Heterogeneity of biologically active deadenylated protamine mRNA components isolated from rainbow trout testes

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ABSTRACT

Poly(A)$^+$ protamine mRNA's were isolated from rainbow trout testes and deadenylated by treatment with calf thymus RNase H. Four subcomponents of deadenylated PmRNA (PmRNA$_{1-4}$) were purified by electrophoresis on a 6% polyacrylamide gel in 8 M urea. Translation of each PmRNA subcomponent in the wheat germ S-30 cell-free system showed that all subcomponents are biologically active but each codes for two or more protamine polypeptides suggesting molecular heterogeneity. However, the deadenylated mRNA's can be categorized into two groups based on the spectrum of protamines whose synthesis they stimulate.

INTRODUCTION

Protamines comprise a family of (3-4) basic proteins synthesized at the spermatid stage of development during the terminal differentiation of rainbow trout testes (1). In order to understand the control of the expression of protamine genes during development of the testis it was necessary to characterize the corresponding family of protamine mRNA's in detail. The mRNA's have been isolated and purified as a group (2,3) and partially characterized by their biological activities upon translation in various cell-free systems (2-4) and upon injection into Xenopus oocytes (5) and chemically by partial sequence analysis (6-8). The mRNA's are distributed in the polysomal and the post-ribosomal supernatant fractions in polyadenylated [poly(A)$^+$] and non-adenylated [poly(A)$^-$] forms (3, 9) and exist as discrete mRNP particles (10). The poly(A)$^+$ protamine mRNA's (PmRNA's) fall into four separable size classes upon electrophoresis in 6% polyacrylamide gels in 8 M urea and the length of their poly(A) segments was shown to be heterogeneous ranging in size from 65-110 A-residues (11). Translation in cell-free systems (12) and hybridization studies with cDNA to total poly(A)$^+$ PmRNA (11) suggested that

while all the mRNA's coding for the protamine components are present in each size class, there is some enrichment of particular template activities in certain components (12). Part of this overlap of activities was attributed to the heterogeneity of the poly(A) lengths in different components. However, when the poly(A) segment was removed from the poly(A)$^+$ PmRNA's by RNase H treatment (13), four separable RNA bands were still obtained suggesting the existence of length heterogeneity among the deadenylated mRNA's and hence in their non-poly(A) regions.

In this paper, we have separated four deadenylated protamine mRNA bands and characterized them on the basis of their translational activity in a wheat germ S-30 cell-free system. Product analysis indicated that PmRNA's in both bands 1 and 2 [poly(A)$^-$ $PmRNA_1$ and $PmRNA_2$ respectively] mainly direct the synthesis of protamine components C_I and C_{III} in approximately equal amounts. Protamine component C_{II} was synthesized in negligible quantities by $PmRNA_1$ and in greater amount when $PmRNA_2$ was used as a template. In contrast, PmRNA's in bands 3 and 4 ($PmRNA_3$ and $PmRNA_4$) mainly direct the synthesis of protamine component C_{II} although significant amounts of C_I and C_0 were also present. Component C_{III} was not found in the translation products of $PmRNA_3$ or $PmRNA_4$.

MATERIALS AND METHODS

A. Large Scale Preparation of Deadenylated PmRNA Components:

Poly(A)$^+$ PmRNA's were purified in milligram quantities by a procedure described previously (14) and were characterized extensively (2-14). The poly(A) segments were removed by RNase H (from calf thymus) from the poly(A)$^+$ PmRNA's (13) and the deadenylated PmRNA's were characterized by electrophoresis in a 6% polyacrylamide gel in 8 M urea (13). Conditions for electrophoresis were as described previously (10) except that potential gradients of 27-33 v/cm (high voltage conditions) were employed during the electrophoretic runs in most experiments. For the purification of reasonable quantities of the four deadenylated PmRNA bands, 200 µg of poly(A)$^+$ PmRNA (2 nanomoles of mRNA having an average molecular weight of 100,000 daltons) was deadenylated in a reaction volume of 2 ml using the conditions described previously (13) except that the nitrocellulose/Sephadex G-25 combination column step was omitted. Instead, the total deadenylated PmRNA was adjusted to 0.24 M with ammonium acetate and precipitated with 3 volumes of ethanol. The precipitate was extracted twice with phenol-chloroform-isoamyl alcohol to

remove RNase H and bovine serum albumin from the reaction mixture and the RNA was precipitated from the aqueous phase with 3 volumes of ethanol. After washing the precipitate twice with 70% ethanol, it was dried in vacuo and stored at $^{-}80^{o}C$. Approximately 150 µg of the RNA was dissolved in 50-75 µl of 99% deionized formamide containing a xylene cyanol FF and Bromophenol blue dye marker mixture (13), heated to $60^{o}C$ for a few minutes and fractionated by electrophoresis under denaturing conditions on a preparative slab gel (20 x 30 x 0.3 cm, with a three slot maker comb) (13). The deadenylated PmRNA bands were localized by running in a parallel slot deadenylated PmRNA's labelled at their 5'-ends with [γ-^{32}P] ATP in the presence of T_4 polynucleotide kinase after removing "cap" structures with tobacco acid pyrophosphatase (15). Regions of the gel corresponding to the labelled markers were excised.

B. Elution of RNA from Gel and Translation in the Wheat Germ Cell-free System:

RNA was eluted by cutting the excised gel regions into small cubes and soaking them in 1 ml buffer containing 0.5 M ammonium acetate, 0.1% SDS and 0.1 mM EDTA for 4-5 hours at $20^{o}C$. After pelleting the polyacrylamide pieces by centrifugation at 10,000 x g, the unlabelled RNA in the supernatant was precipitated with ethanol without adding carrier RNA (repeated twice), dissolved in water at a concentration of 0.1 mg/ml and stored at $-80^{o}C$. Translation of mRNA in the wheat germ S-30 followed by the isolation and characterization of cell-free products was as described previously (14).

RESULTS

The poly(A)$^{+}$ PmRNA's have been previously shown to be free of other RNA's and were translated into 3-4 trout testis protamine components in various cell-free extracts (3,4) and upon injection into Xenopus oocytes (5). Recently we attempted to isolate the mRNA's coding for each of the protamine components by separating the total poly(A)$^{+}$ PmRNA fraction into four components by preparative electrophoresis, however each of the isolated poly(A)$^{+}$ PmRNA bands gave rise to a mixture of the protamine polypeptides although an enrichment of template activities for particular protamine components within each band was observed (12). We attributed such heterogeneity of mRNA's within a given single band as being due to heterodispersity of the poly(A) tracts which range from 65 to 110 nucleotides in length (11). A given mRNA coding sequence could have a poly(A) tract of 65, 85 or 110 residues and thus migrate as three different size classes of RNA in a polyacrylamide gel.

In order to simplify the situation, the poly(A) portion of the poly(A)$^{+}$

PmRNA's was removed from the 3'-end enzymatically (13) and the resulting deadenylated PmRNA's have been separated and their template activities determined. Figure 1A indicates that the deadenylated PmRNA's migrate as 3 major and one minor band (total of 4 bands) upon electrophoresis in a 6% polyacrylamide-8 M urea gel under conditions described in the Materials and Methods section (13). These deadenylated PmRNA bands will be termed poly(A)$^-$ $PmRNA_1$ to $PmRNA_4$, band 1 having the highest molecular weight and band 4 the lowest (13). The four poly(A)$^-$ PmRNA components have nucleotide lengths of approximately 275, 250, 235 and 215 (13). Unfractionated deadenylated PmRNA's have been shown previously to be translated with efficiencies equal to poly(A)$^+$ PmRNA's in the wheat germ S-30 (13). The poly(A)$^-$ $PmRNA_1$ to the poly(A)$^-$ $PmRNA_4$ bands were isolated following preparative denaturing gel electrophoresis and their purity was tested by re-electrophoresis in a 6% polyacrylamide-8M urea gel (Fig 1B). Slot 1 shows the starting material while slots 2-5 indicate the electrophoretic patterns of poly(A)$^-$ $PmRNA_1$ to $PmRNA_4$. It may be seen that four isolated RNA components (slots 2-5) migrate at distinctly different positions and correspond with the four components in the starting material. Poly(A)$^-$ $PmRNA_1$ was the only sharp band obtained while the other RNA components [poly(A)$^-$ $PmRNA_{2-4}$] migrated as broad bands. The individual unlabelled and 5'-end labelled poly(A) PmRNA components have been also shown to co-migrate upon electrophoresis in 8M urea and 99% formamide denaturing polyacrylamide gels (15, L. Gedamu, unpublished data).

The mRNA in each band was assayed for its ability to stimulate the incorporation of [^{3}H] arginine into hot TCA-tungstate acid precipitable material in the wheat germ S-30. Table 1 shows that adding increasing concentrations of each of the poly(A)$^-$ $PmRNA_1$ to poly(A)$^-$ $PmRNA_4$ resulted in an increase in the acid precipitable counts. Poly(A)$^-$ $PmRNA_4$ to poly(A)$^-$ $PmRNA_3$ are translated with comparable efficiencies and to the same extent as the total deadenylated PmRNA's. There are some differences in specific activities with $PmRNA_2$ having the highest and $PmRNA_1$ the lowest. However, poly(A)$^-$ $PmRNA_4$ was apparently not translated as efficiently as the other mRNA's but since it is present in very limited quantities, the concentration could have been measured with less accuracy and, therefore, overestimated in the reaction mixture. The [^{3}H] arginine labelled products of translation of the poly(A)$^-$ $PmRNA_1$ to $PmRNA_4$ were isolated and characterized by ion-exchange chromatography on carboxymethyl-cellulose columns (CM-52, Whatman) under the conditions previously described for the separation of the protamine polypeptides (14). Protamine components C_I and C_{III} (Fig 2) are the major products synthesized

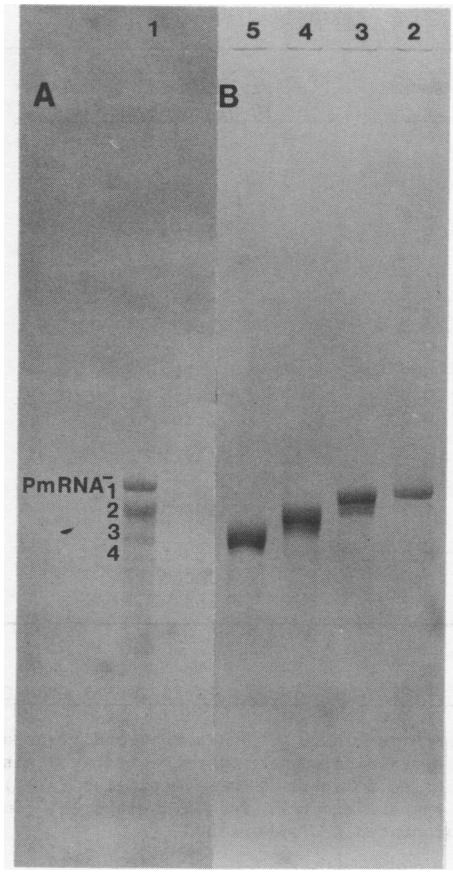

Figure 1A Analysis of total deadenylated PmRNA (3 µg) in a 6% polyacrylamide 8 M urea gel at high voltage.

Figure 1B Re-electrophoresis of deadenylated PmRNA components on a slab gel (6% polyacrylamide - 8 M urea). Migration is from top to bottom. Approximately 150 µg of total deadenylated PmRNA was applied on a preparative slab gel and poly(A)$^-$ $PmRNA_{1-4}$ were extracted as described in the Materials section. The following samples were applied and electrophoresis was performed for 5 hours at 800 volts (10mA). Slot 2, Poly(A)$^-$ $PmRNA_1$ (1 µg); slot 3, poly(A)$^-$ $PmRNA_2$ (1.5 µg); slot 4, poly(A)$^-$ $PmRNA_3$ (1.85 µg) and slot 5, poly(A)$^-$ $PmRNA_4$ (1.35 µg). The gels in 1A and 1B were run at different times and the mobilities of the separated components do not correspond exactly with those in the starting material (1A).

TABLE 1

Poly(A)$^-$ protamine mRNA component	Amount in µg	[^{3}H] arginine incorporated per 10 µl reaction (cpm)	Average specific activity (cpm/µg) per 50 µl reaction
$PmRNA_1$	0.2	3445	
	0.4	10664	123,925
	0.7	21329	
$PmRNA_2$	0.2	8047	
	0.4	15835	194,782
	0.7	25870	
$PmRNA_3$	0.2	7927	
	0.4	11465	167,329
	0.7	22569	
$PmRNA_4$	0.2	2623	
	0.4	5728	69,748
	0.7	10090	
Total deadenylated PmRNA	0.2	5331	
	0.4	10045	125,275
	0.7	16399	

ASSAY FOR ACTIVITY OF PROTAMINE mRNA'S IN THE WHEAT GERM CELL-FREE SYSTEM

All reactions were performed in 50 µl under the conditions described previously (14). 10 µl of the reaction mixture was precipitated with hot TCA-tungstate and [^{3}H] arginine labelled polypeptide were estimated (14). The amount of RNA's indicated were present in 50 µl reaction mixtures. In the absence of mRNA 4245 counts were incorporated and subtracted.

when poly(A)$^-$ $PmRNA_1$ and $PmRNA_2$ are used as templates (Fig 2A and 2B); C_I is the predominant product in each case (49% for $PmRNA_1$ and 43% for $PmRNA_2$) and C_{III} comprises approximately one-third of the total product in each case. Protamine component C_0, which is present in only trace amounts in intact trout testes and upon translation of total PmRNA in a wheat germ cell-free extract, represents one-tenth of the total product (Fig. 2A and 2B). However, protamine component C_{II} which is present in the product directed by poly(A)$^-$ $PmRNA_1$ in very small amounts increases to 17% when poly(A)$^-$ $PmRNA_2$ was used as a template (Fig 2A and 2B). In strong contrast, with poly(A)$^-$ $PmRNA_3$ and poly(A)$^-$ $PmRNA_4$ as templates, protamine component C_{II} becomes the predominant product (45-49%) synthesized by the wheat germ S-30 (Fig 2C and 2D). In addition a decrease in the synthesis of C_I (22-27%) and C_{III} (6-11%) was observed (Fig 2C and 2D). Thus, from Figure 2 it appears that there is a gradient of decreasing synthesis

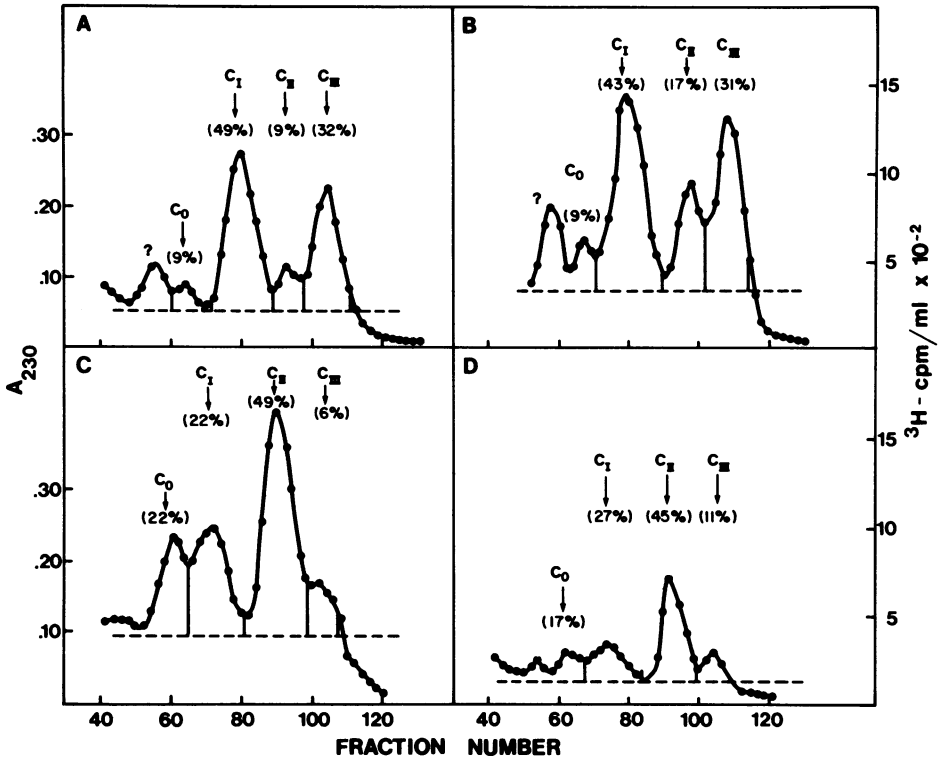

Figure 2 Translation Products of the Deadenylated Protamine mRNA Components

After testing the template activity of the deadenylated PmRNA components in the wheat germ cell-free system (Table 1), the products formed in 0.50 g of each protamine mRNA component were analyzed by chromatography on CM-52 columns (2,14). Radioactivity was estimated in every second fraction. Fractions which contained unincorporated [^{3}H] arginine and optical density protamine markers are not included (Ref. 14,15). All protamine components synthesized in the presence of total deadenylated PmRNA is already presented by Gedamu and Dixon (15).

of C_I and C_{III} and increasing synthesis of C_{II} and C_0 as the length of the mRNA component decreases. We have also observed a [^{3}H] arginine labelled polypeptide eluting before C_0, from the carboxymethyl-cellulose column, in the presence of each of the PmRNA components (Fig. 2A-D) as well as when the total

PmRNA was used as a template (3,14). The nature of this basic polypeptide is unknown although its elution position indicates that it may be less than half the size of components C_I and C_{III} and it may be a degradation product.

DISCUSSION

The biological characteristics of the deadenylated protamine mRNA's [poly(A)$^-$ $PmRNA_1$ to $PmRNA_4$] have been investigated by analyzing the products of their translation in the wheat germ cell-free system [this system in contrast to the rabbit reticulocyte lysate, supports the translation of all the separable protamine mRNA's into protamine polypeptides of characteristic chromatographic behaviour (12)]. All the isolated deadenylated PmRNA components ($PmRNA_1$ to $PmRNA_4$) can stimulate the incorporation of [^{3}H] arginine into protamine polypeptides (Table 1). When the translational products synthesized in the presence of the individual poly(A)$^-$ PmRNA components are analyzed by chromatography on CM-52 columns (Figure 2), the mRNA's fall into two major groups. The first group consists of the two longer components, poly(A)$^-$ $PmRNA_1$ and $PmRNA_2$ and the second group the shorter components, poly(A)$^-$ $PmRNA_3$ and $PmRNA_4$. The first group directs mainly the synthesis of protamine components C_I and C_{III} with minor amounts of C_{II} and C_0, while the RNA's in the second group stimulate predominantly the synthesis of protamine components, C_{II} and C_0 and only trace amounts of C_I and C_{III}. However, there is still heterogeneity in the template response of each of the mRNA bands [poly(A)$^-$ $PmRNA_1$ to poly(A)$^-$ $PmRNA_4$]. From *in vitro* studies, total deadenylated PmRNA has been shown to be translated with an efficiency equal to the poly(A)$^+$ PmRNA's from which they are derived (13) indicating, at least, that endonucleolytic cleavage of the chain or exonucleolytic removal of nucleotides from the 5'-end of protamine mRNA's, where recognition by the ribosomes takes place, can not have occurred. Moreover, studies on the 5'-end structure of the deadenylated PmRNA's by labelling with [γ-^{32}P]ATP in the presence of T_4 polynucleotide kinase before and after treatment of the RNA's with tobacco acid pyrophosphatase, an enzyme used to remove "cap" structures from mRNA's (15), showed that poly(A)$^-$ $PmRNA_1$ and $PmRNA_2$ are almost completely capped while poly(A)$^-$ $PmRNA_3$ and $PmRNA_4$, although they lacked a 7-MeG "cap," showed an intact penultimate 5'-PmRNA sequence (Gedamu et al., in preparation).

The eukaryotic mRNA's so far studied show various structural features at their 5'-ends (16-18). Although most of them have been shown to contain "cap" structures at the 5'-ends and the penultimate nucleotide shows different

degrees of methylation (19-21),there are other mRNA's without such structural features (17). Moreover, each of the various forms of these mRNA's has been shown to be translated in the wheat germ S-30 relatively efficiently. The protamine mRNA's also show both the structural features described above and "uncapped" forms such as poly(A)$^-$ $PmRNA_3$, are translated with efficiencies equal to those of the "capped" mRNA's, poly(A)$^-$ $PmRNA_1$ and $PmRNA_2$, in the wheat germ S-30 (Table 1). Translation of "uncapped" mRNA's with structure pppX... in the wheat germ cell-free system may well only occur following "recapping" by enzymes present in the translation system itself (21). However, we have recently demonstrated that enzymatically "decapped" but otherwise intact protamine mRNA's, with structure pX...., fail to form 80S initiation complexes and their translation in the wheat germ cell-free system is greatly diminished (15). The 5'-termini of such "decapped" molecules are rapidly attacked by an endogenous nuclease and the terminal pA mononucleotide is removed. The loss of translational efficiency may be related to impaired ribosome recognition (15).

The electrophoretic mobilities of the deadenylated mRNA components were observed to change as a function of the voltage during the electrophoretic runs. When electrophoresis was performed at 5-8.3 volts/cm at room temperature, only two RNA bands were observed (Figure 3); but gels run at 27-33 volts/cm resulted in the four bands presented in Figure 1A. Thus, the mRNA's run under low voltage conditions might not be fully denatured in 8 M urea and the high voltage gels may have promoted complete denaturation of the RNA because of the heat generated during the electrophoretic run. This hypothesis is supported by the high G-C content and high melting temperatures of the protamine mRNA's (6,11). The inability of 8 M urea to promote complete denaturation of RNA has also been previously reported (23). This evidence strongly supports the existence of pmRNA with differing degrees of secondary structure (base pairing). Such structural heterogeneity must arise from base sequence heterogeneity. Thus there is evidence for sequence heterogeneity as well as differences in chain lengths.

More recently protamine mRNA's have been sequenced partially after digestion with ribonucleases T_1 (Davies et. al., in preparation) and U_2 (Gedamu and Dixon, in preparation) and after cloning the double stranded protamine cDNA in plasmids pBr322 (A. Dugaicyk, unpublished) and pmB9 (Gedamu, et.al., in preparation). The results indicated that the 3'-end non-coding regions so far sequenced, which represents approximately half the length of the deadenylated mRNA's (13) i.e. 100-110 nucleotides out of 225-250, show greater than 90% sequence homology with few base changes. From the amino acid sequences of the

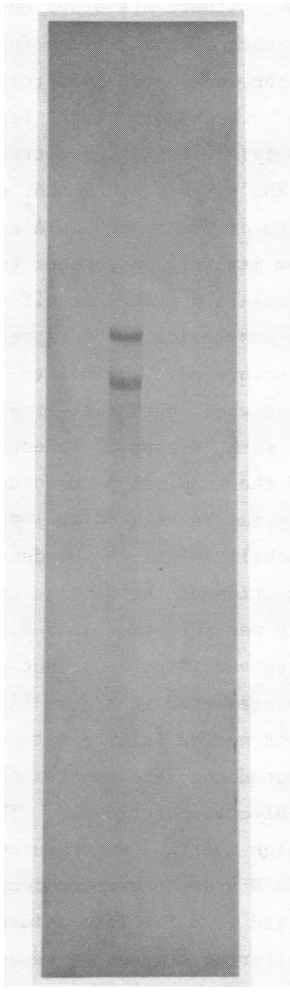

Figure 3 Analysis of deadenylated PmRNA in 6% polyacrylamide slab gel in 8 M urea

Conditions of electrophoresis is described previously (9,13). Sample (3 μg) was dissolved in 99% formamide containing XCFF and BPB and applied in gel. Electrophoresis was performed at 220 volts for 18 hours. Migration is from top to bottom.

protamines (24), it is very likely that the coding sequences of the mRNA's will be homologous, although some nucleotide sequence differences are expected. When the sequence diversity of the poly(A)$^+$ PmRNA components was examined by

molecular hybridization experiments, it was found (11) that all these components probably possess a common sequence of substantial length, calculated to represent approximately 92% of the length determined previously for poly(A)$^-$ $PmRNA_4$, the smallest mRNA component or 206 nucleotides [poly(A)$^-$ PmRNA = 226 x 0.92]. Using similar techniques, Sakai et al. (25) have recently confirmed our results on the homology of the different protamine mRNA sequences and have predicted the presence of at least six different messages with unique sequences. This is compatible with our results.

The translation data indicates that one protein may be coded by more than one mRNA component, for example, poly(A)$^-$ $PmRNA_1$ and $PmRNA_2$ code predominantly for protamine components C_I and C_{III}. Nucleotide sequence analysis of the partial digestion of the products of the 5'-end labelled poly(A)$^-$ $PmRNA_1$, which in all our preparations migrated as one single band both on 8 M urea and 99% formamide gels, showed two major sequences probably representing the mRNA's coding for C_I and C_{III} (Gedamu, L., Chaconas, G., van de Sande, J.H. and Dixon, G.H., manuscript in preparation) while three major sequences are included in the poly(A)$^-$ $PmRNA_2$ and each may represent the mRNA's coding for C_I, C_{II} and C_{III} (see Fig. 2). Studies on the reiteration frequency of the protamine genes in a population of trout testis cells indicate that there are between 2-4 genes for each protamine polypeptide per the diploid content of the DNA in the sperm (26).

Although four distinct bands can be separated from enzymatically deadenylated total PmRNA on preparative denaturing polyacrylamide gels, each isolated poly(A)$^-$ PmRNA component when translated *in vitro* using a wheat germ S-30 still gave rise to more than one protamine polypeptide. In view of the magnitude of the separation problem for such a family of closely related microheterogeneous mRNA's, we have recently approached the problem by cloning double stranded protamine cDNA and have successfully isolated single clones which contain sequentially pure DNA's corresponding to individual members of the protamine gene family.

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