Transcriptional regulation of the antifreeze protein genes in winter flounder

by

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In loving memory of

Amy Yi-Ray Shen

Some people only dream of angels. I held one in my arms.

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Ab	antibody
ACF	ATP-utilizing chromatin assembly and remodeling factor
AEP	antifreeze enhancer-binding protein
AFP	antifreeze protein
AFGP	antifreeze glycoprotein
AP-1	activating protein-1
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
bZIP	basic region/leucine zipper protein
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
CHRAC	chromatin accessibility complex
cpm	counts per minute
DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetatic acid

EST	expressed sequence tag
GH	growth hormone
GSK3	glycogen synthase kinase 3
GTF	general transcription factor
HAT	histone acetylation transferase
HDAC	histone deacetylase
HDP	helicase domain-containing protein
HEPES	N-2-hydroxyethylpiperazine-N -2-ethanesulfonic acid
HNF-1	hepatocyte nuclear factor-1
IBM	ice-binding motif
IGF-1	insulin like growth factor-1
IGF-1R	IGF-1 receptor
IPTG	isopropyl-β-D-thiogalactoside
IRS-1	insulin receptor substrate-1
kb	kilobase pairs
kDa	kilo-Dalton
LAP	liver-enriched transcriptional activator protein
LB	Luria-Bertani Medium
LIP	liver-enriched transcriptional inhibitor protein
MAP	mitogen-activated protein
MBP	maltose-binding protein
MEM	minimal essential medium
mRNA	messenger ribonucleic acid

NRD	nuclecsone remodeling and deacetylating
NURD	nucleosome remodeling histone deacetylase complex
NURF	nucleosome remodeling factor
PCAF	P300/CBP-associated factor
PCR	polymerase chain reaction
PIC	pre-transcription initiation complex
PI3-kinase	phosphatidylinositol 3 phosphate-kinase
PKA	cAMP dependent protein kinase
PKC	protein kinase C
PMSF	phenylmethanesulfonyl fluoride
Pol II	RNA polymerase II
RSF	remodeling and spacing factor
RT-PCR	reverse transcription PCR
SAGA	Spt-Ada-Gcn5 acetyltransferase
SDS	sodium dodecyl sulfate
SH2	Src homology 2
SRB	suppressor of RNA polymerase B mutations
SWI /SNF	switch/sucrose non-fermenting
TAF	TBP-associated factor
TBP	TATA-binding protein
TBS-T	Tris-buffered saline-Tween
TK	thymidine kinase
PAGE	polyacrylamide gel electrophoresis

wfAFP	wihter flounder AFP
wflAFP	winter flounder liver-type AFP
wfsAFP	winter flounder skin-type AFP

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Abstract

Winter flounder, *Pleuronectes americanus*, produces two types of antifreeze proteins (AFPs) to avoid freezing during winter. The expression of the liver-type AFPs (wflAFPs) and skin-type AFPs (wfsAFPs) are differentially regulated by environmental factors and growth hormone. The only intron of the wflAFP gene acts as a liver-specific enhancer and a more refined core sequence Element B interacts with a liver-enriched transcription factor C/EBPα and a presumptive antifreeze enhancer-binding protein (AEP). In the present study, the specific binding of C/EBPα to Element B and the presumptive AEP was confirmed in both rat and flounder liver extracts. Element B of the wflAFP gene was shown to be the core enhancer. Mutations of Element B significantly decreased the enhancer activity of the wflAFP gene intron. Recombinant C/EBPα interacted specifically with Element B and transactivated the wflAFP gene expression, indicating its pivotal role in the liver-specific expression of wflAFP gene. Also, C/EBP proteins were shown to be present in winter flounder. Moreover, IGF-1 and PI3-kinase were found to inhibit the wflAFP gene expression, suggesting their involvement in wflAFP gene regulation. In addition, the intron of a wfsAFP gene was shown to be a ubiquitous enhancer. Element S, the corresponding region of Element B of the wfsAFP intron, did not interact with C/EBPα, while it still bound

to the antifreeze enhancer-binding protein. As expected, a TA insertion in Element B of the wflAFP gene intron destroyed its liver-specific enhancer activity. Finally, the identity of rat AEP was revealed as a novel protein with 988 residues in length. It is homologous to Smubp-2/Rip-1 with DNA-binding domain and putative helicase motifs. AEP interacted specifically with Element B and was necessary for the transactivation of the enhancer of the wflAFP gene. Furthermore, AEP was shown to be widely expressed in various tissues and its homologous gene was present in flounder. The present study provides a mechanism(s) by which the differential tissue distribution of the wflAFP and wfsAFP gene expression can be achieved. A model is proposed integrating the involvement of GH and IGF-1 signaling pathway as well as the roles of C/EBPα and AEP in mediating the seasonal and hormonal regulation of the AFP genes in winter flounder.

Chapter One

Introduction

1.1 Biochemistry of fish antifreeze proteins

Many species of marine teleosts inhabiting the polar oceans often encounter sea water temperature as much as -1.2°C below the equilibrium freezing point of their body fluid. These fish have evolved an adaptative mechanism, the production of specific protein molecules, to protect themselves from freezing. These proteins, antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs) depress the freezing point in a non-colligative manner (Davies and Hew, 1990; Hew and Yang, 1992; Davies and Sykes, 1997; Cheng, 1998). Also, different AFPs have been found in plants and insects, and their distritution appears to be limited to species that are exposed to cold temperatures such as winter rye, budworm and beetle (Hon et al, 1995; Graham et al, 1997; Doucet et al, 2000; Graether et al, 2000).

Presently, there are four types of AFPs (Type I-IV) and one type of AFGP been characterized in fish. These proteins have similar ice binding and crystal growth inhibition characteristics despite their structural diversity and evolutionary divergence: type I, alanine-rich, α-helical proteins; type II, cystine-rich, disulfide linked proteins containing β-sheet; type III, globular proteins; type IV, glutamate- and glutamine-rich helix bundle proteins; and AFGP, carbohydrate-rich proteins with $(Ala\text{-}Ala\text{-}Thr)_n$ repeat. Table 1-1 summarizes the properties of these distinct types of fish AFPs and the species that produce these proteins. Among them, the type I AFPs produced from the winter flounder has been the most extensively studied fish AFPs with respect to protein structure and function, as well as gene regulation.

1.1.1 Structure and function of winter flounder antifreeze proteins

Winter flounder (*Pleuronectes americanus*) is an inshore, shallow-water bottom fish with oval-shaped and laterally compressed body. It resides along the Atlantic coast of North

Table 1-1 Classification and occurrence of fish antifreeze proteins. (Hew & Yang, 1992; and Davies & Sykes, 1997)

	Type I AFP	Type II AFP	Type III AFP	Type IV AFP	AFGP
Mass	3–5kDa	14-24kDa	7kDa	12.3kDa	2.7-32kDa
Primary structure	alanine-rich 11 a.a. repeats	cystine-rich disulfide-linked			(A-A-T)n disaccharide
Secondary structure	amphipathic α-helical	β-sheet	short β strands	mostly α-helix	amphipathic polyproline II helix
Tertiary structure	100% α-helix	globular C-type lectin fold	globular with some flat surfaces	helix bundle?	extended
Species	winter flounder yellow tail flounder shorthorn sculpin grubby sculpin longhorn sculpin	sea raven smelt Atlantic herring	ocean pout wolffish	longhorn sculpin	Antarctic nototheniids northern cods

America from Labrador to Georgia (Scott and Scott, 1988). It produces type I AFPs, that have more than 60% alanine and high helical content with 11-residue repeat sequences (Harding et al, 1999). Two distinct types of type I AFPs are found in winter flounder: the liver-type AFPs (wflAFPs), and more recently, the skin-type AFPs (wfsAFPs) (Gong et al, 1996). The wflAFPs from flounder serum, which are produced from liver, were resolved by reverse phase high-performance liquid chromatography into several components and two of them, wflAFP-6 and 8 (HPLC-6 and 8), comprise 55% and 35% of the total plasma wflAFPs, respectively (Fourney et al, 1984a; Hew et al, 1984). They are both 37 amino acids in length and contain three 11 amino acid repeats of Thr-X2-Asp/Asn-X7 where X is predominantly Ala (Fig.1-1A). Circular dichroism, NMR and X-ray diffraction studies of the wflAFP-6 have shown that it is a single, slightly curved, 100% α-helical peptide stabilized by N- and C-terminal cap structures (Fig.1-1B) (Yang et al, 1988; Sicheri and Yang, 1995; Gronwald et al, 1996; Sonnichsen et al, 1998). The major wfsAFPs (wfsAFP-1, wfsAFP-2 and wfsAFP-3) isolated from flounder skin are 38 or 39 amino acid polypeptides blocked with an acetyl group at the N-terminus. These peptides are also enriched with alanine and contain similar 11 amino acid repeats (Fig. 1-1A).

The AFPs exert their antifreeze activity by depressing freezing point without affecting the equilibrium melting point, and inhibiting ice crystal growth in a non-colligative manner. That means they do not follow the colligative property of the solution that depends on the mole fraction of solute particles present but not on the molecular mass or chemical properties of those particles. Ice can exist in several polymorphic forms, but only the hexagonal ice Ih is stable at normal atmosphere at 0°C. Its lattice unit may be characterized by four axes, a1, a2, a3 and c and comprises eight faces including two basal faces normal to the c-axis and six

A

```
                  2             13            24
wflAFP-6    DTASDAAAAAALTAANAKAAAELTAANAAAAAAATAR

wflAFP-8    DTASDAAAAAALTAANAKAAAKLTADNAAAAAAATAR


wfsAFP-1      MDAPARAAAATAAAAKAAAEATKAAAAKAAAATKAAAH

wfsAFP-2      MDAPAKAAAATAAAAKAAAEATAAAAAKAAAATKAGAAR

wfsAFP-3      MDAPAKAAAATAAAAKAAAEATAAAAAKAAADTKAKAAR
                           11            22
```

B

Fig. 1-1 Amino acid sequences and tertiary structure of wfAFPs. A: Amino acid sequence comparison of major wflAFPs (wflAFP-6 and 8) and wfsAFPs (wfsAFP-1,2 and 3). Conserved amino acids are in bold. B: The X-ray structure of wflAFP-6 (Sicheri & Yang, 1995).

prism faces (Fig. 1-2A). Normally, the ice growth takes place on prism faces, while type I AFP binds to prism faces and prevents crystal growth in a-axes direction. Ice grows on the unordered basal plane continuously with the binding of AFP to prism faces results in bipyramidal ice crystal, which is the typical ice morphology in the presence of AFPs (Fig. 1-2B) (Hew and Yang, 1992; Harding et al, 1999). Also, the antifreeze activity is measured by thermal hysteresis, which is the difference between the melting point and the ice growth temperature. A comparison of the antifreeze activity between wflAFP and wfsAFP is shown in Fig.1-2C (Gong et al, 1996). Earlier ice-binding models for wflAFP-6 assumed that hydrogen bonding was the major interaction for ice binding, while more recent studies suggested that Van der Waals interactions and hydrophobic interactions might be the major driving force instead (DeVries and Lin, 1977; Knight et al, 1991; Chao et al, 1997; Haymet et al, 1998; Harding et al, 1999). Also, putative ice-binding motifs (IBM) have been proposed from the structure of wflAFP-6 (Sicheri and Yang, 1995).

1.1.2 Biosynthesis and gene organization of winter flounder antifreeze proteins

The wflAFPs are synthesized mainly in the liver as large precursor polypeptides of 82 amino acids. The presequences are removed cotranslationally while the prosequences are removed soon after their secretion into blood (Davies et al, 1982; Pickett et al, 1984; Hew et al, 1986). In contrast, wfsAFP mRNAs were found to be more ubiquitously distributed including liver and stomach, but mainly in exterior tissues such as skin, scale, fin and gill (Gong et al, 1992). Those proteins are produced as mature polypeptides without the pre and prosequences, indicating that they are intracellular proteins and that a distinct mechanism of freezing protection by wfsAFPs might exist.

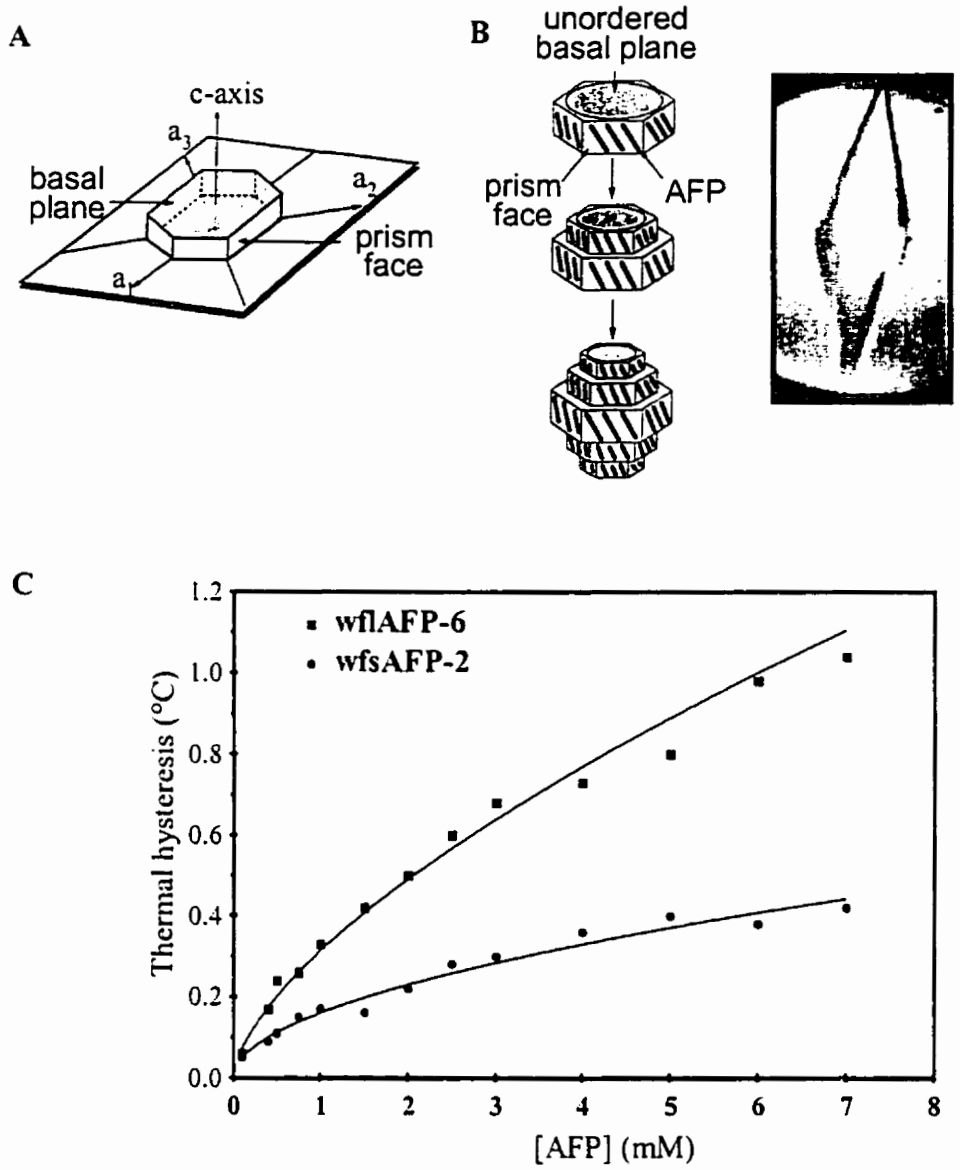


Fig. 1-2 Binding of AFPs to ice surface and thermal hysteresis of wfAFPs. A: Schematic representation of an hexagonal ice crystal. B: AFPs accumulate on prism faces of ice crystal and inhibit ice crystal growth along *a*-axes, resulting in the characteristic bipyramidal ice crystal structure (Hew and Yang, 1992). C: The concentration dependent thermal hysteresis of wflAFP-6 and wfsAFP-2 from flounder serum and skin, respectively (Gong et al, 1996).

Flounder AFPs are found to be encoded by a family of multiple genes. Many of them are organized in direct tandem repeats and the rest linked but irregularly spaced (Scott et al, 1985). Approximately 40 copies of these AFP genes encode the wflAFPs and the majority of genes from the tandem repeats sequenced so far codes for the wflAFP-6 component (Davies, 1992). The sequence of a representative wflAFP gene, 2A-7b, which encodes the most abundant wflAFP-6 component, is shown in Fig. 1-3. It is a tandemly repeated gene with less than 1 kb in length and consists of two exons and a single intron of 497 bp (Scott et al, 1988; Davies, 1992). It contains a cap site, a TATA and CAAT boxes which are 32 bp and 84 bp upstream from the cap site, respectively, as well as initiation and stop codons, a conventional splice junction sequence and a polyadenylation signal.

Similarly, there are approximately 40 copies of wfsAFP genes in flounder genome which are distinct from the genes encoding wflAFPs (Gong et al, 1996). The sequences of wfsAFP cDNA were found to be closely related to two previously identified genomic sequences F2 and 11-3, which belong to the subset of AFP genes that are linked but irregularly spaced (Davies and Gauthier, 1992). They were previously assigned as pseudogenes since they lack a typical TATA box in the putative promoter region and contain stop codons in the 5' upstream region. Two transcriptional start sites were mapped on the F2 gene, which encodes wfsAFP-2, and a putative TFIID binding motif AATAAAT is found 30 bp upstream of the first start site (Fig. 1-4). The organization of the wfsAFP genes, which also consists of two exons and an intron, is very similar to the liver 2A-7b gene. The intron of F2 shares 95% identity to that of 2A-7b except that F2 intron has an extra 241 bp insertion at position +254. The presence of two sets of AFP genes within winter flounder that exhibit distinct tissue

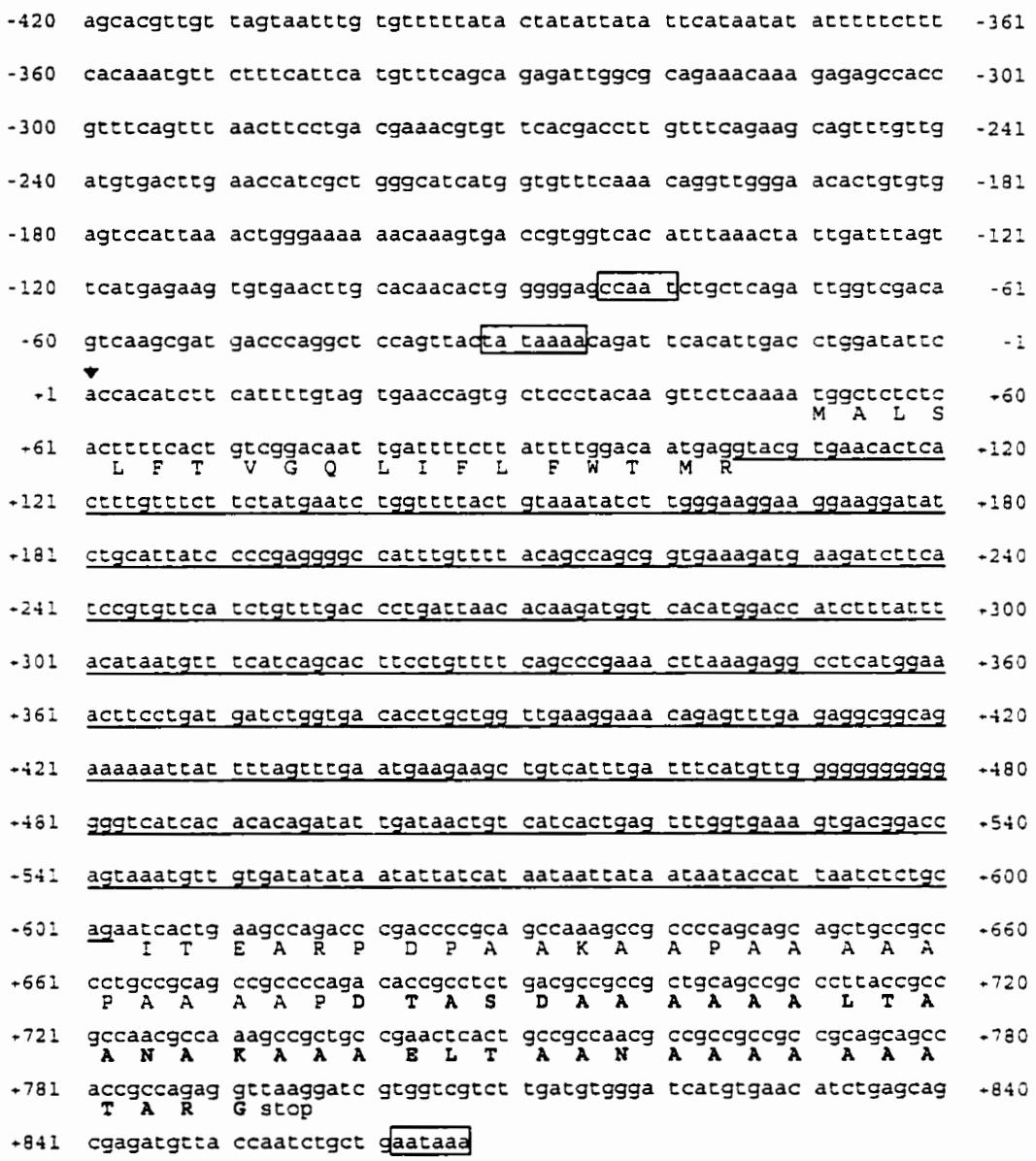


Fig. 1-3 Sequence of a wflAFP gene, 2A-7b. The CAAT and TATA sequences and polyadenylation signal are boxed. The transcription start site (+1) is indicated by an arrow and the intron sequences are underlined. Amino acid sequence encoded by 2A-7b is in capital letters and the mature peptide is in bold. The GenBank accession number is M62415 (Scott et al,1988) .

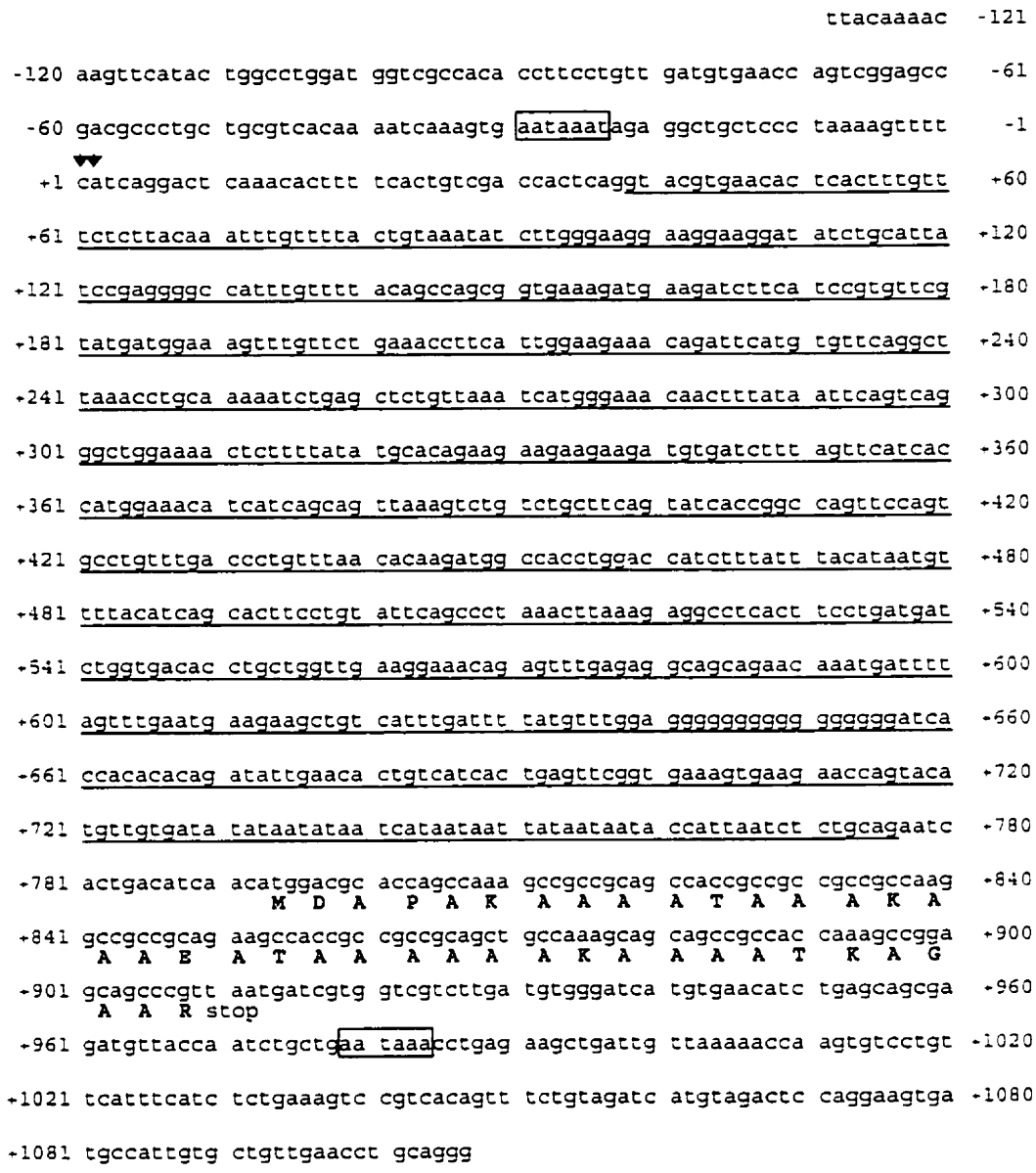


Fig. 1-4 Sequence of a wfsAFP gene, F2. The putative TFIID binding site and polyadenylation signal are boxed. The transcription start sites (+1 and +2) are indicated by arrows and the intron sequences are underlined. Amino acid sequence encoded by F2 is in capital letters. The GenBank accession number is M63479 (Davies and Gauthier, 1992).

specificity has raised interesting questions on how the differential gene expression is achieved and what DNA element(s) and protein factor(s) are involved in this regulation.

1.1.3 Environmental and hormonal control of wfAFP production

The production of AFPs in winter flounder of the Newfoundland population exhibits dramatic seasonal variations. The serum wfAFP level, which is reflected by the thermal hysteresis of the fish blood, starts to increase in November and reaches its peak of approximately 5-10 mg/ml in February. Then it starts to decline in May to its minimal level through the summer (Fig. 1-5) (Slaughter and Hew, 1982; Chan et al, 1993). Also, the seasonal fluctuations of wfAFP mRNA level in liver match closely, but slightly precede the rise and fall of the AFP level (Pickett et al, 1983a; Fourney et al, 1984b; Fletcher et al, 1989). The variations of these AFP mRNA and protein levels were found to be inversely correlated to water temperature as well as the growth rate of the fish. Several studies have been conducted to investigate the environmental and hormonal factors that play roles in this seasonal production of wfAFPs.

Environmental regulation

Though the variations of the serum wfAFPs correlate with the annual cycle of water temperatures, cold temperature does not appear to serve as the signal to initiate the production of wfAFPs in the fall (Fletcher, 1981; Price et al, 1986). When flounders were continuously maintained at 18°C, the AFP mRNA still increased above basal level in the fall. In contrast, the plasma AFP level did not rise in fish exposed to 0°C in fall and even a delay of AFP accumulation was observed. Also, the transcription rate of wfAFPs was not affected by water temperature as shown by nuclear run-on experiments (Vaisius et al, 1989).

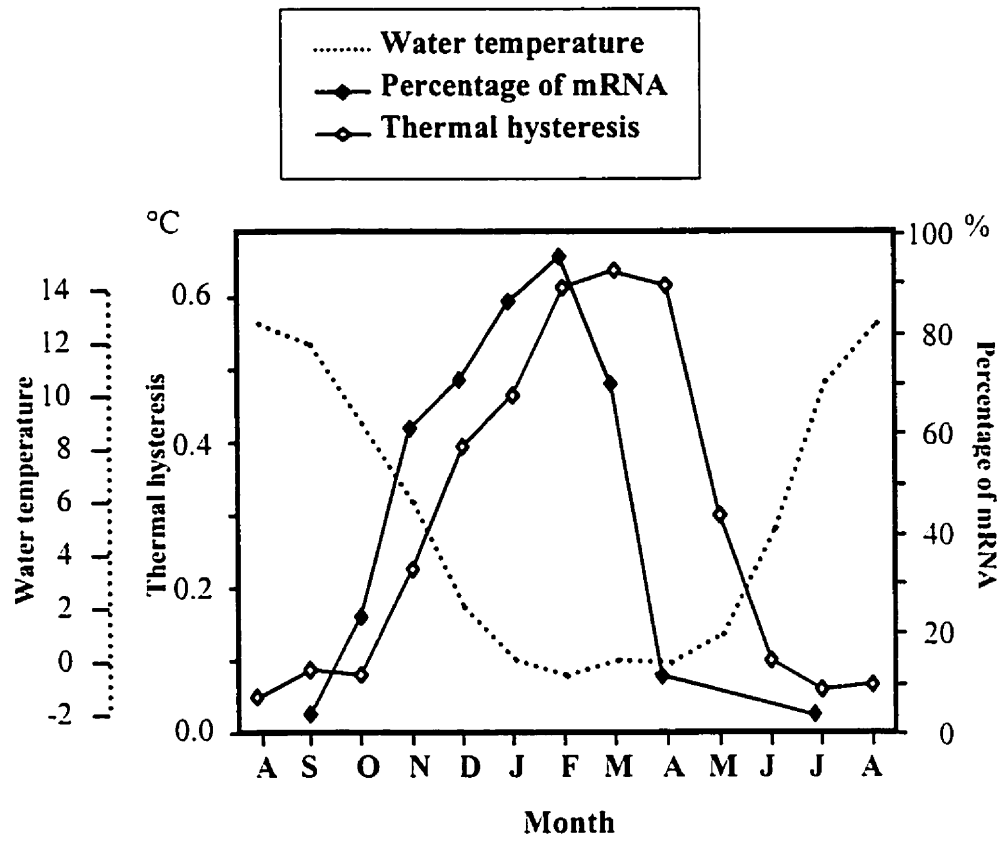


Fig. 1-5 Seasonal variations of the plasma hysteresis and liver AFP mRNA level in winter flounder. The thermal hysteresis in plasma and the AFP mRNA in liver of the winter flounder are shown with a one-year seasonal cycle. The mRNA levels were calculated as a percentage of peak winter values in February (Fletcher et al,1989; Chan et al, 1993).

However, flounders exposed to high seawater temperatures had low levels of plasma AFPs as well as mRNAs, suggesting that cold temperature is still required for wfAFP accumulation in the fall. Since the rate of transcription does not respond to temperature changes, its effect might be post-transcriptional to alter the rates of mRNA degradation and/or metabolic clearance of the serum proteins. The presence of a temperature-dependent nuclease and/or more efficient translation at low temperature has been suggested as possible mechanisms for the control of wfAFP production by water temperature (Chan et al, 1993). Transgenic flies expressing wfAFP genes were shown to have more persistent AFP and AFP mRNA levels when reared at lower temperature and this effect was believed to be mediated by cold-specific mRNA stability that increases the half-life of mRNA (Duncker et al, 1995).

Photoperiod is the cue for triggering the activation of wfAFP synthesis in the fall (Fletcher, 1981; Fourney et al, 1984c). It was shown that long day length could delay the appearance, and significantly suppress the liver AFP mRNA as well as plasma AFP levels. However, short day length did not have any effect on the onset of AFP appearance in the plasma. Therefore, it was suggested that it is the loss of long day length in the fall that switches on the AFP production on time, and this effect of photoperiod may be mediated through the central nervous system (CNS) on pituitary gland via a photoreceptor (Fletcher et al, 1989).

Hormonal regulation

Several studies have demonstrated that the pituitary gland and growth hormone (GH) in particular play important roles in controlling the AFP synthesis in winter flounder (Fletcher, 1979; Hew and Fletcher, 1979; Fletcher et al, 1984; Fourney et al, 1984b; Fletcher et al, 1989; Idler et al, 1989). In hypophysectomized flounder, the plasma antifreeze activity as well as the biosynthesis of AFPs in the liver remained high during summer. Injection of

pituitary extracts significantly reduced the plasma AFP levels in these hypophysectomized fish. Similarily, injection of the GH fractions from pituitary extracts had similar results. The involvement of GH was further supported by nuclear run-on experiments that the AFP gene transcription in liver was suppressed in flounder treated with GH (Chan and Hew, unpublished data). In addition, the seasonal cycle of growth in flounder is inversely correlated with the liver AFP mRNA level. And GH mRNA in pituitary also showed a seasonal cycle with the lowest level occuring during the winter when liver AFP mRNA levels were at their peak (Gill and Fletcher, unpublished data). These results indicate that GH in the pituitary gland plays a direct role in regulating the synthesis of serum AFPs in winter flounder.

Differential regulation of the skin-type wfAFPs

Due to historical reasons, the environmental and hormonal studies discussed above were mainly focused on the plasma AFP level and liver AFP mRNAs, in other words, the liver-type AFPs. It was interesting to find out later that the wflAFPs and wfsAFPs are differentially regulated (Gong et al, 1995). In contrast to the wflAFP mRNA that exhibits 500-700 fold differences between summer and winter, the mRNA of wfsAFPs only exhibit a modest 5-10 fold seasonal variations. Also, while the mRNA of wflAFPs in hypophysectomized fish increased over 40 fold, no significant change was found for the wfsAFPs upon hypophysectomy, suggesting that the skin-type wfAFPs are not under GH control. Table 1-2 summarizes the distinct characteristics between wflAFPs and wfsAFPs. Their differential tissue distributions as well as their differences in seasonal and hormonal regulations have provided an interesting model to study eukaryotic gene expression.

Table 1-2 Comparison of the distinct characteristics between wflAFP and wfsAFP.

	Liver-type (wflAFP)	Skin-type (wfsAFP)
Tissue distribution	liver-specific	widely expressed, mainly in exterior tissues
Gene organization	approx. 40 copies, tandem repeats	approx. 40 copies, linked but irregularly spaced
Seasonal variation	dramatic, 500-700 fold	moderate, 6-10 fold
Hormonal control	inhibited by GH	no significant effect
Biosynthesis	prepro AFP	mature AFP
Cellular localization	extracellular	intracellular

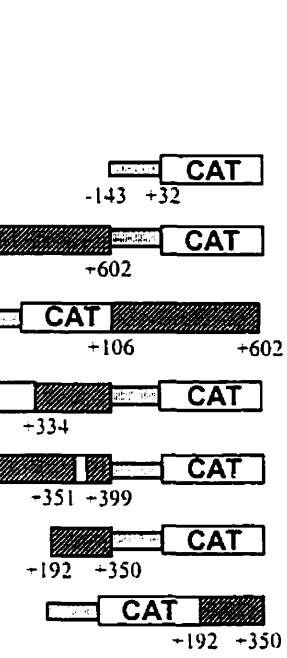
1.1.4 Transcriptional regulation of the liver-type wfAFP (wflAFP) gene

It has been established that transcription is the key mechanism in regulating the seasonal production of wflAFPs (Pickett et al, 1983a; Fourney et al, 1984b; Vaisius et al, 1989). The AFP mRNA contributes 0.5% of the total liver RNA during winter while declining to a mere 0.0007% during summer, which leads to the rise and fall of plasma AFP levels. In addition, the transcriptional rate and accumulation of wflAFP mRNAs in hypophysectomized fish were dramatically reduced by the GH treatment, indicating that the negative GH control is mainly at the transcriptional level. However, post-transcriptional regulation cannot be completely excluded. One study revealed that the capacity of tRNA acceptors for alanine in winter fish increases 40% over summer fish (Pickett et al, 1983b). Also, the alanyl-tRNA synthetase functions preferentially at a lower temperature, indicating that translation efficiency may also be controlled by season and water temperature.

To investigate the DNA regulatory elements in wflAFP gene that are involved in transcriptional regulation, the 5'-upstream region and the only intron of the liver-type 2A-7b gene were examined for their ability to transactivate the chloramphenicol acetyltransferase (CAT) reporter gene in transient expression assays (Gong and Hew, 1993; Chan et al, 1997). Various lengths of the 5'-upstream sequence from 66 bp to –2.3 kb were found to drive similar but very low levels of expression in RTH (rainbow trout hepatoma) cells. On the other hand, the intron of the wflAFP gene (+106 to +602), when linked to the wflAFP basic promoter (-143 to +32), showed more than 10 fold of transactivation activity compared to that of the basic promoter in HepG2 (human hepatoma) cells (Fig. 1-6A). The enhancer activity of the intron was similarly demonstrated in flounder hepatocytes in an *in vitro* transcription assay (Miao et al, 1998a). Moreover, it was shown that the intron acts as a

A

Constructs	Fold enhancement		
	HepG2	HeLa	Rat2
*f*AFP-CAT	1	1	1
In-*f*AFP-CAT	11.0 ± 0.8	0.7 ± 0.1	1.0 ± 0.2
In3'-*f*AFP-CAT	13.6 ± 1.7	0.7 ± 0.1	1.5 ± 0.3
InΔ(192-334)-*f*AFP-CAT	0.91 ± 0.04	ND	ND
InΔ(351-399)-*f*AFP-CAT	9.9 ± 1.2	ND	ND
In(192-350)5-*f*AFP-CAT	15.8 ±1.7	1.4	ND
In(192-350)3'-*f*AFP-CAT	14.5 ± 0.6	0.81	ND



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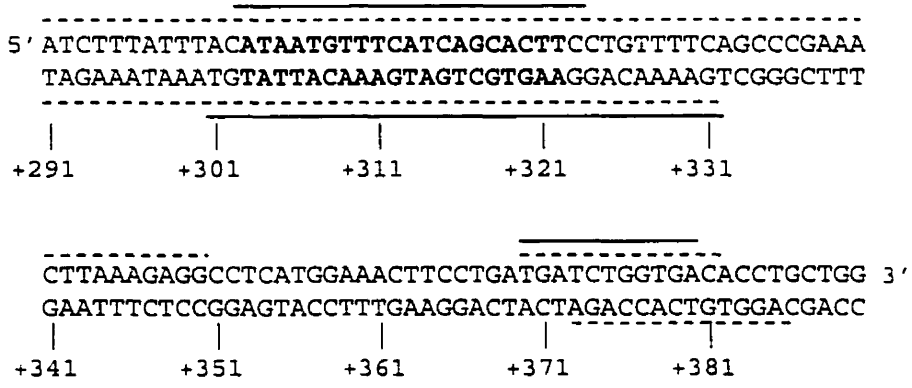


Fig. 1-6 The intron of the wflAFP gene acts as a liver-specific enhancer and interacts with nuclear proteins. A: Results of the transient expression assays in HepG2, HeLa and Rat2 cells. wflAFP constructs with intron sequence linked to the AFP basic promoter and CAT reporter gene were used as indicated (Chan et al, 1997). B: Results of the DNase I footprinting analysis. Regions of the wflAFP intron protected from rat and flounder liver extracts are indicated by dashed and solid lines, respectively. Element B is in bold letters (Miao et al, 1998).

liver-specific enhancer since it only functioned in cells of hepatic origin (HepG2), but not in non-hepatic cells (HeLa, cervical carcinoma, and Rat2, rat embryonic fibroblast). Deletion of the intron sequence from +192 to + 334 completely destroyed the enhancer activity and a truncated intron sequence from +192 to + 350 exhibited similar activity to the full-length intron. Together, these results indicate that the intron of the 2A-7b wflAFP gene acts as a liver-specific enhancer, and the enhancer region is further refined to the sequence +192 to +334 of the gene. Regions in the enhancer of wflAFP gene intron that interact with nuclear proteins were mapped by DNase I footprinting assays with similar results using both rat or flounder liver nuclear extracts (Fig. 1-6B) (Chan et al, 1997; Miao et al, 1998a). One of the regions, designated as Element B (+303 to +322) was shown to form two specific complexes with both rat and flounder extracts by gel retardation assays. One of the complexes could be competed out specifically by oligonucleotide containing C/EBP (CCAAT/enhancer binding protein) binding site. Members of the C/EBP family including C/EBPα, β, γ, δ and ε exhibit similar DNA binding specificity and among them, C/EBPα is expressed only in a limited number of tissues and is present in high concentration in terminally differentiated hepatocytes (Landschulz et al, 1988; Takiguchi, 1998). An antibody to C/EBPα supershifted the specific C/EBP complex formed between Element B and rat liver extract (Chan et al, 1997). These studies suggest that the liver-enriched factor C/EBPα interacts with Element B of the wflAFP gene and might be involved in its tissue-specific expression.

In addition, an oligonucleotide containing activating protein-1 (AP1) consensus was able to compete with another specific complex formed between Element B and rat or flounder liver extracts in gel retardation assays (Chan et al, 1997). However, the protein that binds to this "AP1 binding site" in Element B was distinct from the AP1 components (the Jun and Fos

family transcription factors). Therefore, the presence of a novel "AP-1 like" protein has been proposed that interacts specifically to Element B of the wflAFP gene enhancer. This presumptive protein was designated as the "antifreeze enhancer-binding protein" (AEP) (Chan et al, 1997).

1.2 Transcription of eukaryotic genes

Eukaryotic genes utilize RNA polymerase II (pol II) to transcribe mRNA. The processes of transcription initiation and transcriptional control are regulated by complex mechanisms. The basal transcription involves the interaction of core promoters and general transcription factors, whereas the transcription level of individual gene is regulated by DNA elements, namely enhancers and silencers, and their binding to sequence-specific transcription factors in response to the cellular environment. Recent identifications of many auxiliary complexes, termed coactivators and mediators, as well as the involvement of chromatin structure in transcriptional regulation have added more complexity to the transcription control mechanism (Hampsey, 1998).

1.2.1 Initiation of transcription by RNA polymerase II

Initiation of transcription is the major event directing gene expression and the process of transcriptional initiation by RNA pol II is highly conserved among eukaryotic organisms. Initiation occurs at the core promoter, which is the site for assembly of the pre-transcription initiation complex (PIC) and is defined within –40 to +40 nucleotides relative to the RNA start site. It comprises DNA sequence motifs such as TATA box, TFIIB recognition element,

initiator element, a pyrimidine-rich sequence, and the downstream promoter element (DPE). A core promoter may contain one or some of these elements. Factors interact with the core element are defined as general transcription factors (GTFs), including TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Orphanides et al, 1996). Together with RNA pol II, they are sufficient to direct accurate, basal-level transcription initiation. The assembly of PIC is in a series of ordered steps *in vitro*. The TFIID complex is responsible for the promoter recognition. The TATA-binding protein (TBP) of the complex binds to the TATA box, while the TBP-associated factors (TAFs) interact with flanking sequences to confer additional specificity. Bending of TATA-box DNA around TBP creates a context for interaction with TFIIB, which in turn recruits TFIIF and Pol II complex. The additional recruitment of TFIIH, which includes ATP-dependent helicases that unwind the promoter sequence around the start site, triggers the initiation of transcription (Fig. 1-7A). In contrast, the finding that a subset of GTFs other than TFIID associates with pol II in the absence of DNA suggested the existence of a "RNA pol II holoenzyme" (Fig. 1-7B) (Koleske and Young, 1994; Koleske and Young, 1995). Holoenzyme has been purified from many eukaryotic organisms but its exact composition differs somewhat from different preparation protocols. A significant property of the holoenzyme is its ability to respond to activators. Transcription reactions reconstituted *in vitro* with highly purified RNA pol II and GTFs are not responsive to activators; while when a SRB (suppressor of RNA polymerase B mutations)/Mediator complex, which is a subcomplex of the holoenzyme, is supplemented, the holoenzyme becomes activator-responsive.

In addition, several typical multiple recognition sites are commonly present at promoter-proximal region of many genes and are also considered as components of the basal promoter.

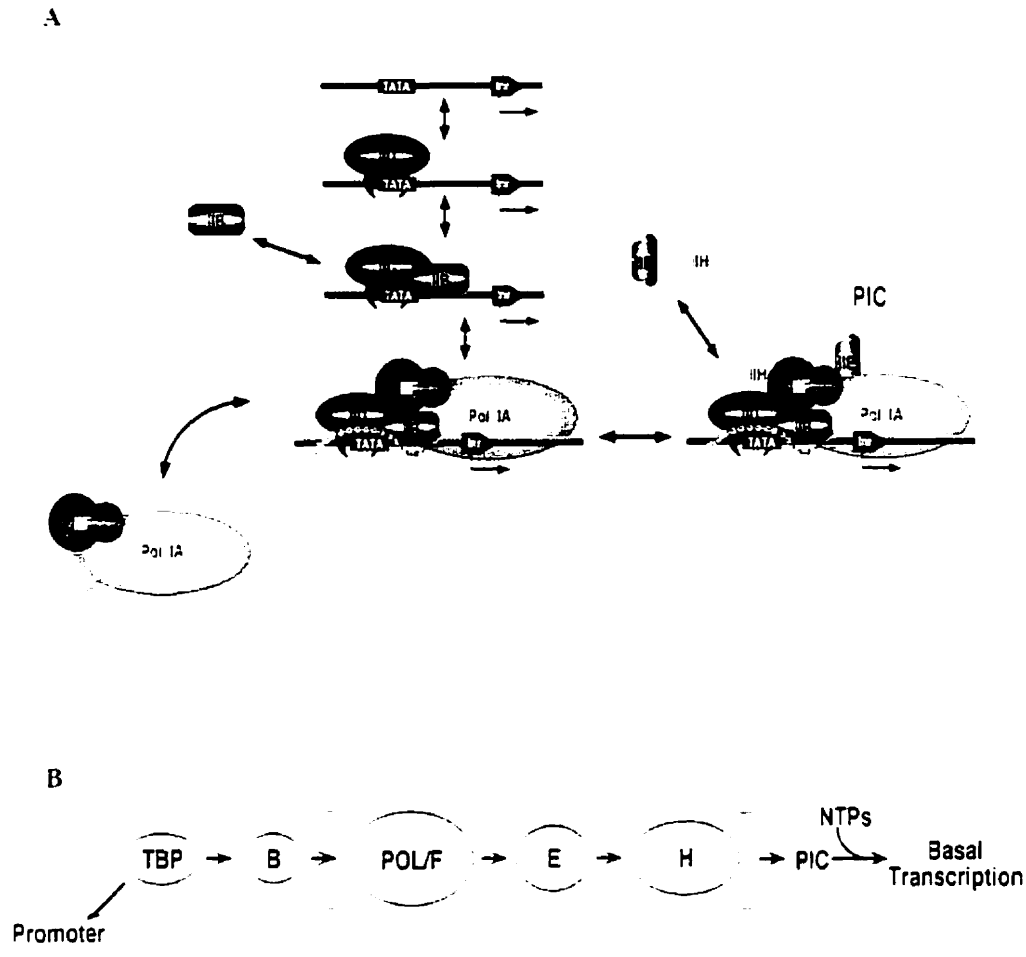


Fig. 1-7 The assembly of PIC in the RNA polymerase II-mediated transcription. A: The step-wise PIC assembly begins with TFIID recognizing the TATA element, followed by coordinated accumulation of TFIIB, Pol II (IIA) plus TFIIF, TFIIE and TFIIH. B: Schematic representation of the basal transcription initiation with the "Pol II holoenzyme" (enclosed by square brackets) (Nikolov & Burley, 1997).

They are located immediately upstream of the core element, about –50 to –200 nucleotides relative to the start site. Sequence-specific factors interact with these sites including Sp1, CCAAT-binding transcription factor (CTF) and CCAAT-box-binding factor (CBF) (Blackwood and Kadonaga, 1998).

1.2.2 Transcriptional control by enhancers and activators

The core promoter and PIC assembly are essential to transcription initiation, while they only achieve minimal level of transcription. Many complex mechanisms and factors have been discovered that are involved in transcriptional control of numerous genes in a cell-type specific, developmentally regulated and /or hormonal-responsive manner.

Studies of eukaryotic transcriptional control have been focused on positive DNA elements, known as enhancers, and proteins, known as activators, that bind to them and stimulate transcription. Enhancers are defined as regulatory sequences that increase transcription independent of their orientation and distance to the RNA start site. They could reside either close to or far away from their target promoters, in introns, exons, or untranslated regions downstream of the promoters (Blackwood and Kadonaga, 1998). Activators are specific to individual genes and typically couple transcription to the physiological need of the cells. They are divided into distinct families with different DNA-binding and activation domains. The activity of these activators as well as their DNA-binding affinity and specificity are controlled by many parameters including dimerization, phosphorylation and ligand binding. For example, the AP-1 family of transcription factors consists of homodimers and heterodimers of Jun, Fos or activating transcription factor (ATF) basic region/leucine zipper (bZIP) proteins (Karin et al, 1997). Jun-Jun and Jun-Fos dimers prefer to bind to TPA-

responsive element (TRE, TGACTCA), while Jun-ATF or ATF homodimers prefer to bind to cAMP-responsive element (CRE, TGACGTCA). Phosphorylation of cJun by the Jun kinase (JNK) significantly increases its transcriptional activity (Smeal et al, 1994).

Enhancers are believed to carry out their functions through interactions with RNA pol II, though the interaction is indirect and involves coactivators (discussed in 1.2.3). Several hypotheses on how enhancer-binding proteins and associated coactivators establish an effective interaction with their cognate promoter were proposed that include DNA looping and DNA scanning models (Wu, 1993; Ptashne and Gann, 1997). More recently, a "facilitated tracking" mechanism that incorporates both models has been proposed (Fig. 1-8) (Blackwood and Kadonaga, 1998). An enhancer-bound complex containing DNA-binding factors and coactivators tracks via small steps (and perhaps scanning) along the chromatin until it encounters the cognate promoter, at which a stable looped structure is established. An important feature of this model is the chromatin structure of the template. Coactivators with chromatin remodeling and modification activities may facilitate the interaction of DNA-binding factors with the enhancer and the enhancer-promoter communication.

1.2.3 Involvement of coactivators and mediators

Numerous coactivators have been subsequently identified to interact with the DNA-bound activators that stimulate transcription activation. They are proteins dispensable for basal-level transcription *in vitro* and are either not directly bound to DNA or not in a sequence-specific manner with the binding (Hampsey, 1998).

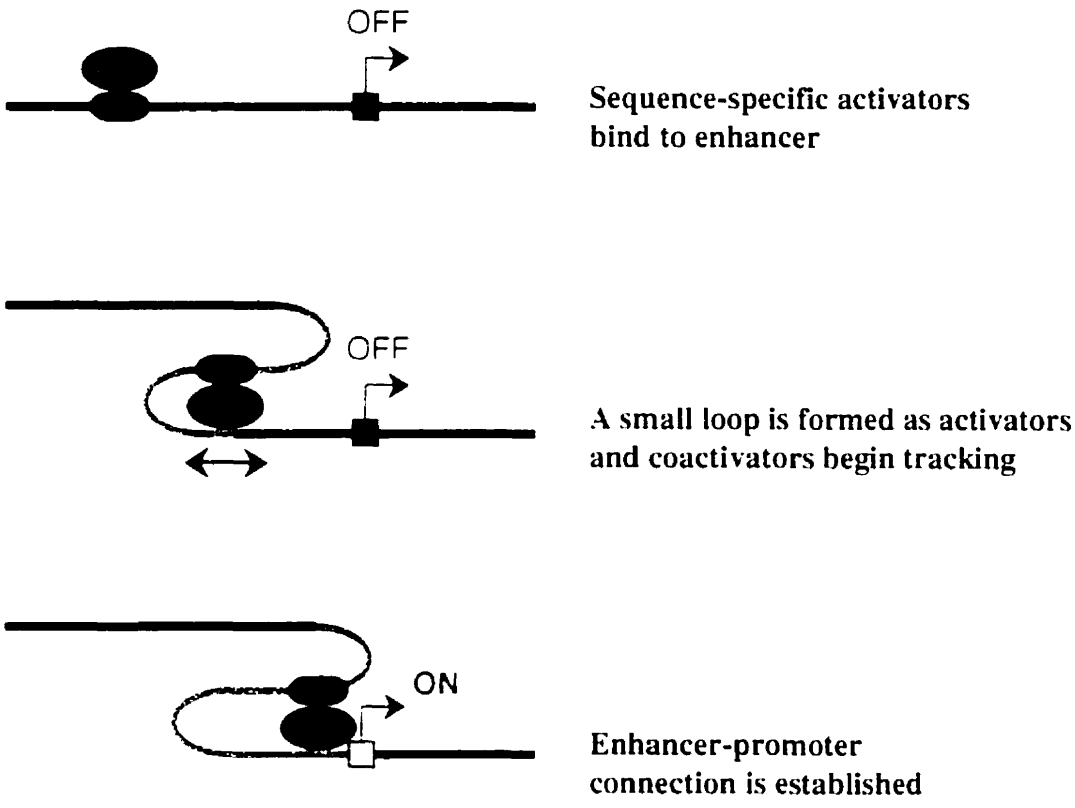


Fig. 1-8 A facilitated tracking model for enhancer function. A complex containing activators and coactivators binds to enhancer and tracks via small steps, perhaps scanning, along the chromatin until it encounters the cognate promoter. A stable looped structure with enhancer-promoter connection is then established (Blackwood & Kadonaga, 1998).

Histone modification complex

Several coactivators were discovered to possess catalytic activities directed at the histones, indicating a role of chromatin structure in transcription control (Kadonaga, 1998). DNA is wrapped around histone octomer in nucleosome and packaged into chromatin structure where transcription is repressed. To turn on transcription, one has to overcome this chromatin-mediated repression. Coactivators such as CBP (cAMP-responsive-element-binding (CREB)-binding protein) and p300 that interact with activators which are involved in many cellular activities, stimulate transcription by acetylation on the lysine residues of the histone tails. Histone acetylation is a modification characteristic of transcribed chromatin and it has been suggested that the charge neutralization by acetylation causes a reduction in the affinity of histone-DNA interactions and thus leads to increased access of factors to the repressed chromatin template (Kadonaga, 1998). The histone acetylation transferase (HAT) activity has since been found in many coactivator complexes including SAGA (Spt-Ada-Gcn5 acetyltransferase) and PCAF (P300/CBP-associated factor). Coincidentally, the histone deacetylase (HDAC) activities were found in corepressor complexes such as mSin3 and NURD (nucleosome remodeling histone deacetylase complex). Thus, it is established that one transcriptional control mechanism is achieved by coactivators with HAT activities which acetylate the histone tail to relieve nucleosome repression, whereas corepressors with HDAC activities deacetylate histones to re-establish repression.

Chromatin-remodeling complex

In addition, multiproteins with ATPase component, such as SWI/SNF (switch/sucrose non-fermenting) and RSC complexes from yeast; and NURD, NRD (nucleosone remodeling and deacetylating) and RSF (remodeling and spacing factor) from human, were found in some

coactivator complexes that can remodel the structure of chromatin and facilitate transcription (Burns and Peterson, 1997; Bjorklund et al, 1999). They are able to perturb the core particle of the nucleosome in an ATP-dependent manner as demonstrated by increased susceptibility of the DNA to nuclease attack or shifted location of nucleosomes on DNA. Thus, these chromatin-remodeling complexes can mediate the formation of nucleosome-hyposensitive sites that the enhancers and promoters of genes are exposed to various factors for transcriptional activation. Also, a connection of the chromatin-remodeling complex with the RNA pol II holoenzyme was suggested since the SWI/SNF complex was found to be an integral component of the holoenzyme (Wilson et al, 1996).

SRB/Mediator complex

Also, as mentioned above, the presence of Mediator complex was revealed by its role as an important interface between activators and RNA pol II holoenzyme *in vivo* (Bjorklund and Kim, 1996). Mediators form a specific complex with the C-terminal domain (CTD) of RNA pol II and stimulate activator-mediated transcription as well as CTD phosphorylation by the TFIIH kinase. The complex has been purified from yeast, and its counterpart in mammals has been identified in mouse and in the human Med1-containing coactivator complexes including SMCC (SRB/MED-containing cofactor complex) and CRSP (cofactor required for SP1 activation complex).

TAF-containing complex

TAFs were found to be associated with TBP in the TFIID complex and were considered to be essential for the initiation of transcription (Pugh and Tjian, 1992). However, studies revealed that depletion of several TAFs did not compromise transcriptional activation *in vivo* (Hampsey and Reinberg, 1997). A TBP-free TAF-containing complex (TFTC) contains a

subset of TAFs and proteins known as components of the PCAF/SAGA complex can substitute for TBP and TFIID *in vitro* in supporting transcriptional activation for TATA-containing and TATA-less promoters. These studies suggested that TAFs may function as coactivators for transcription of a subset of genes that is independent of their association with TBP (Albright and Tjian, 2000). Moreover, one subunit of TFIID, TAFII250, was shown to be a protein kinase as well as a histone acetyltransferase. It appears to play multiple roles in transcription activation including phosphorylation of TFIIF and acetylation of histones (Dikstein et al, 1996; Mizzen et al, 1996). In addition, some TAFs are structurally similar to histones, though the significance of a histone octamer-like structure present within TFIID is unclear (Hoffmann et al, 1997).

More and more factors have been identified that play important roles in transcriptional control. A simplified model was proposed to illustrate the complex regulatory mechanism (Fig. 1-9) (Kornberg, 1999). An activator may stimulate transcription by interaction with a coactivator, which recruits an acetyltransferase to acetylate the histone tails, or a chromatin-remodeling factor to alter the chromatin structure, as well as by interaction with mediator. Conversely, a repressor interacts with corepressor, which recruits a histone deacetylase.

1.2.4 Liver-enriched transcription factors and the C/EBP family

Liver exhibits a variety of tissue-specific functions including gluconeogenesis, urea synthesis, bile acid formation, cholesterol synthesis, drug metabolism, as well as plasma protein synthesis. Regulation of liver-specific genes at the transcriptional level serves as one

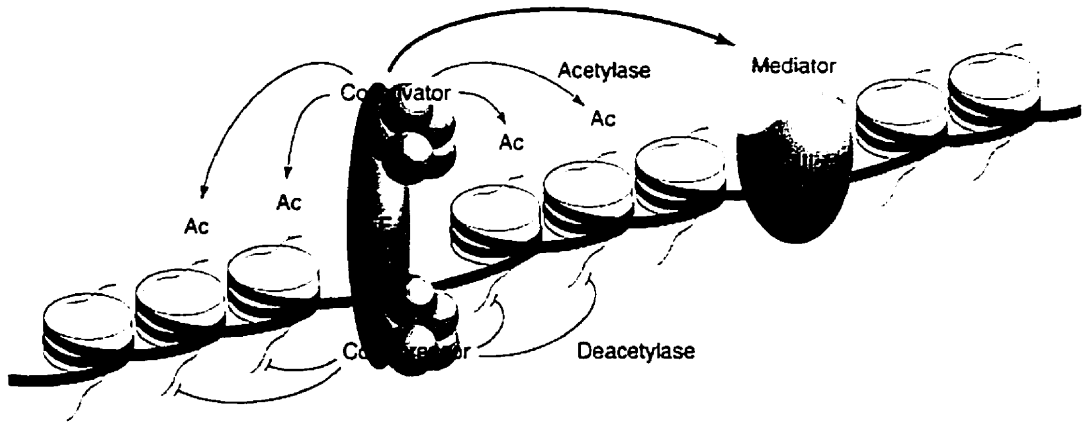


Fig. 1-9 Model for the regulation of eukaryotic gene transcription. A transcription factor (TF), either an activator or a repressor, binds to a regulatory element while RNA polymerase II and general transcription factors binds with a promoter. An activator stimulates transcription both by interaction with a coactivator, which recruits an acetylase to acetylate the histone tail, and by direct interaction with Mediator. A repressor interacts with a co-repressor, which recruits a histone deacetylase (Kornberg, 1999).

of the most important steps to control these processes and a number of hepatocyte-specific transcription factors have been identified participating in these functions.

Liver-specific factors can be classified into five groups based on the properties of their DNA-binding domain: the CCAAT/enhancer-binding protein (C/EBP) family contains a basic region/leucine zipper (bZIP) domain; the hepatocyte nuclear factor-1 (HNF-1) family contains an extra large homeodomain; the HNF-3 family has a winged helix domain; the HNF-4 family belongs to the steroid receptor superfamily; and another bZIP family is characterized by a proline-and acidic-amino-acid-rich (PAR) domain adjacent to the basic region. These factors are highly enriched in liver, however, they are not exclusively expressed in hepatocytes, but in a limited number of tissues and cell types. Also, they are usually capable of dimerizing with other members of proteins in the same family (De Simone and Cortese, 1992).

The C/EBP family is characterized by the bZIP domain consist of a basic DNA-binding region and a dimerization interface, the leucine zipper, located in the C-terminal of the protein (Takiguchi, 1998). The basic region interacts with the major groove of DNA in a sequence specific manner. A C/EBP binding consensus sequence RTTGCGYAAY (R=A or G; Y=C or T) was postulated (Osada et al, 1996), though many actual DNA binding site sequences are derived from this sequence. The leucine zipper is an amphipathic α-helix with a hydrophobic surface containing repeated leucine residues at intervals of seven amino acids, and electrostatic interaction between charged residues flanking the hydrophobic surface contributes to determination of the dimerization specificity (Vinson et al, 1993). In addition, activation and inhibition domains have been found in the N-terminal portion of the proteins.

Their capability of homodimerization and heterodimerization contributes to their differential DNA binding specificity and ability to modulate transcriptional activity of targeted genes. There are six members of the C/EBP family found so far that include C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and CHOP/GADD153 (Lekstrom-Himes and Xanthopoulos, 1998). C/EBPα is largely expressed in differentiated liver and adipose tissues and is involved in many hepatocyte and adipocyte-specific gene expressions (Birkenmeier et al, 1989; Umek et al, 1991). Two isoforms of C/EBPα have been found due to different initiation codon usage. The full-length protein is a 42 kDa polypeptide containing transactivation domains that mediates its binding to TBP and TFIIB. The truncated 30 kDa form retains its dimerization and DNA binding domains; however, it exhibits altered transcriptional activity compared to the full-length isoform (Ossipow et al, 1993). Changes in the relative abundance of the two C/EBPα forms were observed during liver development and preadipocyte differentiation (Lin et al, 1993). C/EBPβ is found mainly in hepatocytes with three isoforms. The 32 kDa full-length form was named as the liver-enriched transcriptional activator protein (LAP), whereas the shortest form of 20 kDa C/EBPβ (liver-enriched transcriptional inhibitor protein, LIP) possesses only the DNA-binding and leucine zipper domains but lacks activation domains. Heterodimerization of the LIP with LAP attenuates transcriptional activity, indicating a dominant negative mechanism of transcriptional regulation (Descombes and Schibler, 1991). C/EBPγ is a 16.4 kDa ubiquitous factor most abundant in non-differentiated cells with dimerization and DNA-binding regions. It also plays a role in dominant negative transcriptional regulation since heterodimerization with C/EBPα and C/EBPβ reduces transactivation (Cooper et al, 1995). C/EBPδ is present in a limited number of tissues including liver with the ability to heterodimerize with C/EBPα

and C/EBPβ. The sequence difference in its DNA binding domain may contribute to its diminished DNA binding affinity (Cao et al, 1991). Three C/EBPε species have also been found encoded by four mRNA isoforms by alternative promoter usage in myeloid and lymphoid lineages and differential splicing and distinct transactivation activity was observed (Yamanaka et al, 1997a; Yamanaka et al, 1997b). CHOP/GADD153 is ubiquitously expressed and heterodimerizes with other C/EBPs. However, it acts as a dominant negative inhibitor of C/EBP transactivation by preventing heterodimer binding of C/EBPα and C/EBPβ to classic C/EBP enhancer sequences (Ron and Habener, 1992).

Moreover, phosphorylation/dephosphorylation is a significant feature in regulating C/EBP family function. Dephosphorylation of C/EBPα by insulin in 3T3-L1 adipocytes is thought to result in reduced expression of the target gene, glucose transporter 4 gene (MacDougald et al, 1995; Hemati et al, 1997). Also, C/EBPβ is phosphorylated in various cells by distinct stimuli through pathways containing different kinases such as protein kinase A (PKA), protein kinase C (PKC) and mitogen-activated protein (MAP) kinase (Nakajima et al, 1993; Trautwein et al, 1994; Takiguchi, 1998). These phosphorylation events generally cause nuclear accumulation and transcriptional activation, while *in vitro* phosphorylation was found to result in attenuation in DNA-binding activity. Increased DNA binding and transactivation has also been reported upon C/EBPδ and CHOP/GADD153 phosphorylation (Ray and Ray, 1994a; Ray and Ray, 1994b; Wang and Ron, 1996; Lacorte et al, 1997).

In addition, transcriptional regulation by C/EBP family members is carried through their direct and/or indirect interactions with other factors. For example, C/EBPα, C/EBPβ and C/EBPδ were all found to bind directly to NF-κB. When cooperating with other liver-enriched factors, C/EBPα and HNF-4 or HNF-1 can synergistically activate the

apolipoprotein B and albumin gene promoters, respectively (Metzger et al, 1993; Wu et al, 1994). Cooperation of C/EBP proteins with ubiquitous factors was also reported. Synergistic activation by C/EBPα with NF-Y was implicated in dramatic activation of the albumin gene in the late fetal stage that the role of NF-Y is believed to generate an open chromatin configuration before the appearance of C/EBPα (Milos and Zaret, 1992).

1.2.5 Helicase domain-containing proteins in transcriptional regulation

DNA and RNA helicases are defined as a diverse group of enzymes with capacity to separate two complementary strands of a duplex nucleic acid molecule. They are involved in many aspects of nucleic acid metabolism in both prokaryotes and eukaryotes including DNA replication, repair and recombination as well as transcription, RNA processing and translation (Matson et al, 1994). Helicase unwinds duplexes using the energy produced from the hydrolysis of a nucleoside 5'-triphosphate (NTPs) to catalyze the breakage of the hydrogen bonds holding the strands together. Sequence analysis of helicases from diverse organisms have revealed a series of short, conserved amino acid motifs, the so-called helicase motifs. An important notion is that while several proteins with the helicase motifs have been demonstrated with *in vitro* helicase activity, some of them do not exhibit helicase activity or the activities have not been tested. Thus, they are called the helicase domain-containing proteins (HDP).

Models to explain the mechanism of how helicases catalyze the unwinding of duplex nucleic acids have been proposed. The "rolling" model is observed from a helicase dimer that two subunits bind to single- and double-stranded DNA alternatively using cycles of ATP binding, hydrolysis and product release to "roll" through duplex DNA (Wong and Lohman, 1992;

Lohman and Bjornson, 1996). In contrast, in the "inchworm" model, which is based on a monomeric helicase, the ATP binding, hydrolysis and product release cycle the protein through a series of conformational states as the protein moves along one step at a time (inches) the DNA substrate (Velankar et al, 1999).

Four superfamilies of helicases and HDPs have been classified based on the extent of amino acid similarity and the organization of these conserved regions (Hall and Matson, 1999). Among these, superfamily 1 contains seven conserved amino acid motifs found from viral, prokaryotic to eukaryotic organisms (Fig. 1-10). The first and third motifs of the Superfamily 1 and 2 (motifs I and II), also known as Walker boxes A and B, are not specific to helicases and were believed to bind NTP (Gorbalenya et al, 1989). Also, crystal structure studies and mutational analysis have demonstrated that the seven conserved motifs are clustered in tertiary structure. They bind to and hydrolyze an NTP molecule and couple the resulting energy to activity that is directed towards the ligand in the nucleic acid binding site. However, distinct function has not been able to be assigned to a particular motif (Hall and Matson, 1999).

The involvement of helicases in transcriptional regulation has been established (Eisen and Lucchesi, 1998). In the RNA pol II basal transcriptional machinery, TFIIE and TFIIH are necessary for full-length transcription (Holstege et al, 1996). Among the nine subunits of TFIIH, at least two of them, XPB and XPD (the xeroderma pigmentosum B and D gene products), were demonstrated as ATP-dependent DNA helicase. Antibodies specific to XPB and XPD inhibit transcription *in vitro* (Drapkin and Reinberg, 1994). TFIIE recruits TFIIH to the transcription initiation complex for open complex formation and DNA melting, which is mediated by XPB. Also, TFIIH is involved in promoter clearance to stimulate escape of

Fig. 1-10 Schematic presentation of the Superfamily I helicase family of proteins. Open boxes represent the conserved helicase motifs and letters inside the boxes are the consensus amino acid sequences of each motif. The relative positions of motifs and spacing between motifs are not to scale. +: hydrophobic residue; o: hydrophilic residue and x: residue that is not restricted to be hydrophobic or hydrophilic (Hall & Matson, 1999).

transcription elongation complexes. Both promoter melting and escape involve single-stranded DNA and it has been suggested that TFIIH functions through a conventional DNA-helicase mechanism with direct interactions between TFIIH and single-stranded DNA (Robert et al, 1998). Alternatively, a recent study on XPB has revealed that it does not interact with the promoter region which undergoes melting but instead it interacts with DNA downstream of this region. Therefore, it is proposed that TFIIH functions as a molecular wrench that rotates downstream DNA relative to fixed upstream protein-DNA interactions, thereby generating torque, and melting the intervening DNA (Kim et al, 2000). In addition, studies have demonstrated that sequences outside of the helicase motifs of XPD are required for transcription, indicating the possible interaction between helicase and other transcription factors (Guzder et al, 1995). p53, a key protein involved in transcriptional activation and apoptosis, was shown to interact with both XPB and XPD and inhibited their helicase activities (Leveillard et al, 1996).

Interestingly, HDPs are found in many complexes, known as facilitators or coactivators. They are required to regulate unrelated genes and are found in a variety of organisms from yeast to mammal, suggesting that they play a common role in transcriptional regulation. The SWI/SNF complex in yeast consists of at least 11 components and one of the subunits, SWI2/SNF2 is a HDP. Its related protein in Drosophila, ISWI, was found in many facilitator complexes involved in chromatin remodeling including nucleosome remodeling factor (NURF), chromatin-accessibility complex (CHRAC) and ATP-utilizing chromatin assembly and remodeling factor (ACF) (Elfring et al, 1994; Ito et al, 1997; Varga-Weisz et al, 1997). It is believed that these complexes use the energy generated by the hydrolysis of ATP to alter chromatin structure and increase the accessibility of DNA to the promoter by transcription

factors. It was shown that binding of TBP to TATA box and GAL4 to its promoter were promoted by SWI/SNF complex in an ATP-dependent manner (Cote et al, 1994; Imbalzano et al, 1994). Models have been presented for the HDP action in these complexes that HDPs could target the coactivator complex to its site of action by associating with the DNA template, the nascent mRNA, or some other component of the chromatin, or they bind to newly-transcribed mRNA molecules and stabilize them (Fig. 1-11). Facilitator complexes may aid the transcriptional machinery in targeting a promoter and then move with or ahead of the polymerase, altering chromatin structure to enhance transcription (Eisen and Lucchesi, 1998). Furthermore, a number of proteins that contain both helicase and DNA-binding motifs have been identified. A homolog to SNF/SWI, HLTF (helicase-like transcription factor) was found to bind and activate plasminogen activator inhibitor-1 gene and to physically associate with Sp1 (Ding et al, 1996; Ding et al, 1999). Also, Rip-1 was found to be involved in the binding of rat insulin II gene (Shieh et al, 1995). CHD1, a SNF2-related protein, has affinity to Ig promoters and is conserved from yeast to mammals. It was shown to contain a chromo (chromatin organization modifier) domain in addition to helicase and DNA binding domains, and can alternate gene expression (Woodage et al, 1997). Together, these studies further support that the HDPs play important roles in transcriptional regulation.

1.3 Growth hormone and IGF-1 actions

Growth hormone exerts its wide effects on body growth and metabolism control via multiple and complicated mechanisms. GH binds to its receptor (GHR) and causes GHR

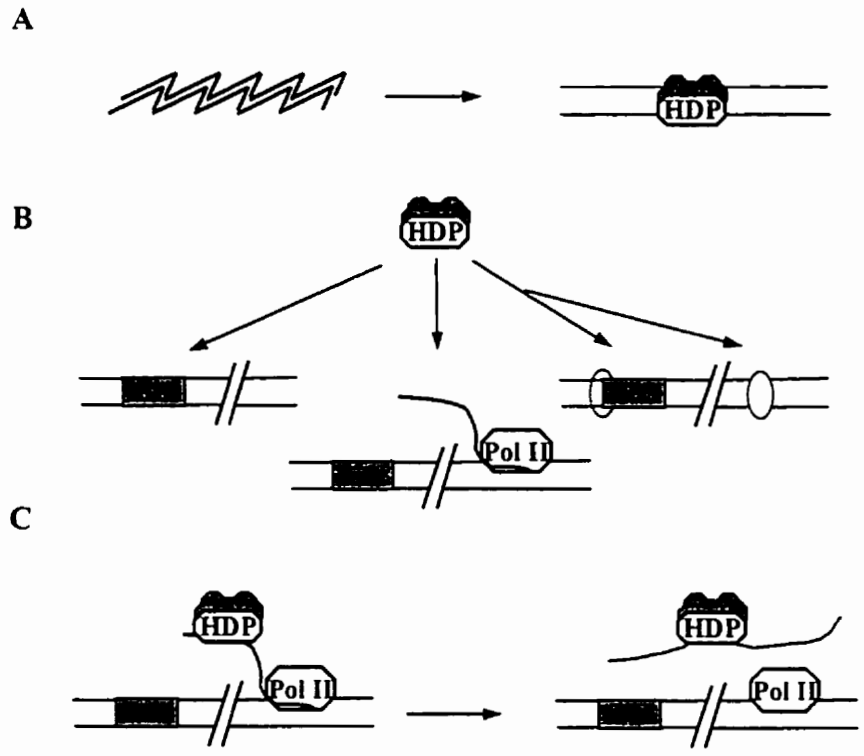


Fig. 1-11 Models for the action of HDPs in the context of transcription facilitator complexes. A: HDPs within the complex alters chromatin structure to make it more accessible to other transcription factors. The jagged line indicates inactive chromatin. B: HDPs target the complex by binding either promoter sequences, nascent RNA, or the chromatin components indicated with white ovals. C: HDPs bind mRNA and help stabilize and perhaps, transport it (Eisen & Lucchesi, 1998).

dimerization, activation of the GHR-associated JAK2 tyrosine kinase, and tyrosyl phosphorylation of both JAK2 and GHR. These events recruit and/or activate a variety of signaling molecules including MAP kinase, insulin receptor substrates, phosphatidylinositol 3′ phosphate-kinase (PI3-kinase), diacylglycerol, protein kinase C, intracellular calcium and STAT transcription factors, and contribute to the GH-induced regulation in enzymatic activity, transport function and gene expression (Carter-Su et al, 1996). Furthermore, the insulin-like growth factor-1 (IGF-1) has been found to represent a major route of GH action. GH stimulates IGF-1 synthesis, mainly from the liver, and consequently increases the circulating IGF-1 level (Daughaday and Rotwein, 1989; Pankov, 1999). Studies in teleosts have also demonstrated the liver as a major site of IGF-1 production in response to GH (Duguay et al, 1994; Shamblott et al, 1995; Shepherd et al, 1997). Other tissues including adipocytes and chondrocytes also exhibit increased IGF-1 mRNA expression in response to GH, suggesting that local IGF-1 contributes to some GH responses. IGF-1 has a high affinity for a family of IGF-1-binding proteins (IGF-BPs), which can moderate its biological actions. Regulation of the IGF-BP genes by GH adds another level of complexity to the relationship between GH and IGF-1.

The IGF-1 function is mediated via its receptor (IGF-1R). IGF-1R is a heterotetrameric protein that consists of two α– and two β–subunits. Binding of IGF-1 to the extracellular α-subunit leads to the phosphorylation of tyrosine residues on the β-subunit. Subsequently, IGF-1R phosphorylates insulin receptor substrate-1 (IRS-1) and IRS-2. Tyrosine phosphorylation of IRS-1 provides binding sites for several distinct Src homology 2 (SH2) proteins and mediates multiple signal pathways. IRS-1 binds the 85 kDa subunit of PI3-kinase through interaction with Tyr-X-X-Met motifs, thereby activating the p110 catalytic

subunit of PI3-kinase, which phosphorylates the 3'-hydroxyl group of phosphatidylinositols and leads to the downstream signaling events. IRS-1 also binds growth factor receptor bound protein 2 (GRB2), a 23 kDa protein with one SH2 domain and two SH3 domains. GRB2 associates with IRS-1 by its SH2 domain and associates with Sos with its SH3 domain. This signaling activates the Ras/Raf/MAP kinase cascade. Moreover, IRS-1 has binding sites for a tyrosine-specific phosphatase Syp, Nck and the 14-3-3 proteins, which interaction requires receptor kinase activity (Lopaczynski, 1999). In addition, IGF-1R directly phosphorylates Shc and Crk, which also activate the GRB2 and/or Sos that leads to the Ras/Raf/MAP kinase pathway (Fig. 1-12) (Blakesley et al, 1996; Petley et al, 1999). Similar to IRS-1, the tyrosine phosphorylated IRS-2 has been identified to interact with both p85 of PI3-kinase and GRB2 (Tobe et al, 1995). In IRS-1 deficient mice, the insulin-stimulated PI3-kinase as well as MAP kinase activities was reduced in muscle, consistent with reduced glucose transport, glycogen synthesis and protein synthesis in these animals. However, the amount of IRS-2 in the liver of the IRS-1 deficient mice was equal to that of IRS-1 in wild-type mice, with normal PI3-kinase and MAP kinase signaling and glycogen synthesis. This work suggests that IRS-2 may play a distinct role in the liver (Kadowaki et al, 1996; Yamauchi et al, 1996). Disruption of IRS-2 impairs both peripheral insulin signalling and pancreatic beta-cell function. IRS-2-deficient mice show progressive deterioration of glucose homeostasis because of insulin resistance in the liver and skeletal muscle and a lack of beta-cell compensation for this insulin resistance (Withers et al, 1998).

IGF-1 stimulates cell proliferation via the Ras/Raf/MAP kinase pathway and cell differentiation via the PI3-kinase pathway in several systems including myoblasts and adipocytes (Coolican et al, 1997). However, contrasting results for mitogenic and myogenic

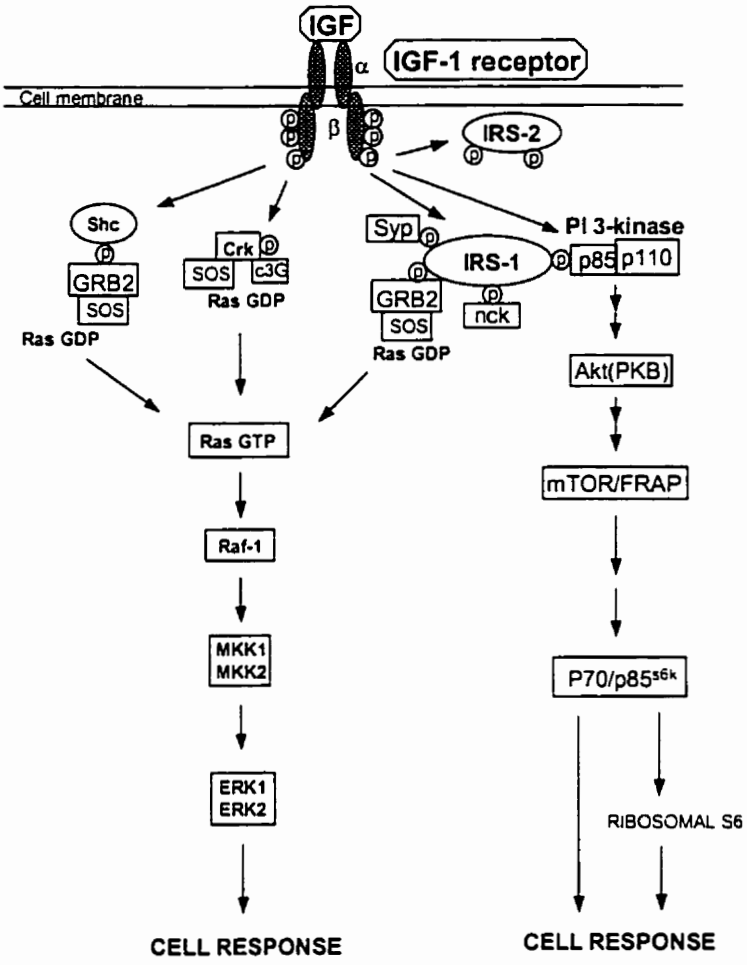


Fig. 1-12 General outline of pathways stimulated by IGF-1 in target cells. The most widely described intermediates are presented in these pathways. All the pathways shown here may not occur in all cell types and cross-reaction between individual members of the pathways is possible (Petley et al, 1999).

responses to IGF-1 were obtained from studies in other cell types such as breast cancer and neuroblastoma cells, suggesting that there is no obvious generalization to predict the signaling pathway that will be involved in a specific cellular response to IGF-1 (Petley et al, 1999).

1.4 Objectives of the present study

Previously, a model was presented to illustrate the seasonal and hormonal regulation of the AFP genes in winter flounder based on the AFP gene expression studies discussed in 1.1.3 and 1.1.4 (Chan et al, 1993, Fig. 1-13). The expression of AFP genes is controlled mainly at the transcriptional level, which is affected by photoperiod. GH represses transcription of the wflAFP genes in the summer and with the loss of long day length during the fall, this repression is removed, resulting in active transcription of the wflAFP genes. The liver-specific expression of the wflAFP gene is mediated by a tissue-specific enhancer located in the intron of the wflAFP gene. This enhancer interacts with nuclear proteins, one of them being the liver-enriched factor C/EBPα, which is considered to be responsible for its tissue-specificity. In addition, the discovery of wfsAFPs, which have similar gene structure but are differentially regulated, has added additional complexity to this model. Questions have been raised on what is the GH signaling pathway(s) that control the wflAFP expression and how the differential regulation of wflAFP and wfsAFP is achieved. Together with the investigation on the biochemical identity of the presumptive AEP, these studies should lead to a better understanding of the transcriptional control of the winter flounder AFP genes.

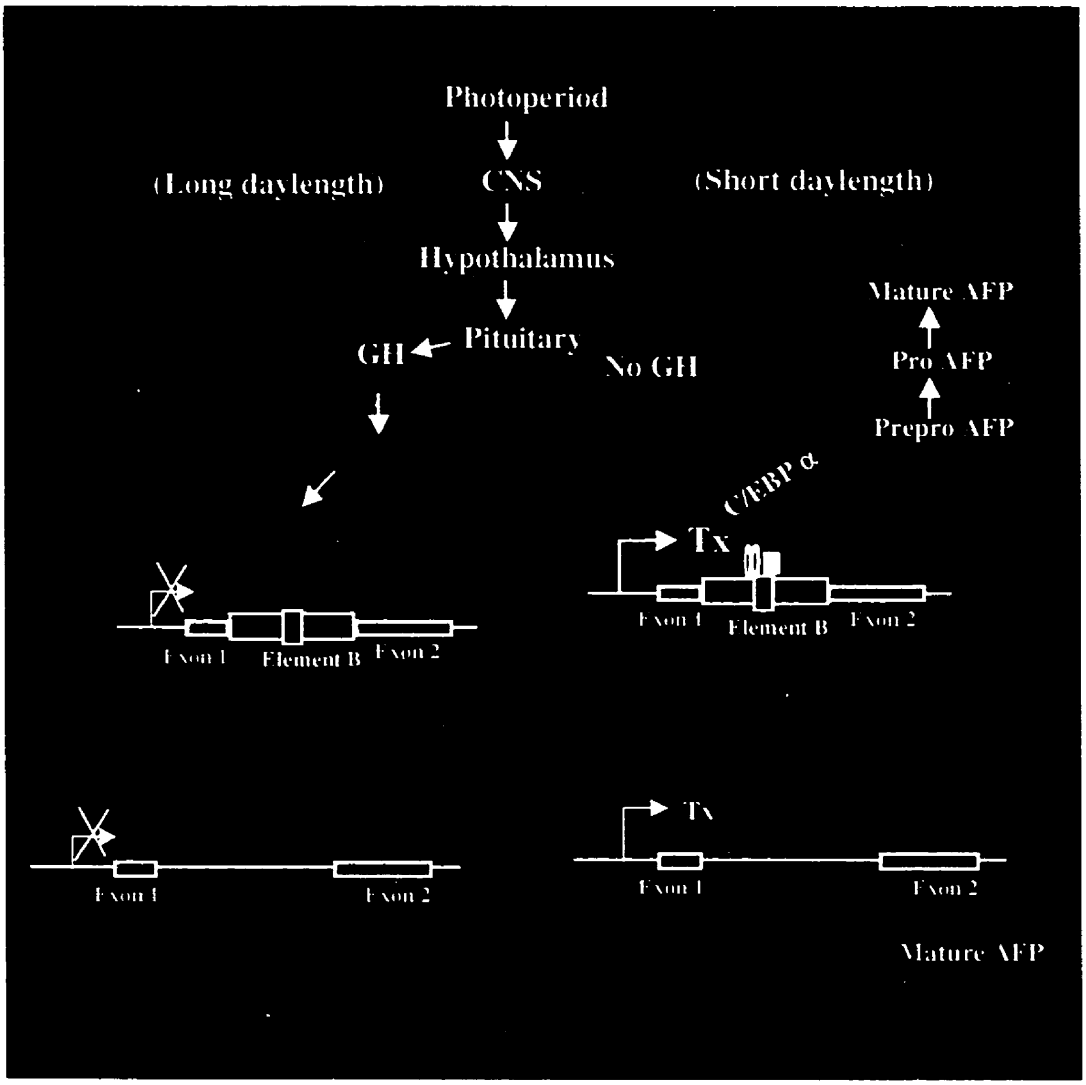


Fig. 1-13 Working model for the hormonal and tissue-specific regulations of the wflAFP and wfsAFP genes. During summer, GH causes the transcriptional inhibition of the wflAFP genes. During winter, the production of GH is inhibited, hence releasing the transcription repression. C/EBPα and the presumptive AEP bind to Element B and activate the wflAFP gene expression. The wfsAFP genes are ubiquitously expressed and are less influenced by GH and seasonal change. The questions on what the molecule(s) is in mediating the GH signaling, what the identity of AEP is, how C/EBPα and AEP are involved in the wfAFP gene regulation and what the role of the wfsAFP gene intron is in its differential regulation remained to be answered.

Therefore, the studies undertaken in this thesis have the following objectives:

1. To further establish the role of the enhancer and C/EBPα in the transcriptional regulation of the wflAFP gene.
2. To investigate the signaling pathways and intermediate molecules involved in the hormonal regulation of the wflAFP gene expression.
3. To examine the mechanism controlling the distinct skin-type wfAFP gene expression.
4. To characterize the biochemical identity of the presumptive antifreeze enhancer-binding protein (AEP) and its role in antifreeze protein gene regulation.

Chapter Two

Liver-specific and hormonal regulated expression of the liver-type AFP gene in winter flounder

Eur J Biochem (1997) 247:44-51. Chan, S. L., Miao, M., Fletcher, G. L. and Hew, C. L.

Mol Mar Biol Biotechnol (1998) 7:197-203. Miao, M., Chan, S. L., Hew, C. L. and Fletcher, G. L.

*Results presented in this chapter were the authour's contributions to these publications.

2.1 Introduction

The biochemistry and gene structure of the winter flounder AFPs have been studied extensively. However, due to the lack of an appropriate fish liver cell line and minimal knowledge on fish transcription factors, our understanding of the molecular machinery that regulates the expression of these genes was limited. To elucidate the molecular mechanism(s) of AFP gene expression, a mammalian model system was used as the first step to characterize the DNA regulatory elements and protein factors that confer its tissue specificity. The only intron (+106 to +602) of the flounder wflAFP gene 2A-7b was shown to contain cis-acting elements important for its liver-specific transcription and a core sequence, Element B (+303 to +322), was found to interact with liver-enriched transcription factor C/EBPα, and a novel "AP-1 like" protein. Interactions between the enhancer and liver nuclear extracts have been demonstrated from both rat and flounder by DNase I footprinting assays (Chan et al, 1997; Miao et al, 1998a). The objectives of the studies in this chapter were to further delineate the transcriptional mechanism of the liver-specific wflAFP gene. DNA-protein interaction was further examined by gel retardation and methylation interference assays. Structural and functional analyses were carried out to study the important residues for DNA-protein interactions and their roles in transactivation of the wflAFP gene. Also, the involvement of C/EBPα in wflAFP gene regulation was investigated.

Several earlier studies have established that the wflAFP gene is regulated under GH control. During summer, GH blocks the transcription of the AFP gene. With the reduction of day length in the fall, the release of GH was decreased or inhibited, thus allowing AFP gene transcription in the liver to proceed (See 1.1.3 and references within). Since GH-stimulated

IGF-1 synthesis and consequent increase in the circulating IGF-1 level represents a major route of GH action, IGF-1 appeared to be a plausible intermediate in the GH signaling pathway in controlling the wflAFP gene expression (Daughaday and Rotwein, 1989). Studies have also demonstrated the liver as a major site of IGF-1 production in response to GH in teleost (Gray and Kelley, 1991; Duguay et al, 1994; Shamblott et al, 1995; Shepherd et al, 1997). There are two major pathways downstream of the IGF-1 signaling, the PI3-kinase pathway and the MAP kinase pathway. The involvement of IGF-1 and its downstream molecule PI3-kinase in the control of wflAFP gene was investigated in transient expression assays in this study.

2.2 Experimental procedures

2.2.1 Preparation of nuclear extracts

Crude liver nuclear extracts were prepared as described by Gorski et al (1986) from male Sprague-Dawley rats (150-200 g) and male winter flounders (400-500 g). Briefly, minced liver was homogenized in homogenization buffer containing 10 mM HEPES, pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml of aprotinin, 0.5 μg/ml of leupeptin and 1 mM benzamidine. The homogenate was centrifuged at 24,000 rpm for 60-90 min at -2°C. The pelleted nuclei were resuspended in nuclear lysis buffer containing 10 mM HEPES, pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol and 2 μg/ml of aprotinin. The nuclear suspension was diluted to approximately 10 A_{260} units/ml followed by the dropwise addition of one-tenth volume of 4 M $(NH4)2SO4$ (brought to pH 7.9 with NaOH). After 30 min of gentle shaking, the viscous lysate was centrifuged at

40,000 rpm for 60 min at 0°C to pellet the chromatin. Solid $(NH_4)_2SO_4$ (0.3 g/ml) was added to the supernatant and was slowly dissolved. After an additional 30 min on ice, the samples were centrifuged at 35,000 rpm for 25 min. The protein pellet was resuspended in dialysis buffer consisting of 25 mM HEPES, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT and 10% glycerol and dialyzed for 4 h. Protein concentrations of liver nuclear extracts were determined in the range of 8-10 mg/ml and 5-8 mg/ml for rat and flounder, respectively.

2.2.2 Gel retardation assay

Single-stranded oligonucleotides were annealed, and end labeled using [γ-^{32}P]ATP (>5000 mCi/ml, Amersham Pharmacia Biotech, Baie d'Urfé, PQ) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). In the binding reaction mixture, 8 μg of nuclear extract was preincubated in a 20μl reaction, containing 25 mM HEPES, pH 8.0, 12.5 mM $MgCl_2$, 20% glycerol, 25 mM KCl, 1 μg calf-thymus DNA and various amounts of unlabeled competitor DNA as indicated for 10 min. Then 5000-10,000 cpm of 5′ end-labeled probe was added for another 10 min on ice. Free DNA and DNA-protein complexes were resolved on a 4% polyacrylamide gel in 0.5x Howley buffer (1x Howley contains 40 mM Tris, 1 mM EDTA, 20 mM sodium acetate, pH 7.2). Double-stranded oligonucleotides used as probes or competitors were as follows (only the sense strand sequences in 5′ to 3′ direction are shown):

Element B: ATAATGTTTCATCAGCACTT (+303 to +322)

AP1 consensus: CGCTTGATGACTCAGCCGGA (Hai and Curran, 1991)

C/EBP site in SV40 promoter: TGTCAGTTAGGGTGTGGAAAGTCCCCAGGC (Johnson et al, 1987)

HNF1 site in the rat albumin promoter: TGTGGTTAATGATCTACAGTTA (Cereghini et al, 1987)

HNF3 site in the transthyretin gene: CCTGATTCTGATTATTGACTTAGTCA (Costa et al, 1989)

In the supershift assay, antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were preincubated with nuclear extracts for 10 min on ice before the probes were added.

Recombinant rat C/EBPα was produced from the pMal-C/EBP-CRI construct (rat C/EBPα fused with maltose-binding protein), kindly provided by Dr. C. Mueller, Queen's University, Kingston, Ontario. The plasmid was transformed into *E.coli* strain DH5α and the fusion protein was induced with IPTG at a final concentration of 0.3 mM. Crude protein extract after lysozyme and sonication treatment was checked for the presence of C/EBPα on SDS-PAGE by antibody against rat C/EBPα and used in the gel retardation assay.

2.2.3 Methylation interference assay

Single-stranded oligonucleotides (Element B of upper or lower strand) were labeled with [γ-^{32}P]ATP (>5000 mCi/ml, Amersham Pharmacia Biotech) and T4 polynucleotide kinase (New England Biolabs) before annealing with 2x unlabeled complementary oligonucleotides. The 5′ end-labeled, double stranded oligonucleotide probes were partially methylated with dimethyl sulfate as described by Maxam and Gilbert (1980) and used for preparative gel retardation assays. Protein bound and free probes were extracted from the polyacrylamide gel of the mobility shift assay and recovered by filtration onto a NA-45 DEAE membrane (Schleicher & Schuell, Keene, NH). The probes were then treated with 1 M piperidine at 90°C for 30 min for the cleavage at methylated guanine residues. The cleaved probes were

lyophilized, separated onto a 15% polyacrylamide-8M urea gel and subjected to autoradiography.

2.2.4 Site-directed mutagenesis

In vitro site directed mutagenesis was performed by the method of Deng and Nickoloff (1992) on the In(192-350)-*f*AFP-CAT construct which contains the defined intron enhancer sequence from +192 to +350 linked with the basic promoter of the 2A-7b AFP gene and the chloramphenicol acetyltransferase (CAT) reporter gene (Chan et al, 1997). A selection primer (ATCGATCCCCGG**AT**CCCGAGCTCTCGAAT) was used to convert the unique *Sma*I and *Kpn*I sites in the plasmid into a *BamH*I site. The mutagenesis primers are (substituted nucleotides are indicated in bold):

mA: CTTTATTTACATAAT**T**TTTCATCAGCACTTCCTG

mC: TTACATAATGTTTCATCAGCA**A**TTCCTGTTTCAG

m_{A+C} CTTTATTTACATAATGTTT**A**ATCAGCACTTCCTGT

m_3: TTACATAAT**T**TTT**T**ATCAGCA**A**TTCCTGTTTTTCAG

The In(192-350)-*f*AFP-CAT plasmid was denatured and annealed with the selection primer and a mutagenesis primer. After elongation and ligation by T4 DNA polymerase and T4 DNA ligase (Clontech, Palo Alto, CA), the DNA pool that contained the circularized wild-type and mutated plasmids was used to transform the repair-deficient BMH 71-18 *mutS* strain of *E. coli*. The plasmid pool isolated from transformed cells was digested with *Kpn*I and used for another round of transformation to amplify the mutated plasmids. In all cases, the mutations were verified using the T7 DNA sequencing kit (Amersham Pharmacia Biotech).

2.2.5 Cell culture and transfection experiments

HepG2 and HeLa cells were maintained in Minimum Essential Medium (MEM) with 0.1 mM nonessential amino acids and MEM, respectively, supplemented with 10% heat-inactivated fetal bovine serum and 100 IU of penicillin and streptomycin/ml. For transfection, cells were plated at 50% confluence and cultured for 24 h prior to transfection with a change of medium after 22 h of incubation. DNA was transfected into cells by calcium phosphate precipitation method (Graham and Eb, 1973), glycerol shocked at 4 h and harvested at 48 h post transfection for analysis. A reference plasmid containing the bacterial β-galactosidase gene under the control of SV40 early promoter was cotransfected with the test plasmid to normalize the efficiency of transfection. CAT assays were performed by the method of Eastman (1987) and Morency et al (1987), and the β-galactosidase activity was determined by the method of Hall et al (1983). All transfection experiments were repeated at least three times, and for each construct at least two independent plasmid preparations were tested. The fold enhancement relative to the activity achieved with the *f*AFP-CAT construct is shown. Treatment of IGF-1 (human IGF-1, Sigma Biosciences, St. Louis, MO) with/without wortmannin (Sigma Biosciences) was performed 4 h after transfection. The thymidine kinase promoter (TK) linked to pBLCAT3 was used as positive control and the *f*AFP-CAT activity was approximately 5% of TK-CAT.

2.2.6 Western blot analysis

Crude rat and winter flounder liver extracts were separated on 12% SDS-PAGE. Then the proteins were transferred to the Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech) using the Bio-Rad electroblotting system. The membrane was blocked

in 2% milk and 5% bovine serum albumin in TBS-T (10 mM Tris, 150 mM NaCl and 0.1% Tween-20) and incubated with antibodies against C/EBPα or C/EBPβ (Santa Cruz Biotechnology) for 1 h at room temperature. The specific signals were detected by ECL chemiluminescence (Amersham Pharmacia Biotech) following the manufacturer's instructions.

2.2.7 Ultraviolet crosslinking

UV crosslinking was performed as described by Chodosh et al (1986). Briefly, Upper and lower strands of Element B with Bromo-dUTP substitution were annealed and end labeled as probes. The binding mixture was 5x the amount used in gel retardation assay as described in 2.2.2 and incubated on ice for 30 min. A UV transilluminator of 305 nm was used to irradiate the mixture for 60 min. The proteins were precipitated and analyzed on 12% SDS-PAGE.

All oligonucleotides were synthesized by the Centre of Applied Genomics, Hospital for Sick Children, Toronto, Ontario.

2.3 Results

2.3.1 Specific complexes are formed between Element B and flounder liver extracts

Gel mobility shift assay was employed to test for specific protein-DNA interaction of Element B with flounder nuclear proteins. Previously, it has been shown that rat liver nuclear extract formed complexes with Element B which could be competed out specifically by an excess of unlabeled Element B, while oligonucleotides for HNF1 and HNF3 were

ineffective (Chan et al, 1997; Fig. 2-1A, lane 3, 4, 7 and 8). Formation of the lower complex was prevented by an excess of AP-1 oligonucleotide, indicating that a protein with similar binding specificity as AP-1 was involved in the formation of the complex (Fig. 2-1A, lane 5). Similarly, a protein with binding specificity related to that of C/EBP was involved in the formation of the upper complex since its formation was competed out by an excess of oligonucleotide containing the C/EBP binding site found in the enhancer of Simian Virus 40 (Fig 2-1A, lane 6). The type of C/EBP involved in this complex was determined by using antibodies specific to C/EBPα (no cross-reaction with other C/EBP family of proteins). The upper complex was supershifted by the antibody that recognizes C/EBPα , but not the preimmune, non-specific antibody (Fig. 2-1A, lane 9 and 10). To confirm that the specific binding was also present in winter flounder, mobility shift assays were performed using flounder liver nuclear extracts. In a titration experiment, a distinct band was found at lower part of the gel when less nuclear extract was added. By increasing the amount of nuclear extracts, this band became diffused and a strong upper band appeared (data not shown). Therefore, two separate mobility shift assays were carried out at low and high concentration of flounder extract from a winter fish (Fig. 2-1B). Both the lower and upper bands that appeared at low and high amount of extract, respectively, were specific to Element B since they could be competed out by an excess of cold Element B but not by HNF3 consensus (Fig. 2-1B, Lane 3, 6, 9 and 12). Moreover, formation of the lower complex was prevented by an excess of AP1 consensus but not by C/EBP consensus. Similarly, formation of the upper band was prevented by an excess of C/EBP consensus but not by AP1 consensus (Fig.2-1B, lane 4, 5, 10 and 11). These data revealed that comparable to the complexes formed between

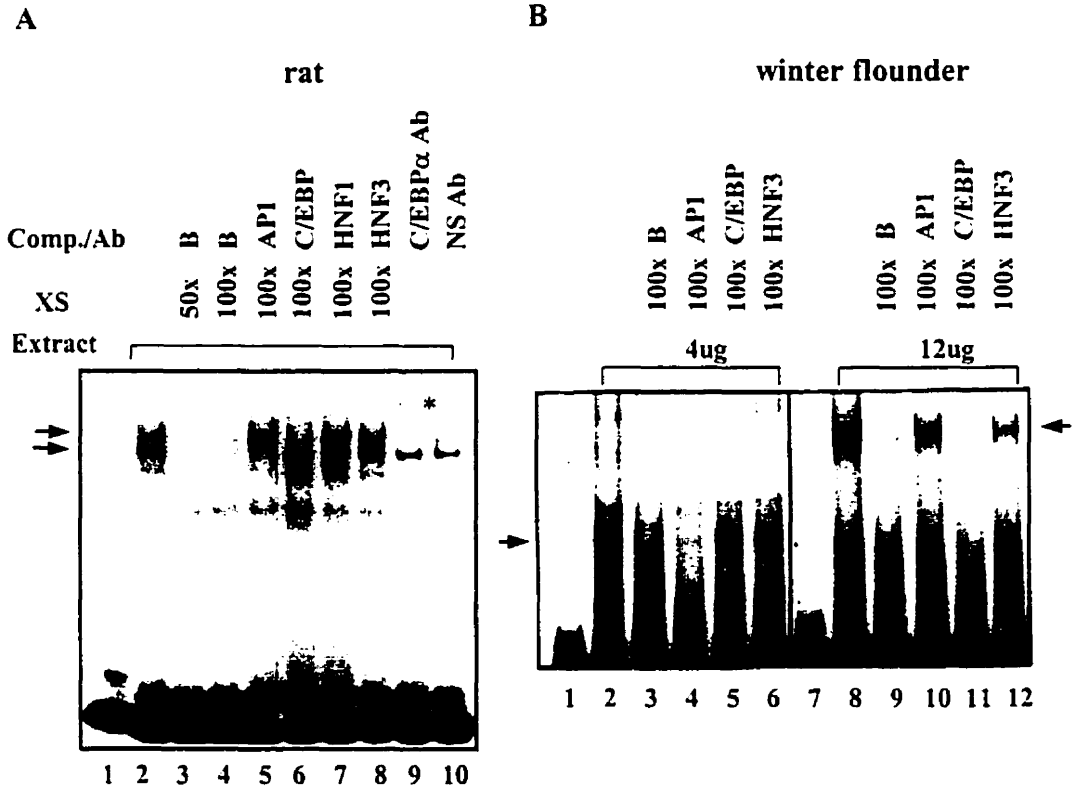


Fig. 2-1 Element B forms specific complexes with rat and flounder liver extracts in mobility shift assay. Gel retardation assay was carried out with (A) rat liver nuclear extract and (B) flounder liver nuclear extract. XS: molar excess of competitor; Comp.: competitors; Ab: specific antibody; NS Ab: non-specific antibody. Arrows indicate specific complex formation. Supershifted band is indicated by an asterisk.

Element B and rat liver extracts, proteins with similar binding specificity were also present in the winter flounder liver nuclear extracts.

2.3.2 Element B mediates the enhancer effect of the intron

To further identify the nucleotides in Element B which were in close contact with rat liver nuclear proteins, Element B (+303 to +322) was analyzed by methylation interference assay (Fig. 2-2). Binding of the "AP-1 like" protein was affected by methylation of guanine residues at positions +308 in the upper strand and +312 in the lower strand. Interference of C/EBP binding was observed by methylation of guanine residues at positions +312 and +320 in the lower strand. This methylation interference pattern showed that these factors recognized overlapping sites of Element B, but did not make exactly the same contacts. The results of methylation interference are summarized in Fig. 2-3A and mutagenesis of Element B was designed accordingly. These substitutions were shown to disrupt the DNA-protein interaction of C/EBPα and/or the putative AP-1 since mutated Element Bs with these substitutions were unable to compete effectively for binding of rat liver nuclear proteins with Element B (data not shown). In addition, In(192-350)-*f*AFP-CAT constructs containing these mutations were generated by site-directed mutagenesis to examine the roles of these residues in the enhancer activity. In transient expression assays, substitutions in these three guanine positions indicated by methylation interference in the m3 construct totally abolished the enhancer effect of the 159 bp intron fragment (+192 to +350) (Fig. 2-3B). Moreover, the single G–>T mutation at +308 of the upper strand (m_A) and +320 of the lower strand (m_C) also decreased the activity to a basal level. The m_{A+C} construct has a single G–>T mutation

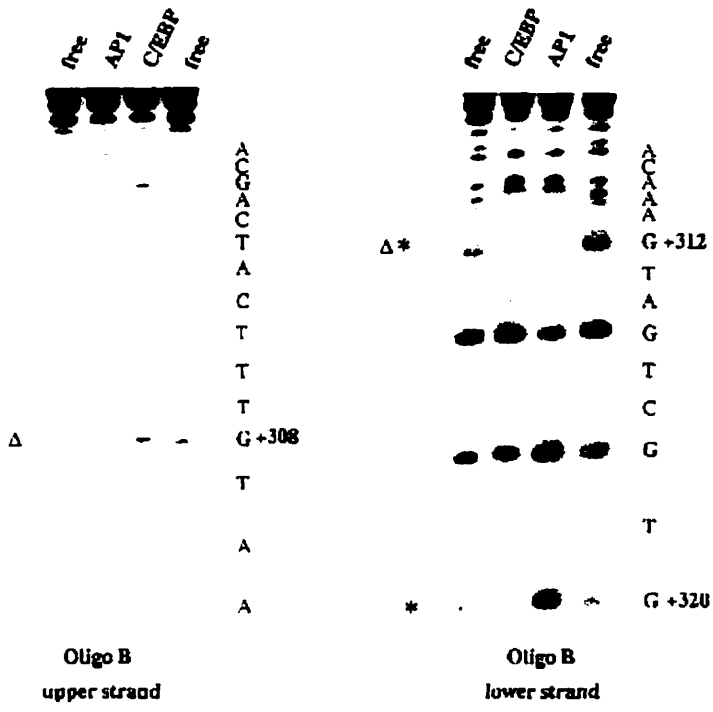


Fig. 2-2 Methylation interference assay of Element B and rat liver extract. Preparative gel-shift assays were performed with oligonucleotide B labeled in the 5' end of either the upper or lower strand and partially methylated. DNA recovered from the putative "AP-1" and C/EBP complexes were analyzed. The triangles and asterisks indicate the position of guanine (G) residue which interferes with binding in the "AP-1" and C/EBP complexes, respectively, when methylated.

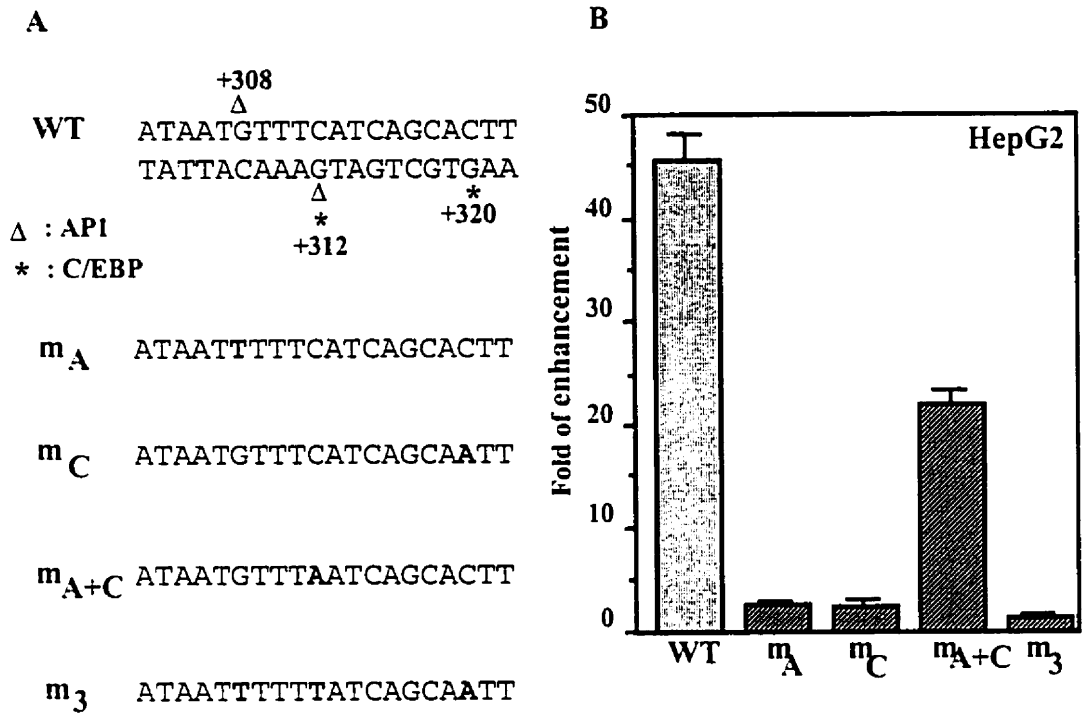


Fig. 2-3 The effect of mutated Element B in the enhancer activity of the wflAFP gene intron. A: Summary of the methylation interference study and site directed mutagenesis. Mutants of In(192-350)-*f*AFP-CAT were constructed based on the interference results. B: Transfection studies. HepG2 cells were transfected with In(192-350)-*f*AFP-CAT (WT) and the various mutation constructs. The fold enhancement relative to the activity achieved with the *f*AFP-CAT construct is shown. The mean values and standard errors of three independent experiments are given.

at +312 (methylation at this guanine residue interfered both AP1 and C/EBP binding). A significant reduction in enhancer activity was observed when this m_{A+C} construct was introduced into HepG2 cells in the transient expression assays. These results clearly indicated that the binding of Element B to nuclear proteins is important to its transactivation ability and an intact Element B is indispensable for the enhancer activity in the wflAFP gene intron.

2.3.3 C/EBPα interacts specifically with Element B and transactivates the wflAFP gene intron

In order to verify the interaction between Element B and C/EBPα, a gel shift assay was carried out using recombinant rat C/EBPα expressed in bacteria. Interaction between Element B and C/EBPα became prominent as the amount of extract increased. This complex could be competed out by Element B and C/EBP consensus, but not by AP1 consensus (Fig. 2-4). These data indicated that Element B of the wflAFP gene interacts specifically with the liver-enriched factor C/EBPα. Furthermore, the effect of C/EBPα on transactivation of the wflAFP genes was investigated by transient expression assays. Rat C/EBPα expression vector (pMSV-C/EBP) was cotransfected with the wflAFP intron construct In(192-350)-*f*AFP-CAT (Fig. 2-5). C/EBPα was able to increase the enhancer activity of the wflAFP intron dramatically in HepG2 cells while no significant increase was observed in HeLa cells. These results showed that C/EBPα is involved in the transcriptional regulation of the wflAFP gene, that it interacts with the core wflAFP enhancer, and transactivates the wflAFP gene in cells of liver origin.

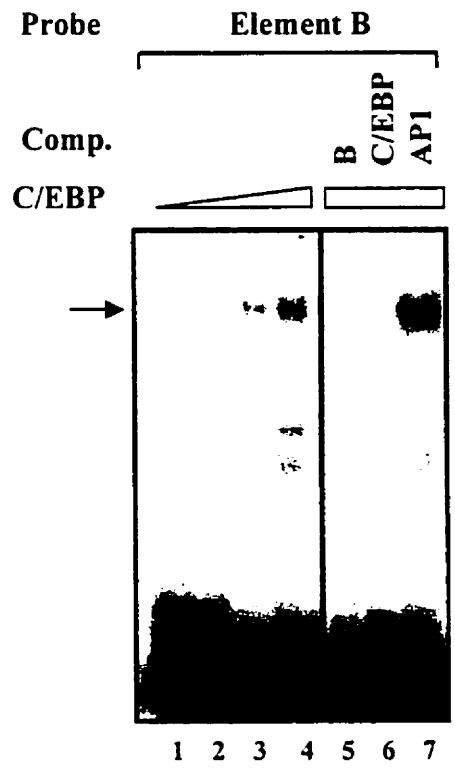


Fig. 2-4 Element B forms a specific complex with recombinant rat C/EBPα in mobility shift assay. Gel retardation assay was carried out using crude extract of bacteria expressed rat C/EBPα. Specific oligonucleotide competitors (100x molar excess) were added into the reaction mixtures as indicated. The arrow indicates the specific complex formation.

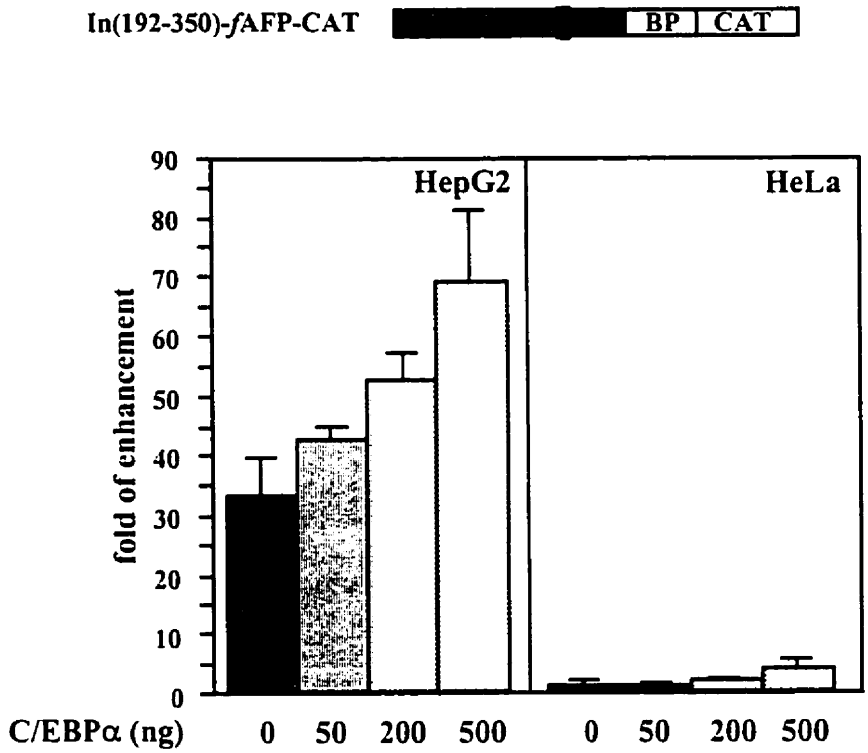


Fig. 2-5 Cotransfection of C/EBPα stimulates the wflAFP gene enhancer activity in HepG2 cells. HepG2 cells were transfected with In(192-350)-*f*AFP-CAT. The amounts of DNA (pMSV-C/EBP) used in cotransfection are indicated. The fold enhancement relative to the activity achieved with the *f*AFP-CAT construct is shown. The mean values and standard errors of three independent experiments are given.

2.3.4 The C/EBP family of proteins is present in the liver of winter flounder

Since the AFP gene is expressed in the liver of winter flounder, it is important to show that the transcription factors implicated in its regulation are present in the homologous system. Immunoblot analysis was performed using rat and winter flounder liver extracts with antibodies raised against rat C/EBPα or C/EBPβ (Fig. 2-6). The C/EBPα antibody raised against the amino acid sequence from 253 to 265, which is unique for the C/EBPα subfamily, was able to recognize proteins in rat liver (rat C/EBPα is 42 kDa with smaller isoforms) but not in the winter flounder liver. However, the C/EBPβ antibody was able to recognize proteins in rat (rat C/EBPβ is about 32 kDa) as well as in winter flounder with molecular mass approximately 34 kDa and 29 kDa. This antibody was raised against a longer peptide from 199 to 345, that is conserved among the C/EBP family, and was cross reactive to C/EBPα, β, δ and ε. These data suggest the existence of C/EBP family of proteins in the liver of winter flounder.

2.3.5 Two proteins of approximately 43 kDa and 80 kDa in rat liver interact with Element B

UV crosslinking was carried out to further characterize the proteins interacting with Element B (Fig. 2-7). Two complexes of approximately 80 and 43 kDa were detected. No specific bands were shown in reaction mixtures without the rat liver nuclear extracts or no UV irradiation (Fig. 2-7, lane 1 and 2); however, high background was observed in lane 2 which may be due to the exposure of sample to light during experimental manipulation. Upon UV irradiation, two protein complexes were visible. These two bands were competed out by cold

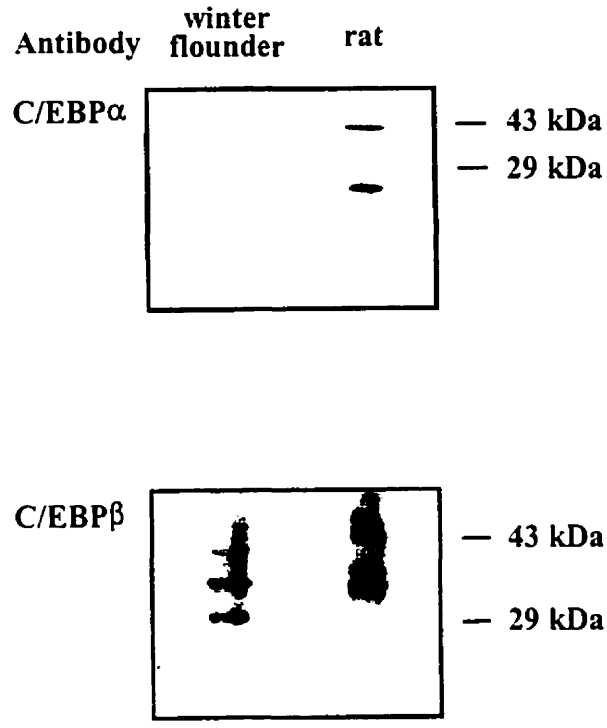


Fig. 2-6 The C/EBP family proteins are present in winter flounder liver. Western blot analysis was performed using winter flounder or rat liver extracts. Antibodies used in the assays were raised against rat C/EBPα (specific to C/EBPα) and C/EBPβ (cross reactive with C/EBPα, β, δ and ε) as indicated.

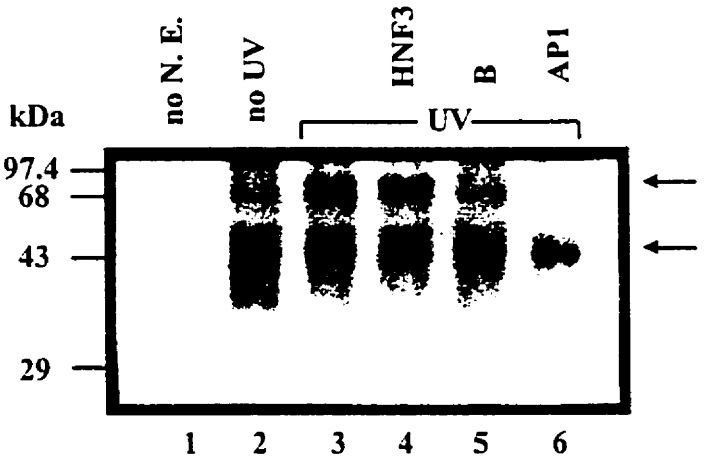


Fig. 2-7 UV crosslinking assay of Element B and rat liver extract. End labeled Bu-dUTP substituted Element B was incubated with rat extract and UV irradiated for 60 min. Proteins were precipitated and separated on 12% SDS-PAGE. 100x molar excess of specific competitors were used as indicated. The arrows indicate the specific binding complexes.

Element B but not by HNF3 consensus, suggesting that the binding of these proteins to Element B was specific (Fig. 2-7, lane 3 to 5). The rat C/EBPα is known as a 42 kDa protein with several smaller isoforms. Mobility shift data demonstrated the C/EBP and Element B complex was able to be supershifted by antibody specific to C/EBPα. Therefore, the crosslinking studies again suggested that one of the rat liver nuclear proteins interacting with Element B was C/EBPα. The upper band with a molecular mass of approximately 80 kDa could be competed out by AP-1 consensus (Fig. 2-7, lane 6), and might represent the presumptive antifreeze enhancer-binding protein (AEP).

2.3.6 The involvement of IGF-1 in wflAFP gene regulation

The mechanism of how the wflAFP gene is regulated by GH is an interesting topic to study. Since IGF-1 mediates a major part of GH action, the effect of IGF-1 on wflAFP gene expression was investigated in HepG2 cells by transient expression assays (Fig. 2-8). Cells transfected with the whole intron of wflAFP gene construct In-*f*AFP-CAT were treated with human IGF-1. IGF-1 was able to decrease the transactivation ability of the wflAFP gene intron in a dose-dependent manner, but had no effect on the basic promoter of the wflAFP gene (-143 to +32). This result suggested that IGF-1 might be an important factor in mediating the GH effect on the wflAFP gene regulation.

2.3.7 Element B mediates the effect of IGF-1 on the wflAFP gene expression

To further define the enhancer region that mediates IGF-1 response, the truncated intron construct In(192-350)-*f*AFP-CAT was used in transient expression assays. IGF-1 was able to inhibit the transcriptional activity of this more refined intron construct containing Element B.

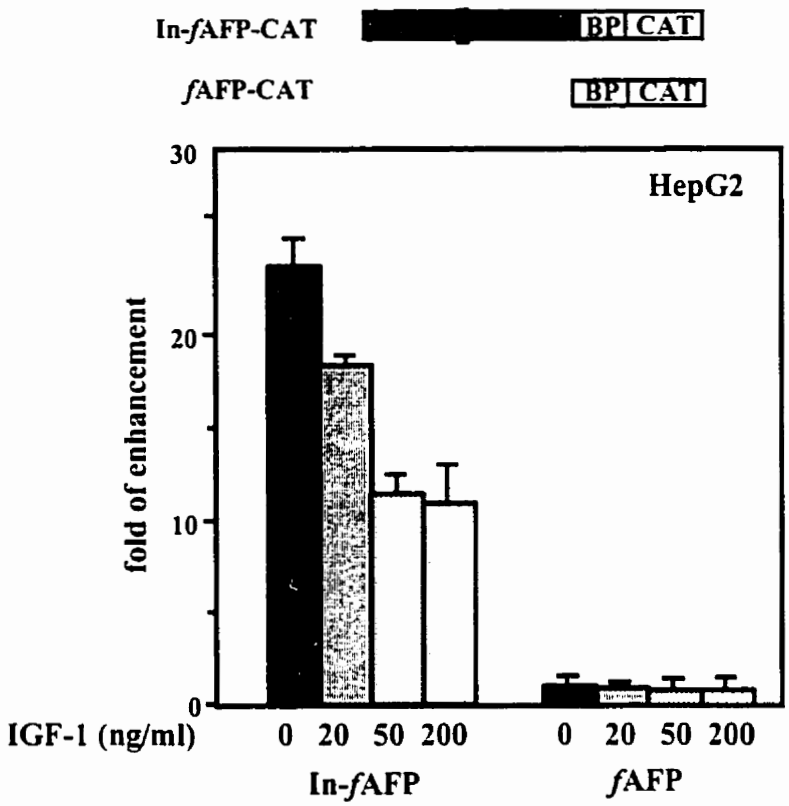


Fig. 2-8 IGF-1 decreases the wflAFP gene expression in transient expression assays. HepG2 cells were transfected with In*f*AFP-CAT or *f*AFP-CAT with IGF-1 treatment as indicated. The fold enhancement relative to the activity achieved with the *f*AFP-CAT construct is shown. The mean values and standard errors of three independent experiments are given.

However, when cells transfected with mSIn(192-350)- *f*AFP-CAT, which contains a TA insertion in Element B and is not interactive with C/EBPα (see 3.3.3), little effect was observed with IGF-1 treatment (Fig. 2-9). These results suggested that the inhibitory effect of IGF-1 on the enhancer activity of the wflAFP gene intron is mediated by Element B.

2.3.8 The involvement of PI3-kinase in the wflAFP gene regulation

As mentioned above, PI3-kinase is one of the downstream signaling molecules in the IGF-1 pathway. To explore its possible involvement in wflAFP gene regulation, an inhibitor specific for PI3-kinase, wortmannin, was used in the transient expression assays (Fig. 2-10). Dramatic inhibition was observed in HepG2 cells transfected with In(192-350)-*f*AFP-CAT when treated with IGF-1. Addition of wortmannin was able to restore the transactivation activity of the wflAFP gene enhancer in a dose-dependent manner, indicating that PI3-kinase was also involved in the signaling pathway for the control of the wflAFP gene expression.

2.4 Discussion

In this study, the regulation of the wflAFP gene was first examined using a mammalian system. Several studies have suggested the conservation of regulatory processes dictating liver specificity between phylogenetically diverse vertebrate species. For example, the promoter of the Xenopus vitellogenin gene was able to direct efficient and accurate initiation of transcription in reaction mixture containing transcriptionally active extracts derived from rat liver nuclei (Kaling et al, 1991). Moreover, the structure of transcription factors is generally conserved among species. The basic region of the Drosophila C/EBPα shares more than 90% identity with the rat sequence and is able to bind DNA with a similar specificity

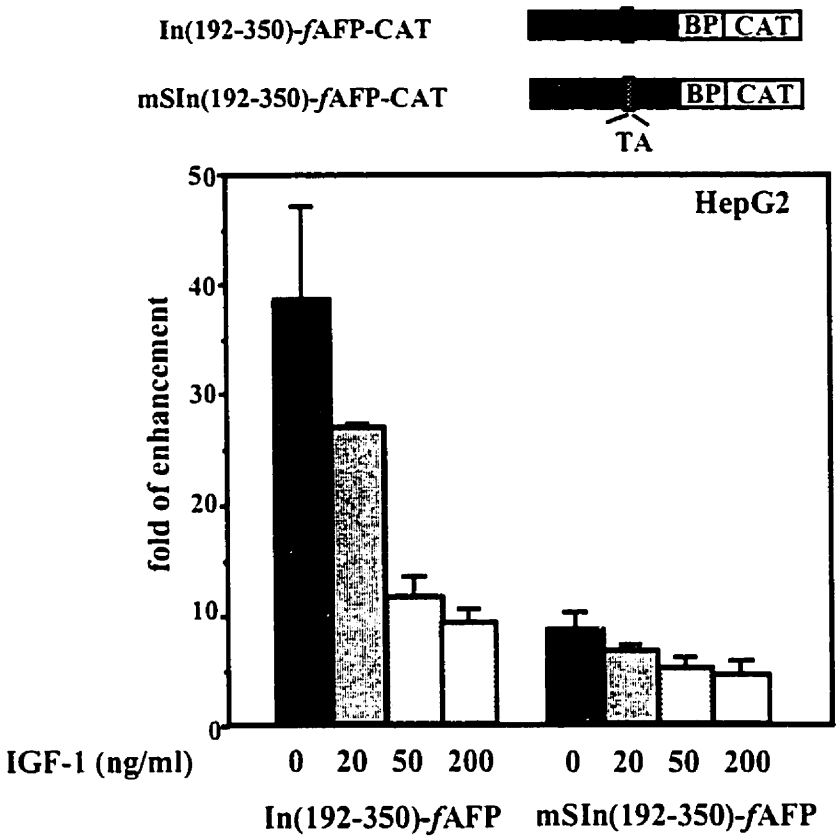


Fig. 2-9 The effect of IGF-1 on the wflAFP gene expression is mediated by Element B. HepG2 cells were transfected with In(192-350)-*f*AFP-CAT or mS In(192-350)-*f*AFP-CAT with IGF-1 treatment as indicated. The fold enhancement relative to the activity achieved with the *f*AFP-CAT construct is shown. The mean values and standard errors of three independent experiments are given.

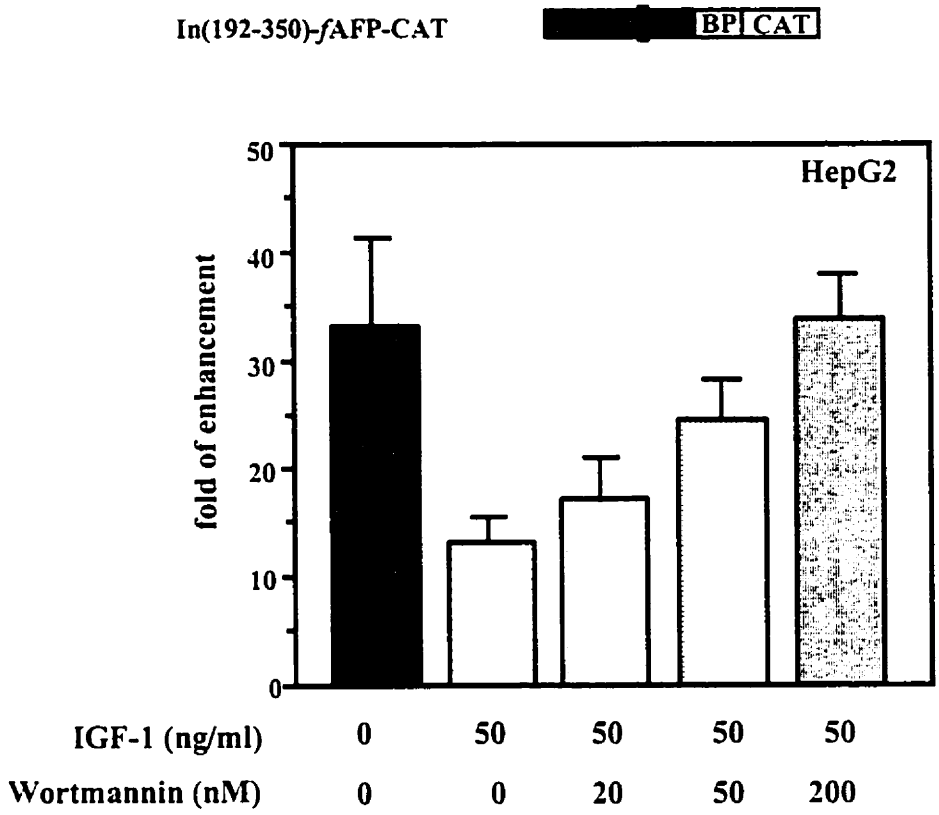


Fig. 2-10 Wortmannin treatment restores the wflAFP gene enhancer activity. In(192-350)-*f*AFP-CAT construct were transfected into HepG2 cells with various IGF-1 and wortmannin treatment as indicated. The fold enhancement relative to the activity achieved with the *f*AFP-CAT construct is shown. The mean values and standard errors of three independent experiments are given.

(Montell et al, 1992). Also, studies on the regulation of wflAFP gene have provided evidence that the mechanism for wflAFP gene regulation is very conserved between mammals and flounder. In addition to the mammalian cell lines, the enhancer activity of the wflAFP intron has been demonstrated in flounder hepatocytes in an *in vitro* transcription assay (Miao et al, 1998a). Similar enhancer regions were shown to interact with nuclear proteins from both rat and flounder liver as demonstrated by DNase I footprinting assays (Chan et al, 1997; Miao et al, 1998a). In this chapter, the interaction between flounder C/EBP and the presumptive AEP to the core enhancer Element B was shown to be consistent with what was observed earlier from rat in gel retardation assays. There is other supportive evidence showing that the AFP genes, which are present only in a subpopulation of fish species, can be expressed in other fish species including Atlantic salmon, goldfish, as well as in Drosophila and tobacco by transgenic technology (Hew et al, 1992; Wang et al, 1995; Rancourt et al, 1987; Kenward et al, 1993). Also, the tissue-specificity of the wflAFP gene was demonstrated in salmon. Transgenic salmon harboring the wflAFP gene exhibited liver-specific and seasonal wflAFP expression (Hew et al, 1999).

Since C/EBPα is well characterized to be a liver-enriched factor mediating the transactivation of several liver-specific genes, the involvement of C/EBPα in the wflAFP intron is suggested to be responsible for the liver-specific expression of the wflAFP gene. C/EBPα can form homodimers or heterodimers with other members of the C/EBP family of proteins. Our data does not exclude the possible involvement of other C/EBPs. Three C/EBP isoforms have been found to regulate the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) gene transcription in the liver (Croniger et al, 1998). The C/EBP protein family has been found from Drosophila to mammal (Montell et

al, 1992; Landschulz et al, 1988), while it has not been demonstrated in fish. Our data predicts that the C/EBP protein family is present in flounder liver. However, the identity of flounder C/EBP interacting with Element B would require further investigation.

Previous studies have shown that the protein that binds to the "AP1 binding site" in Element B is distinct from the AP1 components (the Jun and Fos family transcription factors) (Chan et al, 1997). Antibodies raised against the DNA-binding domains of Jun and Fos which specifically recognize Jun family (cJun, JunB and JunD) and Fos family (cFos, Fra-1, FosB and Fra-2) proteins, respectively, were unable to supershift or abolish the putative "AP-1" complex formed between protein(s) found in the rat liver nuclear extract and Element B. And more specifically, Element B was incapable of forming specific complexes with the DNA binding domains of cJun and cFos heterocomplex. This presumptive "AP1-like" binding protein was designated as the antifreeze enhancer-binding protein (AEP). According to the UV crosslinking result, AEP is a fairly big protein with a molecular mass of at least 80 kDa. AEP is able to interact with both AP-1 consensus and Element B, however, Element B interacts with AEP but has no affinity for the AP-1 protein. The identity of the presumptive AEP and its involvement in wflAFP gene regulation will be discussed in Chapter 4.

This study has further demonstrated that IGF-1 and one of its downstream signal molecules PI3-kinase may play an important role in the regulation of the wflAFP gene expression. Also, the core enhancer Element B appears to mediate the IGF-1 action, possibly via the interaction with C/EBPα. IGF-1 and insulin have been demonstrated to cause C/EBPα dephosphorylation, which is correlated with the repression of GLUT4 gene transcription in adipocytes (Hemati et al, 1997). Together with the fact that GH regulates the seasonal expression of the wflAFP gene and IGF-1 is a major route of GH action, a working model

has been proposed to explain the hormonal regulation of the wflAFP gene via IGF-1, PI3-kinase and C/EBPα through a repression mechanism (Fig. 2-11). The inhibitory mechanism is turned on during summer when GH stimulates the production of IGF-1, and through the PI3-kinase signaling, C/EBPα becomes dephosphorylated and inactivated, resulting in the transcriptional inhibition of wflAFP gene. On the other hand, the active form of C/EBPα and/or other factors, such as the presumptive AEP, interact with Element B and activate the wflAFP gene expression during winter.

In addition to causing dephophorylation of C/EBPα, insulin also stimulates a decline in C/EBPα mRNA and protein levels via the MAPK pathway. At the same time, it increases the expression of a dominant-negative form of C/EBPβ (LIP) that competes for the C/EBP binding site (Hemati et al, 1997). Whether IGF-1 exhibits the same effects remain to be determined. Also, the effect of IGF-1 and insulin on dephosphorylation of C/EBPα was shown to be more dramatic on the truncated 30 kDa isoform than the 42 kDa full-length protein, implying that the two isoforms may have differential roles in transcriptional regulation.

C/EBP has been shown to participate in hormonal responsiveness of other genes. The amount of C/EBPα protein in liver was found to be rapidly and transiently decreased by GH, and the binding of C/EBPα to the promoter of the hepatocyte nuclear factor-6 gene inhibits its expression (Rastegar et al, 2000). Moreover, the stimulation of thyroid hormone (T3) on the PEPCK gene was shown to require its binding to C/EBPα or β (Park et al, 1995; Park et al, 1999).

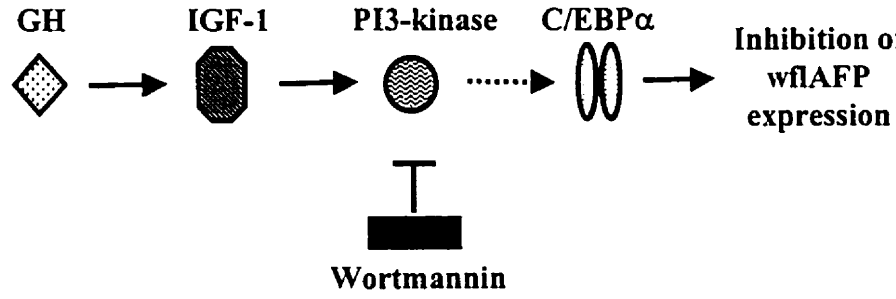


Fig. 2-11 Signaling pathway of the growth hormone regulated wflAFP gene expression. GH stimulates the production of IGF-1 and through the PI3-kinase signaling, C/EBPα becomes dephosphorylated and inactivated, resulting in transcriptional repression of the wflAFP gene. The specific PI3-kinase inhibitor, wortmannin, blocks the signaling pathway.

Much evidence supports the notion that phosphorylation of C/EBP factors is a common mechanism in regulating their transcriptional activation ability. Phosphorylation of C/EBPα *in vitro* by protein kinase C (PKC) results in an attenuation of C/EBP binding to DNA (Mahoney et al, 1992). Glycogen synthase kinase 3 (GSK3) was shown to phosphorylate C/EBPα both *in vitro* and *in vivo* and it was suggested that the insulin stimulated dephosphorylation of C/EBPα is mediated through the inactivation of GSK3 (Ross et al, 1999). Furthermore, *in vitro* phosphorylation of C/EBPβ by PKC or cAMP-dependent protein kinase A (PKA) was shown to alter its binding affinity. The increase of phosphorylation within the activation domain of C/EBPβ by the stimulation of PKC pathway was shown to enhance its transcriptional efficacy (Trautwein et al, 1993; Trautwein et al, 1994). In pituitary cells, C/EBPβ is phosphorylated by the activation of a calcium-calmodulin-dependent protein kinase and this was shown to confer the calcium-regulated transcriptional stimulation of a promoter containing C/EBP binding sites (Wegner et al, 1992). Phosphorylation of C/EBPβ by TGFα-induced activation of the p90 ribosomal S kinase (RSK) is also concomitant with the proliferation of differentiated hepatocytes (Buck et al, 1999).

Other signaling pathways may also be involved in the hormonal regulation of the wflAFP gene. As mentioned earlier, GH exerts regulatory effects on C/EBPs, which may be independent of the IGF-1 signaling pathway, while the MAP pathway down stream of IGF-1 may also be involved in the regulatory process. Other inhibitors, such as PD098059, a MEK inhibitor, can be used to further investigate the role of other signaling molecules involved in the seasonal and hormonal control of winter flounder AFP genes.

Chapter Three

Differential regulation of the skin-type AFP gene in winter flounder

FEBS lett (1998) 426:121-5. Miao, M., Chan, S. L., Hew, C. L. and Gong, Z.

*Results presented in this chapter were the authour's contributions to this publication.

3.1 Introduction

The presence of AFPs in tissues other than liver has been reported in winter flounder. The wfsAFP mRNAs are found mainly in exterior tissues including skin, fin, filaments and scales, and to a lesser extents in stomach, intestine, kidney, spleen as well as liver (Gong et al, 1996). They are encoded by a distinct set of genes from that of the wflAFPs and their expression is under differential seasonal and hormonal control (see 1.1.3 for details). The presence of two distinct AFPs within flounder has raised important questions on the relative role of these proteins in freezing protection, their evolutionary relationships, as well as their regulatory mechanisms. The tissue-specific expression of the liver-type AFPs is conferred by its enhancer element, Element B, which interacts with the liver-enriched C/EBPα, and the presumptive antifreeze enhancer-binding protein (AEP). The present investigation was undertaken to examine the differential molecular mechanisms controlling the expression of the skin-type AFP genes and in particular, the contribution of C/EBPα, if any, in activating these genes.

3.2 Experimental procedures

3.2.1 Plasmid construction

The F2 genomic clone encoding a representative skin-type AFP gene (Davies and Gauthier, 1992) was kindly provided by Dr. P. Davies, Queen's University, Kingston, Ontario. To generate constructs of the skin-type intron with CAT reporter gene, the 2.3 kb *Pst*I fragment of the F2 genomic clone was subcloned into pBluescript (Stratagene, La Jolla, CA). The 750 bp *Pst*I/*Hinc*II intron fragment from the 2.3 kb fragment was further purified and ligated to the *Pst*I site of the *f*AFP-CAT construct which contains the basic promoter sequence from –

143 to + 32 of 2A-7b AFP gene upstream of pBLCAT3 (Chan et al, 1997). Constructs with the 750 bp F2 intron fragment in forward and reverse orientation were designated as F2In-*f*AFP-CAT and RF2In-*f*AFP-CAT, respectively. All constructs were verified using the T7 DNA sequencing kit (Amersham Pharmacia Biotech).

3.2.2 Cell culture and transient expression assay

New born rat keratinocytes (NBRK) were maintained in Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 10% calf serum and 2.5% fetal bovine serum, HeLa cells in Minimum Essential Medium (MEM) with 10% calf serum and HepG2 cells in MEM with 10% fetal bovine serum. DNA was transfected into cells by calcium phosphate precipitation method (Graham and Eb, 1973) and cells were glycerol shocked 4 h and harvested 48 h after transfection. CAT assays were performed using a mixed-phase assay by the method of Eastman (1987) and Morency et al (1987). A β-galactosidase gene driven by SV40 early promoter was used in transfection and the β-galactosidase activity was determined as the internal control (Hall et al, 1983). All transfection experiments were repeated at least three times and pBLCAT3 with or without TK promoter was used as a positive and negative control, respectively. The *f*AFP-CAT activity was approximately 25%, 10% and 4% of the positive control TK-CAT activity in NBRK cells, HeLa cells and HepG2 cells, respectively.

3.2.3 Gel retardation assay

Crude liver nuclear extract from male Sprague-Dawley rats was prepared as described in 2.2.1. Recombinant rat C/EBPα was expressed from pMal-C/EBP-CRI construct (rat

C/EBPα fused with maltose-binding protein) by bacterial expression system. Single-stranded oligonucleotides were annealed and end-labeled using [γ-^{32}P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (New England Biolabs). In the binding reaction, rat liver nuclear extract or bacteria expressed MBP-C/EBPα was incubated with unlabeled competitor DNA as indicated for 10 min in binding buffer containing 25 mM HEPES, pH 8, 12.5 mM $MgCl_2$, 20% glycerol, 25 mM KCl and 40 ng/μl calf-thymus DNA. Antibody specific to C/EBPα (Santa Cruz Biotechnology) was also added to the nuclear extract 10 min before the probe was added. Then the 5'-end-labeled probe (15,000 cpm) was added and incubated for another 20 min on ice. Free DNA and DNA-protein complexes were resolved on 4% polyacrylamide gel in 20 mM Tris, 0.5 mM EDTA, 10 mM sodium acetate, pH 7.2. Double-stranded oligonucleotides used as probes or competitors were as follows:

Element S: ATAATGTTTTACATCAGCACTT

Element B: ATAATGTTTCATCAGCACTT

AP1 consensus: CGCTTGATGACTCAGCCGGA (Hai and Curran, 1991)

C/EBP consensus: CTAGGCATATTGCGCAATATGC (Osada et al, 1996)

3.2.4 Site-directed mutagenesis

In vitro site-directed mutagenesis was performed by the method of Deng and Nickoloff (1992). The mutant mSIn(192-350)-*f*AFP-CAT was constructed using In(192-350)-*f*AFP-CAT with a selective primer which converts the *Kpn*I site into a *BamH*I site (ATCGATCCCCGG**AT**CCCGAGCTCTCGAAAT) and a mutagenic primer which has a TA insertion in Element B (ATTTACATAATGTTT**TA**CATCAGCACTACTTCCTG). The In(192-

350)-*f*AFP-CAT plasmid was denatured and annealed with the selection and mutagenesis primers. After elongation and ligation by T4 DNA polymerase and T4 DNA ligase (Clontech), the DNA pool that contained the circularized wild-type and mutated plasmids was used to transform the repair-deficient BMH 71-18 *mutS* strain of *E. coli*. The plasmid pool isolated from transformed cells was digested with *KpnI* and used for another round of transformation to amplify the mutated plasmids. The mutation was confirmed using the T7 DNA sequencing kit (Amersham Pharmacia Biotech).

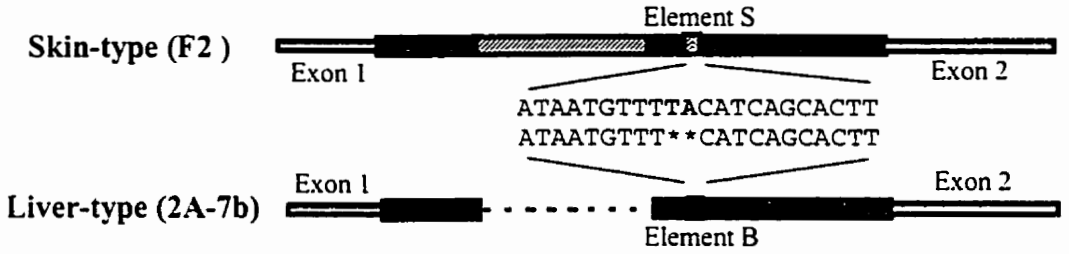
All oligonucleotides were synthesized by the Centre of Applied Genomics, Hospital for Sick Children, Toronto, Ontario.

3.3 Results

3.3.1 The skin-type AFP intron is homologous to the liver-type AFP intron

To explore the different mechanisms controlling skin-type and liver-type AFP gene expression, the gene organization of a representative wfsAFP gene, F2, was compared with the wflAFP gene, 2A-7b (Fig. 3-1). Despite their distinct tissue expression patterns, the two types of AFPs have similar gene structures with two exons and a single intron (Fig. 3-1A). The sequence of the skin-type intron shared 95% identity with the liver-type intron except that it contains an extra 241 bp insertion at position +254 (Davies and Gauthier, 1992) (Fig. 3-1B). The region of skin-type intron corresponding to the Element B of the liver-type intron was designated as Element S. Interestingly, a TA dinucleotide insertion was found within this element. Since the Element B was known to mediate the enhancer activity of the liver-

A



B

```
F2     gtacgtgaacactcactttgtttctcttacaaatttg*ttttactgtaaatatcttggga   +97
2A-7b  ------------------------tc--tg---c--g---------------------   +165

F2     aggaaggaaggatatctgcattatcc**gaggggccatttgttttacagccagcggtgaa   +155
2A-7b  --------------------------cc-------------------------------   +225

F2     agatgaagatcttcatccgtgttcgtatgatggaaagtttgttctgaaaccttcattgga   +215
2A-7b  ----------------------a-c--****************************----   +254

F2     agaaacagattcatgtgttcaggcttaaacctgcaaaaatctgagctctgttaaatcatg   +275
2A-7b  ************************************************************   +254

F2     ggaaacaactttataattcagtcagggctggaaaactcttttatatgcacagaagaagaa   +335
2A-7b  ************************************************************   +254

F2     gaagatgtgatctttagttcatcaccatggaaacatcatcagcagttaaagtctgtctgc   +395
2A-7b  ************************************************************   +254

F2     ttcagtatcaccggccagttccagtgcctgtttgaccctgtttaacacaagatggccacc   +455
2A-7b  ****************************----------a----------t---a------   +284

F2     tggaccatctttatttacataatgttttacatcagcacttcctgtattcagccctaaact   +515
2A-7b  -----------------*********----------**----------t--------g-----   +342

F2     taaagaggcctca******cttcctgatgatctggtgacacctgctggttgaaggaaaca   +569
2A-7b  ------------tggaaa-----------------------------------------   +402

F2     gagtttgagaggcagcagaacaaatgattttagtttgaatgaagaagctgtcatttgatt   +629
2A-7b  ------------g------a----t---------------------------------   +462

F2     ttatgtttggaggggggggggggggatcaccacacacagatattgaacactgtcatca   +689
2A-7b  -c-----g--g----------*******---t---------------ta----------   +515

F2     ctgagttcggtgaaagtgaagaaccagtacatgttgtgatatataatataatcataataa   +749
2A-7b  -------t----------c-g-------a------------------t----------   +575

F2     ttataataataccattaatctctgcag                                   +776
2A-7b  ---------------------------                                   +602
```

Fig. 3-1 Comparison of the skin-type F2 gene and the liver-type 2A-7b gene. A: The homologous introns in wflAFP and wfsAFP genes and the additional intron in wfsAFP genes are indicated by dotted and striped boxes, respectively. B: The sense strands are presented and individually numbered. Dashes indicate sequences identical to F2 and asterisks represent gaps. Element S and B are boxed (Scott et al, 1988; Davies and Gauthier, 1992).

type intron, it is possible that there is a specific effect for the presence of the TA insertion in Element S of the wfsAFP gene.

3.3.2 The intron of the skin-type AFP gene acts as a ubiquitous enhancer

To study whether the skin-type AFP intron, like the liver-type AFP intron, also exhibits transactivation activity, the F2In-*f*AFP-CAT and RF2In-*f*AFP-CAT constructs which contained the skin-type F2 gene intron in the natural or reverse orientation, respectively, upstream of the *f*AFP-CAT, were constructed (Fig. 3-2A). The transient expression assay in NBRK (new born rat keratinocytes) revealed that the F2In-*f*AFP-CAT exhibited a three fold enhancer activity (Fig. 3-2B). However, the reversed F2 intron had little effect on transactivation, indicating that the enhancer activity might be orientation-dependent. The enhancer activity of the skin-type intron was further tested in non-skin derived cell lines. Significant enhancer activity was also observed using the skin-type intron in the HeLa (cervical carcinoma) cells. In contrast, the liver-type intron exhibited no transactivation activity in both NBRK and HeLa cells. Together, these studies suggested that the skin-type intron, like its homologous liver-type intron, also functions as an enhancer. However, the transactivation activity of the skin-type intron was not limited only in skin cells.

3.3.3 Element S of the skin-type intron does not interact with C/EBPα

Since Element B of the liver-type intron is known to mediate its liver-specific enhancer activity, we speculated that the TA insertion in Element S of the skin-type intron may alter its transactivation ability and tissue-specificity. First, gel retardation assays were carried out to investigate the interactions between Element S and rat liver nuclear extracts (Fig. 3-3). In the

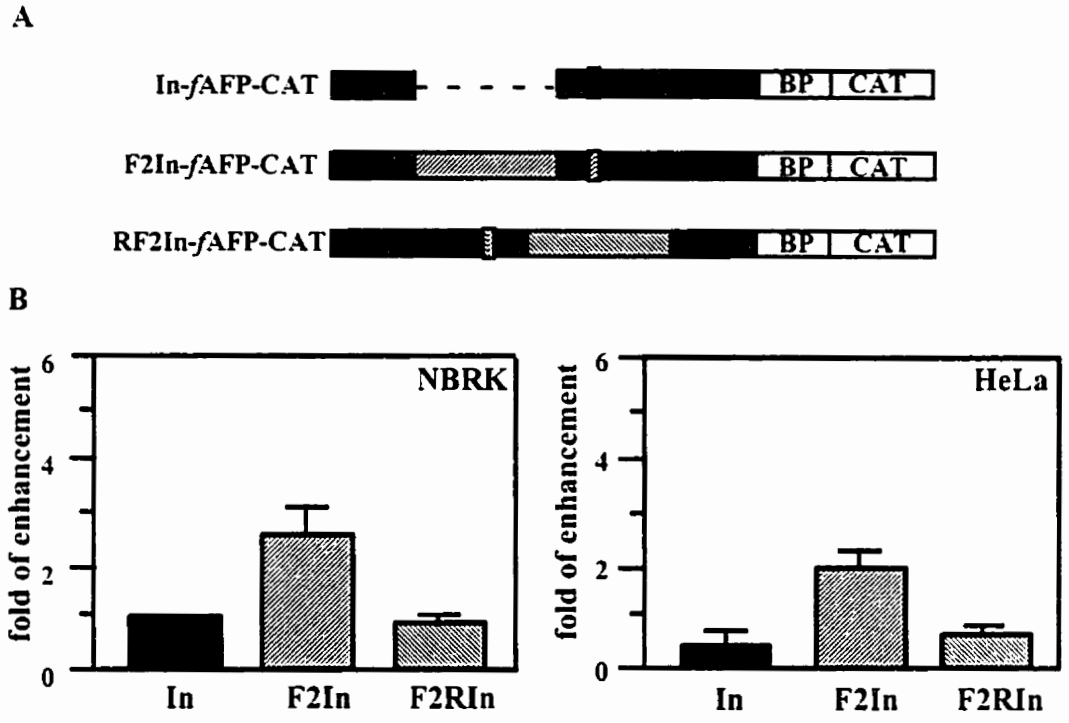


Fig. 3-2 The intron of the wfsAFP gene is a ubiquitous enhancer. A: Schematic illustration of the liver-type AFP intron construct (In-*f*AFP-CAT) and the skin-type intron constructs in nature and reverse orientation (F2In-*f*AFP-CAT and RF2In-*f*AFP-CAT, respectively). B: The constructs were transfected into NBRK and HeLa cells and their activities normalized for transfection efficiency using β-galactosidase activity. The mean values and standard errors of the fold of enhancement relative to the activity achieved with the *f*AFP-CAT construct is shown.

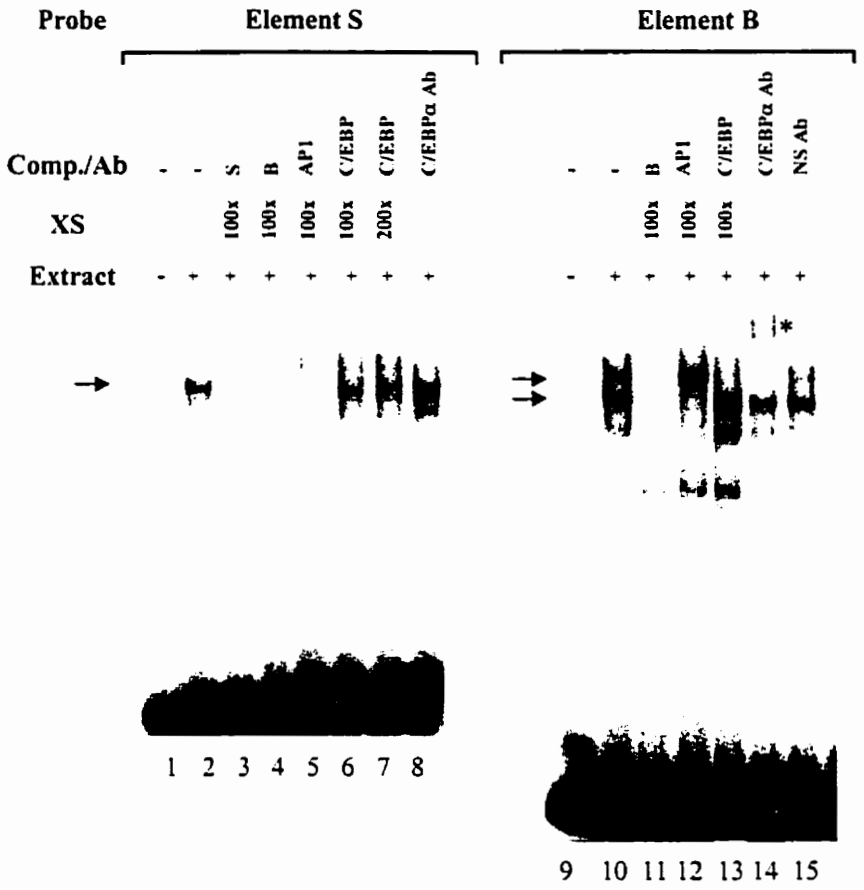


Fig. 3-3 Element S does not form any C/EBP-specific complex with rat liver extract in mobility shift assay. Gel retardation assays were carried out with rat liver nuclear extract. Comp, competitor; XS, molar excess; Ab, specific antibody; NS, non-specific antibody. Arrows indicate specific complex formation. Supershifted band is indicated by an asterisk.

control experiment using Element B as probe, a specific band was competed out by an excess of unlabeled Element B or the oligonucleotide containing the C/EBP binding site, and was supershifted by antibody specific to rat C/EBPα (Fig. 3-3, lane11, 13 and 14). In contrast, though Element S was able to form DNA-protein complex with the rat liver extracts which can be competed out by oligonucleotides containing AP1 consensus (Fig. 3-3, lane 5), excess of C/EBP consensus oligonucleotide was ineffective in competing for binding with any of the complexes. Furthermore, none of the complexes was recognized by antibody specific for C/EBPα (Fig. 3-3, lane 5 to 8). These data indicated that there was no interaction between Element S and C/EBPα. To further examine the C/EBPα binding specificity, rat C/EBPα protein (fused with maltose binding protein) was also used in gel-shift assays (Fig. 3-4). Using the liver-type Element B as a probe, a shift was gradually formed with increasing amounts of extract (Fig. 3-4, lane 2 to 4). This specific band was competed out by an excess of cold Element B or C/EBP consensus but not AP1 consensus, in contrast, there was no specific shift when Element S was used as probe (Fig. 3-4, lane 5 to 7 and 9 to 11). Therefore, these results further confirmed that Element S of the skin-type gene does not interact with the liver-enriched transcription factor C/EBPα.

3.3.4 The TA dinucleotide insertion in Element B of the liver-type intron decreases its liver-specific enhancer activity

To further study the effect of Element S (Element B with a TA insertion) in transactivation, a mutated construct, mSIn(192-350)-*f*AFP-CAT, was generated by site-directed mutagenesis to insert a TA dinucleotide directly into the Element B sequence of the In(192-350)-*f*AFP-CAT

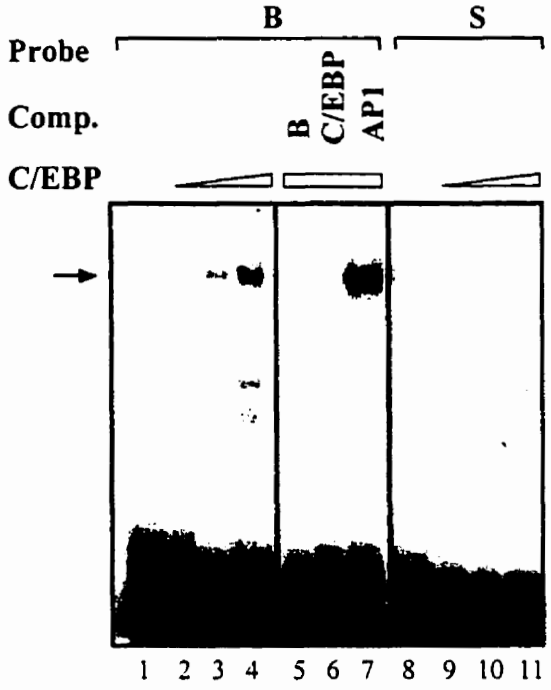


Fig. 3-4 Element S does not interact with recombinant rat C/EBPα in the mobility shift assay. Gel retardation assays were carried out using increasing amounts of crude extract of bacteria expressing rat C/EBPα. Specific oligonucleotide competitors (100x molar excess) were used as indicated. The arrow indicates the formation of specific complex.

construct (Fig. 3-5A). Transient expression assays revealed that the TA insertion dramatically decreased the enhancer activity of the liver-type intron in HepG2 (human hepatoma) cells (Fig. 3-5B). Moreover, the enhancer activity of these two constructs was examined in two non-liver cell lines. Both the wild-type (WT) and the mutated (mS) constructs exhibited no significant transactivation activity in HeLa and NBRK cells, implying that the sequences responsible for skin-type intron activity might reside outside the +192 to +350 region. Together, these data suggested that the TA dinucleotide insertion in Element S, which destroyed the C/EBPα binding site, diminished the liver-specific enhancer activity of the skin-type intron. In comparison, the interaction between Element B and C/EBPα would confer the liver-specific enhancer activity of the liver-type AFP intron.

3.4 Discussion

Our study has demonstrated that the overall gene structures of the skin-type AFP and the liver-type AFP are similar. The major differences between the only intron of the skin-type AFP gene and the liver-type AFP gene include an insertion of a TA dinucleotide in the skin-type gene corresponding to Element B and the presence of a 241 bp large fragment in the skin-type AFP gene. Like the liver-type intron, the skin-type AFP intron also exhibits enhancer activity. However, its transactivation ability is ubiquitous, which is consistent with previous Northern analysis studies that the skin-type AFP mRNAs are widely distributed in many tissues including skin, gill, fin, and scales, as well as in liver, stomach, intestine, kidney and spleen (Gong et al, 1996). Therefore, it is postulated that the intron of the two respective wflAFP and wfsAFP genes plays an important role in regulating their differential tissue expression.

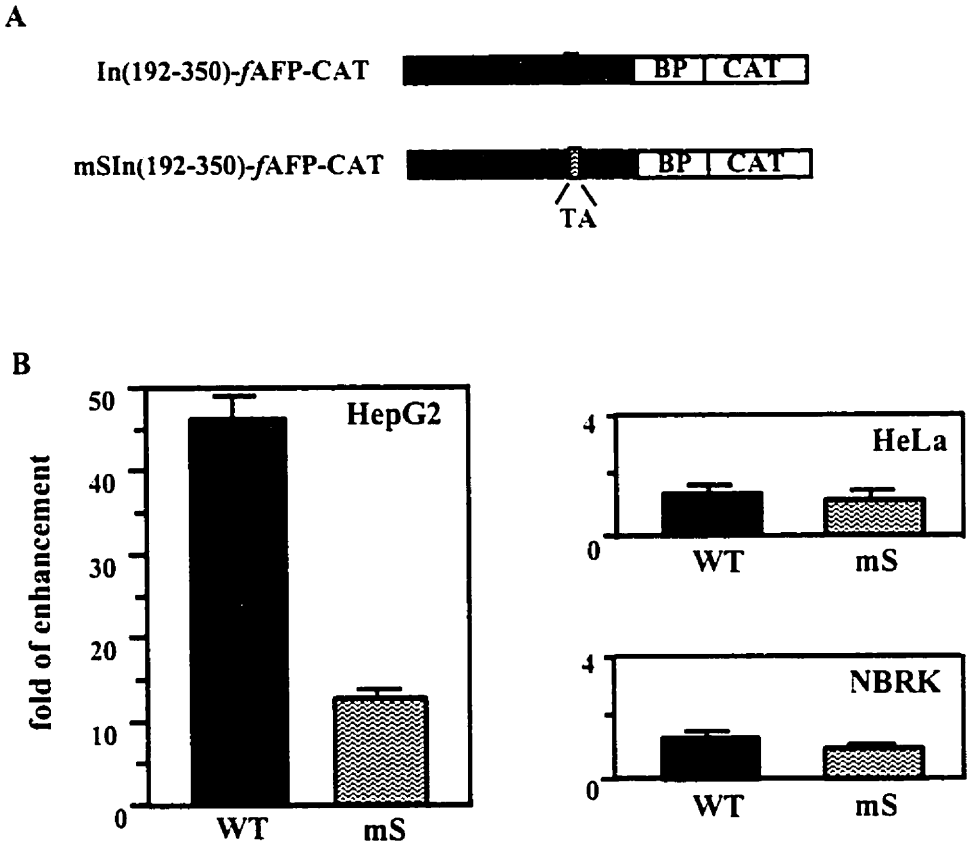


Fig. 3-5 TA dinucleotide insertion of Element B destroys its liver-specific enhancer activity. A: Schematic illustration of the In(192-350)-*f*AFP-CAT (WT) and mutated mSIn(192-350)-*f*AFP-CAT (mS) constructs. The constructs were transfected into HepG2, NBRK and HeLa cells. The enhancement relative to the activity of the *f*AFP-CAT construct is shown. The mean values and standard errors of three independent experiments are given.

In addition, the observation of a TA insertion in the skin-type intron was of particular interest because it is located within Element B which mediates the enhancer activity of the liver-type intron. This dinucleotide insertion effectively destroys the C/EBPα binding specificity, as demonstrated by mobility shift assays using both the rat liver nuclear extract and the C/EBPα fusion protein. In the transfection studies, the dinucleotide insertion in Element B of the liver-type intron substantially reduced its expression in HepG2 cells, further confirming the role of C/EBPα in liver-specific transactivation ability. Since C/EBPα is present primarily in terminally differentiated cells such as hepatocytes and adipocytes (Birkenmeier et al, 1989), the presence of the C/EBPα binding motif in Element B is believed to mediate the liver-specificity of the liver-type AFP intron. In contrast, the skin-type intron losses its interaction to C/EBPα in Element S and a broader tissue expression pattern is observed for the wfsAFPs. The disruption of C/EBP binding site has also been reported in the clotting factor IX promoter of haemophilia B patients that significantly reduced its transactivation activity (Crossley and Brownlee, 1990).

However, it should be pointed out that the additional 241 bp fragment in the skin-type intron might be important in its transcriptional regulation. It is possible that some other cis-acting sequences in this skin-type fragment interacts with transcription factor(s) to dictate the skin-type AFP expression. It is interesting to note that, similar to F2, another skin-type AFP genomic clone (11-3) shares common features including an additional 288 bp fragment which is 97% identical to the 241 bp fragment in the F2 gene and the presence of Element S in the corresponding region (Davies and Gauthier, 1992). Sequences similar to GRE and Oct-1 consensus binding sites were found in these additional fragments of the wfsAFP

introns. The role of these additional fragments and factors involved, if any, would require further investigation.

In addition to the distinct tissue distributions between the wflAFPs and wfsAFPs, their gene expressions are differentially regulated by season and hormone (Gong et al, 1995). There is a 500-700 fold of increase in the wflAFP mRNA level in winter compared to that in summer time. And the wflAFP mRNA elevation is inhibited by growth hormone. The wfsAFP mRNAs, however, only exhibit a 10 fold seasonal variation and are refractory to growth hormone treatment.

The presence of two distinct types of AFPs within a single species is intriguing. With the presumed intracellular localization, protection of internal organelles has been proposed to be one of the physiological roles of the wfsAFPs. In conjunction with the extracellular wflAFPs, they might act to protect cell membrane and maintain its stability which are especially important at peripheral tissues for protection from freezing (Low et al, 2000).

Chapter Four

Identification and characterization of the antifreeze enhancer-binding protein

Eur J Biochem (2000) 267:7237-46. Miao, M., Chan, S. L., Fletcher, G. L. and Hew, C. L.

*Results presented in this chapter were the authour's contributions to this publication.

4.1 Introduction

As described in the previous chapters, we have postulated the presence of the antifreeze enhancer-binding protein (AEP) that interacts specifically with the core enhancer Element B of the wflAFP gene. The nucleotides important for these interactions were identified by methylation interference assays (see 2.3.2). Also, in addition to C/EBPα, a protein with molecular mass of approximately 80 kDa was shown to interact specifically with Element B by UV crosslinking assay (see 2.3.5). The interaction between the liver-enriched C/EBPα and Element B is believed to contribute to the liver-specificity of the wflAFP genes. However, the nature and role of the presumptive AEP was unknown.

The present study was undertaken to investigate the identity of AEP. Due to the lack of appropriate fish cell lines expressing the AFP gene and limited information on fish transcription factors, we have earlier successfully utilized mammalian cell lines such as HepG2 to dissect the cis and trans factors controlling AFP gene expression. The rat ortholog of the AEP was obtained from a rat liver cDNA expression library screened by its binding to Element B. It is a novel helicase domain-containing protein homologous to Smubp-2/Rip-1 and exhibits a strong binding specificity to Element B. Its potential role in AFP gene regulation is proposed.

4.2 Experimental procedures

4.2.1 Library screening and RT-PCR

Upper and lower strands of Oligonucleotides (Element B: ATAATGTTTCATCAGCACTT, or Element S: ATAATGTTT**TA**CATCAGCACTT, only the upper strands are shown) were concatenated by phosphorylating the 5' termini of two complementary strands using T4

polynucleotide kinase (New England Biolabs), followed by annealing and ligation using T4 polynucleotide ligase (New England Biolabs). Then the concatenated oligonucleotides were ^{32}P-labeled using Nick Translation system (Life Technologies, Gaithersburg, MD) and used to screen a rat liver λgt11 cDNA expression library (Clontech, Palo Alto, CA) as described by Vinson et al (1988) with minor modification. Briefly, LB plates containing phage grown for 2.5 h at 42°C were overlaid with nitrocellulose filters that had been soaked with IPTG and air-dried. These plates overlaid with filters were subsequently incubated at 37°C for 4 h. The filters were lifted and air-dried for 15 min and blocked in Blotto buffer (5% milk, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA and 1 mM DTT) at room temperature for 1 h. After washing with Binding buffer (BB, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA and 1 mM DTT), the filters were incubated with ^{32}P-labeled, concatenated oligonucleotide (10^6 cpm/ml) and denatured calf thymus DNA (5 μg/ml) in BB for 1 h and washed twice with BB for 4-5 min. After air-drying, the filters were subjected to autoradiography. The *EcoR*I fragment of a positive clone after three screenings (λAEP1.6) was released from the phage arms and ligated into the *EcoR*I site on pBluescript (Stratagene, pBluescript-AEP1.6). Purified plasmid was subjected to DNA sequencing analysis using the T7 DNA sequencing kit (Amersham Pharmacia Biotech).

To obtain the full length cDNA sequence, RT-PCR was performed. Total RNA from various tissues of male Sprague-Dawley rats were isolated using TRIZOL™ reagent (Life Technologies). For the 5′ sequence, a degenerate AEP left primer, ATGGCCTT/CGTC/ACACCGTGGAGAGC/TTT, corresponding to the start site based on the alignment of the cDNA sequences of its homologs, and a AEP1.6 right primer#1, CTGAGCAGATCCACCTGAAGGTTG, were synthesized. And for the 3′ sequence, two rat EST

sequences (EST216459 Normalized rat lung and EST210210 Normalized rat brain) were found in the Non-redundant Database of GenBank non-mouse and non-human EST entries of NCBI by their homology with Rip-1. The two cDNAs are mostly overlapping with identical sequences and a rat EST right primer, ATGGCTAGCTGGCCATGCAAGGCA, was synthesized accordingly. An AEP1.6 left primer, CCATGTACCACGGACAGCTCACTG, was also synthesized for the 3′ sequence. Eight μg of total RNA from rat liver were incubated with the AEP1.6 right primer#1, or the rat EST right primer, together with 0.5 mM of each dNTP, 15 U RNAguard (Amersham Pharmacia Biotech), and 400 U M-MLV reverse transcriptase (Life Technologies) in 20 μl of 1x reverse transcriptase buffer (50 mM KCl, 3 mM $MgCl_2$, 0.1 mg/ml BSA, 10 mM DTT, 20 mM Tris-HCl, pH 8.4) at 37°C for 1 h. The PCR was carried out in 50 μl reaction volume containing 4 μl of reverse transcriptase reaction mixture, 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each of dNTP, 2 μl of DMSO and 5 U Pfu DNA polymerase (Stratagene). One μM each of the AEP1.6 right primer#1 and the degenerate AEP left primer, or the rat EST right primer and the AEP left primer, were also added into the AEP1.6 right primer#1 or the rat EST right primer reaction mixtures, respectively. The PCR reaction was started by denaturing DNA at 95°C for 1 min, followed by 35 cycles of 95°C, 30 sec and 68°C, 3 min, and a 68°C for 3 min to complete the reaction. Purified PCR products were ligated into the pT7Blue vector (Novagen, Madison, WI) to obtain the pT7-AEP-G#5 (5′ cDNA fragment) and pT7-AEP-D#2 (3′ cDNA fragment) constructs. Purified plasmids were sequenced by the Centre of Applied Genomics, Hospital for Sick Children, Toronto, Ontario.

4.2.2 Southwestern binding assay

A pMal-AEP1.6 construct was made by cloning the 1.6 kb *EcoR*I fragment from pBluescript-AEP1.6 into the *EcoR*I site of pMal-c2 (New England Biolabs). The plasmid was transformed into *E.coli* strain DH5α and the fusion protein was induced with IPTG at a final concentration of 0.3 mM. After lysozyme and sonication treatment, the presence of MBP-AEP1.6 in crude protein extracts was confirmed by Western blotting analysis using antibody against MBP (Santa Cruz Biotechnology). Bacteria expressed maltose-binding protein (MBP), MBP-AEP1.6 and MBP-Smubp-2 (pMal-c-Smubp-2 construct was kindly provided by Dr. T. Honjo, Kyoto University Faculty of Medicine, Kyoto, Japan) were spotted onto nitrocellulose filters and air-dried for 15 min. The filters were blocked in Blotto buffer at 4°C for 1 h and washed with BB. The filters were incubated with ^{32}P-labeled Element B (10^6 cpm/ml) and denatured calf thymus DNA (5 μg/ml) with or without cold competitor in BB for 2 h and washed three times in the same buffer for 5 min each. An oligonucleotide containing the C/EBP consensus sequence: CTAGGCATATTGCGCAATATGC, was used for control as a non-specific competitor (Osada et al, 1996). Filters were air-dried and subjected to autoradiography.

4.2.3 Gel retardation assay

Complementary single-stranded oligonucleotides (Element B, or Element BmA) were annealed, ^{32}P-labeled with T4 polynucleotide kinase (New England Biolabs) and used as probes. In a 10 μl binding mixture, crude protein extract was incubated with competitor DNA and 100 ng of poly dI-dC for 10 min. Then the probe (1.6×10^4 cpm) was added and incubated for another 20 min on ice. Cold competitors used were as follows:

Element B: ATAATGTTTCATCAGCACTT

Element S: ATAATGTTT**TA**CATCAGCACTT

Element BmA: ATAA**TT**TTTCATCAGCACTT

AP1 consensus: CGCTTGATGACTCAGCCGGA (Hai & Curran, 1991)

C/EBP consensus: CTAGGCATATTGCGCAATATGC (Osada et al, 1996)

HNF3 consensus: CCTGATTCTGATTATTGACTTAGTCA (Costa et al., 1989)

Free DNA and DNA-protein complexes were resolved on 4% polyacrylamide gel.

4.2.4 Methylation interference

Single-stranded oligonucleotides (Element B of the upper or lower strand) were ^{32}P-labeled with T4 polynucleotide kinase (New England Biolabs) before annealing with 2x unlabeled complementary oligonucleotides. The 5′ end-labeled double-stranded oligonucleotide probes were partially methylated with dimethyl sulfate (Maxam and Gilbert, 1980), and used for preparative gel retardation assays with the MBP-AEP1.6 protein extracts. Protein bound and free probes were extracted from the polyacrylamide gel, recovered by filtration onto a NA-45 DEAE membrane (Schleicher & Schuell, Keene, NH) and treated with 1 M piperidine at 90°C for 30 min. The cleaved probes were lyophilized, separated onto a 15% polyacrylamide-8M urea gel and subjected to autoradiography.

4.2.5 Cell culture and transient expression assay

HepG2 cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum. To make an AEP expression vector, a PCR fragment was obtained using AEP-*BamH*Istart left primer, ATGGATCCATGGCCTCGTACACCGTGGAGAGTTT, and

AEP-*Avr*II right primer, AGCCTAGGGGCTTCTCACTTGGCTT, then digested with *BamH*I and *Avr*II. This fragment was ligated into the *BamH*I and *EcoR*I digested pcDNA3 vector (Invitrogen, San Diego, CA) together with the *Avr*II and *EcoR*I digested fragment from pT7-AEP-D#2 to make the pcDNA3-AEP construct. DNA was transfected into cells by the calcium phosphate precipitation method and cells were glycerol shocked after 4 h and harvested 48 h after transfection. A β-galactosidase gene under the control of SV40 early promoter was used as internal control. CAT assays were performed by the method of Eastman (1987) and Morency et al (1987), and the β-galactosidase activity was determined by the method of Hall et al (1983). All transfection experiments were repeated at least three times, and for each construct at least two independent plasmid preparations were tested. The fold enhancement relative to the activity achieved with the *f*AFP-CAT construct is shown. The TK promoter linked to pBLCAT3 was used as positive control and the *f*AFP-CAT activity was approximately 5% of TK-CAT.

4.2.6 Northern analysis

Thirty μg of total RNA from rat liver and skin were separated in a denaturing 1% agarose gel and transferred to Hybond™-N (Amersham Pharmacia Biotech) nylon membrane. After UV crosslinking, the membrane was prehybridized in 50% formamide, 1 M NaCl, 10% dextran sulfate, 1% SDS and 200 μg/ml denatured calf thymus DNA for 3 h. The AEP cDNA probe (10^6 cpm/ml), a ^{32}P-labeled *Hinc*II/*Pst*I fragment from the pBluescript-AEP1.6 construct, was added into the prehybridization bag and incubated at 42°C overnight. The membrane was washed twice in 1x SSC, 1% SDS at room temperature for 5 min, twice at 42°C for 15

min, and in 0.5x SSC, 1% SDS at 50°C for 15 min, at 60°C for 15 min, and was subjected to autoradiography.

4.2.7 RT-PCR and Southern analysis

For reverse transcription, 4 μg of total RNA from various tissues was combined with a AEP1.6 right primer#2, CTGAGCAGATCCACCTGAAGGTTG, dNTP, RNAguard, and M-MLV reverse transcriptase in 20 μl of 1x reverse transcriptase buffer as described above and incubated at 37°C for 1 h. PCR was performed as described above with 6 μl of reverse transcriptase reaction mixture, the AEP1.6 right primer#2 and the AEP1.6 left primer without DMSO. The reaction was started by denaturing DNA at 94°C for 10 min, followed by 30 cycles of annealing (70°C, 1.5 min), extension (72°C, 2 min) and denaturation (94°C, 30 sec), then 72°C for 10 min to complete the reaction. Electrophoresed PCR products were transferred to Hybond™-N nylon membrane (Amersham Pharmacia Biotech) and the filter was prehybridized for 2 h and hybridized with the ^{32}P-labeled AEP cDNA probe (10^6 cpm/ml) in 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulfate and 200 μg/ml of denatured calf thymus DNA at 42°C for 18 h. The blots were washed with 2x SSC at room temperature for 15 min, 2x SSC and 1% SDS at 65°C for 30 min, and 0.2x SSC and 0.5% SDS at 65°C for 30 min, then subjected to autoradiography.

4.2.8 Genomic Southern analysis

Genomic DNA was isolated from winter flounder testis by the method Blin and Stafford (1976). DNA was digested with restriction enzymes and separated by electrophoresis on a 0.7% agarose gel. The gel was soaked in 0.4 M NaOH, 0.6 M NaCl for 30 min and blotted

onto Hybond™-N membrane (Amersham Pharmacia Biotech). Prehybridization and hybridization procedures were performed as described in 4.2.7. The membrane was subjected to autoradiography for 72 h.

4.3 Results

4.3.1 Isolation of a partial cDNA clone that binds to Element B

The presence of the presumptive AEP and its interaction with the core enhancer Element B has been demonstrated in both mammalian cell lines and flounder hepatocytes (see 2.3.1). A screening procedure was carried out using a rat cDNA expression library to identify the presumptive AEP. Using concatenated, double-stranded Element B as probe, approximately 380,000 plaques from a rat liver λgt11 cDNA expression library were screened. There were 14 positive clones after the first screening, however, only one clone (λAEP1.6) was positive after the second screening. In addition to Element B, Element S (Element B with a TA insertion, known to interact with AEP but not C/EBPα) (see 3.3.3) was used as probe on the third screening. As expected, the clone interacted with Element B. Furthermore, its binding to Element S suggested that it did not encode C/EBPα (Fig. 4-1). Sequence data of the λAEP1.6 clone revealed that it is 1644 bp in length. It encodes a partial open reading frame of 548 amino acid residues without the initiation and stop codons, suggesting that it was not a complete cDNA clone. Sequence search using BLAST from NCBI revealed that it is a novel protein with high level of sequence identity to hamster Rip-1, human and mouse Smubp-2 and a truncated human Smubp-2, GF-1 (Shieh et al, 1995; Kerr and Khalili, 1991; Fukita et al, 1993; Mizuta et al, 1993).

Element B

ATAATGTTTCATCAGCACTT

Element S

ATAATGTTTTACATCAGCACTT

Fig. 4-1 Library screening of the rat AEP clone using Element B and S as probes. Screening of the rat liver λgt11 library. Binding of Element B and S to the λAEP1.6 clone at the third screening. Individual plates and filters were used for Element B and S as probes, and 100% of plaques were bound to Element B or S, respectively.

4.3.2 Cloning of the full AEP open reading frame sequence

To obtain the complete AEP open reading frame, RT-PCR was employed. Two PCR products approximately 1.6 and 1.9 kb in length were obtained. These PCR fragments overlapped with the AEP1.6 cDNA clone and together provided a complete open reading frame and 3'-untranslated sequence of 3266 bp in total (Fig. 4-2). The deduced polypeptide sequence of rat AEP is 988 amino acid residues in length with seven putative helicase motifs (Fig. 4-3). It shares 91.5%, 89.1 % and 76.1% sequence identity with its counterparts in mouse, hamster and human, respectively, and the putative helicase domains are characteristic to this family of proteins (Fig. 4-4). Most of the N-terminus of the protein (two-thirds of the sequence, including helicase motifs I-IV) is highly conserved among species. The DNA binding domain (DBD), as mapped in human Smubp-2 (Fukita et al, 1993) and containing helicase motifs V and VI, however, is less conserved. The nuclear localization signal (NLS) is located C-terminus to the DBD and the region between DBD and NLS is the most variable segment of the polypeptide chain.

4.3.3 The AEP forms a specific complex with Element B

The rat AEP1.6 cDNA clone (amino acid residues 314-861) was found to contain the DBD based on its homology with Smubp-2 (amino acid residues 638-786) (Fukita et al, 1993). To confirm its specific binding ability to Element B, a fusion protein (MBP-AEP1.6) containing the maltose binding protein and AEP1.6 was produced in bacteria. Southwestern analysis was carried out using crude cell extracts from bacteria expressing MBP-AEP1.6 as well as MBP- human Smubp-2 and MBP (negative control) (Fig. 4-5A). The MBP-AEP1.6 bound strongly to Element B while weaker binding was observed with MBP-Smubp-2 fusion

ATGGCCTCGTACACCGTGGAGAGTTTTGTGGCCCAGCAGCTACAGCTGTTGGAGCTAGAGCGGGACGCCGAGGTGGAGGA 80

Δ AEP left primer

GCGCAGGTCCTGGCAGGAACACAGTTCTCTGAAAGAGCTTCAGAGCCGAGGGGTGTGTTTGCTGAAGCTTCAGGTATCGG 160

GCCAGCGCACCGGGTTGTATGGACAGCGGCTGGTCACCTTTGAGCCCAGGAAGTTTGGGCCTGCAGTGGTGCTTCCCAGC 240

AACAGCTTCACCTCTGGTGATATCGTGGGTCTGTATGATACTAATGAAAGCAGCCAACTGGCCACTGGGGTCCTGACCCG 320

CATCACCCAAAAATCGGTCATAGTGGCCTTTGATGAGTCCCATGATTTCCAGTTGAACCTGGACCGAGAAAATACCTACA 400

GACTGCTGAAGCTTGCCAATGACGTCACCTACAAACGCCTGAAAAAAGCTCTGCTGACACTGAAGAAGTACCATTCTGGG 480

CCAGCATCCTCGCTCATTGATGTCCTGTTGGGTGGCTCCACCCCCAGTCCTGCCACTGAAATACCCCCGCTCACTTTCTA 560

CAACACGACCCTGGACCCTTCCCAGAAAGAAGCTGTGTCTTTTGCGCTGGCGCAGAAAGAAGTTGCCATCATCCATGGGC 640

CTCCTGGCACTGGGAAAACCACAACTGTGGTGGAAATAATCCTTCAAGCTGTGAAGCAAGGCTTAAAGGTTCTATGCTGT 720

GCTCCCTCCAACATCGCTGTGGACAACCTGGTGGAGCGTCTGGCTCTGTGCAAGAAGCAGATTCTTCGCCTGGGTCACCC 800

CGCCCGCCTCCTGGAGTCTGTTCAGCAGCACTCACTGGACGCAGTGCTAGCACGCAGTGACAATGCCCAGATTGTTGCTG 880

ACATCAGAAGGGACATTGACCAGGTCTTTGGCAAGAACAAAAAGACCCAAGATAAGAGAGAAAAAAGTAATTTTCGAAAT 960

GAAATTAAGCTGCTAAGGAAGGAACTGAAGGAAAGGGAAGAAGCAGCCATAGTTCAGAGCCTCAGTGCAGCAGATGTGGT 1040

TCTAGCCACCAACACAGGTGCATCTACTGATGGCCCCCTGAAGCTGCTGCCTGAGGACTACTTTGATGTGGTGGTGGTGG 1120

ACGAGTGCGCCCAGGCCCTAGAAGCCAGCTGCTGGATTCCCCTGCTGAAGGCCCCTAAGTGCATCCTAGCTGGAGACCAC 1200

AAACAGCTGCCACCCACCACTGTCTCTCACAAGGCAGCACTGGCTGGGCTGTCCCGCAGCCTGATGGAGCGTCTGGCAGA 1280

GAAGCATGGTGCTGCTGTGGTAAGGATGCTGGCGGTCCAGTACCGAATGCACCAGGCCATCACGCGCTGGGCCTCGGAAG 1360

CCATGTACCACGGACAGCTCACTGCCCATCCCTCTGTGGCAGGACACCTTCTGAAGGACCTCCCAGGTGTGGCTGACACA 1440

AEP1.6 left primer

GAGGAGACGAGTGTCCCTCTGCTGCTCATAGACACAGCTGGCTGCGGGCTGCTGGAGCTGGAGGAGGAAGACAGCCAGTC 1520

CAAGGGAAACCCCGGTGAAGTTCGCCTCGTCACTTTGCACATCCAGGCTCTGGTGGATGCTGGGGTCCAGGCTGGTGACA 1600

TTGCCGTCATCGCACCCTACAACCTTCAGGTGGATCTGCTCAGACAGAGCCTCTCTAACAAGCACCCTGAGCTGGAGATC 1680

AEP1.6 right primer#1

AAGTCTGTTGACGGCTTTCAAGGCCGAGAGAAGGAGGCTGTGATCCTGACCTTTGTCAGGTCCAACAGGAAAGGTGAAGT 1760

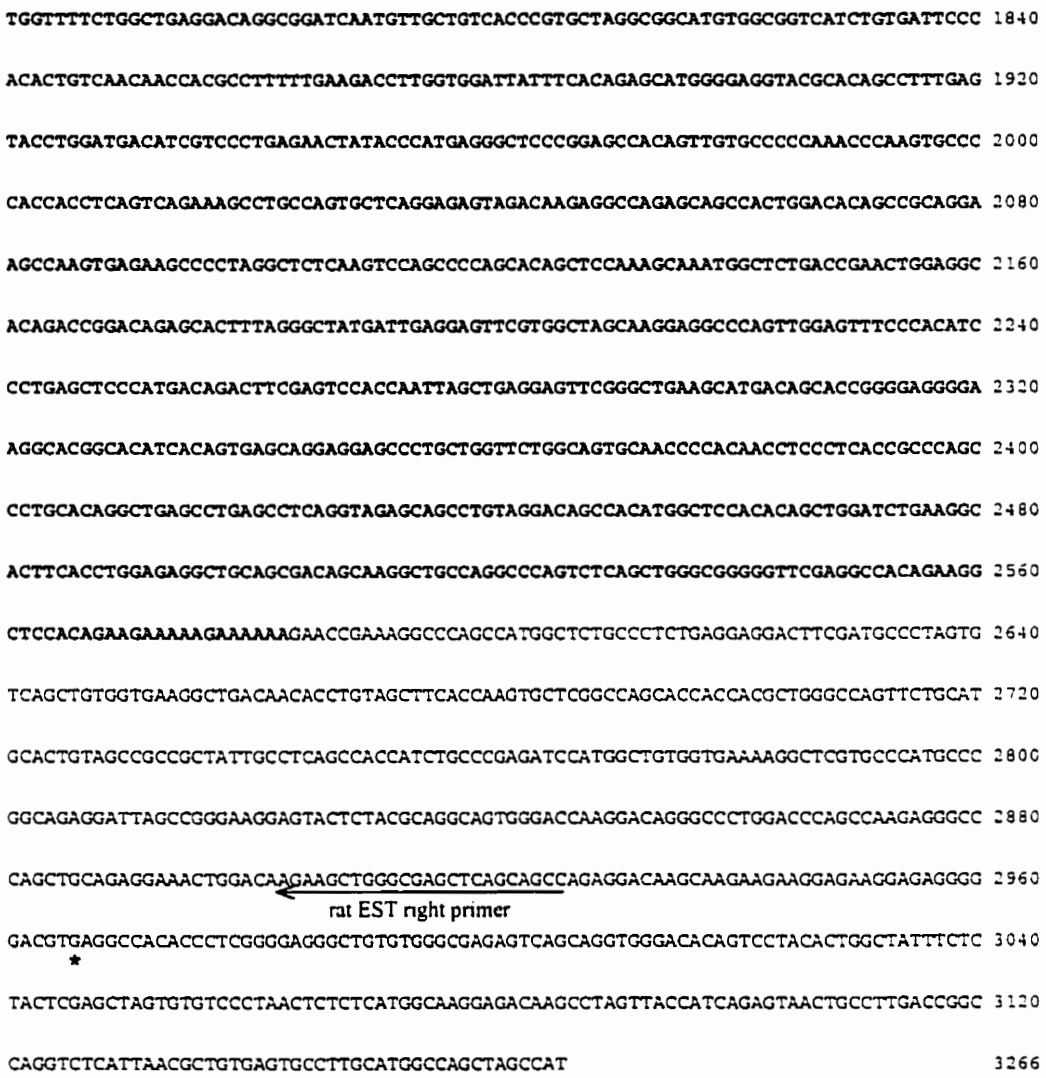


Fig. 4-2 cDNA sequence of the rat ortholog of the presumptive AEP. The sequence obtained from the λAEP1.6 clone is in bold. Primers used in RT-PCR to obtain the full ORF and the 3′-untranslated region are underlined. The start and stop codons for the ORF are indicated by triangle and asterisk, respectively. The GenBank accession number for the rat AEP is AF199411.

A

```
MASYTVESFVAQQLQLLELERDAEVEERRSWQEHSSLKELQSRGVCLLKLQVSGQRTGLY   60

GQRLVTFEPRKFGPAVVLPSNSFTSGDIVGLYDTNESSQLATGVLTRITQKSVIVAFDES  120

HDFQLNLDRENTYRLLKLANDVTYKRLKKALLTLKKYHSGPASSLIDVLLGGSTPSPATE  180

IPPLTFYNTTLDPSQKEAVSFALAQKEVAIIHGPPGTGKTTTVVEIILQAVKQGLKVLCC  240
                                I

APSNIAVDNLVERLALCKKQILRLGHPARLLESVQQHSLDAVLARSDNAQIVADIRRDID  300
  Ia

QVFGKNKKTQDKREKSNFRNEIKLLRKELKEREEAAIVQSLSAADVVPATNTGASTDGPL  360

KLLPEDYFDVVVVDECAQALEASCWIPLLKAPKCILAGDHKQLPPTTVSHKAALAGLSRS  420
            II                          III

LMERLAEKHGAAVVRMLAVQYRMHQAITRWASEAMYHGQLTAHPSVAGHLLKDLPGVADT  480
                    IV

EETSVPLLLIDTAGCGLLELEEEDSQSKGNPGEVRLVTLHIQALVDAGVQAGDIAVIAPY  540

NLQVDLLRQSLSNKHPELEIKSVDGFQGREKEAVILTFVRSNRKGEVGFLAEDRRINVAV  600

TRARRHVAVICDSHTVNNHAFLKTLVDYFTEHGEVRTAFEYLDDIVPENYTHEGSRSHSC  660
                                                          V

APKPKCPTTSVRKPASAQESRQEARAATGHSRRKPSEKPLGSQVQPQHSSKANGSDRTGG  720
                      VI

TDRTEHFRAMIEEFVASKEAQLEFPTSLSSHDRLRVHQLAEEFGLKHDSTGEGKARHITV  780

SRRSPAGSGSATPQPPSPPSPAQAEPEPQVEQPVGQPHGSTQLDLKALHLERLQRQQGCQ  840

AQSQLGGGSRPQKAPQKKKKKEPKGPAMALPSEEDFDALVSAVVKADNTCSFTKCSASTT  900
                    NLS

TLGQFCMHCSRRYCLSHHLPEIHGCGEKARAHARQRISREGVLYAGSGTKDRALDPAKRA  960

QLQRKLDKKLGELSSQRTSKKKEKERGT*                                  988
```

B

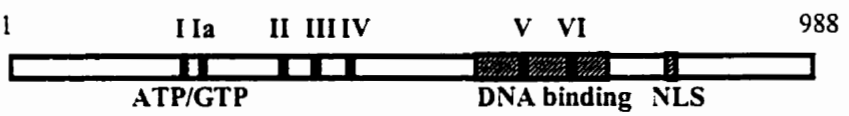


Fig. 4-3 The presumptive antifreeze enhancer-binding protein (AEP). A: The amino acid sequence of the rat AEP. NLS, nuclear localization signal. B: Schematic representation of the rat AEP. The DNA binding domain and the NLS are shown as striped boxes. Locations of the seven putative helicase motifs are defined by their homologous sequences to the motifs in mouse Smubp-2 (Mizuta et al, 1993).

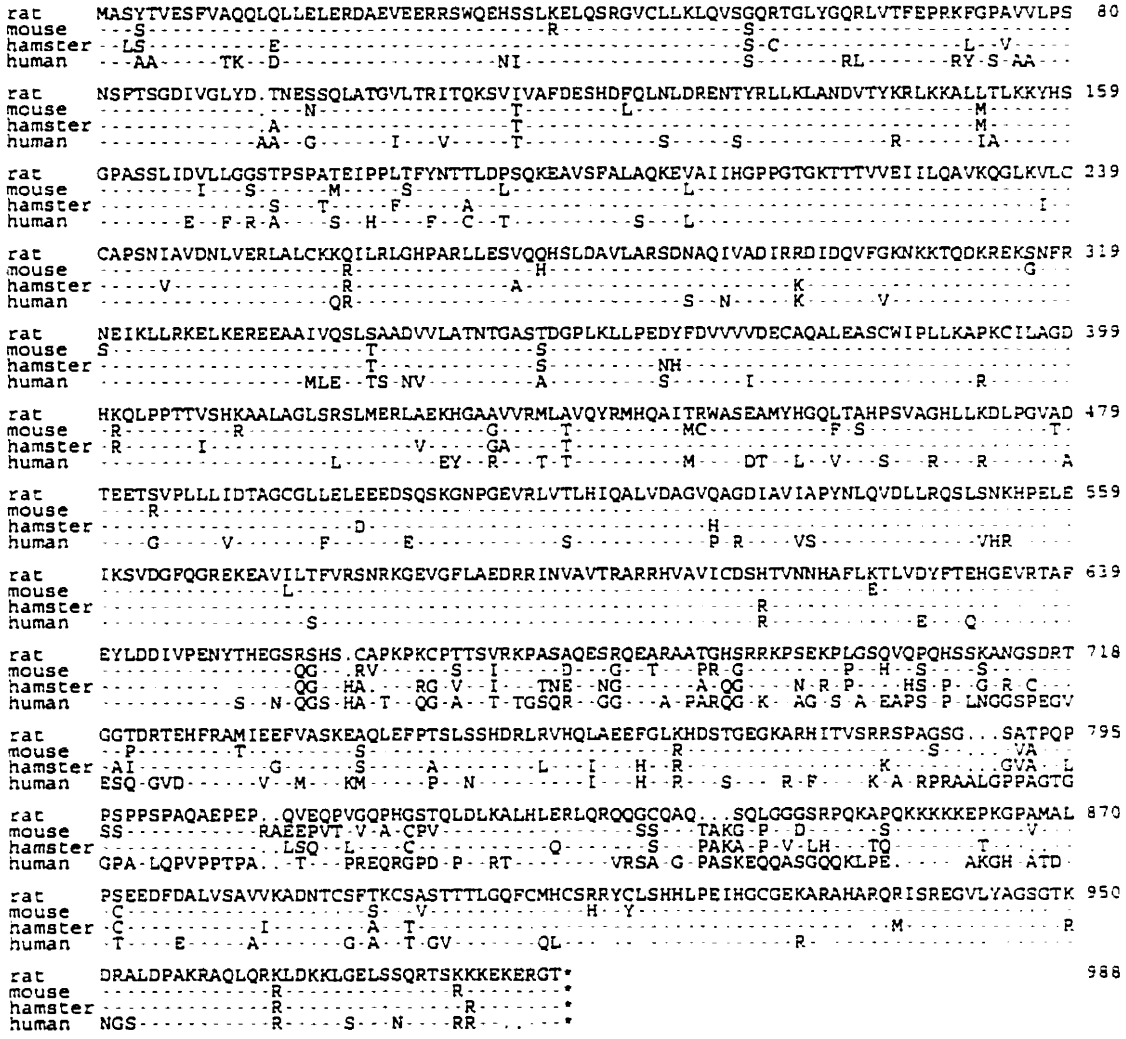


Fig. 4-4 Comparison of the rat AEP with the homologous mouse Smubp-2, hamster Rip-1 and human Smubp-2. Identical amino acid residues are indicated as dashed lines. To maximize the alignment, gaps were introduced shown by dots.

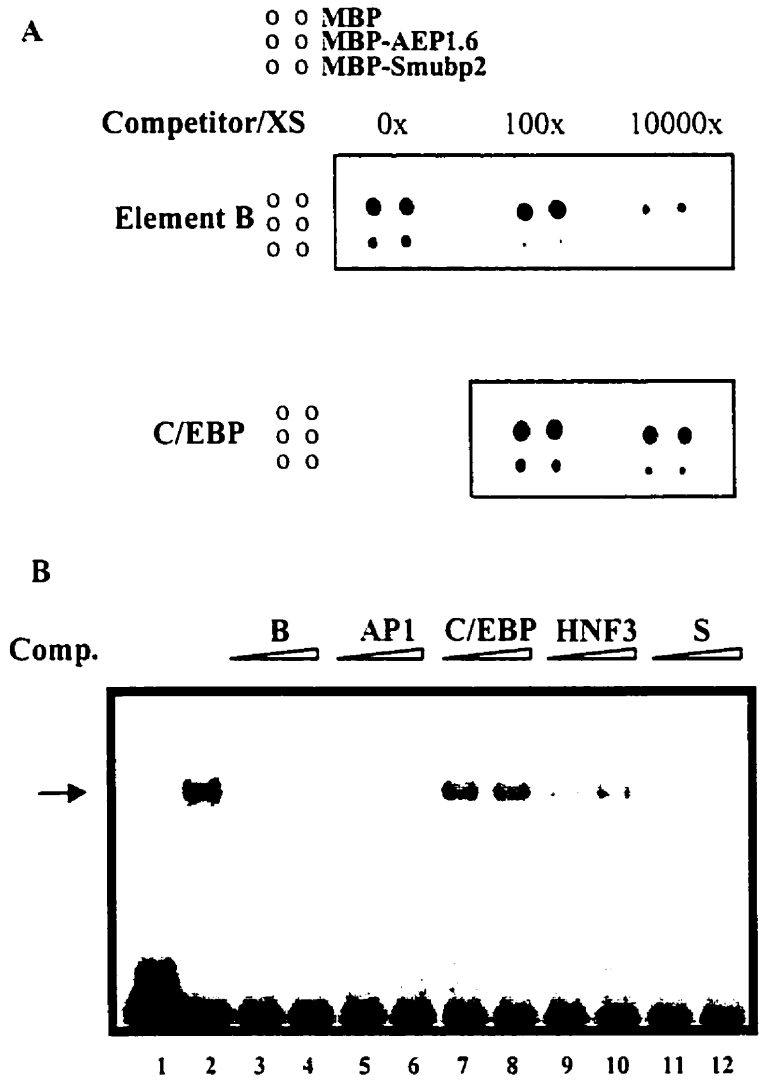


Fig. 4-5 Element B interacts specifically with MBP-AEP1.6 fusion protein. A: Southwestern analysis. End-labeled Element B was used as probe. Ten μg of MBP, MBP-AEP1.6 or MBP-Smubp-2 crude extracts were loaded onto a nitrocellulose filter. The arrangement of those dots is illustrated by circles. Non-labeled Element B and C/EBP consensus oligonucleotides with indicated excess amounts were used as competitors. B: Gel retardation assay. MBP-AEP1.6 protein extracts and end-labeled Element B were used. 100x and 200x molar excess of oligonucleotides were used as competitors. Arrow indicates the specific complex formation.

protein. MBP alone did not interact with Element B. Moreover, increasing molar excess of unlabeled Element B was able to compete the binding between MBP-AEP1.6 and Element B, while the oligonucleotide containing C/EBP consensus was ineffective.

Complex formation between MBP-AEP1.6 fusion protein and Element B was also demonstrated by gel retardation assays (Fig. 4-5B, lane 2). Molar excess of unlabeled Element B, AP1 consensus and Element S were able to compete out the complex (Fig. 4-5, lane 3 to 6, 11 and 12), while excess of C/EBP and HNF3 consensus were relatively ineffective (lane 7 to 10). These results demonstrated that AEP1.6 consists of a DNA binding domain that can interact specifically with Element B.

To further investigate its binding specificity, methylation interference was carried out to map the nucleotides important to complex formation in Element B (Fig. 4-6). These results indicated that the G residues at position +308 of the upper strand and position +312 of the lower strand of the intron enhancer Element B were involved in its interaction to AEP. Previous methylation interference data has demonstrated that G residues +308 and +312 were crucial for binding between Element B and the presumptive AEP in rat liver nuclear extract (see 2.3.2). Together, these results confirmed the identity of the rat ortholog of the antifreeze enhancer-binding protein (AEP) and its specific interaction with the enhancer Element B of the wflAFP gene.

4.3.4 The AEP is essential for the transactivation activity of wflAFP enhancer

To study the role of AEP in the wflAFP gene expression, transient expression assays were conducted in HepG2 cells. The wild-type (WT) enhancer of the wflAFP gene showed strong transactivation activity, while overexpression of AEP, however, did not increase the activity

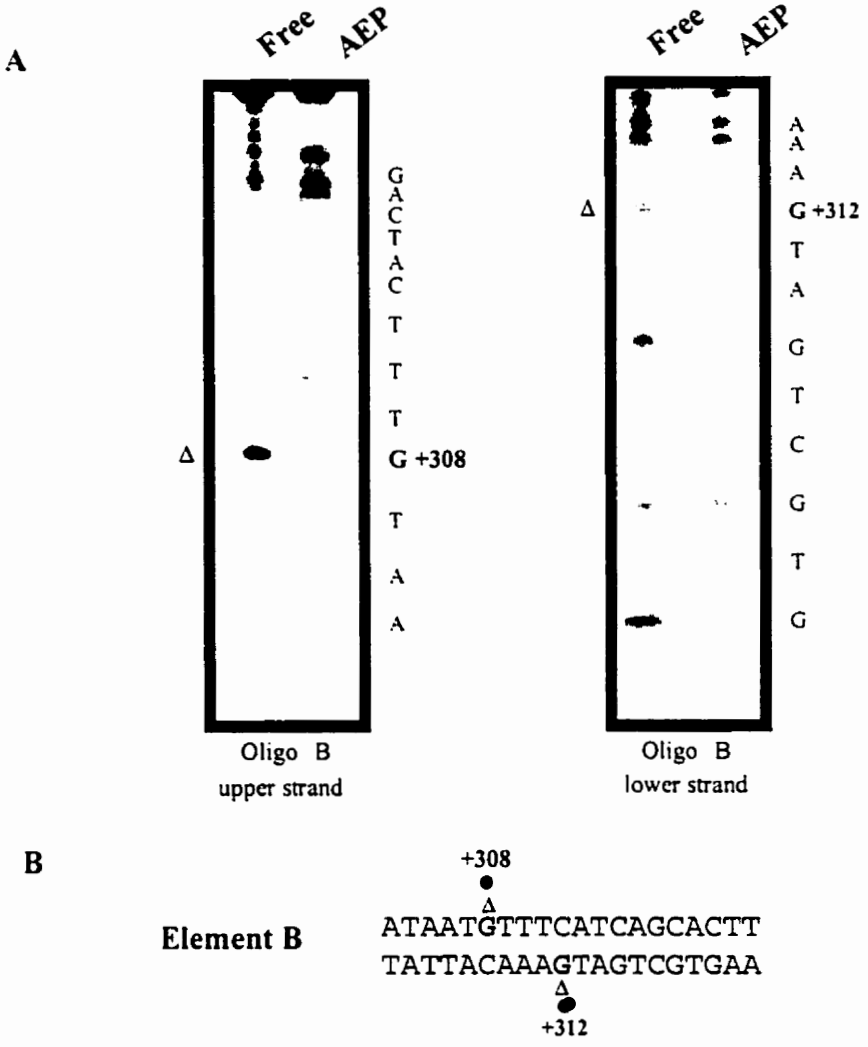


Fig. 4-6 Specific nucleotide residues in Element B are important to its interaction with MBP-AEP1.6 protein. A: End-labeled upper or lower strand Element B were partially methylated and used as probe in the methylation interference assay with MBP-AEP1.6. Free and shifted band (AEP) from the preparative gel shift assay were recovered and analyzed on a polyacrylamide gel. Methylated residues interfering with the Element B and protein binding are indicated by triangles. B: Methylated nucleotide residues interfered the binding of Element B to MBP-AEP1.6 protein and the presumptive AEP complex in rat liver nuclear extracts are indicated by triangles and dots, respectively.

to any significant extent (Fig. 4-7A). The AEP levels may be saturated in these cells since there is endogenous AEP expression in HepG2 cells (Chen et al, 1997). The level of AEP in these transfected cells should be verified when antibody against rat AEP becomes available. Also, no significant effect was observed when AEP was co-transfected with C/EBPα (data not shown). In contrast, a single point mutation at position +308 (mA:G->T) of the wflAFP enhancer dramatically decreased the activation ability of the enhancer. The position at +308 was shown to be an important residue for the DNA-protein interaction between AEP and the enhancer Element B by methylation interference assay. Element B with a mutation at the corresponding position (Element BmA) was unable to compete out the specific binding between AEP and Element B (Fig.4-7B, lane 2-4), while AEP did not interact with Element BmA when the latter was used as probe in the gel retardation assays (Fig. 4-7B, lane 6). A single mutation in Element B of the wflAFP gene enhancer was therefore able to interrupt the specific interaction between Element B and AEP, and destroyed the transactivation activity of the enhancer. Together, these data suggested that the interaction between AEP and Element B is required for the activation of the wflAFP gene enhancer in HepG2 cells.

4.3.5 The AEP is ubiquitously expressed and its gene is present in flounder

To investigate the expression of AEP in rat, Northern analysis was performed (Fig. 4-8A). A major transcript of approximately 3.7 kb was found in liver and skin which is comparable with its homologs in hamster (3.7 kb), mouse (3.7 kb) and human (4.3 kb) (Shieh et al, 1995; Mizuta et al, 1993; Mohan et al, 1998). Moreover, AEP was ubiquitously expressed in all the tissues that were examined including liver and skin as demonstrated by RT-PCR (Fig. 4-8B). This result also agrees with the earlier findings that virtually all the cell lines and tissues

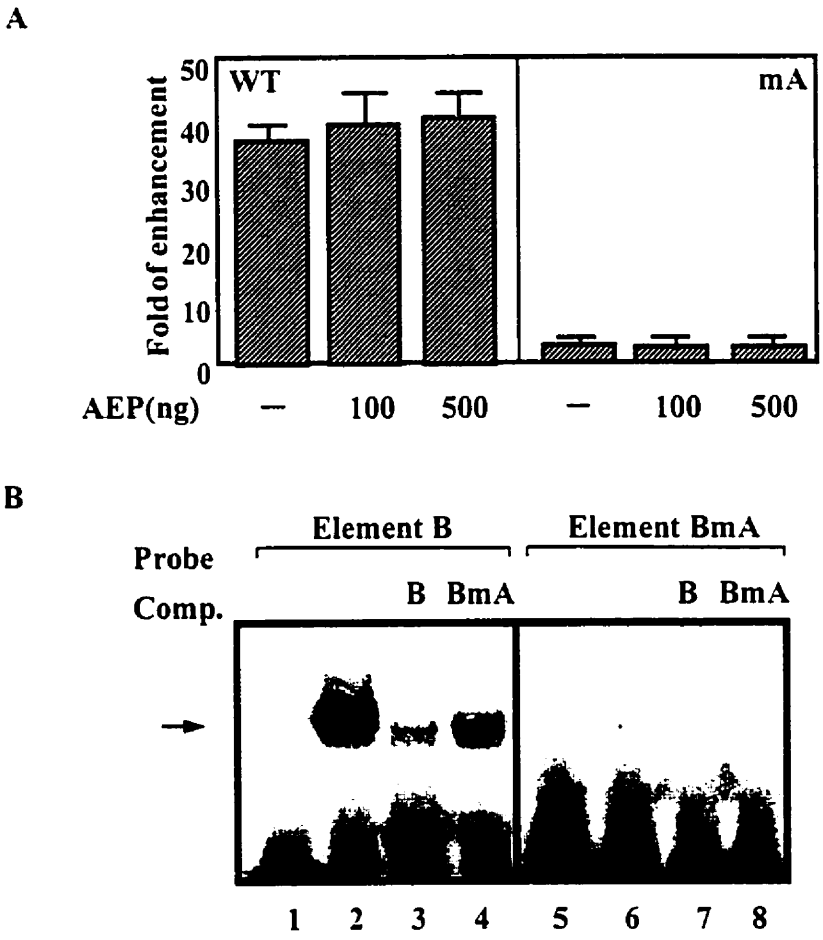


Fig. 4-7 Binding of AEP to Element B is required for the transactivation activity of wflAFP enhancer. A: Transient expression assays. pcDNA3-AEP was cotransfected with In(192-350)-*f*AFP-CAT (WT) or mutated mAIn(192-350)-*f*AFP-CAT (mA) into HepG2 cells. The amounts of DNA (pcDNA3-AEP) used in cotransfection are indicated. The fold of enhancement relative to the activity achieved with the *f*AFP-CAT construct is shown. The mean values and standard errors of three independent experiments are given. B: Gel retardation assay was carried using MBP-AEP1.6 protein extracts (lane 2-4 and 6-8). End-labeled Element B or Element BmA were used as probes and 100x molar excess of oligonucleotides were used as competitors as indicated. Arrow indicates the specific complex formation.

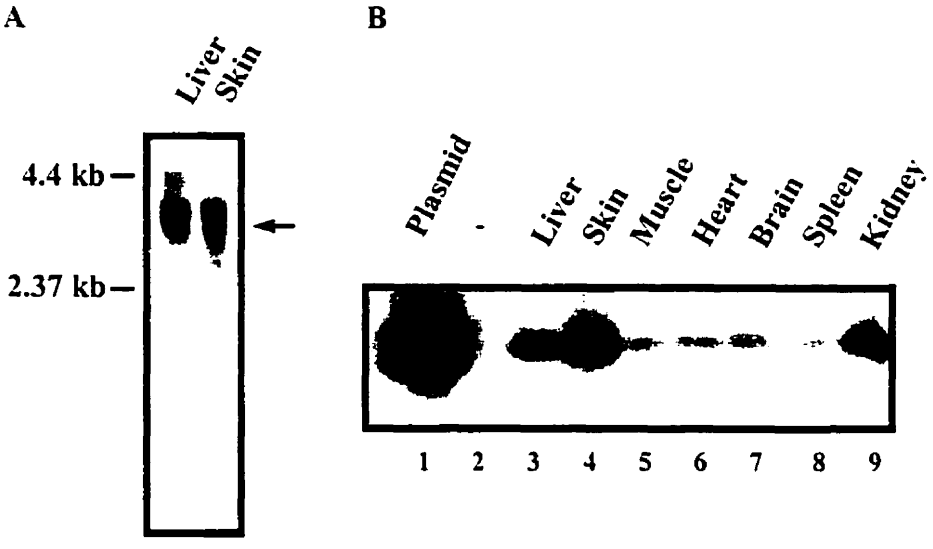


Fig. 4-8 The AEP is ubiquitously expressed in the rat. A: Northern analysis was performed using 30 μg of total RNA from rat liver and skin. B: RT-PCR was carried out using total RNA from various rat tissues as indicated. The 651 bp *Hinc*II/*Pst*I fragment of rat AEP cDNA was used as probe.

examined expressing these proteins (Fukita et al, 1993; Mizuta et al, 1993; Shieh et al, 1995; Mohan et al, 1998).

Lastly, the presence of the AEP gene family in the winter flounder was examined. Genomic Southern analysis was carried out using the rat AEP cDNA as probe (Fig. 4-9). Unique DNA fragments upon enzyme cleavage were hybridized with rat AEP sequence, suggesting the existence of AEP gene in flounder. Its simple banding pattern indicates that it may be a single copy gene. This is also supported by the chromosomal mapping data that human Smubp-2 is localized to a single locus on chromosome 11 at position q13.2-q13.4 and rat Catf1 (cardiac transcription factor-1) is localized to mouse chromosome 19 (Fukita et al, 1993; Sebastiani et al, 1995). Catf1 was identified by its interaction with the enhancer of the artrial natriuretic factor gene and is also homologous to Smubp-2, whether it is identical to rat AEP is unknown. In addition, it has been demonstrated that this family of genes is present in various vertebrates including chicken and salmon, but not yeast and Drosophila (Mizuta et al, 1993).

4.4 Discussion

In this study, we have identified the rat ortholog of the presumptive flounder antifreeze enhancer-binding protein (AEP) as a helicase domain-containing factor. It exhibits specific binding ability to the core wflAFP gene enhancer. The protein is ubiquitously expressed but plays an role in the liver-specific enhancer activity of the wflAFP gene in conjunction with C/EBPα and possibly other proteins.

The AEP/Smubp-2/Rip-1 have been found to interact with DNA sequences including the single-stranded human and mouse immunoglobin μ chain switch region (Fukita et al, 1993;

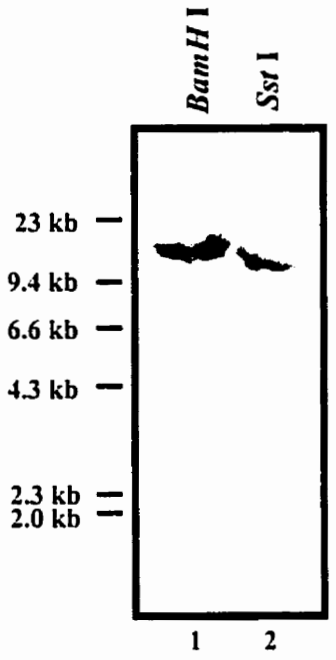


Fig. 4-9 The homolog of rat AEP gene is present in winter flounder. Genomic DNA (20 μg each lane) from flounder testis was digested with restriction enzymes as indicated. The 651 bp *Hinc*II/*Pst*I fragment of rat AEP cDNA was used as probe.

Mizuta et al, 1993), rat insulin II promoter and atrial natriuretic factor gene enhancer (Shieh et al, 1995; Sebastiani et al, 1995), and human Apo-1 promoter, neurotropic virus JCV and Epstein-Barr virus lytic switch promoter (Kerr and Khalili, 1991; Mohan et al, 1998; Zhang et al, 1999). Together with the flounder AFP enhancer sequence reported here, no apparent sequence similarity between their binding elements could be identified. It was suggested that they may recognize the structure or conformation rather than the sequence of DNA (Shieh et al, 1995). Alternatively, different DNA binding domains may exist. The DNA binding domain of human Smubp-2 to the immunoglobin μ chain switch region was mapped to amino acid residues 638 to 786 (Fukita et al, 1993). Similarly, the rat AEP1.6 clone, which contains amino acids 314 to 861 and has specific binding to wflAFP enhancer, overlaps with the DNA binding domain of human Smubp-2. These data indicate that at least one of the DNA binding domains resides in this region, although sequences in this region share less identity among these proteins. More detailed mapping to the DNA binding domain(s) and its preferences for different DNA elements is required to understand the structural/functional relationship between DNA and AEP/Smubp-2/Rip-1. The conservation of AEP/Smubp-2/Rip-1 in vertebrates from human to fish, as well as its wide tissue distribution indicates that it may hold significant functional importance. A mutation of Smubp-2 was identified in *nmd* mouse that causes neuromuscular degeneration (Cox et al, 1998). The primary structure of AEP/Smubp-2/Rip-1 indicates potential helicase and ATPase activity, although neither of these activities has been demonstrated. It is known that DNA helicases can utilize nucleotide triphosphate hydrolysis as energy to break hydrogen bonds between base pairs and to unwind duplex DNA. Factors with helicase activity and/or helicase motifs have been found to regulate transcription including XPB and XPD of the TFIIH and SW12/SNF2 of the

chromatin remodeling complex SWI/SNF complex (see 1.2.5 and Cairns et al, 1994; Drapkin et al, 1994; Peterson et al, 1994). It has been proposed that helicase domain-containing proteins facilitate transcription by altering chromatin structure, thus increasing the accessibility of other transcription factors to the basic transcription apparatus (Eisen and Lucchesi, 1998). The flounder AFP genes are arranged in tandem repeats or with multiple linked copies (Scott et al, 1985). The AEP might function to alter these well-packed chromatin structures, in order to promote the accessibility for other transcription factors such as C/EBPα to DNA, and to achieve rapid and efficient gene expression.

The predicted molecular weight of the rat AEP is approximately 110 kDa, while our previously UV crosslinking data showed that the presumptive AEP was approximately 80 kDa (see 2.3.5). It is possible that there was some error in estimating the size of DNA-bound protein in the gel. Alternatively, full-length form (Smubp-2) and N-terminal truncated form (GF-1) of human Smubp-2 has been reported. The existence of different forms of AEP/Smubp-2/Rip-1 proteins may add additional complexity to its function in transcriptional regulation. The Smubp-2 and GF-1 have no significant differences in transactivating the apoA-I promoter in HepG2 and Hep3B cells (Mohan et al, 1998). We did not observe notable variations in the transient expression assay when Smubp-2 or GF-1 was overexpressed with wflAFP enhancer in HepG2 cells (data not shown). In contrast, GF-1 stimulated the JCV early promoter while Smubp-2 had no effect, and GF-1 had higher induction to Smubp-2 on the viral late promoter (Chen et al, 1997). More studies are required to investigate the presence of the truncated form in species other than human, and the significance of the N- and C-terminal portions of this protein in transactivation.

We have demonstrated that the tissue-specific enhancer of the wflAFP gene interacts with both AEP and the liver-enriched transcription factor C/EBPα. Moreover, it has been reported that Smubp-2 was able to associate with the basal transcription factor TATA binding protein (TBP) to regulate gene expression (Zhang et al, 1999). Therefore, the AEP/Smubp-2/Rip-1 proteins may serve as a co-factor that cross-talks between tissue-specific factors and the basic transcription apparatus. Moreover, Smubp-2 has been shown to activate as well as to repress specific gene promoters (Chen et al, 1997; Zhang et al, 1999), suggesting that the role of AEP/Smubp-2/Rip-1 proteins is versatile depending on its partners.

Chapter Five

Summary and future directions

Studies on the seasonal and hormonal regulation of AFP gene expressions in winter flounder are important for our understanding of the adaptive mechanisms of many organisms to survive in a freezing environment. Based on our earlier studies, we have established that the enhancer of the wflAFP gene confers its liver-specific specificity, and it interacts with the liver-enriched transcription factor C/EBPα and the presumptive AEP protein (Chan et al, 1997). Therefore, the stage was set to further elucidate the role of C/EBPα and the core enhancer in the transcriptional regulation of the wflAFP gene. In the present study, we have demonstrated that the core enhancer Element B (+303 to +322) binds to C/EBPα and the presumptive AEP in both rat and flounder liver extracts. It is a crucial element mediating the transactivation ability of the wflAFP gene enhancer since mutations in Element B destroyed its enhancer activity. The involvement of C/EBPα has been further demonstrated using recombinant C/EBPα. In addition, the identity of the presumptive AEP has been revealed. It is a helicase domain-containing protein (HDP) and is essential for the enhancer activity of the wflAFP gene.

Our results from methylation interference studies have suggested that the C/EBPα and AEP share an overlapping binding site in Element B. The entire Element B sequence was protected from rat or flounder liver nuclear extracts in DNase I footprinting studies. Also, our data from transient expression experiments indicated that the enhancer of the wflAFP gene requires the binding of Element B with both C/EBPα and AEP to achieve significant transactivation activity. Together, these data indicate that C/EBPα and AEP might occupy the Element B sequence simultaneously, however, no direct evidence has been provided. To further address this point, detailed footprinting analysis can be employed. The DNA sequences protected by recombinant C/EBPα or AEP only should be more restricted when

compared to the footprinted regions made by both C/EBPα and AEP or the crude liver nuclear extract. In addition, antibody specific to C/EBPα can be used to deplete its binding with Element B, and a more refined footprint should be observed. A composite element in mouse proliferin gene was shown to interact with both glucocorticoid receptor and AP1, which are believed to co-occupy simultaneously the single element to achieve either positive or negative regulation (Diamond et al, 1990).

The overlapped binding sites for C/EBPα and AEP suggest that there might be direct physical contact between these two factors. Bacterial expressed AEP can be used to raise antibody against rat AEP. Co-immunoprecipitation experiments can then be employed to determine whether there are direct protein-protein interactions between these factors. HDPs were found to be members of the basal transcriptional machinery complex TFIIH as well as many coactivator complexes involving in chromatin remodeling (Drapkin and Reinberg, 1994; Elfring et al, 1994; Ito et al, 1997; Varga-Weisz et al, 1997). It is reasonable to suggest that AEP and/or C/EBPα interact with other coactivators that can alter chromatin structure or act as the link between the DNA-binding proteins and the basal transcription apparatus. Further protein-protein interaction studies including a yeast two-hybrid system, immunological and affinity chromatography can lead to the identification of those important players in the regulation of the wflAFP gene expression.

Also, as a first step to dissect the signaling pathway that mediates the hormonal regulation of the wflAFP gene, both IGF-1 and PI3-kinase have been demonstrated to be involved in controlling wflAFP gene expression. Various inhibitors are available to further explore the GH->IGF-1 signaling pathway(s) mediating wflAFP gene expression. For example, in addition to wortmannin, LY294002 is also a PI3-kinase blocker, and rapamycin works

downstream of the PI3-kinase pathway on the p70S6 kinase (Han et al, 1995; Wiederrecht et al, 1995). Besides, PD098059 is a highly specific inhibitor for the activation of MEK kinase by Raf-1 (Alessi et al, 1995). By using these inhibitors, the involvement of PI3-kinase and possibly MAP kinase signaling pathways can be further delineated.

To address the regulation of the wfsAFP gene expression, we have shown that the intron of the wfsAFP acts as a ubiquitous enhancer. There is an additional 241 bp fragment in the wfsAFP gene intron, which may contain important DNA elements responsible for its enhancer activity (Davies and Gauthier, 1992). More studies are required to define the enhancer element for the wfsAFP gene and DNA-protein interaction studies can be employed to investigate the factors that are involved in the differential regulation of wfsAFP gene.

Moreover, we have demonstrated that the intron of wfsAFP does not interact with C/EBPα. A TA insertion in the core enhancer Element B in the intron of wflAFP gene destroys its liver-specific enhancer activity. These results indicate that C/EBPα plays an important role in wflAFP gene regulation. However, how C/EBPα exerts its regulatory effect remains to be examined. Post-translational regulation, especially phosphorylation is a favorite model since phosphorylation is a common theme in regulating the transactivation ability as well as DNA binding affinity of the C/EBP family of proteins (for review: Takiguchi, 1998). Moreover, it was shown that increased IGF-1 level resulted in the dephosphorylation of C/EBPα (Hemati et al, 1997), which agrees with our result that treatment of IGF-1 suppressed the expression of the wflAFP gene. Alternative mechanisms such as reduction of the C/EBPα mRNA and its protein levels and/or the production of dominant-negative form of C/EBP competing for the C/EBP binding site may exist to control the effect of C/EBPα on wflAFP gene expression (MacDougald et al, 1995). Western analysis can be conducted to examine whether the

C/EBPα protein is phosphorylated or dephosphorylated with cells treated with IGF-1, wartamannin or GH. Also, the variation of C/EBPα mRNA levels under these treatments can be examined by Northern analysis or quantitative PCR. These studies should provide a better understanding on how the expression of wflAFP gene is regulated by C/EBPα.

So far, we have been successful in using mammalian cell lines and rat liver extract as tools to investigate the transcriptional regulation of the wfAFP genes. Studies from the mammalian system have been consistent with results obtained from flounder (Miao et al, 1998a). However, it is important to characterize the transcription factors in winter flounder that mediate the regulation of the wfAFP gene expressions. The presence of C/EBP and AEP counterpart in winter flounder has been supported by our studies. The identification and characterization of these factors in flounder can be facilitated by the application of mass spectrometry (Nguyen et al, 1995; Kuster and Mann, 1998). Preparative gel retardation assays can be performed using Element B and flounder liver nuclear extract to obtain specific protein complexes that are involved in DNA binding. After recovering from the polyacrylamide gel, these factors can be further separated on SDS-PAGE and the protein bands of interest can be subjected to MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometry). MALDI-MS has a practical mass range up to 300 kDa and sensitivity to picomole amount of molecules. Also, sequence information of these proteins can be provided by tandem mass spectrometry. Specific PCR primers could be designed accordingly to screen a flounder liver cDNA library to obtain the clones encoding the flounder C/EBP and AEP and possibly other interacting factors. Alternatively, an expression cloning approach, which has been used for the identification of the rat AEP, can be employed using flounder liver cDNA library. Once these flounder factors involved in wflAFP gene

regulation are characterized, specific antibodies against these flounder proteins can be raised. The protein levels as well as their post-translational modification such as phosphorylation/dephosphorylation of these factors between summer vs winter or between normal vs hypophysectomized fish can be investigated by immunobloting studies. These studies will bring much important insight on how the AFP genes are regulated seasonally and hormonally in winter flounder.

In conclusion, we have established the importance of the core enhancer Element B and C/EBPα in the transcriptional regulation of the wflAFP gene. Also, the antifreeze enhancer-binding protein (AEP) has been identified and its role assessed. The signaling molecules involving in the wflAFP gene regulation and the differential mechanism for the regulation of the wfsAFP gene expression have been revealed. Based on the present studies, a working model has been proposed to integrate the complex seasonal and hormonal, as well as the tissue-specific regulation of AFP genes in winter flounder (Fig. 5-1) (Miao et al, 2000). During summer, GH released from the pituitary stimulates the production of IGF-1, which may dephosphorylate and deactivate C/EBPα and/or affect the level of AEP, resulting in transcriptional inhibition of the wflAFP genes. Alternatively, GH may exihibit direct effect on C/EBPα. With the shortening of day length in the fall, the production of GH is inhibited by the CNS, thereby releasing its transcriptional repression on AFP genes. This would result in the increase of the concentration or activity of C/EBPα and AEP. Their interaction with the core enhancer Element B would lead to a significant increase in wflAFP gene expression. The liver-enriched factor, C/EBPα plays an important role not only in the seasonal and hormonal control, but also in the tissue-specificity of the wflAFPs. On the other hand,

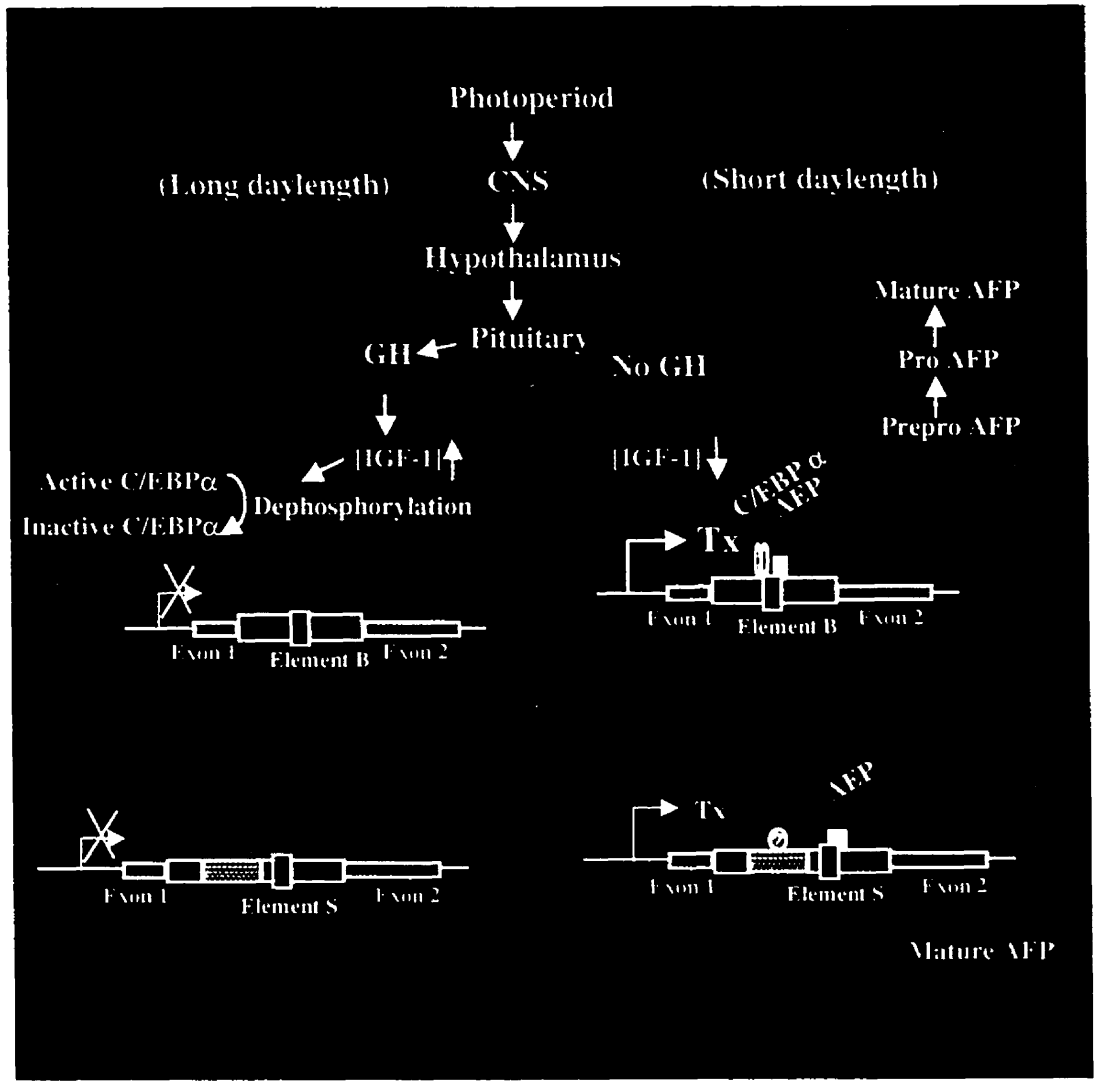


Fig. 5-1 Model for the hormonal and tissue-specific regulations of the wflAFP and wfsAFP genes. During summer, GH released from the pituitary stimulates the production of IGF-1, which dephosphorylates and deactivates C/EBPα and/or affects the level of AEP, resulting in transcriptional inhibition of the wflAFP genes. During winter, the production of GH is inhibited, hence releaseing the transcriptional repression. C/EBPα and AEP become active and/or their levels elevated, and their interaction with the core enhancer Element B activates the wflAFP gene expression. The wfsAFP genes, lacking the C/EBP binding motif, are more ubiquitously expressed and less influenced by GH and seasonal change. Other factor(s) may also involved in the wfsAFP gene regulation.

lacking the C/EBP binding motif, the wfsAFP is more ubiquitously expressed and is less influenced by GH and seasonal change. The fact that AEP also interacts with Element S of the wfsAFP gene suggests that the AEP may be involved in the basal transcription of AFP genes while the tissue-specificity is achieved primarily via C/EBPα.

References

Albright, S. R. and Tjian, R. (2000). TAFs revisited: more data reveal new twists and confirm old ideas. *Gene* **242**: 1-13.

Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen- activated protein kinase kinase in vitro and in vivo. *J Biol Chem* **270**: 27489-94.

Birkenmeier, E. H., Gwynn, B., Howard, S., Jerry, J., Gordon, J. I., Landschulz, W. H. and McKnight, S. L. (1989). Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes Dev* **3**: 1146-56.

Bjorklund, S., Almouzni, G., Davidson, I., Nightingale, K. P. and Weiss, K. (1999). Global transcription regulators of eukaryotes. *Cell* **96**: 759-67.

Bjorklund, S. and Kim, Y. J. (1996). Mediator of transcriptional regulation [see comments]. *Trends Biochem Sci* **21**: 335-7.

Blackwood, E. M. and Kadonaga, J. T. (1998). Going the distance: a current view of enhancer action. *Science* **281**: 61-3.

Blakesley, V. A., Scrimgeour, A., Esposito, D. and Le Roith, D. (1996). Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? *Cytokine Growth Factor Rev* **7**: 153-9.

Blin, N. and Stafford, D. W. (1976). A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res* **3**: 2303-8.

Buck, M., Poli, V., van der Geer, P., Chojkier, M. and Hunter, T. (1999). Phosphorylation of rat serine 105 or mouse threonine 217 in C/EBP beta is required for hepatocyte proliferation induced by TGF alpha. *Mol Cell* **4**: 1087-92.

Burns, L. G. and Peterson, C. L. (1997). Protein complexes for remodeling chromatin. *Biochim Biophys Acta* **1350**: 159-68.

Cairns, B. R., Kim, Y. J., Sayre, M. H., Laurent, B. C. and Kornberg, R. D. (1994). A multisubunit complex containing the SWI1/ADR6, SWI2/SNF2, SWI3, SNF5, and SNF6 gene products isolated from yeast. *Proc Natl Acad Sci U S A* **91**: 1950-4.

Cao, Z., Umek, R. M. and McKnight, S. L. (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev* **5**: 1538-52.

Carter-Su, C., Schwartz, J. and Smit, L. S. (1996). Molecular mechanism of growth hormone action. *Annu Rev Physiol* **58**: 187-207.

Cereghini, S., Raymondjean, M., Carranca, A. G., Herbomel, P. and Yaniv, M. (1987). Factors involved in control of tissue-specific expression of albumin gene. *Cell* **50**: 627-38.

Chan, S. L., Fletcher, G. L. and Hew, C. L. (1993). Control of antifreeze protein gene expression in winter flounder. Biochemistry and molecular biology of fishes. Hochachka and Mommsen, Elsevier Science Publishers. **2:** 293-305.

Chan, S. L., Miao, M., Fletcher, G. L. and Hew, C. L. (1997). The role of CCAAT/enhancer-binding protein alpha and a protein that binds to the activator-protein-1 site in the

regulation of liver- specific expression of the winter flounder antifreeze protein gene. *Eur J Biochem* **247**: 44-51.

Chao, H., Houston, M. E., Jr., Hodges, R. S., Kay, C. M., Sykes, B. D., Loewen, M. C., Davies, P. L. and Sonnichsen, F. D. (1997). A diminished role for hydrogen bonds in antifreeze protein binding to ice. *Biochemistry* **36**: 14652-60.

Chen, N. N., Kerr, D., Chang, C. F., Honjo, T. and Khalili, K. (1997). Evidence for regulation of transcription and replication of the human neurotropic virus JCV genome by the human S(mu)bp-2 protein in glial cells. *Gene* **185**: 55-62.

Cheng, C. H. (1998). Evolution of the diverse antifreeze proteins. *Curr Opin Genet Dev* **8**: 715-20.

Chodosh, L. A., Carthew, R. W. and Sharp, P. A. (1986). A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. *Mol Cell Biol* **6**: 4723-33.

Coolican, S. A., Samuel, D. S., Ewton, D. Z., McWade, F. J. and Florini, J. R. (1997). The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J Biol Chem* **272**: 6653-62.

Cooper, C., Henderson, A., Artandi, S., Avitahl, N. and Calame, K. (1995). Ig/EBP (C/EBP gamma) is a transdominant negative inhibitor of C/EBP family transcriptional activators. *Nucleic Acids Res* **23**: 4371-7.

Costa, R. H., Grayson, D. R. and Darnell, J. E., Jr. (1989). Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and alpha 1-antitrypsin genes. *Mol Cell Biol* **9**: 1415-25.

Cote, J., Quinn, J., Workman, J. L. and Peterson, C. L. (1994). Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. *Science* **265**: 53-60.

Cox, G. A., Mahaffey, C. L. and Frankel, W. N. (1998). Identification of the mouse neuromuscular degeneration gene and mapping of a second site suppressor allele. *Neuron* **21**: 1327-37.

Croniger, C., Leahy, P., Reshef, L. and Hanson, R. W. (1998). C/EBP and the control of phosphoenolpyruvate carboxykinase gene transcription in the liver. *J Biol Chem* **273**: 31629-32.

Crossley, M. and Brownlee, G. G. (1990). Disruption of a C/EBP binding site in the factor IX promoter is associated with haemophilia B. *Nature* **345**: 444-6.

Daughaday, W. H. and Rotwein, P. (1989). Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr Rev* **10**: 68-91.

Davies, P. L. (1992). Conservation of antifreeze protein-encoding genes in tandem repeats. *Gene* **112**: 163-70.

Davies, P. L. and Gauthier, S. Y. (1992). Antifreeze protein pseudogenes. *Gene* **112**: 171-8.

Davies, P. L. and Hew, C. L. (1990). Biochemistry of fish antifreeze proteins. *FASEB J* **4**: 2460-8.

Davies, P. L., Roach, A. H. and Hew, C. L. (1982). DNA sequence coding for an antifreeze protein precursor from winter flounder. *Proc Natl Acad Sci U S A* **79**: 335-9.

Davies, P. L. and Sykes, B. D. (1997). Antifreeze proteins. *Curr Opin Struct Biol* **7**: 828-34.

De Simone, V. and Cortese, R. (1992). Transcription factors and liver-specific genes. *Biochim Biophys Acta* **1132**: 119-26.

Deng, W. P. and Nickoloff, J. A. (1992). Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal Biochem* **200**: 81-8.

Descombes, P. and Schibler, U. (1991). A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* **67**: 569-79.

DeVries, A. L. and Lin, Y. (1977). Structure of a peptide antifreeze and mechanism of adsorption to ice. *Biochim Biophys Acta* **495**: 388-92.

Diamond, M. I., Miner, J. N., Yoshinaga, S. K. and Yamamoto, K. R. (1990). Transcription factor interactions: Selectors of positive or negative regulation from a single DNA element. *Science* **249**:1266-72.

Dikstein, R., Ruppert, S. and Tjian, R. (1996). TAFII250 is a bipartite protein kinase that phosphorylates the base transcription factor RAP74. *Cell* **84**: 781-90.

Ding, H., Benotmane, A. M., Suske, G., Collen, D. and Belayew, A. (1999). Functional interactions between Sp1 or Sp3 and the helicase-like transcription factor mediate basal expression from the human plasminogen activator inhibitor-1 gene. *J Biol Chem* **274**: 19573-80.

Ding, H., Descheemaeker, K., Marynen, P., Nelles, L., Carvalho, T., Carmo-Fonseca, M., Collen, D. and Belayew, A. (1996). Characterization of a helicase-like transcription factor involved in the expression of the human plasminogen activator inhibitor-1 gene. *DNA Cell Biol* **15**: 429-42.

Doucet, D., Tyshenko, M. G., Kuiper, M. J., Graether, S. P., Sykes, B. D., Daugulis, A. J., Davies, P. L. and Walker, V. K. (2000). Structure-function relationships in spruce budworm antifreeze protein revealed by isoform diversity. *Eur J Biochem* **267**: 6082-8.

Drapkin, R., Reardon, J. T., Ansari, A., Huang, J. C., Zawel, L., Ahn, K., Sancar, A. and Reinberg, D. (1994). Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. *Nature* **368**: 769-72.

Drapkin, R. and Reinberg, D. (1994). Transcription. The essential twist [news; comment]. *Nature* **369**: 523-4.

Duguay, S. J., Swanson, P. and Dickhoff, W. W. (1994). Differential expression and hormonal regulation of alternatively spliced IGF-I mRNA transcripts in salmon. *J Mol Endocrinol* **12**: 25-37.

Duncker, B. P., Koops, M. D., Walker, V. K. and Davies, P. L. (1995). Low temperature persistence of type I antifreeze protein is mediated by cold-specific mRNA stability. *FEBS Lett* **377**: 185-8.

Eastman, A. (1987). An improvement to the novel rapid assay for chloramphenicol acetyltransferase gene expression. *Biotechniques* **5**: 730-2.

Eisen, A. and Lucchesi, J. C. (1998). Unraveling the role of helicases in transcription. *Bioessays* **20**: 634-41.

Elfring, L. K., Deuring, R., McCallum, C. M., Peterson, C. L. and Tamkun, J. W. (1994). Identification and characterization of Drosophila relatives of the yeast transcriptional activator SNF2/SWI2. *Mol Cell Biol* **14**: 2225-34.

Fletcher, G. L. (1979). The effects of hypophysectomy and pituitary replacement on the plasma freezing point depression, chloride ion concentration, glucose and protein antifreeze in winter flounder (*Pseudopleuronectes americanus*). *Comp Biochem Physiol* **63**: 535-7.

Fletcher, G. L. (1981). Effects of temperature and photoperiod on the plasma freezing point depression, Cl^- and protein antifreeze in winter flounder (*Pseudopleuronectes americanus*). *Can J Zool* **59**: 193-201.

Fletcher, G. L., Idler, D. R., Vaisius, A. and Hew, C. L. (1989). Hormonal regulation of AFP gene expression in winter flounder. *Fish Physiol Biochem* **7**: 387-93.

Fletcher, G. L., King, M. J. and Hew, C. L. (1984). How does the brain control the pituitary release of antifreeze synthesis inhibitor? *Can J Zool* **62**: 839-44.

Fourney, R. M., Fletcher, G. L. and Hew, C. L. (1984b). Accumulation of winter flounder antifreeze messenger RNA after hypophysectomy. *Gen Comp Endocrinol* **54**: 392-401.

Fourney, R. M., Fletcher, G. L. and Hew, C. L. (1984c). The effects of long day length on liver antifreeze messenger RNA in the winter flounder, Pseudopleuronectes americanus. *Can J Zool* **62**: 1456-60.

Fourney, R. M., Joshi, S. B., Kao, M. H. and Hew, C. L. (198a). Heterogeneity of antifreeze polypeptides from the Newfoundland winter flounder, Pseudopleuronectes americanus. *Can J Zool* **62**: 28-33.

Fukita, Y., Mizuta, T. R., Shirozu, M., Ozawa, K., Shimizu, A. and Honjo, T. (1993). The human S mu bp-2, a DNA-binding protein specific to the single- stranded guanine-rich sequence related to the immunoglobulin mu chain switch region. *J Biol Chem* **268**: 17463-70.

Gong, Z., Ewart, K. V., Hu, Z., Fletcher, G. L. and Hew, C. L. (1996). Skin antifreeze protein genes of the winter flounder, Pleuronectes americanus, encode distinct and active polypeptides without the secretory signal and prosequences. *J Biol Chem* **271**: 4106-12.

Gong, Z., Fletcher, G. L. and Hew, C. L. (1992). Tissue distribution of fish antifreeze protein mRNAs. *Can J Zool* **70**: 810-4.

Gong, Z. and Hew, C. L. (1993). Promoter analysis of fish antifreeze protein genes. Biochemistry and molecular biology of fishes. Hochachka and Mommsen, Elsevier Science publishers. **2:** 307-24.

Gong, Z., King, M. J., Fletcher, G. L. and Hew, C. L. (1995). The antifreeze protein genes of the winter flounder, Pleuronectus americanus, are differentially regulated in liver and non-liver tissues. *Biochem Biophys Res Commun* **206**: 387-92.

Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P. and Blinov, V. M. (1989). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res* **17**: 4713-30.

Gorski, K., Carneiro, M. and Schibler, U. (1986). Tissue-specific in vitro transcription from the mouse albumin promoter. *Cell* **47**: 767-76.

Graether, S. P., Kuiper, M. J., Gagne, S. M., Walker, V. K., Jia, Z., Sykes, B. D. and Davies, P. L. (2000). Beta-helix structure and ice-binding properties of a hyperactive antifreeze protein from an insect. *Nature* **406**:325-8.

Graham, F. L. and Eb, A. J. v. d. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**: 456-67.

Graham, L. A., Liou, Y. C., Walker, V.K. and Davies, P. L. (1997). Hyperactive antifreeze protein from beetles. *Nature* **388**: 727-8.

Gray, E. S. and Kelley, K. M. (1991). Growth regulation in the gobiid teleost, Gillichthys mirabilis: roles of growth hormone, hepatic growth hormone receptors and insulin-like growth factor-I. *J Endocrinol* **131**: 57-66.

Gronwald, W., Chao, H., Reddy, D. V., Davies, P. L., Sykes, B. D. and Sonnichsen, F. D. (1996). NMR characterization of side chain flexibility and backbone structure in the type I antifreeze protein at near freezing temperatures. *Biochemistry* **35**: 16698-704.

Guzder, S. N., Sung, P., Prakash, S. and Prakash, L. (1995). Lethality in yeast of trichothiodystrophy (TTD) mutations in the human xeroderma pigmentosum group D gene. Implications for transcriptional defect in TTD. *J Biol Chem* **270**: 17660-3.

Hai, T. and Curran, T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci U S A* **88**: 3720-4.

Hall, C. V., Jacob, P. E., Ringold, G. M. and Lee, F. (1983). Expression and regulation of Escherichia coli lacZ gene fusions in mammalian cells. *J Mol Appl Genet* **2**: 101-9.

Hall, M. C. and Matson, S. W. (1999). Helicase motifs: the engine that powers DNA unwinding. *Mol Microbiol* **34**: 867-77.

Hampsey, M. (1998). Molecular genetics of the RNA polymerase II general transcriptional machinery. *Microbiol Mol Biol Rev* **62**: 465-503.

Hampsey, M. and Reinberg, D. (1997). Transcription: why are TAFs essential? *Curr Biol* **7**: R44-6.

Han, J. W., Pearson, R. B., Dennis, P. B. and Thomas, G. (1995). Rapamycin, wortmannin, and the methylxanthine SQ20006 inactivate p70s6k by inducing dephosphorylation of the same subset of sites. *J Biol Chem* **270**: 21396-403.

Harding, M. M., Ward, L. G. and Haymet, A. D. (1999). Type I 'antifreeze' proteins. Structure-activity studies and mechanisms of ice growth inhibition. *Eur J Biochem* **264**: 653-65.

Haymet, A. D., Ward, L. G., Harding, M. M. and Knight, C. A. (1998). Valine substituted winter flounder 'antifreeze': preservation of ice growth hysteresis. *FEBS Lett* **430**: 301-6.

Hemati, N., Ross, S. E., Erickson, R. L., Groblewski, G. E. and MacDougald, O. A. (1997). Signaling pathways through which insulin regulates CCAAT/enhancer binding protein alpha (C/EBPalpha) phosphorylation and gene expression in 3T3-L1 adipocytes. Correlation with GLUT4 gene expression. *J Biol Chem* **272**: 25913-9.

Hew, C., Poon, R., Xiong, F., Gauthier, S., Shears, M., King, M., Davies, P. and Fletcher, G. (1999). Liver-specific and seasonal expression of transgenic Atlantic salmon harboring the winter flounder antifreeze protein gene. *Transgenic Res* **8**: 405-14.

Hew, C. L., Davies, P. L. and Fletcher, G. (1992). Antifreeze protein gene transfer in Atlantic salmon. *Mol Mar Biol Biotechnol* **1**: 309-17.

Hew, C. L. and Fletcher, G. L. (1979). The role of pituitary in regulating antifreeze protein synthesis in the winter flounder. *FEBS Lett* **99**: 337-9.

Hew, C. L., Joshi, S. and Wang, N.-C. (1984). Analysis of fish antifreeze polypeptides by reversed-phase high performance liquid chromatography. *J Chromatogr* **296**: 213-9.

Hew, C. L., Wang, N. C., Yan, S., Cai, H., Sclater, A. and Fletcher, G. L. (1986). Biosynthesis of antifreeze polypeptides in the winter flounder. Characterization and seasonal occurrence of precursor polypeptides. *Eur J Biochem* **160**: 267-72.

Hew, C. L. and Yang, D. S. (1992). Protein interaction with ice. *Eur J Biochem* **203**: 33-42.

Hoffmann, A., Oelgeschlager, T. and Roeder, R. G. (1997). Considerations of transcriptional control mechanisms: do TFIID-core promoter complexes recapitulate nucleosome-like functions? *Proc Natl Acad Sci U S A* **94**: 8928-35.

Holstege, F. C., van der Vliet, P. C. and Timmers, H. T. (1996). Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIH. *Embo J* **15**: 1666-77.

Hon, W. C., Griffith, M., Mlynarz, A., Kwok, Y. C. and Yang, D. S. (1995). Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. *Plant Physiol* **109**: 879-89.

Idler, D. R., Fletcher, G. L., Belkhode, S., King, M. J. and Hwang, S. J. (1989). Regulation of antifreeze protein production in winter flounder: a unique function for growth hormone. *Gen Comp Endocrinol* **74**: 327-34.

Imbalzano, A. N., Kwon, H., Green, M. R. and Kingston, R. E. (1994). Facilitated binding of TATA-binding protein to nucleosomal DNA [see comments]. *Nature* **370**: 481-5.

Ito, T., Bulger, M., Pazin, M. J., Kobayashi, R. and Kadonaga, J. T. (1997). ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. *Cell* **90**: 145-55.

Johnson, P. F., Landschulz, W. H., Graves, B. J. and McKnight, S. L. (1987). Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev* **1**: 133-46.

Kadonaga, J. T. (1998). Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. *Cell* **92**: 307-13.

Kadowaki, T., Tobe, K., Honda-Yamamoto, R., Tamemoto, H., Kaburagi, Y., Momomura, K., Ueki, K., Takahashi, Y., Yamauchi, T., Akanuma, Y. and Yazaki, Y. (1996). Signal transduction mechanism of insulin and insulin-like growth factor- 1. *Endocr J* **43**: S33-41.

Kaling, M., Kugler, W., Ross, K., Zoidl, C. and Ryffel, G. U. (1991). Liver-specific gene expression: A-activator-binding site, a promoter module present in vitellogenin and acute-phase genes. *Mol Cell Biol* **11**: 93-101.

Karin, M., Liu, Z. and Zandi, E. (1997). AP-1 function and regulation. *Curr Opin Cell Biol* **9**: 240-6.

Kenward, K. D., Altschuler, M., Hildebrand, D. and Davies, P. L. (1993). Accumulation of type I fish antifreeze protein in transgenic tobacco is cold-specific. *Plant Mol Biol* **23**: 377-85.

Kerr, D. and Khalili, K. (1991). A recombinant cDNA derived from human brain encodes a DNA binding protein that stimulates transcription of the human neurotropic virus JCV. *J Biol Chem* **266**: 15876-81.

Kim, T. K., Ebright, R. H. and Reinberg, D. (2000). Mechanism of ATP-dependent promoter melting by transcription factor IIH. *Science* **288**: 1418-22.

Knight, C. A., Cheng, C. C. and DeVries, A. L. (1991). Adsorption of alpha-helical antifreeze peptides on specific ice crystal surface planes. *Biophys J* **59**: 409-18.

Koleske, A. J. and Young, R. A. (1994). An RNA polymerase II holoenzyme responsive to activators [see comments]. *Nature* **368**: 466-9.

Koleske, A. J. and Young, R. A. (1995). The RNA polymerase II holoenzyme and its implications for gene regulation. *Trends Biochem Sci* **20**: 113-6.

Kornberg, R. D. (1999). Eukaryotic transcriptional control. *Trends Cell Biol* **9**: M46-9.

Kuster, B. and Mann, M. (1998). Identifying proteins and post-translational modifications by mass spectrometry. *Curr. Opin. Structural Biol.* **8**: 393-400.

Lacorte, J. M., Ktistaki, E., Beigneux, A., Zannis, V. I., Chambaz, J. and Talianidis, I. (1997). Activation of CAAT enhancer-binding protein delta (C/EBPdelta) by interleukin-1 negatively influences apolipoprotein C-III expression. *J Biol Chem* **272**: 23578-84.

Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J. and McKnight, S. L. (1988). Isolation of a recombinant copy of the gene encoding C/EBP [published erratum appears in Genes Dev 1994 May 1;8(9):1131]. *Genes Dev* **2**: 786-800.

Lekstrom-Himes, J. and Xanthopoulos, K. G. (1998). Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J Biol Chem* **273**: 28545-8.

Leveillard, T., Andera, L., Bissonnette, N., Schaeffer, L., Bracco, L., Egly, J. M. and Wasylyk, B. (1996). Functional interactions between p53 and the TFIIH complex are affected by tumour-associated mutations. *Embo J* **15**: 1615-24.

Lin, F. T., MacDougald, O. A., Diehl, A. M. and Lane, M. D. (1993). A 30- kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. *Proc Natl Acad Sci U S A* **90**: 9606-10.

Lohman, T. M. and Bjornson, K. P. (1996). Mechanisms of helicase-catalyzed DNA unwinding. *Annu Rev Biochem* **65**: 169-214.

Lopaczynski, W. (1999). Differential regulation of signaling pathways for insulin and insulin- like growth factor I. *Acta Biochim Pol* **46**: 51-60.

Low, W. K., Lin, Q., Ewart, K. V., Fletcher, G. L. and Hew, C. L. (2000). The skin-type antifreeze polypeptides, A new class of type I AFPs. Fish Antifreeze Proteins. Hew, C. L. and Ewart, K. V., World Scientific Press. **1**: In preparation.

MacDougald, O. A., Cornelius, P., Liu, R. and Lane, M. D. (1995). Insulin regulates transcription of the CCAAT/enhancer binding protein (C/EBP) alpha, beta, and delta genes in fully-differentiated 3T3-L1 adipocytes. *J Biol Chem* **270**: 647-54.

Mahoney, C. W., Shuman, J., McKnight, S. L., Chen, H. C. and Huang, K. P. (1992). Phosphorylation of CCAAT-enhancer binding protein by protein kinase C attenuates site-selective DNA binding. *J Biol Chem* **267**: 19396-403.

Matson, S. W., Bean, D. W. and George, J. W. (1994). DNA helicases: enzymes with essential roles in all aspects of DNA metabolism. *Bioessays* **16**: 13-22.

Maxam, A. M. and Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol* **65**: 499-560.

Metzger, S., Halaas, J. L., Breslow, J. L. and Sladek, F. M. (1993). Orphan receptor HNF-4 and bZip protein C/EBP alpha bind to overlapping regions of the apolipoprotein B gene promoter and synergistically activate transcription. *J Biol Chem* **268**: 16831-8.

Miao, M., Chan, S. L., Fletcher, G. L. and Hew, C. L. (2000). Control of the Antifreeze Protein Gene Expression in Winter Flounder. Fish Antifreeze Proteins. Hew, C. L. and Ewart, K. V., World Scientific Press. **1**: In preparation.

Miao, M., Chan, S. L., Hew, C. L. and Fletcher, G. L. (1998a). Identification of nuclear proteins interacting with the liver-specific enhancer B element of the antifreeze protein gene in winter flounder. *Mol Mar Biol Biotechnol* **7**: 197-203.

Miao, M., Chan, S. L., Hew, C. L. and Gong, Z. (1998b). The skin-type antifreeze protein gene intron of the winter flounder is a ubiquitous enhancer lacking a functional C/EBPalpha binding motif. *FEBS Lett* **426**: 121-5.

Milos, P. M. and Zaret, K. S. (1992). A ubiquitous factor is required for C/EBP-related proteins to form stable transcription complexes on an albumin promoter segment in vitro. *Genes Dev* **6**: 991-1004.

Mizuta, T. R., Fukita, Y., Miyoshi, T., Shimizu, A. and Honjo, T. (1993). Isolation of cDNA encoding a binding protein specific to 5'- phosphorylated single-stranded DNA with G-rich sequences. *Nucleic Acids Res* **21**: 1761-6.

Mizzen, C. A., Yang, X. J., Kokubo, T., Brownell, J. E., Bannister, A. J., Owen-Hughes, T., Workman, J., Wang, L., Berger, S. L., Kouzarides, T., Nakatani, Y. and Allis, C. D. (1996). The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. *Cell* **87**: 1261-70.

Mohan, W. S., Chen, Z. Q., Zhang, X., Khalili, K., Honjo, T., Deeley, R. G. and Tam, S. P. (1998). Human S mu binding protein-2 binds to the drug response element and transactivates the human apoA-I promoter: role of gemfibrozil. *J Lipid Res* **39**: 255-67.

Montell, D. J., Rorth, P. and Spradling, A. C. (1992). slow border cells, a locus required for a developmentally regulated cell migration during oogenesis, encodes Drosophila C/EBP. *Cell* **71**: 51-62.

Morency, C. A., Neumann, J. R. and Russian, K. O. (1987). A novel rapid assay for chloramphenicol acetyltransferase. *Biotechniques* **5**: 444-8.

Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T. and Akira, S. (1993). Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc Natl Acad Sci U S A* **90**: 2207-11.

Nguyen, D. N., Becker, G. W. and Riggin, R. M. (1995). Protein mass spectrometry: applications to analytical biotechnology. *J. Chromatogr. A* **705**: 21-45.

Orphanides, G., Lagrange, T. and Reinberg, D. (1996). The general transcription factors of RNA polymerase II. *Genes Dev* **10**: 2657-83.

Osada, S., Yamamoto, H., Nishihara, T. and Imagawa, M. (1996). DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. *J Biol Chem* **271**: 3891-6.

Ossipow, V., Descombes, P. and Schibler, U. (1993). CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. *Proc Natl Acad Sci U S A* **90**: 8219-23.

Pankov, Y. A. (1999). Growth hormone and a partial mediator of its biological action, insulin- like growth factor I. *Biochemistry (Mosc)* **64**: 1-7.

Park, E. A., Jerden, D. C. and Bahouth, S. W. (1995). Regulation of phosphoenolpyruvate carboxykinase gene transcription by thyroid hormone involves two distinct binding sites in the promoter. *Biochem J* **309**: 913-9.

Park, E. A., Song, S., Vinson, C. and Roesler, W. J. (1999). Role of CCAAT enhancer-binding protein beta in the thyroid hormone and cAMP induction of phosphoenolpyruvate carboxykinase gene transcription. *J Biol Chem* **274**: 211-7.

Peterson, C. L., Dingwall, A. and Scott, M. P. (1994). Five SWI/SNF gene products are components of a large multisubunit complex required for transcriptional enhancement [see comments]. *Proc Natl Acad Sci U S A* **91**: 2905-8.

Petley, T., Graff, K., Jiang, W., Yang, H. and Florini, J. (1999). Variation among cell types in the signaling pathways by which IGF-I stimulates specific cellular responses. *Horm Metab Res* **31**: 70-6.

Pickett, M., Scott, G., Davies, P., Wang, N., Joshi, S. and Hew, C. (1984). Sequence of an antifreeze protein precursor. *Eur J Biochem* **143**: 35-8.

Pickett, M. H., Hew, C. L. and Davies, P. L. (1983a). Seasonal variation in the level of antifreeze protein mRNA from the winter flounder. *Biochim Biophys Acta* **739**: 97-104.

Pickett, M. H., White, B. N. and Davies, P. L. (1983b). Evidence that translational control mechanisms operate to optimize antifreeze protein production in the winter flounder. *J Biol Chem* **258**: 14762-5.

Price, J. L., Gourlie, B. B., Lin, Y. and Huang, R. C. C. (1986). Induction of winter flounder antifreeze protein messenger RNA at 4°C *in vivo* and *in vitro*. *Physiol Zool* **59**: 679-95.

Ptashne, M. and Gann, A. (1997). Transcriptional activation by recruitment. *Nature* **386**: 569-77.

Pugh, B. F. and Tjian, R. (1992). Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. *J Biol Chem* **267**: 679-82.

Rancourt, D. E., Walker, V. K. and Davies, P. L. (1987). Flounder antifreeze protein synthesis under heat shock control in transgenic Drosophila melanogaster. *Mol Cell Biol* **7**: 2188-95.

Rastegar, M., Rousseau, G. G. and Lemaigre, F. P. (2000). CCAAT/enhancer-binding protein-alpha is a component of the growth hormone-regulated network of liver transcription factors. *Endocrinology* **141**: 1686-92.

Ray, A. and Ray, B. K. (1994a). Serum amyloid A gene expression under acute-phase conditions involves participation of inducible C/EBP-beta and C/EBP-delta and their activation by phosphorylation. *Mol Cell Biol* **14**: 4324-32.

Ray, B. K. and Ray, A. (1994b). Expression of the gene encoding alpha 1-acid glycoprotein in rabbit liver under acute-phase conditions involves induction and activation of beta and delta CCAAT-enhancer-binding proteins. *Eur J Biochem* **222**: 891-900.

Robert, F., Douziech, M., Forget, D., Egly, J. M., Greenblatt, J., Burton, Z. F. and Coulombe, B. (1998). Wrapping of promoter DNA around the RNA polymerase II initiation complex induced by TFIIF. *Mol Cell* **2**: 341-51.

Ron, D. and Habener, J. F. (1992). CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant- negative inhibitor of gene transcription. *Genes Dev* **6**: 439-53.

Ross, S. E., Erickson, R. L., Hemati, N. and MacDougald, O. A. (1999). Glycogen synthase kinase 3 is an insulin-regulated C/EBPalpha kinase. *Mol Cell Biol* **19**: 8433-41.

Scott, G. K., Davies, P. L., Kao, M. H. and Fletcher, G. L. (1988). Differential amplification of antifreeze protein genes in the pleuronectinae. *J Mol Evol* **27**: 29-35.

Scott, G. K., Hew, C. L. and Davies, P. L. (1985). Antifreeze protein genes are tandemly linked and clustered in the genome of the winter flounder. *Proc Natl Acad Sci U S A* **82**: 2613-7.

Scott, W. B. and Scott, M. G. (1988). Atlantic fishes of Canada, University of Toronto Press.

Sebastiani, G., Durocher, D., Gros, P., Nemer, M. and Malo, D. (1995). Localization of the Catf1 transcription factor gene to mouse chromosome 19. *Mamm Genome* **6**: 147-8.

Shamblott, M. J., Cheng, C. M., Bolt, D. and Chen, T. T. (1995). Appearance of insulin-like growth factor mRNA in the liver and pyloric ceca of a teleost in response to exogenous growth hormone. *Proc Natl Acad Sci U S A* **92**: 6943-6.

Shepherd, B. S., Sakamoto, T., Nishioka, R. S., Richman, N. H., 3rd, Mori, I., Madsen, S. S., Chen, T. T., Hirano, T., Bern, H. A. and Grau, E. G. (1997). Somatotropic actions of the homologous growth hormone and prolactins in the euryhaline teleost, the tilapia, Oreochromis mossambicus. *Proc Natl Acad Sci U S A* **94**: 2068-72.

Shieh, S. Y., Stellrecht, C. M. and Tsai, M. J. (1995). Molecular characterization of the rat insulin enhancer-binding complex 3b2. Cloning of a binding factor with putative helicase motifs. *J Biol Chem* **270**: 21503-8.

Sicheri, F. and Yang, D. S. (1995). Ice-binding structure and mechanism of an antifreeze protein from winter flounder. *Nature* **375**: 427-31.

Slaughter, D. and Hew, C. L. (1982). Radioimmunoassay for the antifreeze polypeptides of the winter flounder (Pseudopleuronectes americanus): Seasonal profile and immunological cross-reactivity with other fish antifreezes. *Can J Biochem* **60**: 824-9.

Smeal, T., Hibi, M. and Karin, M. (1994). Altering the specificity of signal transduction cascades: positive regulation of c-Jun transcriptional activity by protein kinase A. *Embo J* **13**: 6006-10.

Sonnichsen, F. D., Davies, P. L. and Sykes, B. D. (1998). NMR structural studies on antifreeze proteins. *Biochem Cell Biol* **76**: 284-93.

Takiguchi, M. (1998). The C/EBP family of transcription factors in the liver and other organs. *Int J Exp Pathol* **79**: 369-91.

Tobe, K., Tamemoto, H., Yamauchi, T., Aizawa, S., Yazaki, Y. and Kadowaki, T. (1995). Identification of a 190- kDa protein as a novel substrate for the insulin receptor kinase functionally similar to insulin receptor substrate-1. *J Biol Chem* **270**: 5698-701.

Trautwein, C., Caelles, C., van der Geer, P., Hunter, T., Karin, M. and Chojkier, M. (1993). Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature* **364**: 544-7.

Trautwein, C., van der Geer, P., Karin, M., Hunter, T. and Chojkier, M. (1994). Protein kinase A and C site-specific phosphorylations of LAP (NF-IL6) modulate its binding affinity to DNA recognition elements. *J Clin Invest* **93**: 2554-61.

Umek, R. M., Friedman, A. D. and McKnight, S. L. (1991). CCAAT-enhancer binding protein: a component of a differentiation switch. *Science* **251**: 288-92.

Vaisius, A., Martin-Kearley, J. and Fletcher, G. L. (1989). Antifreeze protein gene transcription in winter flounder is not responsive to temperature. *Cell Mol Biol* **35**: 547-54.

Varga-Weisz, P. D., Wilm, M., Bonte, E., Dumas, K., Mann, M. and Becker, P. B. (1997). Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II [published erratum appears in Nature 1997 Oct 30;389(6654):1003]. *Nature* **388**: 598-602.

Velankar, S. S., Soultanas, P., Dillingham, M. S., Subramanya, H. S. and Wigley, D. B. (1999). Crystal structures of complexes of PcrA DNA helicase with a DNA substrate indicate an inchworm mechanism. *Cell* **97**: 75-84.

Vinson, C. R., Hai, T. and Boyd, S. M. (1993). Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. *Genes Dev* **7**: 1047-58.

Vinson, C. R., LaMarco, K. L., Johnson, P. F., Landschulz, W. H. and McKnight, S. L. (1988). In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. *Genes Dev* **2**: 801-6.

Wang, R., Zhang, P., Gong, Z. and Hew, C. L. (1995). Expression of the antifreeze protein gene in transgenic goldfish (Carassius auratus) and its implication in cold adaptation. *Mol Mar Biol Biotechnol* **4**: 20-6.

Wang, X. Z. and Ron, D. (1996). Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science* **272**: 1347-9.

Wegner, M., Cao, Z. and Rosenfeld, M. G. (1992). Calcium-regulated phosphorylation within the leucine zipper of C/EBP beta. *Science* **256**: 370-3.

Wiederrecht, G. J., Sabers, C. J., Brunn, G. J., Martin, M. M., Dumont, F. J. and Abraham, R. T. (1995). Mechanism of action of rapamycin: new insights into the regulation of G1-phase progression in eukaryotic cells. *Prog Cell Cycle Res* **1**: 53-71.

Wilson, C. J., Chao, D. M., Imbalzano, A. N., Schnitzler, G. R., Kingston, R. E. and Young, R. A. (1996). RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling. *Cell* **84**: 235-44.

Withers, D. J., Gutierrez, J. S., Towery, H., Burks, D. J., Ren, J. M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G. I., Bonner-Weir, S. and White, M. F. (1998). Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* **391**: 900-4.

Wong, I. and Lohman, T. M. (1992). Allosteric effects of nucleotide cofactors on Escherichia coli Rep helicase-DNA binding. *Science* **256**: 350-5.

Woodage, T., Basrai, M. A., Baxevanis, A. D., Hieter, P. and Collins, F. S. (1997). Characterization of the CHD family of proteins. *Proc Natl Acad Sci U S A* **94**: 11472-7.

Wu, C. T. (1993). Transvection, nuclear structure, and chromatin proteins. *J Cell Biol* **120**: 587-90.

Wu, K. J., Wilson, D. R., Shih, C. and Darlington, G. J. (1994). The transcription factor HNF1 acts with C/EBP alpha to synergistically activate the human albumin promoter through a novel domain. *J Biol Chem* **269**: 1177-82.

Yamanaka, R., Barlow, C., Lekstrom-Himes, J., Castilla, L. H., Liu, P. P., Eckhaus, M., Decker, T., Wynshaw-Boris, A. and Xanthopoulos, K. G. (1997a). Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proc Natl Acad Sci U S A* **94**: 13187-92.

Yamanaka, R., Kim, G. D., Radomska, H. S., Lekstrom-Himes, J., Smith, L. T., Antonson, P., Tenen, D. G. and Xanthopoulos, K. G. (1997b). CCAAT/enhancer binding protein epsilon is preferentially up-regulated during granulocytic differentiation and its functional versatility is determined by alternative use of promoters and differential splicing. *Proc Natl Acad Sci U S A* **94**: 6462-7.

Yamauchi, T., Tobe, K., Tamemoto, H., Ueki, K., Kaburagi, Y., Yamamoto-Honda, R., Takahashi, Y., Yoshizawa, F., Aizawa, S., Akanuma, Y., Sonenberg, N., Yazaki, Y. and Kadowaki, T. (1996). Insulin signalling and insulin actions in the muscles and livers of insulin-resistant, insulin receptor substrate 1-deficient mice. *Mol Cell Biol* **16**: 3074-84.

Yang, D. S., Sax, M., Chakrabartty, A. and Hew, C. L. (1988). Crystal structure of an antifreeze polypeptide and its mechanistic implications. *Nature* **333**: 232-7.

Zhang, Q., Wang, Y. C. and Montalvo, E. A. (1999). Smubp-2 represses the Epstein-Barr virus lytic switch promoter. *Virology* **255**: 160-70.