Vol. 206, No 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 5, 1995 Pages 387-392

THE ANTIFREEZE PROTEIN GENES OF THE WINTER FLOUNDER, *PLEURONECTUS AMERICANUS*, ARE DIFFERENTIALLY REGULATED IN LIVER AND NON-LIVER TISSUES

Zhiyuan Gong[a], Madonna J. King[b], Garth L. Fletcher[b] and Choy L. Hew[a1]

[a]Research Institute, Hospital for Sick Children, Departments of Clinical Biochemistry and Biochemistry, University of Toronto, Toronto, Canada

[b]Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, Newfoundland, Canada

Received October 28, 1994

The synthesis of winter flounder (*Pleuronectus americanus*) antifreeze protein (AFP) mRNAs in the liver is seasonally regulated by the pituitary gland. With the recent discovery that AFP mRNAs are also present in several non-liver tissues, the aim of the present investigation was to compare the regulatory mechanisms of AFP genes in liver and non-liver tissues. Northern blot analyses indicate that the level of liver AFP mRNA undergoes a several hundred fold difference between the winter and summer months, while AFP mRNAs from gills and kidneys exhibit only a modest 5-10 fold seasonal variation. As expected, the liver AFP mRNA in the hypophysectomized fish was increased by over 40 fold. However, no significant increase was observed for the non-liver AFP mRNAs upon hypophysectomy. These investigations suggest that AFP mRNAs in liver and non-liver tissues are differentially regulated.

The Newfoundland winter flounder (*Pleuronectus americanus*) produces a class of alanine-rich, α-helical antifreeze proteins (AFPs) to survive in the subzero water temperatures during the winter months (1). The AFPs appear in the blood in November when the seawater temperature is approximately 4°C to 6°C. The proteins reach a peak value of 1-10 mg/ml during the coldest winter months with seawater temperatures of 0° to -1.5°C and decline to minimal levels in summer months when the temperature rises above 0°C (2). Concomitant changes of the liver AFP mRNA occur over the same period with its concentration ranging from a 0.5% of the total liver RNA in the winter to 0.0007% during the summer (3).

It has been demonstrated that the seasonal accumulation of AFP is regulated primarily by photoperiod acting through the central nervous system on the pituitary gland (4). Hypophysectomy, or removal of the pituitary, of the winter flounder during the summer months resulted in a significant increase of AFP mRNA in the liver and antifreeze activity in the plasma (5). Recently, we have found that, in addition to the liver transcript, a substantial amount of AFP mRNA was also detected in many other tissues, notably the skin, scales and gills (6). To address

[1]Corresponding author: Dr. Choy L. Hew, Fax :416-978-8802.

0006-291X/95 $5.00

the question as to whether these non-liver AFP mRNAs are regulated in a similar fashion as the liver AFP mRNAs, we have examined the seasonal accumulation of AFP mRNAs in several selected non-liver tissues and the effect of hypophysectomy on AFP mRNAs in these tissues as compared to liver. Our investigations suggest that the liver and non-liver AFP mRNAs are differentially regulated.

MATERIALS AND METHODS

Collection of animals--The winter flounder (*Pleuronectus americanus*) were collected from Conception Bay, Newfoundland and maintained in sea water aquaria at seasonally ambient photoperiod and temperature. Two fish were sacrificed each month (February 22, March 28, April 19, June 12, July 26, September 3, October 17, December 3, 1991 and January 14, 1992) and the various tissues were removed immediately, frozen in liquid nitrogen, and kept at -70°C prior to RNA isolation.

Hypophysectomy--Hypophysectomy and sham operations were conducted in June as previously described (7).

RNA isolation and Northern blot hybridization--Total RNA was isolated from various tissues by the acid guanidium thiocyanate-phenol-chloroform extraction method (8) as modified (6). Formaldehyde agarose gel electrophoresis of RNA was performed as previously described (9). Conditions for RNA blotting and hybridization were described previously (6). The probe used was pkenc17, which encodes HPLC-6, kindly provided by Dr. P. Davies of Queen's University, Kingston. For quantitation, the major radioactive bands (~0.7 kb) were excised and counted using a liquid scintillation counter.

RESULTS

It has been established that the level of flounder AFP mRNAs in the liver varies seasonally with the highest level in the winter months and the lowest in the summer months (3). We have previously demonstrated that AFP mRNAs in the winter flounder, although predominantly expressed in the liver, are present in several non-liver tissues (6). Whether these non-liver AFP mRNAs are similarly regulated is unknown. In order to compare the seasonal accumulation of AFP mRNAs in both the liver and non-liver tissues, flounders were collected throughout the year with two fish selected each month . Total RNA was extracted from the liver, gill and kidney to study the seasonal expression of AFP mRNA. The relative level of AFP per μg of RNA in winter fish has been estimated to be 100, 4.5, and 0.3 for liver, gill and kidney respectively, representing abundant, moderate and low AFP-expressing tissues (6). Northern blot analysis of these RNA samples are shown in Fig. 1. Quantitation of these hybridization data is summarized in Table 1. It is interesting to note that the gill (Panel B) and kidney (Panel C) AFP mRNAs, like the liver AFP mRNA (Panel A), undergo similar seasonal variations with their highest levels in the winter (December) and lowest in the summer (July). However, the variations of these two non-liver AFP mRNAs are much less dramatic than that of the liver AFP mRNA, being about 6 and 10-fold in kidneys and gills, respectively; in contrast, the liver AFP mRNA exhibits as much as a 700 fold seasonal variation. The moderate seasonal variation of AFP mRNAs in other non-liver tissues such as heart, spleen, intestine and scales can also be concluded from Fig. 2 by the comparison of sham-operated summer fish (Panel C and D) with the winter fish (Panel E).

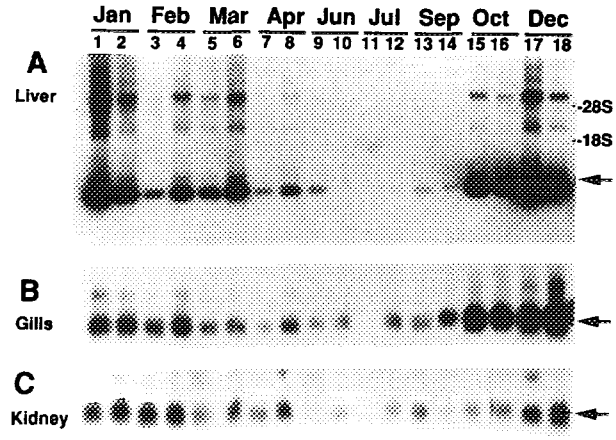

Figure 1. Northern blot analysis of the seasonal accumulation of AFP mRNAs in livers (A), gills (B) and kidneys (C). 18 fish (numbered 1-18) were collected throughout the year and two fish from each of the selected months as indicated at tops of the autoradiograms. Total RNA was prepared from these fish and analysed as described in Materials and Methods. Equal amounts of RNA (5 μg for liver RNA and 30 μg for gill and kidney RNAs) were loaded in each lane. The final wash after hybridization was 0.03M Na^+ at 68°C for liver RNAs and 0.3M Na^+ at 65°C for gill and kidney RNAs. As different amounts of RNAs were loaded for different tissues and the time for autoradiography varies for the three blots, no direct comparison should be made between the blots. Only the major AFP mRNA signals (~0.7kb), indicated by arrows, are shown in Panels B and C.

The accumulation of the liver AFP mRNAs is negatively regulated by growth hormone produced in the pituitary gland (4). Removal of the pituitary, or hypophysectomy, during the summer months causes an increase in the liver AFP mRNAs to a level comparable to the winter level (5). The induction of the liver AFP mRNA by hypophysectomy is due to an increase in AFP gene transcription (10). In order to further compare the regulations of liver and non-liver AFP mRNAs, three flounders were hypophysectomized and two were sham-operated in June and sacrificed in August or September for RNA preparation. Total RNA was prepared from

Table 1
Quantitation of the Annual Accumulation of AFP mRNAs in the Liver, Gill and Kidney

	Liver	Gill	Kidney
January	44.0	36.3	88.7
February	10.9	29.1	106.9
March	16.9	16.0	51.9
April	0.9	13.5	53.1
June	0.9	11.4	24.4
July	0.3	10.4	17.8
September	1.7	19.2	29.5
October	20.0	89.6	35.8
December	100.0	100.0	100.0

The levels of AFP mRNA in December were arbitrarily set at 100.0.

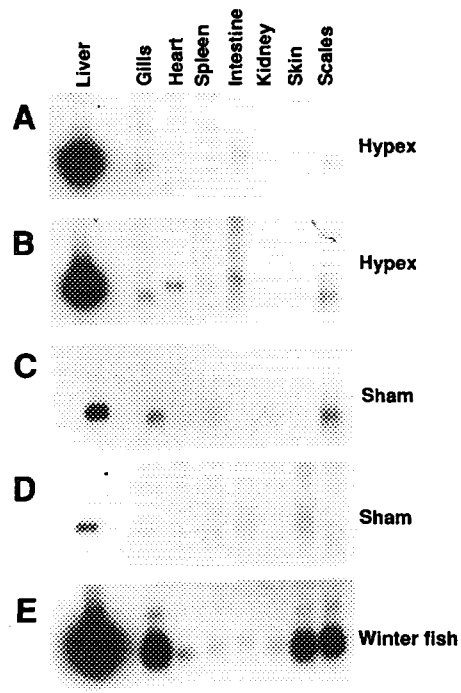

Figure 2. Tissue distribution of AFP mRNAs in hypophysectomized (A, B), sham-operated (C, D) and a control winter fish (E). Total RNA was prepared from 8 different tissues from each individual fish as indicated above each lane. 1 μg of liver RNA and 10 μg of each non-liver RNA were loaded each lane. All blots were washed at the stringency of 0.3M Na^+ and 65°C. Only the major AFP mRNA signals (~0.7kb) are shown.

livers, gills, hearts, spleens, intestines, kidneys, skins, and scales. As a control, total RNA was also prepared using the same set of tissues from a winter fish (collected in January). The tissue distribution of AFP mRNA in these fish was examined by Northern blot hybridization as shown in Fig. 2. Consistent with our earlier study, AFP mRNA id rxpressed predominantly in the liver (6). In addition, AFP mRNAs were also easily detectable in some peripheral tissues such as gills, intestine, skin and scales (panel E). In hypophysectomized fish (Panels A and B), the level of liver AFP mRNAs was significantly induced compared to sham-operated animals (Panels C and D). In contrast, there is little corresponding increase of AFP mRNA in non-liver tissues. Quantitation of these hybridization data, as shown in Table 2, indicated that on average, the

Table 2
Fold Induction of AFP mRNAs by Hypophysectomy

Liver	42.2 ± 14.9
Gill	2.5 ± 0.9
Heart	1.9 ± 1.1
Spleen	0.9 ± 0.4
Intestine	2.6 ± 1.9
Kidney	1.9 ± 1.4
Skin	0.6 ± 0.3
Scales	2.2 ± 1.6

levels of liver AFP mRNAs were induced by 42-fold while non-liver AFP mRNAs increased by only 1 to 2-fold, as compared to the sham-operated fish. These observations further suggest that AFP mRNAs in liver and non-liver tissues response differently to the photoperiod and hormonal regulation.

DISCUSSION

The seasonal variation of the liver AFP mRNA concentration is known to be regulated through the hypothalmo-hypophyseal axis (4). Long day lengths (>14 hrs.) significantly depress the AFP mRNA in the liver and delay its appearance in winter months (11). Hypophysectomy results in a significant increase in the liver AFP mRNA (5), while injection of the pituitary extract or purified growth hormone in the hypophysectomized fish inhibits increase (4). Using an RNA run-on assay with isolated liver nuclei, Vaisius et al. (10) have found that growth hormone blocks the transcription of the AFP genes in hypophysectomized flounders. Therefore, a model has been elaborated for the seasonal regulation of AFP gene expression. Based on this model, the central nervous system, with the loss of long day lengths in the fall, inhibits the release of growth hormone, thus allowing AFP gene transcription to proceed. In the spring, growth hormone is released from the pituitary and the AFP gene transcription is blocked (4).

However, this model is based on the studies only for the liver AFP mRNA. In the present study, we have determined the annual accumulation of AFP mRNA in some non-liver tissues and observed a modest seasonal variations for these non-liver AFP mRNAs. Furthermore, hypophysectomy had no apparent effect on non-liver AFP mRNAs. These observations suggest that the regulation of AFP mRNA is different in the liver and non-liver tissues. So far, only two environmental factors are known to regulate the level of AFP mRNA. The first one is the photoperiod acting through the hypothalmo-hypophyseal axis and the second one is temperature. While it is apparent that photoperiod is not a major factor in the regulation of non-liver AFP mRNA, temperature may be important in controlling the accumulation of non-liver AFP mRNAs. There is no evidence that low temperature stimulates AFP gene transcription either in an *in vitro* RNA run-on analysis (10) or *in vivo* promoter analyses of the AFP gene (Gong and Hew, unpublished observation). Thus, the effect of temperature on AFP gene expression may be posttranscriptional at the level of RNA stability with low temperature increasing the half-life of AFP mRNA, in contrast to some heat shock protein mRNAs which are stabilized at high temperature (12). Consistent with this, the seasonal accumulation of the liver AFP mRNAs requires low temperatures (13) while higher temperatures reduces the amount of AFP mRNAs (10). By acclimating flounders to different temperature, Price et al. (14) observed that more liver AFP mRNAs were accumulated at cold temperatures such as 4^{o}C. Recently, Kenward et al. (15) reported that AFP mRNA accumulation was posttranscriptionally enhanced at cold temperature in transgenic plants expressing the winter flounder AFP genes.

In the winter flounder, there are 40-50 copies of AFP genes in the genome. Two third of the genes are tandemly repeated and the rest are randomly dispersed in the genome (16). The discovery that AFP genes are also expressed in some non-liver tissues has raised the question of

whether this multigene family is differentially expressed in different tissues. The facts that different sizes of AFP mRNAs were detected in livers and other tissues (Fig. 2) and that the liver and non-liver AFP mRNAs hybridized at different stringencies to a AFP cDNA probe isolated from the liver (Gong and Hew, unpublished observations) suggest that the liver and non-liver AFP mRNAs are not transcribed from identical sets of genes. We postulate that there are two groups of AFP genes in the genome of the winter flounder. The first group is primarily expressed in the liver and is subjected to the regulation by the hypothalmo-hypophyseal axis. This group of AFP gene is most likely those localized in the tandem repeats since the AFP cDNA clones isolated so far from the liver match the tandemly repeated genes, encoding the two major AFP components (HPLC-6 and HPLC-8) found in the serum (17). The other group of AFP genes are primarily expressed in the non-liver tissues and may be those genes dispersed in the genome. The latter group of AFP genes apparently is not regulated by the pituitary hormone(s). Another interesting question remaining unanswered is whether the pituitary hormone-dependent regulation of AFP genes is tissue-specific (only in the liver) or gene-specific (only for certain AFP genes). These possibilities are now being examined.

ACKNOWLEDGMENTS

We thank Dr. Vanya Ewart for proof-reading the manuscript. This work is financially supported by Medical Research Council of Canada (to C.L.H.) and Natural Sciences and Engineering Research Council of Canada (to G.L.F).

REFERENCES

1. Davies, P.L. and Hew, C.L. (1990) FASEB J. 4, 2460-2468.
2. Fletcher, G. L. (1977) Can. J. Zool. 55, 789-795.
3. Pickett, M. H., Hew, C. L. and Davies, P. L. (1983) Biochim. Biophys. Acta, 739, 97-104.
4. Fletcher, G. L., Idler, D. R., Varsius, A. and Hew, C. L. (1989) Fish Physiol. Biochem. 7, 387-393.
5. Fourney, R. M., Fletcher, G. L. and Hew, C. L. (1984) Gen. Comp. Endocrinol. 54, 392-401.
6. Gong, Z., Fletcher, G. L. and Hew, C. L. (1992) Can. J. Zool. 70, 810-814.
7. Campbell, C.M., and Idler, D.R. (1976) Gen. Comp. Endocrinol. 28, 143-150.
8. Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
9. Gong, Z. (1991). Biotechniques, 12, 74-76.
10. Vaisius, A., Martin-Kearley, J. and Fletcher, G. L. (1989) Cell. Mol. Biol. 35, 547-554.
11. Fourney, R. M., Fletcher, G. L. and Hew, C. L. (1984) Can. J. Zool. 62, 28-33.
12. Lindquist, S., and Petersen, R. (1990) Enzyme 44, 147-166.
13. Fletcher, G. L. (1981) Can. J. Zool. 59, 193-201.
14. Price, J.L., Gourlie, B.B., Lin, Y., and Huang, R.C.C. (1986) Physiol. Zool. 59, 679-695.
15. Kenward, K.D., Artschuler, M., Hildebrand, D., and Davies P.L. (1993) Plant Mol. Biol. 23, 377-385.
16. Scott, G. K., Hew, C. L. and Davies, P. L. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 2613-2617.
17. Davies, P. L. (1992) Gene. 112, 163-170.