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Purification and Characterization of Winter Flounder Antifreeze Peptide Messenger Ribonucleic Acid†

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ABSTRACT: The serum of winter flounder contains a group of small antifreeze peptides which lower the freezing point of their body fluids during the winter months. The poly(A)-containing mRNA coding for these peptides has been isolated from livers of the winter specimens. When the isolated antifreeze mRNA was analyzed by a denaturing polyacrylamide gel electrophoresis, at least two distinct bands ~450 nucleotides in length are visible. In a wheat germ cell-free protein synthetic system these mRNAs direct the synthesis of small peptides which can be precipitated by antisera against purified winter flounder antifreeze peptides. Full-length cDNA was synthesized from the isolated antifreeze mRNA by avian myeloblastosis reverse transcriptase. From the RNA excess hybridization kinetic analysis, there are probably three different mRNAs coding for the antifreeze peptides. Using the radioactive cDNA probe, it was estimated that 1% of the total RNA in liver of a January specimen is antifreeze mRNA. RNA from a summer specimen showed no significant hybridization even at high concentrations of RNA. These results indicate that the control of antifreeze peptide biosynthesis relies at least in part on the synthesis or degradation of translatable mRNA.

Fishes inhabiting ice-laden seawater often encounter the danger of freezing during the winter. Many families of cold-adapted fishes have evolved antifreeze compounds, either glycopeptides or peptides, in their body fluids to protect them from freezing (DeVries et al., 1970; Duman & DeVries, 1974a; Raymond et al., 1975). It was proposed that the antifreeze compounds might bind to the surface of ice crystals and prevent the water molecules from joining the ice lattice, thus lowering the freezing point of a solution (Raymond & DeVries, 1977; DeVries & Lin, 1977).

Three small antifreeze peptides have been isolated from the serum of winter flounder (Duman & DeVries, 1976; DeVries & Lin, 1977). They have very similar amino acid compositions and sequences. Peptide 3, which has a molecular weight of 3300, is composed of 24 alanine, 5 aspartate, 4 threonine, 1 serine, 2 leucine, and 1 lysine (DeVries & Lin, 1977). In addition to these amino acids, peptides 1 and 2 contain arginine and are probably slightly larger in size. There is a seasonal variation in the concentration of these peptides in the serum, with the highest concentration observed in January and the lowest in the summer months (Petzel et al., 1979). Acclimation studies have indicated that the biosynthesis of these peptides is probably regulated by both temperature and photoperiod (Duman & DeVries, 1974b), but the molecular mechanism underlying this regulation is not known.

Using an in vitro cell-free protein synthetic system, it was shown that livers of winter flounder collected in January contain translatable mRNA of a major peptide; livers from summer specimens do not contain such mRNA. This peptide

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was identified as the presursor(s) of the serum antifreeze peptides (Lin, 1979). In order to understand the cellular events which lead to the biosynthesis of antifreeze peptides under the influence of environmental factors such as temperature and photoperiod, it is necessary to isolate and characterize the mRNA coding for the antifreeze peptides. In this paper we describe the procedures for the purification of the antifreeze peptide mRNA from which a complementary deoxyribonucleic acid (cDNA) was synthesized. The cDNA probe was then used to quantitate the concentration of antifreeze mRNA relative to total RNA isolated from the winter and summer specimens.

Experimental Procedures

Specimen. Winter flounder were collected from Shinnecock Bay, Long Island, NY, by trawling. The livers were removed and frozen with dry ice. The frozen livers were stored at −70 °C until use.

Isolation of Total RNA from Livers of Winter Flounder. The frozen liver was lyophilized prior to the extraction of RNA. The total RNA was extracted from the liver according to procedures described previously (Lin, 1979). Lyophilized liver was ground to a fine powder with a mortar and pestle and extracted with equal volumes of phenol [saturated with 0.01 M Tris-HCl (pH 7.4), 0.5% $NaDodSO_4$,[1] 0.1 M NaCl, and 1 mM EDTA] and chloroform containing 4% isoamyl alcohol and Tris–$NaDodSO_4$–NaCl–EDTA buffer (Perry et al., 1972; Kemper, 1976). RNA in the final aqueous phase was precipitated with 2 volumes of ethanol at −20 °C overnight. The precipitate was dissolved in water, extracted with sodium acetate and EDTA in a final concentration of 3 M and 7.5 mM, respectively, and reprecipitated with ethanol.

Isolation of Poly(A)-Containing RNA from Total RNA. Total RNA was dissolved in a low-salt sample buffer (25 mM KOH–Hepes, pH 7.5, 10 mM EDTA, 1 mM EGTA, and 1% $NaDodSO_4$) and heated in a boiling water bath for 3 min and rapidly cooled to room temperature to disaggregate the RNA (Shapiro & Baker, 1977). LiCl of 5 M was added to the RNA solution (0.6 mg of RNA per mL) to a final concentration of 0.5 M LiCl. The RNA solution was applied to an oligo(dT)–cellulose column equilibrated with binding buffer (25 mM KOH–Hepes, pH 7.5, 10 mM EDTA, 1 mM EGTA, 1% $NaDodSO_4$, and 0.5 M LiCl). After the RNA solution was passed twice through the column, the poly(A)-containing RNA was eluted with 10 mM KOH–Hepes (pH 7.5), 1 mM EDTA, and 0.1% $NaDodSO_4$ and 1-mL fractions were collected. Fractions with high A_{260} absorption were pooled and precipitated with 3 volumes of ethanol in the presence of 20 μg/mL *Escherichia coli* tRNA and 0.23 M NaCl. The precipitate was collected and redissolved in sample buffer and passed through another oligo(dT)–cellulose column as described above.

Fractionation by Isokinetic Sucrose Gradient Centrifugation. Three to five A_{260} units of poly(A)-containing mRNA was dissolved in 200 μL of the low-salt sample buffer and heated in a boiling water bath for 3 min before layering on a 5–29.9% isokinetic sucrose gradient (McCarty et al., 1974; Shapiro & Baker, 1977). The sucrose gradient was centrifuged at 41 000 rpm for 4.5 h in a Beckman SW41 rotor at 25 °C. Four fractions from the gradient, each of which corresponded to a peak of absorption at 260 nm, were collected and precipitated with 2 volumes of ethanol.

Formamide–Polyacrylamide Gel Electrophoresis. RNA isolated from the sucrose gradient was further separated on cylindrical 3% polyacrylamide gels which contained 98% formamide buffered with 0.1 M phosphate buffer (Duesburg & Vogt, 1973). Electrophoresis was carried out at 100 V for 4 h with bromophenol blue and xylene cyanol as markers. One of the gels was stained with ethidium bromide and used as a marker. The rest of the gels were sliced into 1-mm sections. Sections with a high concentration of RNA were pooled, and the RNA was extracted with phenol–cresol–$NaDodSO_4$ buffer (Stolarsky & Kemper, 1978).

A 5% polyacrylamide slab gel containing 98% formamide and 0.1 M phosphate buffer (pH 7.5) (Efstratiadis & Kafatos, 1976) was used to analyze the purity and size of mRNA and to isolate the [^{32}P]cDNA. The electrophoresis was carried out at 150 V for 5.5 h with the circulation of electrode buffers. After electrophoresis, the gel was exposed to a Kodak X-ray film to locate the region of full-length antifreeze cDNA by using *Hin*cII-treated fragments of φX 174 DNA as standards. A small piece of the gel where the cDNA was located was removed, and cDNA was extracted from the gel, as described above for the extraction of RNA.

Cell-Free Protein Synthesis. Both wheat germ S30 extract and rabbit reticulocyte lysate were used to analyze the translatable activities of RNA at various steps of isolation. Isolation of wheat germ S30 and its subsequent use for protein synthesis were carried out according to the procedures of Roberts & Paterson (1973). One microgram of *E. coli* RNA was added to each reaction (Stolarsky & Kemper, 1978). Rabbit reticulocyte lysate was prepared and used for cell-free protein synthesis according to the procedure of Pelham & Jackson (1976). Four microcuries of radioactive amino acid, [^{35}S]methionine or [^{3}H]leucine, was added to the reaction mixtures. The proteins were analyzed on a 17.5% polyacrylamide slab gel which contained 4.4 M urea and 1% $NaDodSO_4$ (Lin, 1979). Radioactive proteins were detected by autoradiography with Kodak XR-5 X-ray film. When [^{3}H]leucine was used, the gel was treated with 2,5-diphenyloxazole prior to its exposure to X-ray film (Bonner & Laskey 1974).

Preparation of cDNA. DNA complementary to antifreeze mRNA was prepared by the addition of avian myeloblastosis reverse transcriptase according to the method of Kacian & Myers (1976). The reaction mixture contained 3 μM (50 μCi) [^{32}P]dCTP, 200 μM dCTP, 500 μM each of dATP, dGTP, and dTTP, 80 μg/mL oligo$(dT)_{10-12}$, 50 mM Tris buffer (pH 8.3 at 42 °C), 140 mM KCl, 10 mM $MgCl_2$, 30 mM β-mercaptoethanol, 17 units of reverse transcriptase, and 1 to 2 μg of mRNA in a total volume of 25 μL. The reaction mixture was incubated at 42 °C for 1 h (Shapiro & Baker, 1977; Buell et al., 1978). At the end of the incubation, the single-stranded RNA was hydrolyzed by treatment with 0.3 N NaOH at 37 °C for 16 h. The solution was neutralized by addition of 1 M Tris (pH 7) and passed through a Sephadex G-50 column that was equilibrated with 20 mM Tris (pH 7.5), 65 mM NaCl, and 10 mM EDTA. The void volume peak which contains the radioactive cDNA was collected, and the cDNA was precipitated with 2 volumes of ethanol in the presence of 20 μg/mL carrier tRNA. mRNA isolated from the polyacrylamide gel gave a very low yield of cDNA presumably due to the incomplete removal of acrylamide which might inhibit the activity of the reverse transcriptase. We therefore used mRNA isolated from isokinetic sucrose gradients as a template for cDNA synthesis. The cDNA thus

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

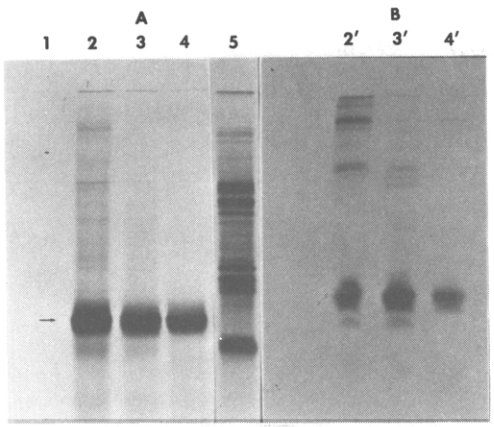

FIGURE 1: Autoradiograms of cell-free protein synthetic products directed by mRNA from different stages of purification. (A) represents products when wheat germ S30 lysate was used and (B) represents products when the reticulocyte lysate was used. Track 1 is a control where no mRNA was added; tracks 2 and 2′ are proteins directed by total poly(A)-containing RNA after oligo(dT) affinity chromatography; tracks 3 and 3′, by 9.4S RNA from sucrose gradient fractionation; tracks 4 and 4′, by mRNA extracted from formamide–polyacrylamide gel; track 5, by the mRNA RNA from a summer specimen. Proteins were labeled with [^{35}S]methionine which is present only in the presequence of the antifreeze molecule. The arrow indicates the position of the peptides which are precipitable by the antibody against purified antifreeze peptides.

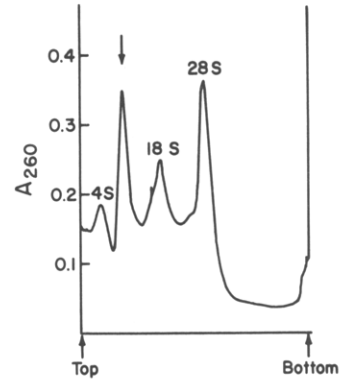

FIGURE 2: Sedimentation profile of an isokinetic sucrose gradient. 120 μg of RNA eluted from an oligo(dT) column was dissolved in 200 μL of sample buffer and layered on a 12-mL 5–29.9% isokinetic sucrose gradient. Centrifugation was carried out at 41 000 rpm for 4.5 h in a Beckman SW41 rotor at 25 °C. The gradient was scanned with an IsCo density gradient fractionator. 10 μg of *E. coli* tRNA (4 S) was added. 18S and 28S peaks contain rRNA eluted with poly(A)-containing RNA from an oligo(dT) column (see Discussion). The arrow indicates the 9.4S fraction which contains the majority of the antifreeze mRNA activity.

prepared was further purified by the polyacrylamide slab gel electrophoresis as described under formamide gel electrophoresis. Only the area of gel corresponding to the full-length of antifreeze mRNA was excised from the slab and extracted with phenol–cresol. Globin cDNA was prepared in the same manner without purification by the polyacrylamide gel electrophoresis.

Hybridization of cDNA to Purified Antifreeze mRNA and Total RNA. Hybridization was carried out in a reaction mixture which contained 300 mM NaCl, 10 mM Tris (pH 7.1), 2 mM EDTA, 0.1% $NaDodSO_4$, 0.5 mg/mL *E. coli* tRNA, and 2.5 pg of antifreeze cDNA (specific activity 200 cpm/pg) in a total volume of 10 μL (McKnight & Schimke, 1974; Shapiro & Schimke, 1975). The hybridization was carried out by a large excess of RNA (as indicated in the figure legend) at 68 °C for different lengths of time. After incubation, the single-stranded DNA was digested with 2 μL of *Aspergillus* S1 nuclease (15 units/μL) for 2 h at 37 °C. The S1 nuclease resistant DNA was precipitated with 10% cold trichloroacetic acid and collected on Whatman GF/C glass filters.

Immunoprecipitation of Wheat Germ Translation Products with the Antibody against Antifreeze Peptides. The immunological assay was performed according to the procedure described previously (Lin, 1979). A preformed precipitate (Lomedico & Saunders, 1976) made with goat antirabbit IgG and rabbit anti-antifreeze was incubated with the wheat germ translational products. The precipitate was washed extensively with buffer, dissolved in 8 M urea, and analyzed by polyacrylamide gel electrophoresis.

Results

Isolation of Winter Flounder Antifreeze mRNA. Tracks 2 and 5 in Figure 1A indicate the patterns of proteins synthesized by the poly(A)-containing mRNA isolated from flounder collected during the winter and summer, respectively. In January, mRNA from livers of winter flounder directs the synthesis of mainly one or possibly two peptides which were identified to be precursors of the antifreeze peptides found in the serum (Lin, 1979). In June, antifreeze peptides are absent from the serum and there is no translatable mRNA coding for these peptides in the liver. Antifreeze mRNA was purified by phenol–isoamyl alcohol–chloroform extraction, oligo(dT)–cellulose affinity chromatography, and isokinetic sucrose gradient fractionation. mRNA activities of fractions from each step of the purification were translated in both wheat germ lysate and rabbit reticulocyte lysate (Figure 1). When the poly(A)-containing RNA was fractionated on a 5–29.9% isokinetic sucrose gradient, a peak corresponding to 9.4 S was observed (Figure 2). This peak contains mainly the mRNA coding for antifreeze peptide (Figure 1A, track 3; Figure 1B, track 3′). RNA from this peak was further purified on a cylindrical formamide gel. Table I summarizes the translational activity, percent recovery, and purification calculated from the wheat germ cell-free protein synthesis.

Table I: Purification of Winter Flounder Antifreeze mRNA

fraction	RNA (μg)	translational act.[a] (cpm/μg of RNA)	total act.[b] (cpm × 10^{-8})	% recovery[c]	purifn (x-fold)
(1) total RNA[d]	18 200	12 000	2.18	100	1
(2) first oligo(dT)–cellulose					
bound	520	68 000	0.35	16.1	6
unbound	17 600	8 760	1.55	71.1	
(3) second oligo(dT)–cellulose					
bound	129	200 000	0.26	11.9	17
unbound	390	7 060	0.06	2.8	
(4) sucrose gradient[e]	39	554 000	0.21	9.6	45

[a] RNA from each step of purification was used to direct the protein synthesis in the wheat germ cell-free protein synthetic system. [^{35}S]Methionine was added to the reaction mixture; the translational activity was determined by radioactivity present in the trichloroacetic acid insoluble fraction. [b] Total activity was calculated by multiplying cpm/μg and μg of RNA. [c] % recovery was calculated by comparing the total activity of each fraction with that of total RNA. [d] Isolated from 0.37 g of lyophilized January liver. [e] Only the 9.4S fraction which contains mostly the antifreeze mRNA is presented.

Cell-Free Protein Synthesis. When the mRNAs isolated from the sucrose gradient (9.4S peak) and gel electrophoresis

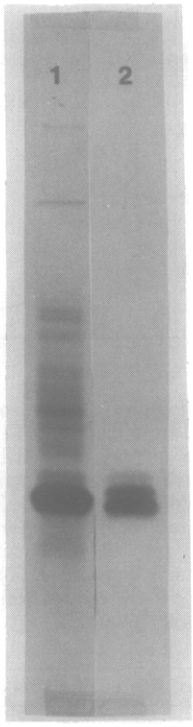

FIGURE 3: Fluorograms of wheat germ cell-free protein synthetic products before (track 1) and after (track 2) immunoprecipitation. mRNA from a January specimen was to direct the protein synthesis, and the synthetic products were precipitated with a preformed precipitate made with goat anti-IgG and rabbit anti-antifreeze. Proteins were labeled with [^{3}H]leucine.

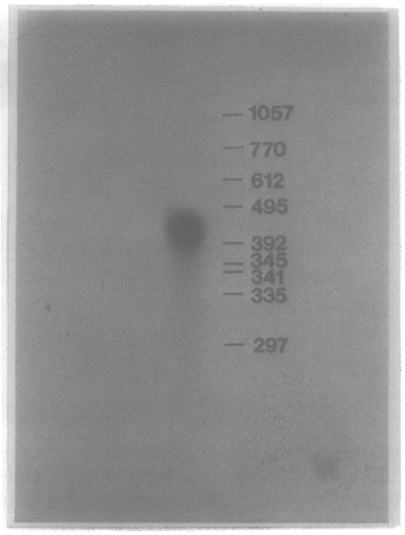

FIGURE 4: Full-length antifreeze cDNA synthesized from antifreeze mRNA by reverse transcriptase. 9.4S RNA from the isokinetic sucrose gradient was used as template for cDNA synthesis in the presence of [^{32}P]dCTP. The [^{32}P]cDNA was electrophoresed on a 5% polyacrylamide gel containing 98% formamide. The portion of the gel containing the 450-nucleotide length of cDNA was removed from the gel, and cDNA was extracted with phenol–cresol. The isolated cDNA was analyzed on the same gel with *Hin*cII-treated ϕX 174 DNA fragments as standards. The numbers indicate the length of fragments in nucleotides.

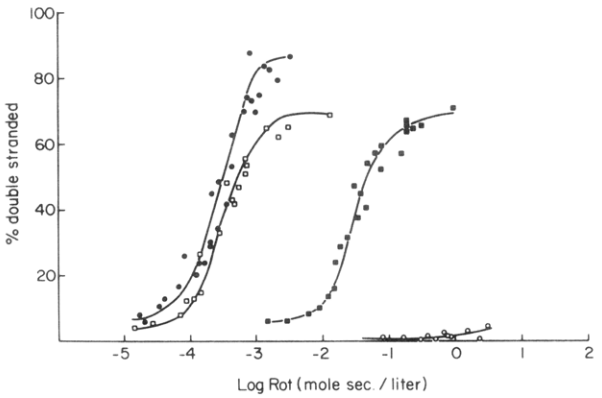

FIGURE 5: Hybridization kinetic analysis of globin mRNA and globin cDNA (●), antifreeze cDNA and antifreeze mRNA (□), and antifreeze cDNA and total RNA from January (■) and June (○) specimens. Hybridization was carried out in the presence of a large excess of RNA. 2.5 pg of antifreeze cDNA was used in each reaction with 2 ng of purified antifreeze mRNA; 0.2 μg of total RNA from January specimens and 5 μg of total RNA from June specimens were used. The reaction was carried out in a total volume of 10 μL which was covered with 3 drops of mineral oil in a 1.5-mL microfuge tube. The hybridization was carried out at 68 °C and stopped by freezing in a dry ice-acetone bath. The single-stranded cDNA was digested with S1 nuclease. The S1 nuclease resistant cDNA was precipitated with 10% trichloroacetic acid and collected on GF/C glass filters.

were used to direct the protein synthesis in both wheat germ and rabbit reticulocyte lysates, over 90% of the translational activity was in one dark band (or two bands when less protein was loaded on the gel) with a molecular weight of ~12 000 according to marker proteins of known molecular weight run on the same gel. These bands can be precipitated by antibodies against purified serum antifreeze peptides. When the precipitated peptides were analyzed on the same gel system, two distinct heavy bands with a third faint band slightly above were observed (Figure 3). Whether these bands are precursors of the three serum antifreeze peptides is not known. Antifreeze peptides do not bind $NaDodSO_4$ to the same extent as do most proteins; thus, their molecular weights cannot be accurately estimated by $NaDodSO_4$ gel electrophoresis (DeVries & Lin, 1977).

Hybridization of Antifreeze cDNA to RNA. When the 9.4S antifreeze mRNA from the sucrose gradient was used for cDNA synthesis, the main product is ~450 nucleotides in length but there are considerable amounts of smaller molecular weight DNA and a smaller amount of larger DNA observed on a formamide slab gel. Addition of actinomycin D in the reaction mixture does not change the pattern of cDNA synthesized. cDNA 450 nucleotides in length was extracted from the gel and used for hybridization experiments (Figure 4). Globin cDNA–globin mRNA hybridization was carried out under the same experimental conditions. The $R_0t_{1/2}$ values for globin and purified antifreeze mRNA were 2.5×10^{-4} and 2.8×10^{-4} mol of nucleotide·s/L, respectively (Figure 5). In both cases, the hybridization was completed in two log intervals of R_0t.

When the purified antifreeze mRNA was analyzed by gel electrophoresis under denaturing conditions, two bands near the region of 450 nucleotides in length were observed (Figure 6). RNA in the upper band was ~10–20 nucleotides larger than that in the lower band and stained more intensively with ethidium bromide. By use of globin cDNA as a standard [the α and β chains give a complexity of 1200 nucleotides (Williamson et al., 1971)], the complexity of winter flounder antifreeze mRNA is 1340 nucleotides which will accommodate three mRNAs 450 nucleotides in length.

When the total RNA extracted from January specimens was used for the hybridization, the value of $R_0t_{1/2}$ was 2.8×10^{-2}, indicating that antifreeze mRNA constitutes 1% of the total RNA. When the total RNA from June specimens was used for hybridization, no detectable hybridization was observed even with a RNA concentration as high as 10 mg/mL (Figure

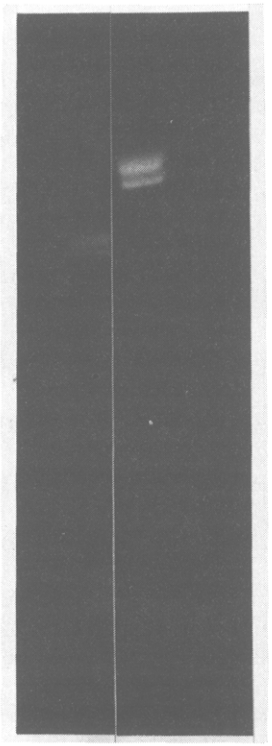

FIGURE 6: Patterns of antifreeze mRNA (left) and globin mRNA (right) after electrophoresis on a polyacrylamide gel containing 98% formamide. The doublets for globin mRNA represent the α and β chains. Two bands near 450 nucleotides in length were visible for antifreeze mRNA. The gel was strained with 1 μg/L ethidium bromide. The photograph was taken with Polaroid type 55 film. The gel was exposed with short-wavelength UV light.

5). Both the hybridization experiments and the cell-free protein translation experiment indicated that the isolated mRNA is relatively free of contamination from other mRNAs.

Discussion

The percent recovery (Table I) was calculated by comparing the total translatable activity of each fraction to that of the total RNA. In the last step of purification, isokinetic sucrose gradient centrifugation, only the 9.4S peak which contains mainly antifreeze mRNA was collected, while in earlier steps all poly(A)-containing RNA was pooled. The 9.6% recovery thus is somewhat misleading. On the basis of the result of hybridization, there is 182 μg of antifreeze mRNA in the total RNA (1%), a final recovery of 39 μg of RNA after sucrose gradient gives an $\sim$19% yield by assuming that 90% of this fraction is antifreeze mRNA. The large loss in recovery occurred in the first oligo(dT) affinity chromatography. It is possible that antifreeze mRNA might exist in two forms, one with and one without the poly(A) sequence as observed in protamine mRNA (Gedamu & Dixon, 1976). The low yield could also be due to the high GC content in antifreeze mRNA since the antifreeze peptides contain over 60% of alanine (coded by GCX). Samples were heated and quick cooled in low-salt buffer to avoid aggregate formation between mRNA and rRNA after phenol–isoamyl alcohol–chloroform extraction, but a considerable amount of antifreeze mRNA activity was found to associate with both 18S and 28S RNA in the isokinetic sucrose gradient fractions after the sample had been passed through the oligo(dT) column twice. The binding of these mRNA to rRNA could mask the poly(A) segment and reduce the effectiveness of oligo(dT)–cellulose to remove the non-poly(A)-containing RNA and could also cause a small amount of the rRNA to be retained by the oligo(dT) and eluted with the antifreeze mRNA. Attempts were made to further purify the 9.4S antifreeze mRNA from the sucrose gradient by formamide–polyacrylamide gel electrophoresis. A slight improvement in removing the contaminants (Figure 1) was handicapped by the low yield, i.e., less than 20%. When the RNA extracted from the gel was used as a template for cDNA synthesis, the reverse transcriptase activity was completely inhibited. The inhibition might have been caused by the residual acrylamide which was coprecipitated with RNA in 70% ethanol. The acrylamide was removed by precipitation of RNA with cetyltrimethylammonium bromide (Young & Young, 1974). This treatment effectively removed the inhibitor for the cDNA synthesis but further decreased the recovery of the RNA by 60%. Thus, an alternative approach was used to prepare full-length antifreeze cDNA for hybridization experiments. The 9.4S RNA fraction from the sucrose gradient was used as a template for the synthesis of cDNA which was further purified by gel electrophoresis. Only the cDNA which migrates near 450 nucleotides in length was removed and extracted from the gel. The small amount of acrylamide present in the cDNA does not seem to interfere with the hybridization.

Results from the RNA excess cDNA–RNA hybridization suggested that there are probably two or three non-cross-reacting species of antifreeze mRNA coding for antifreeze peptides. Since the three isolated serum antifreeze peptides have very limited differences in both composition and sequence, one would expect the coding sequences of the mRNA to be very similar. However, if the noncoding sequences which make up $\sim$60% of the mRNA molecules differ significantly from one another, it is likely that the cDNA of one antifreeze mRNA does not cross-react with cDNA from another antifreeze mRNA. A similar observation was made in protamine mRNA from trout testis. During the late stage of spermatogenesis in rainbow trout a group of structurally similar basic peptides, protamines, was synthesized (Louie & Dixon, 1972). These peptides were synthesized from a family of three to six different mRNAs (Iatrou & Dixon, 1977; Levy & Dixon, 1977; Sakai et al., 1978; Jenkins, 1979). A large percentage of homology of nucleotide sequence exists in coding sequences of the mRNA but not in the noncoding sequence (Jenkins, 1979). In cDNA–RNA hybridization they behave as independent, non-cross-reacting species of mRNA.

Among six species of cold-adapted fish studied, each was shown to synthesize a group of three to eight structurally and functionally similar antifreeze compounds (DeVries, 1976). Whether these compounds are products of a family of genes is not known. Since the hybridization analysis of winter flounder mRNA indicated that there is more than one species of antifreeze mRNA, the antifreeze peptides are probably products of different genes. Many recent studies (Dawid & Wahli, 1979) have shown that it is not uncommon for a set of similar proteins to be coded by a family of genes such as hemoglobin (Williamson et al., 1971), actin (Whalen et al., 1976), vitellogenin (Wiley & Wallace, 1978), and protamine (Sakai et al., 1978). Some of the protein variants are shown to have different biological functions, and others are not. All antifreeze compounds share similar biological activity even though some variants in a set of antifreezes are more effective in depressing the freezing point than others. The significance of employing a family of genes to produce a group of functionally similar antifreezes is not clear. It is possible that such a multiple gene system might provide the fish with a mechanism to be able to produce large amounts of antifreeze in a short period of time.

The biosynthesis of antifreeze peptides in winter flounder responds directly to seasonal change. The antifreeze concentration reaches 30 mg/mL in the body fluid in the winter and is negligible in the summer (Petzel et al., 1979). From the patterns of proteins synthesized by the translatable mRNA, liver of a winter specimen synthesizes mainly the antifreeze peptides. The fact that 1% of total RNA in January specimens is antifreeze mRNA and there is no detectable amount of antifreeze mRNA in the summer indicated that the control of the biosynthesis of antifreeze peptides lies at least in part at the transcriptional level or at the level of the posttranscriptional processing and degradation of mRNA. With the full-length antifreeze cDNA probe, one can quantitate the amount of antifreeze mRNA at various stages of induction and facilitate the understanding of the molecular basis of gene regulation in the antifreeze biosynthesis.

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