The skin-type antifreeze protein gene intron of the winter flounder is a ubiquitous enhancer lacking a functional C/EBPα binding motif

Ming Miao[a], Shing-Leng Chan[b], Choy L. Hew[b,*], Zhiyuan Gong[c]

[a] *Biochemistry Division, Research Institute, Hospital for Sick Children, University of Toronto, Toronto, Ont., Canada*
[b] *Departments of Laboratory Medicine and Pathobiology, and Biochemistry, 100 College Street, Room 351, Toronto, Ont. M5G 1L5, Canada*
[c] *School of Biologial Sciences, National University of Singapore, Singapore, Singapore*

Received 14 January 1998; revised version received 11 March 1998

Abstract The winter flounder antifreeze protein (AFP) intron contains a liver-specific enhancer (Element B) which was shown earlier to bind CCAAT/enhancer binding protein (C/EBP)α. In contrast, as demonstrated in the present studies, the intron of the skin-type AFP gene acted as a ubiquitous enhancer and contained a TA insertion at similar region to Element B (Element S) which destroyed its interaction with C/EBPα. Furthermore, a TA insertion of Element B by site-directed mutagenesis decreased its liver enhancer activity. The presence or absence of C/EBPα binding motifs in Element B and Element S, respectively, may provide a mechanism for their differential expression.

Key words: Winter flounder; Antifreeze protein; Skin-type; Liver-type; CCAAT/enhancer binding protein

1. Introduction

The winter flounder, *Pleuronectes americanus*, is a common teleost inhabiting the shallow seawater of the Atlantic coast of North America. It frequently encounters seawater temperatures below −0.8°C during the winter months. To avoid freezing to death, the animal produces antifreeze proteins (AFPs) to lower its plasma freezing temperature below that of the surrounding environment. These AFPs are able to bind to ice crystals, and inhibit ice crystal growth [1]. The winter flounder AFPs have been extensively characterized (for review, see [2]). These AFPs are synthesized from a multigene family of approximately 80 copies and occur in several isoforms. The most abundant AFP component (HPLC-6) found in the serum is a helical polypeptide of 37 amino acids rich in alanine, and its three-dimensional structure has been elucidated [3,4].

More recently, our laboratories have demonstrated that there are two distinct classes of AFPs in the winter flounder. HPLC-6 and several other AFPs isolated from the sera are produced individually from liver as a preproAFP encoded by the liver-type AFP genes. The second type of polypeptides, the skin-type AFPs, lack both the Pre and Pro sequences and are synthesized from a different set of AFP genes as mature proteins primarily in skin and other external tissues. Furthermore, these two sets of AFP genes are found to be differentially regulated by the environment and somatotropin [5]. Thus, the flounder is the only fish species presently known to contain both the extracellular, liver-type AFPs and the intracellular, skin-type AFPs [6].

The presence of two distinct AFPs within a single species of animal has raised many important questions on the relative role of these proteins in freezing protection, their evolutionary relationships, as well as their regulationary mechanisms. The tissue-specific expression of the liver-type AFPs is conferred by an enhancer element, Element B (from +303 to +322), in the only intron of the AFP gene [7]. Element B contains DNA binding motifs for CCAAT/enhancer binding protein (C/EBP)α, a liver enriched transcription factor, and a novel AP-1 binding protein, tentatively designated as the Antifreeze Enhancer binding Protein (AEP). The present investigation was undertaken to examine the molecular mechanisms controlling the expression of the skin-type AFP genes which are expressed in many tissues and in particular the contribution of C/EBPα, if any, in activating these genes.

2. Materials and methods

2.1. Plasmid construction

The F2 genomic clone encoding the skin-type AFP gene [8] was kindly provided by Dr. P. Davies, Queen's University. To generate constructs of the skin-type intron with chloramphenicol acetyltransferase (CAT) reporter gene, the 2.3-kbp *Pst*I fragment of the F2 genomic clone was subcloned into pBluescript. The 750-bp *Pst*I/*Hin*cII intron fragment from the 2.3-kbp 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 [7]. Constructs with the 750-bp F2 intron fragment in forward and reverse orientation were designated as F2In-fAFP-CAT and RF2In-fAFP-CAT, respectively. The In-fARP-CAT construct, which contains the complete 2A-7b liver-type intron, was generated as described previously [7]. The constructs were verified by DNA sequencing.

2.2. Cell culture and transient expression assay

Newborn rat keratinocytes (NBRK) were maintained in Dulbecco's modified Eagle'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 [9] and cells were harvested 48 h after transfection. The CAT assay was performed using a highly sensitive mixed-phase assay [10,11]. A β-galactosidase gene driven by SV40 early promoter was used in transfection and the β-galactosidase activity was determined [12] as the internal control. All transfection experiments were repeated at least three times and the pBLCAT3 with or without thymidine kinase promoter (TK) were used as positive and negative control, respectively. The fAFP-CAT activity was approximately 25% in NBRK cells, 10% in HeLa cells and 4% of TK-CAT activity in HepG2 cells.

*Corresponding author. Fax: (1) (416) 978-8802.
E-mail: choy.hew@utoronto.ca

Abbreviations: AFP, antifreeze binding protein; C/EBP, CCAAT/enhancer binding protein; AP-1, activator protein-1; AEP, antifreeze enhancer binding protein; CAT, chloramphenicol acetyltransferase

PII S0014-5793(98)00327-5

2.3. Gel retardation assay

Crude liver nuclear extract from male Sprague-Dawley rats (150–200 g) was prepared as described by Gorski et al. [13]. The protein concentration was approximately 8 μg/μl. Rat C/EBPα protein (pMal-C/EBP) was expressed from pMal-C/EBP-CRI construct (rat C/EBPα fused with maltose-binding protein, kindly provided by Dr. C. Mueller, Queen's University, Canada) by bacterial expression system. Single-stranded oligonucleotides were annealed and end-labeled using [γ-^{32}P]ATP and polynucleotide kinase. In the binding reaction, rat liver nuclear extract or bacterial expressed pMal-C/EBP 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. 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. OligoS: ATTTACATAATGTTTTACATCAGCACTTCCTG; OligoB: ATAATGTTTCATCAGCACTT; AP1 consensus: CGCTTGATGACTCAGCCGGA [14] and C/EBP consensus: CTAGGCATATTGCGCAATATGC [15]. All oligonucleotides were synthesized by the Biotechnology Service Centre, Hospital for Sick Children, Toronto. Antibody specific to C/EBPα was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.4. Site-directed mutagenesis

In vitro site-directed mutagenesis was performed by the method of Deng and Nickoloff [16]. The wild-type In(192–350)5′-fAFP-CAT plasmid was constructed as previously described [7]. The mutant mIn(192–350)5′-fAFP-CAT was constructed using In(192–350)5′-fAFP-CAT with a selective primer which converts the *Kpn*I site into *Bam*HI site (ATCGATCCCCGGaTcCCGAGCTCTCGAAAT) and a mutagenic primer which has a TA insertion in Element B (ATTTACATAATGTTTtaCATCAGCACTACTTCCTG). The mutation was confirmed by DNA sequencing.

3. Results

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 skin-type AFP gene, F2 [8], was compared with the liver-type AFP gene (2A-7b) (Fig. 1). Despite their distinct tissue expression patterns, the two types of AFPs had similar gene structures with two exons and a single intron. The sequence of the skin-type intron shared 95% identity with the liver-type intron except that it contained an extra 241-bp insertion at position +254 [8]. The region of skin-type intron corresponding to the Element B of the liver-type intron was designated as Element S (Fig. 1) and interestingly, a TA dinucleotide insertion was found within this element. Since Element B was known to mediate the enhancer activity of the liver-type intron [7], it is likely that there is a specific effect for the presence of the TA insertion in Element S of the skin-type AFP gene.

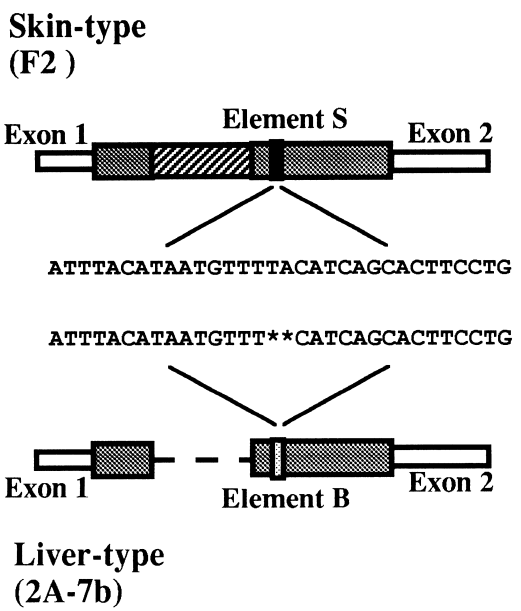

Fig. 1. Comparison of the skin-type and liver-type AFP genes. The approximate locations of the additional fragment are shown by hatched box and Element S and B are shown by solid box and dotted box, respectively. The asterisks indicate the gap in the DNA sequence. The figure is not to scale.

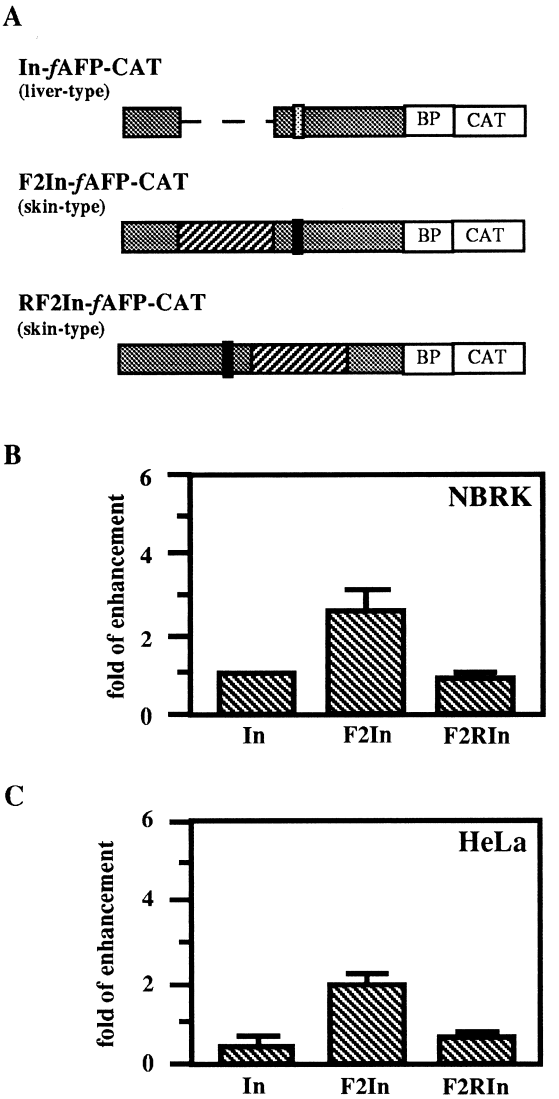

Fig. 2. The skin-type intron is a ubiquitous enhancer. A: Schematic illustration of the liver-type and the two skin-type constructs. The constructs were transfected into NBRK (B) and HeLa (C) cells and their activities normalized for transfection efficiency using β-galactosidase activity. The mean values and standard errors of the fold of enhancement relative to the activity achieved with the fAFP-CAT construct is shown.

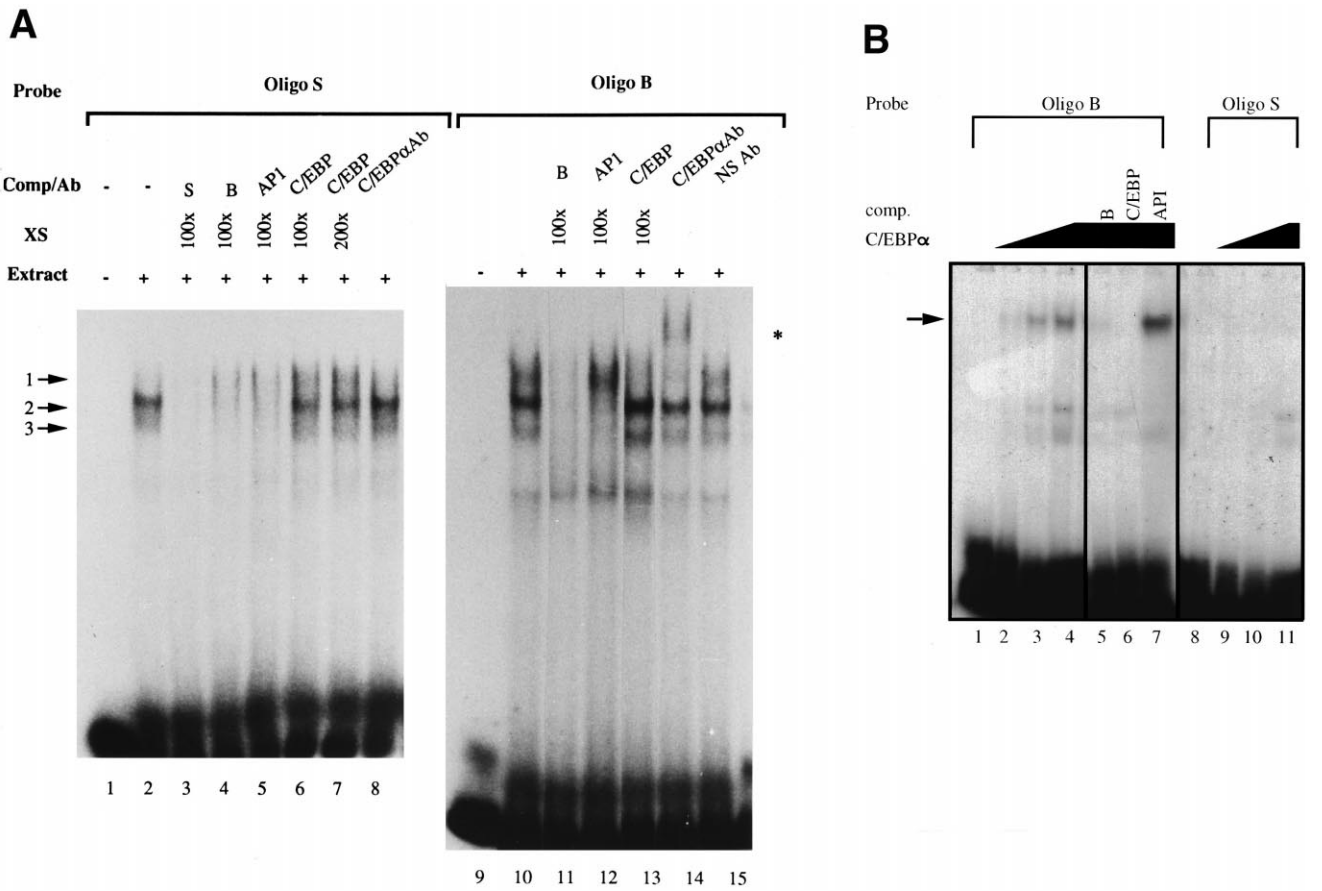

Fig. 3. Element S does not interact with rat C/EBPα. Gel retardation assays were carried out with rat liver nuclear extract (A) or recombinant rat C/EBPα (B). XS, molar excess of competitor; Ab, specific antibody; NS, non-specific antibody. Supershifted band is indicated by an asterisk.

3.2. *The intron of the skin-type AFP gene acts as a ubiquitous enhancer*

To study whether the skin-type intron, like the liver-type intron, also has the transactivation activity, the F2In-*f*AFP-CAT and RF2In-*f*AFP-CAT constructs which contain the skin-type F2 intron in the natural or reverse orientation, respectively, upstream of the *f*AFP-CAT, were constructed (Fig. 2A). The transient expression assay in NBRK revealed that the F2In-*f*AFP-CAT exhibited a three fold enhancer activity (Fig. 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 cells. Significant enhancer activity was also observed using the skin-type intron in the HeLa (cervical carcinoma) cells (Fig. 2C). In contrast, the liver-type intron exhibited no transactivation activity in both NBRK and HeLa cells (Fig. 2B,C). 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 more widely distributed.

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 extract (Fig. 3A). In the control experiment using Element B as probe (lanes 9–15), a specific band was competed out by excess of unlabeled Element B (lane 11) or the oligonucleotide containing the C/EBP binding site (lane 13), and was supershifted by antibody specific to rat C/EBPα (lanes 14 and 15). In contrast, though Element S was able to form DNA-protein complex such as the AP1 complex with the rat liver extract, 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 (lanes 5–8). These data indicate that there is 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. 3B). The presence of recombinant C/EBPα protein was verified using Western analysis with antibody specific to C/EBPα (data not shown). Using the liver-type Element B as a probe, a shift was gradually formed with increasing amounts of extract (lanes 2–4). This specific band was competed out by excess of cold Element B or C/EBP consensus but not by AP1 consensus (lanes 5–7). On the other hand, there was no specific shift when Element S was used as probe

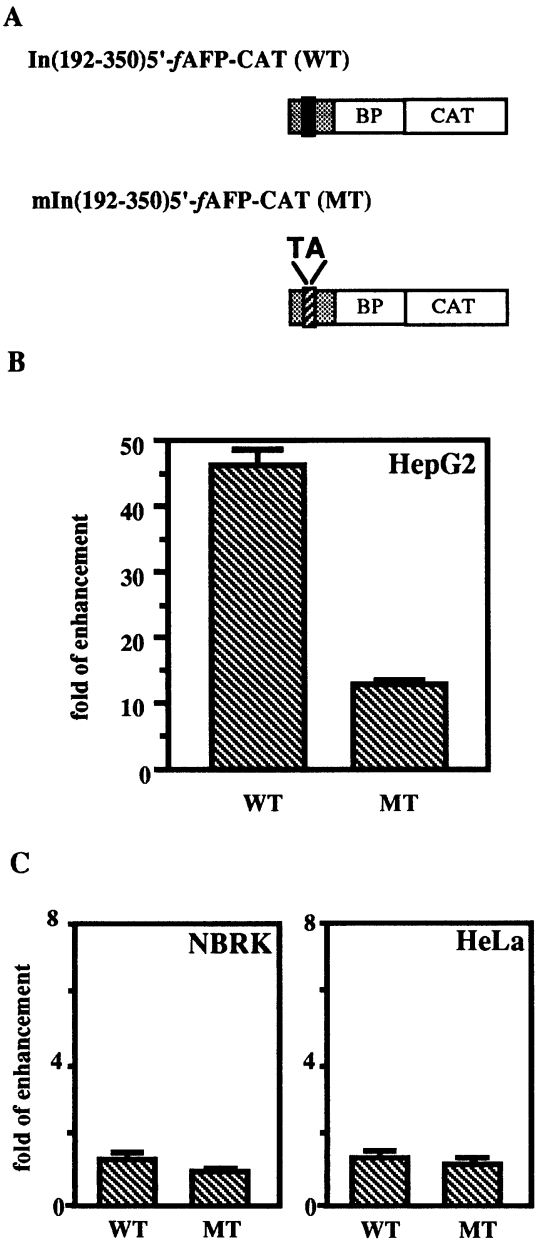

Fig. 4. TA dinucleotide insertion of Element B destroys its liver-specific enhancer activity. In(192-350)5′-fAFP-CAT (WT) and mutated mIn(192-350)5′-fAFP-CAT (MT) (A) were transfected into HepG2 (B) as well as NBRK and HeLa (C) cells. The enhancement relative to the activity achieved with the fAFP-CAT construct is shown. The mean values and standard errors of three independent experiments are given.

(lanes 9–11). Therefore, these results further confirm that Element S of the skin-type gene does not interact with the liver-enriched transcription factor C/EBPα.

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 (that is, the TA insertion to Element B) in transactivation, a mutated construct, mIn(192–350)5′-fAFP-CAT, was generated by site-directed mutagenesis to insert a TA dinucleotide directly into the Element B sequence of the In(192–350)5′-fAFP-CAT construct (Fig. 4A) [7]. Transient expression assays revealed that the TA insertion dramatically decreased the enhancer activity of the liver-type intron in HepG2 (human hepatoma) cells (Fig. 4B). Moreover, the enhancer activity of these two constructs was examined in two non-liver cell lines (Fig. 4C). Both the wild-type (WT) and the mutated (MT) constructs exhibited no significant degree in transactivation activity in HeLa and NBRK cells, implying that the sequences responsible for skin-type intron activity may reside outside the +192 to +350 region. Together, these data suggested that the TA dinucleotide insertion in Element S, which does not interact with C/EBPα, diminishes the liver-specific enhancer activity of the skin-type intron. In contract, the interaction between element B and C/EBPα mediates the liver-specific enhancer activity of the liver-type intron.

4. Discussion

Our study has demonstrated that the overall gene structure of the skin-type AFP and the liver-type AFP is similar. The major differences between the only intron of the skin-type AFP gene and the liver-type AFP gene include an insertion of TA dinucleotide in the skin-type gene corresponding to Element B of the liver-type gene 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 the 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 liver, stomach, intestine, kidney and spleen [6]. Transactivation of the skin-type intron was also observed in HepG2 cells (data not shown). Therefore, it is believed that the intron of the two AFP genes plays an important role in regulating their differential tissue distribution.

In addition, the observation of a TA insertion in the skin-type intron was of particular interest because it is located in the middle of Element B which is important for the enhancer activity of the liver-type intron. The insertion effectively destroys the C/EBPα binding specificity, as demonstrated by mobility shift assays using both the rat liver nuclear extract and 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 to its liver-specific transactivation ability. Since C/EBPα is present primarily in terminally differentiated cells such as hepatocytes and adipocytes [17], the presence of the C/EBPα binding motif in Element B is believed to mediate the liver-specificity of the liver-type AFP intron, while the loss of the interaction to C/EBPα in Element S might explain the broader tissue expression patterns of the skin-type AFP mRNAs. The disruption of C/EBP binding site has also been reported in the clotting factor IX promoter of hemophilia B patients that significantly reduced its transactivation activity [18]. Therefore, the participation of C/EBPα and its disruption by naturally occuring mutation may be a common mechanism in controlling differential gene expression in liver.

On the other hand, the additional 241-bp fragment in the skin-type intron may also be involved in its transcriptional regulation. It is possible that some other *cis*-acting sequences in this skin-type fragment interact with transcription factor(s) to dictate the skin-type AFP expression in external tissues. It

is interesting to note that, similar to F2, the other 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 [8]. Sequences similar to GRE and Oct-1 consensus binding site were found in these additional fragments of the skin-type AFP introns. The role of these additional fragments and factors involved, if any, are under investigation.

In addition to their distinct tissue distribution, the skin-type and liver-type AFP mRNAs are differentially regulated [5]. There is a 500–700-fold variation between the summer and winter months of the liver-type mRNA and it is inhibited by growth hormone. The skin-type AFP mRNA, on the other hand, only exhibits a 10-fold seasonal variation and is refractory to growth hormone treatment. Whether this is mediated by Element B and/or Element S and due to the fluctuation of the C/EBPα and/or other mechanisms remains to be investigated.

The presence of two distinct types of AFPs within a single species is intriguing. The skin-type AFP is structurally very similar to the liver-type but is significantly less active in 'antifreeze' activity [6]. However, whether the skin-type AFP confers selective freeze protection to subcellular organelles or cell milieu is unclear. Both the skin-type AFP and mRNA have been localized to the winter flounder gill cells (Murray et al., manuscript in preparation). It appears that the skin-type AFP might offer the first line of defense against ice invasion.

Acknowledgements: We thank Linda Mark for the preparation of the manuscript and Dr. C. Rosen for providing the NBRK cell line. This work is financially supported by the Medical Research Council of Canada (to C.L.H.). M.M. is a recipient of the University of Toronto Open Fellowship.

References

[1] Hew, C.L. and Yang, D.S.C. (1992) Eur. J. Biochem. 203, 33–42.
[2] Davies, P.L. and Hew, C.L. (1990) FASEB J. 4, 2460–2468.
[3] Yang, D.S.C., Sax, M., Chakrabartty, A. and Hew, C.L. (1988) Nature 333, 232–237.
[4] Sicheri, F. and Yang, D.S.C. (1995) Nature 375, 427–431.
[5] Gong, Z., King, M.J., Fletcher, G.L. and Hew, C.L. (1995) Biochem. Biophys. Res. Commun. 206, 387–392.
[6] Gong, Z., Ewart, K.V., Hu, Z., Fletcher, G.L. and Hew, C.L. (1996) J. Biol. Chem. 271, 4106–4112.
[7] Chan, S.L., Miao, M., Fletcher, G.L. and Hew, C.L. (1997) Eur. J. Biochem. 247, 44–51.
[8] Davies, P.L. and Gauthier, S.Y. (1992) Gene (Amsterdam) 112, 171–178.
[9] Graham, F.L. and van der Eb, A.J. (1973) Virology 52, 456–467.
[10] Neumann, J.R., Morency, C.A. and Russsian, K.O. (1987) BioTechniques 5, 445–448.
[11] Eastman, A. (1987) BioTechniques 5, 730–732.
[12] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning: A Laboratory Manual, pp. 1666–1667. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[13] Gorski, K., Carneiro, M. and Schibler, U. (1986) Cell 47, 767–776.
[14] Hai, T. and Curran, T. (1991) Proc. Natl. Acad. Sci. USA 88, 3720–3724.
[15] Osada, S., Yamamoto, H., Nishihara, T. and Imagawa, M. (1996) J. Biol. Chem. 271, 3891–3896.
[16] Deng, W.P. and Nickoloff, J.A. (1992) Anal. Biochem. 200, 81–87.
[17] Birkenmeier, E.H., Gwynn, B., Howard, S., Jerry, J., Gordon, J.I., Landschulz, W.H. and McKnight, S.L. (1989) Genes Dev. 3, 1146–1156.
[18] Crossley, M. and Brownlee, G.G. (1990) Nature 345, 444–446.