J Mol Evol (1994) 39:134–143

Journal of Molecular Evolution

Convergent Evolution of Crystallin Gene Regulation in Squid and Chicken: The AP-1/ARE Connection

Stanislav I. Tomarev, Melinda K. Duncan, H. John Roth, Ales Cvekl, Joram Piatigorsky

Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Building 6, Room 203, Bethesda, MD 20892, USA

Received: 16 September 1993 / Revised: 16 February 1994

Abstract. Previous experiments have shown that the minimal promoters required for function of the squid SL20-1 and SL11 crystallin genes in transfected rabbit lens epithelial cells contain an overlapping AP-1/antioxidant responsive element (ARE) upstream of the TATA box. This region resembles the PL-1 and PL-2 elements of the chicken βB1-crystallin promoter which are essential for promoter function in transfected primary chicken lens epithelial cells. Here we demonstrate by site-directed mutagenesis that the AP-1/ARE sequence is essential for activity of the squid SL20-1 and SL11 promoters in transfected embryonic chicken lens cells and fibroblasts. Promoter activity was higher in transfected lens cells than in fibroblasts. Electrophoretic mobility shift and DNase protection experiments demonstrated the formation of numerous complexes between nuclear proteins of the embryonic chicken lens and the AP-1/ARE sequences of the squid SL20-1 and SL11 crystallin promoters. One of these complexes comigrated and cross-competed with that formed with the PL-1 element of the chicken βB1-crystallin promoter. This complex formed with nuclear extracts from the lens, heart, brain, and skeletal muscle of embryonic chickens and was eliminated by competition with a consensus AP-1 sequence. The nonfunctional mutant AP-1/ARE sequences did not compete for complex formation. These data raise the intriguing possibility that entirely different, nonhomologous crystallin genes of the chicken and squid have convergently evolved a similar cis-acting regulatory element (AP-1/ARE) for high expression in the lens.

Key words: Lens — Crystallin — Squid — Chicken — Gene — Regulation — AP-1 — Evolution

Introduction

Complex eyes containing photoreceptors, a pigmented epithelium, and a transparent lens were formed independently in the course of vertebrate and invertebrate evolution (Land 1984; Land and Fernald 1992). Among the invertebrates, cephalopod (squid, octopus, and cuttlefish) eyes are best known for their striking similarity to those of vertebrates (Packard 1972). In both cases, a transparent lens is used to focus incoming light onto photoreceptor cells. The major soluble proteins in the eye lens of vertebrates and invertebrates are called crystallins. Vertebrate crystallins are a very diverse group of proteins which often differ in composition between different species (Wistow and Piatigorsky 1988; de Jong et al. 1989). Some crystallins are present in the lenses of all vertebrates (the α and β/γ-crystallins), while others, generally related or identical to metabolic enzymes, are restricted to certain species (the taxon-specific crystallins). S-crystallins are the major lens proteins of squids (Siezen and Shaw 1982). These are not present in vertebrate lenses and are related to the metabolic enzyme, glutathione S-transferase (GST) (Wistow and Piatigorsky 1987; Tomarev and Zinovieva 1988; Tomarev et al. 1992, 1993).

The basis for recruiting a lens crystallin during evo-

Correspondence to: S.I. Tomarev

lution is not known. The fact that many, if not all, of the crystallins are either derived from or identical to proteins providing stress protection or detoxification functions suggests that their recruitment may have involved, at least during the early stages of selection, the induction of gene expression by some form of metabolic stress or environmental signal (Piatigorsky and Wistow 1989; de Jong et al. 1989; Wistow and Kim 1991). The possibility has also been advanced that transcription factors required for the optimal expression of crystallin genes in the lens may have common properties despite differences in the nature of the encoded crystallins (Piatigorsky 1992). If this idea is correct, one would expect to find similar transcription factors utilized for high expression of different crystallin genes in the lens of the same and different species.

In the course of our investigations on the squid, we noted that a consensus AP-1 binding sequence overlapping an antioxidant response element (ARE) was present in the minimal functional 5′ flanking sequences of the SL20-1 and SL11 crystallin genes. This element is required for activity of the squid promoters fused to the bacterial chloramphenicol acetyltransferase (CAT) gene in transfected N/N1003A rabbit lens epithelial cells (Tomarev et al. 1992). This was of special interest since AP-1 has been implicated in the expression of numerous crystallin genes in vertebrates (see Piatigorsky and Zelenka 1992) and both AP-1 and ARE are involved in the induction of GST genes by oxidative stress in mammals (Daniel 1993). Moreover, the AP-1/ARE element in the squid promoters resembles the PL-1 and PL-2 functional elements of the chicken βB1-crystallin promoter (Roth et al. 1991). Consequently, we used site-directed mutagenesis, transfection experiments, electrophoretic mobility shift assays (EMSA), and DNase I footprint analysis to compare the functional requirements and binding properties of the AP-1/ARE sequences found in the squid promoters with the PL-1 element of the chicken βB1-crystallin promoter in chicken lens cells. The results indicate that the squid AP-1/ARE sequences are essential for promoter activity in transfected chicken lens cells and fibroblasts. Moreover, the squid AP-1/ARE sequences form a complex similar to that formed with the PL-1 element of the chicken βB1-crystallin promoter with a chicken lens nuclear extract. These data are consistent with the possibility that the SL20-1 and SL11 crystallin genes of the squid have at least one functional element in common with that of the βB1-crystallin gene of the chicken.

Materials and Methods

Nucleic Acid Isolation. Plasmid DNAs were isolated using Qiagen columns (QIAGEN Inc.). For transfection, plasmid DNAs were purified by the alkaline method (Birnboim and Doly 1979) and subsequently banded twice in a cesium chloride/ethidium bromide density gradient. RNA was isolated by the acidic guanidinium thiocyanate–phenol–chloroform extraction method (RNazol B, Cinna/Biotecx; Chomczynski and Sacchi 1987) from transfected and nontransfected primary lens epithelial cells.

Recombinant DNA Constructions. Plasmid DNAs containing squid S-crystallin gene promoter fragments coupled to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene were described previously (Tomarev et al. 1992). Site-directed mutations were introduced into the squid S-crystallin promoters by using the oligonucleotide-directed mutagenesis method as described by the manufacturer (Amersham Corp.). All constructs were confirmed by dideoxynucleotide sequencing (Sanger et al. 1977) using Sequenase version 2.0 (U.S. Biochemical).

Transfection. Transfections were performed on patches of primary lens epithelial (PLE) cells obtained from 14-day-old chicken embryos (Borras et al. 1988). A minimum of two separate experiments were performed in duplicate for each construct tested. The test plasmid (10 μg) was cotransfected with a control SV40 promoter/β-galactosidase plasmid (pCH110) (Herbomel et al. 1984) (2 μg) as a calcium phosphate precipitate as described elsewhere (Dubin et al. 1989). Cells were assayed for CAT activity by the fluor-diffusion method (Neumann et al. 1987) and for β-galactosidase activity (Borras et al. 1988) 48 h after transfection.

Primer Extension Analysis. Primer extension analysis was performed with 50–75 μg of total RNA from transfected PLE cells. Primer 3761 (5′-CAACGGTGGTATATCCAGTG-3′), whose 3′-end corresponds to CAT gene position +15, was used. The primer was radioactively labeled with [γ-^{32}P]ATP (7,000 Ci/mmol, INC) using T4 polynucleotide kinase (BRL). The labeled oligonucleotide primer was mixed with RNA, heated at 68° C for 5 min, and incubated at 37° C for 30 min to 1 h after addition of 10–20 units of AMV reverse transcriptase (Stratagene). RNA was hydrolyzed by NaOH treatment, and extended cDNAs were extracted with phenol-chloroform and ethanol precipitated. cDNAs were analyzed on 6% polyacrylamide/7 M urea gels. Sequencing reaction ladders generated from primer 3761 were used as a size marker.

Nuclear Extracts, Gel Mobility Shift Assays, and DNase I Footprinting. All synthetic oligonucleotides were synthesized on an Applied Biosystem 380A DNA synthesizer and purified on an 8% denaturing polyacrylamide gel. The sequences of the sense strand oligonucleotides used as probes and competitors were as follows:

SL20-1: 5′-ATTGTTTAAAGTGACTCATCTATT-3′
SL11: 5′-AGGCCAGCGTGACTCATCAAAGAATACA-3′
PL-1: 5′-GGATGTGATGACTGGGCGGCCGCA-3′
SL20-1M6A: 5′-ATTGTTAAAGCACACTCATCTATT-3′
SL11M6A: 5′-AGGCCAGCCACACTCATCAAAGAATACA-3′
PL-1M6A: 5′-GGATGAGCACACTGGGCGGCCGCA-3′
ARE: 5′-TGCTAATGGTGACAAAGCAACTTT-3′
Rat AP-1: 5′-CGAGTGTCTGACTCATGCTTTCGA-3′
Chicken AP-1: 5′-CTGGCTTCTGAGTCAGCACGGACA-3′

Oligodeoxynucleotides were labeled by using [γ-^{32}P]ATP (ICN) and polynucleotide kinase to a specific activity of about 5×10^7 cpm/μg DNA; unincorporated nucleotides were removed by passage through G-25 spin columns. Double-stranded labeled probes were prepared by annealing with a threefold molar excess of the unlabeled complementary strand. Nonradiolabeled competitors were prepared by annealing equimolar amounts of the complementary oligonucleotides. Nuclear extracts were prepared from the indicated tissues of 14-day-old embryonic chickens (Shapiro et al. 1988). Extracts were titrated with various oligonucleotides within the chicken αA-crystallin enhancer (−162 to −88) and probes containing Sp1 (GGGGCGGGCT) and octomer (ATGCAAAT) binding sites to control for the quality of

the nuclear proteins (Klement et al. 1993). Electrophoretic mobility shift assays were performed in the total volume of 12.5 µl by mixing 2 µl of crude nuclear extract (approximately 48 µg of protein) with 40,000 cpm of probe (Cerenkov counting, about 1 ng), 500 ng of poly(dI-dC) in a solution containing 8.5 mM HEPES, pH 7.9, 30 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM DTT, 0.3 mM PMSF, and 4% w/v Ficoll 400. For the competition experiments, 50 ng of cold competitor oligonucleotide (50-fold excess) was added to this mixture. Following a 15-min incubation at room temperature, the products were analyzed by electrophoresis (10 V/cm, 2 h, 4° C) in 5% polyacrylamide gels (acrylamide/bisacrylamide ratio of 29:1), using 0.5 × TBE electrophoretic buffer (45 mM Tris-borate, pH 7.8, 1 mM EDTA). The gels were dried under vacuum and autoradiographed. For footprinting experiments, lens nuclear extracts were further fractionated by heparin-Ultrogel A4-R chromatography as described (Klement et al. 1993). The proteins eluted from the resin with 600 mM KCl (H600CE) (Klement et al. 1993) were used in all footprinting experiments. DNase footprinting experiments were performed as described (Klement et al. 1993) using −202/+37 (SL20-1) or −163/+89 (SL11) probes uniquely labeled at either the Sal site or the HindIII site (approximately 20,000 cpm per assay).

Results

Activity of the Squid Crystallin Promoters in Transfected Chicken PLE Cells

We initially transfected fusion genes containing various promoter fragments of the SL20-1 and SL11 genes linked to the CAT gene into PLE cells and fibroblasts of 14-day-old embryonic chickens. Cotransfections using a SV40 promoter/β-galactosidase fusion gene were performed in every case as an internal control to normalize for transfection efficiency. (See Materials and Methods.) In addition, the results were normalized relative to those obtained in parallel transfection tests using an RSV promoter/CAT fusion gene (Borras et al. 1988). The results are shown in Fig. 1. As was the case for transfected N/N1003A cells derived from rabbit lens epithelial cells (Tomarev et al. 1992), the −84/+37 fragment of the SL20-1 gene and the −111/+89 fragment of the SL11 gene were the minimal sequences demonstrating promoter activity in transfected chicken PLE cells. Each of these minimal promoter fragments contains an AP-1/ARE sequence. The −38/+37 fragment of the SL20-1 gene and the −60/+89 fragment of the SL11 gene, both of which lack the AP-1/ARE sequence, did not drive the CAT gene in transfected chicken cells. The promoterless parent plasmid (pSVOAT-CAT) gave virtually no background CAT activity in transfected PLE cells (data not shown). Reversing the −761/+37 fragment of the SL20-1 gene and the −425/+89 fragment of the SL11 gene (the largest fragments that were tested) eliminated activity of the squid promoters in the transfected cells (Fig. 1). As expected, the squid promoters were much less active in chicken PLE cells than a chicken αA-crystallin promoter fragment (−162/+77) cloned into the same parent vector. The squid promoter fragments were also less active in transfected fibroblasts than in transfected PLE cells.

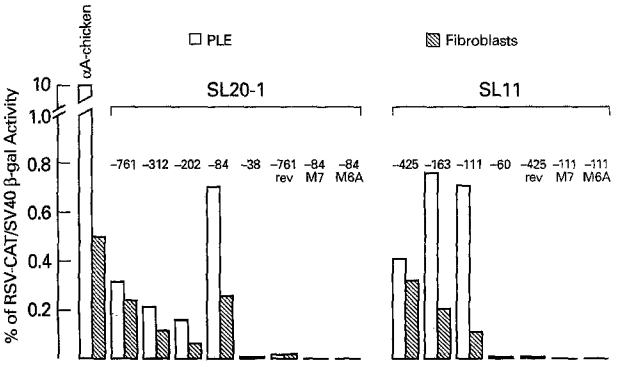

Fig. 1. Relative CAT activity in extracts obtained from transfected chicken PLE cells and fibroblasts. The activity of the Rous sarcoma virus promoter was taken as 100%. (See Materials and Methods.) The results of a typical experiment are presented. The height of each *bar* is an average of measurements using duplicate plates. The M7, M6A, and reverse orientation constructs were tested twice, while the other plasmids were tested three to six times, depending on the construct, with similar relative values obtained each time. The *numbers* above the bars represent the position of the 5′ flanking nucleotide of the construct being tested; the 3′ nucleotides of the SL20-1 gene and SL11 gene fragments were +37 and +89, respectively, in each construct. M7 and M6A are mutant promoters (see Fig. 3); rev is reverse orientation.

Primer extension experiments were performed in order to map the transcription initiation sites of the squid crystallin promoter/CAT fusion genes in transfected PLE cells. RNA was isolated from PLE cells 48 h after transfection and a 20-mer synthetic oligonucleotide (primer 3761) complementary to the beginning of the CAT coding sequence was used to initiate reverse transcription. (See Materials and Methods.) Analysis of the extended cDNAs with a parallel sequencing ladder generated from the 5′ region of the SL20-1 and SL11 crystallin genes showed that both fusion genes initiated transcription at two closely spaced sites near, but not identical to, those used by the endogenous genes in the squid lens (Fig. 2).

The AP-1/ARE Sequence is Essential for Activity of the Squid Promoters in Transfected PLE Cells

As shown in Fig. 3, the AP-1/ARE sequences found in the SL20-1 and SL11 promoters are similar to the PL-1 and PL-2 sequences of the chicken βB1-crystallin promoter. Site-directed mutagenesis experiments have shown that the PL-1 and PL-2 sequences are both essential for activity of the βB1-crystallin promoter in transfected chicken PLE cells (Roth et al. 1991). Thus, we created mutations in the AP-1/ARE sequences of the SL20-1 and SL-11 promoters which resembled those known to reduce the activity of the βB1-crystallin promoter (Fig. 3). The M6A mutation of the PL-1 element of the βB1-crystallin gene was previously shown to reduce promoter activity approximately ninefold, while the M7 mutation of the PL-2 element of the βB1-crystallin gene reduced promoter activity about twofold (Roth et al. 1991).

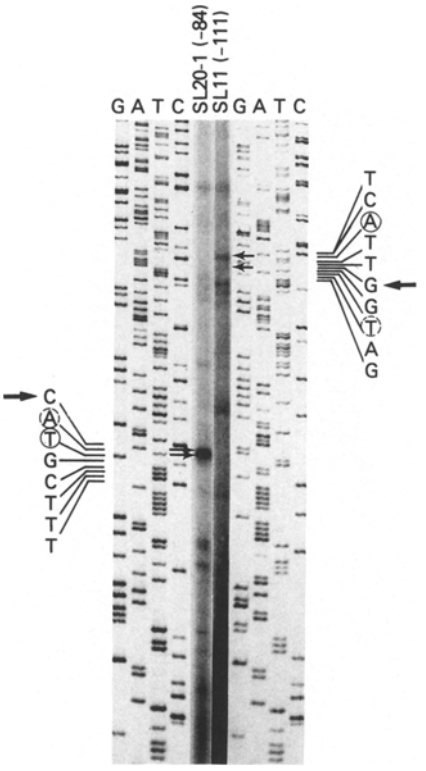

Fig. 2. Primer extension analysis of the transcription initiation sites of the squid SL20-1(−84/+37) and SL11(−111/+89) promoters fused to the CAT gene using RNA obtained 48 h after transfection of PLE cells; 75 μg of total RNA was annealed with primer 3761, elongated, and analyzed as described in Materials and Methods. Sequencing reactions with the same plasmids and primers were used as size markers and are shown on the left for the SL20-1 promoter and on the right for the SL11 promoter. The initiation sites are shown by *arrows* in the middle lanes and are *circled* in the corresponding nucleotide sequences along the sides. *Solid and dotted circles* correspond to the major and minor initiation sites, respectively. The major initiation sites observed in vivo are shown by *large arrows* in the sequences.

	G	Positions
AP-1 consensus	**TGACTCA**	
SL20-1WT	TTAAAG**TGACTCA**TC	-49/-35
SL20-1M7	TTAAAG**GCCTTCA**TC	-49/-35
SL20-1M6A	TAAAGCAC**ACTCA**TC	-49/-35
SL11WT	CCAGCG**TGACTCA**TC	-82/-68
SL11M7	CCAGAG**GCCTTCA**TC	-82/-68
SL11M6A	CCAGACAC**ACTCA**TC	-82/-68
PL-1WT	ATGTGA**TGACT**GGGC	-116/-102
PL-1M6A	ATGTGCAC**ACT**GGGC	-116/-102
PL-2WT	CACTGA**TGAG**CTGGC	-90/-76
PL-2M7	CACTAGGCCT**CT**GGC	-90/-76
ARE consensus	**GTGAC**NNNGC	

Fig. 3. Comparison of AP-1 and ARE (Rushmore et al. 1991) consensus sequences in the promoter regions of the squid SL20-1 and SL11 (Tomarev et al. 1992) and chicken βB1 (Roth et al. 1991) crystallin genes. M6A and M7 are mutations which were made previously in the PL-1 and PL-2 elements, respectively, of the chicken βB1-crystallin promoter (Roth et al. 1991). Nucleotides identical to the AP-1 consensus are *underlined,* while those identical to the ARE are shown in *bold letters.* The position numbers of corresponding sequences in the squid and chicken promoters are shown on the right. Two deletions of one nucleotide (positions −56 and −51) and the A for T substitution at position −48 of the SL20-1 (M6A) promoter mutant were inadvertently created and not studied further.

These mutations in the AP-1/ARE sequences of the squid promoters completely abolished promoter activity in transfected PLE cells and fibroblasts (Fig. 1). We thus conclude that the AP-1/ARE elements found in the SL11 and SL20-1 promoters are important for their function in chicken PLE cells and fibroblasts. This corresponds to similar results obtained for the related PL-1 and PL-2 sequences of the chicken βB1 promoter (Roth et al. 1991).

EMSAs Using AP-1/ARE Sequences from Crystallin Promoters

EMSAs were conducted with synthetic double-stranded oligonucleotides to test for the presence of lens nuclear factors from 14-day-old chicken embryos which bind to the AP-1/ARE sequences found in the squid SL20-1 and SL11 and the chicken βB1 promoters. Competition experiments were performed with nonlabeled self-oligonucleotides, and a variety of oligonucleotides containing the ARE (Rushmore et al. 1991) site, the chicken (Koller and Trueb 1992) and rat (Chiu et al. 1989) AP-1 sites, and the PL-1M6A, SL20-1M6A, and SL11M6A mutant sequences. (See Materials and Methods for the oligonucleotide sequences.) The results are shown in Fig. 4A–D.

The PL-1 sequence of the chicken βB1-crystallin promoter formed two major and several weaker complexes with lens nuclear proteins (Fig. 4A). Both major complexes were eliminated by self-competition, while only the faster-migrating major complex (arrow) was eliminated by competition with the AP-1, ARE, SL11, and SL20-1 oligonucleotides (Fig. 4A,B). By contrast, the PL-1M6A mutant oligonucleotide competed only with the upper complex, and neither complex was reduced by competition with the SL11M6A and SL20-1M6A mutant oligonucleotides (Fig. 4A).

The SL20-1 oligonucleotide formed one major complex (arrow) and several less-intense complexes with the lens nuclear proteins, and all (except for the fastest-migrating complex) were eliminated or markedly reduced by competition with the nonlabeled SL20-1, SL11, PL-1 and rat AP-1 oligonucleotides (Fig. 4C). The major complex identified with an arrow in Fig. 4C migrates very similarly to that specified with an arrow in Fig. 4A and B. The mutant SL20-1M6A and SL11M6A oligonucleotides competed somewhat for formation of the minor complexes and competed either poorly or not at all for formation of the major complex with the wild-type SL20-1 oligonucleotide; the PL-1M6A oligonucleotide did not compete at all with SL20-1 oligonucleotide. Similarly, the wild-type SL11 oligonucleotide formed several complexes with the lens nuclear proteins, one of which (arrow) appeared to comigrate with the major complex formed with the SL20-1 oligonucleotide (Fig. 4D). All the complexes were self-competed with the

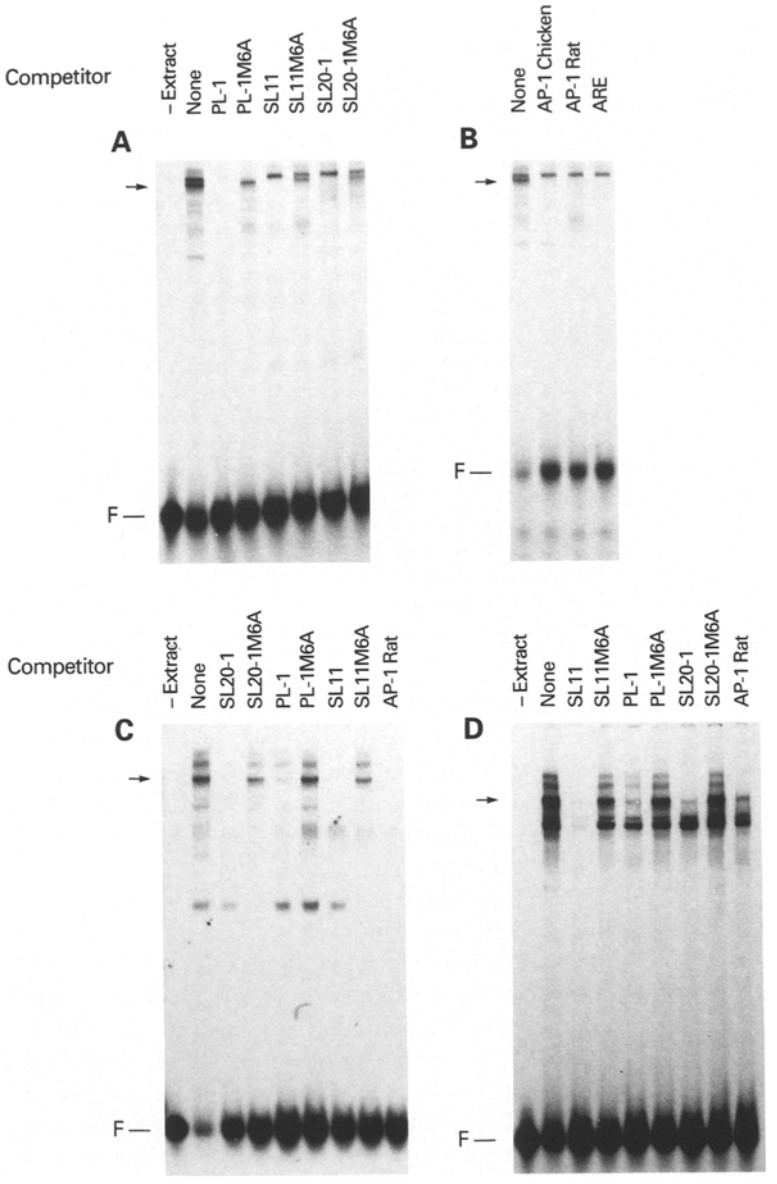

Fig. 4. Gel mobility shift assays. The assays were conducted as described in Materials and Methods, using the indicated radiolabeled and unlabeled competitor oligonucleotides. The ratio of labeled to unlabeled oligonucleotides was 1:50. **A, B** Binding of chicken lens nuclear proteins to an oligonucleotide containing the chicken PL-1 element. The *arrow* points to the major complex, which has similar mobility to and cross-competes with all the tested oligonucleotides except the nonfunctional mutants. F, free probe. **C, D** Binding of chicken lens nuclear proteins to an oligonucleotide containing the squid SL20-1 (C) and SL11 (D) AP-1/ARE element. The oligonucleotide sequences are given in Materials and Methods.

nonlabeled SL11 oligonucleotide, and the intensity of the major complex was strongly reduced by competition with the PL-1, SL20-1, and rat AP-1 oligonucleotides. By contrast, the mutant SL11M6A and PL-1M6A oligonucleotides competed slightly, while SL20-1M6A did not compete for formation of any of the complexes.

Finally, the wild type PL-1, SL11, and SL20-1 oligonucleotides were used to investigate whether the complexes formed above were lens-specific or also formed with nuclear extracts from the brain, heart, and skeletal muscle of chicken embryos (Fig. 5). In general, the results were similar for all tissues tested, indicating the absence of tissue specificity. Moreover, the major, cross-competing complex formed with each oligonucleotide (arrow) comigrated on the gel, consistent with each binding similar nuclear proteins, as expected from the competition experiments. We conclude from these experiments that the PL-1, SL20-1 and SL11 oligonucleotides form at least one similar complex with nuclear proteins of the embryonic chicken lens, and this complex appears similar to that formed with an AP-1 or ARE binding site.

DNase I Footprint Analysis of the SL11 and SL20-1 5′ Flanking Sequences

DNase I footprint analyses were conducted using a partially purified embryonic chicken lens nuclear extract in order to investigate whether the protein:nuclear complexes observed by EMSA also formed within the context of the longer, natural 5′ flanking sequences of the squid SL11 and SL20-1 crystallin genes. The footprints showed that the lens nuclear proteins protected numerous regions of the 5′ flanking sequence of both the SL20-1 (Fig. 6) and SL11 (Fig. 7) genes from DNase I digestion. Importantly, the AP-1/ARE sequence of the

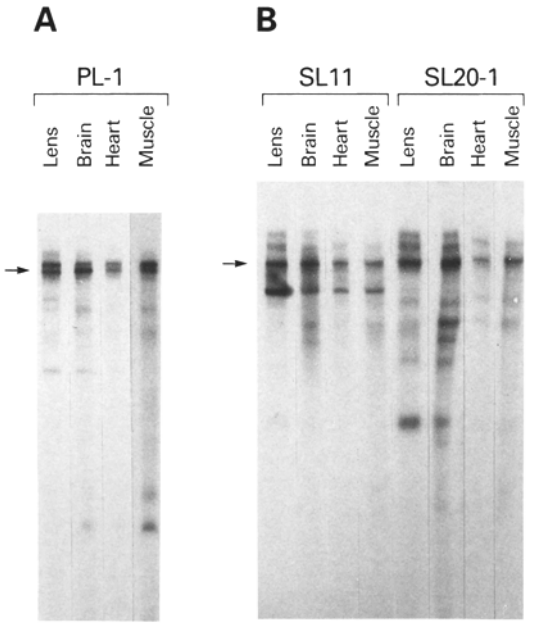

Fig. 5. Gel mobility shift assays using either the chicken PL-1 element **(A)** or the squid SL20-1 and SL11 AP-1/ARE **(B)** as probes with chicken nuclear extracts prepared from different tissues. The oligonucleotides are given in Materials and Methods. The *arrows* point to the same major complex indicated in Fig. 4.

SL11 and SL20-1 genes was protected on both strands from DNase I digestion although the protection of this sequence in the upper strand of the SL20-1 gene was not strong (Fig. 6). These results are consistent with the EMSA experiments indicating specific interactions between nuclear proteins of the embryonic chicken lens and the AP-1/ARE sequence of the SL20-1 and SL11 crystallin promoters.

Discussion

One of the puzzles of evolution is that although the transparent eye lenses of different vertebrates have similar functions (Walls 1967), their crystallins are diverse, structurally different, and often differ among species (Wistow and Piatigorsky 1988; Bloemendal and de Jong 1991). Thus, it has been difficult to identify the unifying characteristics of the crystallins required for optical properties of the lens. Certainly, the ability to accumulate to high concentration in the lens and great stability are essential features of these water-soluble proteins used for refraction. An intriguing connection between the crystallins is that many are able to provide protection against physiological stress (Piatigorsky and Wistow 1989; de Jong et al. 1989). For example, αB-crystallin is a small heat-shock protein (Klemenz et al. 1991; see de Jong et al. 1993) and many of the taxon-specific crystallins are detoxification enzymes (Piatigorsky 1992). This suggests that crystallin recruitment may have involved the induction of high-level gene expression by environmental stress, at least during the early stages of lens evolution. Subsequent adaptive changes leading to developmentally controlled and high constitutive expression of crystallin genes in the lens must have occurred, establishing their encoded proteins as crystallins with specialized refractive functions.

Studies on the cellular lenses of cephalopods (Siezen and Shaw 1982; Chiou 1984; Tomarev and Zinovieva 1988; Tomarev et al. 1991, 1992), gastropods (Cox et al. 1991), and cubomedusan jellyfish (Piatigorsky et al. 1989, 1993) have shown that crystallin diversity extends to the invertebrates, whose eyes have evolved independently from those of vertebrates (Eakin 1972; Ali 1984; Cronin 1986). In general, invertebrates and vertebrates have used different proteins as crystallins. However, it is noteworthy that aldehyde dehydrogenase has been recruited as a crystallin in both octopus (Ω-crystallin: Tomarev et al. 1991; Zinovieva et al. 1993) and elephant shrews (η-crystallin: Wistow and Kim 1991), indicating that convergent evolution may result in the selection of a similar protein for refraction in the lens of an invertebrate and a mammal. The present experiments provide evidence that a similar cis-acting regulatory element (the AP-1/ARE sequence) is required for promoter activity of the squid SL20-1 and SL11 crystallin genes and the chicken βB1-crystallin gene in transfected embryonic chicken lens cells. An overlapping AP-1/ARE sequence is also present in the promoter of the squid SL20-3 crystallin gene (Tomarev et al. 1992) which was not examined here. The chicken AP-1/ARE sequences in the PL-1 and PL-2 elements were shown previously to be required for activity of the chicken βB1-crystallin promoter in these homologous lens cells (Roth et al. 1991).

The squid promoters functioned more efficiently in transfected chicken lens cells than in transfected fibroblasts. This cannot be interpreted as lens preference, since heart-specific α-actin and erythrocyte-specific β-globin promoters were also expressed more efficiently in chicken PLE cells than in fibroblasts in previous transfection experiments. (See Discussion in Tomarev et al. 1992.) The deletion mutations and the gel retardation experiments presented here suggest that the AP-1/ARE sequence is necessary for the activity of the squid promoters in fibroblasts as well as lens cells. It is possible that the low levels of promoter activity found in fibroblasts are due to the lower-than-usual levels of c-jun found in these cells (Hadman et al. 1993).

The nature of the chicken lens nuclear proteins interacting with the AP-1/ARE sequence in the promoters of the squid SL20-1 and SL11 and chicken βB1 crystallin genes is not known. The observation that all of these AP-1/ARE sequences are able to form a similar, comigrating, and cross-competing complex suggests that they bind the same or extremely similar proteins. The lack of tissue specificity for these complexes and the ability to compete with AP-1 binding sequences are consistent with the possibility that this complex

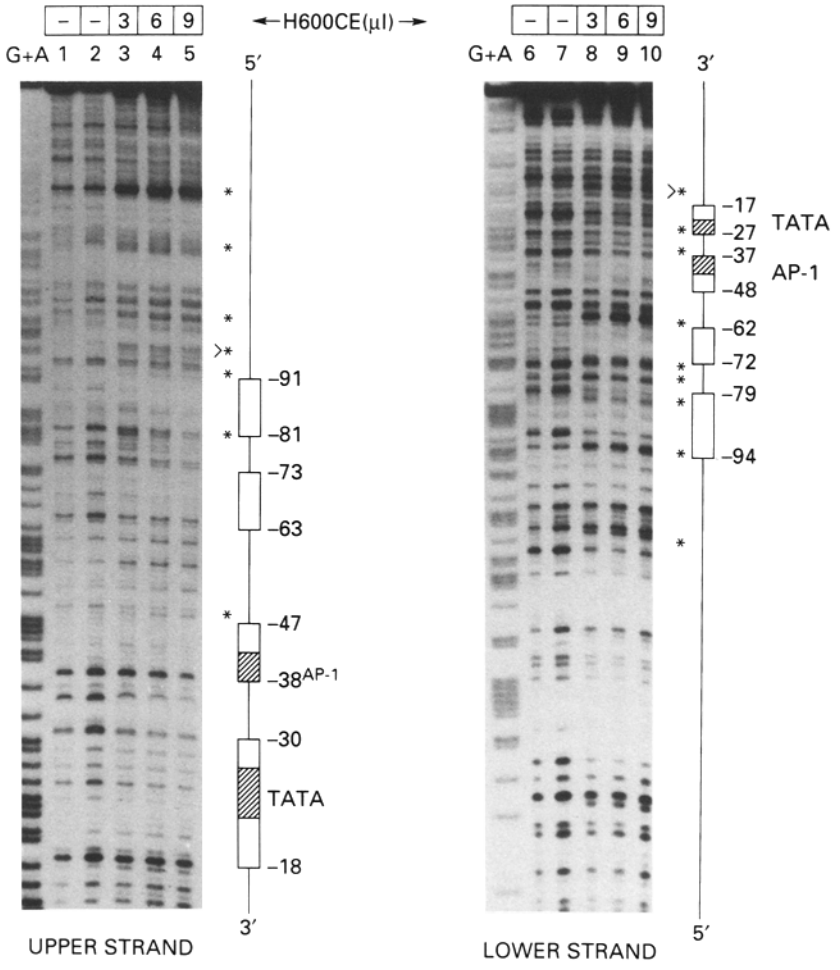

Fig. 6. In vitro DNase I footprinting of the squid SL20-1 promoter (−202/+37) using partially purified nuclear proteins from embryonic chicken lenses. **Upper (left)** coding and **lower (right)** noncoding strand 3′-end-labeled probes were incubated with the indicated volumes of H600CE fraction and analyzed as described (Klement et al. 1993). Regions protected and enhanced from DNase I digestion are shown *boxed* and marked by *asterisks*, respectively. The *hatched* regions correspond to AP-1/ARE and TATA-box sequences.

contains proteins belonging to the c-fos and c-jun families (Curran and Franza 1988; Vogt and Bos 1990; Angel and Karin 1991). AP-1 has been implicated in the expression of a number of crystallin genes (Piatigorsky and Zelenka 1992), and c-fos and c-jun are expressed and differentially regulated in the embryonic chicken lens (Rinaudo and Zelenka 1992). A number of different proteins are able to interact with an AP-1 binding sequence in a phenomenon called cross-talk. Transcription factors complexing with sequences containing an AP-1 binding core include CREB and CREM (Masquilier and Sassone-Corsi 1992), the thyroid hormone receptor (T_3R) (Wondisford et al. 1993), and the retinoic acid and vitamin D_3 receptors (Schule et al. 1990; De Luca 1991). Indeed, retinoic acid can increase crystallin accumulation in cultured lens epithelial cells of chicken (Patek and Clayton 1990) and activate the lens-specific mouse γF-crystallin promoter via a novel retinoic acid receptor (Tini et al. 1993). Recently, we have observed that the PL-1 element forms at least five discrete complexes in a photoaffinity cross-linking experiment with chicken lens nuclear extract (Duncan and Piatigorsky, unpublished), indicating that there are a number of different proteins which could form complexes with the AP-1/ARE-like sequence. Clearly, further experiments are necessary in order to identify the chicken nuclear proteins in these complexes.

Further investigations are also required to determine if the squid AP-1/ARE sequences are necessary for promoter activity of the SL20-1 and SL11 crystallin genes within the squid lens. To date it has not been possible to transfect squid lens cells or to obtain enough squid lens nuclear extract to perform gel mobility shift experiments. AP-1 homologs have been cloned from *Drosophila* (Perkins et al. 1990; Zhang et al. 1990) and yeast (Schnell et al. 1992), consistent with the possibility that proteins homologous to AP-1 are present in squid. Although not homologous to AP-1, the yeast transcription factor, GCN4, recognizes the AP-1 binding sequence. (See Georgakopoulos and Thireos 1992 for references and further discussion.) Thus, the AP-1/ARE sequence may be recognized by an entirely different protein in the squid than in the chicken. Vertebrate retinoic acid receptor homologs are also present in invertebrates (De Luca 1991). It is tempting to speculate that the AP-1/ARE sequence in the squid crystallin promoters is important for expression of these genes in the lens. This would provide a putative link between the recruitment of a crystallin gene and a regulatory pathway involving induction of gene expression by oxida-

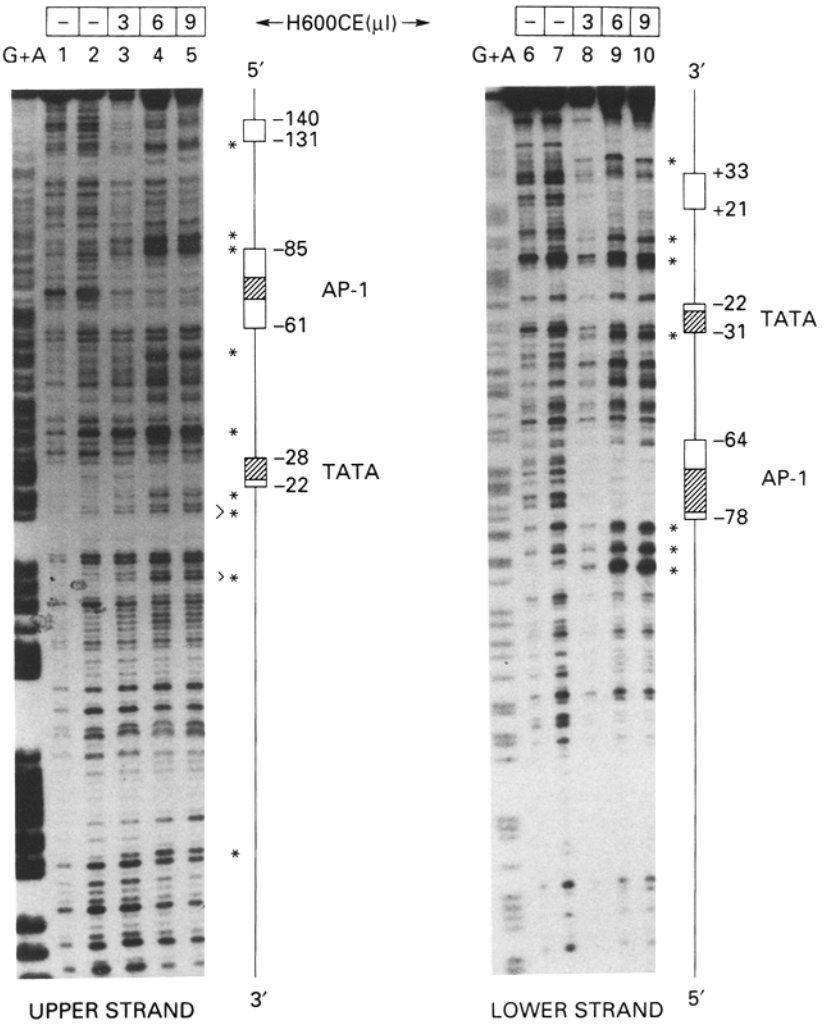

Fig. 7. In vitro DNase footprinting of the squid SL11 promoter (−163/+89) using partially purified nuclear proteins from embryonic chicken lenses. See legend to Fig. 6 for further details.

tive stress. AP-1 is an antioxidant-responsive transcription factor that is subject to redox regulation (Brach et al. 1992; Xanthoudakis et al. 1992; Xanthoudakis and Curran 1992; Meyer et al. 1993). The ARE sequence was identified in experiments involving the induction of GST gene expression by oxidative stress in vertebrates by phenolic antioxidants and planar aromatic compounds (Rushmore et al. 1990, 1991; Daniel 1993). Recent cross-linking and gel mobility shift experiments have ruled out jun homodimers or jun-fos heterodimers in complexes formed with the ARE in the rat GST Ya gene, although it is still possible that a jun family member may be a part of the ARE binding complex (Nguyen and Pickett 1992).

There are several examples supporting the idea that different or convergently evolved genes utilize similar regulatory pathways for their expression in organs which have been derived independently. An impressive example is the expression of alcohol dehydrogenase (ADH) in the liver of humans and the fat body of *Drosophila,* which are analogous structures. The ADH genes in humans and *Drosophila* both employ a similar positive and negative regulatory element (Falb and Maniatis 1992). The CREB/ATF transcription factor (BBF-2) which binds to the fat-body-specific enhancer of the *Drosophila* ADH gene is also able to interact specifically with the regulatory elements required for the liver-specific expression of the human ADH gene (Abel et al. 1992). Two other examples providing evidence for similarities in gene regulation during convergent evolution include the apparent involvement of the COUP steroid receptor transcription factor in vertebrates and svp, its *Drosophila* homolog, in neurogenesis and eye development (Fjose et al. 1993), and the use of retinoid receptors for transcriptional activation during morphogenesis in vertebrates and invertebrates (De Luca 1991). Among the invertebrates, the major chorion proteins of the silkmoth, *Bombyx,* and the fruitfly, *Drosophila,* are not homologous, yet genes of the former are correctly expressed in the latter when introduced into the germ line by P-element transformation (Mitsialis and Kafatos 1985). Finally, the P25 silk-encoding gene of *Bombyx* is expressed in the anterior salivary gland of *Drosophila* in P-element transformation experiments although *Drosophila* does not produce silk (Bello and Couble 1990). The present experiments have

raised the possibility that entirely different crystallin genes in chicken and squid may have convergently evolved similar cis-acting elements for high expression in the eye lens.

Acknowledgments. We thank Drs. R. Kim for providing the chicken αA-CAT construction and M. Kantorow for useful discussions in the course of this work.

References

Abel T, Bhatt R, Maniatis T (1992) A *Drosophila* CREB-ATF transcriptional activator binds to both fat body- and liver-specific regulatory elements. Genes Dev 6:466–480

Ali MA (ed) (1984) Photoreception and vision in invertebrates. NATO ASI Ser A, vol 74. Plenum Press, New York

Angel P, Karin M (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1072:129–157

Bello B, Couble P (1990) Specific expression of silk-encoding gene of *Bombyx* in the anterior salivary gland of *Drosophila.* Nature 346:480–482

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Bloemendal H, de Jong WW (1991) Lens proteins and their genes. Prog Nucleic Acids Mol Biol 41:259–281

Borras T, Peterson CA, Piatigorsky J (1988) Evidence for positive and negative regulation in the promoter of the chicken δ1-crystallin gene. Dev Biol 127:209–219

Brach MA, Herrmann F, Yamada H, Bauerle PA, Kufe DW (1992) Identification of NF-jun, a novel inducible transcription factor that regulates c-jun gene transcription. EMBO J 11:1479–1486

Chiou S-H (1984) Physicochemical characterization of a crystallin from the squid lens and its comparison with vertebrate lens crystallins. J Biochem (Tokyo) 95:75–82

Chiu R, Angel P, Karin M (1989) Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. Cell 59:979–986

Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acidic thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Cox RL, Glick DL, Strumwasser F (1991) Isolation and protein sequence identification of *Aplysia californica* lens crystallins. Biol Bull (Woods Hole) 181:333–335

Cronin TW (1986) Photoreception in marine invertebrates. Am Zool 26:403–415

Curran T, Franza BR (1988) Fos and Jun: the AP-1 connection. Cell 55:395–397

Daniel V (1993) Glutathione S-transferases: gene structure and regulation of expression. Crit Rev Biochem Mol Biol 28:173–207

de Jong WW, Hendriks W, Mulders JWM, Bloemendal H (1989) Evolution of eye lens crystallins: the stress connection. Trends Biochem Sci 14:365–368

de Jong WW, Leunissen JAM, Voorter CEM (1993) Evolution of the α-crystallin/small heat-shock protein family. Mol Biol Evol 10: 103–126

De Luca LM (1991) Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. FASEB J 5:2924–2933

Dubin RA, Wawrousek EF, Piatigorsky J (1989) Expression of the murine αB-crystallin gene is not restricted to the lens. Mol Cell Biol 9:1083–1091

Eakin RM (1972) Structure of invertebrate photoreceptors. In: Dartnall HJA (ed) Photochemistry of vision (handbook of sensory physiology, vol. VII/1). Springer, Berlin pp 625–684

Falb D, Maniatis T (1992) A conserved regulatory unit implicated in tissue-specific gene expression in *Drosophila* and man. Genes Dev 6:454–465

Fjose A, Nornes S, Weber U, Mlodzik M (1993) Functional conservation of vertebrate *seven-up* related genes in neurogenesis and eye development. EMBO J 12:1403–1414

Georgakopoulos T, Thireos G (1992) Two distinct yeast transcriptional activators require the function of the GCN5 protein to promote normal levels of transcription. EMBO J 11:4145–4152

Hadman M, Loo M, Bos TJ (1993) *In vivo* viral and cellular Jun complexes exhibit differential interaction with a number of *in vitro* generated "AP-1- and CREB-like" target sequences. Oncogene 8: 1895–1903

Herbomel P, Bourachot B, Yaniv M (1984) Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell 39:653–662

Klement JF, Cvekl A, Piatigorsky J (1993) Functional elements DE2A, DE2B, and DE1A and the TATA box are required for activity of the chicken αA-crystallin gene in transfected lens epithelial cells. J Biol Chem 268:6777–6784

Klementz R, Frohli E, Steiger RH, Schafer R, Aoyama A (1991) αB-crystallin is a small heat shock protein. Proc Natl Acad Sci U S A 88:3652–3656

Koller E, Trueb B (1992) Characterization of the chicken α1(VI) collagen promoter. Eur J Biochem 208:769–774

Land MF (1984) Molluscs. In: Ali MA (ed) Photoreception and vision in invertebrates. Plenum Press, New York pp 699–725

Land MF, Fernald RD (1992) The evolution of eyes. Annu Rev Neurosci 15:1–29

Masquilier D, Sassone-Corsi P (1992) Transcriptional cross-talk: nuclear factors CREM and CREB bind AP-1 sites and inhibit activation by Jun. J Biol Chem 267:22460–22466

Meyer M, Schreck R, Baeuerle PA (1993) H_2O_2 and antioxidants have opposite effects on activation of NF-B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. EMBO J 12: 2005–2015

Mitsialis SA, Kafatos FC (1985) Regulatory elements controlling chorion gene expression are conserved between flies and moths. Nature 317:453–456

Neumann JR, Morency CA, Russian KO (1987) A novel rapid assay for chloramphenicol acetyltransferase gene expression. Biotechniques 5:444–448

Nguyen T, Pickett CB (1992) Regulation of rat glutathione S-transferase Ya subunit gene expression. DNA-protein interaction at the antioxidant responsive element. J Biol Chem 267:13535–13539

Packard A (1972) Cephalopods and fish: the limits of convergence. Biol Rev 47:241–307

Patek CE, Clayton RM (1990) Age-related changes in the response of chick lens cells during long-term culture to insulin, cyclic AMP and a bovine retinal extract. Exp Eye Res 50:444–450

Perkins KK, Admon A, Patel N, Tjian R (1990) the *Drosophila* Fos-related AP-1 protein is a developmentally regulated transcription factor. Genes Dev 4:822–834

Piatigorsky J (1992) Innovation associated with changes in gene regulation. J Biol Chem 267:4277–4280

Piatigorsky J, Wistow GJ (1989) Enzyme/crystallins: gene sharing as an evolutionary strategy. Cell 57:197–199

Piatigorsky J, Horwitz J, Kuwabara T, Cutress CE (1989) The cellular eye lens and crystallins of cubomedusan jellyfish. J Comp Physiol A 164:577–587

Piatigorsky J, Zelenka PS (1992) Transcriptional regulation of crystallin genes: cis elements, trans-factors, and signal transduction systems in the lens. In: Advanced developments in biochemistry, vol 1, pp 211–256

Piatigorsky J, Horwitz J, Norman B (1993) J1-crystallins of the cubomedusan jellyfish lens constitute a novel family encoded in at least three intronless genes. J Biol Chem 268:11894–11901

Rinaudo JA, Zelenka PS (1992) Expression of c-fos and c-jun

mRNA in the developing chicken lens: relationship to cell proliferation, quiescence, and differentiation. Exp Cell Res 199:147–153

Roth HJ, Das GC, Piatigorsky J (1991) Chicken βB1-crystallin gene expression: presence of conserved functional polyomavirus enhancer-like and octamer binding-like promoter elements found in non-lens genes. Mol Cell Biol 11:1488–1499

Rushmore TH, King RG, Paulson KE, Pickett CB (1990) Regulation of glutathione S-transferase Ya subunit gene expression: identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. Proc Natl Acad Sci U S A 87:3826–3830

Rushmore TH, Morton MR, Pickett CB (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem 266:11632–11639

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74:5463–5467

Schnell N, Krems B, Entian K-D (1992) The PAR1 (YAP1/SNQ3) gene of *Saccharomyces cerevisiae,* a c-jun homologue, is involved in oxygen metabolism. Curr Genet 21:269–273

Schule R, Umesono K, Mangelsdorf DJ, Bolado J, Pike JW, Evans RM (1990) Jun-Fos and receptors for vitamin A and D recognize a common response element in the human osteocalcin gene. Cell 61:497–504

Shapiro DJ, Sharp PA, Wahli WW, Keller MJ (1988) A high-efficiency HeLa cell nuclear transcription extract. DNA 7:47–55

Siezen RJ, Shaw DC (1982) Physicochemical characterization of lens proteins of the squid *Nototodarus gouldi* and comparison with vertebrate crystallins. Biochim Biophys Acta 704:304–320

Tini M, Otulakowski G, Breitman ML, Tsui L-C, Giguere V (1993) An everted repeat mediates retinoic acid induction of the γF-crystallin gene: evidence of a direct role for retinoids in lens development. Genes Dev 7:295–307

Tomarev SI, Zinovieva RD (1988) Squid major lens polypeptides are homologous to glutathione S-transferase subunits. Nature 336:86–88

Tomarev SI, Zinovieva RD, Piatigorsky J (1991) Crystallins of the octopus lens. Recruitment from detoxification enzymes. J Biol Chem 266:24226–24231

Tomarev SI, Zinovieva RD, Piatigorsky J (1992) Characterization of squid crystallin genes. Comparison with mammalian glutathione S-transferase genes. J Biol Chem 267:8604–8612

Tomarev SI, Zinovieva RD, Guo K, Piatigorsky J (1993) Squid glutathione S-transferase. Relationships with other glutathione S-transferases and S-crystallins of cephalopods. J Biol Chem 268: 4534–4542

Vogt P, Bos TJ (1990) Jun: oncogene and transcription factor. Adv Cancer Res 55:1–35

Walls GL (1967) The vertebrate eye and its adaptive radiation. Hafner Publishing, New York

Wistow GJ, Piatigorsky J (1987) Recruitment of enzymes as lens structural proteins. Science 236:1554–1556

Wistow GJ, Piatigorsky J (1988) Lens crystallins: the evolution and expression of proteins for a highly specialized tissue. Annu Rev Biochem 57:479–504

Wistow GJ, Kim H (1991) Lens protein expression in mammals: taxon-specificity and recruitment of crystallins. J Mol Evol 32:262–269

Wondisford FE, Steinfelder HJ, Nations M, Radovick S (1993) AP-1 antagonizes thyroid hormone receptor action on the thyrotropin β-subunit gene. J Biol Chem 268:2749–2754

Xanthoudakis S, Miao G, Wang F, Pan Y-C E, Curran T (1992) Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. EMBO J 11:3323–3335

Xanthoudakis S, Curran T (1992) Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. EMBO J 11:653–665

Zhang K, Chaillet JR, Perkins LA, Halazonetis TD, Perrimon N (1990) *Drosophila* homolog of the mammalian jun oncogene is expressed during embryonic development and activates transcription in mammalian cells. Proc Natl Acad Sci U S A 87:6281–6285

Zinovieva RD, Tomarev SI, Piatigorsky J (1993) Aldehyde dehydrogenase-derived Ω-crystallins of squid and octopus. Specialization for lens expression. J Biol Chem 268:11449–11455