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AVIAN THYMIC HORMONE AND CHICKEN (MUSCLE) PARVALBUMIN ARE DISTINCT PROTEINS: ISOLATION OF A MUSCLE PARVALBUMIN cDNA FRAGMENT BY PCR†

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Access to the nucleotide sequence of parvalbumin from chicken muscle was gained via the polymerase chain reaction. In the absence of specific amino acid sequence data, the PCR primers were based on concensus data for the two parvalbumin Ca^{2+}-binding sites. The 137 bp fragment obtained by amplification clearly codes for a parvalbumin, as judged by the presence of 10 invariant codons within the sequence flanked by the primers. When used to probe poly(A)+ RNA from chicken muscle, the fragment recognizes an 800 nucleotide transcript. The translated nucleotide sequence of the muscle protein is unmistakably distinct from that of the thymus-specific parvalbumin known as avian thymic hormone. Of the 31 amplified residues, the two proteins differ at 14. The presence of a distinct parvalbumin in chicken thymus is consistent with the potent effector role proposed for the protein.

Parvalbumins are small (M_r=10,000-12,000), vertebrate-specific proteins possessing high-affinity sites for two Ca^{2+} ions (1). Both Ca^{2+}-binding domains display the trademark of the calmodulin superfamily, a helix-loop-helix structural motif known as the "EF-hand" (2,3) or "calmodulin fold" (4). The highest levels of parvalbumin occur in skeletal muscle, but the protein has also been detected in several non-muscle tissues in the rat (for a review, see 5). Parvalbumins are generally regarded as Ca^{2+} ion buffers and may function in muscle and neuronal relaxation (e.g., 6,7).

Recently, high levels of a parvalbumin were observed in chicken thymus tissue (8). This protein has been designated avian thymic hormone (ATH), in recognition of an apparent capacity for stimulating differentiation of T lymphocytes (9,10). Circumstantial evidence -- amino acid composition data (8,11), apparent absence of immunological cross-reactivity (12), and Northern analysis (11) -- suggested that ATH was distinct from the muscle parvalbumin. However, in view of the identity of the muscle and non-muscle isoforms in the rat (5), it was important to verify that the two avian parvalbumins were distinct before proceeding with further investigation of ATH.

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Abbreviations: ATH, avian thymic hormone; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactose; SSC, 0.15 M NaCl, 0.015 sodium citrate, pH 7.0.

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In this communication, direct evidence is presented for the nonidentity of the two proteins. We have succeeded in amplifying a fragment of the nucleotide sequence for chicken (muscle) parvalbumin using the polymerase chain reaction. The PCR primers consisted of highly degenerate mixed oligomers based on the concensus amino acid sequence for the two ion–binding sites. These primers enabled us to selectively amplify a 137 bp fragment of the parvalbumin coding sequence from a chicken leg muscle cDNA preparation. The translated sequence of the cloned fragment differs from that of avian thymic hormone at multiple positions.

MATERIALS AND METHODS

Chicken leg- and breast muscle and thymus glands were obtained from Pel-Freez, Inc. At our request, the tissues were placed on dry ice immediately upon collection. Poly(A)+ RNA was isolated from each of the frozen tissues using the Fast Track mRNA isolation kit marketed by Invitrogen. The thermostable DNA polymerase called Replinase (Dupont) was employed for PCR amplification, and Sequenase (US Biochemical Co.) was employed for DNA sequencing. All other enzymes were obtained from Fisher-Promega. The Bluescript KS+ cloning vector was purchased from Stratagene, Inc. Lambda DNA used to generate molecular weight markers was obtained from Promega, while RNA markers were purchased from GIBCO–BRL. Custom PCR primers were ordered from Genosys, Inc. Agarose was purchased from IBI, Inc. The nitrocellulose membrane used for Northern analysis was manufactured by Schleicher & Schuell. IPTG and the chromogenic β-galactosidase substrate X–gal were purchased from Sigma.

Double-stranded cDNA was produced from leg muscle mRNA by a minor modification (13) of the Gubler and Hofmann method (14), employing reverse transcriptase for both first- and second-strand synthesis. The resulting preparation was then used as a template for the polymerase chain reaction. PCR amplification was carried out in an Eppendorf thermal cycler following the protocol supplied with the Replinase but scaling the suggested 20 µl reaction volume up to 100 µl. Thirty cycles of amplification were performed, each cycle consisting of denaturation (94° C, 2 minutes), annealing (37° C, 3 minutes), and polymerization (72° C, three minutes). Additional enzyme was added after 15 cycles.

The desired PCR product was purified by electrophoresis through 3.0% agarose in TBE buffer and recovered by binding and elution from powdered glass (15). After blunting the ends of the fragment with T4 DNA polymerase (16) and phosphorylating the 5' ends with T4 polynucleotide kinase, the fragment was inserted into the Sma I site of Bluescript KS+. The ligation, conducted at 15° C, employed 1 unit of T4 DNA ligase. An aliquot of the ligation reaction was used to transform *E. coli* DH5α (Library Efficiency , from GIBCO-BRL), and the cells were plated on LB agar in the presence of ampicillin, IPTG, and X-gal.

Preparative quantities of the desired construction (pCPV1) were obtained by the alkaline lysis method (17). The fragment was sequenced with the Sequenase system (US Biochemical Co., Inc.), which is based on the dideoxy termination strategy (18). For Northern analysis, the cDNA insert from pCPV1 was released by digestion with BamH I and Pst I, electrophoretically purified, and labeled with [α–^{32}P]dCTP using the random primer method (19). A fragment of the avian thymic hormone cDNA, coding for the first 101 residues of the protein, was obtained by EcoR I digestion of pATH8 (11) and was similarly purified and labeled for use as a probe.

RESULTS

The goal of this work was to obtain the amino acid sequence for a fragment of the chicken (muscle) parvalbumin amino acid sequence and compare it to the known sequence of ATH (11, 20). PCR technology appeared to offer the most efficient approach to the problem. We had

previously employed the polymerase chain reaction to gain access to the nucleotide sequence of ATH (21).

In the case of the muscle protein, the absence of amino acid sequence data for the muscle protein made the problem somewhat more challenging. However, since the two calcium–binding loops were known to display a high degree of conservation (3,22), we decided to base our PCR primer sequences on the concensus amino acid sequences for these regions.

The sequences for the mixed oligomers employed in this work are displayed in Figure 1. The primers labeled CPV1 and CPV2 were both based on the amino acid sequence for the CD binding loop, while the two labeled ATH4 and CPV3 are based on the EF binding loop sequence. All four primers were highly degenerate, with CPV3 actually including some 3072 distinct sequences.

Initial efforts to amplify a fragment of the chicken muscle parvalbumin sequence were undertaken with the CPV1 and ATH4 primers. We had previously used ATH4 to amplify a fragment of the avian thymic hormone cDNA sequence (21). In retrospect, our success with ATH4 seems somewhat serendipitous, since the ATH4 sequence was derived under the assumption that residue 95 was an aspartyl residue, whereas it was subsequently determined to be a glycine. As a result, perhaps, of this misassignment, all attempts to amplify detectable amounts of the muscle cDNA fragment with the ATH4 primer were fruitless. Initially, we attributed the

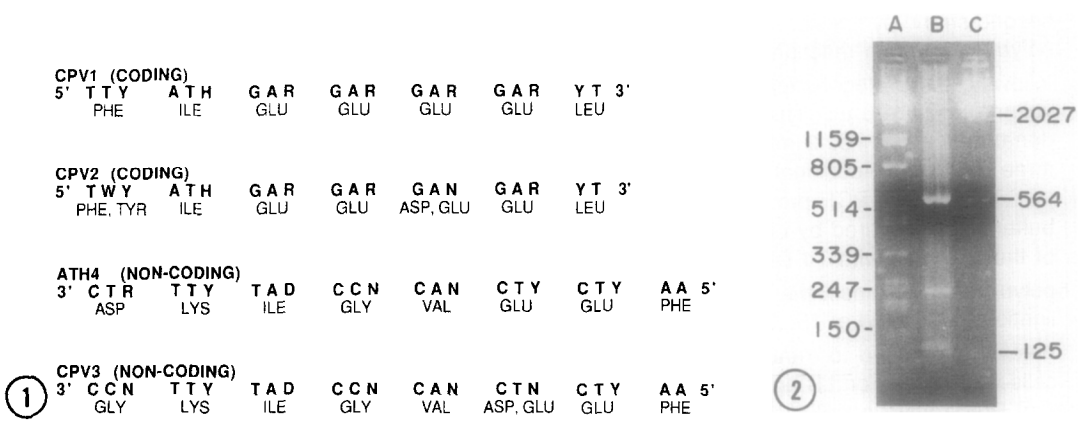

Figure 1. Mixed oligonucleotides employed as PCR primers. The four mixed oligomers shown above were used as PCR primers in attempts to amplify a parvalbumin cDNA fragment from chicken muscle cDNA. The sequences of CPV1 and CPV2 were based on the most common amino acid sequence at the C-terminal end of the CD ion-binding loop in the parvalbumins. The sequences of ATH4 and CPV3 were derived from the concensus amino acid sequence for the C-terminal end of the EF ion-binding loop. The following abbreviations are used to indicate multiple nucleotides: D (G,A,T); H (A,C,T); N (A,G,C,T); R (A,G); W(A,T); Y (C,T).

Figure 2. Amplification of a fragment of the chicken (muscle) parvalbumin coding sequence. 100 ng of double-stranded chicken leg muscle cDNA was subjected to PCR amplification, followed by re-amplification, as described in the text, employing the CPV2 and CPV3 primers. 10% of the reaction volume was then subjected to electrophoresis through a 3.0% agarose gel, in the presence of ethidium bromide (lane B). When photographed with ultraviolet transillumination, a faint band is visible, migrating just behind the 125 bp fragment generated by digestion of lambda DNA with Hind III (lane C). Lambda markers, generated by digestion with Pst I, are shown in lane A.

lack of success to our failure to allow for both TYR and PHE at position 57 and both ASP or GLU at residue 61. However, the combination of ATH4 and CPV2, which allows for the alternative residues, was likewise unsuccessful.

Far more often than not (22), residue 95 is a glycine. We therefore obtained a primer, CPV3, which was compatible with this assignment. Using this primer, in combination with CPV2, we were successful in generating an amplification product of the expected size. The putative fragment, although detectable, was very faint after the first round of PCR. Thus, four additional reactions were performed (30 cycles of amplification), each employing 10 µl of the primary PCR reaction for a template. After this second round of PCR, the amplification product --running just behind the 125 bp fragment obtained by digestion of lambda DNA with Hind III -- was clearly discernible (Figure 2). Interestingly, the combination of the CPV1 and CPV3 primers did not yield detectable fragment, suggesting that the presence of the aspartate codon at residue 61 was critical.

The four secondary PCR reactions were pooled, concentrated, and subjected to agarose gel electrophoresis through a 3.0% gel. The band of interest was excised, and a small amount of the desired DNA was recovered with powdered glass. After blunting with T4 polymerase and phosphorylating the 5' ends, the fragment was ligated into the Sma I site of the Bluescript KS+ cloning vector. Since the Bluescript multiple cloning region falls within the β-galactosidase coding sequence, recombinant colonies could be identified by transforming a *lacZ*$^-$ strain and plating in the presence of IPTG and X-gal.

Although transformation of *E. coli* DH5α afforded more than 5,000 colonies, only eight of these were white. Plasmid was isolated from all eight and digested with BamH I and Pst I. Two of the plasmids were found to harbor detectable inserts. One of these was slightly larger than anticipated and was subsequently found to contain a sequence of unknown origin. The other, however, apparently corresponded to the protein of interest. The nucleotide and translated amino acid sequences of this 137 bp fragment are displayed in Figure 3, together with the corresponding data for avian thymic hormone.

The insert is unmistakably a parvalbumin cDNA fragment. It contains the requisite number of codons between the CD and EF ion-binding domains. Moreover, parvalbumins have been shown to contain numerous invariant residues (3). The codons for all ten of those occurring in the region spanning residues 64-94 are present in the amplified fragment. The corresponding amino acid residues have been underlined in Figure 3.

It is also apparent from Figure 3 that the cloned cDNA fragment codes for a parvalbumin which is distinct from ATH. Of the 31 residues, excluding the primers, that were amplified by PCR, the two sequences are nonidentical at 14. These have been identified with bold-faced type. Notice that our sequence data indicates that residue 101 of the muscle parvalbumin is a lysine. We assume that this is an artifact of the amplification process, since this residue is an invariant glutamate in all parvalbumins examined to date (22). The side-chain of GLU-101 is coordinated to the bound metal ion and is absolutely required for high–affinity Ca^{2+} binding.

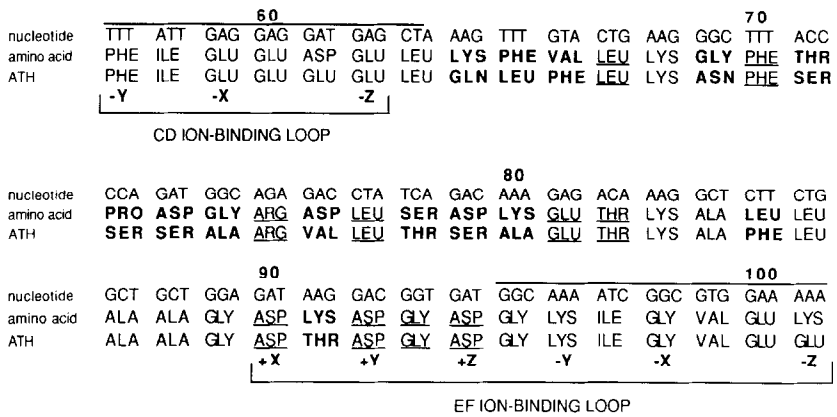

Figure 3. Nucleotide sequence and translated amino acid sequence of the cloned cDNA fragment. The nucleotide sequence of the cloned PCR fragment was obtained by a modification of the Sanger dideoxy termination method. Assignments were confirmed by sequencing both strands. The translated amino acid sequence is displayed below the DNA sequence, together with the corresponding sequence of ATH (18,27). Nonidentities are displayed in bold, and invariant parvalbumin residues are underlined. The lines above the nucleotide sequence indicate the regions spanned by the PCR primers. The borders of the two ion–binding loops are indicated, as are the residues which participate in coordination of metal ions.

To demonstrate that the amplified sequence was actually derived from a chicken muscle gene product, we probed leg muscle mRNA with the ^{32}P-labeled cDNA fragment from pCPV1. Samples of poly(A)+ RNA isolated from chicken leg muscle and chicken breast muscle were subjected to denaturing agarose gel electrophoresis and transferred to nitrocellulose membranes. The labeled CPV1 probe hybridized to a transcript in leg muscle having an average length of 800 nucleotides (Figure 4, panel A, lane 1). No hybridization to the RNA sample from breast muscle was observed (panel A, lane 2), consistent with the reported absence of parvalbumin in that tissue (23,24). A labeled fragment of the avian thymic hormone cDNA failed to hybridize to the leg muscle RNA, attesting to the specificity of the CPV1-mRNA interaction (panel B, lane 2).

DISCUSSION

Parvalbumins from fish and amphibians exhibit characteristic polymorphism (e.g.,25,26). By contrast, the skeletal muscles of birds (27,28) and mammals (29,30) contain a single parvalbumin isoform, which can be expressed in non-muscle tissues as well.

A protein from chicken thymus with the ability to stimulate maturation of T–lymphocyte precursors (9,10) was recently sequenced and shown to be a parvalbumin (8). In the absence of sequence data for the muscle-associated parvalbumin, the possibility that the chicken muscle and thymic parvalbumins were identical could not simply be ignored. In the rat, for example, the muscle isoform has also been detected in specific neurons, kidney, adipose tissue, and testis (5). In the chicken, moreover, the muscle isoform has also been detected in brain (23).

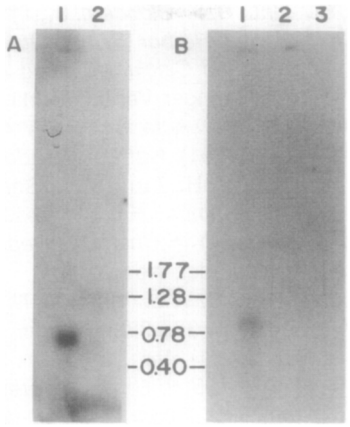

Figure 4. Hybridization of labeled PCR fragment to poly(A+) RNA from chicken leg muscle. Samples of poly(A)+ RNA (10 μg) from chicken leg and breast muscle were subjected to electrophoresis through a 1.0% denaturing agarose gel, then transferred to nitrocellulose membranes by capillary blotting. After prehybridization, the membranes were probed either with the ^{32}P–labeled fragment amplified from leg muscle cDNA (Panel A) or a ^{32}P-labeled fragment of avian thymic hormone cDNA (Panel B). Panel A: lane 1, leg muscle mRNA; lane 2, breast muscle mRNA. Panel B: lane 1, thymus mRNA; lane 2, leg muscle mRNA; lane 3, breast muscle mRNA. The thymus mRNA was included in Panel B as a test of the effectiveness of the ATH cDNA probe. Blots were washed in 0.2 x SSC, 0.1% SDS at 63° C prior to autoradiography.

The failure of antisera to the thymus-specific parvalbumin to recognize the muscle parvalbumin on Western blots of chicken muscle extracts (12) and the failure of a cDNA probe to hybridize to poly(A)+ RNA from chicken muscle (11) suggested that the proteins were nonidentical. However, a rat skin calcium-binding protein -- judged to be novel by various criteria (31) -- was subsequently shown to be identical to the muscle protein (32). With the cloning and sequencing of a muscle parvalbumin cDNA fragment, it is now clear that chicken muscle parvalbumin and avian thymic hormone are distinct proteins.

Because they contain only Ca^{2+}/Mg^{2+} sites, and because their tertiary structures appear to preclude calcium–dependent interactions analogous to those that occur with calmodulin or troponin C (33), parvalbumins are not believed to function as calcium-dependent modulators. Traditionally, they have been viewed as Ca^{2+} ion buffers. Suggested roles include: facilitating relaxation of skeletal muscle (e.g.,6); facilitating recovery of specific neurons following excitation (7); and acting as cytoplasmic Ca^{2+} ion reservoirs to prevent Ca^{2+} toxicity in tissues subject to frequent or continuous excitation (34). Judging from the parvalbumin distribution in the rat, a single polypeptide is capable of fulfilling these roles in diverse tissues. Thus, the finding that avian thymic hormone is distinct from chicken muscle parvalbumin is consistent with the potent physiological effector role proposed for this protein.

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