Differential Expression of Neuroendocrine-Specific Protein in Form-Deprived Chick Eyes

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Purpose. To identify genes that are highly expressed in form-deprived retina–retinal pigment epithelium–choroid tissues. Neuroendocrine-specific proteins were found to be highly expressed.

Methods. mRNAs enriched in retina–retinal pigment epithelium–choroid tissues from 3-, 7-, and 14-day form-deprived chick eyes were isolated by differential display technique with cDNA library screening. Neuroendocrine-specific protein A and C were cloned in control and form-deprived eyes. mRNA and protein levels, with respective regional localizations, were examined by Northern blot, Western blot, and immunohistochemical analyses, respectively.

Results. The isolated clone included an insert with a sequence homologous to both chick neuroendocrine-specific proteins A and C. The increases in mRNA and protein levels were confirmed by Northern and Western blot analyses, respectively. Immunohistochemical localization of neuroendocrine-specific proteins A and C was detected in the layer of photoreceptor inner segments, presumably in the cone cells. Northern blot analysis using negative lenses showed that levels of neuroendocrine-specific protein A and C mRNAs were not altered using negative lenses.

Conclusions. The expression of both neuroendocrine-specific proteins A and C mRNAs in cone photoreceptor cells was upregulated within 14 days of form deprivation, but not in response to negative spectacle lenses. These data suggest that the increase in induction of neuroendocrine-specific proteins is not a secondary consequence of ocular elongation or myopic refraction. Induction of neuroendocrine-specific proteins in form-deprived eyes may be causally related to the development of myopia or may be an unrelated effect of form deprivation. (*Invest Ophthalmol Vis Sci.* 2000;41:1533–1541)

Form-deprivation myopia (FDM) in animal models has been used extensively in research on myopia.[1–4] In chicks, occlusion of the eye during the early postnatal ocular development period impairs form vision and leads to myopia.[5–9] Eye enlargement stems from vitreous chamber elongation, the molecular mechanism of which is not well understood. Recently, transforming growth factor (TGF)-β,[6] basic fibroblast growth factor,[10,11] and three nitric oxide synthase (NOS) isoforms,[5] inducible NOS, brain NOS, and endothelial NOS, were reported to be involved in the development of FDM.

Isolation and identification of genes expressed in form-deprived ocular tissues may aid in the molecular evaluation and understanding of possible mechanisms involved in FDM. Such an attempt also makes it possible to identify genes involved in postnatal ocular development.

To identify which mRNA transcripts are more abundant in retina–retinal pigment epithelium (RPE)–choroid tissues from form-deprived eyes, a differential display method[12] was used with cDNA library screening using the chick spinal cord. One clone, designated pM29, contained the cDNA insert with a sequence homologous to neuroendocrine-specific protein (NSP)-A and -C mRNAs. NSPs in human subjects were first cloned as genes with a neuroendocrine-specific expression pattern.[13,14] Although three isoforms were found in humans, only two isoforms, NSP-A and -C, were cloned in the embryonic chick brain tissues.[15] These isoforms were found to be associated with the endoplasmic reticulum,[14,16,17] although their physiological roles and distribution in chick ocular tissue has not yet been investigated.

We induced myopia using two well-known techniques, form deprivation by goggles and negative spectacle lenses, and examined the expression pattern of NSP and its possible relation to the development of myopia.

Materials and Methods

Induction of Myopia

Chicks (male white leghorns) were obtained from a local hatchery (Shiroyama Hatchery, Hyogo, Japan) and maintained at 30°C under a 12-hour alternating light:dark cycle (6 AM–6 PM light; 6 PM–6 AM dark). All the chicks were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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Form Deprivation or Lens-Induced Myopia

Form deprivation was induced by fixing translucent plastic goggles over the right eyes of the chicks, as described elsewhere.[5] Briefly, hemispheric goggles cut from the bottoms of 15-ml plastic test tubes were attached to the right eyes with cyanoacrylate adhesive. The left eye served as the internal control.

Lens-induced myopia was accomplished according to a previously reported method.[18] Briefly, −15-D lenses made from poly(methyl methacrylate) (PMMA) material with a back optic radius of 7 mm and an optic zone diameter of 10 mm were used. Lenses glued between rigid plastic, and Velcro support rings were attached to the right eyes with cyanoacrylate adhesives. Lenses were cleaned every 3 hours during the 12-hour light cycle. The left eyes served as an internal control.

RNA Isolation

Ten chicks were killed between 10 AM and 1 PM. Eyes were enucleated and divided into anterior and posterior hemispheres. The vitreous and sclera were gently cleaned out. For differential display method, the retina–RPE–choroid tissues from both the control and 7-day form-deprived eye were used. For Northern and Western blot analyses, retinas including the RPE carefully separated from choroid were used. Total RNA was obtained by guanidine-isothiocyanate-cesium chloride precipitation, followed by phenol and chloroform-isoamyl alcohol purification and ethanol precipitation.[19] The final RNA preparations were dissolved in diethyl pyrocarbonate–treated water. The amount of the purified RNA was determined by spectrophotometry (optical density 260/280; ratio higher than 1.9).

Calibration of the Amount of Total RNA

A mixture of 0.1 μl total RNA (1 μg/μl) and 2.5 μl oligo(dT) (20 picomoles/μl) was incubated at 65°C for 10 minutes and then cooled on ice for 5 minutes. After 10 μl 5× reverse transcriptase buffer (Toyobo, Osaka, Japan), 5 μl 2.5 mM dNTP, 1 μl RNase inhibitor (Toyobo), and 1 μl 200 U/μl MMuLV (Toyobo) to a total volume of 50 μl were added, the mixture was incubated at 37°C for 1 hour and then at 95°C for 5 minutes to inactivate the reverse transcriptase. The amount of RNA was controlled by monitoring the intensity of ribosomal RNA bands on a 1% agarose-formaldehyde–ethidium bromide gel. Furthermore, variation in the amount of RNA was minimized by monitoring the expression of β-actin mRNA, a housekeeping gene. No alteration in β-actin mRNA in FDM was confirmed by reverse transcription–polymerase chain reaction (RT-PCR; data not shown) using a set of original primers designed from a published sequence (sense primer; TAAGGATCTGTATGCCAACACAGT and antisense primer; GACAATGGAGGGTCCGGATTCATC).[20] The primers were synthesized by the Biologica Co. (Nagoya, Japan) and purified by precipitation. Five-microliter aliquots of the RT mix were added to the PCR master mix consisting of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U rTaq polymerase (Takara, Shiga, Japan) and 0.2 μM each of β-actin primer. The PCR cycle (performed in a thermocycler; Takara) consisted of 1 minute at 94°C and 1.5 minutes at 72°C. The sequence of the amplified products was confirmed as corresponding to chick β-actin cDNA after force-cloning into pGEM-T (Promega, Madison, WI).

Differential Display

Differential mRNA display was performed using kits (RNAimage; GenHunter, Brookline, MA) according to the manufacturer's instructions. In both the reverse transcription and PCR steps all the reaction reagents were prepared as master mixes and then aliquoted equally into individual tubes to provide uniform reaction conditions and minimize intertube variation. Briefly, 0.2 μg total RNAs from retina–RPE–choroid tissues of control and 7-day form-deprived eyes were reverse-transcribed for 1 hour at 37°C and incubated for 10 minutes at room temperature in a final volume of 20 μl containing 25 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 37.5 mM KCl, 5 mM dithiothreitol, 20 μM of each dNTP, and 100 U MMuLV reverse transcriptase with 2 μM of $T_{12}G$, $T_{12}A$, or $T_{12}C$ (GenHunter) as primers. Two-microliter aliquots of the RT mix were added to the PCR master mix consisting of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2 μM dNTP, 0.25 μl of α[^{32}P] dATP (2000 Ci/mmol; Amersham, Buckinghamshire, UK), 0.2 μM each of $T_{12}G$, $T_{12}A$, or $T_{12}C$ (GenHunter) as a 3′ primer, 0.2 μM each of the arbitrary 13mers (AP 49–56; GenHunter) as the 5′ primer, and 1 U rTaq polymerase (Takara) to a final volume of 20 μl. After an initial incubation of 7 minutes at 94°C, 40 cycles of PCR amplification were performed (94°C for 30 seconds, 40°C for 2 minutes, and 72°C for 1 minute, followed by a final primer extension step at 72°C for 7 minutes). Two microliters of formamide containing 0.01% bromphenol blue and xylene cyanol were added to 3.5 μl of each PCR mixture and incubated at 80°C for 2 minutes. The fragments were size-separated in a 6% denaturing polyacrylamide gel in TBE (Tris base, boric acid, Na_2 EDTA $2H_2O$) buffer.[19] The gel was blotted on a piece of nitrocellulose hybridization filter (Schleicher & Schuell, Dassel, Germany) wrapped in a plastic sheet, vacuum dried, and exposed to x-ray film (Amersham) for 16 to 48 hours.

The fragments reproducibly showing higher expression in the samples from form-deprived eye than those from control were excised from the gel with a clean razor blade, soaked in 100 μl distilled H_2O for 10 minutes, and then boiled for 15 minutes. The extracted PCR products were ethanol precipitated, and then dissolved in 10 μl distilled H_2O. Four-microliter aliquots were subjected to reamplification using the same procedures mentioned earlier, except for the dNTP concentration of 20 μM. Aliquots (0.5 μl) of reamplified PCR products were separated on 1.5% ethidium bromide–stained agarose gel and were confirmed to consist of a single band. After unincorporated nucleotides were removed by a Sephacryl column, the remainder of the products were used as probes for cDNA library screening.

Library Screening

A commercially available primary chick spinal cord cDNA library (Stratagene, La Jolla, CA), specific for chick tissues, was used for screening of the posthatch chick eye. The library was diluted in a suspension medium buffer (according to the Stratagene protocol) and spread on a 150-mm plate with XL-1 Blue MRF′ cells (Stratagene) at a titer of approximately 500,000 plaque-forming units[pfu]/plate. The plaques were grown overnight at 37°C and were blotted on duplicate nitrocellulose hybridization filters (Schleicher & Schuell, Dassel, Germany). The filters lifted from each plate were denatured in 1.5 mM NaCl and 0.5 mM NaOH for 2 minutes, neutralized in 1.5 mM

NaCl and 0.5 M Tris-HCl (pH 8.0) for 5 minutes, rinsed briefly in 0.2 M Tris-HCl (pH 7.5) in 2× SSC,[19] air dried, and ultraviolet cross-linked. The ^{32}P-labeled PCR products were added to the filters in hybridization buffer nitrocellulose (NC).[19] Hybridization was allowed to proceed overnight at 42°C. The filters were washed twice with 2× SSC and 1% sodium dodecyl sulfate (SDS) at room temperature for 30 minutes and then twice with 0.1× SSC and 1% SDS at 65°C for 20 minutes. After drying, the filters were exposed to x-ray film (Amersham) for 16 to 48 hours. Positive clones were picked from the primary plates, transferred into suspension medium buffer with a drop of chloroform, vortexed, and subjected to secondary screening. Single plaques were selected and excised in vivo into phagemids (pBluescript; Stratagene) using SOLR cells and helper phages (ExAssist; Stratagene).

Sequencing

The nucleotide sequence of the cDNA inserts of isolated clones was verified with the a dye terminator cycle sequencing core kit (ABI Prism; Perkin Elmer, Alameda, CA) with DNA polymerase (AmpliTaq FS; Perkin Elmer) on a DNA sequencing system (model 377; Applied Biosystems, Chiba, Japan). The sequences obtained were compared with the GenBank EMBL and DDBJ DNA database. The sequence analysis of clone pM29 was 100% identical with the hydrophobic domain of chick NSP-A and -C (Fig. 1).

Northern Blot Analysis

For Northern blot analysis, 20 μg of each total RNA sample was size separated on a 1% agarose-formaldehyde gel, transferred onto a nitrocellulose filter by wicking, and fixed by ultraviolet cross-linking (Stratagene). The pM29 cDNA insert was excised by *Eco*RI (Toyobo) and *Eco*RV (Toyobo), which allowed the inserts to have no corresponding recognition sites in the vector sequences. It was then isolated by gel electrophoresis, eluted, and radiolabeled using a DNA labeling system (Megaprime; Amersham) with α[^{32}P] dCTP (Amersham) before it was used as a probe.

Hybridization and stringency washes were performed as described except for the usage of the ^{32}P-labeled insert of pM29 as a probe. After drying, the filters were mounted on an imaging plate (BAS-III; Fuji, Kanagawa, Japan), then exposed on an image analyzer machine (BAS-2000; Fuji). For quantity and quality control of the RNA samples, total RNA separated on an agarose-formaldehyde gel were visualized by ethidium-bromide staining and photographed under ultraviolet illumination (337 film; Polaroid, Cambridge, MA). To allow for the differences in the actual quantity of total RNA loaded on the gel, the filter was stripped for 10 minutes at 95°C using a solution containing 1 mM EDTA, 40 mM Na_2HPO_4 (pH 7.2), and 5% SDS. Hybridization was performed using the amplified product ^{32}P-labeled chick β-actin cDNAs as probes.[20]

To check whether increased NSP expression is involved in the development of myopia or may be a consequence of form deprivation, we looked at the time course of the NSP changes in both form-deprived and lens-induced myopic retinal tissues. For Northern blot analysis only retinal tissues were used to rule out the possibility that an increase in gene expression in one tissue may be masked by a decrease in another tissue.

Camera images from the Northern blot analysis data were captured by a black-and-white camera (ACMEL CRT, model M-085-D; Polaroid, Tokyo, Japan) and film (337; Polaroid). Images were saved and converted to Macintosh (Apple Computer, Cupertino, CA) tagged-image file format (TIFF) files (360 and 720 pixels with 8 bits of gray level). TIFF files of all the numerical densities (arbitrary units) were exported to the public domain NIH image 1.55 program for further analysis on a Macintosh (Apple) computer.

Western Blot Analysis

Western blot analysis was performed using monoclonal antibodies (MON-160, MON-161, and MON-162) against human NSP-A and rabbit antiserum POL-8 against human NSP-C (kindly provided by Wilm J. M. Van de Ven, University of Leuven, The Netherlands). Among the three monoclonal antibodies, MON-162 recognized 80% of the human amino acid residues 338 to 442 (GenBank accession number L10333), which is a highly conserved region in human and chick (Fig. 1). MON-160 and -161 recognized 53% of the amino acid residues 174 to 337 (GenBank accession number L10333), which were also conserved among animal species with slightly lower homology. To check for the specificity of the anti-NSP-A antibodies, Western

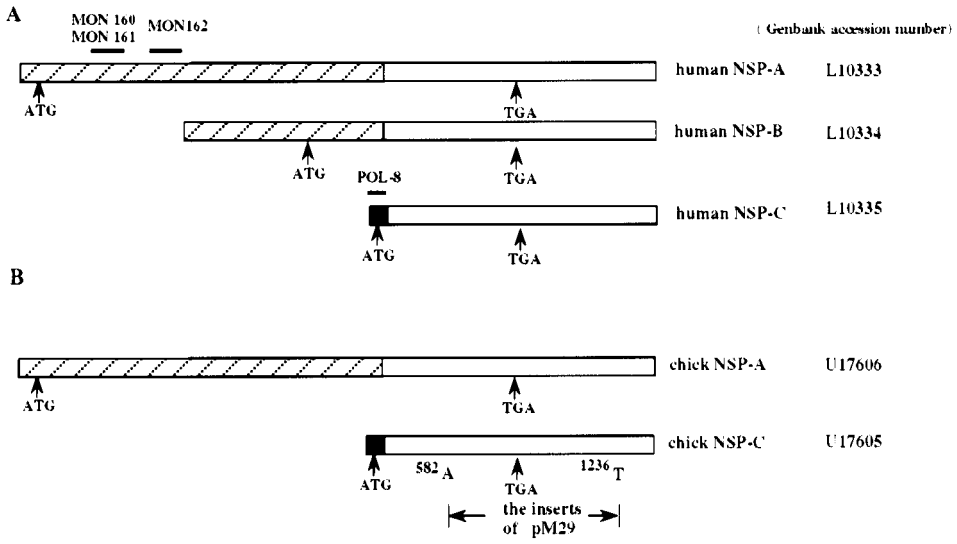

FIGURE 1. Schematic representation of the human and chick NSP transcripts and location of the pM29 inserts. **(A)** Corresponding sequences of human NSP-A, -B, and -C in the 3′ terminal of the coding region and 3′ untranslated region (*open bar*). *Hatched areas* indicate the 5′ terminals. NSP-C included a unique 5′ terminal (*black area*). The regions corresponding to the amino acids used to generate human anti-NSP-A antibodies, MON-160, -161, and -162, and anti-NSP-C antibody, POL-8, are *underlined*. **(B)** Whole open reading frames for chick NSP-A (3.5 kb) and chick NSP-C (1.5 kb) with location of pM29 cDNA insert.

blot analysis was performed with MON-162 only and with a mixture of MON-160 and MON-161. These cross-reacted with an approximately 150-kDa chick retinal protein. No other bands were observed in the Western blot. POL-8 was raised against the first 20 unique N-terminal amino acids of human NSP-C, the amino acid sequence of which was not present in NSP-A and -B (Fig. 1). This sequence (MQATADSTKMDCVWSNWKSQ),[14] however, shares 17 identical amino acids and 2 amino acids similar to the N-terminal amino acids of chick NSP-C (MQASADSTKMDCLWSNWKCQ). POL-8 was diluted 1:100 and preabsorbed overnight at 4°C with a 10^{-4}-M concentration of the chick 20-amino acid peptide (Biologica). This complex was then used for incubation with the membranes followed by secondary antibody detection. POL-8 cross-reacted with a 23-kDa chick protein. The 23-kDa band was eliminated by preabsorption with the immunizing peptide synthesis. Therefore, both POL-8 and the monoclonal antibodies (MON-160, -161, and -162) seemed to be suitable for Western blot analysis and immunohistochemistry. Their cross-reactivities to chick NSPs were confirmed by Western blot analysis. That is, the retina–RPE tissues were sonicated in buffer containing 10 mM Tris-HCl (pH 7.4), 1% (wt/vol) NP-40, 150 mM NaCl, 1 mM EDTA, 20 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride at a concentration of 0.1 g wet wt/ml. Each preparation was boiled with Laemmli's solution containing 5% 2-mercaptoethanol and loaded into a 5% SDS-polyacrylamide gel containing 0.1% SDS. Separated proteins were transferred electrophoretically onto a polyvinylidine membrane (Millipore, Bedford, MA). The membranes were soaked for 30 minutes in phosphate-buffered saline (PBS) containing 5% (wt/vol) skim milk and 0.2% (vol/vol) Tween 20 and then washed with PBS. The membranes were then incubated in a 1:6 dilution of MON-160, -161, and -162 or 1:100 rabbit antiserum POL-8[17] for 24 hours at 4°C. This was followed by incubation for 2 hours at 4°C with the 1:1000 secondary anti-mouse IgG horseradish peroxidase (HRP)–linked antibodies (Amersham) for MON-160, -161, and -162 or 1:1000 anti-rabbit IgG HRP-linked antibodies (Amersham) for POL-8. For the detection of immobilized specific antigens conjugated to HRP-labeled antibodies, a Western blot detection system (ECL Plus; Amersham) was used. The filters were exposed to x-ray films (Hyperfilm MP; Amersham) for 16 to 48 hours. Images were digitized and processed as described earlier.

Immunohistochemistry

Immunohistochemical examination was performed as described elsewhere.[5] Briefly, enucleated eyes (control and form-deprived eyes) were fixed at room temperature for 24 hours in 4% paraformaldehyde containing 0.2% picric acid, washed with 30% sucrose in 0.1 M PBS, and frozen at −20°C. Five-micrometer-thick frozen sections were cut on a cryostat, mounted on poly-L-lysine–coated slides, and air dried. The sections were soaked in PBS containing 10% normal goat serum and 0.03% Triton X-100 for 20 minutes, then incubated in a 1:5 dilution of a mixture of MON-160, -161, and -162 antibodies or 1:100 rabbit antiserum POL-8 for 24 hours at 4°C. The sections were then washed with PBS.

For the immunoperoxidase procedure, sections incubated in MON or POL-8 antibodies were reincubated in 1:1000 HRP-conjugated goat anti-mouse immunoglobulins or HRP-conjugated horse goat anti-rabbit immunoglobulins (both from Vector, Burlingame, CA), respectively. After rinsing in PBS, sections were incubated for 10 minutes in 0.05% 3,3-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05% TB (Tris-HCl buffer, pH 7.6).

For fluorescence immunostaining, the sections incubated in the MON antibody mixture were reincubated in fluorescein isothiocyanate (FITC)–labeled rabbit anti-mouse IgG antibody (Dako; Kyoto, Japan) for 2 hours at 4°C. Those incubated in 1:100 rabbit antiserum POL-8 were reincubated in 1:50 FITC-labeled swine anti-rabbit IgG (Dako) for 2 hours at 4°C. After washing in PBS, sections were mounted in 50% glycerol in PBS. Identification of cone photoreceptors was performed using FITC-labeled peanut agglutinin (PNA) lectin (Biomeda, Foster City, CA).[21] The sections were viewed and photographed under a microscope (Carl Zeiss, Thornwood, NY) equipped with epifluorescence and a standard automatic photographic attach-

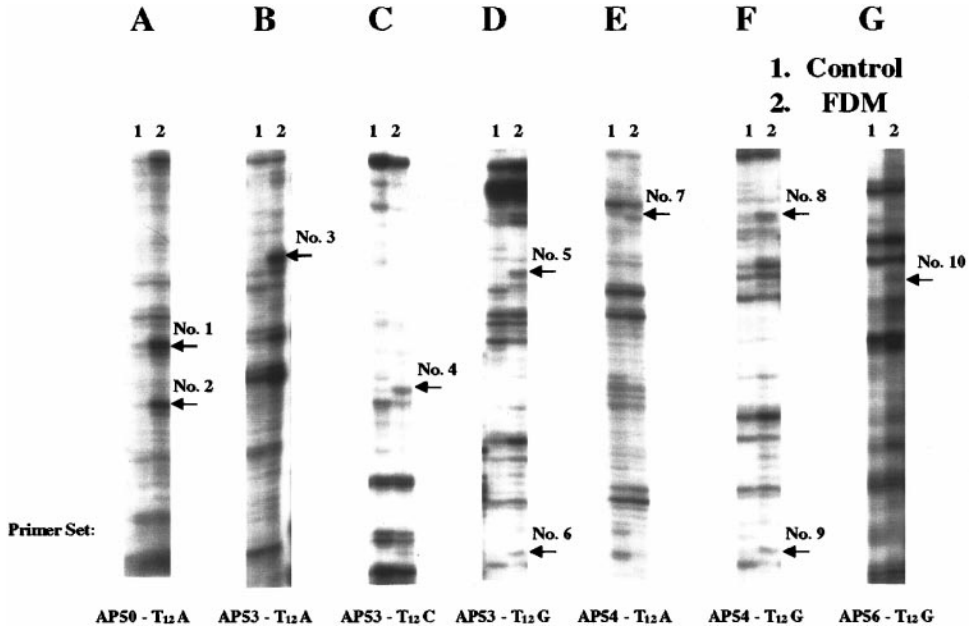

FIGURE 2. Differentially expressed mRNAs (differential display) using total RNA from 7-day retina–RPE–choroid tissues of control and form-deprived eyes. Only 10 (numbers 1–10) of 15 fragments could be amplified by secondary PCR. *Lanes A* through *G*: differential display using primer sets shown *below* the lanes. The approximate band sizes (numbers 1–10) were 150 bp, 150 bp, 150 bp, 300 bp, 250 bp, 200 bp, 200 bp, 100 bp, 150 bp, and 400 bp, respectively. 1, control; 2, FDM.

ment with film (ASA 400; Fuji). For the negative controls, procedures were the same as for the Western blot analysis.

Statistical Analysis

The data obtained from the densitometric readings of the Northern and Western blot analyses were analyzed statistically with an unpaired Students' *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Isolation of Differentially Expressed mRNAs Using Differential Display

When compared with the control group, 15 fragments reproducibly showed higher expression in retina–RPE–choroid tissues from 7-day form-deprived eyes. Secondary amplification by PCR was performed on these 15 fragments using the same set of primers. Ten of the 15 fragments were reamplified (Fig. 2).

Library Screening and Sequence Analysis

The 10 reamplified cDNAs were mixed, purified, ^{32}P-labeled, and used as probes to screen the chick spinal cord cDNA library. Secondary screening resulted in the appearance of 10 putative positive plaques/500,000 pfu. Although 32 clones were isolated, Northern blot analysis confirmed that only one clone, designated pM29, contained the mRNA inserts induced by form deprivation. Northern blot analysis using the inserts of the 31 other clones did not show significant differences in expression between retina–RPE–choroid tissues from control and form-deprived eyes.

pM29 had a cDNA insert size of 700 bp. Its sequence was 100% identical with the hydrophobic domain of both chick NSP-A and -C deposited in GenBank (EMBL and DDBJ DNA databases; Accession Nos. U17606 and U17605, respectively). Although three isoforms of NSP—namely, NSP-A, -B, and -C—were reported in humans,[14] the chick gene corresponding to human NSP-B has not been detected. Because multiple promoters rather than alternative splicing caused the diversity of NSPs,[22] these three isoforms shared 100% homology in the hydrophobic domain of the carboxyl-terminal and 3′ untrans-

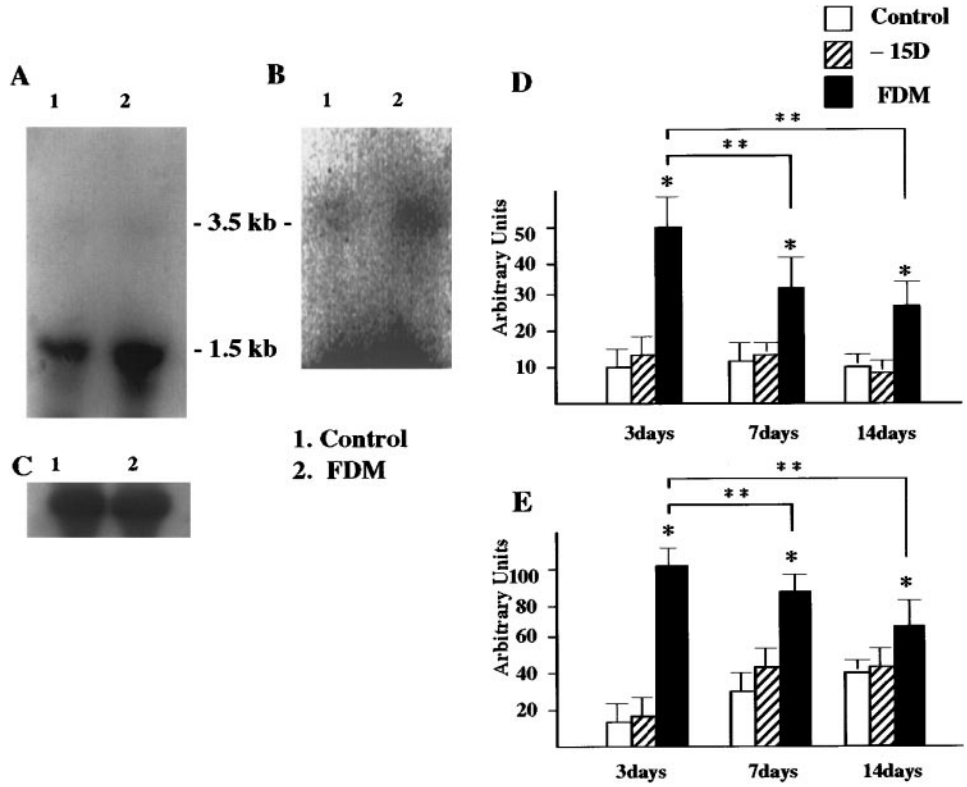

Figure 3. Northern blot for the detection of NSP transcripts. (**A**) Hybridization to a 1.5-kb mRNA species corresponding to NSP-C and a 3.5-kb mRNA species corresponding to NSP-A. (**B**) Overexposure showing the increased expression of NSP-A retinal mRNA at 7 days from the initiation of form deprivation. (**C**) Expression of β-actin mRNA. (**D**) Time course of NSP-A mRNA expression in form-deprived or lens-induced myopic retinal tissues during 14 days of FDM treatment. NSP-A mRNA expression in the retinal tissue was highest at 3 days, followed by 7 days. Expression of NSP-A and -C retinal mRNAs was equal in the lens-induced myopic and control samples. (**E**) Expression of NSP-C mRNA in the retina was also upregulated by form deprivation in the same time course pattern as that of NSP-A mRNA. There was no difference in NSP-C mRNA expression in the lens-induced myopic samples. For (**D**) and (**E**), the *white bar* corresponds to control, *hatched bar* to the −15-D group, and the *black bar* to the FDM group. Values are means ± SD (arbitrary units, $n = 10$). $^{*}P < 0.05$, significant difference in mRNA expression, $^{**}P < 0.05$, versus 7 and 14 days.

lated region (Fig. 1). It is conceivable that the sequence of these regions in chick NSP-B, if any, was identical with those of NSP-A and -C.

Northern Blot Analysis

The inserts of pM29 were radiolabeled and used as probes for Northern blot analysis to confirm which isoforms of NSP were highly expressed in retinal tissues from form-deprived eyes and to check for the existence of NSP-B in chick eyes. The inserts of pM29 were hybridized to mRNA species with sizes of 1.5 kb and 3.5 kb. Considering the differences in sizes of human mRNAs, they corresponded to chick NSP-C and NSP-A, respectively (Fig. 3A). In the control retinal tissues NSP-C mRNA expression was higher than that of NSP-A mRNA (Fig. 3A). In the 7-day retinal tissues from form-deprived eyes, expression of both NSP-A (Figs. 3B) and -C mRNA (Fig. 3A) species were significantly increased (Figs. 3A, 3B). The expression of β-actin mRNA did not appear to change with form deprivation (Fig. 3C). Despite overexposure of the film, no apparent hybridization to a 2.3-kb mRNA species, which would be expected for the transcript of a chick NSP-B homologue, was observed (Fig. 3B).

In the Northern blot analysis using retinal tissues from control, form-deprived, and lens-induced myopic eyes, the increase in retinal NSP-A mRNA expression was significantly higher at 3 days, followed by 7 days, and then 14 days of form deprivation (Fig. 3D). The expression pattern of NSP-A mRNA in the retina was similar to that of NSP-C (Fig. 3E). Although −15-D lenses induced increased ocular growth and myopia during 14 days of negative lens-induced myopic treatment, the expressions of NSP-A and -C mRNA in retinal tissues were not altered (Figs. 3D, 3E).

Western Blot Analysis

The expression of NSP-A and -C mRNAs was altered only with FDM treatment but not with lens-induced myopic treatment. The time course of the NSP protein expression was evaluated only in form-deprived myopic retinal tissues. Human anti-NSP-A antibodies (a mixture of MON-160, -161, and -162) cross-reacted with an approximately 150-kDa chick retinal tissue protein (Fig. 4A). The size of this protein was almost equal to the sizes reported in humans, rats, and mice. Therefore, this seems to reflect chick NSP-A. POL-8 polyclonal antibody, an anti-human NSP-C, cross-reacted with a 23-kDa chick protein (Fig. 4B), which is likely to be chick NSP-C. These bands were absent when the reaction was performed without the primary antibodies for NSP-A or by preabsorption with the immunizing peptide synthesis sequence for NSP-C. (Figs. 4A, 4B).

Form deprivation caused significant increases in the intensity of both NSP-A and -C immunoreactivity in retinal tissues during the 14 days of form deprivation (Figs. 4C and D).

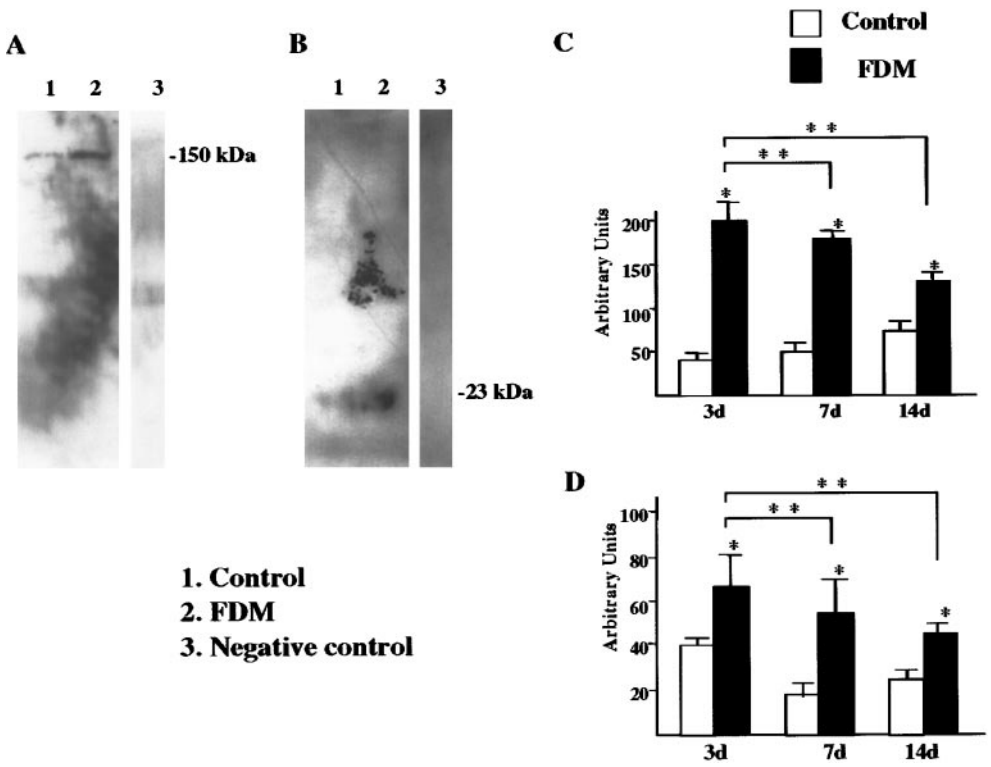

Figure 4. Identification of molecular forms of immunoreactive NSP-A and -C in the Western blot analysis of chick retinal tissue proteins. (**A**) Cross-reaction of human anti-NSP-A antibody, a mixture of MON-160, -161, and -162 with an approximately 150-kDa chick protein in the control (*lane 1*) and FDM groups (*lane 2*) during 7 days of form deprivation. *Lane 3*: negative antibody control. (**B**) Cross-reaction of anti-NSP-C antibody POL-8 with an approximately 23-kDa chick protein in the 7-day control (*lane 1*) and FDM groups (*lane 2*). *Lane 3*: negative control. (**C**) Time course of NSP-A protein expression in retinal tissues from form-deprived eyes during 14 days of form deprivation. (**D**) Time course of NSP-C protein expression in retinal tissues from form-deprived eyes during 14 days of form deprivation. Values are means ± SD (arbitrary units, $n = 10$), $^{*}P < 0.05$, significant difference in protein expression, $^{**}P < 0.05$, vs. 7 and 14 days.

Immunohistochemistry of NSPs in the Chick Ocular Tissue

In the control eyes, immunoreactivity to anti-NSP-A and anti-NSP-C antibodies was detectable in the layer of photoreceptor inner segments (Figs. 5A through 5D). Because of pigmentation, it was difficult to determine whether RPE cells were also immunopositive. Immunoreactivity in the ganglion cell layer, basal layer of the RPE, and choroid was observed in the negative controls (no primary antibody) and therefore does not indicate specific NSP-A or -C immunoreactivity. FDM did not alter the localization of the NSP-A and -C in any of the retinal layers (data not shown).

Figure 6A shows the schematic representation of the four types of cone cells and a rod cell. PNA lectin-binding was observed in the cone cells (Fig. 6B). The abundance and configuration of the PNA-positive cells suggest that cone and not rod photoreceptors in the photoreceptor inner segments were positive to the anti-NSP-A antibody (Fig. 6C). Similarly, only the photoreceptor inner segment, presumably cone photoreceptor cells, was presumably positive to the anti-NSP-C antibody (Fig. 6D).

Discussion

Form deprivation in the chick eye induces axial elongation accompanied by growth and remodeling of the sclera.[5–11] Most researchers have focused on various factors that modulate myopic development, such as neurotransmitters.[2–4] Differential mRNA display has been developed to detect and characterize altered gene expression.[12] A combination of reverse transcription using anchored oligo-dT primer and PCR using arbitrary 5′ primers allows simultaneous identification of genes that are either upregulated or downregulated. Although differential display is an extremely specific and sensitive method, it could only amplify products with sizes of less than 300 bp. Therefore, cloning of products is not always sufficient to identify the amplified mRNA. In the present study, retina–RPE–choroid tissues from form-deprived eyes were compared with control eyes. Those showing higher expression in the form-deprived samples were used as probes for cDNA library screening.

Each probe was positive to the chick spinal cord cDNA library with a frequency of approximately 1/500,000 pfu, indicating that this differential display method could detect mRNA species amounting to approximately 0.001% of all mRNAs. Of 32 positive plaques, pM29 alone contained the cDNA insert whose sequence was 100% identical with the 3′ terminal and 3′ noncoding region of both chick NSP-A and -C.[15] Although the diversity of NSPs is based on multiple promoters rather than alternative splicing,[22] their C-terminals consist of identical hydrophobic domains. NSP-A contains unique N-terminal sequences of 404 amino acids, whereas NSP-C includes only 20 amino acids in its N-terminal. Thus, hybridization to the 1.5-kb and 3.5-kb mRNA species in Northern blot analysis corresponded to the NSP-C and NSP-A transcripts, respectively. The NSP-C transcript was more abundant than the NSP-A transcript in the control chick retinal tissue. It has been reported previously that expression of NSP-C mRNA is greater than that of NSP-A mRNA in several mammalian endocrine tissues and cell lines.[13] In human tissues the existence of NSP-B, which consisted of a unique 5′ terminal of approximately 500 bp and the same 3′-terminal half as NSP-A and -C, was found only in the neuroendocrine small-cell lung cancer cell line NCl-H82.[13] We did not detect any transcript corresponding in size to NSP-B in chick retinal tissues. This may be due to limitations in the

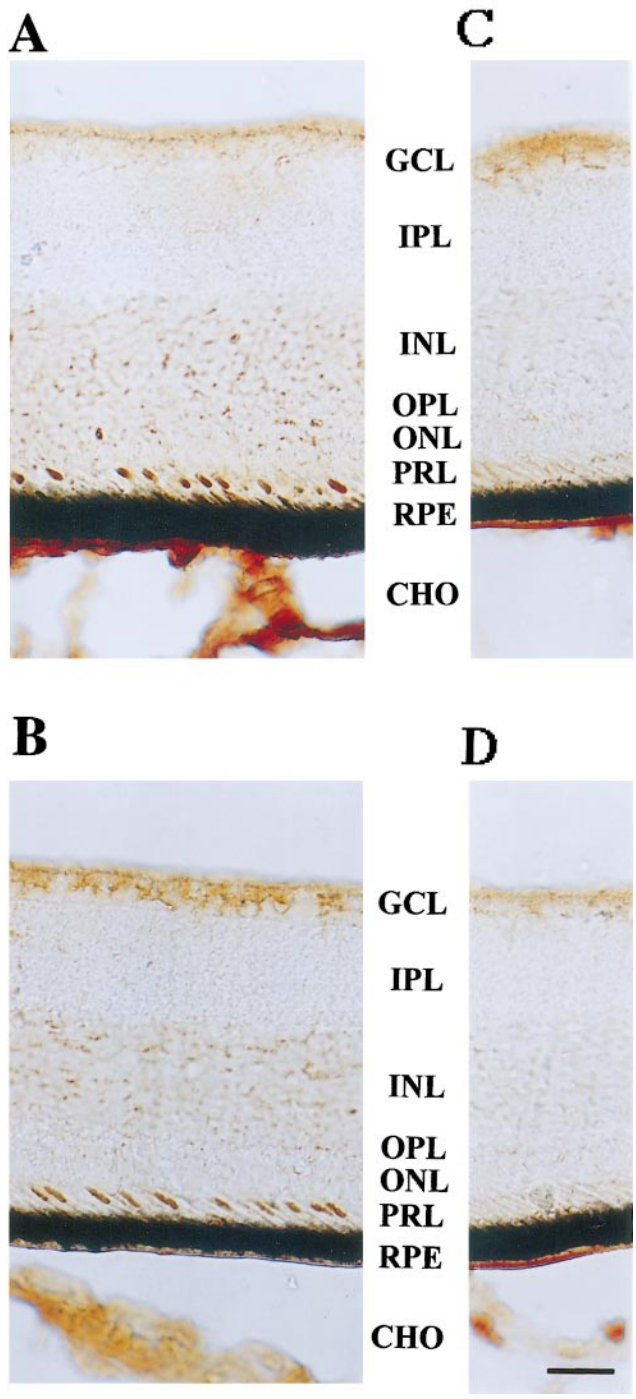

Figure 5. Immunohistochemical localizations of NSP-A and -C in the chick retina. (**A**) Immunoreactivity of NSP-A in the control eye detected mainly in the inner segments of the photoreceptor layer. (**B**) Immunoreactivity of NSP-C in the inner segments of the photoreceptor layer. (**C**, **D**) Negative antibody controls for NSP-A and NSP-C, respectively. Scale bar, 50 μm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer; RPE, retinal pigment epithelium; CHO, choroid.

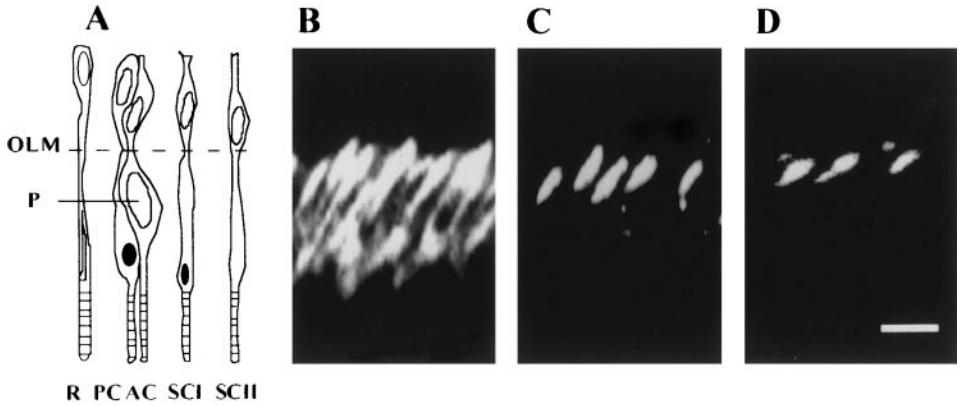

FIGURE 6. Localization of NSP-A and -C immunoreactivity in the inner segments of the photoreceptor layer. (**A**) Types of visual receptor cells in the chick retina. (**B**) PNA lectin showing binding to cone photoreceptor cells. (**C**) Immunoreactivity to anti-NSP-A localized in the inner segments of the photoreceptor cell layer, presumably to cone cells. (**D**) Anti-NSP-C showing similar binding sites. Scale bar, 100 μm. R, rods; PC, principal cones; AC, accessory cones; SCI, single cones type I; SCII, single cones type II; P, paraboloid; OLM, outer limiting membrane.

sensitivity of our methodology. However, these findings demonstrate that the NSP-B promoter was silent, minimally activated, or otherwise absent, and that only NSP-A and -C were expressed in chick ocular tissues. Nonetheless, form deprivation caused a marked increase in both NSP-A and -C mRNAs. We determined whether such increases in mRNAs were responsible for the corresponding increase in NSP proteins.

Although the anti-NSP-A antibody was monoclonal and the anti-NSP-C polyclonal, both anti-human NSP-A and -C antibodies cross-reacted with their chick counterparts. The amino acid residues used to generate anti-NSP-A antibodies were completely conserved in chick, whereas those for anti-NSP-C antibodies were directed to a less well-conserved region accounting for the lower affinity of NSP-C compared with NSP-A in the Western blot analysis. Therefore, the apparent amounts of NSP-A and NSP-C were not comparable. However, both NSP-A and NSP-C proteins were apparently increased in FDM. These increments were associated with an increase in the mRNA levels mainly through upregulation of transcription.

We cloned the NSP-A and -C genes showing higher expression in the 7-day FDM samples by differential display method. Next, we evaluated the time course of NSP expression after initiation of form deprivation. The increase in expression of NSP-A and -C mRNAs was most apparent at 3 days after induction of form deprivation, followed by 7, then 14 days. Although form deprivation caused overexpression of NSP-A and -C through upregulation of transcription during 14 days form deprivation treatment, the increased NSP expression may be an effect of visual deprivation, completely unrelated to myopia. Therefore, we examined the effects of negative spectacle lenses, which also induce increased ocular growth and myopia. Negative lens-induced myopia did not alter the expression of either NSP-A or -C mRNA. This may suggest that increased expression of NSPs reflects morphologic changes in photoreceptors after periods of form deprivation, rather than enhancement of ocular elongation through intracellular signal transduction of the cone cells.

Our results indicate that the changes in NSP expression are specific for form deprivation rather than negative lens defocus and leaves open the question of whether NSP is part of the mechanism by which form deprivation causes excessive ocular elongation. The difference in NSP expression between form deprivation and negative lenses strengthens the possibility that the signaling pathways involved in enhanced ocular elongation brought about by wearing diffusers are different from those involved in the elongation brought about by lenses. The eye, particularly the amacrine cells, has been reported to have the ability to distinguish between form deprivation–induced and lens-induced blur in the development of myopia.[23]

Because expression of NSPs in ocular tissues has not been evaluated in the past, its relationship to eye growth and development remains unknown. However, because NSPs have been reported to be upregulated in nerve cells in association with treatment with growth factors such as β nerve growth factor,[24] the changes in NSP expression with form deprivation treatment may be an unrelated effect.

NSP-A and -C expression were reported to be restricted to neuroendocrine and neural tissues in human,[25] but the distribution of NSPs in human and chick ocular tissues has not yet been investigated. We investigated their distribution in the chick ocular tissues.

Both NSP-A and -C were found to be enriched in the granular endoplasmic reticulum of neuroendocrine and neural tissues.[14,16,17] These endoplasmic reticula lie between cytoplasmic channels of smooth reticula. The shape of the immunoreactive structures suggested that both NSP-A and NSP-C were presumably localized to the cones cells in the inner photoreceptor segment.[26]

NSP-A was ubiquitously expressed in the mature rat brain,[15] whereas NSP-C expression was restricted to the cerebral and cerebellar cortex, excluding Purkinje cells.[27] Only NSP-C was found to be expressed in various peripheral nerves.[27] In contrast to such regional differential expression, the distribution of NSP-A and -C in ocular tissues is identical and may be restricted to cones, suggesting the existence of NSP genes having ocular tissue–specific regulation.

In conclusion, NSP-A and -C were constitutively expressed in chick ocular tissues, although NSP-B was conclusively absent. FDM caused upregulation in expression and translation of

both NSP-A and -C in the photoreceptor inner segments, presumably to cone photoreceptor cells.

Further identification of genes that are highly expressed in both form-deprived and lens-induced myopic ocular tissues by differential display may provide new insights into the mechanisms regulating development of myopia.

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