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# IN VITRO TRANSLATION OF CYTOSKELETAL BEADED-CHAIN FILAMENT PROTEINS FROM CHICKEN LENS mRNA

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The beaded-chain filament is a unique cytoskeletal structure that appears in the elongating fiber cells during the differentiation of lens epithelial cells to form the mature fiber cells. This beaded-chain structure is made up of two proteins of molecular weight 95 kDa and 49 kDa. As a prerequisite for cloning the cDNAs of these proteins, newborn chicken lens total poly(A+) mRNA was translated in vitro, using a rabbit reticulocyte lysate system and [$^{35}$S]-L-methionine. The labelled translation products were analyzed by one-and two dimensional gel electrophoresis followed by autoradiography. Immunoprobing of the translation products on Western blots using specific polyclonal antibodies identified the above proteins, and demonstrated the presence and expression of specific mRNAs in the neonatal chick lens, that code for the in vitro synthesis of these two cytoskeletal proteins. These mRNAs are low abundant mRNAs as compared to the crystallin mRNAs. 

Lens epithelial cells undergo a remarkable morphological and biochemical transformation during the process of differentiation into mature fiber cells. Morphological events include the loss of organelles and the elongation of the cuboidal epithelial cell to more than 100 times its original length (1). Biochemical changes include a switch in the composition of crystallins, a shift from G-actin to F-actin with an increase in the total actin content, decreased vimentin relative to actin, decreased tubulin synthesis, initial appearance of myosin and the accumulation of MP-26, the main intrinsic membrane protein (1). Also reported was a unique cytoskeletal structure, the beaded-chain filament, seen only in the elongating fiber cells of the chick lens (2). The beaded-chain structure contains two specific proteins of molecular weight (mol. wt.) 95 kDa and 49 kDa, referred to as cytoskeletal proteins, CP-95 and CP-49, respectively (2). CP-95 and CP-49 are lens specific, appear only in fiber cells and

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serve as markers of terminal differentiation of lens epithelial cells (3). Immunologically related proteins have been detected in bovine and human lenses (3). In all three species, the cross-reacting proteins consist of multiple isoelectric variants, distinguishable by their extent of phosphorylation (3,4). More recently, it has been shown that adrenergic drugs are capable of not only increasing the phosphorylation of CP-49, but also cause its reorganization in lens fiber cells (5,6).

Although, the biosynthesis of CP-95 and CP-49 has been demonstrated in intact organ cultured chick lenses (7), in vitro biosynthesis of these proteins has not been studied. Since we plan to study the molecular structure and regulation of the genes responsible for these cytoskeletal proteins, the present study was undertaken to demonstrate the presence of specific mRNAs in the chicken lens that code for these proteins in a cell-free translation system.

## MATERIALS AND METHODS

Materials Phenol, guanidine thiocyanate and $^{14}$C-labelled mol.wt. markers, nuclease-treated rabbit reticulocyte lysate translation system were all obtained from Bethesda Research Laboratories, Gaithersberg, Maryland. 2-day chickens were supplied by Townline Poultry Farm, Zeeland, Michigan. Whatman 3 MM filter circle discs (2.3 cm diameter) were bought from Thomas Scientific., Swedesboro, New Jersey. Gel electrophoresis supplies, nitrocellulose sheets (0.45- μm pore), horseradish peroxidase or alkaline phosphatase-conjugated goat anti-rabbit IgG, 4-chloro-1-naphthol, 5-bromo-4-chloro-3-indoylphosphate (BCIP) and P-nitroblue tetrazolium chloride (NBT) were purchased from Bio-Rad, Richmond, California. Oligo (dT)-cellulose Type 3 was purchased from Collaborative Research, Inc., Bedford, Massachusetts. [$^{35}$S]methionine was from Amersham, Arlington Heights, Illinois. Immunobilon-P (PVDF) transfer membranes were obtained from Millipore Corporation, Bedford, Massachusetts.

Preparation of mRNA Lenses from 2-day postnatal chickens were frozen in liquid nitrogen immediately afer extraction and stored at - 80°C until ready to use. Total RNA was isolated using the guanidine thiocyanate-phenol extraction procedure (8). Lenses were homogenized in 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarcosyl, 0.1M β-mercaptoethanol, pH 7.0. The homogenate was mixed with an equal volume of chloroform/iso amyl alcohol mixture (49:1), shaken vigorously for 30 seconds and 0.1 volume of 2 M sod. acetate, pH 4.0 was added. After thorough mixing, the mixture was incubated at 65°C for 10 min. An equal volume of water-saturated hot phenol (65°C) was added, shaken vigorously and kept on ice for 15 min. with occasional stirring. The sample was then centrifuged at 12,000 X g in a Sorval RC-2 centrifuge. The top aqueous layer was reextracted with an equal volume of chloroform/isoamyl alcohol (49:1) and centrifuged. Total RNA was precipitated from the supernatant in

the presence of equal volume of isopropanol at -20°C overnight. The total RNA was dried and dissolved in sterile water to a final concentration of 3 mg/ml and then chromatographed on an oligo (dT)-cellulose (affinity) column to isolate poly (A+) RNA (9). About 200 µg of poly (A+) was obtained from 4.5 grams of lenses and stored frozen at - 80 C in diluted aliquots until ready to use.

In vitro translation A Nuclease-treated rabbit reticulocyte lysate system was used for cell-free in vitro translation (IVT) of the isolated mRNA, using [$^{35}$S]methionine as the label. The reaction mixture (30 µl) contained 12 µl reticulocyte lysate, 50 µCi [$^{35}$S]methionine (1320 µCi/mmol), 0.5-1.0 µg mRNA, 87 mM potassium acetate, 1 mM magnisium acetate, and was incubated at 30 C for 1.5 h. Total incorporation of [$^{35}$S]methionine was determined by precipitation on filter circles and washing in 10% (w/v) trichloroacetic acid followed by counting the filters in a Packard 3320 Tri-Carb liquid scintillation counter in aquasol.

The translation products were analyzed by one- and two dimensional (1D and 2D) gel electrophoresis (10,11,12). $^{14}$C-labelled proteins of known molecular weight served as markers. The gels were dried and autoradiographed using Kodak X-0 Mat AR film for about 7-10 days at -80°C. In some experiments, chick lens water insoluble fraction (WIF) (13) was added to provide known proteins that help to locate the labelled proteins on the autoradiograph. This reticulocyte system generally provided an incorporation of [$^{35}$S]methionine with the chick lens mRNA to a level of about 70-80% of that observed with globin mRNA as the standard mRNA.

Immunological identification of IVT products
IVT products from 1D and 2D gels were electrophoretically transferred onto nitrocellulose sheets in a Bio-Rad Trans-Blot unit, for 5 h at 60 V (about 200 mA) using 0.025 M Tris, 0.192 M Gly, pH 8.3 containing 20% Methanol (14). Following the transfer, blots were autoradiographed to check the efficiency of transfer, as well as to visualize the labelled proteins on the blot before immunostaining. Specific polyclonal antibodies raised in rabbits against the chicken lens proteins (3) were used (at 1:100 dilution). Goat anti-rabbit secondary antibodies coupled to either horseradish peroxidase or alkaline phosphatase (at 1:1000 dilution) were used.

Amino acid sequence determination Adult chick lens WIF was resolved on 2D electrophoresis (11, 12) followed by the transfer of proteins onto Immobilon-P (Polyvinylidene difluoride, PVDF) transfer membrane in 10 mM 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS), 10% (v/v) methanol, pH 11.0 as the transfer buffer (15, 16) at 250 mA for 2 h in a Bio-Rad Transblot unit. After transfer, the blots were washed with deionized water for 5 min, stained for 5 min with 0.1% (w/v) Coomassie-blue in 50% (v/v) methanol with 10% (v/v) acetic acid, until the background is clear. After washing with deionized water, the blots were air-dried, the bands were cut out and stored dry in Eppendorf tubes at -20°C until ready to use.

For amino acid sequence determination, the cut bands were either directly used or eluted with 70% (v/v) isopropanol, 5% (v/v) trifluoroacetic acid (TFA) for 16 h at room temperature followed by drying in a Speed-Vac and reconstitution in 0.1% aqueous TFA. Amino acid microsequence analysis was obtained by

automated Edman chemistry on an Applied Biosystems gas-phase sequenator, (model 470), with on line HPLC (model 120) and a Nelson analytical chromatography data systems.

## RESULTS

Figure 1A shows the Coomassie-blue stained 1D gel pattern of IVT products (lane 3) along with $^{14}$C-labelled protein mol.wt. markers (lane 2) and chick WIF (lane 1). The autoradiograph of this gel (panel 1B) demonstrates the in vitro synthesis of a number of proteins (lane 5) in the mol. wt. range of 200 kDa - 14 kDa (lane 4). Lane 6 shows that in the absence of added chicken mRNA, there was minimal endogenous protein synthesis, since the reticulocyte lysate was pretreated with micrococcal nuclease that destroys most of the endogenous mRNA.

The in vitro synthesized proteins were identified by 2D gel electrophoresis (Figure 2A) followed by autoradiography (Figure 2B). The WIF of adult chick lens was added to the IVT mixture

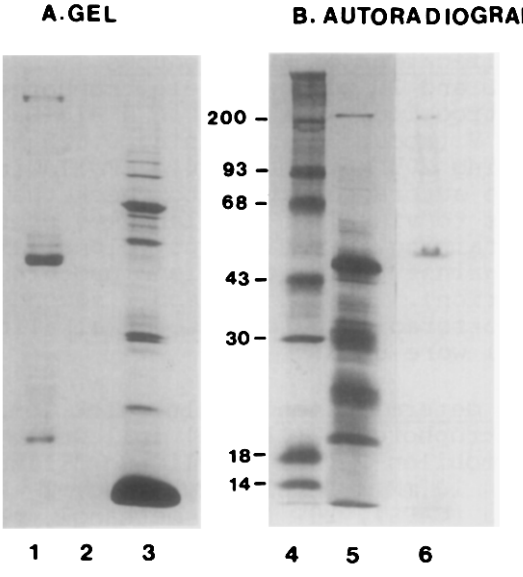


Figure 1. SDS-PAGE of the IVT products of chick lens mRNA. 1A shows Coomassie-blue stained gel pattern of chick lens WIF (lane 1), $^{14}$C-labelled mol. wt. protein markers (lane 2), and IVT products (lane 3). 1B show the corresponding autoradiograph with mol. wt. markers (lane 4) and IVT products (lane 5). Lane 6 shows the endogenous protein synthesis of the control reaction mixture in which chick lens mRNA was omitted. The numbers on the left side of panel 1B indicate the mol. wts. of the marker proteins in kDa.

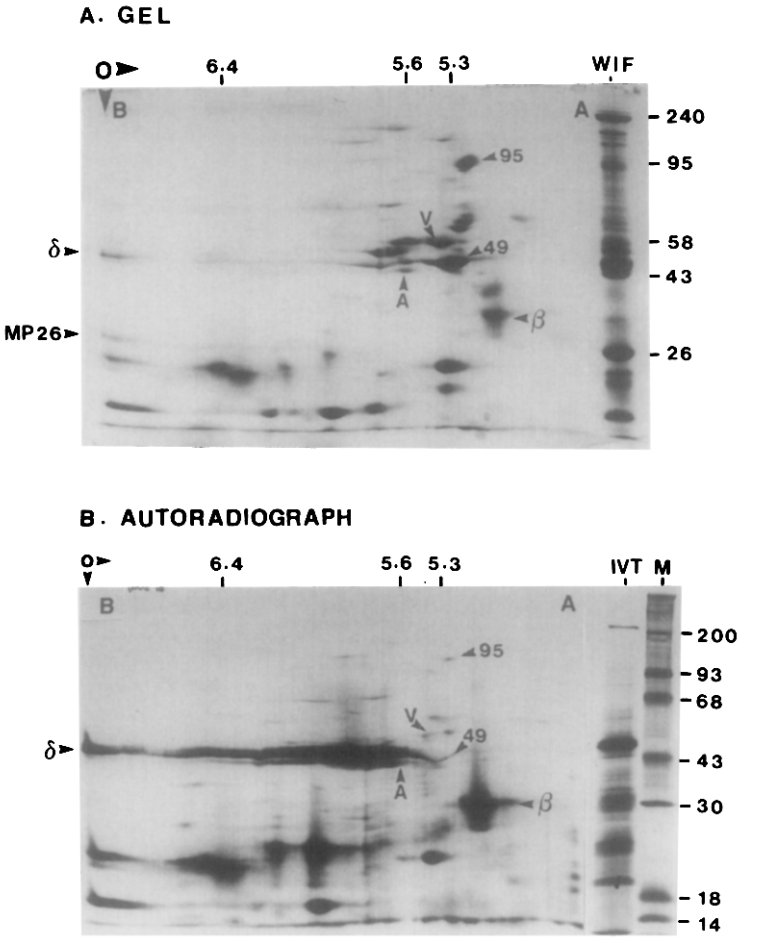


Figure 2. 2D gel electrophoresis of the IVT products. 2A shows the Coomassie-blue stained 2D gel pattern of IVT products mixed with the adult chick lens water insoluble fraction (WIF). The letters A and B on the top corners indicate the acidic and basic ends, respectively, of the 1st dimension gel. 2B shows the corresponding autoradiograph. 0, top of the IEF gel. Horizontal arrow indicates the direction of IEF and the vertical arrow shows the direction of SDS-PAGE. The pI is indicated on the top horizontal margin of the figures. The labels 95 = CP-95, 49 = CP-49, V = Vimentin, A = Actin, β = β-Crystallin, δ = δ-crystallin and MP-26 = major intrinsic protein, 26. M indicates the markers and the numbers on the right correspond to their mol. wt. in kDa. IVT = In vitro translation products.

to provide known proteins, which help to locate the labelled proteins on the autoradiograph. Comparison of the gel with the autoradiograph demonstrates that most of the proteins present in the adult chick lens WIF were synthesized in vitro from their respective mRNAs. The abundant synthesis of

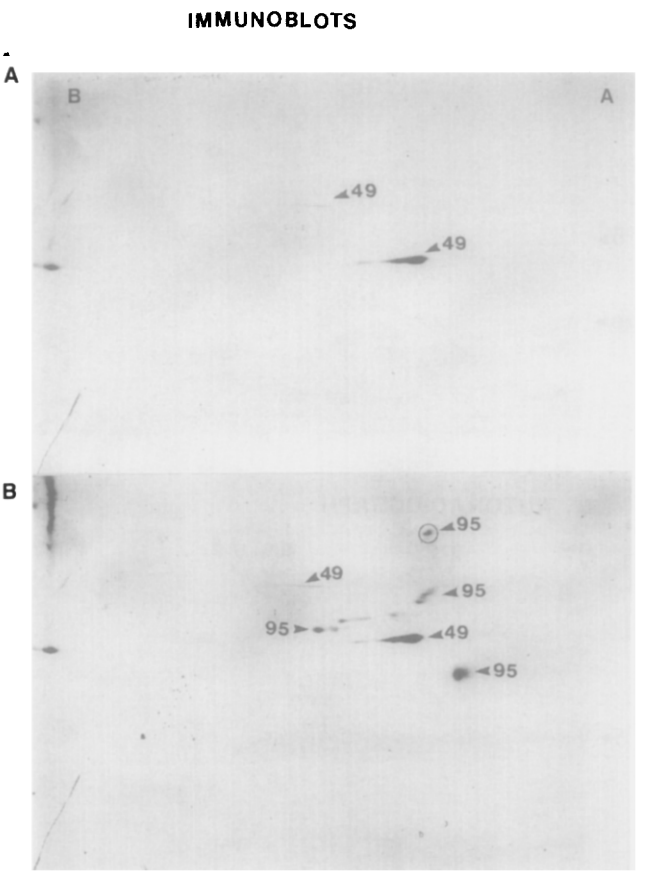


Figure 3. 2D immunoblots of the IVT products. 3A shows the two bands immunoreactive with the anti CP-49 serum (labelled as 49). 3B shows the immunoreaction of the same blot when treated with anti CP-95 serum. In addition to the immunoreaction at the expected location (open circle), other lower mol. wt. bands were also seen (labelled as 95) after the anti CP-95 serum treatment. The letters A and B on the top corners of the blot (panel 3A) indicate acidic and basic ends, respectively.

δ-crystallin is typical of the avian lens. The autoradiograph clearly shows the synthesis of the cytoskeletal proteins CP-95 and CP-49. In addition, the synthesis of vimentin, actin, β-crystallin, and a range of other low mol.wt. crystallins was also observed.

In order to further confirm the synthesis of CP-49 and CP-95 in the cell free system, we used specific polyclonal antibodies as probes. Figure 3 shows the 2D immunoblots of the IVT products alone without added chick lens WIF. The top panel (3A) shows the immunoreaction with the anti CP-49 serum. The

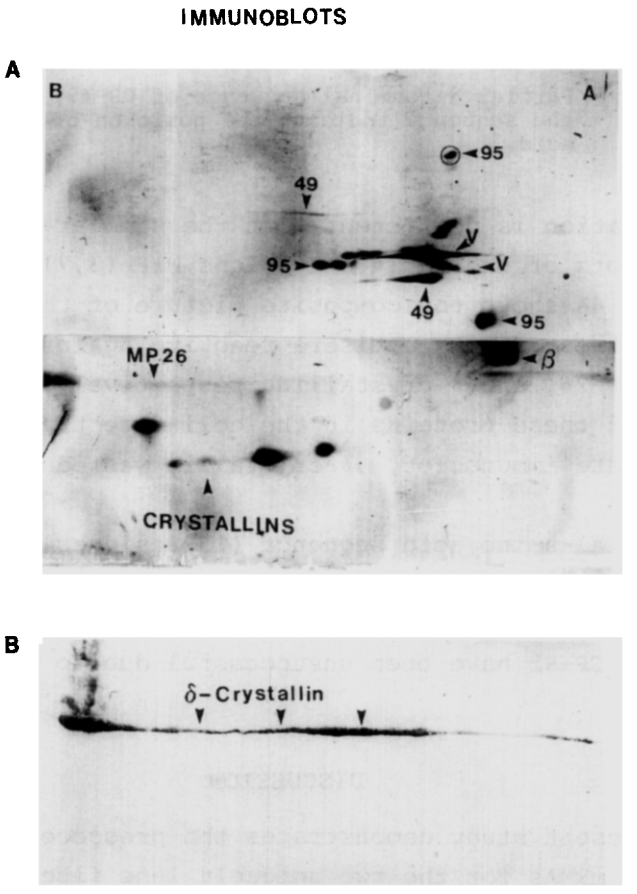


Figure 4. 2D immunoblots of the IVT products. 4A shows the immunoreactive bands (labelled as V) developed on the same blot from Figure 3, by using the anti vimentin serum. The lower part of the blot was cut and treated separately with the anti-chicken lens serum which brought out the immunoreaction with MP-26, β - and other crystallins. 4B shows the immunoreaction of the IVT products with the anti-δ-crystallin on a duplicate blot.

major band is at the expected position with respect to the isoelectric point (pI) and mol. wt., confirming the immunological identity of synthesized CP-49. The trailing associated with this band is due to more basic isoelectric variants. The faint immunoreaction seen at relatively higher mol. wt. and more basic pI, was not observed before in the adult chick lens. Figure 3B (lower panel) shows the immunoreaction with anti CP-95 serum. In addition to the reaction at the expected position (open circle), bands in the lower mol. wt. region were found to be immunoreactive to anti CP-95 serum.

N-TERMINUS

GLN - LEU - PRO - ARG - ARG - ALA - SER - SER - PHE - LEU - GLY - GLN - GLN - ALA - PRO
1 5 10 15

Figure 5. Partial N-terminal sequence of CP-49. Numbers below the sequence indicate the position of the amino acid.

This observation is consistent with the similar finding in the 2D immunoblots of the adult chick lens WIF (3,7).

Figure 4A shows the composite picture of the same immunoblot treated with antisera reactive against chicken vimentin, MP-26, and β-crystallin, respectively, confirming the synthesis of these proteins in the cell-free translation system. In a duplicate immunoblot, δ-crystallin was identified (Figure 4B).

A partial amino acid sequence (15 residues) from the N-terminus of CP-49 was obtained and shown in Figure 5. On the other hand, our efforts to obtain the partial amino acid sequence of CP-95 have been unsuccessful due to its blocked N-terminus.

## DISCUSSION

The present study demonstrates the presence and expression of specific mRNAs for the two uniquely lens fiber cell-specific cytoskeletal proteins CP-49 and CP-95 in the cell-free translation system. These mRNAs may be considered as low abundant mRNAs, as compared to the relative abundance of other mRNAs directing the synthesis of various crystallins.

The minor band seen on the immunoblot using anti CP-49 serum was not observed before with adult chick lens WIF. Since the mRNA used in the present study was obtained from 2-day postnatal chick lens, it may represent a protein homologous to CP-49, that is expressed early during the development and disappears in the adult lens. However, preliminary studies indicated that the higher mol. wt. protein immunologically related to CP-49 may be present constitutively in reticulocyte lysate (data not shown). This raises the interesting possibility that a protein immunologically related to the chick lens CP-49 is expressed in completely unrelated tissue, namely, rabbit reticulocytes. The functional significance of this finding remains to be determined.

The major band identified as CP-49 in the IVT products represents the variant that is normally phosphorylated, based on its acidic pI of 5.3 (3). Since there is evidence for the presence of several phosphorylating kinases in the reticulocyte lysate (17), it appears that the newly synthesized CP-49 is also undergoing post-translational phosphorylation in vitro.

The lower mol. wt. proteins that are immunoreactive to anti CP-95 serum were previously observed in the 2D immunoblots of adult chick lens WIF (3,7), and were thought to be the degradation products derived from CP-95. The in vitro synthesis of these proteins from total chick lens mRNA indicates that they are homologous proteins rather than the degradation products of CP-95. At this stage, it is not clear whether they are the products of separate but related genes, or the products of a single gene derived through differential processing of the transcripts. Further studies are needed to determine whether individual mRNA transcripts actually exist in the chicken lens that code for these homologous proteins. With regards to CP-49, oligonucleotide probes are now being generated corresponding to its partial amino acid sequence.

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