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Regulation of *FABP* in Retinal Development and Malignant Glioma

by

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Abstract

One of the greatest challenges in modern developmental biology is to discover the molecular mechanisms and pathways directing embryogenesis. Thanks to the efforts of many investigators, significant progress has been made in this regard. Nevertheless, much remains to be learned. In the experiments described in the following chapters, we characterize the factors leading to the cell-specific expression of two orthologous genes implicated in neural development: the retinal-fatty acid binding protein (*R-FABP*) gene and the brain-fatty acid binding protein (*B-FABP*) gene. Diverse functions have been proposed for the large family of fatty acid binding proteins related to R-FABP and B-FABP, including lipid transport and sequestration, as well as roles in mammary gland, heart and neural differentiation. B-FABP has been described as a differentiation factor for a class of glial cells called radial glia, involved in the early stages of brain development. We present evidence in Chapters 2 and 3, implicating a role for transcription factors AP-2α and AP-2β in cell-specific *R-FABP* gene repression in the developing retina. The AP-2 family encodes three proteins known to be important for neural development: AP-2α, -β, and –γ. Beyond their involvement in the down-regulation of the *R-FABP* gene, their expression pattern in the retina suggests an important role in the differentiation of a subset of retinal neurons. In Chapter 4, members of the nuclear factor I (NFI) gene family are implicated in the differential expression of the *B-FABP* gene in malignant glioma (MG) cell lines. Moreover, we have found a correlation suggesting that the trans-activating potential of NFI is modulated by differential phosphorylation in these cell lines. NFI phosphorylation appears to be mediated by a cell-specific phosphatase specifically present in B-FABP expressing MG cell lines. Altogether, our results indicate

that R-FABP and B-FABP, although they have a similar tissue distribution, are regulated in very different ways and indicate a role for two well-known transcription factor families in directing cell-specific gene expression.

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List of Abbreviations

a	amacrine cells
A	adenine
aa	amino acid
As	astrocyte
ALBP	adipocyte lipid binding protein
AP-2	activator protein-2
B	bound
B-FABP	brain-fatty acid binding protein
BLBP	brain lipid-binding protein
BLE	basal level element
BSA	bovine serum albumin
bp	base pairs
C	cytosine
CA-II	carbonic anhydrase II
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
CRBP-II	cellular retinol binding protein II
C-terminal	carboxyl terminal

CTF	CCAAT transcription factor
CTPS	copper phthalocyanine-3,4',4'',4'''-tetrasulfonic acid
d	day
da	displaced amacrine cells
dATP	deoxyadenosine triphosphate
dCMP	deoxycytosine monophosphate
dCTP	deoxycytosine triphosphate
DIG	digoxigenin
DMEM	Dulbecco Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNA-PK	dsDNA dependent-protein kinase
DNase I	deoxyribonuclease I
DRGE	dorsal root ganglion enhancer
DSCS	dorsal spinal cord silencer
DTT	dithiothreitol
ED	embryonic day
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EMSA	electrophoretic mobility shift assay
EST	expressed sequence tag
FABP	fatty acid binding protein

FCS	fetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
fmol	femtomole
fp	footprint
G	guanosine
GABA	γ-aminobutyric acid
GCL	ganglion cell layer
GFAP	glial fibrillary acidic protein
h	horizontal cells
H-FABP	heart-fatty acid binding protein
hMTIIa	human metallothionein IIa
HRE	hormone response element
HRP	horseradish peroxidase
HS	hypersensitive site
I-FABP	intestinal-fatty acid binding protein
IgG	immunoglobin G
INBL	inner neuroblastic layer
INL	inner nuclear layer
IPL	inner plexiform layer
IS	inner segment
kb	kilobase pairs
kDa	kilodaltons

LOH	loss of heterozygosity
LRD	lysinated rhodamine dextran
M	molar
MDGI	mammary derived growth inhibitor
MG	malignant glioma
min	minutes
μM	micromolar
mM	millimolar
MMTV	mouse mammary tumor virus
MoMuLV	Moloney murine leukemia virus
MOPS	N-morpholinopropanesulfonic acid
mRNA	messenger ribonucleic acid
MT	mutant
NE	nuclear extract
NFL	nerve fiber layer
NFI	nuclear factor I
nM	nanomolar
nt	nucleotide
N-terminal	amino terminal
ONBL	outer neuroblastic layer
ONL	outer nuclear layer
ONPG	o-nitrophenyl-β-D-galactosidase
OPL	outer plexiform layer

OS	outer segment
PAGE	polyacrylamide gel electrophoresis
PAP	potato acid phosphatase
PBS	phosphate buffered saline
Pbx	paired box
PCL	Purkinje cell layer
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
pTP	terminal protein
PUFA	polyunsaturated fatty acids
RA	retinoic acid
rev.	reviewed
R-FABP	retinal-fatty acid binding protein
RGE	radial glial element
RPE	retinal pigmented epithelium
RPF-1	retina-derived POU-domain factor-1
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SV-40	simian virus -40

T	thymidine
TBP	TATA binding protein
TFIID	transcription factor IID
TRITC	tetramethylrhodamine isothiocyanate
TUNEL	TdT-mediated deoxyuridine triphosphate nick end labeling
V	volts
VZ	ventricular zone
WT	wildtype

Chapter 1 -Introduction

1.1 Eye Development

The complex morphogenic and cellular events that lead to development of the eye have been well described. Much of what is known stems from the work of the Spanish neuroanatomist Santiago Ramón y Cajal whose studies on cells of the brain and retina, their connections and derivation, form the foundation of neurocytology (Ramon y Cajal, 1894). In the chick, eye development begins at embryonic day (ED) 2 (for review, see Mey and Thanos, 1992). Two outpouches called the lateral vesicles develop as evaginations of the diencephalon, or embryonic forebrain (see Fig. 1-1). These vesicles extend towards the surface ectoderm and induce a thickening of the overlying ectoderm. This leads to the formation of the lens placode (the primitive optic lens). Invagination of the lens placode internalizes the lens and leads to involution of the hollow optic vesicle. Like a ball deflating, compression of the optic vesicle creates the optic cup, a bilayered structure grossly resembling the adult eye. The outer layer of the optic cup becomes the pigmented epithelium, while the inner layer develops into the neural retina.

1.1.1 Genesis of Retinal Cells

The retina develops from the simple sheet of primitive neuroepithelial cells which comprise the inner layer of the early optic cup. In the mature vertebrate retina, there are seven distinct cell types, six neuronal types [photoreceptors (rods and cones), horizontal, bipolar, amacrine, interplexiform and ganglion cells] and a single type of glial cell, the Müller glial cell (see Fig. 1-2). The neuroepithelial cells are precursor cells that have the

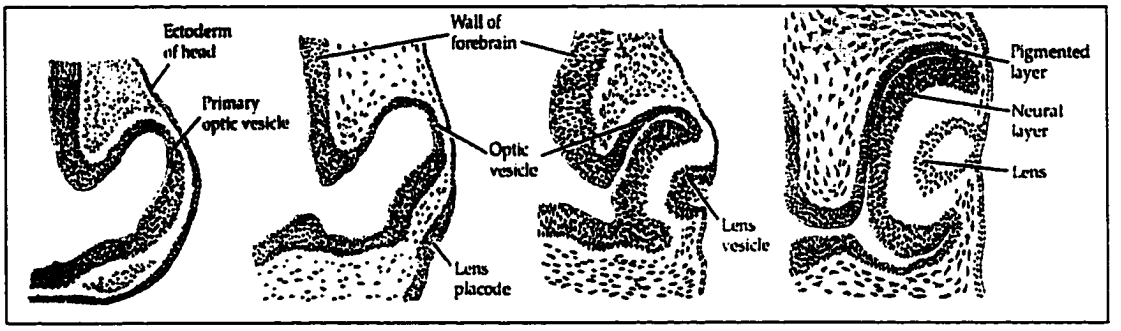

Figure 1-1. Important events in the development of the eye. A. Optic vesicle develops as an extension of the forebrain. **B.** Induction of the lens placode in the overlying surface ectoderm. **C.** Invagination of the optic vesicle to form the optic cup. **D.** Internalization of the lens and apposition of the inner and outer surfaces of the optic cup. This illustration is from Mann in *The Development of the Human Eye*. Grune and Stratton, New York (1964).

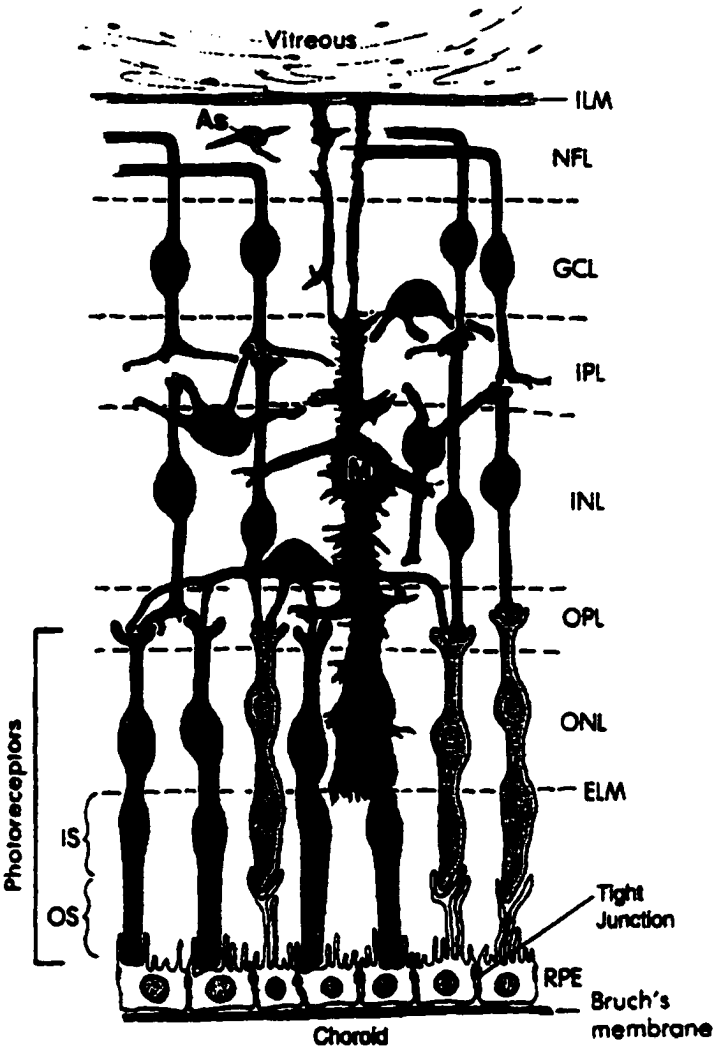

Figure 1-2. The adult vertebrate retina. Diagram of the retinal layers and their synaptic relationships. The main cell types are designated as R, rods; C, cones; H, horizontal cells; G, ganglion cells; M, Müller glial cells; I, interplexiform cells; A, amacrine cells; B, bipolar cells, As, astrocyte. Retinal layers are ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer. ILM and ELM are the inner and outer limiting membranes, RPE is the retinal pigmented epithelium and IS and OS are inner and outer photoreceptor segments. This illustration is from *Retina. Basic Science and Inherited Retinal Diseases.* Stephen J. Ryan, Editor-in-Chief. The C.V. Mosby Company, St. Louis, 1989.

potential of differentiating into all the different cell types found in the retina (Sidman, 1961; Turner and Cepko, 1987). Because the retina is a direct extension of the brain, it is perhaps not surprising that retinal neurogenesis proceeds in a similar manner as the developing brain. Three overlapping stages of neural development have been defined: (i) cell division, (ii) cell migration, and (iii) cellular differentiation. Early stages of retinal development are characterized by extensive cell proliferation. In the chick embryo, >90% of retinal cells are dividing at ED3. By ED7, towards the end of the cell division stage, about 60% of cells are still dividing whereas by ED9, only about 10% of retinal cells are still proliferating (Dütting *et al.*, 1983).

During development, the retina becomes subdivided into two regions, the outer neuroblastic layer (ONBL), a region of proliferating cells adjacent to the retinal pigmented epithelium (RPE), and the inner neuroblastic layer (INBL), the destination of migrating post-mitotic neurons (Fekete *et al.*, 1994). With cell migration and differentiation, the retina takes on the appearance of the mature tissue, consisting of three nuclear layers, two plexiform layers and the ganglion fiber layer.

The order of cell appearance in the developing retina has been extensively studied (Kahn, 1974; Prada *et al.*, 1991). Prada and co-workers (1991) determined the time of appearance of various retinal cell types in the chick retina by comparing the incorporation of [^{3}H]-thymidine in retinal cells at different stages of development. Retinal cell-types are born in overlapping waves, such that different cell-types are being produced at the same time. However, the timing of appearance of each cell-type is distinct, with the earliest ganglion cells beginning to withdraw from the cell cycle at ED2, whereas the earliest

bipolar cells are not detected until ED5. The other cell types (amacrine, horizontal, photoreceptor and Müller glial cell) begin to appear between ED3 and ED4.

1.1.2 Cell Migration

After their birth in the ONBL, the immature retinal neurons migrate radially inwards towards the vitreous humour. Progress has been made towards understanding the mechanism(s) of migration. Retinal neuronal migration has been proposed to involve at least two mechanisms: perikaryal translocation and glial guided migration. In the former, a premigratory neuron extends processes to both the inner and outer surfaces of the retina and then translocates its nucleus from the ventricular zone to its final destination in the INBL. Experiments by Snow and Robson (1995) using retrograde fluorescent dye labelling of retinal ganglion cells have shown that these cells have processes extending to each surface and so appear to be migrating by perikaryal translocation. The second mechanism, glial-guided neuronal migration, is a process whereby immature neurons follow pre-existing Müller glial processes that span the width of the retina. Müller glial cells are present early in the developing retina and are proposed to serve a similar function as the radial glial cells present in other parts of the CNS, which have been shown to guide migrating neurons (for review see Hatten, 1999). Meller and Tetzlaff (1976) have direct evidence based on electron microscopic studies of the developing retina that supports this mechanism for cell migration in retinal development. This latter mechanism would explain the organizing properties of the Müller glial cell on dissociated retinal cultures observed by Willbold and Layer (1998). Regional specialization of Müller glial cells could potentially provide migratory neurons with cues regarding directionality

and appropriate disembarkment zones. Glial-guided migration and perikaryal translocation are not mutually exclusive mechanisms and it seems likely that they function consecutively, as has been suggested by Snow and Robson (1995). Perikaryal translocation may function early in retinal development while the retina is relatively simple, whereas glial-guided migration may be a later process involved in establishment of the mature retinal strata.

1.1.3 Retinal Differentiation

Lineage tracing experiments using dye tracer injections (Holt *et al.*, 1988; Wetts and Fraser, 1988) and retroviral integration markers (Turner and Cepko, 1987; Turner *et al.*, 1990) have revealed that the retinal neuroepithelial cells are pluripotent, capable of differentiating into both neuronal and glial cell types. By injecting rat eyes with a β-galactosidase expressing retrovirus, Turner and Cepko (1987) were able to infect retinal progenitor cells and identify their descendants. The retrovirus they used (BAG) was replication-incompetent, so that only cells which inherited the integrated virus expressed β-galactosidase. Each clone of β-galactosidase positive cells was variable in cell number and type. Some of the largest clones contained 22 cells, while others had only a single cell. Some of the clones contained rods, bipolar, and amacrine cells, while others had rods, bipolar and Müller glial cells, indicating that even at the late developmental stage that these experiments were done (postnatal day 0 to 7), the progenitor cells retained the capability of producing multiple cell types. A similar conclusion was reached by Wetts and Fraser (1988) who followed the descendants of single Xenopus optic vesicle cells injected with the fluorescent dye, lysinated rhodamine

dextran (LRD). Labelled cells representing all retinal cell types were detected with this technique. These experiments indicate that a given progenitor cell is capable of giving rise to any combination of descendant cell types as indicated by the variety of cells in each clone.

Cell fate determination appears to occur during or slightly after terminal cell division since certain retinal cell-type specific markers are first expressed at this stage. Waid and McLoon (1995) have detected RA4 protein, a ganglion cell specific marker, as early as 15 minutes after completion of mitosis suggesting that ganglion cell differentiation begins during or just after mitosis. Similarly, Barnstable and coworkers (1985) were able to detect the amacrine cell marker HPC-1 in migrating amacrine cells. Given the remarkably early expression of these markers, it seems that termination of cell division initiates a rapid series of events promoting cellular differentiation.

The molecular mechanisms governing retinal cell fate determination are still poorly understood. Presumably, cell fate commitment initiates cell-type specific differentiation programs leading to expression of genes involved in cell maturation. These programs are thought to rely on a hierarchy of transcription factors to regulate the many cell-type specific genes expressed in a particular cell type. Expression or activation of a particular master transcription factor, or particular combination of transcription factors, would therefore regulate expression of subordinate transcription factors resulting in a cascade effect. A number of transcription factors have been implicated in retinal development (see Table 1-1). One of the most interesting of these genes is the homeobox-containing transcription factor Pax-6, which has been called the 'master control gene' for eye development. Ectopic expression of murine Pax-6 or its *Drosophila* homolog, *eyeless*

Table 1-1 Transcription Factors Involved in Retinal Development

Gene	Putative Function	Reference
Brn-3b	Ganglion cell survival and/or specification	Erkman *et al.*, 1996 Gan *et al.*, 1996
Chx10	Bipolar cell differentiation	Burmeister *et al.*, 1996
Hes1	Delays retinal differentiation	Tomita *et al.*, 1996
Mash-1	Generation of bipolar cells and Müller glia	Jasoni and Reh, 1996
Math5	Ganglion cell specification	Brown *et al.*, 1998
Crx	Photoreceptor differentiation	Furukawa *et al.*, 1997
Optx2	Retinal specification	Toy *et al.*, 1998
Pax-6	Early eye specification	Halder *et al.*, 1995
Prox1	Horizontal cell specification	Belecky-Adams *et al.*, 1997
RPF-1	Ganglion and amacrine differentiation	Zhou *et al.*, 1996

(ey), leads to ectopic eye formation in *Drosophila*, indicating Pax-6 by itself is sufficient to turn on the early steps involved in eye formation (Halder *et al.*, 1995). Eye formation is disrupted in *Small eye* (*Sey*) mice due to homozygous loss of Pax-6 (Hill *et al.*, 1991). Pax-6 is expressed in the early developing eye (Walther and Gruss, 1991; Grindley *et al.*, 1995). Pax-6 may also play a role later in retinal development as suggested by its presence in differentiated retinal cells. Belecky-Adams and colleagues (1997) suggest that *Pax-6* may be involved in cell-fate decisions based on its early expression in amacrine and ganglion cells.

The Chx10 transcription factor is also important for retinal differentiation. *Chx10* mutations underlie the phenotype of the ocular retardation (*or*) mouse which shows microphthalmia, hypoplastic retina and specific loss of bipolar cells (Burmeister et al., 1996). In the developing retina, Chx10 expression is localized entirely to bipolar interneurons found in inner nuclear layer, suggesting it is involved in their differentiation (Liu et al., 1994; Belecky-Adams et al., 1997). Downstream target genes of Chx10 have not yet been reported.

1.2 Fatty acid binding proteins (FABP)

The first FABP was isolated by Ockner and coworkers (1972) as a ~12 kDa cytosolic protein found in rat liver, intestine, and heart, which reversibly bound long chain fatty acids. Subsequent experiments have revealed a large family of FABP encoding genes and estimate the age of this ancient gene family to be up to one billion years old (Chan *et al.*, 1985). It is now known that vertebrates possess at least 11 genes encoding members of the FABP family. Divergence of these genes has given rise to

specialized family members with distinct ligand binding specificities and tissue- and cell-specific expression (see Table 1-2). For instance, intestinal FABP (I-FABP), highly expressed in the intestine, has highest affinity for long chain unsaturated fatty acids, whereas cellular retinoic acid binding protein (CRABP) is more widely expressed and preferentially binds the hydrophobic morphogen, retinoic acid.

Members of the FABP family have diverse functions in metabolism, signal transduction and development (see Table 1-2). Intestinal and liver FABP function in the solubilization and intracellular transport of fatty acids in preparation for their metabolism. CRABPI and II are involved in the intracellular transport of retinoic acid and are thus a component of the retinoic acid signalling system involved in development (Dong *et al.*, 1999). Several FABP family members have been implicated in cell growth and differentiation including heart FABP (H-FABP, also called mammary derived growth inhibitor (MDGI)), brain FABP (B-FABP) and retinal FABP (R-FABP). MDGI was isolated from bovine mammary gland as a factor that inhibited the growth of mammary carcinoma cells *in vitro* (Böhmer *et al.*, 1984). Yang and coworkers (1994) found that MDGI inhibited the proliferation of mammary epithelial cells and led to the functional and morphological differentiation of mammary gland explants. The function(s) of B-FABP will be discussed in section 1.2.2.

1.2.1 FABP Structure and Ligand Specificity

The three dimensional structures of a number of FABPs have been determined (for review see Börchers and Spener, 1994). Diverse FABP family members conform to a common tertiary structure called the β-clam. The ligand lies in a cavity created by two

Table 1-2. FABPs, their ligands and expression

Protein	Ligand	Tissue Occurrence
(H)eart-FABP or Mammary derived growth inhibitor (MDGI)	fatty acids	Heart, skeletal muscle, mammary gland, kidney, stomach, aorta and lung
(A)dipocyte-FABP or adipocyte lipid binding protein (ALBP)	fatty acids	adipose tissue
(M)yelin-FABP	fatty acids	peripheral nerve myelin
(B)rain-FABP or brain lipid binding protein (BLBP) or (R)etinal-FABP	fatty acids	brain and retina
(E)pidermal-FABP	fatty acids	skin
(I)ntestinal-FABP	fatty acids	intestine
(L)iver-FABP	fatty acids, others	liver, intestine, kidney
Ileal lipid binding protein (ILBP)	Bile acids	intestine
CRABP	retinoic acid	widespread
CRABP II	retinoic acid	widespread
CRBP	retinol	liver, kidney, lung, testis
CRBP II	retinol, retinal	intestine

Table adapted from Börchers and Spener (1994).

orthogonal anti-parallel β sheets, which enclose the ligand similar to the shells of a clam. The ligand binding specificity of each FABP is governed by the amino acid residues lining the binding site. For instance, Jakoby *et al.* (1993) have shown that a single amino acid change in cellular retinol-binding protein II (CRBP-II) is able to change its ligand specificity from retinoids to fatty acids. They converted a glutamine residue (Glu-109) to an arginine, making CRBP-II more similar to I-FABP. In I-FABP, the positively charged arginine side chain at position 106 has been shown to interact with the carboxyl group of the fatty acid providing binding specificity. Similarly, other residues, particularly the arginine residue at position 126 of myelin P2 and H-FABP, have been implicated in ligand binding (Jones *et al.*, 1988; Zanotti *et al.*, 1992).

Analysis of the ligand binding properties of B-FABP by Xu and colleagues (1996) determined that B-FABP preferentially binds long-chain unsaturated fatty acids. Bacterially produced B-FABP did not bind palmitic acid or arachidinic acid, both saturated fatty acids, but bound unsaturated fatty acids such as oleic acid (18:1), arachidonic acid (20:4) with high affinity. Xu *et al.* (1996) suggest a potential endogenous ligand for B-FABP to be docosahexaenoic acid (22:6), since it was bound with highest affinity, enriched in the brain and required for neural development.

1.2.2 B-FABP Function

Fifteen years since its discovery in the developing CNS, the function(s) of B-FABP remain to be established. Since the brain (and retina) rely on glucose rather than metabolize fatty acids for energy, the role of B-FABP likely differs from L-FABP, I-FABP, and H-FABP, which are involved in fatty acid solubilization for catabolism. The

earliest proposed B-FABP function was that it acts as a free fatty acid sink, protecting synaptosomal trans-membrane proteins from their inhibitory effects (Bass *et al.*, 1984). Free fatty acids inhibit sodium dependent amino acid uptake systems, which are responsible for the uptake of neurotransmitter amino acids such as glutamic acid and γ-aminobutyric acid (GABA). Addition of B-FABP to reactions measuring amino acid transport in isolated synaptosomes relieved fatty acid induced inhibition, leading Bass and coworkers (1984) to suggest that B-FABP might be involved in regulating synapse function. However, this mechanism would predict a neuronal location for B-FABP, which does not agree with the newly discovered expression pattern of B-FABP in radial glial cells of the developing brain.

Sellner (1994) has proposed that B-FABP is involved in altering the membrane composition of developing neurons. Retina and brain are characteristically enriched in polyunsaturated fatty acids (PUFA) (Fliesler and Anderson, 1983). Since B-FABP preferentially binds unsaturated fatty acids (Xu *et al.*, 1996), it could act as a storage depot facilitating the incorporation of PUFA into the rapidly expanding embryonic CNS. This hypothesis is consistent with the known binding specificity of B-FABP, its early expression in the developing CNS and the high levels of B-FABP expressed in the developing brain and retina. We have found high levels of chick retina (R)-FABP, the chicken ortholog of B-FABP, as early as ED3.5 in the chick retina and brain (Godbout, 1993). R-FABP has been estimated to compose up to 1% of the total cytosolic protein, indicating that large amounts of this protein are required during retinal differentiation, as might be expected for a protein functioning as a storage facility. Developing cells would

be able to accumulate PUFA in preparation for neurite extension without detrimental effects on their internal environment.

Perhaps the most intriguing hypothesis regarding B-FABP function is that suggested by Feng *et al.* (1994), who propose that it is a signalling protein involved in the establishment and maintenance of the radial glial fiber system. This array of radially orientated glial fibers is involved in glial-guided neuronal migration in the developing brain and spinal cord (for review see Hatten, 1999). Immature neurons use radial glial processes as a substrate for migration, which guides migrating neurons to their correct location. In the presence of neurons, glial cultures extend radial glial-like processes, along which neurons can be seen to migrate (Edmondson and Hatten, 1987). Feng and coworkers (1994) found that addition of anti-B-FABP antibodies to neuronal-radial glial co-cultures prevents normal neuronal-induced differentiation of the radial glial cells. This suggests that B-FABP functions as an extracellular signaling molecule involved in establishing and maintaining radial glial differentiation. Feng *et al.* (1994) demonstrated the presence of B-FABP in the tissue culture media of murine cerebellum cells.

1.2.3 Expression of B-/R-FABP in the Developing CNS

One of the earliest hints that B/R-FABP plays a role in embryonic development came from studies of its expression pattern. B/R-FABP expression is under strict spatiotemporal control. Considerable data have been accumulated regarding B-FABP distribution during murine development. Northern and Western analysis of murine postnatal day 8 tissues has shown that B-FABP is found exclusively in the CNS, particularly the developing cerebellum and to a lesser extent, the forebrain (Kuhar *et al.*,

1993; Feng *et al.*, 1994). In adult mice, *B-FABP* mRNA is detected in whole brain as well as in the olfactory bulb by Northern analysis (Kurtz *et al.*, 1994). By analysing total CNS and cerebral cortex taken at different developmental stages, Feng and coworkers (1994) found that murine *B-FABP* mRNA levels rose from E12 to E14 in the CNS, peaked from E14 to P0/P5, decreasing to undetectable levels in the adult cerebral cortex. B-FABP was not detected in the developing mouse retina (Kurtz *et al.*, 1994).

B-FABP has a similar expression pattern in the developing embryos of other species, with interesting differences. Rat *B-FABP* mRNA is found in P7 brain and P21 liver, but not in heart, kidney, lung, spleen, thymus (Bennett *et al.*, 1994). The retina was not examined. Murine *B-FABP* was not detected in the liver (Kurtz *et al.*, 1994; Kuhar *et al.*, 1993). In humans, *B-FABP* mRNA is found in fetal and adult human brain and perhaps adult skeletal muscle (Shimizu *et al.*, 1997). They did not detect *B-FABP* in fetal or adult liver or kidney. Godbout *et al.* (1998) have demonstrated *B-FABP* mRNA in human brain (fetal and adult) as well as in fetal retina. No *B-FABP* mRNA was detected in human fetal liver and muscle was not tested. Chicken *R-FABP* is expressed in embryonic brain, retina, and to a lesser extent kidney (Godbout, 1993). *R-FABP* is expressed in a very interesting pattern during chick CNS development: high levels of transcript are found in the early retina, falling to undetectable levels prior to hatching, whereas in the developing brain, *R-FABP* levels rise during the same time period, suggesting that its role in retina is earlier that in the brain.

The cell-specific expression pattern of B-FABP in the developing mouse CNS has been extensively described (Feng *et al.*, 1994; Kurtz *et al.*, 1994). B-FABP mRNA and protein is expressed in radial glia throughout the CNS. In the early spinal cord (E10), B-

FABP is localized to two regions, a wide band of cells in the dorsal half and a narrow band of cells adjacent to the floorplate. The floorplate is located along the ventral spinal cord surface and is responsible for inductive events in spinal cord formation. With development, B-FABP expression spreads ventrally such that B-FABP is expressed in the ventricular zone (VZ) throughout the spinal cord. The VZ is a region adjacent to the ventricle, where the neuronal and glial precursor(s) are localized. B-FABP was also found in radial processes emanating from the VZ to the pial surface. These cells are likely spinal cord radial glial cells given that they co-express RC2, a radial glial marker and nestin, which is found in neuronal precursor cells and radial glia (Kurtz *et al.*, 1994; Feng *et al.*, 1994). Postnatally, B-FABP expression is localized to astrocytes, many of which are derived from radial glia.

In the developing cerebellum, *B-FABP* mRNA is found in small cells within the Purkinje cell layer (PCL). This cell layer contains Purkinje cells, immature migrating neurons and the somata of B-FABP positive Bergmann glia, specialized radial glia of the cerebellum. B-FABP is co-expressed in GFAP positive radial fibers which extend from the PCL to pial surface and diffusely in the molecular layer (Feng *et al.*, 1994; Kurtz *et al.*, 1994). These B-FABP positive cells are likely to be Bergmann glia based on their co-expression of GFAP. By immunogold electron microscopy, B-FABP was localized to both the nuclei and cytoplasm of Bergmann glia and migrating granule neurons confirming the immunofluorescence results.

In situ hybridization revealed *B-FABP* transcripts in several regions of the E13 forebrain, including the striatum, ventrolateral VZ, walls of the thalamus and VZ of hypothalamus, hippocampal formation and VZ of the olfactory bulb (Kurtz *et al.*, 1994).

At E17, B-FABP is found in radial glia in VZs throughout the di- and telencephalon. There is some discrepancy between Kurtz *et al.* (1994) and Feng *et al.* (1994): Kurtz *et al.* (1994) found B-FABP in a thin layer of cells along the E17 hippocampal dentate gyrus, whereas Feng *et al.* (1994) state that the hippocampus was negative. Perhaps the difference arises from looking at different section depths. In combination these data indicate that B-FABP is expressed in radial glial cells and perhaps cerebellar migrating granule neurons throughout the developing CNS.

1.2.4 B-FABP Gene Regulation

The intriguing distribution of B-/R-FABP in the developing CNS has led to an interest in its gene regulation. Genes encoding B-FABP orthologs from various species [murine (Kurtz *et al.*, 1994; Feng and Heintz, 1995), human (Godbout *et al.*, 1998), and chick (Bisgrove *et al.*, 1997)] have been cloned, and analysed to assess how they are regulated. Comparison of the mouse and human *B-FABP* genes reveals a high level of sequence conservation extending upstream of the transcriptional initiation site and also within intronic sequences (D.A.B., unpublished). This suggests that their regulatory elements may be conserved as well. Although they retain similar expression patterns in the developing CNS, the promoter sequences of mammalian and chicken *B/R-FABP* have diverged significantly. The chick *R-FABP* promoter appears to be embedded in a G-C island, whereas the promoters of the mammalian orthologs are not. G-C rich promoters tend to be associated with constitutively expressed housekeeping genes, although this is clearly not the case for the *R-FABP* gene given its highly tissue specific expression.

Feng and Heintz (1995) have functionally dissected the murine *B-FABP* promoter by analysis of transgenic mice carrying various *B-FABP* promoter fragments linked to a lacZ reporter gene. They found that 1.6 kb of 5' upstream sequence was sufficient to direct correct spatiotemporal reporter gene expression throughout the developing CNS. They defined gene regulatory domains by comparison of the reporter expression from several 5' deleted constructs. A silencer element (DSCS-dorsal spinal cord silencer) was localized to the –1.2 to –1.6 kb region, without which the reporter gene is inappropriately expressed in the dorsal spinal cord past the time when the endogenous *B-FABP* gene is normally down-regulated. A positive element (DRGE-dorsal root ganglion enhancer) was found within the –0.8 to –1.2 kb region. This enhancer programs *B-FABP* expression in cells of the dorsal root ganglion and notochord. B-FABP positive cells in these regions do not express the reporter gene if this region is missing. A third regulatory element (RGE-radial glial enhancer) was localized to the –0.3 to –0.8 kb region which drives B-FABP expression in Bergmann glia of the cerebellum (representing specialized radial glial cells) and radial glia elsewhere in the CNS. Transgenic mice carrying the –0.8 kb construct expressed the β-galactosidase in radial glia at the proper time, whereas expression was not detected in mice carrying the –0.3 kb construct. Transgenic mice carrying the –0.3 kb construct did not express the reporter gene, indicating that this region is insufficient to direct *B-FABP* transcription. Beyond broadly defining these domains, further experiments to characterize DNA binding sites responsible for the DSCS, DRGE, or RGE elements have not been reported by Feng and Heintz.

Josephson and coworkers (1998) localized three DNA binding sites within the murine *B-FABP* proximal promoter by deletion analysis and *in vitro* DNA binding

assays. A binding site for POU family members, located between –370 to –362 bp, was necessary for wild-type expression of *B-FABP*. Transgenic mice in which these 9 basepairs have been deleted from a reporter construct which has 0.8 kb of *B-FABP* upstream sequence fused to the lacZ gene, show a restricted pattern of *B-FABP* expression, with reduced reporter expression throughout the CNS, particularly the telencephalon and caudal regions of the spinal cord. Josephson *et al.* also found a binding site for members of the paired box (Pbx) family overlapping the POU binding site based on gel shift competition and supershift assays. This site did not appear to be used *in vivo* because its removal (leaving the POU site intact) had no effect on reporter expression. The third DNA binding site was a hormone response element (HRE) located at -286 to -275 bp. Deletion mutagenesis of this site prevented reporter expression in the telencephalon.

Identification of a POU binding site in the *B-FABP* promoter is particularly interesting given the importance of this transcription factor family in nervous system development (reviewed in Latchman, 1999). Members of the POU family are defined by the presence of a POU domain. This region contains two sub-domains, the POU-specific box and the POU homeobox motifs, which are responsible for DNA-protein interaction. Analysis of the function of POU family members by gene knockout experiments indicates a role in neuronal cell type specification and/or cell survival. Xiang and coworkers (1996) have shown that loss of Brn-3a, a POU domain protein, causes loss of motor and sensory neurons. Similar results were obtained by McEvilly and coworkers (1996). Fujii and Hamada (1993) have shown that differentiation of P19 embryonal carcinoma cells into neurons and glia requires expression of Brn-2. Treatment of P19

cells with retinoic acid leads to differentiated neurons and glia. The effect of retinoic acid treatment can be mimicked by Brn-2 overexpression, and blocked by expression of antisense Brn-2. Specification of specific classes of neurons/glia by POU members has been preserved throughout the metazoa. In C. elegans, the POU protein, UNC-86 is involved in the specification of the HSN neuron, which is necessary for egg laying (Finney *et al.*, 1988; Desai *et al.*, 1988). Josephson *et al.* (1998) detected Brn-1 and Brn-2 bound to the *B-FABP* POU binding site. Their mutagenesis experiments indicate a role in *B-FABP* activation as its removal leads to decreased expression of B-FABP in certain regions of the CNS (forebrain, midbrain and caudal spinal cord).

1.3 Activator Protein-2 (AP-2)

1.3.1 Properties

AP-2 was first described by Tjian's group in 1987 as a 52 kDa HeLa nuclear factor that bound to a region within the SV40 enhancer and caused its activation (Mitchell *et al.*, 1987). AP-2 was also characterized simultaneously by Karin's group who showed that AP-2 was involved in the up-regulation of the human metallothionein II_A (hMT-II_A) gene, by interaction with its enhancer (Imagawa *et al.*, 1987). Subsequent cloning experiments have revealed that there are three closely related *AP-2* genes in vertebrates: *AP-2*α (Williams *et al.*, 1988), *AP-2*β (Moser *et al.*, 1995) and *AP-2*γ (initially called *AP-2.2*, Oulad-Abdelghani *et al.*, 1996). In common with other transcriptional regulators, the DNA binding and trans-activation functions of AP-2 are present on two separable domains. Mutational analysis of the cloned *AP-2*α cDNA localized the DNA binding domain to the C-terminal half of the protein, whereas the

trans-activation domain was found in the N-terminal half (Williams *et al.*, 1988). The AP-2 DNA binding domain is divided into two regions, a basic region involved in DNA binding site specificity, and a dimerization domain required for DNA-protein interaction. AP-2 appears to exist as stable homo- and heterodimers in solution (Williams and Tjian, 1991b). Dimerization is mediated by a novel helix-span-helix motif located in the region between amino acids 280-410. Helix 1 (280-307) is separated from helix 2 (390-410) by a span of 82 amino acids (Williams and Tjian, 1991a). Dimerization is proposed to occur by interaction of the hydrophobic surfaces of these amphipathic helices. Dimerization is necessary for DNA-protein interaction, as mutation of amino acids in the helical region abolished dimerization and DNA-protein interaction (Williams and Tjian, 1991a). The function of the span region is unknown. It appears to be involved in DNA-protein interaction, as deletion of 19 amino acids within the span region leads to a protein still able to dimerize but incapable of stable DNA interaction without the aid of wildtype monomers (William and Tjian, 1991a). While the DNA binding domain of AP-2α is best characterized, it is assumed that the other family members adopt a similar configuration given their high sequence similarity, their common DNA binding specificity and their ability to form stable heterodimers. Mohibullah *et al.*, (1999) recently made use of the SELEX technique to better define the binding sequence of the consensus AP-2 binding site. They defined two AP-2 binding sites, $GCCN_4GGC$ and $GCCN_3GGG$, in addition to the previously characterized $GCCN_3GGC$.

The AP-2 trans-activation domain has been defined by protein fusion experiments. Williams and Tjian (1991b) linked portions of AP-2 to a heterologous DNA binding domain (GAL4) and tested the ability of the chimeric protein to activate a

reporter gene. An 80 amino acid section of AP-2 was sufficient to activate transcription and thus contained the activation domain. This region does not have sequence similarity to the trans-activating domains of other known transcription factors. It is very rich in proline residues (30%). In this respect, the AP-2 activation domain resembles that of the proline-rich transcription factor, CTF/NFI. Beyond the high proline content however, there is no sequence similarity between the AP-2 and NFI families. The mechanism whereby the proline-rich domain activates transcription is not known. Tjian's group suggested that the high proline content causes the activation domain to adopt a rigid structure creating a site for interaction with other proteins (co-activators or components of the basal transcriptional machinery). Gerber *et al.* (1994) have found that simple homopolymeric stretches of proline or glutamine are sufficient in themselves to cause transcriptional activation, although the actual mechanism remains unclear. Published data regarding the functioning of the AP-2 activation domain are sparse.

1.3.2 AP-2 Expression During Development

The AP-2 family is expressed in a tissue- and cell-specific manner during development. The expression patterns of AP-2α and -β are quite similar, whereas that of AP-2γ deviates. All three are expressed in neural crest cells and their derivatives (West-Mays *et al.*, 1999). Neural crest cells are migratory cells, born in regions lateral to the neural folds, which contribute cells to a variety of tissues including melanocytes, certain endocrine cells (adrenal medullar cells), neuronal (sensory neurons) and glial (Schwann cells) cells of the dorsal root ganglia, limb bud mesenchyme, connective tissue and skeletal elements of the face and parts of the eye. These areas coincide with the

expression of AP-2 that is in skin, facial mesenchyme and developing limb buds (Mitchell *et al.*, 1991; Moser *et al.*, 1995; Chazaud *et al.*, 1996). In the early mouse embryo, each gene is expressed in the developing brain, which is derived from the neuroepithelium rather than the neural crest. Interesting differences are apparent; for instance, *AP-2γ*, but not *AP-2α*, is expressed in the telencephalon whereas this is reversed in the diencephalon (Chazaud *et al.*, 1996). *AP-2β* is not expressed in the forebrain (Moser *et al.*, 1995). The midbrain is positive for *AP-2β* and shows lesser amounts of *AP-2α* and *AP-2γ*. Transcripts for each of the three genes have been detected in the hindbrain. Both *AP-2α* and *AP-2β* are expressed in the embryonic kidney; however, whereas *AP-2α* is found in cells of the proximal tubules, *AP-2β* is found in the developing distal tubules (Moser *et al.*, 1997). All three *AP-2* genes are expressed in the epidermis (Chazaud *et al.*, 1996; Moser *et al.*, 1997).

1.3.3 AP-2 Function

Gene knockout experiments indicate a crucial role for AP-2α in embryogenesis. *AP-2α -/-* mice die perinatally with severe defects in cranial and body wall closure, skeletal formation and craniofacial development (Schorle *et al.*, 1996; Zhang *et al.*, 1996). Many of the malformations can be attributed to failure to fuse at the midline. *AP-2α*-null embryos exhibit full midline facial clefting stemming from failure of nasal and mandibular midline regions to fuse. The cranial folds (putative forebrain, midbrain and rostral hindbrain) fail to fuse to form the neural tube. This severely perturbs brain formation, and as a result, much of the brain develops inside out, with the ventricular zone (germinal layer) facing outwards instead of towards the ventricles (exencephaly).

During normal development, cells of the dorsoventral body wall migrate ventrally and fuse at the midline forming the sternum. In AP-2α-null mice, the ventral body wall fails to close, leaving the visceral contents exposed (thoracoabdominoschisis). Other congenital defects are apparent, seemingly unrelated to the midline fusion defects. Most neural crest cell derived skeletal bones of the head are missing or present in only a rudimentary form (eg, maxilla and cranial vault). The spine is contorted (scoliosis). Most of the embryos (18/28) lack the radius bone of the forelimbs (Zhang *et al.*, 1996). This further substantiates a role for AP-2α in limb development, which was initially suggested based on the expression of AP-2α in the developing limb bud progress zone (Mitchell *et al.*, 1991; Shen *et al.*, 1997). Sensory organs are also affected: eyes are often missing or abnormal and displaced within the everted forebrain. Lenses are often absent or rudimentary, apparently due to interruption of the contact between the optic vesicle and the surface ectoderm. Auditory system formation is also disrupted, with the malleus, incus and stapes bones of the ear missing.

To further examine the role of AP-2α in murine development, the phenotype of chimeric mice created from wildtype and *AP-2α* null cells were examined (Notoli *et al.*, 1998). These mice were created by microinjecting *AP-2α* null embryonic stem cells in *AP-2α* +/+ blastocysts, followed by implantation into host mothers. The resulting embryos were a mosaic of *AP-2α* +/+ and *AP-2α* -/- cells. The spectrum of defects (i.e. body wall, craniofacial, neural tube, ocular and limb formation defects) found with the *AP-2α* null mice were recapitulated in the chimeric mice. One of the questions arising from the *AP-2α* knockout experiments was to what extent the phenotype was due to loss of normal tissue inductive events rather than inactivation of the *AP-2α* gene. For

instance, it is well known that normal eye development requires interaction of the primitive optic vesicle with the surface ectoderm. The ocular defects of the *AP-2α* knock-out mouse could therefore be a secondary effect of forebrain overgrowth, thereby interrupting normal tissue interactions, rather than to an actual requirement for AP-2α. Notoli and coworkers (1998) found the defects of the *AP-2α* knock-out mice arose independently of each other, suggesting separate roles for AP-2α in each of these events. The ocular defects were observed even in the absence of exencephaly or other defects, suggesting a direct and crucial role for AP-2α in eye development.

With respect to the role of AP-2α in eye development, West-Mays and colleagues (1999) have examined the ocular defects of *AP-2α* null and chimeric mice and found that AP-2α is specifically required for development of the lens, as well as specification of the retina and the development of retinal ganglion cells. The lenses of chimeric mice with defects restricted to the eye, were frequently absent or defective. The abnormalities included misshapen lenses and corneolenticular adhesions, which result from incomplete separation of the primitive lens from the overlying surface ectoderm. These results suggest a role for AP-2α in lens development, although there is still the possibility that the defects in the lens are secondary to defects in the optic cup.

In addition to lens defects, West-Mays *et al.* (1999) found that chimeric embryos exhibited hypotrophy of the iris and ciliary body, as well as malformation of the optic stalk. Particularly interesting was the failure of the dorsal retinal pigmented epithelium (RPE) to properly differentiate, apparently differentiating into dorsal retina instead. This suggests that AP-2α may be involved in dorsoventral patterning of the RPE and/or the retina. Why only the dorsal aspect of optic cup formation is affected remains unclear. As

will be discussed in the next section, AP-2α expression is inducible by retinoic acid (RA), a morphogen distributed in a dorsoventral gradient during retinal development. One way to explain the ocular phenotype of the AP-2α chimeric mice is by postulating that loss of AP-2 interferes with the interpretation of this gradient.

Retinal development was also affected by loss of AP-2α. Chimeric mice had extensive retinal folding and unusual clusters of retinal cells (West-Mays *et al.*, 1999). Some retinal regions were missing ganglion cells, an observation confirmed by the lack of Brn-3b immunoreactivity, a ganglion cell specific marker. West-Mays and co-workers (1999) detected AP-2α and AP-2β protein in the developing mouse retina at E15. Positive cells were located in a subset of cells in the future inner nuclear layer (INL, Fig. 1-2). Later in development (postnatal day 14), both proteins were expressed in a subpopulation of the ganglion cell layer, in addition to the INL. The retinal defects and the pattern of expression of AP-2 family members in the developing retina led West-Mays and colleagues to propose that AP-2 is necessary for retinal differentiation.

Although *AP-2β* knockout mice have a relatively minor morphological defect compared to the *AP-2α* null mice, it is nevertheless a lethal defect (Moser *et al.*, 1997). Only kidney development seems to be affected by loss of AP-2β. *AP-2β*-null mice die postnatally due to polycystic kidney disease, which presents as small cysts in the medullar and cortical regions. Moser and co-workers (1997) determined that cells of the distal tubules and collecting ducts in *AP-2β*-null mice underwent apoptosis at a significantly higher rate than wildtype mice, as measured by TUNEL staining. Moser *et al.* (1997) therefore suggested that AP-2β suppresses apoptosis in these cells.

1.3.4 Regulation of AP-2

The highly specific mRNA distribution patterns of the AP-2 family members indicates that their expression is tightly regulated. Whether the developmental regulation of AP-2 is exerted at the level of transcriptional initiation or at a later control point (elongation, termination, RNA stability) has not yet been fully determined. Williams *et al.* (1988) made the interesting observation that *AP-2α* mRNA (and protein) levels are transiently induced during retinoic acid-induced differentiation of NT2 cells (a human teratocarcinoma cell line) into neuronal cell-types. *AP-2α* mRNA levels peaked within two days of treatment and declined thereafter. Nuclear run-on experiments indicated that elevated levels of *AP-2α* were due to increased transcriptional activity of the *AP-2α* gene. Since retinoic acid is an important morphogen in many of the tissues that express AP-2α (craniofacial and limb development), this suggests that AP-2α may be involved in this developmental signalling system. The lag time between retinoic acid treatment and induction of AP-2α led Williams *et al.* (1988) to postulate that AP-2α induction is a secondary effect, induced by an intermediary transcription factor whose transcription is retinoic acid sensitive. In contrast, AP-2γ transcription appears to be directly responsive to retinoic acid. Philipp *et al.* (1994) determined by nuclear run-on experiments that AP-2γ transcription is rapidly induced in P19 embryonal carcinoma cells treated with retinoic acid, as early as 30 minutes post-induction and reach maximal expression levels within 12 hours. The molecular mechanisms of how retinoic acid upregulates *AP-2α* or γ have yet to be worked out.

Analysis of the *AP-2α* gene has revealed several cis-regulatory elements potentially involved in cell-specific gene expression. Characterization of the human *AP-2α* promoter by Creaser *et al.* (1996) led to the identification of an octamer binding site between residues –53 to –44 bp, which was conserved in the murine and chick *AP-2α* promoters. Mutational analysis of this binding site found it to be crucial for *AP-2α* promoter activity. This site was bound by the ubiquitous Oct-1 POU transcription factor present in HeLa nuclear extracts. This site was also bound by additional unidentified factors present in extracts of PA1 and NT2 teratocarcinoma cell lines.

The human *AP-2α* promoter has another DNA binding site centered at –336 bp (Imhof *et al.*, 1999). This site, called A32, was originally defined as an autoregulatory element, which bound purified AP-2 as detected by footprinting (Bauer *et al.*, 1994; Imhof *et al.*, 1999). Experiments by Imhof and coworkers (1999) have shown that the A32 site is actually a composite binding site. Two zinc finger proteins were isolated that also bind this region by expression cloning from a brain cDNA library: BTEB-1 and AP-2rep. BTEB-1 was previously identified as a transcription factor that bound to a site within the cytochrome P-450IA1 gene and repressed its transcription (Imataka *et al.*, 1992). Although widely expressed, BTEB-1 mRNA is preferentially translated in brain and neuroblasts (Imataka *et al.*, 1994). BTEB-1 has a positive effect on AP-2 transcription since co-transfection of a BTEB-1 expression vector with an AP-2 reporter in a variety of cell lines resulted in six-fold increased promoter activity (Imhof *et al.*, 1999). The other factor binding to the A32 element was a novel factor, AP-2rep. Open reading frame analysis of AP-2rep identified a 402 amino acid zinc finger protein with similarity to the wt-1/egr transcription factor family. AP-2rep appears to function as a

negative regulator of AP-2α transcription as its over-expression down-regulates expression of both a co-transfected *AP-2α* promoter reporter and the endogenous *AP-2α* gene. The results of Imhof and coworkers (1999) suggest a model where the opposing activities of AP-2, BTEB-1 and AP-2rep provide spatial and temporal control of AP-2α expression via the A32 element. Detailed comparison of the expression patterns of AP-2α, BTEB-1 and AP-2rep by immunohistochemistry and/or *in situ* hybridization are needed to assess this model. In this regard, comparison of the factors bound to the A32 element in AP-2α expressing and non-expressing cell lines or tissues might also be informative.

AP-2 expression may also be regulated at the post-transcriptional level. A number of alternatively spliced forms of *AP-2α* have been isolated which may provide an additional level of *AP-2* gene regulation. One such splice variant, called *AP-2α*B, appears to interfere with AP-2 mediated transcriptional activation (Buettner *et al.*, 1993). AP-2αB is generated from a transcript that fails to splice out the fifth intron and as a result lacks the C-terminal dimerization domain encoded by exons 6 and 7. The C-terminal 70 amino acids of AP-2αB are encoded by the upstream end of intron 5 and do not appear to possess homology to any known protein in the GenBank database. The mechanism whereby AP-2αB interferes with AP-2αA-mediated trans-activation is unclear. Conflicting results as to whether AP-2αA and AP-2αB can physically interact make it difficult to evaluate the hypothesis put forth by Buettner and co-workers (1993) that indirect interaction via an unknown factor interferes with transactivation.

Meier and coworkers (1995) identified three additional AP-2α splice variants expressed during mouse embryogenesis. In this paper, the AP-2αA encoding transcript is

called variant 1. Variant 2 lacks exon 2, which encodes the AP-2α activation domain. This variant could therefore potentially function as a dominant negative isoform by interfering with the trans-activating activity of the other isoforms. Splice variants 3 and 4 result from the use of alternative first exons located within the first intron. Variants 1 and 2 use exon 1a, variant 3 uses 1b and variant 4 uses exon 1c. Exons 1a-c encode three different N-terminal ends in frame with exon 2. The functional significance of these differences is not known. Exons 1a, 1b and 1c appear to have their own separate promoters, which would allow for complex transcriptional control mechanisms (Meier *et al.*, 1995). Unique 5' untranslated regions and alternative N-terminal peptide sequences could provide an additional level of control.

1.4 Nuclear Factor I (NFI)

1.4.1 History

CCAAT transcription factor/nuclear factor I (CTF/NFI) is a family of proteins involved in transcriptional regulation and viral DNA replication. Nuclear factor I was originally isolated as a cellular factor that enhanced the initiation of adenoviral DNA replication (Nagata *et al.*, 1982). A necessary step in adenovirus replication is the covalent linking of dCMP to the adenoviral terminal protein (pTP), a virally encoded protein that serves as the primer for viral DNA replication. Nagata and coworkers (1982) purified NFI from HeLa nuclear extracts by conventional chromatography based on its ability to enhance this conjugation reaction. Experiments performed by Jones *et al.*. (1987) later determined that NFI was identical to CTF, a set of proteins involved in transcriptional regulation of the HSV thymidine kinase promoter. Direct comparison of

NFI and CTF preparations found them to be indistinguishable in terms of their peptide composition, DNA replication and transcriptional regulatory activity.

NFI consists of a number of proteins between 52-66 kDa, which bind with high affinity to the sequence TGGA/C(N_5)GCCAA (Rosenfeld and Kelly, 1986). Cloning of vertebrate NFI-encoding cDNAs and genomic sequences has revealed at least four closely related genes (*NFI-A*, *-B*, *-C*, and *–X*). Multiple alternatively spliced mRNA are derived from each gene. Santoro *et al.* (1988) were the first to clone NFI cDNAs. Using a synthetic oligonucleotide derived from purified CTF/NFI amino acid sequence, three related NFI-C cDNAs (called *CTF1*, *CTF2*, and *CTF3*) were isolated from a HeLa cDNA library. Comparison of these cDNAs revealed that they were alternatively spliced versions of the same gene (*NFI-C*). Functional analysis of the encoded proteins, showed that each was competent in sequence specific DNA binding and trans-activation (Santoro *et al.*, 1988; Mermod *et al.*, 1989). *CTF1* and *CTF2* (*CTF3* was not tested) also enhanced the coupling of dCMP to pTP and therefore possessed viral replication enhancing activity. Subsequent isolation of related cDNAs have identified at least three additional differentially spliced NFI-C cDNA (*CTF5-7*, Altmann *et al.*, 1994; Wenzelides *et al.*, 1996). Analysis of the transcripts produced from the other three known NFI genes (*NFI-A*, *-B* and *-X*) has revealed similarly complex differential splicing (Inoue *et al.*, 1990; Liu *et al.*, 1997; Apt *et al.*, 1994).

Some NFI splice forms have been shown to function as repressors. For instance, the NFI-B3 splice variant codes for the DNA binding and dimerization regions of NFI but lacks the trans-activating function (Liu *et al.*, 1997). By itself, NFI-B3 protein does not bind to DNA and has no effect on a reporter gene under the control of an NFI binding

site. However, when coexpressed with other NFI family members, NFI-B3 interferes with trans-activation. This suggests that NFI-B3 may interfere with NFI-mediated trans-activation by non-productive heterodimer formation and in this manner, regulate the activity of the other isoforms.

1.4.2 Structure/Function Studies

DNA binding

A number of studies have been performed to determine which portions of the NFI protein is involved in DNA binding site recognition. Isolation of NFI-C cDNAs allowed Mermod and coworkers (1989) to over-express different parts of the NFI protein in bacteria and assay the DNA binding activity of each. They found that the N-terminal 220 amino acids were sufficient to bind DNA, whereas the N-terminal 150 amino acids were not. Similarly, Meisterernst *et al.* (1989) assayed the activity of *in vitro* translated pig NFI/CTF-2 and found that while residues 11-257 were active in DNA binding, residues 11 to 209 were only partially active and residues 11 to 175 were inactive. Removal of as little as the first 33 amino acids (i.e. residues 1-33) also prevented DNA interaction (Mermod *et al.*, 1989). Gounari *et al.* (1990) found that NFI-A constructs with internal deletions removing residues 24-33 or 24-53 were inactive; however, removal of only the first 11 amino acids of NFI-A had no effect on DNA binding activity. This N-terminal DNA binding domain is the most highly conserved region amongst the NFI family. Considering that the NFI proteins from different genes all bind to the same recognition sequence, this is perhaps not unexpected.

Additional work detailing the workings of the DNA binding domain has been performed by Dekker and coworkers (1996). Through further deletion mapping of the N-terminal 1-236 residue region of rat NFI, they found that the 1-78 region bound DNA non-specifically while the 1-182 and 75-236 regions bound DNA specifically, albeit with low affinity. They propose that NFI has two DNA binding subdomains: a highly basic region at the N-terminus (residues 1-78) which provides low-specificity interaction with the DNA and a second subdomain located between residues 75-182 that provides binding site specificity. In concert, these regions would work together to form the DNA binding domain of NFI. The 75-182 region contains residues important for DNA binding based on analysis of point mutations of human NFI-C [cysteines 99, 115, 152, 158 (Novak *et al.*, 1992) and leucine 112 and arginine 113 (Armentero *et al.*, 1994)]. Based on circular dichroism measurements, the 1-78 region is predicted to be predominantly α-helical. However, the mechanism of binding to DNA remains elusive and awaits more detailed structural studies of the NFI DNA binding domain.

Dimerization

Like most known transcription factors, NFI binds to DNA and exists in solution as a dimer. The region of NFI responsible for dimerization has been localized to the N-terminal half of the protein. Using *in vitro* transcribed/translated NFI, Mermod and colleagues (1989) found that the N-terminal 220 amino acids of NFI (CTF-1ΔC220) is sufficient for dimerization. Mobility shift assay gels showed that when expressed together, CTF-1 and CTF-1ΔC220 appeared to form a heterodimer of intermediate mobility compared to that found when they were expressed separately and then mixed.

Another explanation for this observation is that the two monomers can indirectly interact by binding separately to adjacent sites on the same DNA molecule. To rule this possibility out, glutaraldehyde crosslinking experiments were performed which indicated that CTF-1 and CTF-1ΔC220 exist as dimers even in the absence of DNA (Mermod *et al.*, 1989). Radiolabeled CTF-1 and CTF-1ΔC220 prepared by *in vitro* translation, were treated with glutaraldehyde followed by denaturing PAGE analysis. Bands corresponding in size to monomers (65 kDa for CTF-1 and 35 kDa for CTF-1ΔC220) and dimers (120 kDa and 60 kDa for CTF-1 and CTF-1ΔC220, respectively) were observed following fluorographic detection.

Chaudhry *et al.* (1998) tested if the trans-activation potential of NFI heterodimers differed from the potential of homodimers by testing their ability to activate a reporter gene. Co-transfection of an NFI-X expression plasmid with increasing amounts of an NFI-C expression plasmid led to intermediate levels of activation compared to that achieved when they were separately transfected (even after taking into account that only 50% will be heterodimers). Similarly, reporter trans-activation observed when NFI-B and NFI-C were co-transfected was intermediate to that obtained when they were expressed separately. The situation *in vivo* is likely to be very complex considering the number of NFI spliceforms isolated and the overlapping expression patterns of the NFI genes.

Activation Domain

NFI is capable of activating and/or repressing the expression of a number of genes. NFI has separable DNA binding and trans-activation domains, in common with many other transcription factors. The NFI trans-activating domain appears to reside in the

C-terminal half of NFI. Deletion analysis of CTF-1 by Mermod and colleagues (1989) localized this activation domain to the C-terminal 100 amino acids. While removal of the C-terminal 62 residues reduced its trans-activating activity, removal of 100 residues eliminated it completely. Domain swap experiments were also used to demonstrate that the C-terminal region of NFI has trans-activating activity. Portions of the CTF coding region, fused to the Sp1 DNA binding domain, were assayed for their transactivation potential using a Sp1 responsive reporter (pUCSVCAT). Fusion of either the 150-486 residue region (C-terminal 337 amino acids) or the 400-486 region (C-terminal 87 amino acids) to the Sp1 DNA binding domain led to strong induction of reporter gene expression. The C-terminal 100 amino acids of CTF do not share homology with any known transcription factor. The striking feature of this region is the prevalence of proline residues (25%), leading Mermod and coworkers (1989) to suggest that it is this aspect of CTF that is responsible for its trans-activation activity. As discussed earlier, this suggestion would be consistent with the findings of Gerber *et al.* (1994) who found that homopolymeric stretches of proline are sufficient to cause trans-activation. However, Altmann and coworkers (1994) suggest the NFI trans-activation is not mediated by the proline rich domain, but rather by two domains (I and II) which flank this region. They base this model on their finding that one of their cDNA isolates, CTF7, is highly active in trans-activation, although it lacks exons 7-9 which encode the proline rich domain. In this model, domain I (encoded by exons 4-6) and domain II (exons 10-11) function separately but synergistically. Their reporter assays indicated that CTF7 is significantly more active than CTF1, which contains all exons including exons 7-9. They argue that the proline rich region is simply a flexible linker connecting domains I and II, such that its removal

would not have a negative effect on trans-activation. The increased activity of CTF7 would be derived from the increased proximity of domains I and II. Interestingly, this proline rich domain is conserved amongst the NFI family.

Enhancement of Adenoviral DNA replication

One of the first steps of adenoviral DNA replication, and the step implicated in NFI mediated enhancement, is the covalent linking of a dCMP moiety to the viral preterminal protein (pTP). To localize the region of NFI responsible for this enhancement, Mermod *et al.* (1989) tested the ability of various CTF-1 truncations to stimulate this covalent attachment *in vitro*. They found that the region responsible for increased pTP-dCMP conjugation mapped within the N-terminal 220 amino acids of CTF-1. The N-terminal 150 amino acids were inactive in this respect, as was a truncated protein removing the N-terminal 68 residues. Thus this activity localized to the region containing the DNA binding and dimerization functions. Using a different assay, Gounari *et al.* (1990) obtained similar results. They found that addition of full length or just the N-terminal 240 amino acids of rat NFI-A leads to increased replication of viral DNA. The assay measured viral DNA replication based on the level of incorporation of radiolabeled nucleotide into purified viral DNA with and without added NFI protein. Stimulation of viral replication is a function conserved in Xenopus NFI-X proteins. Addition of Xenopus NFI-X3 or its N-terminal 209 amino acid derivative (xNFI-X3Δ209) to reaction mixtures increased formation of the pTP-dCMP covalent complex in a concentration dependent manner (Roulet *et al.*, 1995).

1.4.3 Expression of NFI

Initially thought to be ubiquitously and constitutively expressed, certain NFI isoforms are now known to be enriched in select tissues/organs. Chaudhry and coworkers (1997) have compared the expression of all four NFI genes during murine development by *in situ* hybridization. NFI-A, the first NFI to be expressed, is detectable by 9 days postcoitum (dpc) in the heart and brain. None of the other NFIs are detected prior to 10.5 dpc. At 10.5 dpc, cells of the distal tips of the developing limb buds express NFI-A. As well, NFI-B can be detected in the developing lungs and NFI-C can be found around the aortic arches and in the dorsal root ganglia. No NFI-X was detected at 10 dpc. Later in development (11.5 dpc), all four genes are expressed in the mandibular arch, developing lungs and liver. NFI-A, -B, and –C are also found in gut mesenchyme. In the CNS, NFI-A, -B and –X are expressed in the telencephalon and metencephalon. At 14.5 dpc, NFI-A, -B and –X are highly expressed in the cerebral cortex. NFI-C is not expressed in the CNS at this stage. All four genes are expressed in the embryonic lungs at 14.5 dpc, with NFI-B having the highest level of expression. Postnatally, NFI-A and –B are expressed in the white matter of the cerebral cortex, whereas NFI-X is concentrated in the grey matter, suggesting mostly glial and neuronal distributions, respectively. All four genes are expressed in the postnatal cerebellum, likely in granule cells (Chaudhry *et al.*, 1997). NFI-A, -B and –X are expressed in the hippocampal dentate gyrus. Certain tissues such as testes and spleen, never express, or express little of any of the NFI genes. Therefore, the expression of the four NFI genes varies, with some overlap, between different tissues

at various stages of development. These differences in expression may contribute to the cell-specific gene regulation attributed to NFI family members.

1.4.4 Regulation of NFI Activity

NFI activity is regulated at the post-translational level by phosphorylation. Yang *et al.* (1993) were the first to demonstrate that NFI is phosphorylated *in vivo* and that phosphorylation decreased NFI trans-activating activity. Based on the ion exchange column elution profile, they noticed that NFI isolated from actively growing or c-Myc overexpressing 3T3-L1 tissue culture cells was more negatively charged than NFI from quiescent or non-transfected 3T3-L1 cells. Furthermore, they found that this difference could be eliminated by treatment with potato acid phosphatase (PAP), a non-specific phosphatase, indicating that the charge difference was due to differences in phosphorylation. Downregulation of NFI activity by phosphorylation did not appear to be due to reduced DNA binding affinity because phosphorylated and unphosphorylated NFI bound DNA with equal affinity. To determine if phosphorylation had an effect on its trans-activation activity, Yang and coworkers (1993) compared the activity of several NFI-dependent reporter gene constructs in normal and c-Myc over-expressing cell lines. NFI phosphorylation suppressed the activation of several NFI dependent promoters, including the SV40, MoMuLV and pro-α2(I) collagen promoters. To ensure that the reduced activation in the c-Myc over-expressing cells was not due to a general non-specific repression of transcription in these cells, Yang *et al.* (1993) also tested the activity of two promoters which are not regulated by NFI, the human β-actin and argininosuccinate synthetase promoters. There was no difference in the activity of these

promoters in normal and c-Myc over-expressing cells, indicating that the observed suppression of NFI-dependent promoters was not simply part of a global reduction in transcriptional activity.

Cooke and Lane (1999) have recently shown that NFI phosphorylation is inducible by insulin in 3T3-L1 cells. Treatment of these cells with insulin results in a rapid and transient phosphorylation of NFI, as indicated by electrophoretic mobility shift changes in gel retardation assays and Western blotting. These changes precede repression of the GLUT4 gene, a trans-membrane glucose transporter, whose expression is known to be down-regulated in response to insulin. These results indicate a physiological role for NFI phosphorylation as a downstream component of the insulin signaling pathway.

The proteins involved in NFI phosphorylation/dephosphorylation have not been identified. Two kinases have been shown to act on NFI *in vitro*. Jackson *et al.* (1990) have reported that dsDNA-dependent protein kinase (DNA-PK) is able to phosphorylate NFI *in vitro*. Kawamura *et al.* (1993) have noted that CDC-2 kinase is also capable of phosphorylating NFI *in vitro*. Incubation of NFI with CDC2 kinase and [γ-^{32}P]ATP results in the transfer of radiolabeled phosphate to NFI. Additional work is needed to determine if these mechanisms function *in vivo*. Other questions remain, such as which NFI residues are phosphorylated, how this interferes with NFI activity and how the process is regulated.

1.5 Malignant glioma

Brain tumors that are derived from glial cells are called gliomas. Gliomas are named according to their probable cell-type of origin; for instance, ependyomas are

derived from ependymal glial cells lining the ventricles, astrocytomas from astrocytes and oligodendrogliomas from oligodendrocytes. Tumors are graded on a four point scale according to their severity, ranging from the most benign (grade I) to the most malignant (grade IV). Malignant gliomas (MG), the most common type of brain tumor, consist of grade III and IV astrocytomas. Even with the best available treatment (surgery, radiation, and/or chemotherapy), most MG patients succumb to their disease within two years of diagnosis. This dismal prognosis has prompted a great deal of work examining the genetic basis of MG, with significant progress being made in this area.

1.5.1 Common Genetic Losses/Mutations Associated with Gliomas

Many genes and chromosomal regions have been identified that are altered or missing in MG tumors. Restriction fragment length polymorphism (RFLP) analysis of MG DNA has detected loss of heterozygosity (LOH) on chromosomes 10, 11, 13, 17 and 22 (James *et al.*, 1988; Fults *et al.*, 1990, 1992), indicating that numerous genes are likely involved in the initiation and/or progression events of MG. Through karyotypic analysis of malignant glioma tumors, Bigner and coworkers (1988) determined that alterations in chromosomes 9p and 19q were statistically significant events. Located on 9p, is the tumor suppressor, *p16*, a cyclin dependent kinase inhibitor potentially involved in many different tumor types (Kamb *et al.*, 1994). Also on chromosome 9p are the interferon genes, *IFNA* and *IFNB*, which are often deleted in MG cell lines (Miyakoshi *et al.*, 1990). Other tumour suppressors involved in MG include RB, PTEN and p53.

Amplification of the epidermal growth factor receptor (*EGFR*) gene is found in 40-50% of malignant gliomas (Libermann *et al.*, 1985; Wong *et al.*, 1987). EGFR is a

receptor tyrosine kinase involved in the reception of exogenous growth factor signals. Some MG produce a truncated version of EGFR which results in a constitutively active protein (Ekstrand *et al.*, 1992). Other genes amplified in MG tumors include; *α-PDGFR* (Fleming *et al.*, 1992), *MET* (Fischer *et al.*, 1995), *MDM2* (Reifenberger *et al.*, 1993) and *CDK4* (Reifenberger *et al.*, 1994; He *et al.*, 1994).

1.5.2 GFAP and Malignant Glioma

The glial fibrillary acidic protein (GFAP) is an intermediate filament protein specifically expressed in differentiated astrocytes which appears to be involved in the extension of glial processes (Eng, 1980; Linskey and Gilbert, 1995). GFAP is often expressed in primary MG tumors. In a study examining GFAP expression in 336 astrocytomas and glioblastomas, GFAP was expressed in a majority of tumors (Gottschalk and Szymas, 1987). As expected for a differentiation marker, there was an inverse correlation between GFAP levels and the degree of MG anaplasia, with the most benign tumors expressing higher amounts of GFAP than the more malignant tumors (Eng and Rubinstein, 1978; Velasco *et al.*, 1980; van der Meulen *et al.*, 1978). In established cell lines derived from MG, GFAP expression tends to be lost with increased passage number (Bigner *et al.*, 1981), although this isn't always the case since KMU100 still expresses GFAP after ten years in culture and U251, established in 1968, continues to express GFAP (Matsumura and Karamoto, 1994; Godbout *et al.*, 1998). Rutka and Smith (1993) have shown that overexpression of GFAP in GFAP-deficient MG cell lines leads to the extension of glial processes. Conversely, transfection of antisense GFAP into the GFAP positive MG cell line, U251, led to decreased glial process extension, which could

be reversed by overexpression of GFAP (Weinstein *et al.*, 1991; Rutka *et al.*, 1994; Chen and Liem, 1994). The antisense GFAP cells showed increased growth and invasive potential compared to controls; these cells grew to a greater cell density, showed a greater degree of cell piling, had a 2-3-fold increase in proliferation rate and were more proficient in penetrating Matrigel barriers as compared to control cells (Rutka *et al.*, 1994). These results suggest that GFAP is not only a marker for differentiated glial cells, but also plays a role in its establishment. Loss of GFAP expression may therefore represent a progression event from lower to higher grade malignant gliomas (Murphy *et al.*, 1998). Reexpression of GFAP and other genes involved in glial differentiation may represent a novel method of treating gliomas. Reinduction of GFAP expression in GFAP negative glioma tumour cells may force these cells to become more differentiated and less malignant, analagous to Rutka's findings in cultured glioma cells (Rutka and Smith, 1993). Toda and coworkers (1999) compared the growth of GFAP expressing and non-expressing C6 glioma cells in athymic mice and found that GFAP positive tumors grew slower than negative tumors suggesting that GFAP expression has similar effects *in vivo*. Differentiation therapy, although in its infancy, appears to be a promising approach for the treatment of malignant gliomas.

1.6 Research Objectives

Chapter 2. Involvement of AP-2 in the Regulation of the *R-FABP* Gene in the Developing Chick Retina

Little is known regarding the molecular pathways that underlie the retinal maturation process. We are studying the regulation of the retinal fatty acid binding protein (*R-FABP*) gene, highly expressed in retinal precursor cells, to identify DNA regulatory elements and transcriptional factors involved in retinal development. Although the upstream sequence of the *R-FABP* gene is extremely GC-rich, CpG methylation in this region is not implicated in the regulation of this gene because the 5' flanking DNA remains unmethylated with tissue differentiation when there is a dramatic decrease in *R-FABP* transcript levels. Using a combination of DNase I hypersensitivity experiments, gel shift assays and DNase I footprinting, we have found three sites of DNA/protein interaction within 205 bp of 5' flanking DNA in the undifferentiated retina and four sites in the differentiated retina. DNA transfection analysis indicates that the first two footprints located within 150 bp of 5' flanking DNA are required for high levels of transcription in primary undifferentiated retinal cultures. The first footprint includes a putative TATA box and Sp1 binding sites while the second footprint contains a consensus AP-2 DNA binding site. Supershift experiments using antibodies to AP-2 and methylation interference experiments indicate that an AP-2-like transcription factor present in both late proliferative stage retina and differentiated retina binds to the upstream region of the *R-FABP* gene. A combination of data including the expression profile of AP-2 during retinal development and DNA transfection analysis using constructs mutated at critical residues within the AP-2 binding site suggest that AP-2 is a repressor of *R-FABP* transcription.

Chapter 3. Differential Expression of AP-2α and AP-2β in the Developing Chick Retina: Repression of *R-FABP* Promoter Activity by AP-2

Retinal fatty acid binding protein (R-FABP) is the avian counterpart of murine brain FABP implicated in glial cell differentiation and neuronal cell migration. R-FABP is highly expressed in the undifferentiated retina and brain of chick embryos. We have previously shown by *in vitro* studies that the AP-2 transcription factor binds to a consensus AP-2 binding site in the *R-FABP* promoter region. Based on the expression pattern of AP-2 in the developing retina and on mutational analysis of the AP-2 binding site in DNA transfection experiments, we proposed that AP-2 could be involved in the down-regulation of *R-FABP* transcription. Here, we describe the cDNA isolation of two members of the AP-2 family expressed in the chick retina, AP-2α and AP-2β. We show that *R-FABP* mRNA and the AP-2 factors are expressed in mutually exclusive patterns in the differentiating retina: whereas AP-2α and AP-2β are selectively expressed either in amacrine or in amacrine and horizontal cells, respectively, *R-FABP* mRNA is found in Müller glial cells and/or bipolar cells. Furthermore, a decrease in *R-FABP*-dependent expression is obtained upon co-transfection of primary retinal cultures with AP-2 expression vectors and a CAT reporter construct. The early and cell-specific expression of AP-2α and AP-2β in the developing retina suggest a role for this transcription factor family in the early steps of amacrine and horizontal cell differentiation. Repression of the *R-FABP* gene in these cells may be an important component of their developmental program.

Chapter 4. Regulation of B-FABP Expression by Differential Phosphorylation of Nuclear Factor I in Malignant Glioma

The human brain-fatty acid binding protein (B-FABP), postulated to be a glial differentiation factor, is expressed in radial glial cells of the developing brain, as well as in a subset of malignant glioma (MG) cell lines. We are studying the regulation of the human *B-FABP* gene in MG cell lines to determine how its cell-specific expression is achieved. By *in vitro* DNA binding assays, we have identified two nuclear factor I (NFI) binding sites within the *B-FABP* proximal promoter. Footprint 1 (fp1) is located in the -35 to -58 base pair (bp) region adjacent to the TATA box (–22 to –28 bp) while footprint 3 (fp3) is located further upstream, spanning the -237 to –260 bp region. Using the mobility shift assay, we show that factors binding to fp1 and fp3 are specifically competed by an NFI consensus oligonucleotide and are supershifted by anti-NFI antibodies. By reporter gene assays, we find that the fp1 NFI site is necessary but not sufficient for *B-FABP* promoter activity in a MG cell line, while the fp3 NFI binding site appears to be dispensable *in vitro*. The NFI family has been shown to mediate cell-specific regulation of a number of genes. We find that the NFI proteins in B-FABP positive and negative cell lines are differentially phosphorylated, a modification previously shown to inhibit NFI-mediated transcriptional activation. Nuclear extracts of B-FABP positive cells contain a phosphatase activity, absent from negative cell lines. which may account for this differential phosphorylation.

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Chapter 2

Involvement of AP-2 in the regulation of the *R-FABP* gene in the developing chick retina[1]

(short title - *R-FABP* gene regulation in the developing retina)

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2.1 Introduction

The fatty acid binding protein (FABP) family consists of a number of structurally related proteins with characteristic cellular, tissue and developmental distribution patterns. Members of this family include heart FABP, intestinal FABP, adipocyte lipid binding protein (ALBP or aP_2), myelin P_2, cellular retinoic acid binding proteins (CRABP) and cellular retinol binding proteins (CRBP) (1,51). Some FABPs, such as adipocyte FABP and myelin P_2, are restricted to one tissue/organ type while others are more broadly expressed. For example, intestinal FABP is found both in intestine and stomach while liver FABP is present in liver, intestine, kidney and stomach (rev. in 51). Roles proposed for these proteins include the uptake, intracellular solubilization, storage and/or delivery of fatty acids and retinoids (1). A role in signal transduction has also been proposed for heart FABP and ALBP via phosphorylation of a tyrosine residue at position 19 by the insulin receptor (8,41). Furthermore, mammary derived growth inhibitor [also called heart FABP (46)] and liver FABP appear to be involved in the differentiation of mammary epithelial cells and in the control of hepatocyte cell proliferation, respectively (35,36,60). Some FABPs are located in both the nucleus and cytoplasm, suggesting that they may play a role in carrying hydrophobic ligands to the nucleus (6,7). Meunier-Durmort *et al.* (38) have recently shown that long chain fatty acids are strong inducers of L-FABP gene expression.

The presence of a mammalian brain FABP was first reported in 1984 (2,4) and the murine gene cloned in 1994 (18,37). The mouse brain FABP (B-FABP or BLBP) has 67% amino acid identity with murine heart FABP and a lower level of homology with other members of the family (37). B-FABP expression correlates with neuronal differentiation in the mouse CNS where the protein is primarily expressed in radial glial cells. Antibodies to

B-FABP prevent both neuronal and glial cell differentiation of primary cerebellar cells *in vitro*. In these cultures, the extension of the radial glial processes is blocked as well as the migration of neuronal cells along the processes (18). Based on these observations, it was proposed that B-FABP is required for the establishment of the radial glial cell fiber system required for the migration of immature neurons in the developing nervous system. Using transgenic mice, Feng and Heintz (19) identified a radial glial cell-specific element located between 300 and 800 bp upstream of the *B-FABP* transcription initiation site.

We have identified a FABP transcript that is highly expressed in the retina and brain of the developing chick embryo (24). The predicted amino acid sequence of R (retina)-FABP is 85% identical to that of the mouse B-FABP. Homology to other members of the FABP family is considerably lower (e.g. 70% identity to bovine heart FABP, 59% to bovine myelin P_2, 31% to rat intestinal FABP). Interestingly, B-FABP was not detected in the retina of the developing mouse embryo (37). We have found *R-FABP* to be highly expressed from day 3 (d3) (the earliest stage tested) to d7 in the chick retina as well as from d5 to d19 in the chick brain (24,26), suggesting a role in chick retinal development/maturation in addition to the roles proposed for B-FABP in the brain.

Retinal development in the chick is divided into three overlapping stages: (i) cell proliferation and migration which occurs primarily from d2 to d8 of incubation, (ii) readjustment to the proper layer (d8-10), (iii) expression of differentiated properties (d11 to hatching at d21). At d3, the majority of cells in the chick retina are proliferating multipotential neuroectodermal cells that can differentiate into the six major classes of neuronal cells (photoreceptors, ganglion, bipolar, amacrine, interplexiform, horizontal) and one class of glial cells (Müller) that form the mature retina (49,50,53,55,56). At d7, 60% of

the cells are proliferating, although the retina has differentiated to some extent and the innermost nerve fiber layer and ganglion cell layer are readily apparent (16,44). By d9, only 10% of the cells are undergoing cell division (16). In the differentiated d19 retina, *R-FABP* mRNA levels are barely detectable, having undergone a 50-100X reduction compared to d3.5 (24,26).

Here, we study the regulation of the *R-FABP* gene in order to gain insight into the molecular mechanisms that underlie retinal development. We have identified four DNA elements in the 5' flanking region of the *R-FABP* gene that bind nuclear proteins. One of these elements contains a binding site for AP-2, a retinoic-acid-regulated transcriptional factor that plays an important role in the regulation of gene expression during mammalian embryogenesis.

2.2 Materials and Methods

2.2.1 Primer Extension Analysis

Poly(A)$^+$ RNA was isolated from d5 retina, d16 retina and d16 heart as described previously (24). The 20 nt primer 5'-GTTGTGGCTGTCCGCCAGCT-3' corresponding to positions 38 to 57 bp of the *R-FABP* cDNA previously described (24) was end-labelled with γ^{32}P-ATP (3000 Ci/mmol) and T4 polynucleotide kinase. End-labelled primer was annealed to poly(A)$^+$ RNA at 42°C for 90 min and the cDNA extended at 50°C for 30 min using AMV reverse transcriptase, following the protocols supplied by Promega. Samples were run on a 8% polyacrylamide gel containing 7M urea in 1X TBE buffer. Marker lanes consisted of either labelled sequencing reactions or end-labelled ϕX174 phage digested with HinfI.

2.2.2 DNase I Hypersensitivity Analysis

Procedures related to DNase I hypersensitivity experiments were as described (27). Briefly, nuclei were isolated from 4 dozen d7 chick retinas by homogenization in cold 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.5 M sucrose, 0.15 mM spermine, 0.5 mM spermidine and layering on a 0.88 M sucrose cushion in homogenization buffer. The nuclei were spun through the cushion at 5000 rpm for 5 min in a HB-4 rotor. The nuclei were resuspended in 10 mM Tris-HCl pH 7.5, 2.5 mM $MgCl_2$, and 0.25 M sucrose. DNase I (Worthington) was added to aliquots of nuclei to final concentrations of 0.1, 1, 2.5, 5, 10 and 25 μg/ml and incubated at 37°C for 10 min. An undigested control was included with each experiment. Digestion was stopped by adding two volumes of 10 mM Tris-HCl pH 7.5, 1% SDS, 5 mM EDTA, and 50 μg/ml proteinase K (Boehringer Mannheim). Samples were incubated

overnight at 37°C, extracted with chloroform and phenol, and precipitated in ethanol. The purified DNAs (10 μg for each time point) were then digested with appropriate restriction enzymes and electrophoresed in a 1% agarose gel in 40 mM Tris-acetate and 1 mM EDTA (pH 7.2). The DNA was transferred to nitrocellulose and hybridized with probes a, b and c (described in figure legend) labeled by nick-translation.

2.2.3 DNA Transfection Analysis

R-FABP 5' flanking DNA fragments of different lengths were ligated to the 5' end of the *CAT* gene in the pCAT basic vector (Promega): the -3.5 kb construct contains a 3562 bp fragment from -3545 (BanI) to +17 (BanI), the -234 bp construct contains a 245 fragment from -234 (PvuII) to +11 (RsaI), the -135 bp construct contains a 145 bp fragment from -135 (SstII) to +11 (RsaI), the -51 bp construct contains a 61 bp fragment from -51 (NaeI) to +11 (RsaI). The -65 bp construct was obtained by ligating a PCR generated fragment from -65 to +11 bp into the pCAT basic vector. To test the activity of the AP-2 binding site 5'-**GCC**GTG**GGC**-3' (conserved residues in bold), we generated two mutant constructs with base pair substitutions in the residues required for the binding of AP-2. In mutant 1, the AP-2 site (located at -75 to -67 bp) was mutated to G<u>TT</u>GTGGGC by PCR amplification of a DNA fragment spanning the AP-2 site using a primer containing the two mutated bases indicated by the underline 5'-CGTTG<u>TT</u>GTGGGCGGCTCCCCTC CC-3'. In mutant 2, the AP-2 site was mutated to G<u>TT</u>GTG<u>TT</u>C using the following primer 5'-CGTTG<u>TT</u>GTG<u>TT</u>CGGCTCCCCTCCC-3' and mutant 1 DNA as the template DNA. Both mutant fragments were introduced into the -135 bp construct. All constructs were sequenced to ensure that only the intended mutations were introduced. Transient expression

assays were as described by Graham and van der Eb (29) and Godbout *et al.* (25). To prepare primary retinal cultures, d5 retinas were dissected using an inverted microscope and digested with trypsin at 37°C for 5 min. The equivalent of three to four retinas/100 mm plate were cultured in Dulbecco Modified Eagle Medium (DMEM) plus 10% fetal calf serum. The cells were transfected when approximately one-third of the plate surface was covered (6-7 days after plating). For each transfection, five μg of CAT vector DNA and five μg of pSV-β-galactosidase control vector (internal control) (Promega) were precipitated in calcium phosphate and left on the cells for 16 hours. The cells were harvested 48 to 50 hours later. At this time, the plate was confluent or almost confluent. Cell extracts were prepared using a freeze/thaw protocol provided by Promega and CAT activity measured using ^{14}C-chloramphenicol in the presence of n-butyryl coenzyme A. Samples were assayed by both liquid scintillation counting and thin layer chromatography. β-galactosidase activity was measured in 96 well plates using a plate reader (405 nm) and ONPG (o-nitrophenyl-β-D-galactopyranoside) as the substrate. As an additional control for transfection efficiency, Hirt DNA was prepared and analysed by Southern blotting using CAT vector DNA as the probe (31).

2.2.4 CpG Methylation Analysis

Genomic DNA was prepared as described by Sambrook *et al.* (45). Cytosine deamination by bisulphite treatment and PCR amplification was according to Frommer *et al.* (20) as modified by Clark *et al.* (13). Briefly, ten μg genomic DNA from d5 chick retina, d16 chick retina and adult chicken liver were digested with BamH1, denatured with sodium hydroxide and treated with sodium bisulphite and hydroquinone for 16 hours at 55°C. The

free bisulphite was removed using a desalting column (Promega Magic DNA Clean-Up System). The DNA was treated with sodium hydroxide, neutralized with ammonium acetate, ethanol precipitated and resuspended in 100 µl TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). PCR amplification was carried out using Taq1 polymerase (Stratagene) and the following primers -- primer 1 5'-TAAAGTTGTGGTTGTT(T/C)G TTAG-3' from positions +60 to +81 where (T/C) represents the location of a C that could potentially be methylated, and primer 2 5'-CACTAAAAATTACCTTCACTTAACC-3' from positions -442 to -418. The PCR products were re-amplified using a nested primer (primer 3) from positions -355 to -335 (5'-CTCCCAAAAACCTTATCCTAC-3') and primer 1. The amplified DNA was ligated into pBluescript with a T overhang (33) and transformed into XL1-Blue. DNA inserts were sequenced using T7 DNA polymerase (Pharmacia) and the M13 forward and reverse primers.

2.2.5 DNA/Protein Binding Assays

Nuclear extracts were prepared from d7 retina and d16 retina as previously described (28,52). Protein concentrations were measured using the Bradford assay (BioRad).

Footprinting analysis. The 245 bp Pvu11/Rsa1 *R-FABP* genomic DNA fragment from -234 to +11 bp was ligated into the Sma1 site of the pBluescript vector. The top and bottom strands were separately labelled by either digesting the construct with Xba1 or Hind111, filling-in with Klenow polymerase and ^{32}P-dCTP, and cutting out the insert with either Hind111 or Xba1. The labelled DNAs were purified on a polyacrylamide gel. Procedures related to DNase I footprinting analysis were as described except that no polyvinyl alcohol was added (34). Briefly, 10 fmol of DNA was combined with 10 to 20 µg

nuclear extract and incubated in 50 µl binding buffer containing 0.25 µg poly(dI-dC) for 15 min on ice, followed by 2 min at room temperature. An equal volume of start buffer containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ was added and the mixture digested with 4 to 6 µl of a 5 µg/ml solution of DNase I (Worthington). The reaction was stopped after 1 min. Samples were purified by phenol/chloroform extraction and ethanol precipitation and resolved on a 7% polyacrylamide sequencing gel. As a negative control, the DNA without nuclear extract was digested with DNase I. The G+A sequencing ladder was prepared according to Belikov and Weislander (3).

Electrophoretic mobility shift assay. The Nae1/Eag1 fragment from -52 to -90 bp (containing footprint II) was labelled by filling-in at the Eag1 site using Klenow polymerase. EMSA were performed using 0.025 picomole of labelled DNA in a final volume of 20 µl containing 2 µg poly(dI-dC) and 15 µg nuclear extract (42). The following DNA fragments (100X molar excess) were used as competitors: the Eag1/Nae1 fragment from -9 to -51 bp that includes footprint 1, the -52 to -90 bp Nae1/Eag1 fragment that includes footprint II, and three double-stranded consensus oligonucleotides to the Sp1, AP-2 and CTF/NF1 transcription factors (Promega). The -52 to -90 bp Nae1/Eag1 fragment containing the two base pair substitutions in the AP-2 site (described as mutant 1 in the DNA transfection section) was tested for its ability to bind nuclear proteins using the same conditions as the wild-type Nae1/Eag1 fragment. For the supershift experiments, we used 2 µl of either anti-human AP-2 antibody (Santa Cruz Biotechnology) or anti-human TFIID [TATA box binding protein (TBP)] antibody (Promega) using the protocol supplied by Santa Cruz Biotechnology. Anti-human NF1 antibody (Santa Cruz Biotechnology) was also used in some experiments.

Methylation interference assay. The noncoding strand of the -52 to -90 bp NaeI/EagI DNA fragment and the coding strand of the -9 to -90 EagI/EagI DNA fragment were individually end-labelled with ^{32}P-dCTP using Klenow polymerase. The labelled DNA fragments were partially methylated at G and A residues with dimethyl sulfate as described (21). Binding reactions were carried out as described for the EMSA using a five-fold scale-up. Free and bound molecules were separated on a 6% 0.5X TBE polyacrylamide gel, eluted from the gel slices by soaking in 0.2 M NaCl, 20 mM EDTA pH 8.0, 1% SDS, 1 mg/ml tRNA and cleaved in 10% piperidine at 90°C for 30 min. Residual piperidine was removed by lyophilization. The cleavage products were analysed on either a 8% or a 12% denaturing polyacrylamide gel.

2.2.6 Northern and Western Blot Analyses

Poly(A)$^+$ RNA was isolated from d5, d7, d10 and d16 retina as described earlier (24). The RNA was transferred to a nitrocellulose filter, hybridized to a 1.5 kb *AP-2* cDNA isolated from a human adult retina cDNA library (Genome Systems Inc., clone 362684, accession numbers AA018570 and AA018571), and washed at 45°C in 0.1X SSC and 0.1% SDS. The ends of the *AP-2* cDNA were sequenced to ensure that it was the correct clone. The filter was hybridized to mouse α-actin cDNA to control for lane to lane variation in RNA levels.

To analyse levels of AP-2 in nuclear extracts, proteins were electrophoresed in a 8% polyacrylamide-SDS gel and electroblotted onto nitrocellulose. The filter was incubated with a 1:1000 dilution of anti-human AP-2 antibody (Santa Cruz Biotechnology). Antigen-antibody interactions were visualized with the ECL Western blotting analysis system

(Amersham) using a 1:100,000 dilution of peroxidase-linked secondary anti-rabbit IgG antibody (Jackson Immuno Research Laboratories).

2.3 Results

2.3.1 Structure of the *R-FABP* Gene

The 709 bp *R-FABP* cDNA isolated from a d3.5 chick retina cDNA library was used to probe a chicken genomic library. Three independent bacteriophages were isolated: GC3-1 with an insert of ~15 kb, GC3-4 (~17 kb) and GC3-5 (~14 kb). Restriction enzyme digestions and partial sequence analyses of the inserts contained in these three bacteriophages generated the physical map shown in Fig. 2-1. The *R-FABP* gene is contained entirely within a 3.5 kb DNA fragment. GC3-1 contains ~14 kb of 5' flanking DNA while GC3-4 contains ~14 kb of 3' flanking DNA. A middle repetitive sequence element, CR1, is located ~4 kb upstream of the *R-FABP* gene. Another repetitive element, opa, is located ~1 kb upstream of the gene. Opa repeats are sometimes translated, forming long tracts of polyglutamine (54). Northern blot analysis of poly(A)$^+$ RNAs isolated from chick tissues (including retina) using a DNA fragment containing the opa repetitive element as the probe suggest that this opa repeat is not transcribed in the chick (data not shown).

The structure of the chick *R-FABP* gene is similar to that reported for mammalian *FABP* genes with exons 1 to 4 encoding aa 1-24, aa 25-82, aa 83-116 and aa 117-132, respectively. The fourth exon is the longest, consisting of 352 bp, 301 bp of which represent the 3' untranslated region. Donor and acceptor splice sites conform to the GT/AG rule.

2.3.2 Identification of the 5' End of the R-FABP Transcript

The *R-FABP* cDNA isolated from a chick retina library contains 9 bp of 5' untranslated sequence. To determine the exact site of transcription initiation, primer extension analysis was carried out using a 20 nt oligomer corresponding to positions 38 to

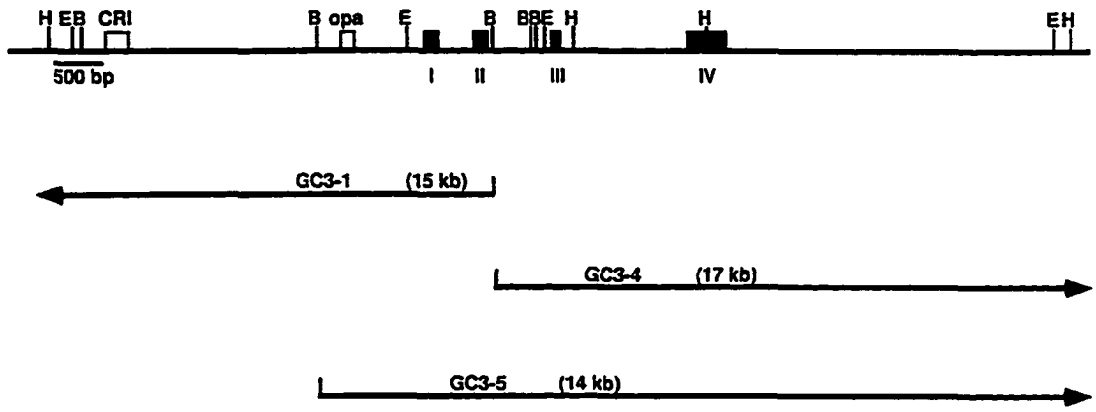


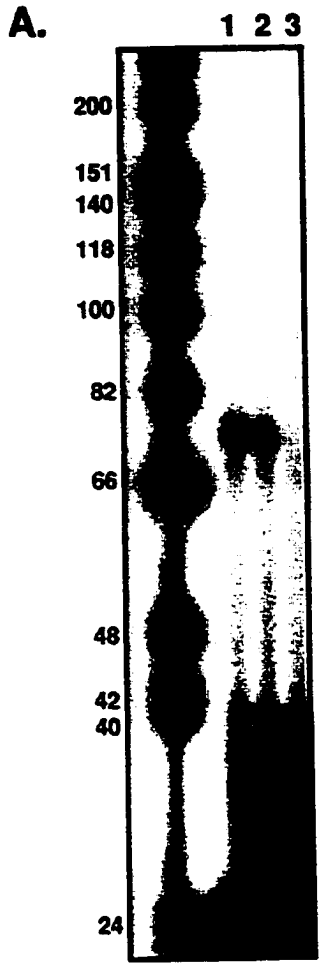
Figure 2-1. Map of the *R-FABP* gene. The three bacteriophages GC3-1, GC3-4 and GC3-5 were selected by screening a chicken genomic library with *R-FABP* cDNA. The size of the genomic DNA insert is indicated in parentheses. The four exons are represented by black boxes while the open boxes represent the location of CRI and opa repetitive elements. Sites recognized by the following restriction enzymes are indicated: H (Hindlll), E (EcoRl), B (BamHl).

57 of the cDNA described earlier (24). Poly(A)$^+$ RNA was isolated from d5 retina and d16 brain, both of which express elevated levels of *R-FABP* transcript and from d16 heart which expresses no (or undetectable) *R-FABP* mRNA. Extended products of ~75 nt were observed in the d5 retina and d16 brain but not in the d16 heart (Fig. 2-2A). To determine the exact site of transcription initiation, the same reaction mixtures were run on a 8% denaturing polyacrylamide gel alongside previously analysed sequencing reactions. Three major extended products of 75, 76 and 77 nt were identified. The transcription initiation sites of *R-FABP* mRNA are therefore located 18-20 nt upstream of the 5' end of the longest cDNA isolated from the chick retina library (Fig. 2-2B). The 5' most nucleotide is designated +1.

A putative TATA box is located 30 bp upstream of the transcription initiation site. However, as shown in Fig. 2-2B, the classic TATAAA sequence is replaced by TTTAAA in the *R-FABP* gene. In 97% of TATA boxes, an "A" is found in the second position. A "T" in this position has only been reported in 2% of TATA box-containing genes. It is therefore not clear whether this sequence represents a bona fide TATA box. A notable feature of the sequence immediately upstream of the transcription initiation site is its extremely high GC content. Over a 200 bp sequence, 162 bp (81%) are G's or C's. These are indicated in bold in Fig. 2-2B.

2.3.3 Localization of DNase I Hypersensitive (HS) Sites Flanking the *R-FABP* Gene

An efficient way to scan relatively large regions of DNA for the presence of regulatory elements is to treat nuclei with increasing concentrations of DNase I and to analyse the digested DNA by Southern blotting. This technique has been used to identify



B.

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                                                  TGATGCCGCCCCGCGAGCGC    -200
CCCAACACCGCCGGCCCCACCAATCCCCGCGCAGCGCGGTTGGCCGCGGGGCGGTGAGAG    -180
CCCTCCCGGCGGGGCGGGCAGACTCCGGCCGCCGTCCCGCCCGTTGCCGTGGGCGGCTCC    -120
CCTCCCGCCGGCCCCCCGCCGGGCGCGGGCTTTAAATGCGGGGCGGTGAGGCGGCCGTGG     -60

***
CGTTTCCCCGTACTCCGGCACCCGCTGCCATGGTTGAGGCTTTCTGCGCGACGTGGAAGC      +1
TGGCGGACAGCCACAACTTTGACGAATACATGAAGGCGCTGG                        +61
```

Figure 2-2. Identification of the 5' end of the *R-FABP* transcript. (A) Primer extension analysis was carried out using a 20 nt primer (5'-GTTGTGGCTGTCCGCCAGCT-3') corresponding to positions 38 to 57 of the *R-FABP* cDNA described in Godbout (24). Poly(A)$^+$ RNAs from d5 retina (lane 1), d16 brain (lane 2) and d16 heart (lane 3) were incubated with the 5' end-labelled antisense primer and the cDNA extended using reverse transcriptase. The cDNA fragments were resolved on a 8% denaturing polyacrylamide gel. Size markers (nt) are indicated on the side. The band at ~75 nt represents the longest cDNA product and is specific to the tissues that express *R-FABP* mRNA. (B) Partial sequence of *R-FABP* gene in the vicinity of the transcription initiation site. The start methionine is underlined and the three sites of transcription initiation are indicated by the asterisks. The G's and C's in the 200 bp 5' flanking DNA are in bold.

both proximal and distal regulatory elements in a large number of genes (30,48,59). Nuclei from d7 chick retina were digested with 0.1, 1, 2.5, 5, 10 and 25 μg/ml DNase I. Genomic DNA was isolated from each nuclei preparation and digested with Hind111, run on a 1% agarose gel and transferred to nitrocellulose. Duplicate blots were hybridized with a 500 bp Hind111/Bgl1 DNA fragment (probe a) from the 5' end of the *R-FABP* gene (Figs. 2-1 and 2-3A,C) and with a 500 bp Sca1/Xho1 fragment (probe b) located between exons 1 and 2 (Figs. 2-3B,C). Using probe a, the expected 6 kb fragment representing intact genomic DNA was obtained as well as a ~4.2 kb fragment that increased in intensity with increasing DNase I concentration. Probe b hybridized to the intact 6 kb genomic DNA fragment as well as to a ~1.8 kb fragment that increased in intensity in a DNase I-dependent manner. Both HS sites correspond to the same location at the 5' end of the *R-FABP* gene, immediately upstream of the transcription initiation site in the GC-rich region shown in Fig. 2-2. This site is indicated by a large asterisk in Fig. 2-3. A weak HS site at ~4.4 kb was also detected when the filter was hybridized with probe b. This HS site is indicated by the small asterisk in Fig. 2-3. Since this HS site was only found in one orientation and was very weak, it was not analysed further.

Regulatory elements have been found not only in the 5' flanking region of genes but also within genes and in their 3' flanking regions. We therefore analysed the downstream region of the *R-FABP* gene for the presence of HS sites. The DNase I treated genomic DNA was digested with EcoR1, generating a 6 kb fragment that includes exons 3 and 4 as well as 3.5 kb of 3' flanking DNA. Using a 250 bp EcoR1/Hind111 fragment as the probe (labelled "c" in Fig. 2-3C), we obtained a strong band at 6 kb but no additional smaller bands that would indicate the presence of HS sites. The strong HS site located at the 5' end of the *R-*

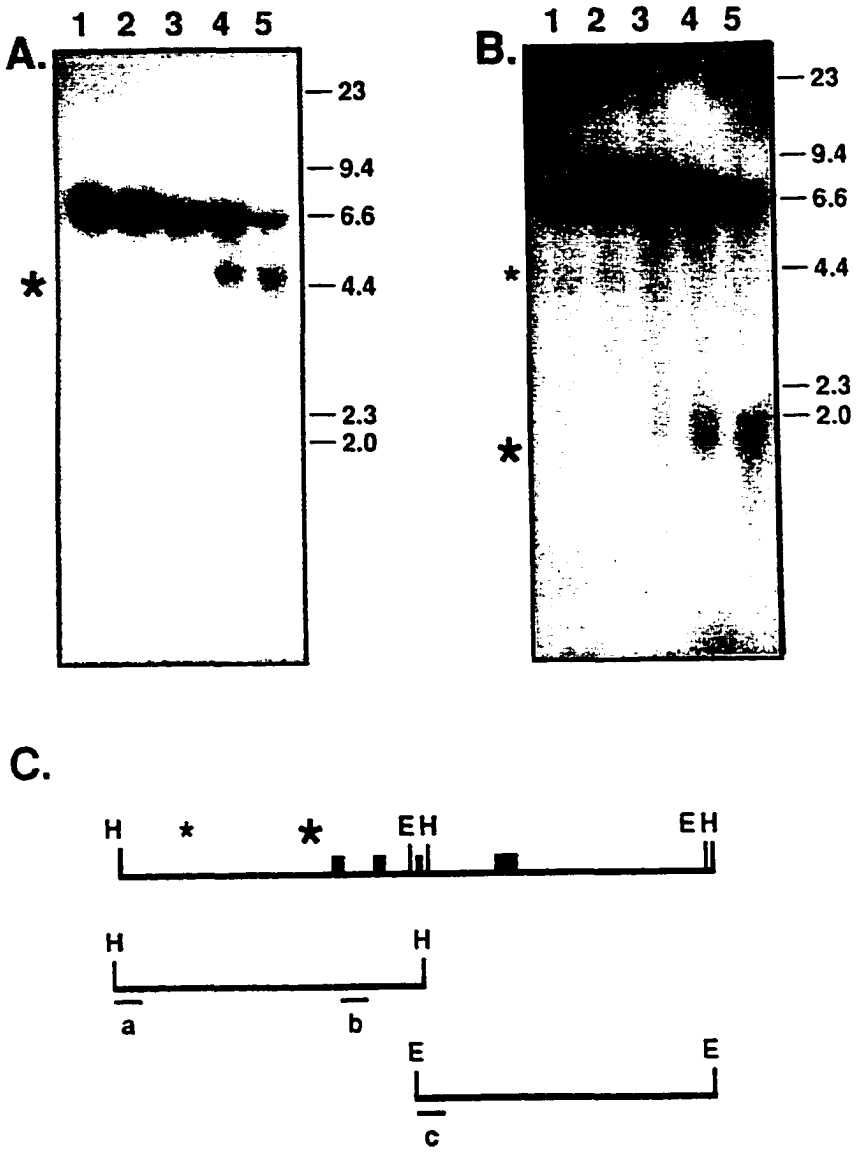

Figure 2-3. DNase I hypersensitivity of the *R-FABP* gene. Nuclei from d7 chick retina were treated with increasing concentrations of DNase I (lane 1 - no DNase I, lane 2 - 0.1 μg/ml, lane 3 - 1 μg/ml, lane 4 - 5 μg/ml, lane 5 - 10 μg/ml). The DNA was extracted from each sample and digested with HindIII. The DNA was transferred to nitrocellulose filters and hybridized with (A) a 500 bp HindIII/BglI fragment from the 5' end of the *R-FABP* gene (probe a), and (B) a 500 bp ScaI/XhoI fragment located between exons 1 and 2 (probe b) (the XhoI site is derived from the phage polylinker region). The HS sites are indicated by the asterisks. (C) Diagrammatic representation of the *R-FABP* gene indicating the location of the probes (fragments a, b and c) used for hybridization. Results obtained with fragment c are described in the text. Restriction enzymes are: H (HindIII) and E (EcoRI). Size markers in kb are indicated on the side.

FABP gene suggests that a major regulatory domain(s) is located immediately upstream of the transcription initiation site.

2.3.4 Methylation Analysis of the *R-FABP* Promoter

DNA methylation in promoter regions has been implicated in the regulation of cell-, tissue- and developmental-specific genes (17). In general, hypomethylation of CpG residues correlates with gene expression while hypermethylation correlates with nonexpression. The 5' end of the *R-FABP* gene is exceedingly GC-rich with 33 CpG dinucleotides within 200 bp of 5' flanking DNA. A recently developed method (13,20) allows the analysis of the methylation status of every C residue. We have used this technique to analyse the methylation state of the region from -334 to +59 bp in preparations of genomic DNA from d5 retina (elevated levels of *R-FABP* mRNA), d16 retina (low levels of *R-FABP* mRNA) and adult chicken liver (no detectable *R-FABP* mRNA by Northern blot analysis). The bisulphite-treated DNAs were PCR-amplified first with primers 1 and 2, and subsequently with primer 1 and the nested primer 3 as described in Materials and Methods. Because of the reported variability in methylation patterns observed by others (40), we sequenced both strands of six independent clones from each of the genomic DNAs. Surprisingly, all of the C's in all of the DNAs sequenced were converted to T's, indicating that none of the C residues in this region were protected by methylation (data not shown). As a result of bisulphite treatment, the two strands of DNA contain many base substitutions which result in considerable differences in the sequence of one strand relative to the other. The three primer PCR strategy described here will therefore amplify only the strand of DNA to which the primers were made. We did not sequence the other DNA strand because there is no

evidence that methylation can occur on only one strand. Based on these data, we conclude that methylation in the immediate upstream region of the *R-FABP* gene is not involved in either the dramatic reduction in *R-FABP* transcript levels observed from d5 to d16 in the chick embryo or for the repression of *R-FABP* gene activity in adult liver.

2.3.5 Analysis of Regulatory Domains by DNA Transfection of Primary Retinal Cultures

We transfected primary chick retinal cultures from d5 embryos to determine whether the 5' flanking region of the *R-FABP* gene has regulatory activity. Four different constructs were tested: either 51 bp, 135 bp, 234 bp or 3.5 kb of 5' flanking DNA were linked to the CAT reporter gene and introduced into primary cultures of retinal cells by calcium phosphate-mediated DNA transfection. A CAT vector (pCAT basic) containing no enhancer and no promoter served as the negative control. The results of four separate experiments are individually tabulated in Fig. 2-4A. Each number presented in this figure is an average of at least two separate measurements of CAT activity. β-galactosidase activity was measured in each case to ensure that the transfection efficiencies were similar for each plate. There was less than a two-fold variation in β-galactosidase activity from sample to sample within each set of experiments. Because we used primary cultures, there was more variation in CAT activity from experiment to experiment than ordinarily observed using established cultures. However, a similar trend was observed in all four experiments: CAT activity increased 8-25X in constructs containing 51 bp of *R-FABP* upstream sequence compared to pCAT basic. An additional ~4-5X increase was obtained with 135 bp of upstream sequence. Little or no further increase in CAT activity was observed with the -234

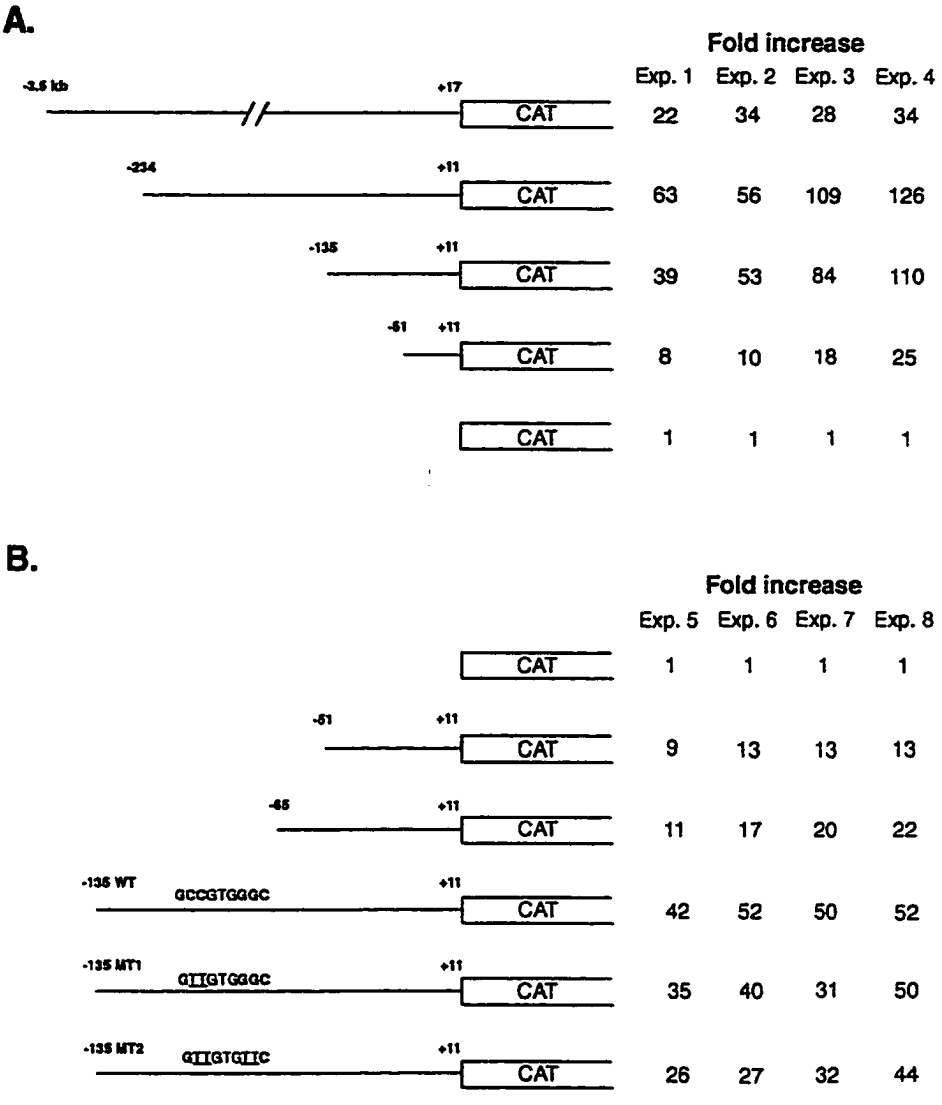

Figure 2-4. Analysis of the regulatory activity of the *R-FABP* 5' upstream region using the CAT assay. (A) Four DNA fragments containing from -3.5 kb to +17 bp, -234 to +11 bp, -135 to +11 bp, -51 to +11 bp were ligated to the pCAT basic vector containing no promoter or enhancer. Primary d5 retinal cultures were transfected and CAT activity measured by the conversion of ^{14}C-chloramphenicol to its butyrylated derivatives. The results from 4 separate experiments are tabulated. Each number represents the average fold increase in CAT activity for each construct compared to pCAT basic. Numbers have been adjusted to account for construct size and for variations in β-galactosidase activity. (B) Wild-type (WT) and mutant (MT) AP-2 recognition sites in the context of the -135 bp construct described above were transfected into primary d5 retinal cultures. In -135 MT1, the AP-2 site (GCCGTGGGC) was mutated to GTTGTGGGC and in -135 MT2, the AP-2 site was mutated to GTTGTGTTC. The -65 bp construct was prepared by PCR amplification of the region from -65 to +11 bp. The fold increase indicated for experiments 5 to 8 have been adjusted for variations in levels of transfected DNA by densitometric scanning of gels containing Hirt DNA.

bp construct while the -3.5 kb construct consistently generated lower CAT activity than either the -135 or -234 constructs, suggesting that the sequence from -234 bp and -3.5 kb has a negative influence on transcription. To ensure that the -3.5 kb was taken in by the cells as efficiently as the other constructs, we measured levels of transfected plasmid DNAs using the method of Hirt (31). There was less than a two-fold variation from construct to construct (data not shown). These data suggest that sequences located within 135 bp of 5' flanking DNA are especially important for the upregulation of the *R-FABP* gene.

2.3.6 DNA/Protein Interaction Analysis of the *R-FABP* Promoter.

Transient DNA transfection of primary retinal cultures using the CAT assay suggest that full activity is obtained using the -234 bp construct and that most of the activity is within 135 bp of 5' flanking DNA. To analyse the sites of protein/DNA interaction within this region, *in vitro* DNase I footprinting analysis was carried out using a PvuII/RsaI genomic DNA fragment from -234 to +11 bp. To compare sites of DNA/protein interaction in the undifferentiated versus differentiated retina, nuclear extracts were prepared from d7 retina and d16 retina. Regions protected from DNase I digestion are shown in Fig. 2-5. When the coding strand was labelled, four distinct footprints were observed in the d16 retinal extracts: from -24 to -52 bp (footprint I), -59 to -80 bp (footprint II), -114 to -140 bp (footprint III), and -199 to -205 bp (footprint IV). The patterns obtained with d7 retina were similar except that footprint II only extended from -63 to -80 bp and footprint III was not detected in these extracts although there was a DNase I hypersensitive site at position -129. DNase I footprinting of the non-coding strand consistently produced footprints that were less clear than those observed on the coding strand. A number of strong bands in the region

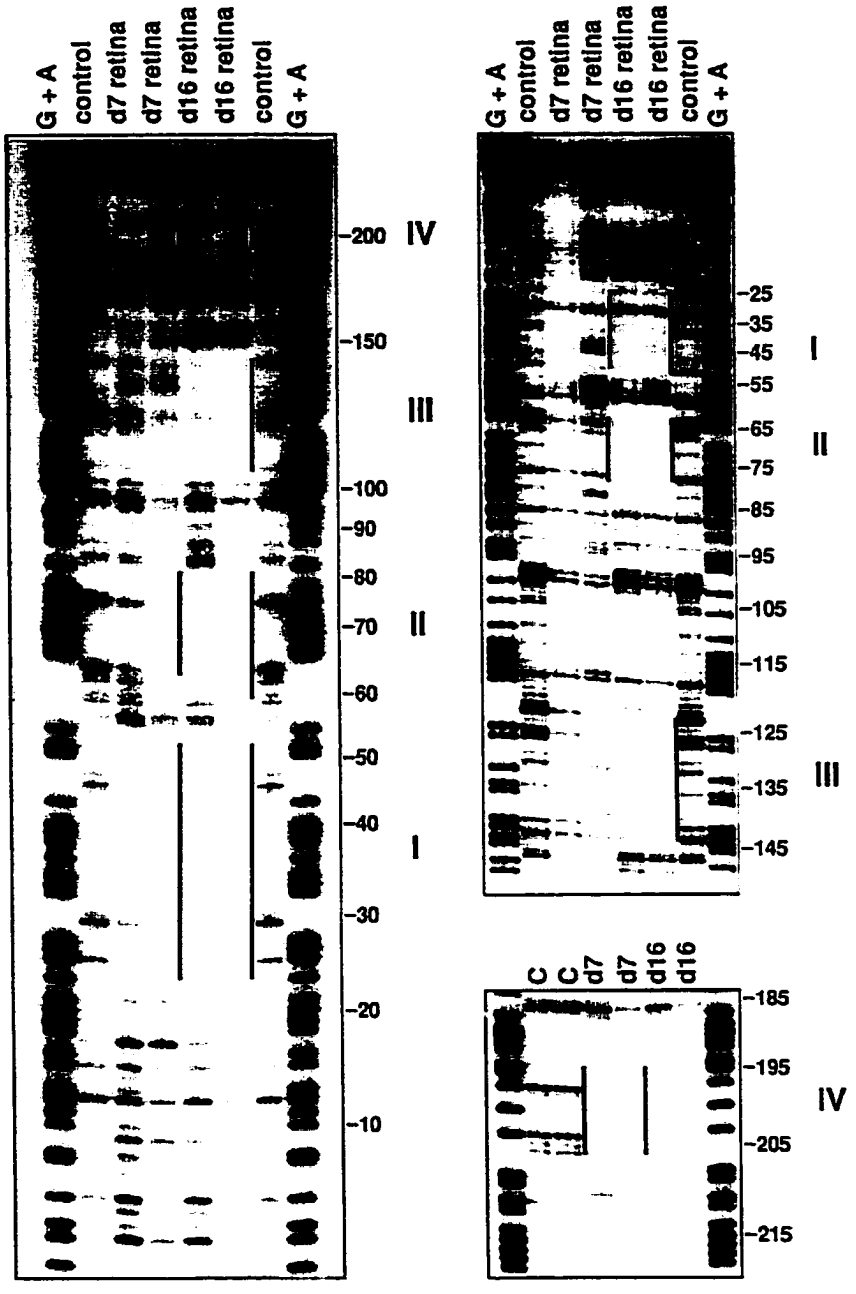

Figure 2-5. DNase I footprinting of the protein binding sites in the *R-FABP* promoter region. The top and bottom strands of the 245 PvuII/RsaI *R-FABP* DNA fragment from -234 to +11 bp were separately labelled, incubated with nuclear extracts prepared from either d7 retina or d16 retina (15 μg nuclear extracts), digested with either 0.2 μg/ml (lanes 3 and 5) or with 0.3 μg/ml (lanes 4 and 6) DNase I and run on a 7% denaturing polyacrylamide gel. The samples were loaded in a different order in the bottom right gel, with 0.2 μg/ml DNase I in lanes 4 and 6 and 0.3 μg/ml DNase I in lanes 5 and 7. The results obtained with the coding strand are shown on the left and those with the non-coding strand on the right. G+A lanes represent the purine sequence of the radiolabelled coding (left) and non-coding (right) strands. Numbers on the right refer to position relative to the transcription initiation site (designated as +1). Control lanes consist of protein-free DNA cut with DNase I. Footprints I to IV are indicated by the lines on the right hand side of lanes 4 (d7 retina) and 6 (d16 retina) of the gels located on the left and top right. Footprint IV on the bottom right gel is indicated on the left of lanes 4 (d7 retina) and 6 (d16 retina).

from -85 to -145 showed a reduction in intensity although extended areas of reduced intensity corresponding to footprints were not obvious. The following footprints were obtained on the non-coding strand using d16 retinal extracts: from -206 to -195 bp (footprint IV), -143 to -122 bp (footprint III), -63 to -80 (footprint II), and -52 to -27 bp (footprint I). Footprints I, II and IV were also observed using d7 retinal extracts. There is therefore a general agreement with the footprints on the coding and non-coding strands.

As mentioned earlier, the 5' flanking region of *R-FABP* is extremely GC-rich (2-2B). The sequence protected in footprint I consists of a stretch of G's and C's followed by the putative TATA (TTTAAA) box (Fig. 2-6). A number of possible Sp1 binding sites are located within this region. Similarly, footprints II and III consist almost entirely of G's and C's, while the nucleotides protected by footprint IV are 5'-GATAAT-3' (Fig. 2-6). Interestingly, there is an AP-2 consensus binding site within footprint II -- 5'-**GCC**GTG**GGC**-3' (the conserved residues are indicated in bold). Footprint II is located within the 135 bp 5' flanking DNA of the *R-FABP* gene that generates a high level of activity in the DNA transfection analysis. To further analyse footprint II, EMSA was carried out using a labelled DNA fragment from -52 to -90 bp as the probe. A similar pattern was obtained using both d7 and d16 retinal extracts although the intensity of the bands observed using d7 extracts was consistently weaker than that observed using d16 extracts (Fig. 2-7). In the absence of specific competitors, a strong retarded band (indicated by the arrow) and multiple weaker retarded bands were observed (lanes 1 and 6 in Fig. 2-7). When the -52 to -90 bp DNA fragment was used as a competitor, these bands either disappeared or were substantially reduced in intensity, including the strong band indicated by the arrow (lanes 2 and 7). With a DNA fragment from -9 to -51 bp as the competitor,

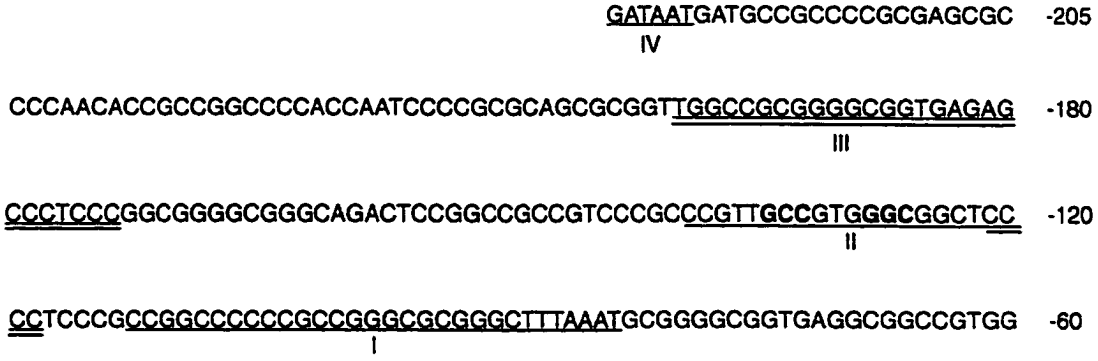

Figure 2-6. Location of the footprints in the *R-FABP* upstream sequence. The 205 bp sequence located upstream of the transcription initiation site is indicated as well as the location of the footprints (single underline) found within this region using d7 and d16 retinal extracts. The double underline represents the extended footprint II observed using d16 retinal extracts. Footprint III is only observed using d16 retinal extracts. The conserved nucleotides in the AP-2 binding site are indicated in bold.

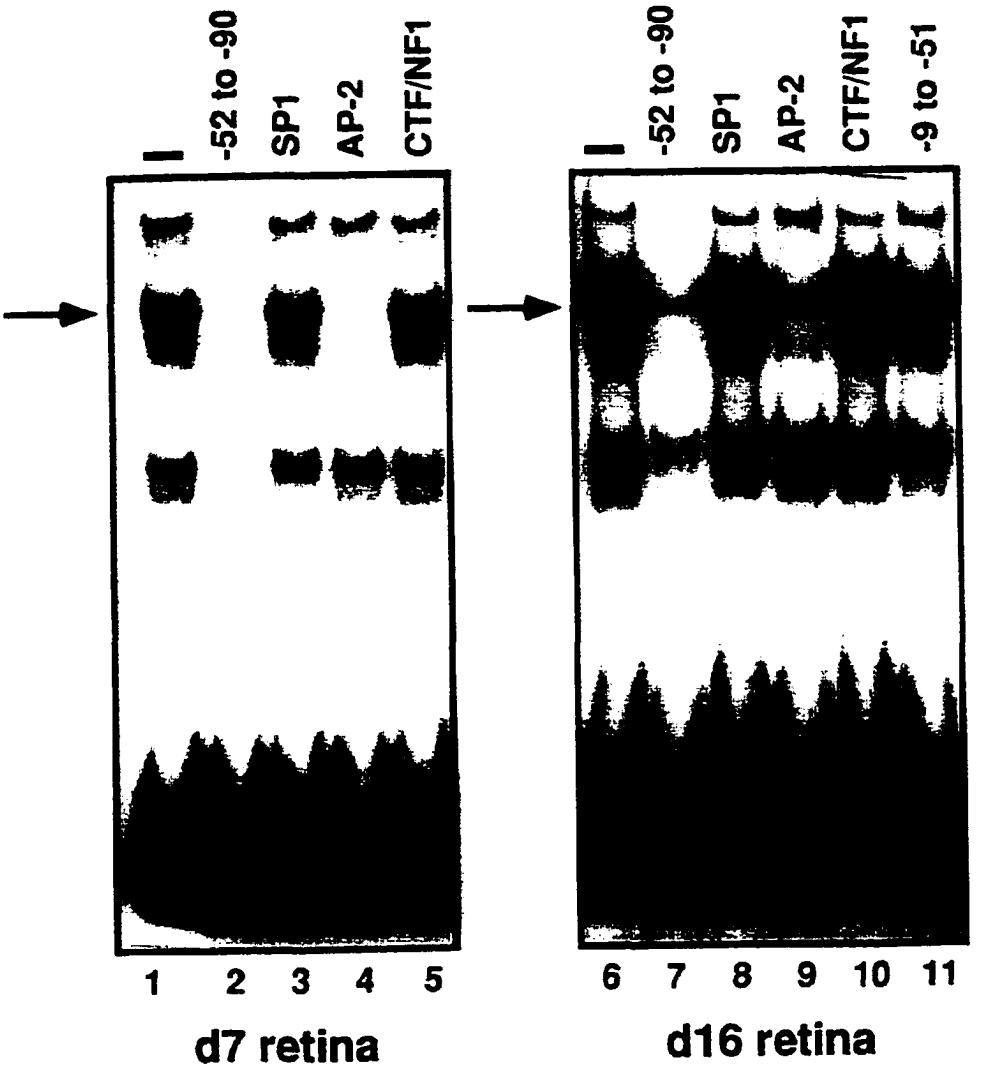

Figure 2-7. Electrophoretic mobility shift assay using a DNA fragment (-52 to -90 bp) containing footprint II as the probe. The ^{32}P-labelled probe (-52 to -90 bp) was incubated with nuclear extracts from either d7 or d16 retina and run on a 6% non-denaturing polyacrylamide gel in 0.5X TBE. The following competitors (in ~100X molar excess) were tested: lanes 1 and 6, no competitor; lanes 2 and 7, unlabelled -52 to -90 bp DNA fragment; lanes 3 and 8, SP1 oligonucleotide; lane 4 and 9, AP-2 oligonucleotide; lanes 5 and 10, CTF/NF1 oligonucleotide; lane 11, -9 to -51 bp DNA fragment. The arrow indicates the location of the strongest specific DNA/protein band. The free probe is at the bottom of the gel.

there was only a slight decrease in the intensity of the bands (lane 11). In the presence of a 100X excess of consensus double-stranded oligonucleotides to SP1, AP-2 and CTF/NF1, competition was observed only in the lanes containing the AP-2 oligonucleotide (lanes 4 and 9) where two of the retarded bands either disappeared or were significantly reduced in intensity (including the band indicated by the arrow). The remaining bands were not affected by the presence of excess unlabelled AP-2 oligonucleotide. To obtain additional evidence that an AP-2 like transcription factor is binding to the -52 to -90 bp region, nuclear extracts from both d7 and d16 retina were incubated with either an antibody to AP-2 or an antibody to TBP prior to addition of the labelled -52 to -90 bp DNA fragment. A supershifted band was observed in the presence of the AP-2 antibody with both d7 (data not shown) and d16 nuclear extracts (Fig. 2-8). Supershifting was not obtained in the presence of either TBP antibody (shown) or NF1 antibody (not shown).

We used the methylation interference assay to determine which purine residues in the -52 to -90 bp region are in close proximity to the transcription factor found in the major EMSA band. Fig. 2-9 shows that methylation of G residues at positions -74, -73 and -67 on the non-coding strand and -75, -72, -70, -69, -68 on the coding strand interferes with protein binding. This pattern was identical for both d7 and d16 retinal extracts. Methylation of a G (indicated by the arrow) located outside the AP-2 binding site at position -64 on the non-coding strand also interfered with protein binding. Similar methylation interference patterns were observed for the AP-2 DNA binding sites in the SV40 enhancer and human metallothionein IIa (hMTIIa) distal basal level element (BLE) except that no extension beyond the AP-2 site was noted (57).

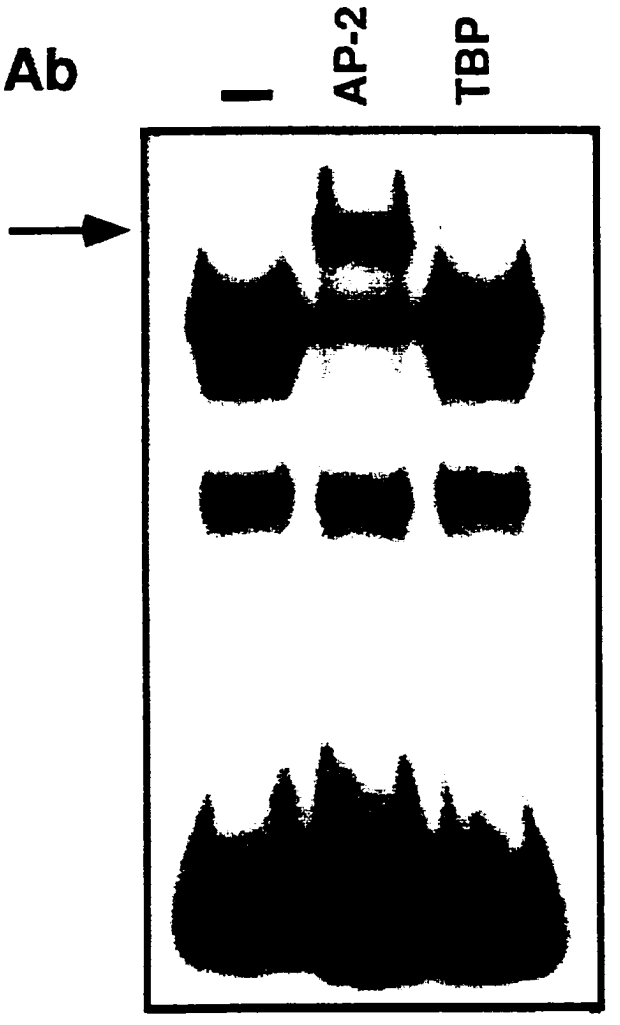

Figure 2-8. Supershift of a DNA/protein band using the AP-2 antibody. EMSA was carried out using the ^{32}P-labelled DNA fragment from -52 to -90 bp and either d7 (not shown) or d16 (shown) retinal extracts in the presence of AP-2 antibody or TBP antibody. A supershifted band indicated by the arrow was observed in the presence of the AP-2 antibody.

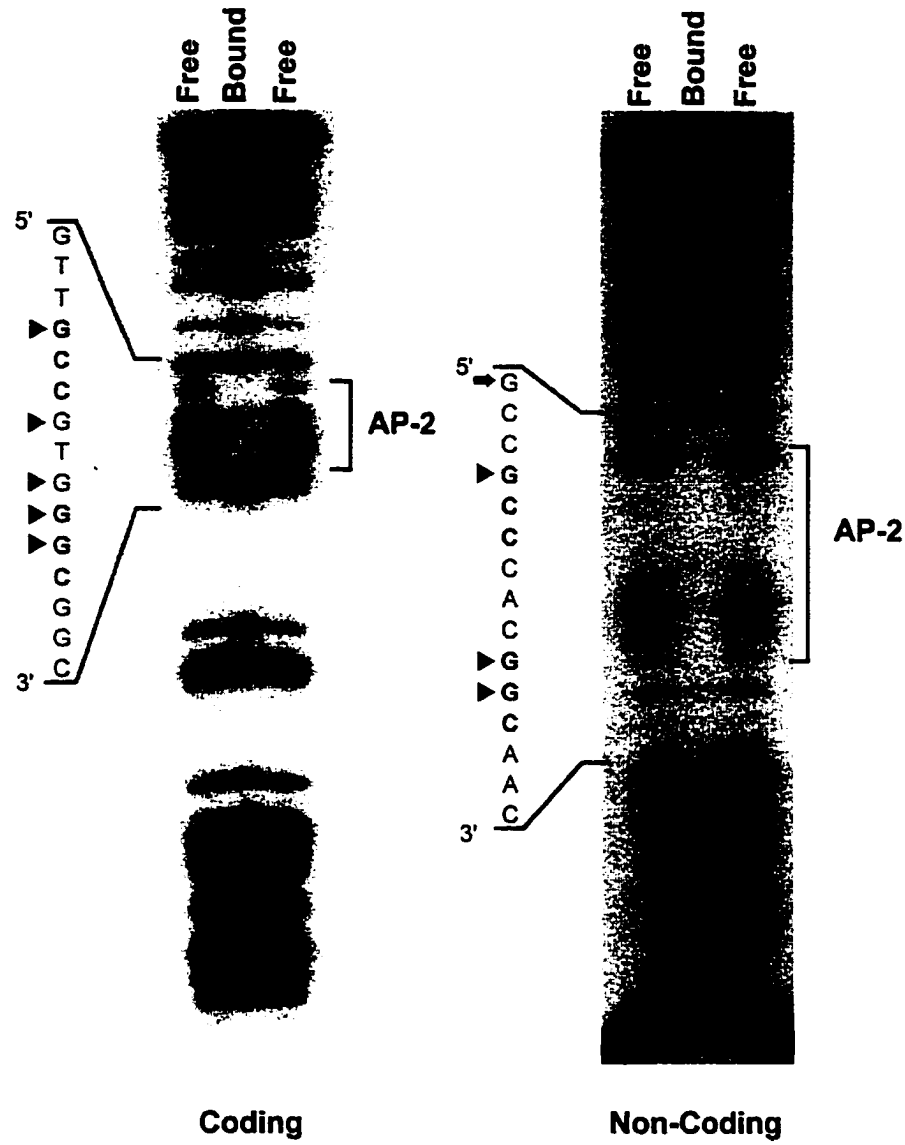

Figure 2-9. Methylation interference using the DNA fragment from -52 to -90 bp (containing footprint II). EMSA was carried out using either d7 (not shown) or d16 (shown) chick retinal extracts with a partially methylated singly endlabelled probe representing the coding and non-coding strands. The bands corresponding to free DNA and protein bound DNA (major band indicated by the arrow in Fig. 7) were excised, the DNA eluted and cleaved with piperidine and electrophoresed in a 8% (coding strand) or 12% (noncoding strand) denaturing polyacrylamide gel. The conserved residues in the AP-2 DNA binding site are indicated in bold (5'-GCCNNNGGC-3'). The arrowheads indicate the G residues that are part of the AP-2 binding site in which methylation interferes with AP-2 binding. The arrow indicates the G residue in the non-coding strand that lies outside the consensus AP-2 binding site.

2.3.7 AP-2 Expression in the Retina.

The DNA transfection results indicate that footprint II, which includes the AP-2 binding site, plays a positive role in the regulation of the *R-FABP* gene. However, the DNA/protein interaction experiments suggest that AP-2 is more abundant (or more active) in the differentiated d16 retina (which expresses low levels of *R-FABP* mRNA) than in d7 retina (which has elevated levels of *R-FABP* mRNA). An *AP-2α* cDNA isolated from a human adult retina library (Genome Systems Inc.) was used to probe a Northern blot containing mRNAs from chick retinas at different developmental stages (Fig. 2-10A). Three *AP-2* transcripts of ~1.8, 3.5 and 4 kb (indicated by the arrows) were detected after washing the blots under moderate stringency with the strongest signal obtained at d10. By d16, *AP-2* transcript levels were significantly reduced. *AP-2* transcripts were not detected at d5 while weak bands at 1.8 kb and 4 kb were observed at d7. The band marked by an asterisk represents residual signal from a previous hybridization and is unrelated to *AP-2* mRNA.

We used a commercially available anti-human AP-2 antibody specific for AP-2α to measure AP-2 levels in the d7 and d16 nuclear extracts used for the footprinting and EMSA experiments. In agreement with the gel shift assays and the Northern blot analysis, we found considerably lower levels of AP-2α in d7 compared to d16 retinal extracts (Fig. 2-10B). Analysis of total cellular lysates indicate that levels of AP-2α are at least 10X lower in d7 retina compared to d10 retina and approximately 2X lower in d16 retina compared to d10 retina (data not shown). AP-2α was not detected in d5 retina.

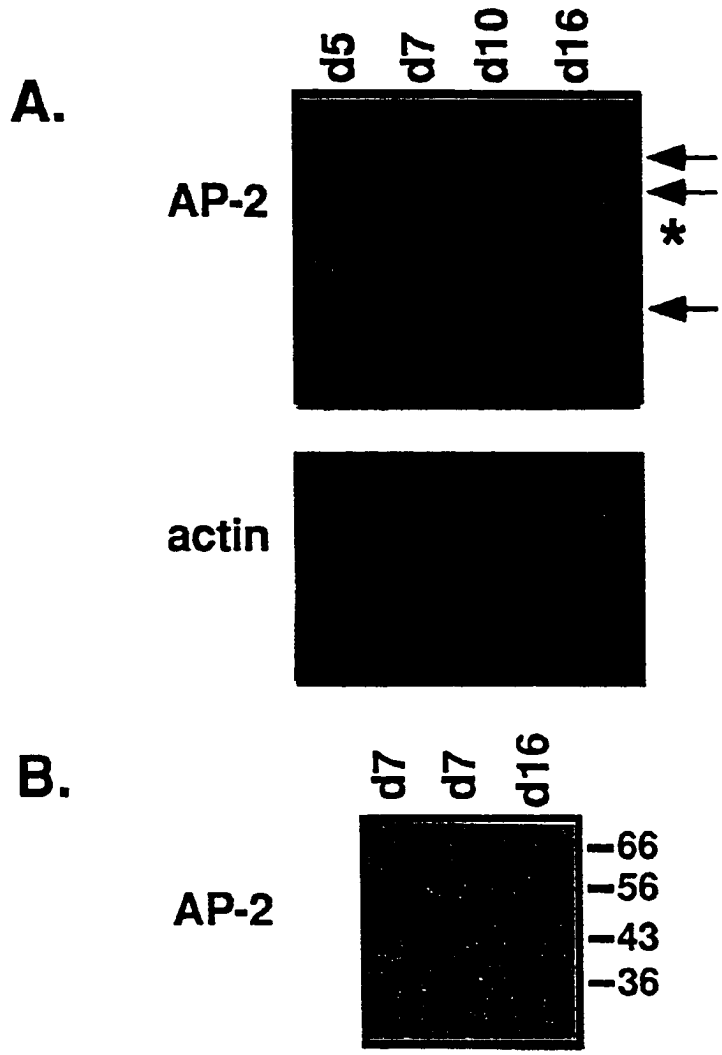

Figure 2-10. Analysis of *AP-2* transcript and protein levels in the chick retina at different developmental stages. (A) Poly(A)$^+$ RNA was extracted from d5, d7, d10 and d16 chick retina. After electrophoresis, the RNA (2 μg/lane) was transferred to nitrocellulose filter and sequentially hybridized with ^{32}P-labelled *AP-2* cDNA isolated from a human adult retina library and actin cDNA. The blot hybridized with *AP-2* cDNA was washed at 45°C in 0.1X SSC and 0.1% SDS. Exposure to X-ray film was 3 days for the *AP-2* cDNA probe and 1 hour for the actin probe. The arrows indicate the position of the *AP-2* transcripts. The band marked by an asterisk represents residual signal from a previous hybridization. (B) Western blots were prepared using nuclear extracts from two different preparations of d7 retina and from d16 retina. Fifty μg protein were loaded in each of the d7 lanes and 25 μg in the d16 lane. AP-2 was detected using a commercially available anti-human AP-2 antibody that specifically recognizes AP-2α. Molecular weight standards (kDa) are indicated on the side.

2.3.8 Mutation Analysis of the AP-2 Binding Site.

To more directly assess the role of the *R-FABP* AP-2 binding site in proliferating retinal cells, we transfected primary d5 retinal cultures with pCAT constructs carrying mutations in the core recognition sequence for AP-2. In mutant 1 (MT1), the AP-2 sequence 5'-**GCC**GTG**GGC** (conserved residues in bold) was mutated to GTTGTGGGC and in mutant 2 (MT2), the AP-2 site was mutated to GTTGTGTTC. The mutations were introduced into the -135 bp construct which contains 135 bp of *R-FABP* upstream sequence. Comparison of the wild type and mutant -135 bp constructs indicates that mutations in the AP-2 binding site have only a slight effect on CAT activity, decreasing levels by <2-fold (Fig. 2-4B). Since there are only two clear footprints in the *R-FABP* proximal regulatory region using d7 retinal extracts, we tested a -65 bp construct to determine whether inclusion of sequences between -51 bp and the AP-2 site (located from -67 to -75 bp) might increase CAT activity to the levels observed with the -135 bp construct. Again, there was only a slight increase (between 1.2- and 1.7-fold) in CAT activity in the -65 bp compared to the -51 bp construct.

We tested whether AP-2 could bind to the mutated AP-2 recognition site by EMSA. Wild-type (WT) and mutant 1 (MT) labelled DNA fragments from -52 to -90 bp served as probes for these experiments. The intense band indicated by the arrow in Fig. 2-7 is readily apparent in the presence of wild-type probe (Fig. 2-11, lanes 2 and 8) but is absent using mutant DNA as the probe (lanes 3 and 9). This band was previously shown to supershift in the presence of AP-2 antibody. Competition experiments indicate that a 50X excess of unlabelled wild-type DNA serves as an effective competitor for binding to this factor (lanes 4 and 10), while 100X excess of mutant DNA fails to reduce the intensity of this band (lanes

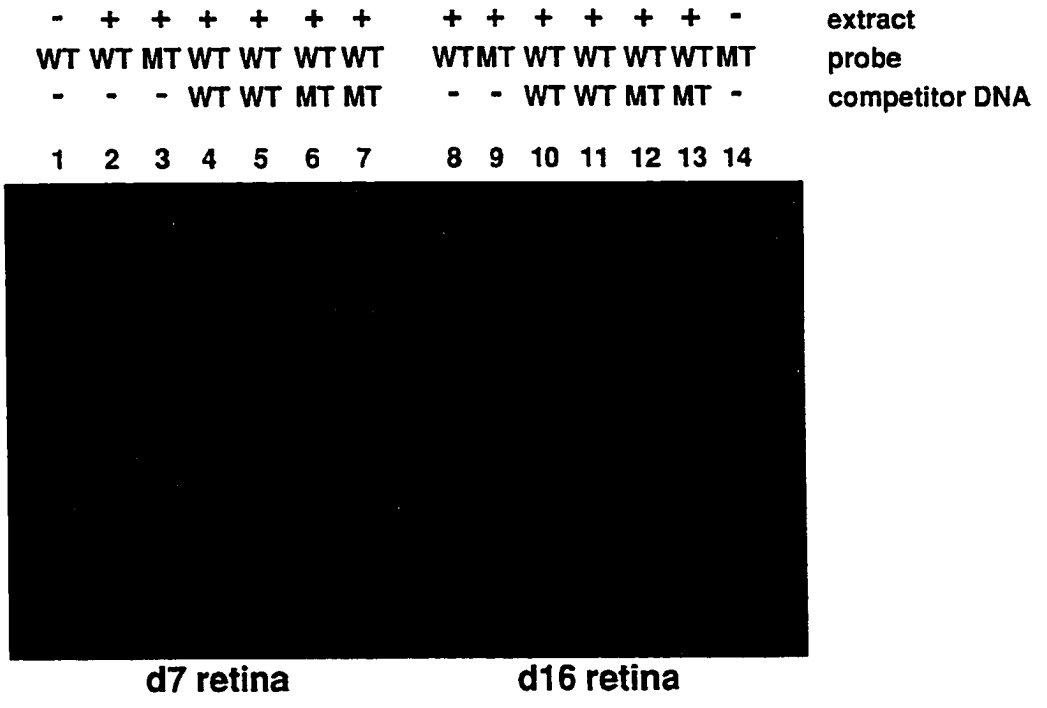

Figure 2-11. Mutation of the core recognition sequence for AP-2 binding prevents DNA/protein interaction. Electrophoretic mobility shift assays were carried out using wild-type (WT AP-2: GCCGTGGGC) and mutant (MT AP-2: GTTGTGGGC) DNA fragments from -52 to -90 bp. The ^{32}P-labelled probes were incubated with nuclear extracts from either d7 or d16 retina in the presence or absence of wild-type or mutant competitor DNA. There was a 50X excess of competitor in lanes 4 (WT), 6 (MT), 10 (WT), 12 (MT), and 100X excess in lanes 5 (WT), 7 (MT), 11 (WT), 13 (MT).

7 and 13). Retarded bands with a faster migration rate were also observed using both the wild-type and mutant DNA as the probe (lanes 2, 3, 8 and 9). A reduction in the intensity of these bands was obtained when wild-type and mutant DNA were used as competitors, although mutant DNA was not as effective as wild-type DNA (compare lanes 6, 7, 12, 13 with lanes 4, 5, 10, 11). It should be noted that these lower bands did not supershift in the presence of AP-2 antibody (Fig. 2-8). Another weak band located near the gel origin (shown not to be competed out with excess unlabelled AP-2 oligonucleotide in Fig. 2-7) was restricted to the lanes containing the wild-type probe (lanes 2 and 8). Qualitatively similar results were observed with d7 and d16 retinal extracts, although the signal intensity was considerably lower using d7 extracts.

2.4 Discussion

The retina is well-characterized morphologically and physiologically. The time of appearance and location of each of the major cell types in the retina as well as the function of these cells has been described in a number of species. However, we still have a poor understanding of the nature and function of the precursor neuroectodermal cells that differentiate into the various classes of neurons and glia found in the mature retina. *R-FABP* is expressed in tissues derived from neuroectodermal cells, including brain and retina. In the retina, *R-FABP* has an expression profile that suggests a role in cell proliferation and early differentiation; i.e., *R-FABP* is expressed at elevated levels in the undifferentiated cells of the retina and tissue maturation is accompanied by a dramatic decrease in mRNA levels (50-100X decrease from d3.5 to d19 of incubation) (24). We analysed the regulation of the *R-FABP* gene in order to gain insight into the molecular mechanisms and transcription factors involved in controlling gene expression in the retinal neuroectodermal cells.

The 5' flanking DNA of the chicken *R-FABP* gene has an extremely high GC content, with >80% G's and C's over 200 bp of 5' flanking DNA including 33 CpGs. The expression of some genes has been shown to be closely related to the methylation status of CpGs in their promoter regions (rev. in 17). Nonmethylated CpG islands are generally believed to be associated with sites of transcribed genes while CpG methylation apparently inhibits transcription. Some transcription factors which contain CpGs in their recognition sites are affected by methylation (e.g. AP-2) (14) while others (e.g. Sp1) (32) are not. DNA methylation appears to function by preventing the binding of some transcription factors and by altering chromatin structure (rev. in 17). We have found no evidence of methylation in the promoter region of the *R-FABP* gene in either retina (differentiated or undifferentiated

tissue) or in the adult liver where *R-FABP* mRNA is not detected. These results suggest that CpG methylation in the immediate upstream region of the *R-FABP* gene is not involved in the repression of *R-FABP* gene transcription at least in the differentiated retina and liver. Similar observations have been made for the human β-globin gene whose promoter is unmethylated not only in tissues expressing the β-globin gene but in non-expressing tissues as well (5).

We used nuclear extracts of d7 and d16 retina to study DNA/protein interactions in the upstream region of the *R-FABP* gene. At d7, 60% of retinal cells are proliferating (16) and *R-FABP* transcript levels are high (24,26). The relatively large size of the eyes at this late proliferative stage facilitates the accumulation of the large quantities of retinal tissue required for these analyses. DNase I hypersensitivity and DNA/protein interaction studies using d7 retina suggest that the major regions responsible for transcriptional regulation of the *R-FABP* gene are located within 205 bp of the transcription initiation site. Three footprints were identified in this region using d7 retinal extracts and four footprints using d16 retinal extracts. Footprint I (-24 to -52 bp) includes a putative TATA box (TTTAAA) located 30 bp upstream of the *R-FABP* transcription start site as well as Sp1 consensus sites and is required for basal transcriptional activity based on transient CAT expression in primary d5 retinal cultures. Of note, another *FABP* gene closely related to *R-FABP*, mouse *H-FABP*, also has a TTTAAA sequence located -21 to -26 bp upstream of the transcription start site (47). A subset of *FABP* genes may show a preference for this sequence, similar to what has been reported for the milk protein genes (61). A construct that includes footprint II (-63 to -80 bp) in addition to footprint I generated a 4-5X increase in CAT activity compared to a construct with footprint I alone. Additional 5' flanking DNA, including footprint IV at -

199 to -205 bp, resulted in a <2-fold further increase in CAT activity. Footprint III (-114 to -140 bp) is found only with d16 retinal extracts and may play a negative role in the regulation of *R-FABP* transcription.

Footprint II is of special interest because different patterns are observed using d7 (footprint from -63 to -80 bp) and d16 (-59 to -80 bp) retinal extracts, suggesting that the interaction of nuclear protein(s) with this DNA region differs in the undifferentiated and differentiated retina. Furthermore, footprint II includes an AP-2 consensus binding site, 5'-GCCNNNGGC-3'. AP-2 is a transcription factor implicated in gene regulation during embryogenesis (57), and has been shown to be involved in the differentiation of P19 embryonal carcinoma cells along the neuroectodermal pathway by retinoic acid (43). A number of genes have been found to be regulated by AP-2, including insulin-like growth factor binding protein-5 which is expressed in the retina (10,15). There are at least three genes that encode members of the AP-2 family in mammals, *AP-2α*, *AP-2β* and *AP-2γ* (39,58). To date, only the *AP-2α* cDNA has been isolated from chicken (EMBL/GenBank accession number U72992). AP-2 factors encoded by all three genes bind as dimers to the same AP-2 DNA recognition site. These factors have similar dimerization domains but differ in their transactivation domains. AP-2α consists of two splice variants, AP-2αA and AP-2αB (9). The latter is a negative regulator of transcriptional activation by AP-2 and functions by blocking DNA binding. The AP-2 family is therefore considerably more complex than originally anticipated suggesting a need for controlling AP-2 activity at multiple levels and in a tissue- or cell-specific manner. Gel shift assays using an AP-2 consensus oligomer as the competitor, supershift experiments using AP-2 antibody and methylation interference experiments all indicate that a member of the AP-2 family binds to

footprint II located upstream of the *R-FABP* gene. Notably, although the d16 footprint extends further than the d7 footprint, the same banding patterns are observed in the EMSA and supershift experiments using either d7 or d16 retinal extracts. Furthermore, methylation interference experiments indicate that the same nucleotides are involved in nuclear protein binding in the two tissues. Taken together, these results suggest that one or more members of the AP-2 family expressed in d7 and d16 retina is binding to the AP-2 consensus site located upstream of the *R-FABP* gene.

One would expect a positive regulator of *R-FABP* transcription to be expressed in the undifferentiated retina when *R-FABP* transcript levels are highest. The absence of detectable *AP-2α* mRNA and protein in d5 retina and its relatively low abundance in d7 retina compared to d10 retina suggest that at least this member of the AP-2 family is not an activator of *R-FABP* transcription. The comparatively weak signal observed in the DNA/protein interaction analyses (footprinting and EMSA) using d7 versus d16 retinal extracts further indicates that overall AP-2-like activity (defined as the ability to bind to the AP-2 recognition site) is significantly higher in the more differentiated retina. Based on these observations, we propose that AP-2 (most likely AP-2α) functions as a repressor rather than an activator of transcription. In support of this hypothesis, it should be noted that a decrease in the steady-state levels of *R-FABP* mRNA is first observed in d10 retina (24) when AP-2α transcript and protein levels peak. Furthermore, although *R-FABP* mRNA is abundant in the d7 retina, only 60% of cells are in the proliferative stage. The remaining cells have already differentiated or are undergoing differentiation. It is therefore possible that the factors in d7 retina that interact with the AP-2 binding site are found exclusively in the differentiated population of cells. Because of the difficulty in obtaining sufficient retinal

material at earlier developmental stages for nuclear extract preparation, this question may best be resolved by identifying the AP-2 factors expressed in the developing chick retina and determining which cell types produce these factors.

Although AP-2 is generally considered to be an activator of transcription, it has also been shown to negatively regulate the transcription of the following genes: stellate cell type I collagen, K3 keratin, acetylcholinesterase, prothymosin and ornithine decarboxylase (11,12,22,23). In all these cases, it was proposed that AP-2 functions as a repressor by displacing or competing with a positive transcription factor that has a binding site that overlaps with or is adjacent to the AP-2 recognition site. If AP-2 functions as a transcriptional activator of the *R-FABP* gene, transfection of a construct carrying a mutated AP-2 site into proliferating retinal cells should result in a decrease in CAT activity. If, on the other hand, AP-2 functions as a transcriptional repressor, an increase in activity would be expected only in cells that express AP-2. DNA transfection of mutant constructs where as many as four of the six conserved AP-2 residues have been altered (5'-**GCCNNNGGC**-3' to either 5'-**GTT**NNN**GGC**-3' or 5'-**GTT**NNN**TTC**-3') generates only a slight decrease in CAT activity compared to the wild-type construct. Gel shift experiments with the mutant AP-2 recognition site show that AP-2 can no longer bind to this site. The DNA transfection results using d5 retinal cultures are therefore consistent with AP-2 functioning as a repressor of *R-FABP* transcription, in agreement with the DNA/protein interaction experiments and the AP-2 expression profile in the developing retina. We have not yet identified the sequences that are responsible for the increase in CAT activity observed between -51 and -135 bp. A positive regulatory element may overlap with the AP-2 binding site and be partially inactivated by the mutations introduced in this region. Gel shift experiments reveal

the presence of a number of bands that are not competed out by excess AP-2 oligomers using a -52 to -90 bp fragment as the probe. Alternatively, sequences upstream of footprint II may be required for optimal transcriptional activity.

The extension in footprint II seen in the d16 extract may reflect the presence of an additional factor binding to this region which is only detected under the conditions used in our footprinting assay. One major difference between the gel shift and footprinting experiments is the comparatively large size of the DNA fragment used for footprinting. More complex DNA/protein interactions may therefore be favored using the latter technique. Whether the AP-2 transcription factor(s) expressed in d7 and d16 retina are the same or represent different members of the family or different splice variants will be the subject of future investigations. It will also be of interest to study footprint III to see if we can identify the transcription factor that binds to this region as it may represent a transcriptional repressor for genes expressed in the differentiating retina.

In conclusion, we have used a combination of techniques to identify the regulatory elements important for the transcription of the *R-FABP* gene in the developing retina. By DNase I hypersensitivity analysis, DNA transfection and DNA/protein interaction assays we have shown that strong *cis* regulatory activity is located within 200 bp of the transcription initiation site of the *R-FABP* gene. The data presented here support a role for the family of AP-2 transcriptional factors in the regulation of the *R-FABP* gene in the developing retina. The AP-2 expression profile in the retina, gel shift experiments and DNA transfection analysis using constructs with mutations in the conserved residues of the AP-2 binding site all suggest that AP-2 is a repressor of *R-FABP* transcription.

2.5 Acknowledgements

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Chapter 3

Differential Expression of AP-2α and AP-2β in the Developing Chick Retina: Repression of *R-FABP* promoter activity by AP-2[1]

(Running title – AP-2 in the Developing Retina)

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R. Godbout: Northern analysis, DNA transfections

3.1 Introduction

To better understand the molecular mechanisms of retinal development, we are studying genes whose expression patterns suggest a role in cell specification and differentiation. Chick retinal development begins shortly after formation of the optic vesicle on embryonic day (ED) 2 and is complete by the time of hatching on ED21. The mature retina contains six different neuronal cell types (photoreceptor, amacrine, horizontal, bipolar, ganglion, interplexiform cells) and a single type of glial cell, the Müller glial cell. These cells arise from multipotent progenitor cells that are predominantly localized to a region of active mitosis called the ventricular zone, located next to the retinal pigment epithelium (Fekete *et al.*, 1994). Post-mitotic cells migrate from the ventricular zone to their appropriate strata, eventually resulting in the characteristic lamellar organization seen in the mature retina.

The chicken retinal fatty acid binding protein (*R-FABP*) gene may play a role in retinal development. Brain FABP, the mammalian ortholog of *R-FABP*, has been implicated in murine brain development, specifically in the establishment and maintenance of the radial glial fiber system guiding neuronal migration (Feng *et al.*, 1994; Kurtz *et al.*, 1994). *R-FABP* is highly expressed in the primitive neuroectodermal cells of the undifferentiated chick retina from ED3 to ED7 as well as in the developing brain. There is a 50-fold decrease in transcript levels from ED7 to ED19 in the retina (Godbout, 1993; Godbout *et al.*, 1995). *R-FABP*, expressed during a period of active neuronal migration in the retina, may play an analogous role to that of B-FABP in the brain.

In a previous study of the regulation of the chicken *R-FABP* gene, we found multiple sites of protein-DNA interaction within 205 bp of 5' flanking DNA (Bisgrove *et al.*, 1997). One of these, located 59 bp upstream of the *R-FABP* transcription initiation site, corresponded to the consensus AP-2 DNA binding site, GCCNNNGGC. Supershift experiments using antibodies to AP-2 and methylation interference experiments indicated that an AP-2-like transcription factor binds to this region. Surprisingly, mutation of the AP-2 consensus site did not result in a decrease in *R-FABP* transcription activity upon DNA transfection of ED5 retinal cultures, suggesting that AP-2 might function as a repressor rather than an activator of *R-FABP* transcription. Further support for this hypothesis came from Western blots showing an increase in AP-2 levels from ED7 to ED16 in the retina, in contrast to *R-FABP* mRNA levels which undergo a dramatic decrease during this time.

Three genes encoding members of the AP-2 family have been isolated in mammals, *AP-2α*, *AP-2β* and *AP-2γ*. The spatial and temporal expression profiles of the AP-2 factors during embryogenesis suggest that they are important for normal development. Severe malformations of the face and skull, as well as abnormalities of the sensory organs, result from inactivation of the *AP-2α* gene in mouse (Schorle *et al.*, 1996; Zhang *et al.*, 1996). Eye development in the *AP-2α*-null embryos is severely perturbed with morphological defects apparent in the optic cups as early as E10.5 (Dr. Pamela Mitchell, University of Zürich, personal communication). *AP-2β* gene knock-out mice die one to two days postnatally because of polycystic kidney disease (Moser *et al.*, 1997a). No ocular defects were reported in this paper. Recently, Drosophila AP-2 has been shown to be expressed in distinct regions of the embryonic head including the visual

system, suggesting a role in the development and differentiation of the central nervous system (Bauer *et al.*, 1998).

The expression pattern of chicken AP-2 in early embryos from stage 5 (ED1) to stage 28 (ED5.5) suggests a role in the budding out of facial prominences and limb buds (Shen *et al.*, 1997). To define the role of the AP-2 transcription factors in retinal development, we have isolated and sequenced the *AP-2* cDNAs present in an ED16 chick retina library. Here, we examine the cellular distribution of AP-2α and AP-2β in the developing chick retina by *in situ* hybridization and immunofluorescence analyses and show that *R-FABP* mRNA is excluded from retinal cells that express AP-2. We also show that overexpression of AP-2 down-regulates *R-FABP* promoter activity.

3.2 Results

3.2.1 Isolation of AP-2α and AP-2β cDNAs from Chick Retina

To identify the *AP-2* genes expressed in the chick retina, we screened an ED16 retina cDNA library with human EST probes specific for *AP-2α* and *AP-2β*, as well as with the previously cloned mouse *AP-2γ* cDNA. Three *AP-2α* cDNA clones, four *AP-2β* clones but no *AP-2γ* clones were obtained upon screening 2 X 10^5 bacteriophage. None of the *AP-2α* cDNAs were full-length based on sequence analysis. The sequence of the coding region was identical to that of the previously described chicken *AP-2α* (Shen *et al.*, 1997) although there were a few alterations in the 3' untranslated region. None of the *AP-2α* clones represented *AP-2αB*, a dominant negative alternative splice form of *AP-2α* described in mouse (Buettner *et al.*, 1993).

Both strands from one of the *AP-2β* clones containing the entire open reading frame were completely sequenced. The portion of chicken AP-2β cDNA encoding the open reading frame is 85% and 83% identical to that of the human and mouse *AP-2β* cDNAs, respectively. Fig. 3-1 shows an alignment of the predicted amino acid sequence of chicken AP-2β with that of mouse and human AP-2β. Chicken and human AP-2β are 98% identical at the amino acid level (440/449 amino acids). Eight of the 9 amino acid substitutions are conservative. Six of the substitutions are located in the N-terminal half of the protein which contains the transactivation domain. The DNA binding/dimerization domain contains 3 amino acid differences, at positions 373, 425, and 427. A similar level of identity is observed with mouse AP-2β except that the murine sequence has one less codon than either human or chicken AP-2β (codon 425).

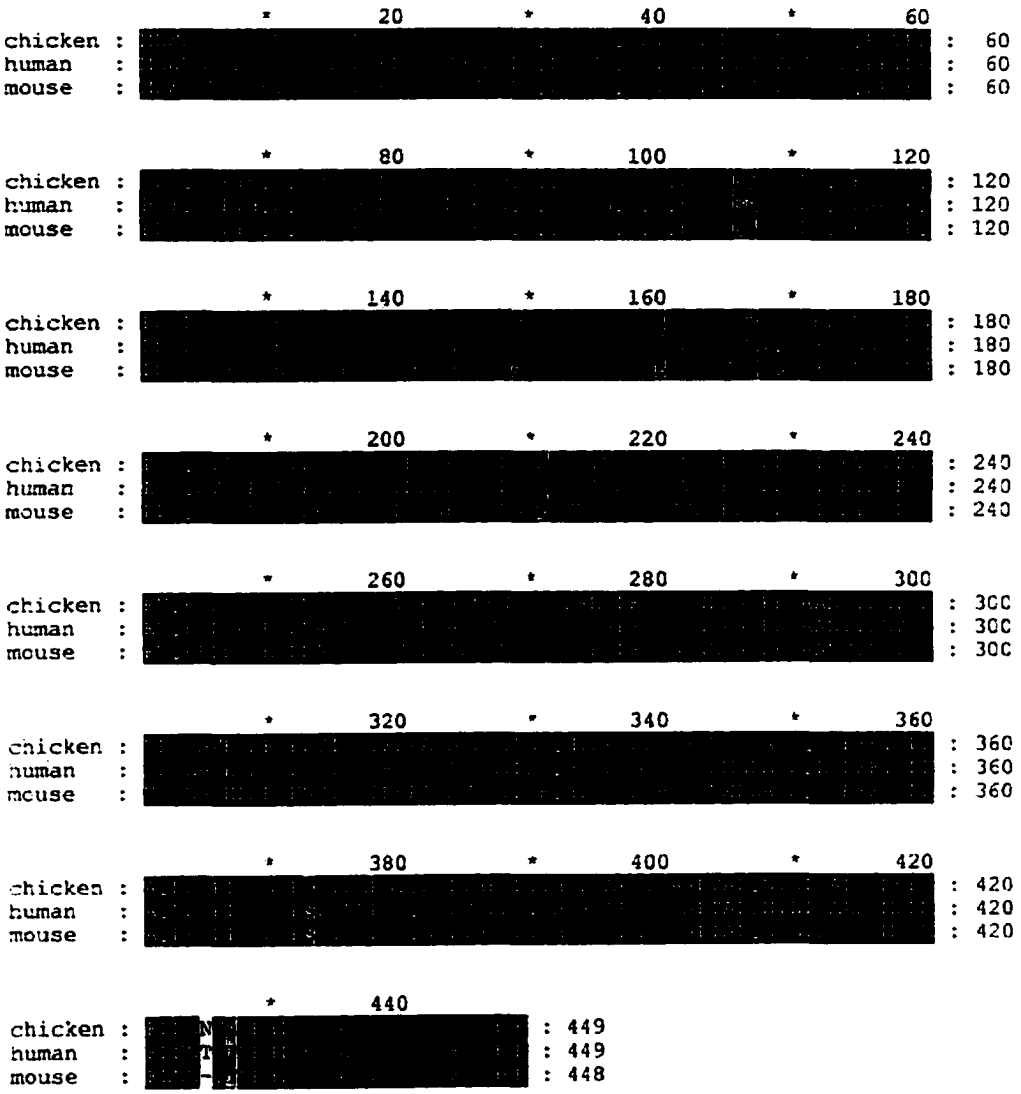

Figure 3-1. Comparison of the chicken, human and mouse AP-2β amino acid sequences. Identical residues are indicated in the black boxes and conserved residues in the shaded boxes. A dash at position 425 of the murine sequence was inserted in order to preserve sequence alignment. The following residues were considered similar: G, A, S, T; E, D, Q, N; R, K, H; V, M, L, I; F, Y, W.

The predicted molecular mass of chicken AP-2β is 49 kDa, similar to that of the 437 amino acid chicken AP-2α, predicted to be 48 kDa. The predicted amino acid sequences of chicken AP-2α and AP-2β are 71% identical (83% similar) over their entire length (Fig. 3-2). The region of highest sequence conservation lies within the C-terminal half (DNA binding/dimerization domain) with 84% identical residues (94% similar). There are also long stretches of conserved residues within the proline-rich N-terminal transactivation domain, particularly between residues 40-100, where AP-2α and AP-2β are 83% identical (87% similar). This region was shown to be essential for the transactivation function of human AP-2α (Williams and Tjian, 1991).

3.2.2 AP-2α and AP-2β mRNA and Protein in the Developing Embryo

AP-2α and *AP-2β* transcript levels in chick tissues at different developmental stages were analysed by Northern blotting (Fig. 3-3A). Probes specific to either *AP-2α* or *AP-2β* transcripts were used for this analysis. Three main forms of the *AP-2α* transcript were observed in the retina, ranging in size from 2.5 to 4 kilobases (kb). Four *AP-2β* transcripts of different sizes were present in the retina (1.8, 2.2, 3.5, >5 kb). Levels of both *AP-2α* and *AP-2β* mRNA peaked at ED10 in the retina. Transcripts were barely detectable at ED5.5, the earliest stage tested. Although at lower levels than in the retina, the *AP-2α* and *AP-2β* transcript patterns in the ED5.5 to ED10 brain were similar to those observed in the retina, with a significant decrease in levels at ED16. *AP-2β* mRNA was relatively abundant in the developing kidney, with 2-3X higher levels at ED16 compared to ED7. Of note, only the 2.2 kb form of *AP-2β* was expressed in kidney, suggesting that the three other forms of *AP-2β* mRNA found in brain and retina

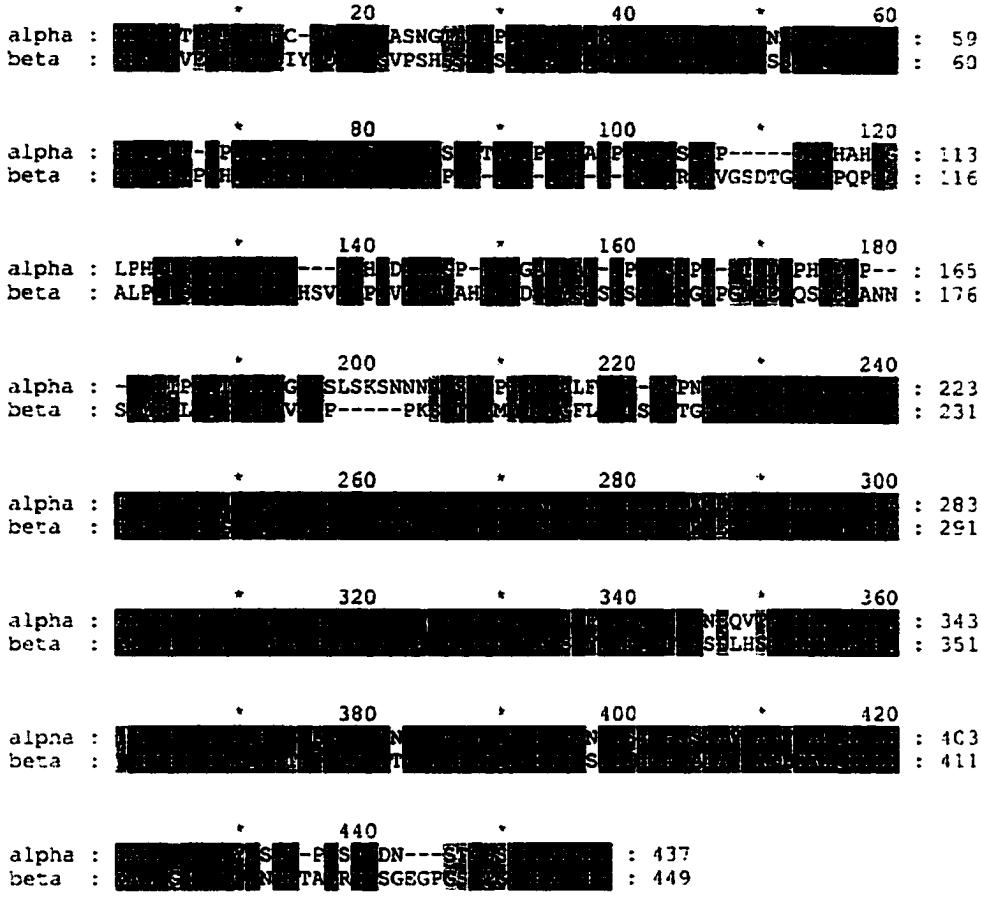

Figure 3-2. Comparison of chicken AP-2α and AP-2β amino acid sequences. The predicted amino acid sequences of chicken AP-2α and AP-2β were aligned, inserting dashes to maximize alignment. Black boxes indicate identical residues while shaded boxes indicate conserved residues. Similar residues are defined in Fig. 1.

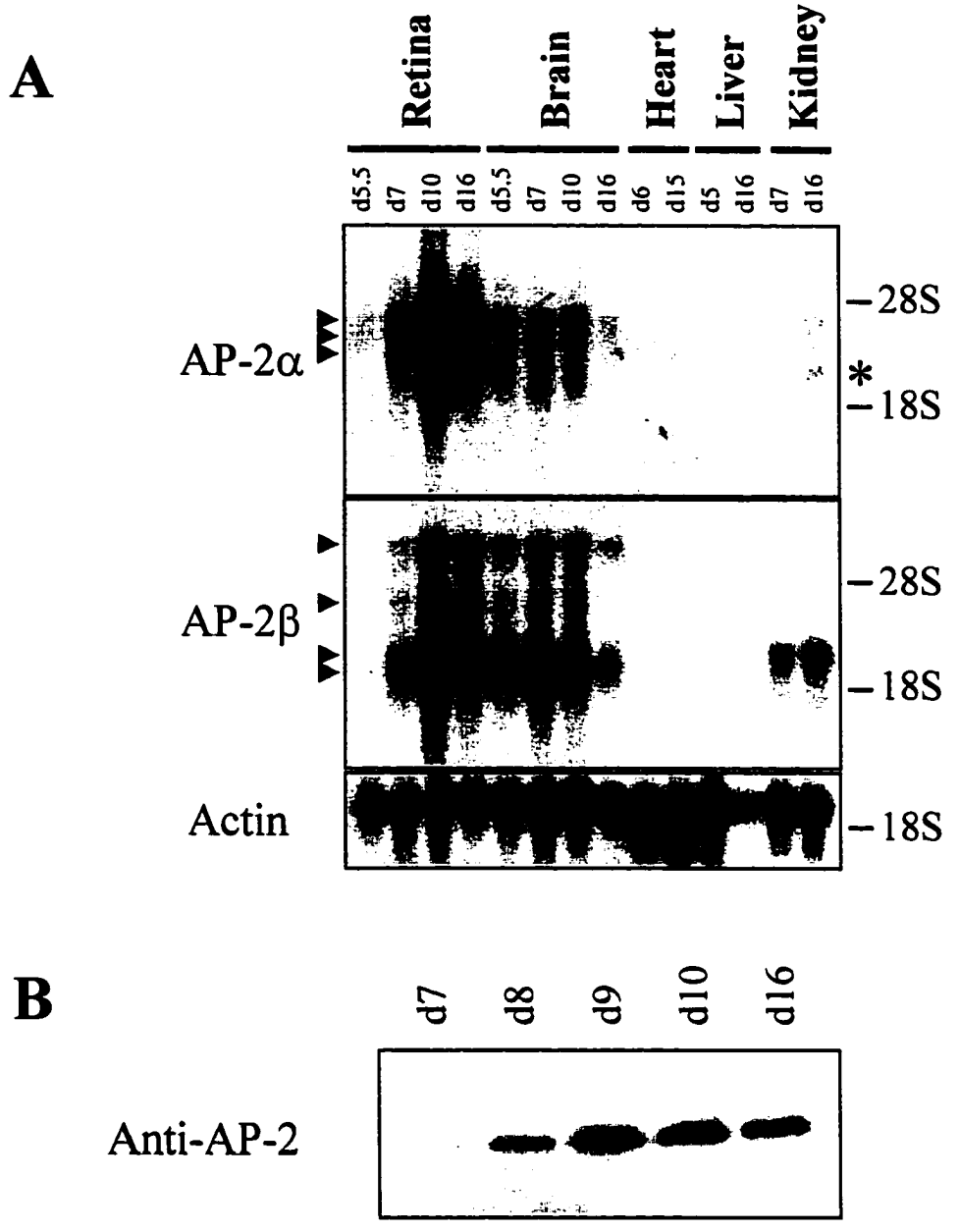

Figure 3-3. Northern and Western blot analyses of AP-2α and AP-2β expression. (A) Northern blots were prepared from poly(A)$^+$ RNA extracted from retina (ED5.5, 7, 10, 16), brain (ED5.5, 7, 10, 16), heart (ED6, 15), liver (ED5, 16) and kidney (ED7, 16). The filter was sequentially hybridized with ^{32}P-labelled: (i) 400 bp *AP-2β* cDNA, (ii) 400 bp *AP-2α* cDNA and (iii) actin DNA. The filter was stripped after each hybridization. The 28S and 18S rRNA markers are indicated on the side. The *AP-2α* and *AP-2-β* transcripts are indicated by the arrowheads. The asterisk indicates residual signal from the AP-2β hybridization. The extra bands obtained with the actin probe represent tissue-specific actin mRNAs. (B) Western blots were prepared from ED7, ED8, ED9, ED10 and ED16 total chick retina extracts (100 μg protein/lane). The proteins were electrophoretically separated by SDS-PAGE and transferred to a nitrocellulose filter. The filter was incubated with polyclonal anti-human AP-2 antibody at a 1:1000 dilution. The molecular mass of AP-2 is ~50 kDa.

are neural tissue-specific. *AP-2β* mRNA was not detected in either heart or liver, while low levels of *AP-2α* mRNA were observed in heart and liver on overexposed films, decreasing from ED5/6 to ED16.

Next, we analysed AP-2 protein levels in the ED7 to ED16 retina by Western blotting of whole cell lysates. The polyclonal AP-2 antibody used for this analysis recognizes both AP-2α and AP-2β based on its ability to immunoreact with either recombinant (our unpublished results) or *in vitro* translated AP-2α and AP-2β (Moser *et al.*, 1997 and Fig. 3 therein). Low levels of AP-2 protein were observed at ED7, followed by a rapid increase at ED8 and ED9, to reach maximal levels at ED9/ED10 (Fig. 3-3B). The reduction in AP-2 levels at ED16 reflects the decrease in *AP-2* transcript levels between ED10 and ED16.

3.2.3 Inhibition of *R-FABP/CAT* Reporter Gene Expression by AP-2

In a previous study, we found that an AP-2-immunoreactive transcription factor was binding to a consensus AP-2 recognition site within the promoter of the *R-FABP* gene (Bisgrove *et al.*, 1997). Transfection of pCAT reporter constructs with mutagenized AP-2 binding sites into undifferentiated ED5 retinal cultures (i.e. AP-2-negative) did not produce a significant decrease in CAT activity, suggesting that AP-2 down-regulates rather than up-regulates *R-FABP* expression. A repressor role for AP-2 was corroborated by Northern and Western blot analyses indicating that increased expression of AP-2 correlated with decreased levels of *R-FABP* mRNA (Godbout, 1993; Bisgrove *et al.*, 1997). To directly test the effect of AP-2 on *R-FABP* promoter activity, we co-transfected AP-2 expression constructs with an *R-FABP/CAT* reporter construct

containing 135 bp of *R-FABP* 5' flanking DNA including the consensus AP-2 recognition site (Fig. 3-4A). Four AP-2 expression constructs were prepared, with the coding regions of AP-2α or AP-2β in the sense or anti-sense orientation. Co-transfection of either AP-2α or AP-2β in the sense orientation decreased CAT activity by 50% compared to the anti-sense expression vectors (Fig. 3-4B) (AP-2α, p=0.01; AP-2β, p=0.0002). No decrease in CAT activity was observed upon co-transfection of either pCAT-basic vector or a construct containing a mutagenized AP-2 recognition site (data not shown). Overexpression of the AP-2 expression vectors was verified by Western blot analysis of transfected cell lysates (Fig. 3-4C). Consistency in amount of transfected DNA from experiment to experiment was monitored by Southern blot analysis of Hirt DNA (Fig. 3-4D). These results indicate that AP-2α and AP-2β are capable of repressing *R-FABP* promoter activity *in vivo*.

3.2.4 Localization of *AP-2α*, *AP-2β* and *R-FABP* Transcripts in the Retina

If AP-2 can down-regulate *R-FABP* transcription, then AP-2 positive cells should have reduced levels of *R-FABP* mRNA. We therefore studied the distribution of *AP-2α*, *AP-2β*, and *R-FABP* mRNA in the developing retina by *in situ* hybridization. No *AP-2* transcripts were detected in the ED3.5 (stage 21) retina, in contrast to *R-FABP* transcripts which were abundantly expressed throughout the tissue (data not shown). By ED5, *AP-2α* and *AP-2β*-positive cells (purple) were scattered throughout the inner neuroblastic layer (INBL) of the posterior retina (Fig. 3-5a,c), while *R-FABP* mRNA remained abundant throughout the retina (Fig. 3-5b). At ED7, the inner plexiform layer is beginning to form in the posterior retina and is not yet present in the anterior retina. The

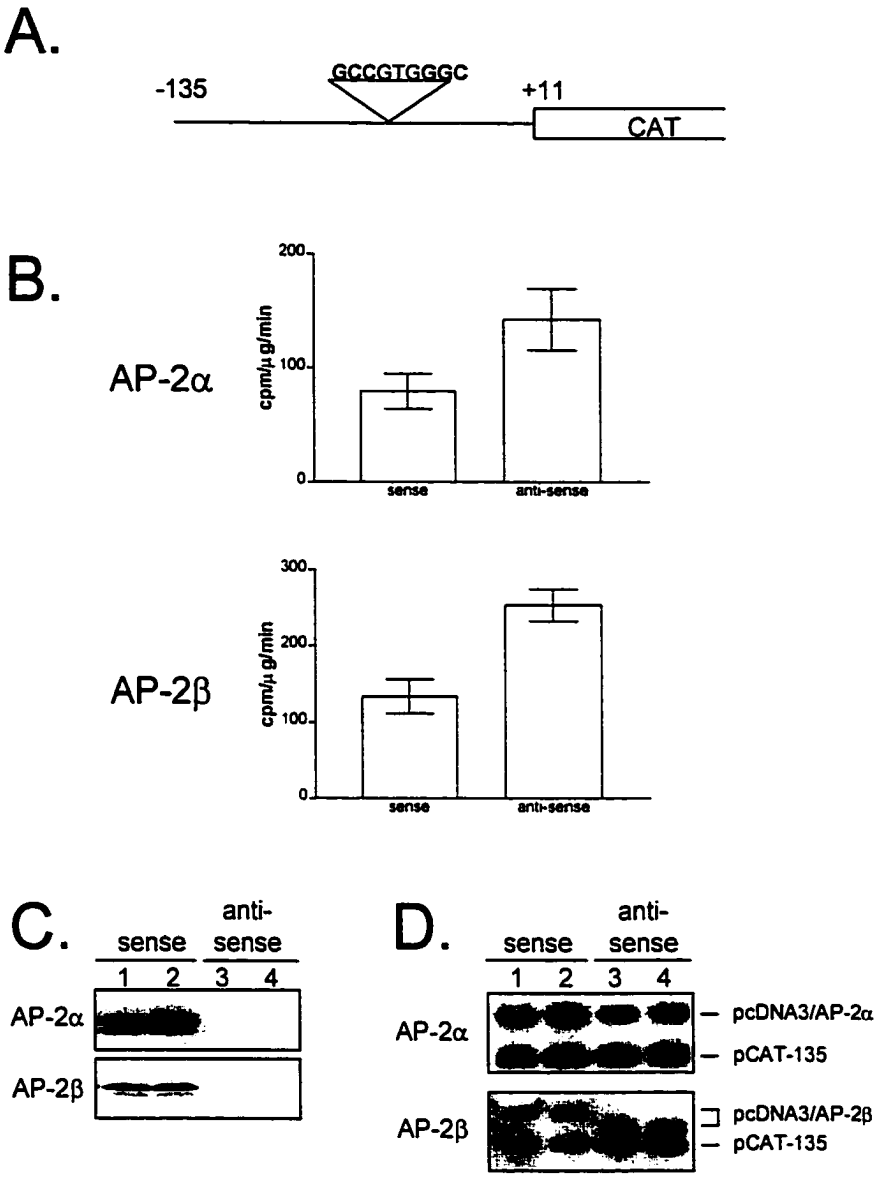

Figure 3-4. Overexpression of AP-2α and AP-2β represses *R-FABP* promoter activity. (A) Map of the pCAT-135 plasmid containing the *R-FABP* promoter region from -135 to +11 bp linked to the CAT reporter gene. The AP-2 consensus binding site (indicated in bold-face type) is located 59 bp upstream of the transcription initiation site. (B) Co-transfection of ED5 primary retinal cultures with AP-2α and AP-2β expression constructs and pCAT-135. Level of CAT activity obtained upon co-transfection of sense and anti-sense pcDNA3/AP-2α and pcDNA3/AP-2β expression constructs with pCAT-135 was measured as cpm/μg cell extract/min. The results shown represent the average of four experiments. The standard deviations are indicated by the error bars. (C) Western blot analysis of AP-2α and AP-2β levels in co-transfected retinal cultures. Retinal culture lysates were electrophoresed in a 10% SDS-PAGE gel and blotted onto a nitrocellulose filter. AP-2α and AP-2β were detected using anti-AP-2 antibody. The results from two separate experiments are shown, with the AP-2 constructs in both sense (lanes 1 and 2) and anti-sense (lanes 3 and 4) orientations. (D) Southern analysis of *Bam*HI-digested Hirt DNA from co-transfected retinal cultures. The blot was probed with pCAT-Basic and hybridizes to both the pCAT-135 construct and to the expression constructs. The results from two experiments are shown with AP-2α and AP-2β in the sense (lanes 1 and 2) and anti-sense (lanes 3 and 4) orientations.

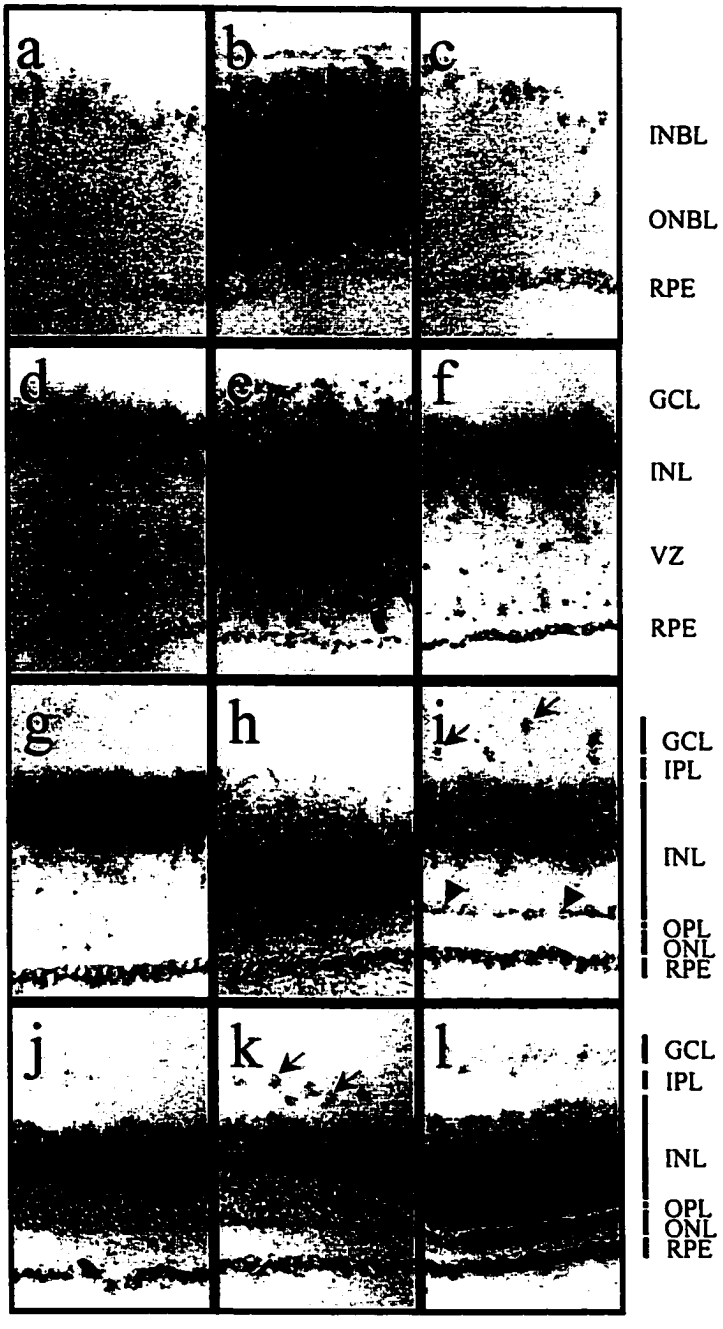

Figure 3-5. Localization of *AP-2α*, *AP-2β* and *R-FABP* transcripts in the developing chick retina. Non-radioactive *in situ* hybridization was performed to determine which cells in the developing retina express *AP-2α*, *AP-2β*, and *R-FABP* mRNA. Adjacent sections of ED5 (a-c), ED7 (d-f), ED11 (g-l) retina were hybridized with DIG- or fluorescein-labelled AP-2α (a,d,g), AP-2β (c,f,i), R-FABP (b,e,h) and CA-II (l) antisense RNA. The signal was detected by alkaline-phosphatase-coupled secondary antibody, generating a purple or brown color. Panel l was counterstained with ethyl green to show the cell layers. Panels j and k represent sections dually hybridized with either AP-2α (purple) (j) or AP-2β (purple) (k), and R-FABP (brown) antisense RNA. Displaced amacrine cells are indicated by arrows and the horizontal cell layer by arrowheads (i,k). The sections are oriented with the retinal pigment epithelium at the bottom and ganglion cells on top. GCL, ganglion cell layer; INBL, inner neuroblastic layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONBL, outer neuroblastic layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RPE, retinal pigment epithelium; VZ, ventricular zone. Photomicrographs were taken at 200X magnification using a Nikon Diaphot 300 microscope.

number of *AP-2α*- and *AP-2β*-positive cells was higher in the ED7 retina, with the zone of positive cells extending further towards the anterior retina (Fig. 3-5d,f). Positive cells were primarily confined to the inner nuclear layer (INL). The retina differentiates from the posterior to the anterior region and from the inner to the outer layer. AP-2-positive cells in both ED5 and ED7 are therefore located in the most differentiated aspect of the retina. In comparison, *R-FABP* transcripts were widespread throughout the INL as well as in the ventricular (proliferative) zone (VZ) of ED7 retina (Fig. 3-5e). By ED11, the inner and outer plexiform layers have formed, generating the multi-layered appearance of the mature tissue consisting of three nuclear layers separated by plexiform layers. The *AP-2α* transcripts were specifically found in the vitreal (inner) half of the INL, where amacrine cell bodies are located (Fig. 3-5g). *AP-2β* transcripts were found in amacrine cells, as well as in a thin layer of cells in the INL along the outer plexiform layer identified as horizontal cells based on their characteristic position within the retina (Fig. 3-5i). *AP-2β* transcripts were also detected in a subset of cells in the ganglion cell layer, likely displaced amacrine cells because of their location immediately next to the inner plexiform layer, if not slightly within it, as previously described (Génis-Gálvez *et al.*, 1977). In contrast to the *AP-2* transcripts, *R-FABP* mRNA in the ED11 retina is confined to the outer half of the INL (Fig. 3-5h). The nuclei of Müller glial cells are found predominantly in the middle of the INL while bipolar cell bodies are in the outer half of the INL. The dual *in situ* hybridization experiments shown in Fig. 3-5j and 3-5k demonstrate the mutually exclusive distribution of *R-FABP* (brown) mRNA and either *AP-2α* (Fig. 3-5j) or *AP-2β* (Fig. 3-5k) (purple) mRNA in the ED11 retina, dividing the INL into an inner AP-2(+)/*R-FABP*(-) region and an outer AP-2(-)/*R-FABP*(+) region.

In the mouse brain, radial glial cells express B-FABP during granule cell migration (Feng *et al.*, 1994; Kurtz *et al.*, 1994). Müller glial cells represent a type of radial glial cell specific to the retina and may serve a role similar to that of brain radial glial cells (Willbold and Layer, 1998). Carbonic anhydrase II (CA-II) has been shown by others to be specifically expressed in the Müller glial cells of the differentiated retina (Vardimon *et al.*, 1986). We carried out *in situ* hybridization with a CA-II riboprobe to determine whether *CA-II* and *R-FABP* mRNA have similar distribution profiles. Fig. 3-5l shows an ED11 retinal section hybridized to CA-II antisense RNA (purple). The section was counterstained with ethyl green in order to visualize all three nuclear layers. *CA-II* mRNA was primarily found in the middle of the INL, the usual location of Müller glial cell nuclei. As shown in Fig. 3-5h, *R-FABP* mRNA is also concentrated in the middle of the INL; however, it extends throughout the outer half of the INL where bipolar nuclei are located, suggesting a broader distribution for the *R-FABP* transcript.

3.2.5 Immunofluorescent Analysis of AP-2 in the Retina

To analyze the cellular distribution of AP-2 during retinal development, we performed immunofluorescence analysis of retinal sections at various developmental stages. As mentioned earlier, the antibody used in these experiments recognizes both AP-2α and AP-2β. There were occasional AP-2 positive cells distributed along the inner border of the ED5 posterior retina (Fig. 3-6a,b). As expected, the AP-2 transcription factor was restricted to the nucleus. A band of AP-2 positive cells, several nuclei deep, was found in the INL bordering the inner plexiform layer of ED7 retina (Fig. 3-6c,d). The AP-2-expressing cells extended into the anterior retina nearly to the ciliary

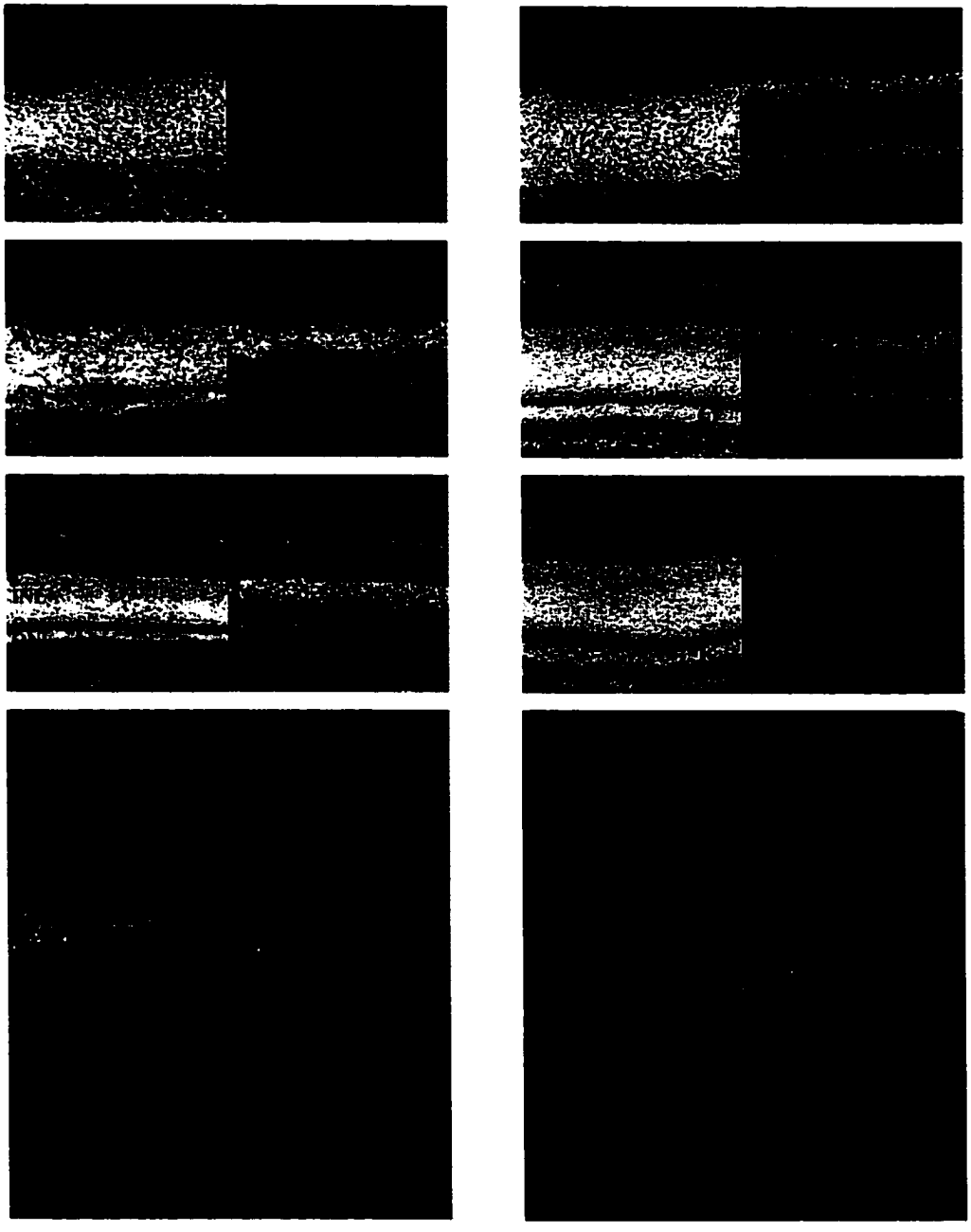

Figure 3-6. Immunofluorescent analysis of AP-2 in the developing chick retina. Sections from each developmental stage were stained with anti-AP-2 antibody (with FITC-conjugated secondary antibody, green) (b, d, f, h, and j) and counterstained with the fluorescent dye Hoescht 33258 to label the nuclei blue (a, c, e, g, and i). Retinal sections were prepared from: (a,b) ED5; (c,d) ED7; (e,f) ED10; (g, h) ED16; and (i, j) ED19 stage embryos. The retinal layers are indicated in (i), while the positive cell layers are labeled in (j). (k,l) ED16 retina stained with Hoescht 33258 (k) and monoclonal anti-syntaxin (TRITC-labeled secondary antibody, red) (l). Photomicrographs were taken at 200X magnification using a Nikon Diaphot 300 microscope. (m,n) Confocal microscope images representing dual AP-2 (green) and syntaxin (red) immunofluorescent staining of ED16 retina are shown at (m) 200X and (n) 600X. Confocal images were obtained using a Molecular Dynamics Multiprobe 2001 microscope. Scale bars, (m) 50 μm and (n) 20 μm. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; da, displaced amacrine cells; a, amacrine cells; and h, horizontal cells.

epithelium, thinning to a single layer of cells in the mid-peripheral retina. A discontinuous band of nuclei was also found immediately vitreal to the presumptive outer plexiform layer, the location of horizontal neurons. Expression in this region was restricted to the posterior third of the retina. The positive cells in the INL between the two bands of AP-2 expression are presumably migrating amacrine cells. Migrating cells would still be expected at ED7 because amacrine neurogenesis continues until ED10 (Prada *et al.*, 1991). As with the *in situ* hybridization experiments, positive cells were also observed in the ganglion cell layer and likely represent displaced amacrine cells.

By ED10, there was a greater number of AP-2-expressing amacrine cells, with most, if not all of the cells in the vitreal half of the INL being AP-2 positive. A positive signal was also detected in the INL bordering the outer plexiform layer where horizontal cells are located (Fig. 3-6e,f). As well, AP-2 was present in the putative displaced amacrine cells that line the outer border of the ganglion cell layer. Similar results were obtained at ED16 when the retinal strata are more defined (Fig. 3-6g,h). There was a sharp band of expression approximately 10 cells wide spanning the inner third of the INL. The staining of the horizontal cell layer was more uniform than at ED10 with a depth of 3-4 cells. The staining of amacrine and horizontal cells at ED19 had condensed into tighter layers, with AP-2-positive amacrine cells spanning approximately 6 nuclei in the posterior retina while AP-2-positive horizontal cells spanned 1-2 nuclei (Fig. 3-6i,j).

Syntaxin, a protein involved in synaptic vesicle docking, is a marker of amacrine and horizontal cells (Barnstable *et al.*, 1985; Alexiades and Cepko, 1997). Immunostaining of an ED16 retinal section with anti-syntaxin revealed extensive staining of the inner plexiform layer, as expected for a protein involved in synaptic function (Fig.

3-6k,l). Staining of the outer plexiform layer was somewhat weaker. Cytoplasmic staining of amacrine and horizontal cells by anti-syntaxin can be observed in the inner nuclear layer. To determine if syntaxin and AP-2 co-localize, we carried out double immunofluorescent labeling. Co-localization of cytoplasmic syntaxin (red) and nuclear AP-2 (green) to the same cells can be seen in Fig. 3-6m,n. These data provide further evidence that the AP-2 positive cells in the INL are amacrine and horizontal cells, as expected based on their position.

3.3 Discussion

In recent years, considerable progress has been made in identifying genes that are developmentally regulated in the retina (Freund *et al.*, 1996). Functional analyses of some of these genes have generated valuable information regarding the molecular mechanisms controlling cell proliferation and cell fate determination in the developing retina (Fini *et al.*, 1997; Reh and Levine, 1998). For example, experiments by Austin *et al.* (1995) have shown that the transmembrane receptor Notch-1 and its ligand Delta affect the growth and differentiation of retinal cells along the ganglion pathway. However, many gaps remain in our understanding of the cascade of events underlying the retinal maturation process.

The elevated levels of *R-FABP* mRNA in the chick retina from ED3 to ED7 followed by a dramatic decrease in transcript levels from ED7 to ED19 suggest that the transcription of the *R-FABP* gene must be tightly regulated in the developing embryo (Godbout, 1993). The presence of a consensus AP-2 binding site in the *R-FABP* promoter and the binding of an AP-2-immunoreactive nuclear protein to this region indicate that one or more members of the AP-2 family are likely involved in the regulation of the *R-FABP* gene (Bisgrove *et al.*, 1997). In our earlier study, we demonstrated the presence of AP-2 mRNA and protein in the chick retina (Bisgrove *et al.*, 1997). At that time, we were not able to identify which *AP-2* genes were expressed in the retina because the different members of the AP-2 family had not been cloned in chicken. The sequence of chicken *AP-2α* cDNA has since been reported by Shen *et al.* (1997) and here we have cloned *AP-2β* from a chick retina cDNA library. We now show that both the *AP-2α* and *AP-2β* genes are expressed at elevated levels in the chick retina.

The temporal profiles of both genes are similar, with barely detectable levels in the undifferentiated ED5 retina and a dramatic increase in transcript and protein levels from ED7 to ED9/10. Maximal levels are observed at ED9/10 when few retinal cells are proliferating and the great majority of cells are committed to a specific differentiation pathway (Prada *et al.*, 1991). Moser *et al.* (1997a) did not detect either *AP-2α* or *AP-2β* mRNA in the undifferentiated retina of day 15 mouse embryos by *in situ* hybridization although transcripts were detected in the adult mouse eye by RT-PCR (Moser *et al.*, 1995). These results suggest a similar expression profile for the AP-2 factors in mammalian and avian retina. Based on their patterns of expression, we propose that the AP-2 factors are required during the early steps of retinal differentiation.

The *AP-2α* and *AP-2β* transcripts are also expressed in the developing brain and kidney, although levels of *AP-2α* mRNA are considerably lower in these tissues than in the retina. The *AP-2* transcripts are either barely detectable in heart and liver (*AP-2α*) or not detectable (*AP-2β*). Multiple forms of the *AP-2α* and *AP-2β* transcripts are observed in retina and brain, likely representing alternative splicing events or usage of different transcription initiation sites as previously observed for murine *AP-2α* (Meier *et al.*, 1995). In contrast, there is only one major form of the *AP-2β* transcript in kidney suggesting that the additional forms found in retina and brain may be neural tissue-specific.

Both *AP-2* genes are selectively expressed in subsets of retinal cells in the chick embryo: AP-2α is restricted to amacrine cells while AP-2β is specifically found in amacrine, horizontal and putative displaced amacrine cells. AP-2α and AP-2β were detected in the ED5 retina but not at ED3.5. Neurogenesis of amacrine and horizontal

cells begins at ED3 and ED4, respectively (Prada *et al.*, 1991). It is unlikely that AP-2 plays a role in the specification of these cell lineages because AP-2 appears to be induced shortly after the cells are committed to a particular lineage. However, the early and cell-specific expression of AP-2α and AP-2β suggest a role for AP-2 in determining the initial events associated with amacrine and horizontal cell differentiation. Others have reported the presence of both AP-2α and AP-2β in specific cell types, leading to the hypothesis that combinations of AP-2 factors may cooperate to regulate the expression of specific sets of genes (Moser *et al.*, 1997b). Some of the genes activated or repressed by the combination of AP-2α and AP-2β in amacrine cells may therefore be different from those activated or repressed by AP-2β in horizontal cells.

Analysis of both *AP-2α*-null mice and chimeric mice indicates a role for AP-2α in several morphogenic pathways including eye formation, limb pattern formation and development of the neural tube, head and body (Schorle *et al.*, 1996; Zhang *et al.*, 1996; Nottoli *et al.*, 1998). The ocular defects in these mice are so severe and appear so early that it has not been possible to assess whether AP-2α plays a role in retinal differentiation. In contrast to *AP-2α* gene knock-outs, *AP-2β*-null mice show no abnormalities other than renal malformation (Moser *et al.*, 1997a). However, the mouse retina is still undifferentiated when the *AP-2β-/-* mice die of polycystic kidney disease one to two days after birth. Expression of amacrine and horizontal cell differentiation markers occurs primarily after birth (Grün, 1982; Cepko *et al.*, 1996). A role in retinal differentiation may therefore only be identified if the lifespan of *AP-2β-/-* mice can be extended. Alternatively, different members of the AP-2 family may be able to

compensate for each other in some cell types or tissues and retinal abnormalities may not be observed unless multiple *AP-2* genes are inactivated.

Although no *AP-2γ* cDNA clones were obtained by screening an ED16 chick retina library, Oulad-Abdelghani *et al.* (1996) detected *AP-2γ* mRNA in the adult mouse retina by *in situ* hybridization, specifically in the inner nuclear layer and ganglion cell layer although the cells expressing this transcript were not identified. *AP-2γ* was not expressed in retinal precursor cells of day 11.5 mouse embryos as determined by *in situ* hybridization (Chazaud *et al.*, 1996). Our Northern blot analysis of chick retina using mouse *AP-2γ* cDNA as the probe generated a barely detectable signal (data not shown), suggesting that AP-2γ is not expressed or weakly expressed in the chick retina.

Examination of the regulation of the *AP-2* genes to determine how AP-2 expression is directed to horizontal and amacrine cells may lead to insight into the nature of the factors involved in the determination and differentiation of these two classes of neurons. In some cell types, such as P19 embryonal carcinoma and NT2 teratocarcinoma, AP-2 expression is inducible by retinoic acid (Philipp *et al.*, 1994; Lüscher *et al.*, 1989). These cells undergo neuronal differentiation in response to retinoic acid. Induction of AP-2 could therefore couple response to retinoic acid with the regulation of genes involved in neuronal differentiation. It will be interesting to determine if retinoic acid, previously implicated in retinal development (Dräger and McCaffery, 1996), can up-regulate AP-2 in differentiating amacrine and horizontal cells. If retinoic acid participates in this process, it is likely to be in concert with differentially expressed proteins involved in retinoic acid metabolism. It is noteworthy that, unlike other retinal cells, the processes of amacrine, horizontal and displaced amacrine cells are

all confined to their neighbouring plexiform layer, providing a commonality between these cell types. Transcription factors restricted to retinal cells with this property may be involved in *AP-2* regulation.

Although the AP-2 family has most commonly been associated with transcriptional activation (Duan and Clemmons, 1995; Dyck and Fliegel, 1995), it has also been shown to repress the transcription of several genes (Gaubatz *et al.*, 1995; Getman *et al.*, 1995; Chen *et al.*, 1996). If members of the AP-2 family are involved in the repression of *R-FABP* transcription, one would expect *R-FABP* mRNA to be excluded from cells expressing AP-2. Northern blot analysis of *AP-2α*, *AP-2β* and *R-FABP* indicates a negative correlation between *AP-2α/β* and *R-FABP* mRNA levels during retinal development, with high *R-FABP*/low AP-2 in the undifferentiated retina and low *R-FABP*/high AP-2 in the differentiated tissue. Furthermore, *in situ* hybridization and immunofluorescent analyses show mutually exclusive expression patterns for the AP-2 factors and *R-FABP* transcripts starting at ED10. While *R-FABP* mRNA is abundantly and widely expressed in the undifferentiated retina from ED3.5 to ED7, the transcript becomes restricted to the outer portion of the inner nuclear layer with differentiation. This region of the retina contains cell bodies from Müller glial cells and from bipolar cells. *R-FABP* transcripts are excluded from amacrine and horizontal cells, in contrast to AP-2α and AP-2β which are selectively expressed in these cells. *R-FABP* mRNA is also absent in photoreceptors and ganglion cells, neither of which expresses AP-2, suggesting that other transcription factors are involved in *R-FABP* repression in these cell types.

In addition to the correlative studies showing that *R-FABP* mRNA is excluded from AP-2-expressing cells, our DNA transfection experiments indicate that AP-2α and AP-2β can directly repress *R-FABP* promoter activity *in vitro*. Overexpression of either AP-2α or AP-2β in primary retinal cultures resulted in a 50% decrease in the activity of the *CAT* reporter gene under the control of the *R-FABP* promoter containing the AP-2 recognition site. There was no decrease in CAT activity when either AP-2α or AP-2β antisense expression constructs were used or when a pCAT construct carrying a mutagenized AP-2 site was tested.

Murine brain FABP, expressed in radial glial cells, is involved in the establishment of the radial glial fiber system guiding neuronal migration and may also play a role in relaying inductive signals for neuronal differentiation (Feng *et al.*, 1994; Kurtz *et al.*, 1994). Similar to B-FABP, R-FABP (initially expressed in neuroectodermal precursor cells and later restricted to Müller glial cells and/or bipolar cells) may be involved in the formation of a migratory system for retinal neurons. The production of R-FABP by Müller glial cells, which span the entire width of the retina, may play a role in the positioning and differentiation of all neuronal cell types in this tissue.

In summary, we have used two approaches to determine whether members of the AP-2 family down-regulate *R-FABP* transcription. First, we identified the AP-2 factors that are expressed in the retina and demonstrated mutually exclusive expression profiles for two members of this family and *R-FABP* transcripts. Second, we showed by DNA transfection analysis that overexpression of either AP-2α and AP-2β in primary retinal cultures results in a decreased activity from the *R-FABP* promoter. These data suggest that these two AP-2 factors can repress *R-FABP* transcription. Given the number of

genes shown to be regulated by AP-2 in different systems, repression of the *R-FABP* gene by AP-2α and AP-2β probably represents only one component of their overall transcriptional regulation program in the retina. A number of transcription factors are expressed in the retina; however, little is known regarding their mechanisms of action and the nature of their target genes. The identification of two AP-2 transcription factors specifically expressed in the amacrine and horizontal cells of the retina, in conjunction with the discovery that *R-FABP* likely represents a target gene for these transcription factors, provide the first steps towards the elucidation of one of the molecular pathways underlying retinal differentiation.

3.4 Experimental Procedures

3.4.1 Screening of the ED16 Chick Retina cDNA Library

Preparation of the cDNA library was as previously described (Godbout, 1993) except that the cDNA was produced from ED16 chick retina poly(A)$^+$ RNA. Approximately 2 X 10^5 bacteriophage were sequentially hybridized with: (i) a 1.2 kb *Eco*RI/*Hin*dIII human *AP-2α* cDNA fragment (IMAGE EST clone ID 142203, Genome Systems Inc), (ii) a 1.5 kb *Eco*RI/*Hin*dIII *AP-2β* cDNA fragment derived from human adult retina (IMAGE EST clone ID 362684, Genome Systems Inc) and (iii) mouse *AP-2γ* cDNA (a kind gift from R. Buettner, University of Regensburg Medical School, Germany). Filters were washed at 45°C in 0.1X SSC and 0.1% SDS. Positive clones were purified by additional rounds of screening. The cDNA ends of positive clones were sequenced using an ABI 310 automated sequencer and the sequences analyzed using the BLAST program of the National Center for Biotechnology Information, NIH. The sequence of the *AP-2β* cDNA was generated by sequencing both strands of a cDNA containing the entire coding region. The *AP-2β* cDNA sequence has been submitted to GenBank (accession # AF065140).

3.4.2 Northern Blot Analysis

Poly(A)$^+$ RNA was isolated from retina, brain, heart, liver, and kidney at the developmental stages indicated in the legend to Fig. 3-3. The mRNAs were electrophoresed in a 6% formaldehyde-1.5% agarose gel in MOPS buffer and transferred to nitrocellulose. The filter was sequentially hybridized to a 400 bp *Pst*I fragment derived from the 3' end of chicken *AP-2α* cDNA and to a 400 bp PCR-amplified DNA

fragment extending from bp 301-700 of chick *AP-2β* cDNA. Both probes were selected on the basis of their low level of similarity to other members of the AP-2 family. Filters were washed at 45°C in 0.1X SSC, 0.1% SDS. The filter was hybridized to mouse actin cDNA to control for lane to lane variation in mRNA levels.

3.4.3 Western Blot Analysis

Chick retinas were dissected at various stages between ED5 and ED19, and were immediately frozen in liquid nitrogen. Whole cell lysates were prepared by lysing thawed retina in 4 volumes of 1X SDS-PAGE buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol). Sample viscosity was reduced by several passes through a 21 gauge syringe and then clarified by ultracentrifugation. The Bradford assay was used to quantitate the protein content of each extract (Bio-Rad Laboratories). Cell lysates (100 μg protein/lane) were electrophoresed in a 10% SDS-PAGE gel and transferred to nitrocellulose. Blots were stained with 0.005 % CTPS (copper phthalocyanine 3, 4', 4'', 4''' tetrasulfonic acid in 12 mM HCl) to mark the position of the standards. The filter was incubated with affinity-purified anti-human AP-2 polyclonal antibody at a 1:1000 dilution (Santa Cruz Biotechnology). Antigen-antibody interactions were visualized with the ECL detection system (Amersham-Pharmacia) using horseradish peroxidase-conjugated anti-rabbit IgG antibody at a 1:100,000 dilution (Jackson ImmunoResearch, Inc.).

3.4.4 *In Situ* Hybridization

Sense and antisense riboprobes labeled with digoxigenin (DIG) or fluorescein were synthesized by *in vitro* transcription with T3 or T7 RNA polymerase according to the manufacturer's protocol (Boehringer Mannheim). An 850 bp chick *AP-2α* partial cDNA extending from bp 826 to 1676 (GenBank Accession No. U72992) served as the template for synthesis of the AP-2α riboprobe. A 1.2 kb *AP-2β* cDNA fragment containing the entire coding region (GenBank Accession No. AF065140) served as the template for the AP-2β riboprobe. The *R-FABP* riboprobe has been described previously (Godbout *et al.*, 1995). Whole embryos (ED5) or eyes (ED7 and ED10) were fixed overnight in 4% PBS-buffered paraformaldehyde at 4°C, cryoprotected overnight in 30% sucrose and embedded into O.C.T. (Tissue-Tek, Miles Inc.). Frozen sections were pretreated essentially as recommended by Boehringer Mannheim except that the sections were dehydrated through an ethanol gradient after acetylation. The sections were then prehybridized at 55°C in hybridization solution minus the probe (40% formamide, 10% dextran sulphate, 1X Denhardt's solution, 4X SSC, 10 mM DTT, 1 mg/ml yeast tRNA, and 1 mg/ml denatured herring sperm DNA). Riboprobes were denatured at 70°C for 10 minutes prior to overnight hybridization with the sections at 55°C. Posthybridization washes were as previously described (Belecky-Adams *et al.*, 1997). Sections were blocked with PBS containing 2% blocking reagent (Boehringer Mannheim) and 0.3% Triton X-100. DIG (or fluorescein) label was detected using an alkaline phosphatase (AP)-conjugated anti-DIG (or anti-fluorescein) antibody at a 1:1000 dilution with polyvinyl alcohol enhancement as described (Jowett, 1997).

3.4.5 Immunofluorescence

Frozen sections were prepared by embedding freshly dissected chick eyes in O.C.T., followed by cryostat sectioning (6 μm). All sections were in the nasolateral plane and intersected the lens. The sections were fixed in acetone, washed with PBS and blocked in 10% skim milk. Manipulations involving fluorescent reagents were performed in the dark. Sections were incubated with a 1:100 dilution of rabbit polyclonal anti-AP-2 antibody (Santa Cruz Biotechnology) or a 1:100 dilution of monoclonal anti-syntaxin antibody (Sigma Immunochemicals). Immunoreactivity was detected using a 1:100 dilution of the secondary antibodies, FITC-conjugated anti-rabbit IgG antibody (Boehringer Mannheim) or TRITC-labeled anti-mouse IgG antibody (Jackson ImmunoResearch, Inc.). Control slides were exposed to the secondary antibodies only. Slides were counterstained with the fluorescent nuclear stain Hoescht 33258 (Molecular Probes). Slides were mounted with Fluorosave (Calbiochem) and visualized using a Nikon Diaphot 300 microscope equipped with epifluorescence attachment. Confocal images were obtained using a Molecular Dynamics Multiprobe 2001 microscope (Biological Sciences Microscopy Unit, University of Alberta).

3.4.6 DNA Transfection Analysis

Expression plasmids were prepared by inserting the coding regions of chicken *AP-2α* and *AP-2β* into pcDNA3 (Invitrogen) in the sense and anti-sense orientations. To prepare the AP-2α construct, an *Eco*RI fragment extending from bp 1-1675 of the previously cloned chicken *AP-2α* cDNA (a kind gift from J. Richman, University of

British Columbia, Canada) was inserted into the *Eco*RI site of pcDNA3. To prepare the AP-2β construct, the coding region of the *AP-2β* cDNA obtained from our ED16 chick retina library was PCR-amplified with *pfu* polymerase (Stratagene) and the primers: 5'-ATGCTCTGGAAACTGGTTG-3' and 5'-TCATTTTCTGTGTTTCTCTTCC-3' (start and stop codons are underlined). The product was cloned into the *Eco*RV site of pcDNA3 and sequenced to verify that no mutations were introduced by PCR. The *R-FABP* promoter reporter construct (pCAT-135) and calcium phosphate mediated transient transfections into ED5 primary retinal cultures have been previously described (Bisgrove *et al.*, 1997). Co-transfections were carried out using 5 μg of reporter plasmid and 10 μg of either pcDNA3/AP-2α or pcDNA3/AP-2β. Overexpression of AP-2α and AP-2β was confirmed by Western blotting of whole cell lysates of transfected cells. Transfection efficiency was monitored by preparation of Hirt DNA from the transfected cells followed by Southern blotting using pCAT-Basic (Promega) as the probe.

3.5 Acknowledgements

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body wall defects in mice lacking transcription factor AP-2. *Nature* **381**: 238-241.

Chapter 4

Regulation of B-FABP expression by Differential Phosphorylation of Nuclear Factor I in Malignant Glioma[1]

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4.1 Introduction

Gliomas comprise a diverse group of central nervous system neoplasms of glial origin. The most common type of glioma, malignant glioma (MG), is usually fatal within two years of diagnosis despite conventional treatment. Improved therapies for this aggressive tumor will be derived from studying the mechanisms of normal glial differentiation and novel therapies designed to force glioma cells to undergo differentiation represent an active area of research. To this end, we are characterizing the pathways governing glial differentiation. Glial fibrillary acidic protein (GFAP), a cytoskeletal protein expressed in mature astrocytes, is often used as a histological marker of glioma differentiation. Expression of GFAP in MG cell lines results in a more differentiated phenotype and decreased invasive potential (Weinstein *et al.*, 1991; Rutka *et al.*, 1994). We have recently shown that GFAP(+) MG cell lines co-express brain fatty acid binding protein (B-FABP), a 15 kDa protein expressed in the developing brain and retina (Godbout *et al.*, 1998). Expression of B-FABP in MG is intriguing because murine B-FABP has been proposed to be a radial glial differentiation factor, implicated in the establishment and maintenance of the radial glial fiber system (Feng *et al.*, 1994). This network of radially oriented glial processes in the developing cerebellum and cerebral cortex, serves to guide immature migrating neurons to their correct location (for review, see Hatten, 1999). Addition of anti-B-FABP antibodies to neuronal-radial glial co-cultures prevents normal neuronal-induced differentiation of the radial glial cells, suggesting that B-FABP functions as an extracellular signaling molecule involved in establishing and maintaining radial glial differentiation.

We are studying the transcriptional regulation of the *B-FABP* gene in malignant glioma cell lines to determine how it is differentially expressed. Here we describe our initial characterization of the *B-FABP* promoter in MG cell lines and present results suggesting that members of the CTF/NFI (CCAAT-binding transcription factor/nuclear factor I) gene family may contribute to its differential expression. We have identified two NFI binding sites in the proximal *B-FABP* promoter using *in vitro* DNA binding assays, one of which is crucial for *B-FABP* promoter activity in transient transfection assays. NFI proteins in B-FABP positive and negative MG cell lines are differentially phosphorylated. We present evidence that expression of a cell-specific phosphatase is responsible for differential expression of the *B-FABP* gene in malignant glioma.

4.2 Materials and Methods

Cell lines. All malignant glioma cell lines were obtained from Drs. Rufus Day III and Joan Turner, Department of Oncology, University of Alberta and Cross Cancer Institute.

Cloning of the human *B-FABP* gene promoter. Isolation of the human *B-FABP* gene has been previously described (Godbout *et al.*, 1998). A 2980 bp EcoRI fragment containing exons I, II and 1.8 kb of upstream flanking sequence was subcloned in pBluescript II (Stratagene) and sequenced using standard techniques. The *B-FABP* transcriptional start site was mapped by primer extension using the Primer Extension Sytem kit (Promega) and the primer 5'- CTC TTT AGA GAC AGG AGC GGG GAT C-3', which corresponds to the region between +43 and +67 bp within the 5' untranslated region.

Transfections and CAT Assay. *B-FABP* promoter activity was monitored in pCAT-basic (Promega) using a series of reporter gene constructs which put the chloramphenicol acetyltransferase (CAT) gene under the control of various portions of the *B-FABP* promoter. A deletion series extending from a common downstream PstI (+24) site to various 5' flanking region restriction sites was prepared: pCAT-1.8 insert extended upstream to the EcoRI (-1765) site, pCAT-1.2 to PstI (-1219), pCAT-660 to DraI (-660), pCAT-240 to AluI (-240) and pCAT-140 to XhoI (-138). Site-directed mutagenesis of the fp1 and fp3 NFI DNA binding sites were carried out by the method of Hemsley *et al.* (1989). To introduce mutations in the fp1 NFI binding site, inverse PCR was performed on pCAT-240 using *pfu* polymerase (Stratagene) and the head-to-head

mutagenic primer set 5'-ATC ACT AAA TTT TTG CCC ACC CTC and 5'-TTA AAT TGC AAA CAC ACC CC (NFI binding site is in bold, underlined nucleotides represent the GG to AA mutation). After gel purification and recircularization, mutagenesis of the NFI binding site was confirmed by automated sequencing (ABI Prism 310). A 190 bp XhoI/XbaI fragment containing the mutagenized fp1 NFI binding site was exchanged for the corresponding wildtype region in pCAT-1.8 to generate pCAT1.8(fp1*). Mutations were introduced into the fp3 NFI binding site in a similar manner, except that the starting template was a 1.2 kb PstI fragment subcloned into pBluescript II and the mutagenic primer set was 5'-AGC CCC ATA AAA TCC CTG CCG AG-3' (NFI binding site in bold, TG to AA mutation underlined) and 5'-GGA GGC AGG GAA CGG GAA AAT GAG-3'. The 1.2 kb PstI fragment containing the mutant fp3 NFI binding site was used to replace the corresponding wildtype fragment in pCAT-1.8 to generate pCAT-1.8(fp3*). The double mutant construct (pCAT-1.8(fp1*3*)) combining both the fp1 and fp3 mutations was prepared by replacing the 1629 bp wildtype EcoRI/XhoI fragment of pCAT1.8(fp1*) with the corresponding region of pCAT-1.8(fp3*) which carries the mutant fp3* region. The resulting clones were sequenced over the mutated region to confirm that only the desired change was introduced.

Transient transfections into U251 MG cells were carried out using the standard calcium phosphate method. Cells were transfected at 20-25% confluency with 10 μg of each plasmid. The cells were harvested 48 hours post-transfection and cytosolic extracts prepared by multiple freeze-thaw cycles. CAT activity was monitored by incubating each extract with D-threo-[dichloroacetyl-1,2-^{14}C]-chloramphenicol (Dupont-NEN Research Products, Boston, MA) and n-butyryl coenzyme A (Sigma) in 250 mM Tris-HCl (pH 8),

followed by extraction of reaction products into xylene and counting by liquid scintillation. Reported values are the average of at least four independent transfection experiments. To control for variation in transfection efficiency, CAT activities were normalized using values obtained by densitometric scanning of Southern blots containing BamHI digested Hirt DNA prepared from each sample (Hirt, 1967). Southern blots were prepared by capillary transfer of DNA onto nitrocellulose filters and probed with nick-translated pCAT-basic.

DNase I footprinting analysis. Singly end-labeled DNA probes were produced by linearizing plasmids containing either a 227 bp AluI fragment (-13 to –240 bp) or a 283 bp EcoO109I/XhoI fragment (-138 to –418 bp) of the *B-FABP* promoter with either XbaI or HindIII (enzymes which cut in the pBluescript polylinker region) and then radiolabeled by filling-in with Klenow polymerase. The radiolabeled insert was released by digestion with either HindIII or XbaI and purified by PAGE and electroelution. The G+A chemical sequencing reactions were prepared as per Belikov and Wieslander (1995). Nuclear extracts were prepared from B-FABP negative (T98) and positive (M016, U251) malignant glioma cell lines as described by Roy *et al.* (1991).

DNase I footprinting was carried out as previously described except that polyvinyl alcohol was omitted from the binding buffer (Jones *et al.*, 1985). Briefly, radiolabeled DNA probe (10 fmol) was incubated with indicated MG nuclear extract (20 μg) in binding buffer for 15 minutes on ice, followed by 2 minutes at room temperature. An equal volume of 5 mM $CaCl_2$/10 mM $MgCl_2$ was then added, followed by DNase I (Worthington, DPFF grade) to 1 μg/mL. DNase I digestion was allowed to proceed for 60 seconds at room temperature before addition of stop buffer (0.2 M NaCl, 20 mM

EDTA, 1% SDS). The DNA was purified by phenol/chloroform extraction and ethanol precipitation. Samples were resuspended in formamide loading buffer and denatured at 90°C for 3 minutes prior to electrophoresis in an 8% sequencing gel.

Electrophoretic mobility shift assay (EMSA). The mobility shift assay was carried out as per O'Brien *et al.* (1995). Complementary oligonucleotides (see Table 4-1) were annealed and radiolabeled by filling-in with Klenow polymerase in the presence of [α–^{32}P]-dCTP or [α–^{32}P]-dATP (Dupont-NEN Research Products, Boston, MA). Unincorporated label was removed by Biogel P6 spin column chromatography (Bio-Rad Laboratories). Nuclear extracts were prepared from five B-FABP negative (A172, CLA, M021, T98 and U87) and five B-FABP positive (M016, M049, M103, U251 and U373) malignant glioma cell lines as described by Roy *et al.* (1991). Nuclear extracts (4 μg) were pre-incubated with 2 μg of poly [dI-dC] in binding buffer (20 mM HEPES pH 7.9, 20 mM KCl, 1 mM spermidine, 10 mM DTT, 10% glycerol, and 0.1% NP-40) for 10 minutes at room temperature. When included, a hundred-fold molar excess of unlabelled competitor oligonucleotide was added during this pre-incubation stage. Competitor oligonucleotides containing binding sites for AP-2, CTF/NFI, or Sp1 were obtained from Promega. For supershift experiments, 2 μL of either anti-NFI antibody (a kind gift from Dr. Naoko Tanese, NYU Medical Center, New York) or control anti-AP-2 (Santa Cruz Biotechnology) was included in the binding reaction. Labelled probe DNA (25 fmol) was added and incubated for 20 minutes at room temperature. DNA-protein complexes were resolved in pre-run 6% polyacrylamide gels (0.5X TBE) at 150V.

Dephosphorylation of nuclear extracts using potato acid phosphatase (PAP) was carried out by incubating 4 μg of T98 or U251 nuclear extract with 0.05, 0.5, 1.0, or 1.5

Table 4-1. Sequences of synthetic oligonucleotides used for this paper

fp1	TTA AAT CAC TGG ATT TTT GCC CAC C A GTG ACC TAA AAA CGG GTG GGA GAA
fp1*	TTA AAT CAC T**AA** ATT TTT GCC CAC C A GTG A**TT** TAA AAA CGG GTG GGA GAA
fp2	GGC CTG AGC CAA TCA CAA AG G GAC TCG GTT AGT GTT TC
fp3	CCC ATT GAA TCC CTG CCG AGC TTT GGG TAA CTT AGG GAC GGC TCG AAA G
fp3*	AGC CCC AT**A** **A**AA TCC CTG CCG AG GGG TA**T** **T**TT AGG GAC GGC TCG AA

units PAP (Sigma) in 0.1 M MES buffer (pH 6.0) at room temperature for 30 minutes. The mobility shift assay was carried out with these nuclear extracts as described above. To detect endogenous phosphatase activity, nuclear extracts were dialysed into phosphate-free buffer (25 mM HEPES pH7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol and 0.2 mM PMSF), and incubated at 30°C for 30 minutes prior to the mobility shift assay. Where indicated, 50 mM K_2PO_4 pH 7.4 was added as a phosphatase inhibitor.

Methylation interference assay. The coding and non-coding strands of the fp1 oligonucleotide probe (see Table 4-1) were separately labeled by filling-in with either [α-^{32}P]-dCTP or [α-^{32}P]-dATP (Dupont-NEN Research Products, Boston, MA). Scaled-up DNA binding reactions (five-fold) using partially methylated probe DNA were performed as described for the mobility shift assay. Methylation of probe DNA and methylation interference assay were carried out as described by Garabedian *et al.* (1993). Briefly, bound and free DNA were excised from the mobility shift gel, eluted overnight in elution buffer (0.2 M NaCl, 20 mM EDTA, 1% SDS, 1 mg/mL yeast tRNA) and purified by phenol/chloroform extraction and ethanol precipitation. Eluted DNA was cleaved with piperidine at 90°C for 30 minutes. Residual piperidine was removed by repeated lyophilization. The DNA was resuspended in formamide loading buffer and heated at 90°C for 3 minutes. Cleavage products were resolved in a 12% denaturing sequencing gel and visualized by autoradiography.

Western and Northern blot analysis. Two micrograms of poly A^+ RNA from five B-FABP negative (A172, CLA, M021, T98 and U87) and five B-FABP positive (M016, M049, M103, U251 and U373) glioma cell lines were fractionated on a 1.5%

agarose/formaldehyde gel. The RNA was transferred to reinforced nitrocellulose (Amersham Life Sciences) and sequentially hybridized with probes specific for NFI-A (2.2 kb EcoRI/XhoI insert from EST clone #71994 (Genome Systems, Inc.)), NFI-B (5'-GCT GGG TTT GTG CAA TGT GGG GAT TTC ATG-3'), NFI-C (0.7 kb EagI/XhoI insert of EST#129328), NFI-X (0.7 kb PstI/XhoI insert of EST#154038), *B-FABP* (0.7 kb PstI/XhoI cDNA insert (Godbout, 1993)), and actin (0.5 kb cDNA insert). Blots were washed at 60°C in 0.1X SSC and 0.1% SDS.

Western blots were prepared by electrophoresing nuclear extracts (25 μg) of B-FABP positive (M016, M049, M103, U251 and U373) and negative (A172, CLA, M021, T98 and U87) MG cells on an 8% polyacrylamide-SDS gel and then electroblotting onto nitrocellulose. Filters were blocked with 5% fat-free powdered milk and then incubated with 1/1000 dilution of anti-NFI antibody or 1/100 dilution of anti-c-myc antibody (N-262, Santa Cruz Biotechnology). Primary antibody was detected with HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories) using the ECL detection system (Amersham Pharmacia Biotech).

4.3 Results

Regulatory regions in the B-FABP promoter. We have previously shown the human *B-FABP* gene resides within a 4.5 kb region on chromosome 6q22-23 (Godbout *et al.*, 1998). To study the regulation of *B-FABP* transcription, we began by mapping the transcriptional initiation site by primer extension. The human *B-FABP* start site position corresponds to that previously reported for the murine *B-FABP* gene (indicated as +1, Fig 4-3, Kurtz *et al.*, 1994; Feng and Heintz, 1995). Sequence analysis of the region upstream of the initiation site revealed a potential TATA box located at position –28 to -22 bp (underlined in Fig. 4-3). To localize regulatory elements of the *B-FABP* promoter, a series of reporter genes were constructed which put the chloramphenicol acetyltransferase (CAT) gene under the control of various portions of the *B-FABP* promoter. These constructs are shown schematically in Fig. 4-1A. The reporter plasmids were introduced into the B-FABP positive MG cell line, U251, by calcium phosphate mediated DNA transfection. The results, expressed as fold-increase over the promoter-less parent vector (pCAT-basic), are summarized in Fig. 4-1B. Background levels of CAT activity were obtained with the construct carrying 140 bp of *B-FABP* 5' flanking DNA (pCAT-140). A slight increase in CAT activity (1.9-fold) was observed with the pCAT-240 construct, which contained 240 bp of *B-FABP* upstream sequence, suggesting that a positive element resides in this region. An additional positive element is located in the -240 to -660 bp region of the *B-FABP* promoter because reporter gene expression increased 6-fold over background with pCAT-660. Reporter activity increased 7-8 fold over background with pCAT-1.2 suggesting that an additional weak positive element may be present in the

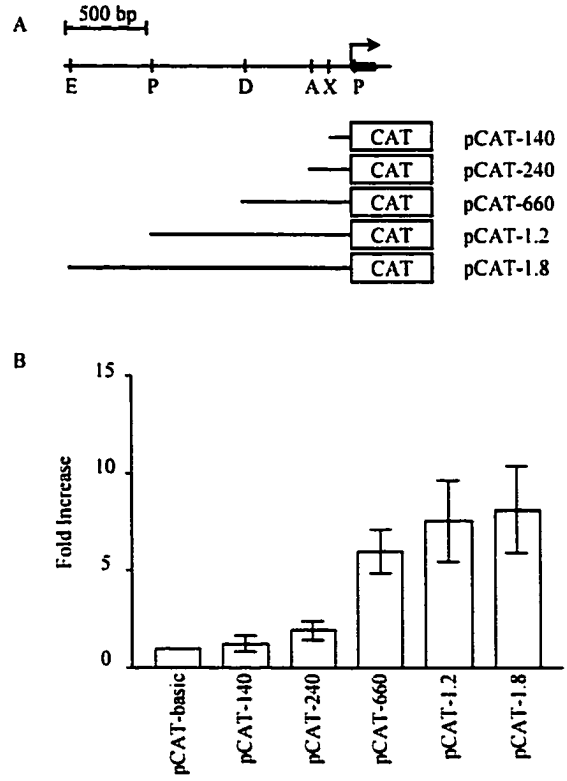

Figure 4-1. Analysis of regulatory elements in the *B-FABP* promoter. A. Schematic diagram of the *B-FABP* promoter showing the transcriptional start site (arrow), exon I (filled box) and restriction enzyme cut sites used for the construction of the pCAT-deletion plasmids. CAT constructs extended from a common 3' PstI (P) site, upstream to the XhoI (X) site (pCAT-140), the AluI (A) site (pCAT-240), the DraI (D) site (pCAT-660), the PstI (pCAT-1.2) and the EcoRI (E) site (pCAT-1.8). **B.** Ten micrograms of the reporter constructs described above were separately introduced into U251 MG cells by calcium phosphate-mediated transfection. Extracts prepared from transfected cells were assayed for CAT activity by monitoring the level of [^{14}C]-chloroamphenicol butyrylation. CAT activities are reported as fold-increase over the promoter-less parent vector (pCAT-basic). The values have been normalized for transfection efficiency as determined by Hirt DNA quantitation. Results are the mean fold increase +/- S.D. for at least four independent experiments.

–660 to –1200 bp region. These transfection experiments suggest the *B-FABP* promoter contains one or more positive elements within each of the following regions: –140 to –240 bp, -240 to –660 bp and –660 to –1200 bp.

DNase I footprint analysis of the *B-FABP* promoter. DNase I footprinting analysis was performed to locate sites of DNA/protein interaction in the *B-FABP* proximal promoter in B-FABP expressing and non-expressing cell lines. The analysis was performed with nuclear extracts from both B-FABP negative (T98) and B-FABP positive (M016 and U251) MG cell lines to determine if gross differences were present in the factors bound to the *B-FABP* promoter. Two overlapping DNA fragments spanning the –13 to –418 bp region were analyzed: probe 1, a 227 bp AluI fragment, extended from –13 to –240 bp and probe 2, a 283 bp EcoO109I/XhoI fragment extended from –138 to –418 bp. Singly end-labeled probe 1 or probe 2 was incubated with nuclear extract and then partially digested with DNase I followed by denaturing gel electrophoresis and autoradiography. As shown in Fig. 4-2A, two DNase I protected regions were detected using probe 1: footprint 1 (fp1), extended from -35 to -58 bp and footprint 2 (fp2) from -155 to –174 bp. Footprint analysis of probe 2 identified three additional protected regions as shown in Fig. 4-2B; footprint 3 (fp3) extended from -237 to –260 bp, footprint 4 (fp4) from -303 to –328 bp, and footprint 5 (fp5) from -340 to –359 bp. Nuclear extracts from both the B-FABP negative (T98) and B-FABP positive (M016 and U251) cell lines produced identical footprint patterns were over the entire region analyzed (–13 to –418). These data are summarized in Fig. 4-3.

The sequences of fp1 to fp5 were analyzed to search for known DNA binding motifs. Fp1, fp2 and fp3 contain putative DNA binding sites for the transcription factor

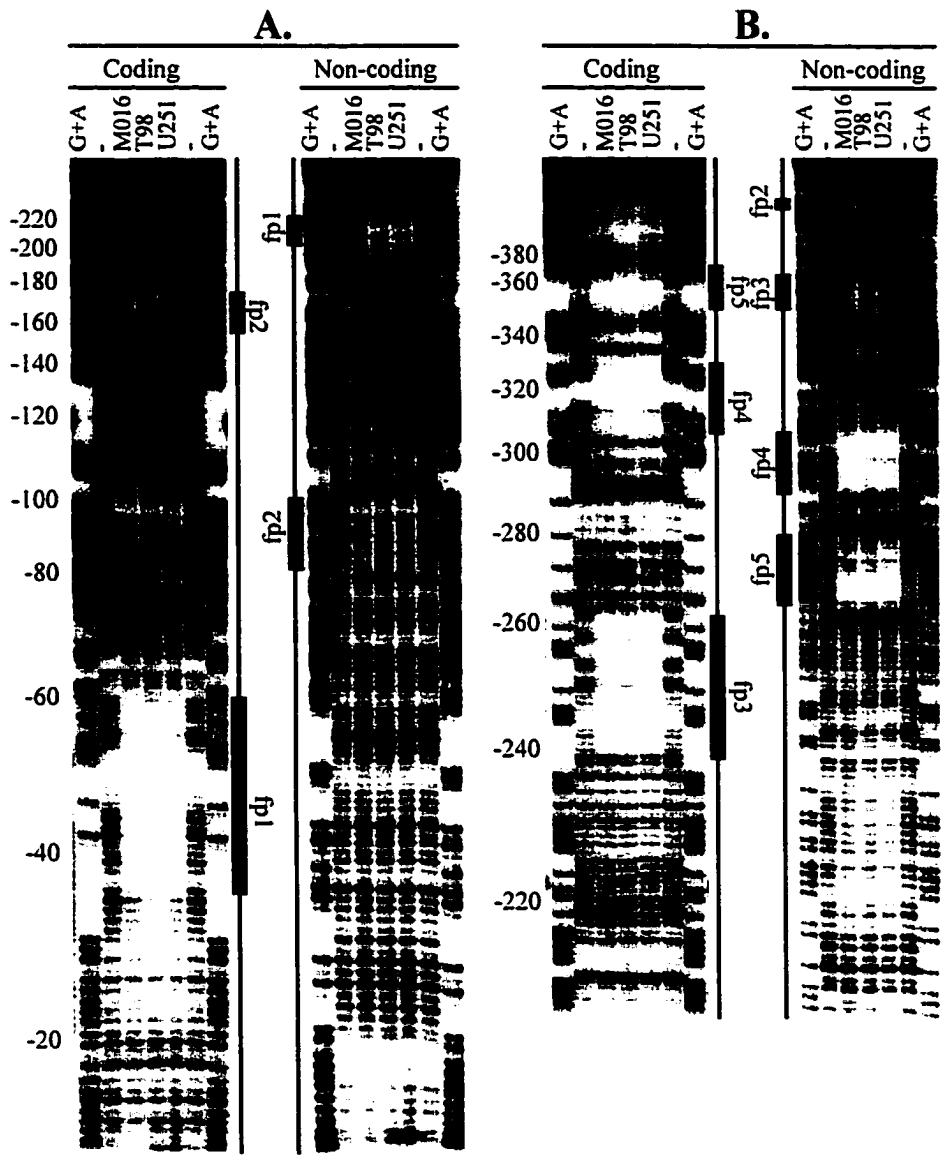

Figure 4-2. DNase I footprint analysis of the *B-FABP* promoter. A. A DNA fragment spanning the –13 to –240 bp region of the *B-FABP* promoter (probe 1) was singly end-labeled on the coding or non-coding strand, incubated with T98, M016 or U251 nuclear extracts, DNase I digested, and then electrophoresed on a denaturing 8% polyacrylamide gel. No nuclear extract was added to control (-) lanes. G+A chemical sequencing reactions of the probe DNA are shown flanking the footprinting reactions. Footprints (fp1 and fp2) are indicated by the filled rectangle. **B.** DNase I footprint analysis of the –138 to –418 bp region revealed three additional footprints indicated with a filled rectangle (fp3, fp4 and fp5). Footprint assay was carried out as described above.

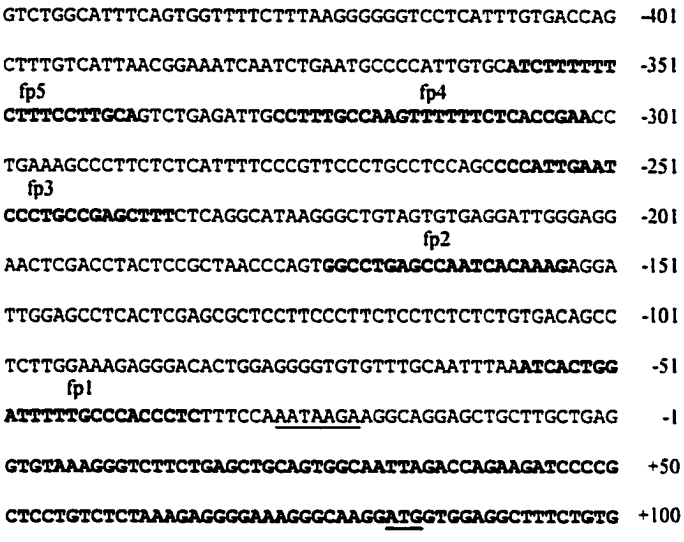

Figure 4-3. Summary of footprint results. The sequence of the –450 to +100 bp region of the *B-FABP* promoter is shown, indicating the location of the five footprints (shaded regions). The transcriptional start site is located at +1. The putative TATA box located at –28 to –22 bp is underlined. The sequence in bold represents the 5' end of exon I and the start methionine is underlined.

nuclear factor I (NFI) based on their similarity to the consensus NFI binding site (TGGM(N_5)GCCAA, where M is A or C). The fp1 region contains the sequence TGGA(N_5)GCCCA, fp2 contains TGGC(N_4)GCCAA and fp3 has the sequence TGAA(N_5)GCCGA.

NFI binds to fp1 and fp3 in B-FABP expressing and non-expressing glioma lines. The mobility shift competition assay was used to determine if the complexes formed on the fp1 and fp3 protected regions were due to NFI as suggested by their sequence (Fig. 4-4). Radiolabelled oligonucleotides corresponding to footprint regions fp1 or fp3 (Table 4-1) were incubated with nuclear extracts from either T98 (B-FABP negative) or U251 (B-FABP positive), along with a hundred-fold excess of unlabelled fp1, fp2, fp3, AP-2, NFI or Sp1 oligonucleotides. As shown in Fig. 4-4, one major DNA-protein complex was observed with T98 nuclear extract using either the fp1 or fp3 probes. This complex was specifically competed by fp1, fp3 and consensus NFI oligonucleotides but not by fp2 or negative control consensus AP-2 or Sp1 oligonucleotides indicating that NFI or an NFI-like factor binds to the fp1 and fp3 regions. The fp2 oligonucleotide was unable to compete for the factor(s) bound to the fp1 and fp3 sites even though it contains a near consensus NFI binding site (TGGC(N_4)GCCAA). The spacing between the perfect half sites is one nucleotide too short. Three major shifted complexes were detected with fp1 and fp3 oligonucleotides when they were tested with nuclear extract from U251, the B-FABP-positive cell line. Although of higher mobility than the T98 complex, these three complexes were also specifically competed by the fp1, fp3 and NFI oligonucleotides suggesting NFI or related factors were also responsible for the U251 complexes. These data suggest that NFI or

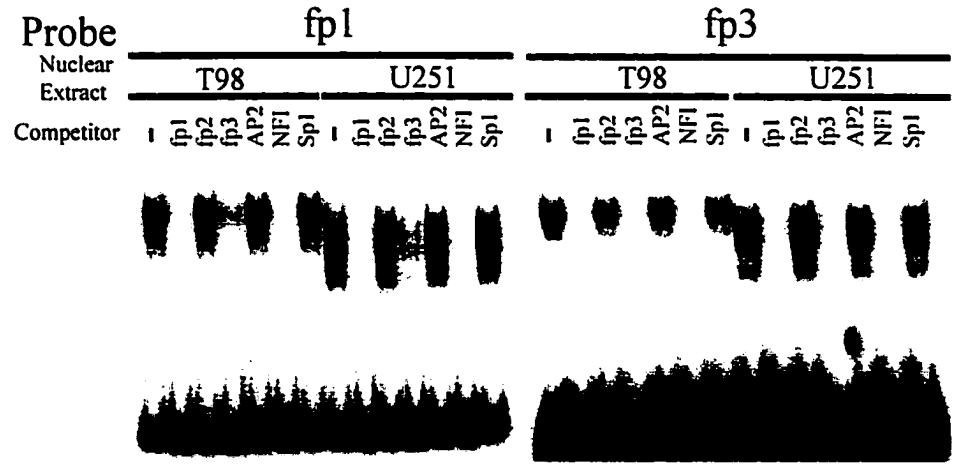

Figure 4-4. NFI binds to the fp1 and fp3 elements. Gel shift assays were carried out with radiolabeled fp1 or fp3 oligonucleotides and T98 or U251 nuclear extracts. DNA binding reactions were electrophoresed in a 0.5X TBE/6% polyacrylamide gels to separate unbound (free) DNA and DNA/protein complexes. Where indicated, a hundred-fold molar excess of unlabeled competitor oligonucleotides corresponding to fp1, fp2, fp3 or AP-2, CTF/NFI or Sp1 consensus DNA binding sites were added to the DNA binding reaction prior to electrophoresis.

NFI-like proteins bind to the fp1 and fp3 regions of the *B-FABP* promoter in both B-FABP expressing (U251) and non-expressing (T98) MG cell lines.

Antibody supershift and methylation interference experiments were carried out to determine whether the fp1 and fp3 DNA-protein complexes were due to NFI or some other factor with a similar DNA binding specificity. Supershift experiments were performed to determine if the factor(s) bound to the fp1 region were recognized by a pan-specific anti-NFI antibody. As shown in Fig. 4-5, addition of anti-NFI antibody to DNA binding reactions containing radiolabelled fp1 oligonucleotide and T98, M016 or U251 nuclear extracts, resulted in a supershifted complex corresponding to the DNA/NFI/anti-NFI complex (arrow). No supershifted band was observed with the control anti-AP2 antibody. This indicates the factor(s) bound to the fp1 region are likely NFI because they share antigenic determinants.

The methylation interference assay was used to map the nucleotides involved in DNA-protein interaction within the fp1 region for comparison with known NFI binding sites. Partially methylated fp1 oligonucleotide, radiolabelled on the coding or non-coding strand, was isolated from the most abundant T98 gel shift complex and the three U251 complexes and subjected to piperidine cleavage followed by denaturing polyacrylamide gel electrophoresis. As shown in Fig. 4-6A, methylation of G residues located at -52, -51 or -44 on the coding strand and -43 or -42 of the non-coding strand prevent formation of the T98 and U251 DNA-protein complexes (arrows). Identical methylation interference patterns were obtained with the T98 complex (B) and the three U251 complexes (B1-B3) indicating that the same or similar recognition sequence is involved in their formation. All the residues showing methylation interference fall within the NFI recognition

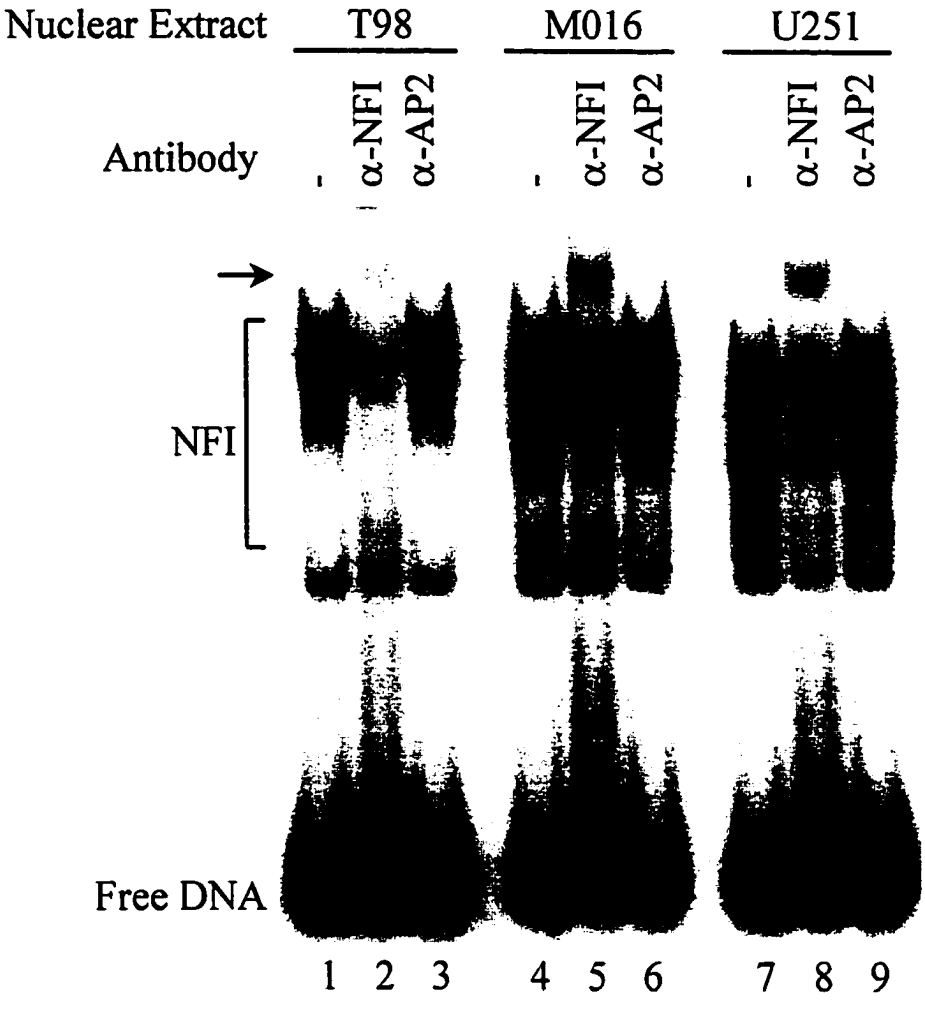

Figure 4-5. Anti-NFI antibody supershifts fp1 DNA-protein complexes. Four μg of T98, M016, or U251 nuclear extract was incubated with anti-NFI antibody (α-NFI), anti-AP-2 antibody (α-AP2) or without added antibody (-) prior to addition of radiolabeled fp1 oligonucleotide probe and gel electrophoresis (0.5X TBE/6% polyacrylamide). Bands corresponding to unbound DNA (free) and NFI/DNA complexes (NFI) are indicated. The arrow indicates the supershifted complex observed with T98, M106, and U251 nuclear extracts in the presence of anti-NFI. This supershifted band is not detected in the absence of antibody nor in the presence of the unrelated control antibody (anti-AP2).

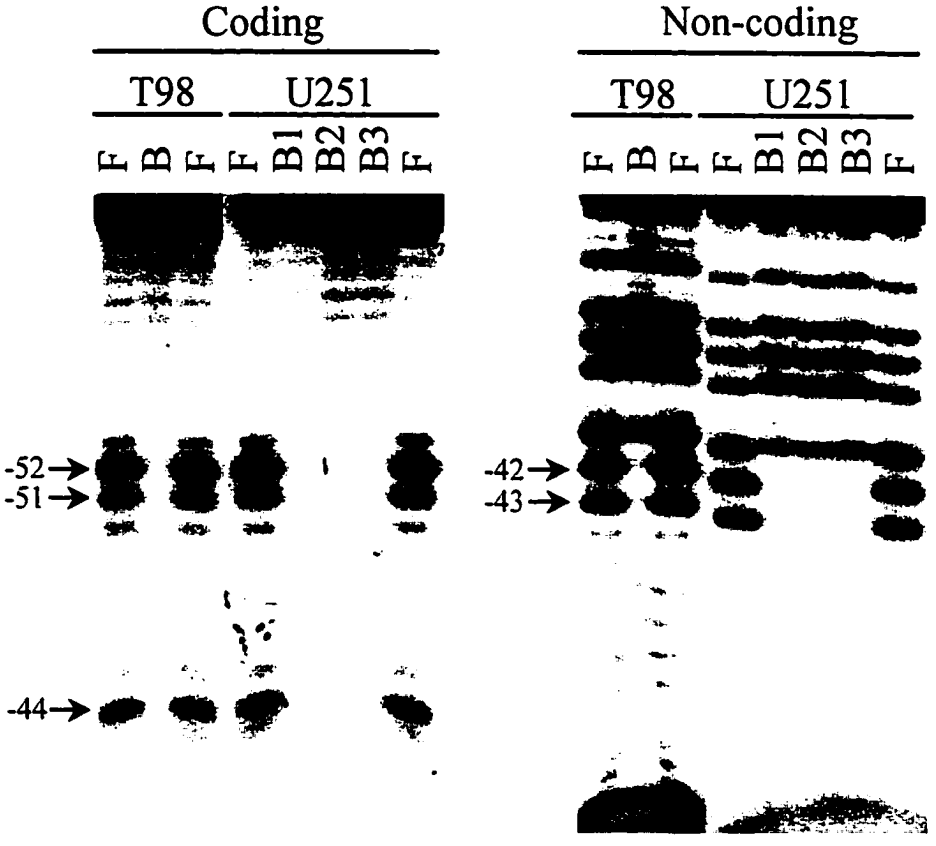

Fig. 4-6. Methylation interference assay of fp1 NFI binding site. fp1 oligonucleotide probes radiolabeled on the coding or non-coding strand were partially methylated with dimethyl sulfate and subjected to the mobility shift assay with T98 or U251 nuclear extracts. DNA isolated from the free (F) and NFI bound (B) bands of the mobility shift gel was subjected to piperidine cleavage prior to electrophoresis on a 12% denaturing polyacrylamide gel. Arrows mark positions of guanosine residues that when methylated, interfere with DNA-protein interaction. B1-B3 indicates DNA isolated from the three major U251 NFI complexes.

sequence in the fp1 footprint region and are consistent with the methylation interference patterns shown for known NFI binding sites (de Vries *et al.*, 1987; Raymondjean *et al.*, 1988). Together, the competition assays, supershifting and methylation interference experiments indicate that NFI proteins bind to the fp1 and fp3 regions.

Alteration of the fp1 NF-I site reduces *R-FABP* promoter activity. To determine the effect of eliminating the fp1 and/or fp3 NFI DNA binding sites, *B-FABP*/CAT reporter gene constructs were prepared with mutations in the fp1 binding site (pCAT-1.8(fp1*)), the fp3 binding site (pCAT-1.8(fp3*)) or both (pCAT-1.8(fp1*3*)) (Table 4-1). We verified that the introduced mutations would eliminate DNA-protein interaction by testing oligonucleotides corresponding to the mutagenized fp1 and fp3 NFI binding sites using the mobility shift competition assay. As shown in Fig. 4-7A, the fp1 oligomer is able to compete for the NFI complexes in both T98 and U251 binding reactions, whereas the fp1 mutant oligomer (fp1*) is not (compare lanes 2 versus 3 and lanes 5 versus 6). Similarly, the mutations introduced into fp3 also eliminated DNA-protein interaction (lanes 8 versus 9 and 11 versus 12).

Reporter gene constructs carrying these mutations, as well as the parent pCAT-1.8, were transfected into U251 cells to determine the effect of the mutations on *B-FABP* promoter activity. The results are summarized in Fig. 4-7B. While the parental construct pCAT-1.8 has about eight-fold increased promoter activity over background, the mutation of the fp1 NFI binding site reduces promoter activity to near background levels (1.4-fold). Elimination of the NFI binding site in fp3 has little effect on *B-FABP* promoter activity, generating CAT levels that were slightly higher (9.2-fold) than the parent construct. Relative CAT levels with the double mutant construct (pCAT-

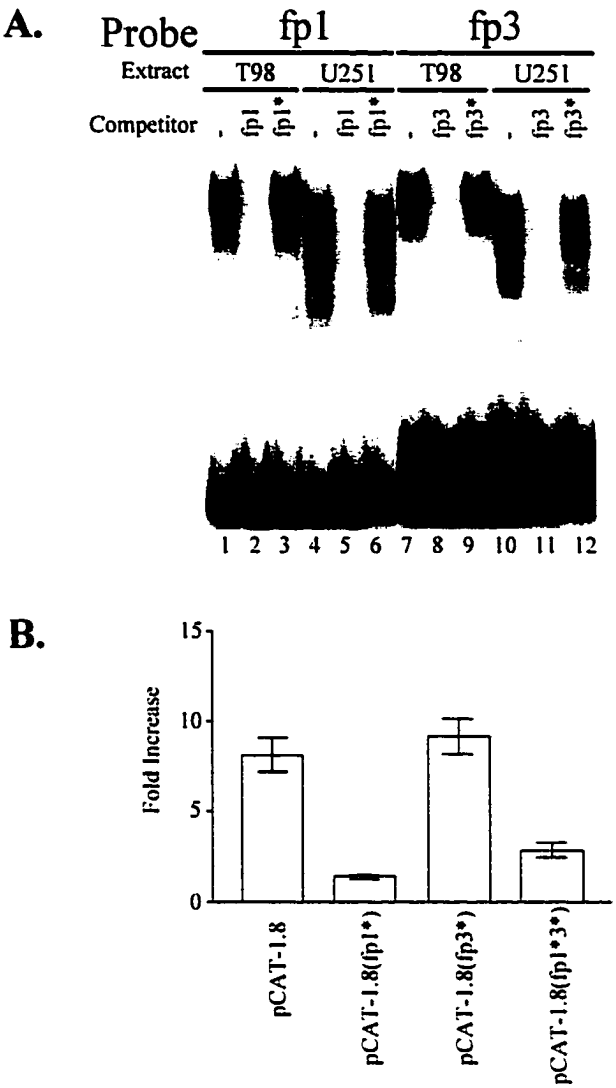

Fig. 4-7. Mutagenesis of fp1 and fp3 NFI binding sites prevents DNA-protein interaction and reduces reporter activity. A. Mobility shift assay was carried out by incubating radiolabeled fp1 (lanes 1-6) or fp3 (lanes 7-12) oligonucleotides with either T98 (lanes 1-3 and 7-9) or U251 (lanes 4-6 and 10-12) nuclear extracts, followed by gel electrophoresis and autoradiography. Where indicated, a 100-fold excess of unlabeled fp1 (lanes 2 and 5) or fp3 (lanes 8 and 11) oligonucleotides or oligonucleotides containing mutations in the NFI binding site (fp1* (lanes 3 and 6) or fp3* (lanes 9 and 12)) were added to the DNA binding reaction. Control binding reactions were carried out in the absence of added competitor DNA (-). **B.** B-FABP/CAT reporter plasmids containing NFI binding site mutations in fp1 (pCAT-1.8(fp1*)), fp3 (pCAT-1.8(fp3*)), both (pCAT-1.8(fp1*3*)) or neither (pCAT-1.8) were transfected into U251 malignant glioma cells. Reporter activity was determined by measuring the conversion of chloramphenicol to its butyrylated derivatives as described. Reported values have been corrected for transfection efficiency by analysis of Hirt DNA and are reported as fold increase over pCAT-basic, the promoter-less parent vector. Values are the mean +/- S.D. of at least four independent experiments.

1.8(fp1*3*)) were 2.9-fold over background. The fp1 NFI site appears to be necessary for *B-FABP* promoter activity in U251 cells as its elimination reduces CAT activity levels to background levels. On the other hand, the fp3 NFI site appears to have little effect on *B-FABP* promoter activity since its removal does not adversely affect reporter activity.

NFI isoforms in B-FABP (+) and (-) MG cell lines.

Differences in the gel shift patterns observed with T98 and U251 nuclear extracts suggested a difference in the NFI isoforms present in each cell line. Nuclear extracts from additional MG cell lines were tested by gel shift assay with the fp1 oligo to determine if B-FABP expression correlated with which NFI isoform(s) was present. As shown in Fig. 4-8, the additional B-FABP negative MG cell lines (A172, CLA, M021, and U87) gave rise to one or two complexes of similar mobility to that of the T98 complex. Nuclear extracts from additional B-FABP positive MG cell lines (M016, M049, M103, and U373) resulted in multiple high-mobility complexes similar to U251 (with the exception of M016). The M016 gel shift pattern varied with different nuclear extract preparations, often appearing to be a hybrid of the two patterns, suggesting this cell line may be a mixture of cell types. We have previously shown by immunofluorescence that both B-FABP expressing and non-expressing cells co-exist in M016 cultures (Godbout *et al.*, 1998). Our results suggest there is a correlation between expression of the *B-FABP* gene and the NFI isoforms interacting with the fp1 binding site, in 5/5 negative cell lines and at least 4/5 positive cell lines.

The observed differences in NFI isoforms present in positive and negative cell lines could reflect differences in which *NFI* gene is expressed in each line, the presence of alternative gene products from a single gene and/or differential post-translational

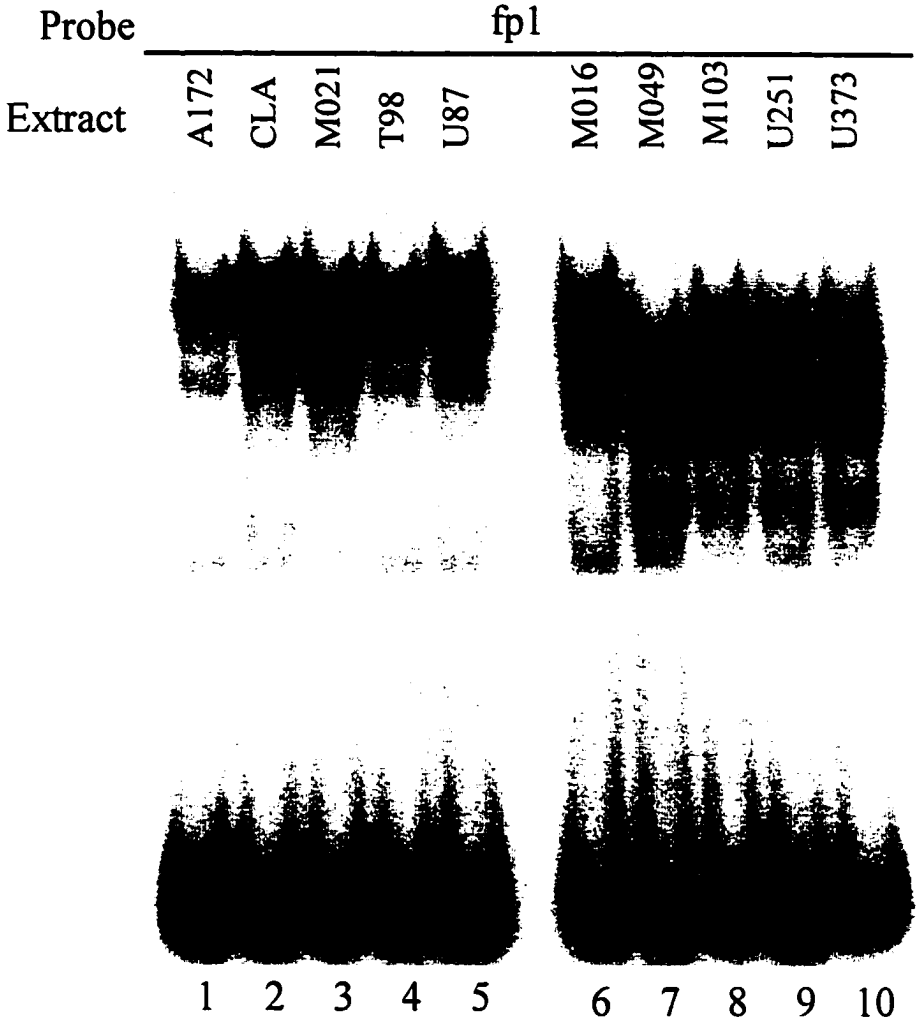

Figure 4-8. Different NFI isoforms in B-FABP positive and negative MG cell lines. Mobility shift assay were carried out using radiolabeled fp1 oligonucleotide and nuclear extracts prepared from five B-FABP negative MG cell lines (A172, CLA, M021, T98 and U87) and five B-FABP expressing lines (M016, M049, M103, U251, and U373). Unbound probe DNA migrates at the bottom of the figure.

modification. To further characterize the NFI proteins, Western blots were prepared containing nuclear extracts from five B-FABP expressing (M016, M049, M103, U251 and U373) and five non-expressing (A172, CLA, M021, T98, and U87) glioma cell lines, and tested by immunoblotting with an anti-NFI antibody. Although raised against NFI-C, the anti-NFI antibody crossreacts with NFI proteins derived from all four NFI genes (Ortiz *et al.*, 1999). As shown in Fig. 4-9A, immunoblotting of nuclear extracts from B-FABP negative and positive cell lines show two sets of NFI immunoreactive bands, one set migrating at around 50 kDa and the other at approximately 60 kDa. These size estimates are similar to reported molecular masses of known NFI family members, which range from 52 to 60 kDa (Rosenfeld and Kelly, 1986). Figure 4-9A shows that the ratio of 50 and 60 kDa isoforms are approximately equal in B-FABP (-) cell lines, while the 50 kDa isoforms predominate in the B-FABP (+) cell lines. These results provide further evidence that B-FABP positive and negative cell lines express distinctive subsets of NFI proteins as suggested by the mobility shift assay. Because gene-specific anti-NFI antibodies are not currently available to determine which gene(s) the 50 and 60 kDa isoforms are derived from, we used Northern blot analysis to further characterize the differences in NFI content between B-FABP positive and negative cell lines.

To test whether the NFI genes were differentially expressed in MG cell lines, a Northern blot containing poly A^+ RNA derived from five B-FABP negative (A172, CLA, M021, T98, and U87) and five B-FABP positive (M016, M049, M103, U251, and U373) cell lines was sequentially probed with ^{32}P labelled probes specific for NFI-A, -B, -C, and -X. As shown in Fig. 4-9B, all four NFI genes were expressed in each glioma cell line, albeit at different levels. Three weak transcripts were detected with the NFI-A probe.

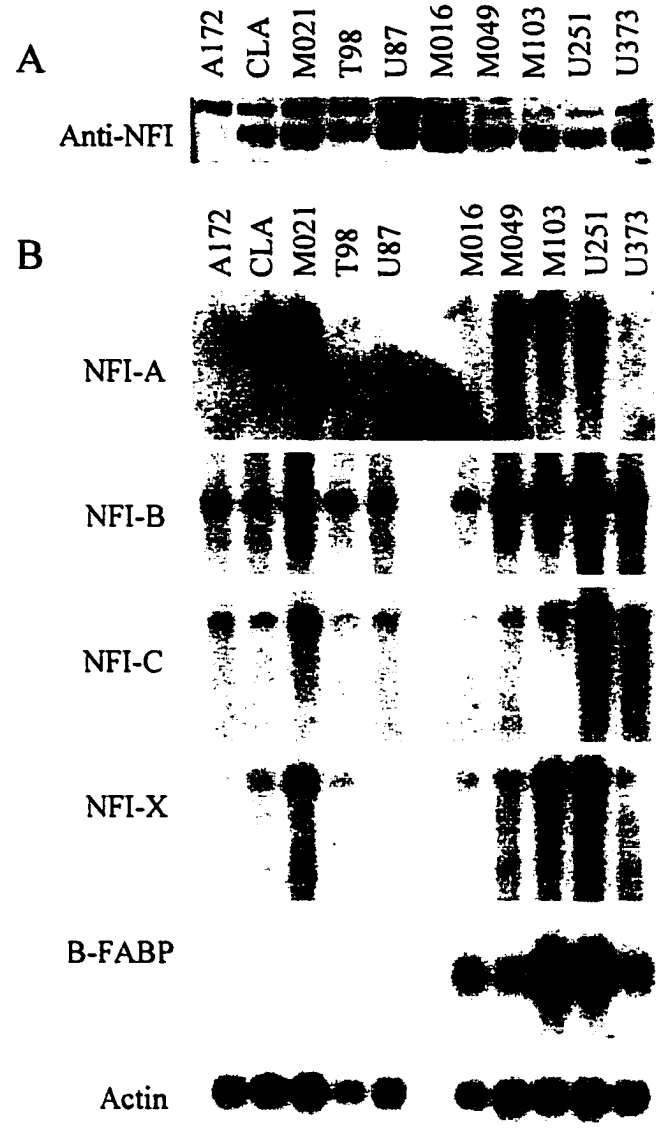

Figure 4-9. Western and Northern analysis of NFI in MG cell lines. A. Western blot was prepared by SDS-PAGE of nuclear extracts (25 μg) of five B-FABP negative (A172, CLA, M021, T98 and U87) and five B-FABP positive (M016, M049, M103, U251, and U373) MG cell lines, followed by electroblotting onto nitrocellulose. Immunoblots were blocked with 5% non-fat milk and incubated with anti-NFI (a kind gift of N. Tanese). Primary antibody was detected with horseradish peroxidase (HRP) conjugated anti-rabbit IgG using the ECL detection system (Amersham). **B.** Northern blot prepared by transferring poly-A^+ RNA prepared from five B-FABP negative (A172, CLA, M021, T98 and U87) and five B-FABP positive (M016, M049, M103, U251, and U373) MG cell lines onto nitrocellulose. Blot was sequentially hybridized with ^{32}P radiolabeled DNA probes specific for NFI-A, -B, -C, -X, B-FABP and actin. After each hybridization, the blot was stripped as described (Thomas, 1980).

with highest expression in M049, M103 and U251 (the blot was exposed for 2 weeks at –80°C, while the typical exposure for the others is 1-3 days). NFI-B was strongly expressed in all ten MG cell lines, with highest expression in M021, M049, M103 and U251. A smaller weaker transcript was also detected in each cell line. High levels of NFI-C transcript were found in M021 and U251, with lower levels found in the other lines. NFI-X was expressed mostly in M021, M103, and U251, with a weaker signal in the other lanes. A second smaller NFI-X transcript was also detected. Although we observed differences in the relative levels of NFI transcripts between the cell lines, no clear correlation was found between B-FABP expression and the presence or absence of a given NFI transcript.

NFI is differentially phosphorylated in B-FABP positive and negative cell lines. Phosphorylation of NFI has been shown to down-regulate its trans-activating activity (Yang *et al.*, 1993; Cooke and Lane, 1999). We therefore determined if the difference between expressing and non-expressing lines was due to differences in protein phosphorylation. Potato acid phosphatase (PAP)-treated and -untreated T98 and U251 nuclear extracts were compared using the mobility shift assay as shown in Fig. 4-10. Treatment of T98 or U251 extracts with increasing amounts of PAP results in the conversion of the normally observed NFI complexes into a single high mobility complex not observed with either untreated extract. These results indicate that the T98 and U251 NFI isoforms are phosphorylated. The differences in electrophoretic mobility of the untreated T98 and U251 complexes suggest that NFI in T98 is normally phosphorylated to a greater extent than in U251. The T98 NFI complex may represent a hyperphosphorylated isoform whereas the U251 complexes are phosphorylated to a lesser

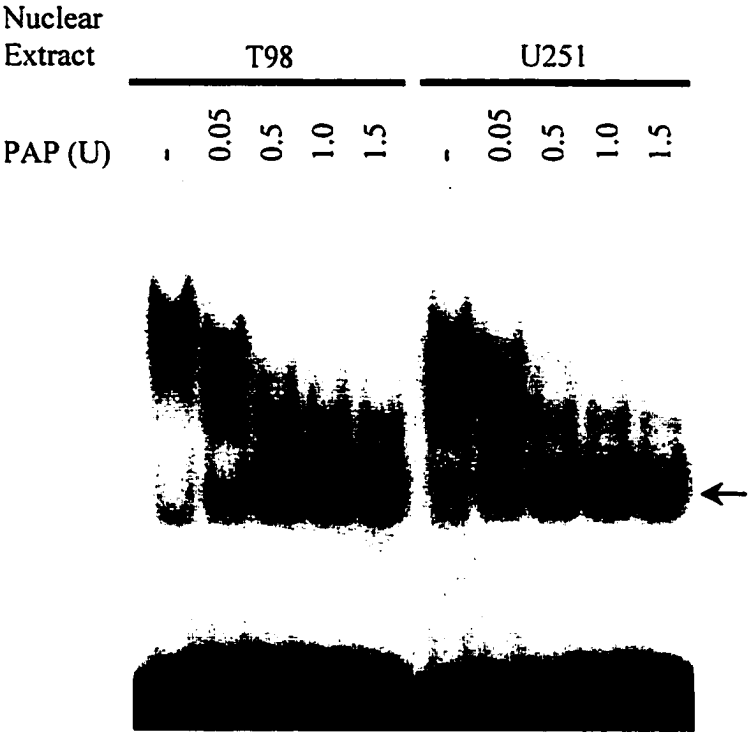

Fig. 4-10. NFI is differentially phosphorylated in T98 and U251 nuclear extracts. A. Mobility shift assay performed using radiolabeled fp1 oligonucleotide and four micrograms of T98 or U251 nuclear extracts, which were untreated (-) or incubated with 0.05, 0.5, 1.0 or 1.5 units of potato acid phosphatase (PAP, Sigma) at room temperature for 30 minutes. Arrow marks the position of the dephosphorylated NFI/fp1 complex.

extent.

Experiments by Yang *et al.* (1993) demonstrated that overexpression of c-myc in 3T3-L1 cells leads to increased levels of NFI phosphorylation. We therefore compared the level of c-myc expression in B-FABP positive and negative MG cell lines by Northern blot analysis as shown in Fig. 4-11A. With the exception of A172 and U373, the B-FABP negative lines contained higher levels of c-myc transcript than B-FABP positive lines. An even better correlation was observed at the protein level, with all five B-FABP (-) cell lines showing higher levels of c-myc than the B-FABP (+) lines (Fig. 4-11B). This suggests that higher levels of c-myc in B-FABP (-) cell lines may contribute to increased levels of NFI phosphorylation and consequently, to decreased *B-FABP* transcription.

Endogenous NFI phosphatase activity is present in U251 but not T98 nuclear extracts. During the preparation of phosphate-free T98 and U251 nuclear extracts we noticed that U251 nuclear extracts contained a phosphatase activity capable of dephosphorylating NFI which was absent from T98 extracts. As shown in Fig. 4-12, incubation of phosphate-free U251 nuclear extracts at 30°C for 30 minutes prior to use in the mobility shift assay resulted in the fully dephosphorylated form of NFI, whereas no detectable NFI dephosphorylation was found using T98 extracts (compare lanes 1 and 3). This phosphatase activity could be inhibited by the addition of 50 mM PO_4^{2-} (compare lanes 3 versus 4). Furthermore, the phosphatase activity in U251 nuclear extracts is capable of dephosphorylating the hyperphosphorylated T98 NFI. As shown in lane 5, the T98 NFI becomes dephosphorylated when incubated with an equal quantity of U251 nuclear extract (compare lanes 5 and 6). These results indicate that U251 nuclear extracts

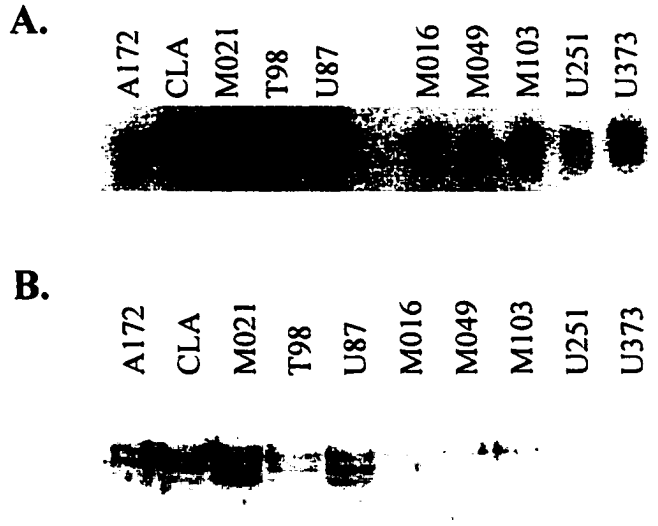

Figure 4-11. Expression of c-myc in malignant glioma cell lines. A. Northern blot of poly-A$^+$ RNA prepared from five B-FABP negative (A172, CLA, M021, T98 and U87) and five B-FABP positive (M016, M049, M103, U251, and U373) MG cell lines, hybridized with radiolabeled fragment of the c-myc cDNA. Blot was washed in 0.1X SSC/0.1% SDS at 60°C. B. Western blot containing nuclear extracts (25 μg) from malignant glioma cell lines described in A was incubated with anti-c-myc (N-262, Santa Cruz Biotechnology). Primary antibody was detected with horseradish peroxidase (HRP) conjugated anti-rabbit IgG using the ECL detection system (Amersham Pharmacia Biotech).

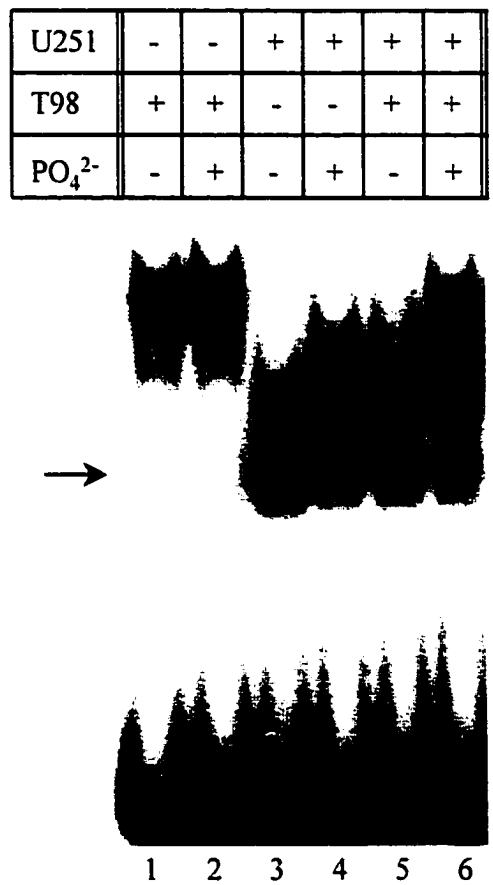

Figure 4-12. Endogenous NFI phosphatase activity is present in U251 but not T98 nuclear extracts. T98 (lanes 1,2) or U251 (lanes 3,4) nuclear extracts (phosphate-free) or an equal mixture of both nuclear extracts (lanes 5,6) were incubated at 30°C for 30 minutes in the absence (lanes 1,3,5) or presence (lanes 2,4,6) of 50 mM K_2PO_4 (pH 7.4) prior to use in mobility shift assay with radiolabeled fp1 oligonucleotide. Arrow marks the position of dephosphorylated NFI/fp1 DNA complex.

possess a phosphatase activity, that is absent or inactive in T98 nuclear extracts, which may underlie the mechanism for differential phosphorylation of NFI in malignant glioma.

4.4 Discussion

We are studying the promoter of the human *B-FABP* gene, a glial-specific gene, to understand how its transcription is specifically upregulated in malignant glioma. In a previous study, we found *B-FABP* expressed in 7/15 of malignant glioma lines tested (Godbout *et al.*, 1998). Using these cell lines, we have now identified two DNA binding sites for members of the NFI family in the *B-FABP* proximal promoter which appear to play a role in B-FABP differential expression. The first, footprint 1 (fp1) is located at –58 to –35 bp, adjacent to the TATA box, while the second, fp3, is located further upstream between –260 to –237 bp. Elimination of the fp1 NFI binding site by site-directed mutagenesis dramatically reduces reporter activity in a B-FABP expressing MG cell line, indicating that it is necessary for B-FABP expression. Comparison of the NFI proteins expressed in a panel of B-FABP positive and B-FABP negative MG cell lines indicates a correlation between the phosphorylation state of NFI and transcription of the *B-FABP* gene. In nuclear extracts of B-FABP negative MG lines, NFI proteins were hyperphosphorylated, whereas they were phosphorylated to a lesser extent in B-FABP positive cells. A phosphatase specifically expressed in B-FABP positive MG cell lines may be responsible for dephosphorylation of NFI resulting in expression of the *B-FABP* gene.

Originally identified as a factor involved in adenovirus DNA replication, NFI is now known to also be a transcription factor involved in the regulation of many cellular genes. Each of the four genes that make up the NFI gene family (NFI-A, -B, -C, and –X) give rise to a number of different transcripts by alternative splicing (Santoro *et al.*, 1988).

A number of mechanisms have been proposed to account for cell-specific gene regulation by the widely expressed NFI family. The multitude of alternative transcripts suggests that functional differences might be present in the NFI proteins. Indeed a number of NFI proteins generated by alternative splicing are capable of interfering with transcriptional activation by their differentially spliced counterparts. For instance, Liu and coworkers (1997) have isolated an NFI-B splice variant, NFI-B3, which lacks the trans-activation domain and interferes with NFI mediated trans-activation by non-productive heterodimer formation. By Northern analysis, we were unable to detect differences in the NFI transcripts present in the MG cell lines that would account for the NFI protein differences observed by gel shift analysis. Therefore, differential expression of the NFI genes or use of alternatively spliced isoforms does not appear to play a role in differential B-FABP expression in MG.

Protein phosphorylation has been shown to modulate NFI mediated trans-activation. Yang *et al.* (1993) have demonstrated that NFI is phosphorylated in actively growing cells and c-myc over-expressing 3T3-L1 cells, whereas it is dephosphorylated in quiescent cells. NFI phosphorylation resulting from c-myc overexpression leads to reduced activation of a number of NFI dependent promoters, including the MoMuLV, SV-40 and MMTV viral promoters. Cooke and Lane (1999) have found that NFI is phosphorylated in response to insulin stimulation, resulting in decreased transcription of the GLUT4 glucose transporter gene. We have found that NFI proteins are hyperphosphorylated in less differentiated glioma cells (B-FABP/GFAP-negative) while they are moderately phosphorylated in B-FABP/GFAP positive cells. The gel shift pattern we obtained with nuclear extracts from the B-FABP positive cells appears to be

the same as the phosphorylated NFI pattern described by Yang *et al.* (1993). Since our nuclear extracts were prepared from actively growing cells, this result is not surprising. These results suggest that the partially phosphorylated NFI proteins are able to trans-activate the *B-FABP* promoter. The multiple phosphoforms of NFI in B-FABP positive cells suggests a model in which NFI trans-activation is attenuated by increasing levels of phosphorylation, where the fully dephosphorylated form is fully active, the partially phosphorylated isoforms are partially active and the hyperphosphorylated isoform is inactive. Given the variety of genes responsive to NFI, its regulation by (de)phosphorylation is likely to have global cellular consequences on gene expression. Our results imply that the level of NFI phosphorylation may be closely linked to the differentiation state of the cell. By modulating the expression of differentiation factors such as B-FABP, NFI phosphorylation may be intimately involved in the differentiation process.

The molecular mechanism(s) determining the phosphorylation state of NFI have not yet been determined. Additional work is needed to identify and characterize the kinase(s) and phosphatase(s) involved in NFI phosphorylation in response to diverse stimuli such as c-myc over-expression and insulin stimulation. Two kinases, DNA-PK and CDC2, have been reported to phosphorylate NFI proteins *in vitro* (Jackson *et al.*, 1990; Kawamura *et al.*, 1993). Interestingly, c-myc has previously been implicated in the upregulation of CDC2 expression; however, whether CDC2 or DNA-PK function *in vivo* to effect NFI phosphorylation remains to be determined (Kim *et al.*, 1994; Born *et al.*, 1994). We have found that B-FABP negative cells have a greater level of c-myc mRNA and protein than B-FABP positive cells, indicating an inverse correlation between c-myc

and B-FABP levels in MG cell lines. Our results therefore support the finding of Yang *et al.* (1993) that c-myc expression leads to decreased gene activation through NFI phosphorylation. Furthermore, our results suggest that an NFI protein phosphatase is present in B-FABP positive cells. This could explain why NFI in these lines is less phosphorylated than in B-FABP negative cells. The identity of this phosphatase is currently under investigation. Cell-specific expression of this phosphatase could provide a basis for cell-specificity of NFI dependent promoters.

Although necessary, the fp1 NFI binding site is unable to direct significant levels of *B-FABP* transcription in isolation (see Fig. 4-1), indicating that it functions in conjunction with other factors interacting elsewhere within the promoter. Our DNA transfection experiments suggest that the human *B-FABP* promoter contains one or more positive regulatory elements located in the –240 to –660 bp region, which helps drive expression in MG cell lines in concert with NFI. Feng and Heintz (1995) localized an element termed the RGE (radial glial element) in the –0.3 kb to –0.8 kb region of the murine *B-FABP* promoter, which was responsible for transcriptional upregulation in radial glial cells. Two regulatory elements within this region of the murine *B-FABP* promoter have recently been characterized by Josephson *et al.* (1998): a POU binding site at –370 to –362 bp, and a non-steroid hormone receptor response element (HRE) at –286 to –275 bp. In the mouse, the POU binding site was necessary for proper B-FABP expression in the developing CNS, while the HRE was required to drive wild-type levels of B-FABP expression in the developing telencephalon. Although the POU and hormone response elements are conserved in the human promoter, we did not detect DNA-protein interactions in these regions by DNase I footprint analysis with MG nuclear extracts. The

reason for these differences is unclear but suggests alternative mechanisms for *B-FABP* gene regulation. We did detect two protected regions (fp4 and fp5) in this region with MG cell extracts, which lie between the putative POU and HRE binding sites. Additional work is needed to characterize the factors bound to fp4 and fp5, and to determine whether these elements are responsible for the reporter gene activation observed with this region.

In summary, we have shown that the promoter of the human *B-FABP* gene contains two binding sites for members of the NFI transcription factor family and that NFI proteins in B-FABP expressing and non-expressing malignant glioma cell lines are differentially phosphorylated. We also presented evidence for the presence of a NFI phosphatase activity specifically in B-FABP positive cells. These results suggest a model in which expression or activation of a phosphatase leads to dephosphorylation of NFI and consequently activation of *B-FABP* transcription. Isolation and characterization of this phosphatase and examining its potential role in glial differentiation will be the subject of further investigation.

4.5 Acknowledgements

We thank Dr. Rufus Day III for supplying the MG cell lines used in this study, Dr. Naoko Tanese for anti-NFI antibody and Elizabeth Monckton for expert technical assistance. This work was supported by a Research Initiative Program grant from the Alberta Cancer Board and an Alberta Cancer Board Studentship to D.A.B.

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Chapter 5 -Discussion

The experiments described in the preceding chapters outline the isolation and partial characterization of the *R-FABP* gene in the developing chick retina (Chapters 2 and 3) and the *B-FABP* gene in human malignant glioma cell lines (Chapter 4) to determine how they are differentially expressed. Chapter 2 describes the characterization of the *R-FABP* promoter and presents evidence that the developmentally regulated transcription factor AP-2 is involved in down-regulating its expression. Chapter 3 extends these findings by showing that the expression patterns of *R-FABP* and members of the AP-2 gene family are mutually exclusive, providing compelling evidence that *R-FABP* is down-regulated by AP-2 in the developing retina. In addition, the early and cell-specific expression patterns of *AP-2α* and *AP-2β* suggest a novel role for these factors in retinal differentiation. In Chapter 4, members of the NFI family of transcription factors are implicated in differential expression of the *B-FABP* gene in malignant glioma. Correlation of B-FABP levels with the phosphorylation state of NFI suggests differential phosphorylation of NFI proteins may regulate the expression of the *B-FABP* gene in malignant glioma. A cell-specific phosphatase activity identified in B-FABP positive cells may be involved in this process.

5.1 Role of R-FABP in Retinal Development

As described in Chapter 1.2.2, Feng *et al.* (1994) have proposed that B-FABP, the murine ortholog of R-FABP acts as a radial glial differentiation factor active during neuronal migration in the brain. We propose that R-FABP may play a similar role during retinal development given its early expression in Müller glial cells, which are the retinal

equivalent of radial glial cells. B-FABP and R-FABP are very similar to each other, with 84% amino acid identity (Feng *et al.*, 1994). Both are similarly expressed in the developing CNS, although B-FABP is reportedly not expressed in the developing mouse retina (Godbout, 1993; Kurtz *et al.*, 1994; Feng *et al.*, 1994). As Kurtz *et al.* (1994) do not specify which developmental stage was tested for B-FABP expression this difference may arise from analysis of different developmental stages. However, the human B-FABP ortholog is present in human fetal retina based on our cDNA library screening, indicating that B-FABP is also expressed in the mammalian retina.

5.2 Regulation of the *R-FABP* Gene during Differentiation: AP-2 and Other Positive and Negative Regulatory Elements

We examined the *R-FABP* promoter to determine the spatial and temporal control elements responsible for its early retinal expression. Identification of a binding site for AP-2 transcription factors in the *R-FABP* promoter and the finding that these factors are expressed in spatiotemporally controlled patterns in the developing retina, suggests that they may play a role in *R-FABP* gene regulation (Chapter 2). Indeed, co-transfection of an AP-2α or AP-2β expression construct with a reporter vector under the control of the *R-FABP* promoter demonstrated that AP-2 expression was able to reduce *R-FABP* promoter activity (Chapter 3). *In vivo* evidence for such a link comes from the comparison of *AP-2α*, *AP-2β* and *R-FABP* expression patterns in the developing retina. Initially expressed in all early retinal cells, *R-FABP* expression is progressively turned off in cells that express AP-2 family members (Chapter 3). Given these data, it is reasonable

to suggest that AP-2 plays a role in *R-FABP* downregulation in the retina, perhaps even a key role.

Although AP-2 is most frequently considered a transcriptional activator (Chapter 1.3.1), it is also capable of functioning as a transcriptional repressor. There are several potential mechanisms whereby AP-2 could repress *R-FABP* expression. AP-2 appears to act directly on the *R-FABP* promoter, since it contains a consensus AP-2 binding site and binds AP-2 *in vitro*, and therefore it seems unlikely that AP-2 would induce expression of a transcriptional repressor, which would then inactivate *B-FABP* transcription. AP-2 has been implicated in the downregulation of a number of genes including the genes encoding: prothymosin and ornithine decarboxylase (Gaubatz *et al.*, 1995), alpha 2(I) collagen (Miao *et al.*, 1999), stellate cell type I collagen (Chen *et al.*, 1996), K3 keratin (Chen *et al.*, 1997), acetylcholinesterase (Getman *et al.*, 1995), insulin-like growth factor II (Rietveld *et al.*, 1999) and C/EBPα (Jiang *et al.*, 1998). In most cases, AP-2 is proposed to displace a positive-acting factor from an overlapping binding site. For instance, Gaubatz and coworkers (1995) have shown that the prothymosin-α and ornithine decarboxylase genes, both normally upregulated by Myc binding to E-box elements in their promoters, are downregulated by AP-2 binding to sites overlapping the E-box elements. Similarly, we propose that AP-2 downregulates *R-FABP* expression by displacing a positive factor(s) bound to the footprint II region. By mobility shift analysis, we have shown that other factors unrelated to AP-2, specifically bind to the footprint II region; these have a different binding site specificity than AP-2 (see Fig. 2-7) and are not recognized by an anti-AP-2 antibody (see Fig. 2-8). The identitity of these other factors has not yet been determined but they are present in the earliest nuclear extract that we

have tested (ED7 retina). Owing to the small size of the embryonic retina, we have not prepared nuclear extracts from earlier timepoints to test for the presence of these binding proteins. This may now be necessary to characterize these other factors binding to the footprint II region so that the displacement hypothesis can be properly tested.

An additional mechanism that might allow AP-2 to downregulate *R-FABP* transcription would be through expression of repressor isoforms of AP-2 via alternative splicing and/or alternative promoter usage (Chapter 1.3.4). We found multiple *AP-2α* and *AP-2β* transcripts in embryonic brain and retina by Northern analysis (Fig 3-3). As noted in Chapter 1.3.4, at least two *AP-2α* transcripts have been characterized that are capable of interfering with AP-2 mediated transactivation; AP-2αB (Buettner *et al.*, 1993) and AP-2α-variant 2 (Meier *et al.*, 1995). We have not characterized the alternative transcripts that are present in embryonic retina and so are unable to properly address whether this mechanism is used alter *R-FABP* expression. Since alternative transcripts are present, this mechanism of regulating *R-FABP* transcription remains a formal possibility. However, based on our Northern blot analysis, there does not appear to be specific modulation of any one AP-2 transcript with retinal development.

AP-2 appears to control only one aspect of the *R-FABP* expression program in the developing retina. The mechanism(s) directing high *R-FABP* expression in the early retina is not known. In early stage chick retina (ED3), all cells initially express *R-FABP*, which later becomes restricted to Müller glial and/or bipolar cells [Godbout *et al.*, 1995; Bisgrove *et al.*, 1999 (Chapter 3)]. Deletion analysis of the *R-FABP* promoter indicates that several regions within the proximal promoter (-234 to +11 bp) together drive high levels of *R-FABP* expression in primary chick retinal cells and we have localized three

regions of DNA/protein interaction within this region by DNase I footprinting [Bisgrove *et al.*, 1997 (Chapter 2)]. Our data suggest that AP-2 binds to one of these elements (footprint 2) and is responsible for downregulating *R-FABP* expression in amacrine, horizontal and displaced amacrine cells. The function of the other binding sites has not been determined. Furthermore, the mechanism downregulating *R-FABP* expression in ganglion and photoreceptor cells, which express neither *R-FABP* or AP-2, has yet to be addressed. These cells may either not produce the necessary factors for *R-FABP* expression, or use another repressive mechanism not involving AP-2. In addition, we do not know how *R-FABP* expression is eventually completely shut down in the adult retina. We have shown that Müller glial cells and perhaps bipolar cells are the only R-FABP positive cells in the ED11 retina (Fig. 3-5). By Northern blot analysis, Godbout (1993) has shown that the ED19 retina contains negligible amounts of *R-FABP* transcripts, indicating that R-FABP expression has been downregulated in Müller and/or bipolar cells. However, AP-2 is not expressed in these cells at ED19 (Fig. 3-6), indicating that it is not involved in the downregulation of *R-FABP* expression and that another mechanism must be used.

5.3 Cell-Specific Expression of AP-2 in the Retina

One of the most interesting features of AP-2α and AP-2β expression in the retina are their strict cell-specific expression, with AP-2α restricted to amacrine neurons and AP-2β expressed in amacrine, displaced amacrine and horizontal neurons. Although a partial sequence for the chicken *AP-2α* gene promoter has been reported (Creaser *et al.*, 1996), little is known regarding its regulation. Since the proximal promoters of the

murine and chick *AP-2α* genes are quite similar (with ~70% identical residues over first 100 bp) and they appear to be similarily expressed in the retina, these genes may be regulated in a similar way (Creaser *et al.*, 1996; Godbout *et al.*, 1995; West-Mays *et al.*, 1999). As discussed in Chapter 1.3.4, several regulatory elements have been experimentally identified in the murine *AP-2α* promoter, including an octamer binding site between –53 to –44 bp, which is conserved in the chicken AP-2α promoter, and a more distal site called A32 which binds AP-2 itself and two novel factors, BTEB-1 and AP-2rep. Expression of BTEB-1 or AP-2rep has not been reported in the retina. Whether either of these sites plays a role in the cell-specific expression of AP-2α in the retina remains to be determined. Creaser *et al.* (1996) found that the –53 to –44 bp octamer binding site was occupied by the transcription factor Oct-1 in HeLa cells. Oct-1 has been reported to be present in the retina, although its spatiotemporal expression pattern in this tissue is not known (Hafezi *et al.*, 1999). An interesting possibility is that the AP-2α octamer site is bound by retina-derived POU-domain factor-1 (RPF-1), which binds to the Oct-1 consensus sequence and is implicated in amacrine and ganglion cell differentiation (Zhou *et al.*, 1996). The chick *AP-2β* gene promoter has not yet been isolated, nor has the transcriptional regulation of any of the mammalian *AP-2β* genes been studied.

Another avenue worth exploring is the possibility that retinoic acid may induce expression of AP-2 family members in the retina. Retinoic acid, a vitamin A derivative, is not involved in the visual cycle, however it is necessary for normal eye development (Hyatt and Dowling, 1997). Retinoic acid is produced in a dorsoventral gradient in the embryonic eye from retinaldehyde by the action of two dorsoventrally distributed

aldehyde dehydrogenases (Dräger *et al.*, 1998). Since the expression of AP-2 family members is inducible by retinoic acid in other developmental systems (Chapter 1.3.4), their expression in the developing retina may be similarly induced by retinoic acid. Our AP-2α and AP-2β *in situ* hybridization experiments using chick retinal sections were not designed to detect dorsoventral variations but simply to determine the cellular distribution of AP-2 in the retina. Our retinal sections were prepared from isolated eyes and so their orientation cannot be determined. This possibility would be best addressed by *in situ* hybridization leaving the eye in its normal context to determine the three-dimensional distribution of AP-2 isoforms in the eye.

5.4 Role of AP-2 in Retinal Development

We have found that AP-2α and AP-2β are expressed in a cell-specific pattern in amacrine, displaced amacrine and horizontal neurons of the embryonic chick retina (Chapter 3). The AP-2 family is known to be involved in the regulation of many different genes and so it is unlikely that its sole function in the retina is to downregulate expression of the *R-FABP* gene. Its early and cell-specific expression suggest that it might play additional roles, either in the specification or the differentiation of these cell types. Comparison of the earliest detectable expression of AP-2α and AP-2β with the known schedule of retinal cell appearance indicates that AP-2 plays a role after cell specification. Whereas we have detected AP-2α and AP-2β in the retina as early as ED5 (with no detectable signal at ED3.5), Prada and coworkers (1991) have shown that amacrine and horizontal cells are born as early as ED3 and ED4, respectively. We therefore propose that AP-2α and AP-2β are involved in the differentiation of amacrine and horizontal cells

and that downregulation of the *R-FABP* gene is only one aspect of its regulatory program. Other candidate genes with AP-2 binding sites that are expressed in the retina include: α-2-adrenergic receptor (Venkatataman *et al.*, 1999), axonin-1 (Giger *et al.*, 1995), ornithine aminotransferase (Zintz and Inana, 1990), insulin-like growth factor binding protein-5 (Duan and Clemmons, 1995) and transforming growth factor-α (Wang *et al.*, 1997). Expression of AP-2α and AP-2β in the developing retina may therefore induce expression of a variety of genes involved in the differentiation process.

AP-2 is also implicated in neuronal differentiation in other systems. Treatment of P19 cells with 1 μM retinoic acid causes their differentiation into neuronal and glial cell types, whereas exposure to 1% dimethylsulfoxide or 10 nM retinoic acid leads to the appearance of mesoendodermal cell types (Rudnicki and McBurney, 1987). Philipp *et al.* (1994) followed the levels of AP-2α in P19 cells induced to differentiate along each pathway to determine if AP-2α induction was specific for neuroectodermal differentiation. AP-2α was induced in cells differentiating along the neuroectodermal pathway but not the mesoendodermal pathway, suggesting that AP-2α expression is specifically involved in the differentiation of neuroectodermal lineage. One way for AP-2 to accomplish this task would be for it to upregulate a subset of genes involved in neural differentiation or conversely to downregulate other genes preventing such differentiation.

As discussed in Chapter 1.3.3, West-Mays *et al.* (1999) were able to use chimeric mice to study the consequences of AP-2α inactivation at later stages of development. they found that loss of AP-2α leads to retinal malformations including: abnormal retinal folds and cell clustering, duplication of dorsal retina and loss of ganglion cells (West-Mays *et al.*, 1999). If AP-2α is involved in the differentiation of amacrine cells, then their

development should be prevented in its absence; however, none of the defects observed by West-Mays and coworkers (1999) appeared to be amacrine cell-specific. Similarily, if AP-2β is involved in the differentiation of amacrine, displaced amacrine and horizontal cells, then its loss may be expected to have negative implications on their development. As the murine retina develops predominantly post-natally, the embryonic lethality exhibited by AP-2β-null mice prevents proper assessment of the role of AP-2β in retinal development (Moser *et al.*, 1997). With their largely overlapping expression patterns in the developing retina, AP-2α and AP-2β may be able to compensate for the loss of each other in gene knock-out experiments. Although, double mutant mice would be informative in this regard, the lethality of each mutation make this a difficult experiment to perform, even using chimeric mice.

Recently, the *Drosophila* AP-2 ortholog, DAP-2, has been cloned and shown to be expressed in the eye-antennal imaginal disc, suggesting that its role in eye development may be conserved from invertebrates to vertebrates (Bauer *et al.*, 1998; Monge and Mitchell, 1998). Although the eyes of vertebrates and *Drosophila* are very different, it has been shown that many of the signaling pathways have been conserved; for instance, the orthologs Pax-6 and *eyeless* are necessary for eye formation in vertebrates and *Drosophila* respectively (Halder *et al.*, 1995; Chow *et al.*, 1999). Misexpression of Pax-6 and *eyeless* has been demonstrated to direct the formation of ectopic eyes in *Xenopus* and *Drosophila*, respectively (Halder *et al.*, 1995; Chow *et al.*, 1999). Moreover, vertebrate Pax-6 is able to direct eye formation in *Drosophila* indicating that the cascade of events downstream of Pax6/*eyeless* have been sufficiently conserved to trigger eye formation (Halder *et al.*, 1995). The function of DAP-2 in

Drosophila eye development may differ somewhat from the role of AP-2 in vertebrates because the structure of the *Drosophila* compound eye and the vertebrate eye are so different.

5.5 AP-2 and Diseases of Eye Development

Given the severe defects of the AP-2α null mouse, it is unlikely that a human embryo possessing homozygous AP-2 mutations would survive to term. On the other hand, AP-2 heterozygous mice are generally unaffected and so human carriers may exist (Zhang *et al.*, 1996; Schole *et al.*, 1996). Notoli *et al.* (1998) noted that AP-2α +/- embryos can exhibit mild defects in craniofacial morphogenesis (dental malocclusion), albeit at a low penetrance (4%), suggesting that loss of one *AP-2α* allele may lead to phenotypic changes which should be observable in the human population. Chromosomal mapping of the human *AP-2α* and *AP-2β* genes localized them to chromosome 6p24 and 6p12, respectively (Williamson *et al.*, 1996). Two disease loci have been mapped to the telomeric end of chromosome 6p that have features expected for mutations in AP-2: orofacial clefting syndrome (Davies *et al.*, 1995, 1998) and Axenfeld-Rieger syndrome (Ozeki *et al.*, 1999). Patients with the orofacial clefting syndrome exhibit cleft palate, while Axenfeld-Rieger patients show a variety of abnormalities including defects in eye formation (anterior chamber defects and microphthalmia), dental and facial abnormalities (Shields *et al.*, 1985). While the molecular basis of the orofacial clefting syndrome has not yet been determined, recent data have implicated mutations in two other transcription factors as the basis for Rieger's syndrome: the homeobox protein, Pitx2 (Amendt *et al.*, 1998) and the forkhead transcription factor FKHL7 (Nishimura *et al.*, 1998).

5.6 B-FABP and Malignant Glioma

B-FABP has been proposed by Feng and coworkers (1994) to function as a differentiation factor involved in the establishment and maintenance of radial glial cell differentiation in the developing brain. We have demonstrated that human B-FABP is expressed in the developing brain, retina and a subset of malignant glioma cell lines (Godbout *et al.*, 1998). In a panel of 15 malignant glioma cells lines, we found that five expressed high levels of *B-FABP* mRNA as determined by Northern blotting, while the others expressed negligible or no *B-FABP*. Interestingly, B-FABP (+) cell lines co-expressed glial fibrillary acidic protein (GFAP), a cytoskeletal protein found specifically in differentiated astrocytes, suggesting that B-FABP is found in MG cells showing the greatest degree of differentiation. As described in Chapter 1.5.2, reducing GFAP levels in MG cells by overexpressing antisense GFAP, leads to cells that have increased growth and invasive potential compared to GFAP (+) cells (Rutka *et al.*, 1994). Expression of both genes in the same cell is curious because they are usually only co-expressed in a few selected cell types *in vivo*: cerebellar Bergmann glia, radial glia of the hippocampal dentate gyrus and gomori-positive astrocytes (Kurtz *et al.*, 1994; Young *et al.*, 1996). Most radial glial cells stop expressing B-FABP, lose their long radial fiber and are converted into mature GFAP-expressing astrocytes after neuronal migration in the developing brain is complete (Schmechel and Rakic, 1979). So, these cells typically undergo a transition from B-FABP-positive/GFAP-negative radial glia into B-FABP-negative/GFAP-positive astrocytes (Hatten, 1999). Co-expression of these proteins may indicate that the tumor arose from a cell that normally expresses both genes or perhaps a

cell in transition between a radial glial cell and an astrocyte. It would be interesting to determine whether nestin and vimentin are also expressed in these GFAP positive MG cell lines. These cytoskeletal proteins are expressed in radial glial cells prior to the transition to astrocytes and then silenced, with a temporal pattern similar to B-FABP

5.7 Regulation of NFI Activity by Phosphorylation: Wider Implications of NFI Phosphorylation in MG and in Cell-Specific Gene Regulation

In Chapter 4, we demonstrated that members of the NFI transcription factor family bind to two elements (fp1 and fp3) in the *B-FABP* promoter and that NFI proteins in B-FABP positive and B-FABP negative malignant glioma cell lines are differentially phosphorylated. Based on these data and the data of Yang *et al.* (1993), we propose that phosphorylation of NFI decreases its transactivating potential. While nuclear extracts from both B-FABP negative and positive cell lines have NFI that can bind to fp1 and fp3, the NFI bound to the *B-FABP* negative promoter is hyperphosphorylated and inactive, whereas the dephosphorylated NFI bound to the *B-FABP* (+) promoter is transcriptionally active.

NFI is known to regulate the expression of many different genes, so its regulation by phosphorylation is likely to have effects in addition to its regulation of the *B-FABP* gene. Other genes whose expression has been shown to be affected by NFI phosphorylation include: the murine pro-α2(I) collagen gene, the murine GLUT4 gene, the metallothionein I gene and several viral promoters (Yang *et al.*, 1993; Cooke and Lane, 1999). Not all NFI responsive genes are down-regulated in response to NFI phosphorylation. For example, the metallothionein I gene is up-regulated by NFI

phosphorylation resulting from c-myc overexpression. NFI phosphorylation may therefore have different effects depending on the context of the gene. Its phosphorylation in response to insulin stimulation, as shown by Cooke and Lane (1999), suggests that NFI activity may be linked to the nutritional state. Insulin stimulation in response to high blood glucose levels leads to cellular uptake of glucose and a variety of anabolic events, such as increased production of fats, glycogen and polypeptide synthesis, indicating that a variety of cellular processes are affected by insulin stimulation.

Even though NFI is widely expressed, differential phosphorylation may enable NFI to function in a cell or tissue-specific manner. We have shown that B-FABP positive MG cell lines specifically express or activate a protein phosphatase activity, not found in B-FABP (-) cell lines (Chapter 4). While not expressed in a cell-specific manner itself, NFI may be active only in cells expressing this phosphatase activity. The kinase activity responsible for NFI phosphorylation does not appear to be cell-specific, at least in MG cell lines, because NFI from both the B-FABP negative and B-FABP positive MG cell lines were phosphorylated to some degree. This kinase activity may be regulated in other systems to provide an additional level of NFI control. The experiments demonstrating NFI phosphorylation have all been carried out in tissue culture-based systems. To determine if NFI is differentially phosphorylated *in vivo*, it will be necessary to compare the phosphorylation state of NFI isolated from different tissues. Once the NFI phosphatase has been identified it will be interesting to study its expression pattern to see if it is the sole mediator of the phosphorylation status of NFI.

We have not yet identified the NFI phosphatase and kinase activities present in the MG cell lines. One candidate phosphatase to be considered is PTEN (phosphatase and

tensin homologue deleted on chromosome ten), also known as MMAC (mutated in multiple advanced cancers). *PTEN* was originally identified as a tumor suppressor gene located on chromosome 10q23 and is predicted to encode a 403 amino acid protein phosphatase based on its sequence similarity with known phosphatases (Steck *et al.*, 1997; Li *et al.*, 1997). Chromosome 10q23 is a region frequently lost or mutated in glioblastoma multiforme, the most malignant form of astrocytoma (Bigner *et al.*, 1988). Sixty to ninety percent of glioblastoma multiforme tumours have deletions or mutations involving chromosome 10 leading to loss of heterozygosity (LOH) (Collins and James, 1993; von Deimling *et al.*, 1995). Myers and coworkers (1997) have demonstrated that PTEN is a dual specificity phosphatase able to dephosphorylate proteins modified on serine, threonine and tyrosine residues, with a preference for highly acidic proteins. As well, PTEN has been shown to dephosphorylate the lipid second messenger, phosphatidylinositol 3,4,5-triphosphate (Maehama and Dixon, 1998). PTEN mutations or deletions have been identified in several of the MG cell lines used in Chapter 4, including the B-FABP negative lines: A172, T98 and U87 and the B-FABP positive lines: U251 and U373 (Steck *et al.*, 1997; Li *et al.*, 1997). The individual mutations are listed in Table 5-1. If PTEN is the NFI phosphatase, then it should be functional in B-FABP positive cell lines and either missing or inactive in B-FABP negative cell lines. The B-FABP negative cell lines either lack PTEN entirely (A172) or have mutations in the N-terminal half (T98 and U87), while the mutation found in the two B-FABP positive cell lines studied (U251 and U373) is located in the C-terminal half. The phosphatase domain is located in the N-terminal half of PTEN between residues 90 and 141, with the critical phosphatase motif [(I/V)-H-C-X-A-G-X-X-R-(S/T)-G] located between 122 to 133 (Steck *et al.*, 1997; Li *et*

Table 5-1. PTEN mutations in malignant glioma cell lines

Cell line	Mutation	Codon	Predicted effect
B-FABP negative			
A172	Homozygous deletion of PTEN	-	No PTEN expression
T98	T125G	42	Leu →Arg
U87	49 bp deletion	54	Frameshift
B-FABP positive			
U251	Insertion of TT	241	Frameshift and truncation at codon 256
U373	Insertion of TT	241	as for U251

Adapted from Steck *et al.*, 1997 and Li *et al.*, 1997.

al., 1997). A172 and U87 certainly lack PTEN activity because A172 lacks the *PTEN* gene entirely and U87 has a 49 bp deletion leading to a frameshift at codon 54 located N-terminal to the phosphatase domain. T98 has a Leu to Arg substitution mutation at codon 42, which Steck *et al.* (1997) suggests would disrupt a predicted secondary structure located in this region and so may not produce a functional phosphatase. The two B-FABP positive cell lines studied also have a frameshift mutation leading to a truncated PTEN protein, however, the frameshift does not occur until after the phosphatase domain, and thus may not affect its phosphatase activity. To determine whether PTEN is the NFI phosphatase, it will be necessary to directly test whether PTEN is capable of dephosphorylating NFI *in vitro* and whether it is specifically active in B-FABP positive cell lines. Tian *et al.* (1999) have recently shown that re-introduction of wild-type PTEN into U87 MG cells leads to reduced proliferation, reduced anchorage-independent growth and increased differentiation including expression of GFAP. This suggests the possibility that PTEN expression underlies the co-expression GFAP and B-FABP in MG cell lines.

There are several different mechanisms whereby phosphorylation could regulate the activity of NFI including: altering its protein/protein interactions, changing its nucleocytoplasmic localization, altering its ability to interact with DNA or directly affecting its trans-activation domain. Since NFI is present in nuclear extracts from both B-FABP positive and negative cell lines, its phosphorylation does not appear to affect its overall subcellular localization. Yang *et al.* (1993) compared the DNA binding affinity of phosphorylated versus dephosphorylated NFI and found no significant difference, indicating that phosphorylation does not prevent NFI from interacting with DNA. It is not possible to rule out interaction of phosphorylated NFI with an inhibitory protein based on

our mobility shift gels. Such interaction would be expected to lead to formation of a low mobility complex, similar to that observed in Fig. 4-8. It is equally likely however that phosphorylation interferes with transcriptional activation by directly modulating the activity of the trans-activation domain. We postulate that there are multiple phosphorylation sites on NFI, generating the multiple gel shift complexes observed with nuclear extracts from B-FABP positive cell lines to the single low mobility complex found in B-FABP negative lines, representing the fully phosphorylated NFI. Further experiments are needed to determine how phosphorylation regulates NFI activity.

5.8 Differential Expression of c-myc Expression in MG Cell Lines: Correlation with B-FABP Expression

The c-myc proto-oncogene is a transcription factor involved in cell proliferation and apoptosis (for review, see Bouchard *et al.*, 1998). Constitutive expression of c-myc leads to growth factor-independent cell proliferation. We have found that c-myc is preferentially expressed in the least differentiated (GFAP/B-FABP negative) MG cell lines (Chapter 4), suggesting that it may be involved in the proliferation of these cells. Yang *et al.* (1993) found that c-myc overexpression led to increased phosphorylation of NFI. Our results suggest that increased NFI phosphorylation is due, not to increased kinase activity, but rather to absence of phosphatase activity. This leads us to propose the model outlined in Figure 5-1. Expression of c-myc leads to repression of the NFI phosphatase, which allows hyperphosphorylation of NFI and consequently reduced expression of B-FABP. In cell lines that do not express c-myc, the NFI phosphatase is free to dephosphorylate NFI leading to expression of B-FABP. One mechanism whereby

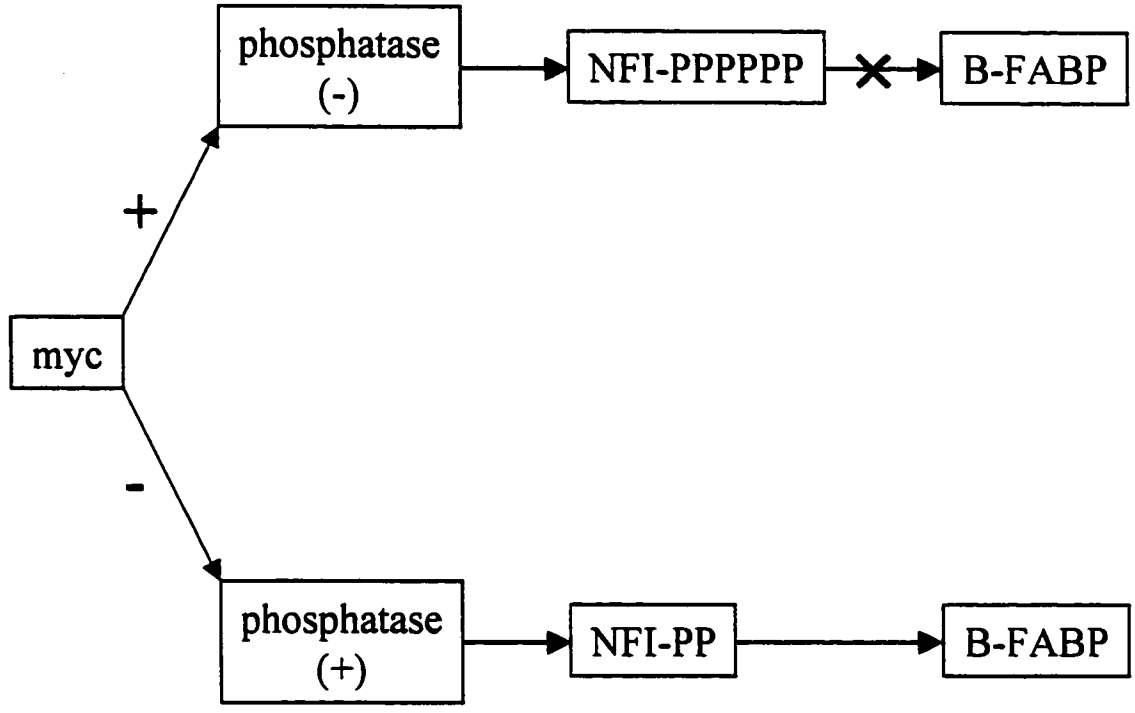

Figure 5-1. Model depicting the interplay between c-myc expression and B-FABP expression. Expression levels of c-myc dictate levels of phosphatase activity. Dephosphorylation of NFI leads to B-FABP expression.

c-myc levels could modulate phosphatase levels might be to have c-myc or a repressor induced by c-myc, down-regulate expression of the phosphatase at the level of transcription. Alternatively, this regulation could also occur at a post-transcriptional stage, for instance, c-myc could induce expression of a phosphatase inhibitor, leading to a reduction in phosphatase activity. Once the NFI phosphatase has been identified, we will be able to pursue the role that c-myc plays in NFI activation.

5.9 Future Experiments

Future experiments will be required to address the myriad of questions remaining and those arising from this work. Of particular interest, in light of the major differences between the promoters of the *R-FABP* and *B-FABP* genes, will be to determine the expression pattern of B-FABP and AP-2 isoforms in the developing human retina. Both B-FABP and AP-2 are known to be expressed in the developing human retina based on our data and the expressed sequence tag (EST) expression data present in GenBank. Another area that should be pursued is to identify the downstream targets of AP-2 in the retina as well as to study the proteins that regulate AP-2α and AP-2β expression in the retina. Analysis of the regulation of the AP-2 genes may lead to further insight into the factors involved in retinal cell specification and/or the early stages of differentiation. Similarly, identification of the genes regulated by AP-2 in the retina will provide insight into the molecular events downstream of AP-2. One possible approach to identify AP-2 target genes would be to isolate cDNAs from retinal cells transfected with AP-2 expression vectors in the sense and anti-sense orientation and to identify differentially expressed transcripts by differential display PCR (ddPCR) or other techniques. The

regulation of the *R-FABP* gene could be further studied by identifying the factors bound to the other footprinted regions identified in Chapter 2.

The correlation that we have found between NFI phosphorylation and *B-FABP* expression is not necessarily a causal one and its effect remains to be tested. To directly test the effect of NFI phosphorylation on *B-FABP* activity, *in vitro* transcription experiments comparing the effects of phosphorylated and dephosphorylated NFI, could be performed. Another area of interest will be the purification and identification of the NFI phosphatase activity detected in U251 nuclear extracts. One way of doing this would be to directly purify this enzyme from U251 nuclear extracts by conventional column chromatography and identify it by amino acid sequencing. If the phosphatase has not been previously cloned, the protein sequence could be used to prepare degenerate oligonucleotide probes for cDNA library screening.

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