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RAPID EXPRESSION OF NOVEL PROTEINS IN GOLDFISH RETINA FOLLOWING OPTIC NERVE CRUSH

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Summary. Polyadenylated messenger RNA was isolated from goldfish retinas at various times following unilateral crush of the optic nerve. RNA was translated in a cell-free system and product proteins analyzed by two-dimensional gel electrophoresis and autofluorography. Poly $(A)^+$ mRNA-directed protein synthesis revealed an 8-fold increase in the labeling of polypeptides of about 30 kd M_r and a pI of 5.5 in retinas 2 d following optic nerve crush, compared with control retina mRNA translation products. In vitro labeling of retinal proteins revealed the enhanced synthesis of comparable 30 kd proteins in 2 d post-crush retinas. Evidence presented suggests that this 30 kd protein cluster may correspond to fish 30 kd stress or heat-shock proteins (hsp-30).

Introduction. The regenerating goldfish optic nerve is a convenient model for the study of regeneration in the vertebrate central nervous system (1,2,3). Studies in this and other laboratories have focused upon induction of specific retinal proteins following crush of the optic nerve (4,5,6). Of particular interest are rapidly transported, growth-associated proteins (GAPs), most fully described in regenerating toad optic nerve (7). A slowly transported 68-70 kd protein doublet in goldfish optic nerve, the labeling of which is greatly increased in regeneration, has also been under investigation (5).

In an effort to characterize some of these protein inductions at the molecular level, we have carried out two-dimensional gel autofluorographic studies of rabbit reticulocyte lysate cell-free translations of polyadenylated mRNAs isolated from post-crush and control retinas at various times following nerve injury. This report deals with the finding of a robust early response to the crush not previously observed.

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Methods

Optic Nerve Crush. Goldfish were anesthetized with tricaine methane sulfonate prior to intraorbital crush of the right optic nerve (4). Fish were housed in aerated tanks at 27 ± 2°C and were fed daily.

RNA Isolation. Sixty to seventy pairs of retinas (post-crush and unoperated) were used for each experiment. Total cellular RNA was isolated by a modification (9) of the guanidinium isothiocyanate (Sigma) CSCI method (10). Polyadenylated RNA was isolated by oligo dT cellulose (Collaborative Research) affinity chromatography (11) and quantitated by absorbance at 260 nm.

Cell-Free Translation. One µg of poly $(A)^+$ RNA was treated with methyl mercury hydroxide (12) and then translated in 20 µl of non-depleted rabbit reticulocyte lysate (13; Amersham) in the presence of 75 µCi of [^{35}S]methionine (1020 Ci/mmol, Amersham). Translations were carried out at 30°C for 60 min before stopping on ice.

In Vitro Protein Labeling. Protein synthesis in post-crush and control retinas was compared by incubating groups of 5 right and left retinas separately, *in vitro*, with 100 µCi/ml of [^{35}S]methionine in the medium of Dunlop et al. (14) at 27°C for 1 h. Whole retinal proteins were prepared in the membrane buffer of Ames and Nikaido (15). Retinas were homogenized in 1 ml of 2% SDS containing 0.5 mM $MgCl_2$ and 50 mM Tris-HCl (pH 6.8), and heated at 70°C for 30 min, followed by centrifugation at 100,000 x g for 1 h.

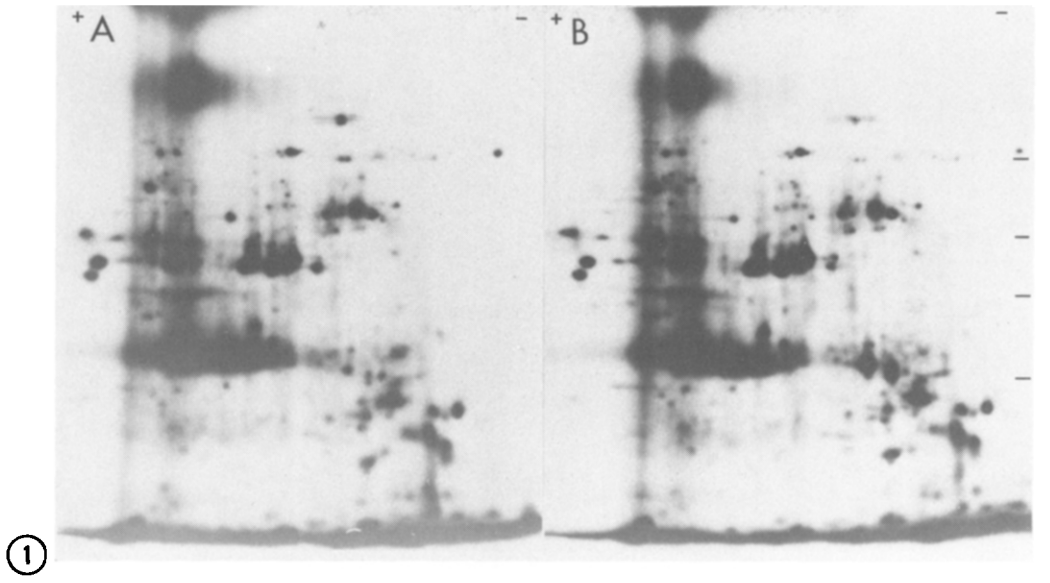
Heat-Shock. Excised whole retinas were heat-shocked *in vitro* following the protocol used by Clark and Brown (16), except that the control temperature was 27°C and the heat-shock temperature was 37°C. Following 30 min of preincubation at 27°C retinas were incubated at 37°C for 5 min. [^{35}S]methionine was added and 10 min later the retinas were incubated at 27°C for an additional 45 min.

Two-Dimensional Gel Electrophoresis. The protocol was modified (17,18) from that of O'Farrell (19). In all cases, 200 k to 300 k TCA-precipitable cpm were loaded onto each two-dimensional gel. SDS gels were 11% acrylamide.

Autofluorography. Gels were treated with Fluoro-Hance (Research Products International), dried and exposed to Kodak XRP-5 film at -70°C for 5-8 d (20).

Quantitation Analysis. Optical densities were determined on a Loats Image Analysis System (Westminster, MD).

Results. Autoradiograms of two-dimensional gels of reticulocyte lysate translations indicated the appearance of mRNAs for several major labeled proteins with M_r of about 30 kd within 2 d of optic nerve crush (Figs. 1 and 2). Selected regions (Fig. 3) were quantitated on a video-based densitometer and post-crush/control ratios computed (Table 1). While 6 nearby regions showed no demonstrable change, 3 regions represented as a triplet (regions 7, 8 and 9) showed 7.5 to 8.4-fold increases. These proteins have been quantitated as a triplet, although there may be additional components. RNA coding for these proteins was elevated through the 4th day following crush, but was attenu-



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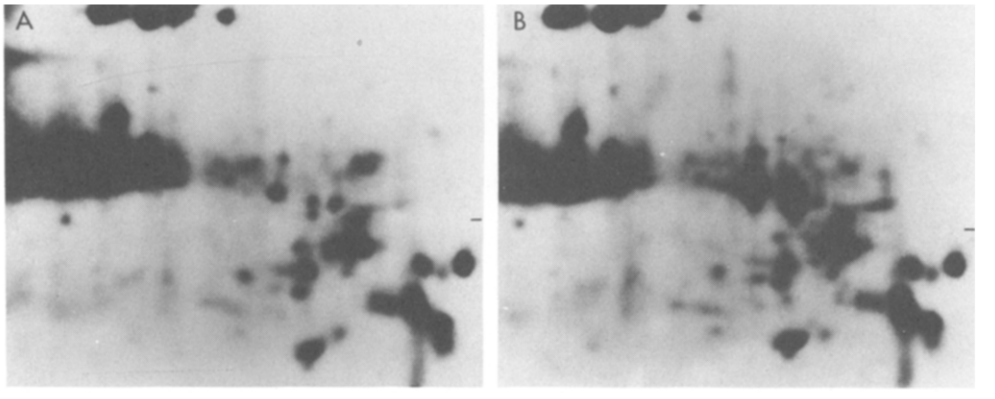

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Figure 1. Fluorographs of two-dimensional gel electrophoresis of goldfish poly $(A)^+$ retinal mRNA cell-free translation products. A) Control (left retina), B) 2 d post-crush (right retina). Molecular weight standards of 66, 45, 36 and 29 kd are indicated on the right.

Figure 2. Enlargements of 30 kd region of retinal fluorographs shown in Fig. 1. A) control, B) 2 d post-crush. The molecular weight standard indicated is 29 kd.

ated by 1 wk, and returned to basal levels by 10 d following crush (data not shown). Retinal proteins labeled *in vitro* with [^{35}S]methionine for 1 h (Fig. 4) showed a comparable increase and similar pattern, indicating that the 30 kd proteins are expressed without extensive post-translational modification. Isolated control retinas that were subjected to a 37°C heat-shock also demonstrated the appearance of labeled protein in the region of the triplet (Fig. 5). This result is compatible with the hypothesis that the labeled protein expressed

Figure 3. Line drawing of fluorograph enlargement (Fig. 2B). The ratios listed in Table 1 express the ratios of the corrected integrated optical density (I.O.D.) of post-crush (PC) to control (C), as determined by the Loats system for each of the numbered spots. The 30 kd triplet is defined as regions 7, 8 and 9.

within 2 d of optic nerve crush is related to, or is perhaps identical with, a teleost stress or heat-shock protein.

Discussion. We have previously observed a number of rapid metabolic changes that may be related to macromolecular synthesis in the goldfish retina following optic nerve crush. Retinal uridine kinase, as well as UMP- and UDP-kinases are elevated within 2 d following crush, and these activities may be regulated transcriptionally (21). Increased retinal uptake of ^{3}H-labeled uridine is seen within 4 d following crush (22). The exact timing of these early events, like regeneration itself, is likely to be dependent upon the temperature at which fish are maintained (23).

In general, alterations in retinal protein synthesis associated with regeneration have been inferred to originate in ganglion cells on the basis of their axonal transport (3,5,7). The focal involvement of the ganglion cells in the retinal response to axotomy is also supported by autoradiographic studies

TABLE 1: CHANGES IN OPTICAL DENSITY OF SELECTED LABELED RETINAL PROTEINS 2 D FOLLOWING NERVE CRUSH

Number	I.O.D. Ratio (PC/C)
1	0.9
2	1.1
3	1.2
4	1.2
5	1.0
6	0.9
7	8.4
8	6.8
9	7.5

Integrated optical density ratios (I.O.D.) of autoradiograms of 2D gels from control (C) and 2 d post-crush (PC) retinas numbered in Fig. 3 were compared. See text.

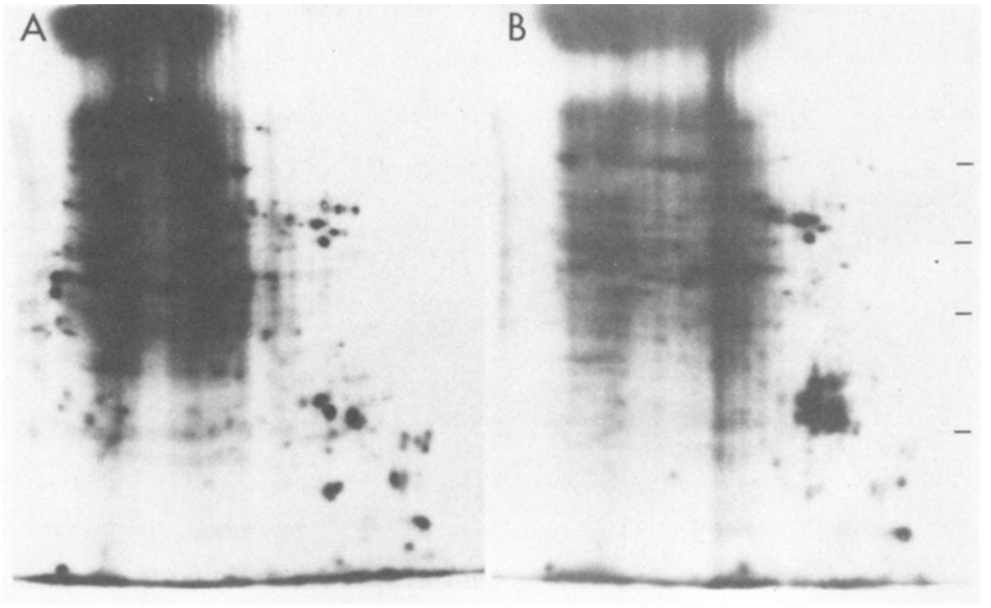

Figure 4. Fluorographs of two-dimensional gel electrophoresis of total retinal proteins, 2 d control (A) and post-crush (B). Following incubation with [^{35}S]-methionine, retinas were homogenized in 2% SDS (see METHODS). Note also the labeling differences in addition to the 30 kd region. A significant post-crush labeling increase at 55 kd migrates in the region of the tubulin subunits. Molecular weight standards of 66, 45, 36 and 29 kd are indicated on the right.

in which labeled uridine and amino acid incorporation patterns have indicated stimulation of RNA and protein synthesis, respectively, in the retinal ganglion cell layer (24,25).

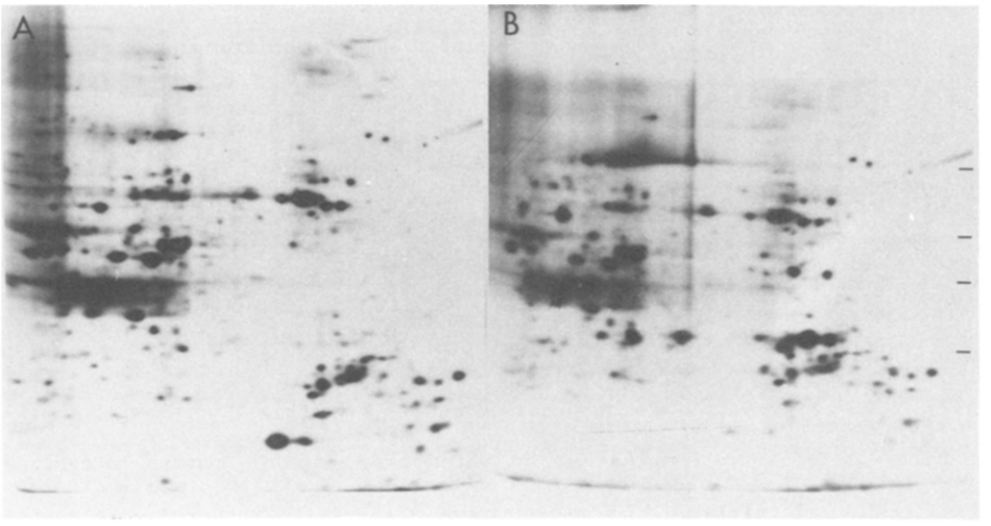

Figure 5. Two-dimensional gel fluorograph of proteins synthesized in control (A) and heat-shocked (B) goldfish retinas. The heat-shock protocol and sample preparation was that of Clark and Brown (16; see METHODS). Molecular weight standards of 66, 45, 36 and 29 kd are indicated on the right.

While axonally transported early 30 kd proteins associated with regeneration have not yet been demonstrated, this might be explained by the fact that paradigms for the study of axonal transport in the regenerating goldfish optic nerve might not reveal their presence. Transport studies are typically initiated 10 or more days following intraorbital optic nerve crush, at which time sufficient nerve regrowth has transpired for measurement of newly transported protein. Thus, an early burst of protein synthesis in the retina would go undetected.

Because of its rapid and robust synthesis prior to significant neurite outgrowth, we have explored the possibility of this 30 kd protein being a stress-induced or heat-shock protein. Fish express a 30 kd heat-shock protein (hsp-30; 26), and this is confirmed in the present study. Stress proteins are expressed in response to a wide variety of stimuli, including brain trauma (27). Studies in the rat have shown the expression of a stress protein in vitro following brain slicing (28). Heat-shock protein synthesis has been inferred to be present in rabbit retinal ganglion cells, since it is axonally transported in the optic nerve (16).

The 30 kd proteins described here appear to constitute the largest, as well as the most rapid, increase in RNA and protein labeling yet observed as a result of nerve crush. A regeneration-related mRNA translation product in the 24-28 kd range has been observed 2 d following crush, but it is reported to remain elevated 35 d post-crush, and the enhancement is relatively small in magnitude (6). The use of methyl mercury hydroxide pre-treatment of mRNA in the present study increased overall incorporation of [^{35}S]methionine into protein, but did not change the pattern or relative spot intensity, and therefore would not likely account for differences with previous observations. It remains to be seen whether a more detailed analysis of this finding will implicate these proteins in a mechanism by which the neuronal genome recognizes axonal crush, and initiates the regenerative response. Further investigation of the interval between nerve crush and appearance of these 30 kd proteins may shed light on the nature of the signal that triggers its onset.

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