CPV3 is a novel avian parvalbumin. It displays an isoelectric point of 4.6, intermediate between that of avian thymic hormone (pI = 4.3) and the muscle parvalbumin isofrom (pI = 5.2). Expression of CPV3, like that of avian thymic hormone (ATH), is restricted to the thymic stroma. However, the CPV3 content of chicken thymus tissue (120 μg/g tissue) is 4 times lower than that of ATH (500 μg/g tissue). The polymerase chain reaction (PCR) was used to gain access to the nucleotide sequence of CPV3. A 147-base pair fragment of the coding sequence, corresponding to residues 48-97, was amplified from total chicken cDNA using degenerate PCR primers. A RACE-PCR strategy was then used to extend the known sequence in both the 5' and 3' directions. The cDNA sequence thus obtained includes 671 base pairs. Primer extension analysis suggests that the cloned cDNA corresponds to a full-length transcript. Northern analysis of chicken mRNA indicates that the average CPV3 transcript is approximately 800 nucleotides in length, significantly smaller than the ATH message (approximately 1000 nucleotides). Southern analysis suggests the presence of a single CPV3 gene in the chicken genome. The translated nucleotide sequence, displaying 108 residues between the initiator and termination codons, is that of a β-parvalbumin. The CPV3 sequence exhibits 58% identity with ATH and 52% identity with the chicken muscle isofrom. Interestingly, CPV3 and the mammalian oncodevelopmental parvalbumin called oncomodulin are identical at 73 of 108 residues (68% identity). Correspondingly, flow-dialysis measurements with 45Ca indicate that the Ca2+-binding domains are inequivalent, as in oncomodulin. The apparent dissociation constants, at pH 7.4 in 150 mM NaCl, are approximately 10 nM and 80 nM.

Ca2+ ion plays a central role in biological signal transduction in eukaryotic cells. Cytosolic Ca2+-concentrations increase transiently in response to a variety of stimuli in diverse cell types (1). In general, the physiological consequences resulting from the Ca2+ signal are mediated by intracellular Ca2+-binding proteins. Many of these proteins share a common metal ion-binding motif consisting of a central ion-binding loop flanked by short segments of helical structure. This helix-loop-helix structure is termed the "EF-hand" or "calmodulin fold" (2, 3). Certain members of this class of proteins, notably calmodulin and troponin C, function as calcium-dependent regulators. Others apparently serve as Ca2+ buffers.

Parvalbumins are small (M, = 11, 500), vertebrate-specific members of the calmodulin superfamily (2, 4). They contain two functional Ca2+-binding sites, commonly referred to as the CD and EF binding sites. Both sites typically belong to the "high-affinity" or "Ca2+/Mg2+" category, exhibiting substantial affinity for both Ca2+ and Mg2+ ions at physiological pH and ionic strength (5, 6). Parvalbumins are expressed at highest levels in skeletal myofibrils and GABA-ergic neurons (7, 8). In mammals, they have been detected in several other tissues as well, e.g. testis, adipose tissue, and kidney (9–11). They are generally viewed as Ca2+ buffers that probably function as soluble relaxing factors and/or cytosolic Ca2+ ion reservoirs.

Whereas fish and amphibians express multiple parvalbumin isofroms (e.g. 12–14), postnatal mammals express a single isofrom, which they employ in both muscle and non-muscle settings (11). Birds likewise express a single muscle parvalbumin (15), but have apparently retained other isofroms to function in extramuscular tissues. In 1989, for example, Ragland and his colleagues (16) reported that birds express a unique parvalbumin in the thymus gland, a finding that has since been verified by two other laboratories (17–21). This thymus-specific parvalbumin is called avian thymic hormone, or ATH,1 for its reported ability to stimulate maturation of T-cell precursors from bone marrow (22, 23).

Two years ago, we isolated a novel calcium-binding protein (24) from chicken thymus tissue and suggested that it represented a novel parvalbumin isofrom. Subsequent studies have confirmed this hypothesis. Reflecting its status as the third parvalbumin to be identified in the chicken, the protein is called CPV3. In this paper, we compare CPV3 with the other two parvalbumin isofroms and describe the cloning of its cDNA.

**EXPERIMENTAL PROCEDURES**

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1 The abbreviations used are: ATH, avian thymic hormone; CPV3, chicken parvalbumin isofrom 3, expressed in thymus; CPV1, chicken parvalbumin isofrom 1, from skeletal muscle; DTT, dithiothreitol; TBE, electrophoresis buffer consisting of 89 mM Tris, 89 mM boric acid, and 2 mM EDTA; MES, 2-(N-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline; TE, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; RAS, RIBI™ adjuvant system; PCR, polymerase chain reaction; RACE-PCR, rapid amplification of cDNA ends by the polymerase chain reaction; CD site, the metal ion-binding site in the parvalbumins flanked by the C and D helical segments; EF site, the metal ion-binding site in the parvalbumins flanked by the E and F helical segments; bp, base pairs; kb, kilobases.*
from Sigma: ampicillin, acrylamide, N,N'-methylenebisacrylamide, ethidium bromide, 2-mercaptoethanol, trifluoroacetic acid, Tween 20, phenylmethylsulfonyl fluoride, MES, HEPES, Tris base, boric acid, and Coomassie Brilliant Blue R-250. RPMI medium was obtained from the University of Missouri Cell and Immunobiology Core Facility. All other chemicals were reagent-grade.

Coomassie Brilliant Blue staining was performed in 0.75-mm mini-slab gels using the Laemmli buffer system (25). Bio-Rad molecular weight standards were employed for calibration. Proteins were visualized with Coomassie Brilliant Blue R-250 or silver staining (26). Nitrocellulose (Schleicher & Schuell) or polyvinylidene difluoride (Bio-Rad) replicas of polyacrylamide gels were prepared by electrophoretic transfer (2 h at 4.5 V/cm), employing a Bio-Rad buffer (27). Coomassie Brilliant Blue autoradiography was performed as described by Maruyama et al. (28), employing Kodak X-AR5 film for detection. Isoelectric focusing was performed in a Bio-Rad analytical isoelectricfocusing cell. The 0.4-mm polyacrylamide slabs (4.85% acrylamide, 0.15% N,N'-methylenebisacrylamide) were prepared with Bio-Rad narrow-range ampholytes (pH 4-6). Gels were prepared at 50 V for 30 min, loaded, then focused for 1 h at 100 V and then for 2 h at 200 V. Gradients were calibrated with standards obtained from Sigma.

HPLC separations were performed with a Pharmacia LKB 2249 gradient pump and a Pharmacia LKB Ultramicro column. Protein standards were separated on a Mono Q column. Anion exchange separations were performed on a Pharmacia Mono Q column, reverse-phase separations on a Pharmacia Pep-S column (in 0.1% trifluoroacetic acid, employing an acetonitrile gradient for elution).

Chicken muscle parvalbumin (CPV1) was isolated by the method of Strehler et al. (29). Avian thymic hormone was isolated by the method described by Serda and Henzl (20). Protein concentrations were determined by the dye-binding method of Bradford (30), employing Bio-Rad protein assay reagent.

Soluble tissue extracts were prepared by homogenizing fresh tissue samples in 3 volumes of lysing buffer (20 mM HEPES-NaOH, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% 2-mercaptoethanol). Taking advantage of the thermal stability of CPV3, the extracts were heated at 80 °C for 5 min, then centrifuged to remove denatured proteins. This step eliminates many extraneous proteins, without altering CPV3 levels, and thereby reduces competition for binding sites on nitrocellulose membranes during Western analyses.

Flow-dialysis measurements were conducted at room temperature (23 °C) as described by Colowick and Womack (31), with minor modifications (32-34). The 0.50-ml samples were prepared in 0.15 M NaCl, 0.025 M HEPES-NaOH, pH 7.4, 0.001 M EDTA. Divalent metal ions were removed from protein and buffer solutions prior to flow dialysis by ultrafiltration, then further purified on a Mono Q column, employing 0-4.0 M NaCl, in 20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DT. The isolated protein exhibits a band on silver-stained polyacrylamide gels.

Yields from 200 g of crude thymus tissue average between 1 and 2 mg. Based on the CPV3 content in thymus tissue (see below), we estimated a 50-75% recovery at 5-10%. The necessity for three chromatographic steps and for retaining only the most highly enriched fractions at each step may help explain the low yield.

Partial Amino Acid Sequence Analysis—Internal peptides of CPV3 were generated by CNBr cleavage (36) or arginylendopeptidase digestion. CNBr cleavage of CPV3 (250 pg) was performed for 24 h at room temperature in 0.5 ml of 70% formic acid, employing 1 mg of CNBr (Alrild). The reaction mixture was diluted to 5 ml, lyophilized, and redissolved in 50 μl of 50 mM Tris-HCl, pH 8.0. A 25-μl aliquot of the solution was then subjected to SDS-PAGE, followed by electrophoretic transfer to polyvinyldene difluoride. The resolved peptides were visualized with Coomassie Brilliant Blue R-250, and the fragment of lowest mobility was excised and sequenced directly.

Digestion of CPV3 (150 μg) with arginylendopeptidase (Takara, Inc.) was performed in accordance with the supplier’s recommendation. Selected fragments were isolated by reverse-phase HPLC on a Pharmacia Mono Q column. Prior to sequencing, the sample was lyophilized and redissolved in 250 μl of water, and allowed to adsorb overnight to a Polybrene-coated glass-fiber filter.

Amino acid sequence analysis was performed on a 470A gas-phase microsequencer from Applied Biosystems. Phenylthiobiotinylated proteins were sequenced "on-line" with an Applied Biosystems model 120 phenylthiohydantoin analyzer.

Immunochemical Techniques—Polyclonal antibodies to CPV3 were produced in mice employing antigen conjugated to keyhole limpet hemocyanin. Titters exceeded 10⁵ as judged by Western blot. Preparation of an anti-ATH antiserum, employing the same strategy, has been described previously (20).

Nitrocellulose replicas used for Western analysis were blocked for 1 h in 5% nonfat dry milk and rinsed with PBS containing 0.05% Tween 20 (PBST). They were then probed sequentially with the appropriate primary antibody and an alkaline phosphatase-linked secondary antibody (Promega), washing in between with PBST. Following a thorough wash with Tris-buffered saline containing 0.05% Tween 20, the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Promega). 1° and 2° antibodies were diluted into an antibody incubation solution that contained 5% (w/v) calf serum, 5% (w/v) nonfat dry milk, and 0.05% Tween 20, with the remaining volume consisting of PBS. Incubations were carried out in sealed pouches overnight at 4 °C or for 4-5 h at room temperature.

For quantitation, immunoblots were photographed with Polaroid Type 55 film, and the resulting 4 x 5-inch negatives were subjected to densitometric analysis. Densitometry was performed using a Hoefer DS 170 densitometer, then 510 nm absorbance was measured. The densitometric signals were proportional to concentration in the range of interest.

Molecular Biology—Poly(A) RNA was isolated from chicken thymus tissue with the Fast-Track kit from Invitrogen. Single-stranded cDNA was synthesized using avian myeloblastosis virus reverse transcriptase from Promega or Boehringer and the protocol supplied by Promega. PCR amplifications were performed in a total volume of 100 μl with a Perkin-Elmer thermal cycler. Taq polymerase, buffers, and dNTP solutions were purchased from Perkin-Elmer. Prior to cloning or additional rounds of amplification, PCR products were either purified directly with the QIAEX PCR purification kit (QIAGEN, Inc., Chatsworth, CA) or with the QIAEX gel extraction kit following agarose gel electrophoresis. Library Efficiency Escherichia coli DH5α cells were purchased from Life Technologies, Inc. pBlueScript SK+ and KS+ were obtained from Stratagene. DNA sequencing was performed either with Sequenase v.2.0 (United States Biochemical) or the fmol® PCR amplification kit (Promega), employing direct incorporation of α-[32P]dATP. Sequencing reactions were resolved on 5% gels prepared with the Long Ranger™ marker formulation (AT Biochemicals). All other restriction enzymes and DNA-modifying enzymes were purchased from Promega. For Northern analysis of chicken thymus mRNA, a fragment of the CPV3 cDNA was labeled with α-[32P]dCTP by the random primer method (37). Prehybridization and hybridization steps were performed in Quik-Hyb solution (Stratagene). The ECL® kit (Amersham) was used for nonradioisotopic detection of genomic CPVS probes on nitrocellulose.

Custom oligonucleotide primers were ordered from Genosys, Inc. or from the University of Missouri DNA Core Facility. These primers were used in the amplification of the CPV3 sequence: TPI, GCGGCGGC-
**RESULTS**

While characterizing the metal ion-binding properties of ATH, we discovered a calcium-binding protein in extracts of chicken thymus tissue that appeared to be a novel parvalbumin isof orm (24). Membership in the parvalbumin family has been confirmed by limited amino acid sequencing. Since CPV3 is N\(^{-}\)acetylated, internal peptides were obtained by CNBr cleavage or digestion with arginylendopeptidase. One of the CNBr fragments displayed the sequence: SKKSSQLKGI-FRILDNDQSFIGIEEDELKYL. The boldfaced type identifies the consensus sequence for a parvalbumin CD binding loop. Arginylendopeptidase, on the other hand, released this fragment: MLTASETKPLAADDHDDGKIGAEEFQ. In this case, the bold type corresponds to a consensus EF binding loop.

CPV3 exhibits an apparent isoelectric point of 4.6. The IEF behavior of the three chicken parvalbumins described to date is shown in Fig. 1. Note that the isoelectric point of CPV3 is intermediate between that of ATH (4.3) and that of chicken muscle parvalbumin (5.1). The pl that we observe for the muscle isof orm is in good agreement with the value of 5.2 reported by Kuster et al. (21).

**CPV3 Distribution and Abundance**—The tissue distribution of CPV3 was examined by Western blot analysis. Extracts were prepared from brain, heart, kidney, leg muscle, liver, lung, spleen, and thymus. Equal volumes were resolved on 7.5–20% gradient SDS-polyacrylamide gels (Fig. 2, top). Identical gels were transferred to nitrocellulose, and the replicas were probed with anti-CPV3 antiserum and an enzyme-linked secondary antibody. Expression of CPV3, like that of ATH (22), is apparently restricted to thymus tissue (Fig. 2, bottom).

To determine whether CPV3 is produced by lymphoid- or nonlymphoid tissue within the thymus, glands from 3-week-old chicks were subjected to gentle mechanical disruption with a wire-mesh tissue sieve, to obtain thymocyte-enriched and thymocyte-depleted cell populations. The cells that were rinsed from the gland with RPMI medium, and collected by centrifugation, constituted the crude thymocyte fraction. The remaining tissue fragments constituted the crude nonlymphoid, or stromal, fraction. Soluble extracts were prepared from both cell populations and analyzed by SDS-PAGE, together with a control extract prepared from whole thymus tissue. (Fig. 3A, top panel). The qualitative differences in the electrophoretic patterns obtained with the thymocyte (lane 2) and stromal (lane 3) fractions suggest that the tissue fractionation procedure was effective.

Nitrocellulose replicas were then prepared from polyacrylamide gels identical with that shown in the top panel of Fig. 3A and subjected to Western blot analysis for CPV3 and ATH (Fig. 3A, middle and bottom panels). Although strong signals are observed for both thymic parvalbumins in the lanes corresponding to the crude stromal fraction (lane 3), CPV3 and ATH are barely detectable in the thymocyte extract (lane 2). The weak signals observed in lane 2 probably reflect contamination of the crude thymocyte fraction by stromal cells.

The anti-CPV3 antiserum used in these experiments was specific for CPV3 (Fig. 3B). When equal amounts of the three chicken parvalbumins were subjected to Western analysis with the anti-CPV3 antiserum, only the CPV3 sample in lane B yielded a signal upon development (Fig. 3B, bottom panel). The additional faint band in lane B, having an apparent molecular weight of \( \sim 29,000 \), may be a disulfide-linked dimer of CPV3. Although the antiserum does not recognize either of the other chicken parvalbumins under these conditions, it displays reactivity toward several other thymic proteins (cf. lane A, Fig. 2, bottom) which may represent minor contaminants in the CPV3 antigen preparation.

CPV3 and ATH levels in chicken thymus tissue were estimated by Western blot analysis (Fig. 4). Samples of pure antigen (10 to 200 ng) were subjected to SDS-PAGE and electrophoretic transfer, together with several dilutions of a thymus extract. The resulting replicas were probed with the appropriate antiserum, followed by an alkaline phosphatase-conjugated secondary antibody. The signals appearing in the extract lanes upon development were compared to those of the standards. For quantitation, the immunobLOTS were photographed, and the resulting negatives were scanned with a video densitometer. According to this analysis, the signal in the top panel from 200 \( \mu \)g of thymus tissue represents 24 \( \pm 3 \) ng of CPV3, and the signal in the lower panel from 100 \( \mu \)g of tissue represents 49 \( \pm 4 \) ng of ATH. Thus, the CPV3 content in the avian thymus is
Cloning of a Novel Avian Thymic Parvalbumin

Cloning of a Novel Avian Thymic Parvalbumin—Acquisition of amino acid sequence data for CPV3 allowed us to access the nucleotide sequence by PCR. Degenerate oligonucleotide PCR primers were designed using the amino acid sequence data for residues 48–54 and residues 91–97. Following the recommendation of McPherson et al. (40), both nucleotides were included at positions of double degeneracy, but deoxyinosine was employed at positions of higher degeneracy. The resulting mixed-oligonucleotides (primers TP1 and TP2) amplified a 147-bp fragment from single-stranded chicken thymus cDNA (Fig. 5A), which was then cloned into E. coli DH5α. Except for residue 76, the translated nucleotide sequence of the insert agreed completely with the amino acid sequence data.

We extended our knowledge of the CPV3 cDNA sequence by applying PCR technology known as “RACE-PCR” (for Rapid Amplification of cDNA Ends, Ref. 41) or “one-sided” PCR (42). This strategy utilizes one nonselective primer in combination with an opposing sequence-specific primer. Although the entire cDNA population undergoes arithmetic amplification during the PCR process, the concentration of the target sequence should increase geometrically. Generally, however, it is not possible to isolate the fragment of interest from the cDNA background after just a single round of amplification. A second round of amplification is usually necessary, employing the nonselective primer once more in combination with a nested sequence-specific primer. Application of this method to the CPV3 cDNA is depicted schematically in Fig. 5B.

To obtain the region of the cDNA upstream from the 147-bp fragment, a sample of single-stranded chicken thymus cDNA was tailed with oligo(dG), employing terminal transferase (43). A preliminary round of amplification was then performed using an oligo(dC)15 primer (TP3) and an anti-sense sequence-specific primer corresponding to residues 91–97 (TP2). Following purification of the PCR reaction mixture, a second round of amplification was performed, employing the oligo(dC)15 primer in combination with a nested CPV3-specific primer (TP4) that spanned residues 79 to 84. The resulting 360-bp fragment included sequence information for residues 1–84, plus 108 nucleotides prior to the start codon.

A similar strategy was used to obtain the 3′ end of the coding sequence and the downstream noncoding region. However, since the original chicken thymus cDNA synthesis had been primed with oligo(dT), it was unnecessary to tail the DNA. Instead, PCR was performed directly on total single-stranded cDNA, employing an oligo(dT)-based primer (TP5) and an opposing CPV3-specific primer (TP1) corresponding to residues 48–54. We included a GC-rich extension at the 5′ end of the oligo(dT)-based TP5 primer in order to raise its melting temperature. The early amplification cycles were performed at a low annealing temperature (30 °C) to allow efficient hybridization of the oligo(dT). However, after five cycles, the annealing temperature was raised to 45 °C, reasoning that most of the target sequences should have incorporated the GC-tail by that point. After purification, an aliquot of the primary reaction mixture was subjected to a second round of amplification, this time with primer TP5 and a second, nested CPV3-specific primer (TP6) corresponding to amino acid residues 68 to 74. This nested amplification scheme yielded a 375-bp fragment harboring the coding information for residues 68–74, as well as 236 nucleotides of 3′ noncoding information.

The cDNA sequence determined for CPV3 is displayed in Fig. 6. There are 324 nucleotides between the initiation and termination codons, 108 nucleotides upstream from the start codon, and 236 nucleotides downstream from the stop codon. The sequence ATATAG, located 11 nucleotides upstream from the poly(A) addition site, probably serves as the polyadenylation signal (44). Thus, the cDNA sequence predicts a transcript size of 571 nucleotides, exclusive of the poly(A) tail.

Primer Extension Analysis—To determine whether the cloned cDNA was full-length, primer extension analysis (45)
was performed. An antisense, 32P-labeled primer, corresponding to amino acid residues 29–36 (TP7), was allowed to hybridize to total poly(A)+ thymus mRNA, after which primer-template complexes were extended with reverse transcriptase. An aliquot of this reaction mixture was then subjected to electrophoresis through a 5% polyacrylamide gel. The size of the major extension product should reflect the distance between the primer binding site and the 5' terminus of the RNA transcript (see Fig. 5B). Its length can be determined quite accurately by running it adjacent to a sequencing ladder, preferably one utilizing the same primer and template.

The results of our analysis, shown in Fig. 7A, indicate that the extension product included just 72 nucleotides upstream from the start codon, significantly shorter than predicted by the cloned cDNA sequence. Inspection of the 5' end of the CPV3 cDNA reveals a sequence, spanning nucleotides 9–31, having...
the potential for forming a stable stem-loop. This putative structure includes 10 Watson-Crick base pairs and a 3-nucleotide loop (Fig. 7B). It is possible that the corresponding element of secondary structure in the CPV3 message could cause premature termination of the primer extension reaction, yielding a truncated product. Conceivably, the reverse transcriptase would stall when it encounters the stem-loop and dissociate from the template. Presumably, premature termination of reverse transcription did not likewise occur during the original cDNA synthesis because the mRNA template was denatured by heating immediately prior to initiating the synthesis. The primer extension protocol, however, does not include a denaturation step. Instead, the radiolabeled primer is hybridized to the mRNA for 4–6 h at 42 °C, and the extension reaction is carried out at the same temperature.

If this hypothesis is correct, the result shown in Fig. 7A suggests that the primer extension reaction usually terminates some six nucleotides short of the stem-loop (vertical bar at left in Fig. 7A). However, a small percentage of the time, the reaction proceeds for a few more nucleotides. In fact, minor extension products are visible in Fig. 7A right up to the 3' end of the putative stem-loop.

There are 31 nucleotides between the 5' end of the cloned cDNA sequence and the 3' end of the putative hairpin. These 31 plus the 72 nucleotides of upstream sequence included in the major primer extension product account for 103 nucleotides of 5' non-coding information. This figure agrees well with the 108 nucleotides that we observe in the upstream region of the cloned cDNA sequence. The agreement is better still if we use the length of the largest minor extension product. We recently detected the stem-loop sequence in the CPV3 gene at the genomic level (data not shown), indicating that it reflects a genuine sequence element of the CPV3 message.

**Cloning of a Novel Avian Thymic Parvalbumin**

**FIG. 8. Northern analysis of chicken thymus mRNA and Southern analysis of chicken genomic DNA.** A, denatured chicken thymus mRNA (5 μg) was subjected to agarose gel electrophoresis in the presence of formaldehyde, blotted onto nitrocellulose, then probed at 60 °C either with a 32P-labeled fragment of the CPV3 cDNA or a fragment of ATH cDNA. Two nonstringent washes were then performed at room temperature in 0.5 × SSC, 0.1% SDS, followed by a single high-stringency wash at 62 °C in 0.1 × SSC, 0.1% SDS. Numbers at right correspond to mobilities of denatured, end-labeled fragments of ADNA obtained by digestion with PsI and EcoRI. A commercially obtained RNA ladder (Life Technologies, Inc.) gave similar results. Lane 1, hybridization with CPV3 cDNA probe; lane 2, hybridization with ATH cDNA probe. B, samples of chicken genomic DNA (10 μg) were digested with either BamHI, EcoRI, or HindIII, concentrated by EtOH precipitation, then resolved on a 1% agarose gel. Numbers at right represent mobilities of ADNA fragments generated by separate digestion with PsI or HindIII.

**Northern Analysis of Chicken Thymus mRNA**—Samples of chicken thymus mRNA (5 μg) were denatured and subjected to electrophoresis through a 2.0% agarose gel, containing 2.2 M formaldehyde, then blotted onto nitrocellulose. The replicas were then probed with 32P-labeled fragments of CPV3 cDNA (Fig. 8A, left) or ATH cDNA (Fig. 8A, right). The CPV3 probe hybridizes to a transcript having an average length of ~800 nucleotides, detectably smaller than the ATH transcript (~1000 nucleotides).

**Southern Analysis of Chicken DNA**—Chicken genomic DNA was subjected to Southern analysis employing a CPV3 cDNA probe. Samples of DNA (10 μg) were digested with either BamHI, EcoRI, or HindIII, subjected to electrophoresis through a 0.4% agarose gel, and transferred to nitrocellulose. Non-radioisotopic detection was performed with the Amersham ECL kit, following the protocol supplied with the kit. A fragment of CPV3 cDNA spanning nucleotides 97–520 was labeled with fluorescein-dUTP by the random primer method, then allowed to hybridize overnight to the nitrocellulose replica. Following two nonstringent washes and a single high stringency wash, the blot was blocked and incubated with a peroxidase-conjugated goat anti-fluorescein antibody. After washing, the chemiluminescent signal was generated with H2O2 and luminol, and the blot was immediately exposed to x-ray film for 30 min.

The resulting autoradiogram is presented in Fig. 8B. Both the EcoRI and HindIII digests afford single bands of 11 kb and 7.2 kb, respectively. The BamHI digest displays two very large bands: one approximately 14 kb in length, the other exceeding 23 kb. The faintness of the upper signal probably reflects in-
complete transfer to the nitrocellulose membrane, as the target DNA was not depurinated prior to blotting.

Homology to Other Parvalbumins—The translated cDNA sequence for CPV3 (Fig. 6) indicates that the protein contains 108 amino acid residues. Like the other two chicken parvalbumins, CPV3 also lacks tryptophan and contains a single tyrosine and 9 phenylalanine residues. Although parvalbumins are characteristically cysteine-deficient, CPV3 contains 2 cysteine residues, at positions 18 and 72.

Fig. 9 compares the amino acid sequence of CPV3 to those from several other parvalbumins. CPV3 shows slightly greater homology to isoforms belonging to the β-lineage. For example, it is identical with ATH at 63 of 108 positions (58% identity) as compared to 56 of 108 for the chicken muscle isoform (52% identity). Surprisingly, the CPV3 sequence shows the greatest similarity to the mammalian oncofetal parvalbumin known as oncomodulin. The two proteins are identical at 73 of 108 residues (68% identity).

Ca²⁺-binding Affinity—At the reviewer's request, we conducted a preliminary study of the Ca²⁺-binding properties of the CPV3 protein, employing flow dialysis with ⁴⁰Ca²⁺. Control experiments were also performed with recombinant ATH and a variant of oncomodulin harboring oncomodulin → rat parvalbumin substitutions at residues 57, 59, 60, and 69. The Ca²⁺-binding constants for the latter have been reported previously (34), as part of an investigation into the determinants of Ca²⁺-binding affinity in oncomodulin. The data for the three proteins, plotted in Scatchard format, are displayed in Fig. 10. As previously observed (18, 20), the two ATH ion-binding sites behave equivalently (A), displaying an average $K_{Ca} = 10$ nM. By contrast, the two binding sites in the oncomodulin variant are strikingly non-equivalent (■), with the CD site displaying $K_{Ca} = 0.3$ μM and the EF site $K_{Ca,EF} = 30$ nM. CPV3 displays intermediate behavior (■). The curvature in the data indicates that the two binding sites are non-equivalent, as in oncomodulin; however, they display substantially greater affinity for Ca²⁺. The data can be modeled by a two-site Scatchard model ($\chi^2 = 0.0091$; $R^2 = 0.992$), with $K_1 = 0.010 \pm 0.001$ μM and $K_2 = 0.079 \pm 0.05$ μM.

**DISCUSSION**

In 1989, Ragland and co-workers (16) reported that the thymus-specific protein known as avian thymic hormone was a parvalbumin. Since then, it has become apparent that birds express a second thymus-specific parvalbumin isoform. The existence of the second calcium-binding protein was first suggested by ⁴⁰Ca²⁺ autoradiographic studies undertaken to monitor the purification of avian thymic hormone (24). Membership in the parvalbumin family has been confirmed by sequence analysis at both the amino acid and nucleotide levels. We have labeled the protein CPV3 to reflect its status as the third parvalbumin isoform to be identified in the chicken.

Western blot analysis (Figs. 2 and 3) indicates that CPV3 expression, like that of ATH, is restricted to the non-lymphoid compartment of the thymus. However, the level of expression is
substantially lower than that of ATH. Whereas the ATH content is roughly 500 µg/g tissue, the CPV3 content is just 120 µg/g. The ATH content that we observe is comparable to the value of 1.2 mg/g reported by Murthy et al. (22).

Interestingly, the mouse antisera to CPV3 used in these immunoblot studies do not cross-react significantly with either of the other two chicken parvalbumins (Fig. 5) under conditions that yield a strong signal for CPV3. This finding is consistent with our previous observations. For example, anti-ATH antisera fail to recognize the chicken parvalbumin isofrom from muscle (20), and polyclonal antibodies to rat oncomodulin do not cross-react with rat muscle parvalbumin, despite substantial (53%) sequence identity in the two proteins. 2 It is anticipated that a globular protein with M<sub>r</sub> ~ 12,000 would exhibit relatively few antigenic determinants. Apparently, these epitopes are not highly conserved in the parvalbumins, despite the overall high degree of sequence homology.

We used PCR to gain access to the nucleotide sequence of CPV3, as we had previously for ATH (17) and chicken muscle parvalbumin (19). The amino acid sequence data for two selected internal fragments of CPV3 enabled us to design protein-specific PCR primers. The coding, or “sense,” primer was based on residues 48–55, and the noncoding, or “antisense,” primer was based on residues 90–97.

With these primers, we successfully amplified a 147-nucleotide fragment of DNA. The nearly perfect agreement between the translated nucleotide sequence of the fragment and the available amino acid sequence conclusively established that we had succeeded in accessing the CPV3 cDNA sequence. We obtained the remainder of the cDNA sequence using a PCR technique that is referred to as “RACE” or “one-sided” PCR (41, 42). The carboxyl-terminal end of the coding sequence and the 3′-noncoding region were obtained by two successive rounds of PCR amplification, employing an oligo(dT)-based primer and nested, CPV3-specific sense primers. A similar strategy was used to obtain the 5′ end of the cDNA. After tailing total single-stranded cDNA with dG, two rounds of PCR amplification were performed, employing oligo(dC) and nested, CPV3-specific antisense primers.

The resulting CPV3 cDNA sequence spans 671 base pairs, including 108 bp of 5′-noncoding sequence, 327 base pairs from the initiation to the termination codons, and 326 base pairs of 3′-noncoding sequence. We believe that this corresponds to the “full-length” cDNA. Interestingly, primer extension analysis indicates that the 5′-untranslated region should include just 72 nucleotides of sequence upstream from the start codon. However, the first 35 nucleotides of the cloned cDNA sequence includes a 27-nucleotide region having the potential for forming a stable stem-loop structure. The presence of this hairpin in the corresponding CPV3 message could cause premature termination of the primer extension reaction, resulting in the truncated product.

Northern analysis of chicken thymus mRNA indicates that the average CPV3 transcript size is about 800 nucleotides. Subtracting the 671 nucleotides between the 5′ end of the message and the polyadenylation site leaves a poly(A) tail having an average size of roughly 130 nucleotides. The CPV3 message is slightly smaller than the ATH message. In this analysis, we measure an average ATH transcript length of approximately 1000 nucleotides, in reasonable agreement with the 1050-nucleotide figure reported previously by Palmisano and Henzl (18).

Southern analysis, employing a fragment of the CPV3 cDNA as a probe, suggests that there is a single copy of the CPV3 gene in the chicken genome. This finding is consistent with data gathered on other parvalbumin genes, notably those from human and rat (50–52).

The parvalbumin family contains two sublineages, α and β (53). α-Parvalbumins display pl values above 5.0, while β-parvalbumins typically have isoelectric points below 4.5. ATH qualifies as a β-parvalbumin on the basis of its isoelectric point (pl = 4.3) and sequence homology to other β-parvalbumins (18, 46). The muscle isoform (pl = 5.2), on the other hand, is an α-parvalbumin (21). Although CPV3 displays an intermediate isoelectric point (pl = 4.6, Fig. 1), the protein clearly belongs to the β-lineage. The translated nucleotide sequence shows 108 residues between the initiation and termination codons, diagnostic for β-parvalbumins. Moreover, Cys-18 and Phe-66 are β-lineage-specific residues that are both present in the CPV3 sequence. Not surprisingly, CPV3 exhibits slightly greater homology to ATH (87 of 108 residues) than to the muscle isoform (61 of 108 residues).

An unexpected finding that emerged from our analysis of the CPV3 cDNA sequence is the strong sequence similarity between CPV3 and the oncofetal mammalian parvalbumin called oncomodulin (54). The two proteins are identical at 73 of their 108 residues, for 68% identity. Oncomodulin was discovered by MacManus (55) in extracts of a rat hepatoma in 1979. Normal expression of the protein is restricted to the extraembryonic tissue of the fetal placenta (56); however, it frequently reappears upon tumorigenesis (57). Its name derives from its frequent expression in neoplasms and its suggested role as a calcium-dependent modulator during early embryological development. Given the high level of sequence identity in the two proteins, it is likely that CPV3 and oncomodulin evolved from a common precursor.

Against the backdrop of high sequence identity, the differences between CPV3 and oncomodulin loom significantly. In particular, the nonidentities at positions 7–10 of the CD ion-binding loop merit consideration. Whereas CPV3 harbors the consensus parvalbumin sequence F-I-E-E, oncomodulin contains Y-L-D-G. To our knowledge, oncomodulin is the only parvalbumin to exhibit this variant sequence. The departure from CPV3 suggests that the oncomodulin sequence evolved after the two proteins diverged. Moreover, the strict conservation of this sequence in mammals as diverse as mice and humans suggests that it has functional implications.

Oncomodulin binds Ca<sup>2+</sup> less tightly than other parvalbumins (32, 58). Whereas the CD and EF sites of typical parvalbumins display identical dissociation constants for Ca<sup>2+</sup> between 2 and 10 nm, the respective Ca<sup>2+</sup>-binding constants for oncomodulin are 0.8 µM and 0.04 µM. It is of interest to determine whether the metal ion-binding properties of CPV3 resemble those of oncomodulin. Although an inability to isolate substantial amounts of the protein has largely frustrated this effort to date, we have been able to estimate the Ca<sup>2+</sup>-binding constants by flow dialysis. Whereas avian thymic hormone possesses two equivalent high-affinity Ca<sup>2+</sup>-binding sites (K<sub>n</sub> = 10 nm), the two sites in CPV3 are demonstrably nonequivalent, as judged by the curvature in the Scatchard plot (Fig. 10). If the CPV3 flow-dialysis data are fit to a two-site model, we extract dissociation constants of K<sub>1</sub> = 10 ± 1 nm and K<sub>2</sub> = 79 ± 5 nm. Thus, as in oncomodulin, the Ca<sup>2+</sup>-binding sites in CPV3 are nonequivalent. However, their affinities for Ca<sup>2+</sup> are substantially higher than the corresponding sites in the oncofetal protein. By analogy to oncomodulin, we would assign K<sub>1</sub> to the EF site and K<sub>2</sub> to the CD site.

Within the vertebrate kingdom, there is a strong evolutionary trend to fewer parvalbumin isoforms. Lower vertebrates, fish and amphibians, express multiple isoforms in a tissue-specific and developmentally regulated manner. By contrast, postnatal mammals seem to rely exclusively upon a single iso-

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2 R. C. Hapak, H. Zhao, J. M. Boschi, and M. T. Henzl, unpublished observations.
form, which is expressed in skeletal muscle and non-muscle tissues alike, as needed. Avian species exhibit yet another pattern of parvalbumin expression. Like the mammals, birds express a single muscle parvalbumin isoform. However, they have retained at least two other parvalbumins, ATH and CPV3, to ostensibly fulfill biological tasks associated with the avian immune system.

Both are β-parvalbumins, and both are expressed in the non-lymphoid tissue of the thymus gland. At present, little is known with certainty concerning the biological functions of these two proteins. ATH was discovered by Ragland and his colleagues at the University of Georgia, and Ragland has suggested that ATH functions as an immunomodulatory protein, triggering maturation of immature T lymphocytes (16). According to this hypothesis, ATH is secreted by the thymic epithelial cells and interacts with peptide receptors on immature T lymphocytes and/or T-cell precursors in bone marrow. Perhaps CPV3 plays a similar role.

In this context, the presence of 2 cysteine residues in CPV3 is interesting. Although x-ray crystallographic data and chemical studies on other parvalbumins indicate that cysteine is inaccess to soluble Ca\(^{2+}\)-bound form of the protein (59-61), the sulfhydryl of cysteine 72 in CPV3 should be solvent-exposed. If CPV3 performs an extracellular role analogous to that proposed for ATH, there is the possibility for a physiologically relevant dimerization. In brain, for example, the Ca\(^{2+}\)-binding protein called S-100β forms a disulfide-linked dimer, yielding a potent nerve growth factor called neurite extension factor (62).

The suggestion that ATH and CPV3 may serve as extracellular signaling factors conflicts with the traditional view of parvalbumins as cytosolic Ca\(^{2+}\) buffers. Until additional evidence for an immunomodulatory role is presented, it would be premature to discount an intracellular function for the thymic parvalbumins. The thymic stroma, a strikingly heterogeneous loci at which the two avian thymic parvalbumins could influence T-cell maturation. Moreover, both positive and negative selection processes require physical contact between the immature lymphocytes and cells of the thymic stroma. There are clearly multiple loci at which the two avian thymic parvalbumins could influence the T-cell developmental process.

Although the muscle parvalbumin isoform has been detected in the myoid cells of the rat thymus gland (63), no additional isoforms have been observed. This absence is somewhat unexpected, given the general similarity of T-cell maturational and functional processes in birds and mammals (for discussion, see Ref. 64). Delineating the functional significance of the thymic parvalbumins and rationalizing their deletion from the mammalian genetic repertoire offer challenging avenues for future investigation.

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