker of BSA (67 kDa), γ -globulin (55 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa).

the native enzyme (Table 2). The polymerization efficiency was maximum for 8 mM Mg^{2+} . When Zn^{2+} is added to the reaction mixture along with Mg^{2+} , TdT activity is stimulated (30% stimulation of activity). This effect of Zn^{2+} on the chicken enzyme was confirmed with both native and recombinant enzyme preparations (data not shown). These activity measurements further confirmed that the cDNA clone we isolated and expressed in bacterial cells encoded the sequence for chicken TdT.

DISCUSSION

A cDNA coding for the chicken thymus TdT protein has been isolated, sequenced and expressed in *E. coli*. Comparison of the sequence and functional analysis of the recombinant protein confirmed its identity as a template independent DNA polymerase. Access to the avian cDNA has permitted a detailed analysis of TdT expression in lymphoid tissues of avians, a biochemical analysis of the avian TdT protein, and a sequence comparison of TdTs from three different genre (mammals, avian and amphibian).

A major difference between TdT activity found in mammalian thymus in comparison to avian thymus was the intrinsic specific activity of the two enzymes. TdT purified from mammalian sources has a specific activity of 80 000 U/mg and a turnover number of 4.6×10^{12} (34). In contrast, avian TdT isolated from thymus has a specific activity of 4 000 U/mg with a turnover number of 2.3×10^{11} (12). Comparable differences in specific activity were observed in recombinant TdTs from these two species.

The reasons for this dramatic difference in specific activity are not clear. The numbers of nucleotides inserted at the junctions of T-cell receptor genes are lower in chickens than mammals, but the differences are not as large as the 20-fold variation in specific activities of the proteins would predict. Also unclear are the structural features of the avian TdT that are responsible for its low specific activity. Significant portions of the amino acid sequence from chickens to humans are conserved, including regions containing the nucleotide (24) and putative DNA binding domains (31). However, access to these two sequences will allow us to probe structure-function relationships of both proteins. Other relative catalytic properties of the enzyme are similar to its mammalian counterparts.

Unlike mammals, in which tissue sites of both pre-B and pre-T cell development contain readily detectable TdT activity and protein, only the thymus in chickens appears to harbor TdT expressing cells. The sole site of avian B-cell development, the bursa of Fabricius, does not contain detectable TdT activity (35). However, when bovine TdT antibodies were used in immunodetection experiments, a single TdT-positive cell was detected at 18 days of embryonic development (11). We have been unable to detect TdT using Northern blot and RT-PCR at 7 and 14 days of embryonic development. This is not surprising since DNA rearrangement is thought to occur in only a few progenitor cells in the bursa. Access to the avian cDNA sequence generated from the current study coupled with a sensitive PCR analysis established that TdT coding sequences were not detectable in the chicken bursa at 7 and 14 day-old embryos or 1 and 6 weeks chickens post hatching (36,37).

The lack of demonstrable TdT activity and TdT mRNA in bursa of Fabricius of the chicken raises interesting questions concerning the development of the avian immune system. Immunoglobulin genes from the chicken, like their counterparts in mammalian species, go through DNA rearrangement before forming the functional immunoglobulin genes present in mature B-cells (10,38). The pattern of rearrangement of the immunoglobulin genes in avian species is different from those of mammalian species. The chicken heavy and light chain Ig repertoires are each created during B-cell development by gene conversion of a single rearranged V gene segment using V segment pseudogenes as donors (38–40). The rearrangement of Ig genes is not an ongoing process in the chicken as it is in mammalian species. Rather, it takes place as a single wave during early embryogenesis (41–43). For the chicken Ig heavy chain, no N-region nucleotide addition is observed at either DJ or VDJ junctions (44). Only P additions (complementary nucleotides to either coding end) are present in 15–20% of the junctions. The length of P additions does not appear restricted to a dinucleotide and they can extend for 3 or 4 bases (44).

For the chicken Ig light chain, studies have suggested that chicken V_{L1} – J_L joints do appear to have non-random nucleotide added to the coding ends during rearrangement (45). The nucleotides in chicken light chain genes appear to be specific addition of a single C nucleotide to the full length V_{L1} and an A nucleotide to the full length J_L segment prior to exonuclease trimming (45). In contrast, in mammalian cells, the N-region insertion contains multiple nucleotides (GC-rich) and is the sole consequence of TdT catalyzed additions (46).

Thus the absence of detectable TdT in chicken B-cells is reflected in absence of its product, the N-region nucleotide. The presence of the specific nucleotides at the joints of rearranged

immunoglobulin genes suggest that different enzymes and/or different mechanisms may be involved. There are several hypotheses proposed to account for the origins of P nucleotide insertions (47–49). Two recent studies suggest a hairpin model for V(D)J recombination. First, Roth *et al.* (1992) had observed the site-specifically cleaved molecules with covalently-closed termini in thymus DNA from adult or new born mice with severe combined immunodeficiency (50). Secondly, Meier and Lewis (1993) found that coding joints formed in lymphoid cells from normal mice have P nucleotides that cover a wide range of lengths (51). Whatever the mechanism, TdT is clearly not involved in a recombinase complex specific for avian immunoglobulin genes.

TdT activity is detected in avian thymus and the levels of specific mRNA are similar pre- and post hatching. Thus, it is likely that TdT activity is responsible for some level of diversity in T-cell receptor genes. The T-cell systems of avians and mammals share many conserved structural and functional features (52). However, studies conducted in the chicken suggest a relatively simple genetic organization of avian $\alpha\beta$ T-cell receptor (TCR) gene. The TCR β -chain gene locus in chickens contains two variable region families, $V_{\beta 1}$ and $V_{\beta 2}$, each of which consists of very few highly homologous members (53). The avian $V_{\beta 1}$ gene segments are rearranged by a deletional mechanism, whereas inversion is apparently the mechanism used to rearrange $V_{\beta 2}$ genes (54). The chicken TCR α -chain gene is also rearranged and expressed only in T-cells, as is true for mammalian TCR genes. One large V_{α} family exists, members of which are used by both $V_{\beta 1}$ and $V_{\beta 2}$ T-cell lines (55). The TCR α repertoire is further diversified by the presence of multiple J_{α} segments, as in mammals (56). Both the TCR α and β chain sequences contain deletions and N-nucleotide additions at the ends of the recombining elements (53,56).

From the data presented herein, we suggest that TdT activity is involved in N-region additions in T-cell receptor genes in chicken but is not responsible for diversity mechanisms in immunoglobulin genes. The intrinsic low activity of the chicken enzyme may be physiologically important. However the structural basis for the apparent differences in specific activity of the mammalian and chicken enzyme is not clear. Access to the complete chicken cDNA will make feasible additional studies about structure-function relationships of the protein and its evolution from chicken to man.

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