Low temperature persistence of type I antifreeze protein is mediated by cold-specific mRNA stability

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Abstract In winter flounder, the levels of type I antifreeze protein (AFP) and its mRNA vary seasonally by as much as 1000-fold. Elevated levels in the fall are prompted by the loss of long day-lengths, while higher spring temperatures correlate with AFP clearance. We have investigated the role of temperature on AFP accumulation using transgenic *Drosophila melanogaster* by expressing multiple AFP genes under control of the heat-inducible *hsp70* promoter. AFP and AFP mRNA persisted far longer in flies reared at 10°C compared to 22°C. This difference appears to be mediated by cold-specific mRNA stability since no such temperature effect was observed with either an endogenous heat-inducible mRNA or a constitutively expressed mRNA.

Key words: Antifreeze protein; mRNA turnover; Cold stability; Transgenic *Drosophila*; Heat shock

1. Introduction

Some fishes of the North Atlantic have evolved to live in cold or even ice-laden seawater. As an example, the winter flounder (*Pleuronectes americanus*) of Newfoundland waters are exposed to temperatures ranging between 14°C in summer and −1.4°C in winter [1]. To help prevent serum freezing at subzero temperatures, these fish synthesize an alanine-rich, α-helical antifreeze protein (AFP), which is able to bind to nascent ice crystals, and thereby inhibit their growth [2]. The result is a net depression of the serum freezing point, while the melting point is virtually unchanged, a phenomenon termed thermal hysteresis.

Flounder AFP (type I) is synthesized on a seasonal basis. It accumulates in the serum during autumn in response to the loss of long day-lengths, mediated through a decrease in the amount of growth hormone secreted by the pituitary gland [3]. AFP message levels follow a similar temporal pattern, representing an estimated 0.0007% of the total liver mRNA in August but as much as 0.5–1% in January [4,5]. Translational control mechanisms may also play a role in the seasonal accumulation of type I AFP, since a 40% increase in liver $tRNA^{Ala}$ levels was observed in the winter compared to the summer, while other tRNAs experienced no such fluctuations [6]. Additionally, it was shown that the optimum temperature for winter flounder alanyl-tRNA synthetase ranged between 0 and 5°C, while other flounder tRNA synthetases had temperature optima between 20 and 30°C [6].

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We are using *Drosophila melanogaster* as a whole animal model system for the transgenic expression of AFPs. Representative genes for each of the three fish AFP types: flounder type I AFP [7], sea raven type II AFP [8] and wolffish type III AFP [9] have been expressed in this organism. Despite the use of the same *Drosophila* yolk protein promoter to drive transgene expression, there was a marked difference in the accumulation of AFPs. The globular AFPs (types II and III) were well produced and gave rise to accurately measurable levels of hemolymph thermal hysteresis, while type I AFP could not be detected, either by this technique or by immunoblotting [10]. Type I AFP was synthesized by placing its gene under the transcriptional control of the heat-inducible *Drosophila hsp70* promoter [7]. Even with this construct, the AFP accumulation was insufficient to result in measurable hemolymph thermal hysteresis.

Although there is no evidence that temperature regulates transcription of the winter flounder AFP gene [11], its effects at the post-transcriptional level have not been evaluated. Here, we report that the clearance rate of type I AFP in transgenic flies is dramatically reduced at 10°C compared to 22°C, and that this is largely mediated by the instability of its mRNA at the higher temperature. We speculate that this property of the mRNA could modulate the level of type I AFP in flounder as a function of seawater temperature.

2. Materials and methods

2.1. *Drosophila melanogaster* stocks

The transgenic fly stock HsAFP-8 has six stably integrated copies of a *Drosophila hsp70*/winter flounder AFP gene chimera in females, five in males, and has been described previously [12]. Mutant ry^8 flies [13], which were the injection stock used to generate type I AFP transgenics, served as non-transgenic controls in all experiments.

2.2. *AFP persistence trials*

Flies were placed in glass bottles and incubated at 36.5 ± 0.2°C for 2 h to induce the heat shock promoter, then allowed to recover for 2 h at 22°C (room temperature) to allow AFP accumulation. The flies were then transferred to bottles containing a 10% yeast-sucrose medium [14] and placed at either 22°C or in a water bath at 10.0 ± 0.2°C. Hemolymph was isolated as previously described [9] and hemocytes were pelleted by centrifugation (10,000 × *g*, 4°C, 5 min). SDS-PAGE and immunoblotting were performed following standard procedures [15]. Rabbit anti-type I AFP antiserum was obtained through immunization with synthetic type I AFP.

2.3. *RNA analysis*

Flies subjected to the same heat shock/recovery routines described in section 2.2, were collected following incubation at either room temperature (22°C) or 10.0 ± 0.2°C. *Drosophila* total RNA was isolated following the method of Ashburner [16]. RNA samples were electrophoresed using formaldehyde as a denaturant in a 1.0% agarose gel by the method of Lehrach et al. [17], with the exception that the formaldehyde concentration was 660 mM. Probe preparation and blot stripping were according to standard procedures [15].

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3. Results and discussion

3.1. Synthesis of type I AFP in transgenic flies

When a stably transformed fly line containing five (male) or six (female) chimeric *Drosophila hsp70*/winter flounder AFP transgenes was subjected to heat shock, flounder type I proAFP was secreted into the hemolymph as shown by immunoblot analysis (Fig. 1). A 6 kDa band in the sample from heat-shocked HsAFP-8 flies (Fig. 1, lane c) co-migrated with the type I proAFP standard (lane a), both of which had small satellite bands underneath. No such protein was visible in hemolymph from HsAFP-8 flies not subjected to heat-shock (lane b), or in hemolymph from heat-shocked (lane e) or untreated (lane d) control ry^s flies. All of the hemolymph samples revealed a number of slow-migrating bands, well removed from the synthesized AFP. It can be concluded that these represent hemolymph proteins which cross-reacted with the polyclonal anti-type I AFP antibodies used. Although AFP was detected by this immunoblotting procedure, the accumulation of this protein was so low that no hemolymph thermal hysteresis could be detected.

3.2. Cold persistence of type I AFP

The levels of type I AFP observed in the transgenic flies were extremely low considering that the *hsp70* sequence is one of the strongest *Drosophila* promoters described [18]. However, the temperature used to rear flies (22°C) is far higher than that encountered by winter flounder in Newfoundland waters [1], and it is possible that there was a loss of secondary structure in the AFP at 22°C, which increased its susceptibility to proteolysis. Indeed, it has been shown that type I AFP is 85% α-helical at −1°C, but only 47% helical when the temperature is raised to 25°C [19]. To investigate the effect of temperature on AFP turnover, we heat-shocked HsAFP-8 flies and then maintained them at either 10 or 22°C for varying lengths of time. Immunoblot analysis of hemolymph samples (Fig. 2) revealed

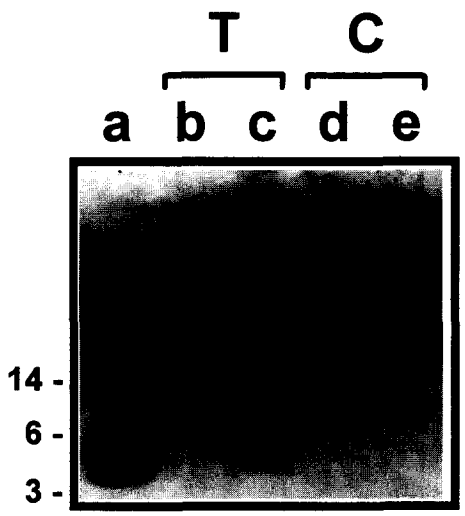

Fig. 1. Immunoblot analysis of hemolymph. Hemolymph samples (1 μl/lane) were obtained from HsAFP-8 flies (T) that were either untreated (lane b), or had been given a heat-shock (lane c) as described in section 2. Hemolymph from non-transgenic ry^s flies (C), untreated (lane d) and heat-shocked (lane e), was similarly analyzed. Purified winter flounder type I proAFP (10 ng) was run as a standard (lane a). Molecular masses (kDa) of the protein markers are indicated on the left.

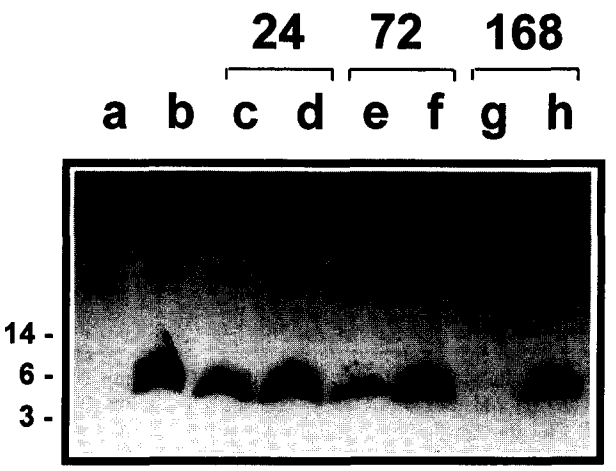

Fig. 2. Immunoblot analysis of hemolymph from heat-shocked flies reared at two temperatures. Samples (1 μl) were from untreated HsAFP-8 flies (lane a), heat-shocked HsAFP-8 flies immediately following recovery from heat-shock (lane b), maintained at 22°C for 24 h (lane c), 72 h (lane e), 1 week (lane g), or at 10°C for 24 h (lane d), 72 h (lane f), 1 week (lane h) following heat-shock treatment. Molecular masses (kDa) of the protein markers are indicated on the left.

that AFP levels were comparable for flies reared for 24 h at either temperature (lanes c and d). By 72 h, however, the amount of AFP in the 22°C sample (lane e) was far less than that in the 10°C sample (lane f). At 1 week, the 6 kDa protein was no longer visible at 22°C (lane g), while a strong band remained at 10°C (lane h). These results suggest that type I AFP persists longer at the colder temperature. Interestingly, it appears that the slower-migrating protein in the AFP doublet, observed shortly after heat shock (lane b), disappears over time (lanes d, f and h). Whether it is degraded or processed into the smaller species is unknown. These findings on the possible temperature dependence of AFP stability are consistent with experiments where a winter flounder AFP gene was expressed in tobacco [20]. In this system a constitutively active cauliflower mosaic virus 19S promoter was used to drive transgene transcription. Type I AFP was not detected at the normal cultivation temperature (25°C) but did accumulate if the plants were incubated for more than a day at 4°C.

3.3. Cold-specific stability of type I AFP mRNA

To determine the role of AFP mRNA levels in the observed cold-dependent persistence of AFP, HsAFP-8 flies were first heat shocked and then transferred to 10 or 22°C prior to the isolation of total RNA. The resulting northern blot was probed with flounder AFP cDNA (Fig. 3A), and as observed previously [7], three distinct AFP transcripts were produced by the transgenic flies (lane b) in response to the multiple polyadenylation signals in the flounder AFP gene. 24 h after heat shock there was already a considerable decrease in the levels of the AFP message in flies kept at 22°C (lane c), relative to those kept at 10°C for the same time interval (lane d). By 72 h, the AFP transcripts were barely visible in the 22°C sample (lane e), while the same three clear bands could still be seen in the 10°C lane (lane f). To verify that equal amounts of RNA were loaded in each lane, the blot from Fig. 3A was stripped and reprobed with chicken α-tubulin cDNA (Fig. 3C). The signal from the *Drosophila* tubulin mRNA was uniform across the blot.

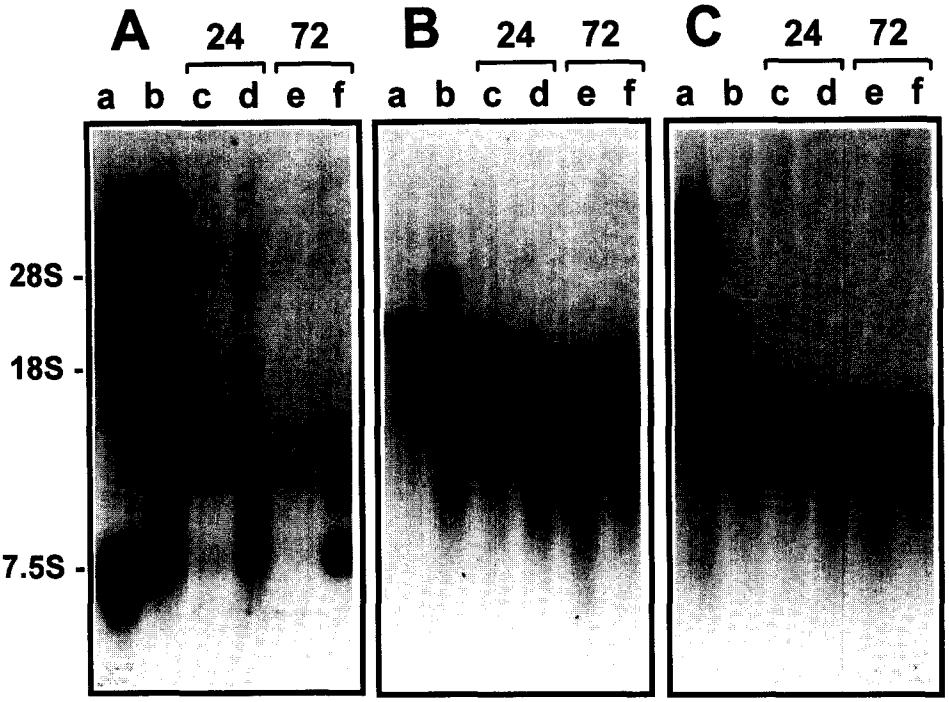

Fig. 3. Northern analysis of total RNA from heat-shocked flies reared at two temperatures. Total RNA (15 μg) from winter flounder liver (lane a), and from heat-shocked HsAFP-8 flies collected immediately following heat-shock recovery (lane b), or after maintenance at 22°C for 24 h (lane c) or 72 h (lane e), and after maintenance at 10°C for 24 h (lane d) or 72 h (lane f) was electrophoresed as described in section 2. The resultant blot was probed with winter flounder AFP cDNA (panel A), stripped and reprobed with chicken a-tubulin cDNA (panel C), stripped once more and reprobed with the *Drosophila hsp83* gene (panel B). RNA size markers are indicated at the left.

Our findings did not preclude the possibility that the lower AFP transcript turnover rate at 10°C relative to 22°C was due to a general metabolic slowdown at the lower temperature. We therefore examined the abundance of another heat shock mRNA under the same conditions. The northern blot was stripped again and reprobed with the heat-inducible *Drosophila hsp83* gene (Fig. 3B). This time the observed pattern of mRNA decay was dramatically different from that observed for the transgene. The *hsp83* message levels at 22 and 10°C were roughly equivalent after 24 h (lanes c and d), and after 72 h (lanes e and f) and, therefore, were independent of the maintenance temperature of the flies. These results strongly suggest that the cold persistence of type I AFP in transgenic *Drosophila* was, at least in part, mediated at the mRNA level. They do not rule out the possibility that AFP levels were additionally influenced by temperature-dependent protein stability. Indeed, similar conclusions were reached from the study in which a flounder type I AFP gene was expressed in transgenic tobacco at both 25 and 4°C [20]. The accumulation of type I AFP with time at 4°C was accompanied by a steady increase in AFP mRNA.

In light of the evidence that low temperatures per se do not stimulate AFP gene transcription either in vitro [11] or in vivo [21], Gong et al. [21] have speculated that low temperatures may act post-transcriptionally to stabilize AFP mRNA and thereby increase its half-life. This could account for several observations that link low temperatures with AFP mRNA accumulation in winter flounder [11,22,23]. Our results here with transgenic *Drosophila* provide direct evidence in a whole animal model system that the half-life of AFP mRNA is selectively and markedly reduced at moderately elevated temperatures. Taken together with the results from the transgenic tobacco study, they imply that the effect is mediated by the mRNA itself since it occurs in such different hosts (plant, insect and fish). It may well have evolved in flounder to delay AFP synthesis in the fall until water temperatures decline.

Few examples exist of temperature-dependent mRNA stability, the best known being the stabilization of certain heat shock mRNAs at high temperatures [24]. It will therefore be of great interest to determine whether the differences in type I AFP mRNA turnover observed here are mediated by RNA secondary structure, RNA-binding proteins, or some other mechanism.

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