Number and Frequency of Protamine Genes in Rainbow Trout Testis†

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ABSTRACT: Protamine messenger RNA (mRNA) was purified from rainbow trout testis and its full-length complementary DNA (cDNA) prepared by reverse transcription. Analyses of hybridization kinetics of the cDNA with excess mRNA and with excess total DNA have led to the following conclusions: (1) there are about six different protamine genes, (2) probably one copy of the gene exists for each kind of protamine peptide in a haploid genome, and (3) considerable stretches of the sequence of different protamine genes are similar to one another, showing cross-hybridization under less stringent conditions.

Protamine is a group of proteins which is present in fish testis whose histone is replaced with this protein in the late stage of spermatogenesis. Because of the small size of these proteins, the isolation and structural analysis of protamine messenger RNAs (mRNAs) appear promising.

Dixon and his colleagues have purified a mixture of these mRNAs from rainbow trout testis and characterized them partially (Gedamu and Dixon, 1976a,b; Iatrou and Dixon, 1977; Davies et al., 1976, 1977). There appear to be multiple mRNA species by the criteria of gel electrophoresis and partial sequence analysis (Davies et al., 1977; Iatrou and Dixon, 1977; Gedamu et al., 1977). However, information on the exact number of mRNA species and their gene frequency is not available at present, although Levy and Dixon (1977a) reported that the gene frequency of protamine was less than four for each protamine.

Although most mRNA genes, including those of globin (Bishop, 1972; Harrison et al., 1972), immunoglobulin (Honjo et al., 1974; Tonegawa et al., 1974; Faust et al., 1974), ovalbumin (Sullivan et al., 1973), and silk fibroin (Suzuki et al., 1972) are unique, those of histone (Kedes and Birnstiel, 1971) and feather keratin (Kemp, 1975) are known to be repeated. It is of special interest to see if protamine genes are reiterated at all in view of the close biochemical relationship of these proteins to histones.

In the present study, we have analyzed the complexity classes of protamine mRNA and the gene frequency of these mRNAs with the methods of cDNA–mRNA and cDNA–total DNA hybridization, respectively.

Hybridization at different temperatures and two different assay systems for detecting hybrids have been employed for detailed analysis of the reaction. Its data indicate that there are about six genes unique for each of the protamines, whose sequences are partially homologous.

Materials and Methods

Preparation of mRNA and Synthesis of cDNA. Rainbow trout (*Salmo gairdnerii*) testes and liver were kindly supplied by Kamiyama Trout Farm, Tokushima, Japan. Protamine mRNA was prepared essentially according to Gedamu and Dixon (1976b). The testis was homogenized in TKM buffer [50 mM Tris[1]-HCl (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$, 0.25 M sucrose] and centrifuged at 10 000*g* for 15 min. The post-mitochondrial supernatant was made to 50 mM NaCl, 0.5% sodium dodecylsarcosinate, and extracted with phenol–chloroform (1:1) containing 0.1% 8-hydroxyquinoline. RNA was precipitated with ethanol and dissolved in 0.7 M NaCl, 10 mM EDTA, 50 mM Tris-HCl (pH 7.6), 20% formamide. The solution was passed through a column (0.5 × 3 cm) of poly(U)-Sepharose (Pharmacia Fine Chemicals) (Lindberg and Person, 1972), and poly(A)-containing RNA [poly(A)$^+$ RNA] was eluted with 90% formamide, 10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.2% sodium dodecylsarcosinate. The poly(A)$^+$ RNA was further purified on a 5–20% sucrose gradient in 10 mM Tris-HCl (pH 7.5), 0.2% sodium dodecylsarcosinate, at 30 000 rpm for 15 h at 20 °C, a 6–7S peak was pooled, and the RNA was precipitated with ethanol. Rat hemoglobin mRNA was prepared from reticulocytes of anemic rats as described by Williamson et al. (1971). The reaction mixture for the cDNA preparation contained, in a total volume of 0.03 mL, 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 2 mM dithiothreitol, 100 μg/mL actinomycin D, 100 μg/mL purified mRNA, 15 μg/mL oligo(dT)$_{10}$, 0.01 mM [^{3}H]dCTP (23 Ci/mmol, Radiochemical Centre) or [^{32}P]dCTP (260 Ci/mmol, Radiochemical Centre), 0.2 mM other nonradioactive deoxynucleoside triphosphates, and about 20 units of avian myeloblastosis virus (AMV) reverse transcriptase. This enzyme was purified from AMV essentially according to Kacian and Spiegelman (1974). After incubation for 90 min at 37 °C, cDNA was purified by phenol–chloroform extraction, alkaline treatment (0.3 N NaOH, 15 h, 37 °C), followed by Sephadex G-50 column chromatography (0.5 × 15 cm). The specific activity of the [^{3}H]cDNA was about 1.2×10^7 cpm/μg and that of [^{32}P]cDNA was $\geq 1 \times 10^8$ cpm/μg.

Preparation of DNA from Trout Testis Nuclei. Total DNA was prepared from trout testis nuclei by a modified procedure of Marmur (1961; Quagliarotti et al., 1970). DNA was fragmented to 300–500 base pairs by sonication with a Kubota sonicator KMS-250 at the maximum output for 5 min. DNA chain length was determined by polyacrylamide gel electrophoresis with restriction endonuclease (*Hap*) fragments of fd phage DNA as markers (Takanami, 1973).

Isolation and Labeling of Single-Copy DNA. Sheared and denatured DNA was incubated to a C_0t of 5×10^3, and single-stranded DNA was isolated by a column of hydroxylapa-

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[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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tite. The same incubation and fractionation were repeated once again on the single-stranded DNA fraction. Finally, about 1% of the total DNA remained as single-stranded DNA, which was hybridized extensively (to a C_0t of 5–6 × 10^4) to obtain double-stranded single-copy DNA. This DNA was labeled by the techniques of "nick translation" (Rigby et al., 1977) with [^{3}H]dCTP as a precursor.

Wheat Germ S-30 Cell-Free Protein Synthesis. Wheat germ S-30 fraction was prepared according to Roberts and Paterson (1973) and used for protein synthesis with protamine mRNA. After incubation, the incorporation of labeled arginine and leucine was determined with trichloroacetic acid–tungstate precipitation (5% trichloroacetic acid–0.25% Na_2WO_4, pH 2.0) as described by Gedamu and Dixon (1976a). For product analysis, the mixture was precipitated with 4 volumes of ethanol, and the precipitate was extracted with cold 0.2 N H_2SO_4 and analyzed by polyacrylamide gel electrophoresis (Gedamu and Dixon, 1976b) or carboxymethylcellulose column chromatography in the presence of marker protamines prepared from rainbow trout testes (Ling et al., 1971).

Hybridization Procedures. cDNA–mRNA hybridization was performed in 30 μL of 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA containing 1000 cpm of cDNA, and an appropriate amount of mRNA. After denaturation at 100 °C for 5 min, the mixture was incubated at 70 °C for an appropriate period. After incubation, the hybridization mixture was diluted with 0.5 mL of S1 buffer [30 mM sodium acetate (pH 4.5), 0.15 M NaCl, 1 mM $ZnCl_2$] and incubated with 400 units of nuclease S1 (Seikagaku Kogyo, Japan) in the presence of 10 μg of denatured calf thymus DNA at 37 °C for 90 min. Nuclease-resistant radioactivity was precipitated with 10% trichloroacetic acid and counted on a glass filter disk. In the case of cDNA-total DNA hybridization, about 700–1000 μg of sheared, alkali-denatured DNA was mixed with about ~0.02 ng of [^{32}P]cDNA at a concentration of 10–15 mg/mL, together with ~0.5 ng of nick-translated unique [^{3}H]DNA as an internal marker. Sealed samples were denatured again in boiling water for 10 min and incubated at 70 or 60 °C up to appropriate C_0t values. The mixture was treated with 4000 units of nuclease S1 and precipitated with 10% trichloroacetic acid. Hybridized cDNA in the precipitate was dissolved in NCS solubilizer (Amersham/Searle) and counted for ^{3}H and ^{32}P radioactivity using respective windows. In the case of the hydroxylapatite column assay, the reaction mixture was diluted to 50 mM phosphate and loaded on a hydroxylapatite column at 60 °C. Single-stranded and hybridized double-stranded DNA were eluted with 0.12 and 0.4 M phosphate, respectively. Trichloroacetic acid insoluble radioactivity was counted on a glass filter disk as above.

Determination of Thermal Stability. cDNA–DNA or cDNA–mRNA duplexes were precipitated using ethanol and dissolved in 10 mM Tris-HCl (pH 7.5). After heating at the indicated temperature for 10 min, nuclease S1-resistant radioactivity was counted as above.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in 99% formamide was performed according to Duesberg and Vogt (1973); 7% acrylamide (acrylamide/methylenebisacrylamide = 6:1) was made on a 20 × 15 cm slab plate in 99% deionized formamide, 25 mM sodium phosphate (pH 6.5). Electrophoresis was done at 50 V for 15 h at room temperature with continuous tray buffer (25 mM sodium phosphate, pH 6.5) circulation.

Results

Size and Functional Heterogeneity of Protamine mRNA. According to Dixon and his colleagues (Iatrou and Dixon, 1977), 6–7S poly(A)$^+$ RNA from the rainbow trout testis migrates as a somewhat broad band corresponding to 270 to 320 nucleotides in a polyacrylamide gel electrophoresis under denaturing conditions. This did not seem to be due to the contamination of other RNA. Several dense portions were discernible in the band but they were still not clearly separated. Our trial with an acrylamide gel electrophoresis in 99% formamide resulted in a similar separation (data not shown). Size heterogeneity was clearly shown. In order to estimate the purity and kinds of messenger RNAs contained in this RNA preparation, the preparation was tested with a wheat germ S-30 protein-synthesizing system.

TABLE I: Incorporation of [^{3}H]Arginine and [^{3}H]Leucine into Proteins by the Protamine mRNA Preparation in the Wheat Germ S-30 Cell-Free System.[a]

mRNA	incorp (pmol) [^{3}H]Leu	[^{3}H]Arg	Arg/Leu
G	8.53	0.90	0.105
HMW	5.15	1.33	0.258
P	0.09	11.73	130.03

[a] Protamine mRNA (P mRNA, 1.6 μg) was incubated in 50 μL of the wheat germ cell-free system with [^{3}H]arginine or [^{3}H]leucine as described under Materials and Methods. Rat globin mRNA (G mRNA, 4.1 μg) and rat cytoplasmic high-molecular-weight RNA (HMW RNA, 2.1 μg) were incubated in the same manner.

Table I presents the incorporation of [^{3}H]arginine and [^{3}H]leucine into trichloroacetic acid–tungustate precipitable protein products.

The very high incorporation of [^{3}H]arginine compared to [^{3}H]leucine indicates a high content of arginine in the in vitro products, which is consistent with the idea that the products are mainly protamines.

The trace amount of leucine incorporation must be due to the contaminating non-protamine-containing mRNA, because protamines do not have leucine residues in their peptides (Ando and Watanabe, 1969). However, 97% of the arginine label and about 50% of the leucine label were extracted with 0.2 N H_2SO_4. Of the acid-soluble product, all of the [^{3}H]arginine label moved with marker protamines on polyacrylamide gel electrophoresis and most of the leucine-labeled product moved with a similar mobility as histones (data not shown). On the assumption that protamine mRNA has an average efficiency for translation in this in vitro system, the purity of this mRNA preparation was calculated to be over 95%.

Product proteins were chromatographed on a carboxymethylcellulose column together with protamines prepared from testis nuclei. Figure 1 shows that the incorporated radioactivity almost coincided with marker protamines, although relative amounts are not the same. It may be concluded from these observations that the prepared mRNA contained templates for several protamine species, including three major ones (Gedamu and Dixon, 1976a). This was corroborated and extended by the experiments described below.

Hybridization of Protamine mRNA with Its cDNA. Protamine cDNA was synthesized using AMV reverse transcriptase. The size of the synthesized cDNA was determined by polyacrylamide gel electrophoresis under denaturing conditions, where both DNA and RNA migrated according to their chain lengths (Manitatis et al., 1975). Figure 2 shows that the major portion of the cDNA had almost the same length (ca. 300 nucleotides) as protamine mRNA used as the template. Very short chains were relatively few. To determine the complexity of the protamine mRNA, the cDNA was hybridized

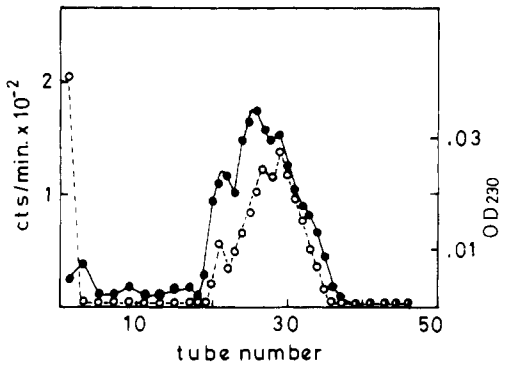

FIGURE 1: Carboxymethylcellulose column chromatography of in vitro protein products. Purified protamine mRNA was translated in a wheat germ S-30 system in the presence of [^{3}H]arginine. Basic protein was extracted, mixed with a protamine fraction prepared from trout testis, and loaded on a carboxymethylcellulose column (1.2 × 15 cm), followed by elution with a 0.6–1.1 M LiCl linear gradient: absorbance at 230 nm (○) and ^{3}H radioactivity (●).

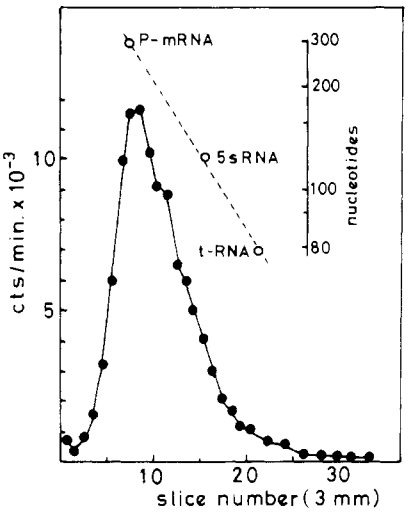

FIGURE 2: Polyacrylamide gel electrophoresis of protamine cDNA. Protamine cDNA was electrophoresed in a 7% polyacrylamide gel, in 99% formamide, 25 mM sodium phosphate (pH 6.5), with 5S, 4S, and protamine mRNA as internal standards. After the run, the gel was sliced into 3-mm pieces and extracted with water. Aliquots were counted in a Bray's scintillator.

with excess template mRNA. At the same time, the reaction between rat globin mRNA and its cDNA was followed as a standard. As shown in Figure 3, globin cDNA–mRNA hybridization had a sharp transition with a $R_0t_{1/2}$ of 5×10^{-4}, whereas protamine cDNA–mRNA hybridization showed a broader pattern with two apparent inflection points. This appears to indicate that protamine mRNA contained at least two sequence components with different complexities, the lower complexity sequences being several times more abundant than the higher complexity ones. If the protamine mRNA is composed of several species, analytical complexity will become over 4×10^5 daltons (300 nucleotides × 320 × 4 ~6 = 3.8×10^5 ~5.8×10^5). From the comparison of the kinetic complexity with that of globin mRNA (α chain + β chain, 4×10^5), the protamine mRNA preparation was regarded as satisfactorily pure also from this criterion.

Thermal stability of mRNA–cDNA duplexes is shown in Figure 4. The melting temperature of the protamine mRNA–cDNA duplex is considerably higher than that of the globin mRNA–cDNA duplex. This is presumably due to the higher G-C content of protamine mRNA than globin mRNA,

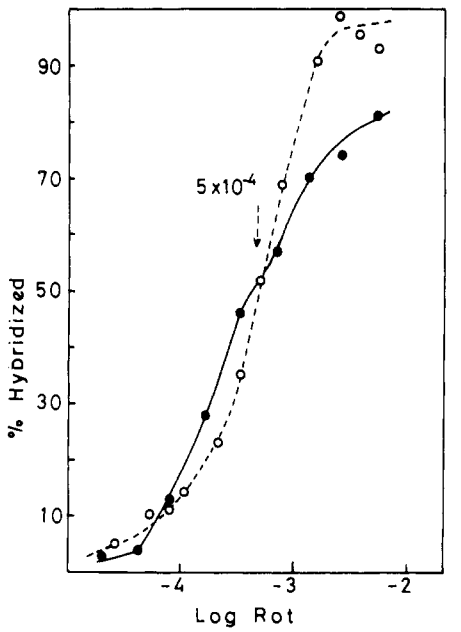

FIGURE 3: Hybridization of protamine cDNA with a vast excess of protamine mRNA. Approximately 1000 cpm of cDNA (about 0.08 ng) was hybridized with various amounts of protamine mRNA up to the indicated R_0t values, and nuclease S1 resistant radioactivity was counted as described under Materials and Methods: protamine cDNA vs. protamine mRNA (●); rat globin cDNA hybridized to its template mRNA at the same time (○). The arrow indicates the $R_0t_{1/2}$ of globin mRNA.

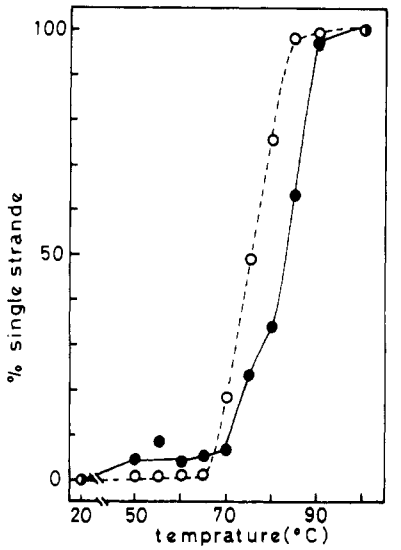

FIGURE 4: Melting profiles of mRNA–cDNA duplexes. After hybridization to an R_0t of 7×10^{-3} for protamine and an R_0t of 5×10^{-3} for globin mRNA as in the legend for Figure 3, duplexes were precipitated with ethanol and dissolved in 10 mM Tris-HCl (pH 7.5). Nuclease S1 resistant duplexes were measured at indicated temperatures: protamine mRNA–cDNA hybrids (●). Globin mRNA–cDNA hybrids (○).

which is expected from the extraordinarily high content of arginine codons in the former. Although the melting profile of the protamine mRNA–cDNA duplex was suggestive of the presence of a sequence of a sequence family with a higher degree of mismatching than the majority of the sequences which appeared to be well matched, it was not conclusive from this pattern alone. However, this inference was, in fact, supported by the experiments of cDNA–total DNA hybridization as described below.

Hybridization of Total DNA with Protamine cDNA. For the gene frequency to be determined accurately, it is essential

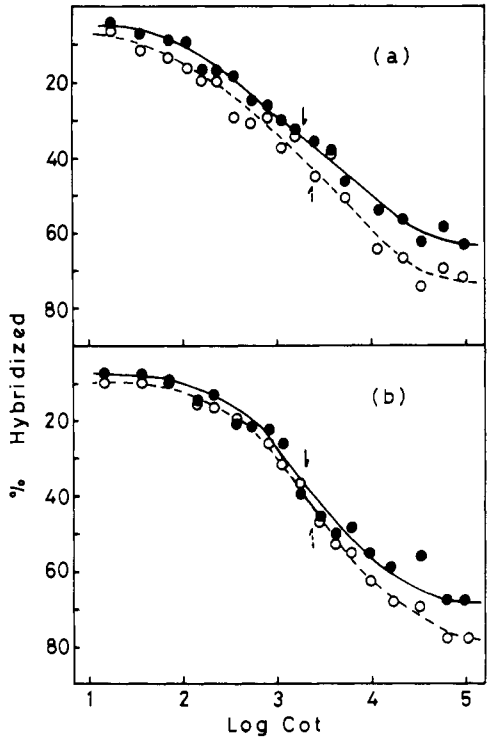

FIGURE 5: Hybridization of protamine cDNA with a vast excess of total DNA at 70 °C. 2500 cpm of protamine [^{32}P]cDNA (0.02 ng) was mixed with 725 μg of total DNA in 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA and incubated at 70 °C up to indicated C_0t values. 3000 cpm (0.5 ng) of unique [^{3}H]DNA was included as internal standard. After incubation, the hybridization mixture was diluted with 0.9 mL of H_2O and divided into three parts. Two of them were diluted with 2 mL of nuclease S1 buffer (see Materials and Methods); one part was digested with nuclease S1 and the other part was incubated in the same manner but without nuclease S1. Trichloroacetic acid insoluble radioactivity was counted for both samples as described under Materials and Methods, from which the percent of total radioactivity that was in the hybrid was calculated (a). The last parts were diluted with 1 mL of 0.05 M sodium phosphate and fractionated on a hydroxylapatite column as described under Materials and Methods (b): (●) protamine [^{32}P]cDNA, (○) unique [^{3}H]DNA. Arrows indicate each $C_0t_{1/2}$.

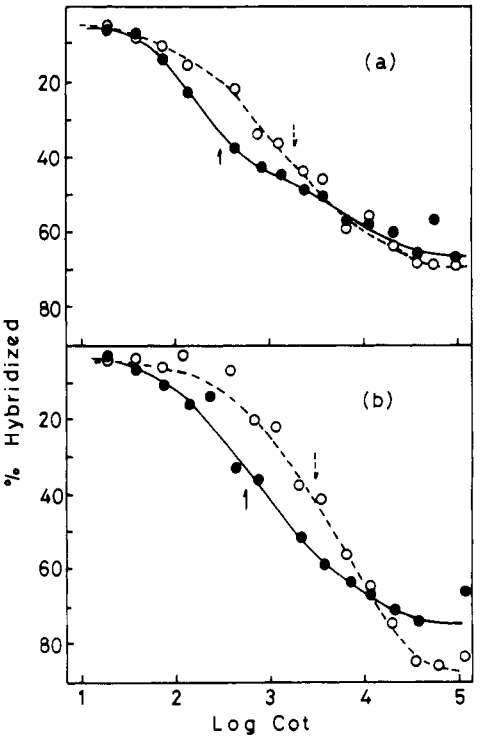

FIGURE 6: Hybridization of protamine cDNA with a vast excess of total DNA at 60 °C. 1600 cpm of protamine [^{32}P]cDNA (0.015 ng) was mixed with 950 μg of total DNA together with 2200 cpm (0.36 ng) of unique [^{3}H]DNA. Hybridization was performed at 60 °C, and hybrids were fractionated in the same manner as in the legends to Figure 5: (a) nuclease S1 fractionation, (b) hydroxylapatite fractionation, (●) protamine [^{32}P]cDNA, (○) unique [^{3}H]DNA. Arrows indicate each $C_0t_{1/2}$.

to know the $C_0t_{1/2}$ value of the single-copy DNA of the relevant genome, preferably in each experiment. However, this was not easy for rainbow trout DNA, since this DNA, like salmon sperm DNA (Britten and Kohne, 1968), appeared to contain many different classes of repetitive sequences (our unpublished results; Levy and Dixon, 1977b), some of which were not so reiterated that their reassociation curve was not well separated from that of unique sequences.

To circumvent this difficulty, we prepared a highly labeled unique DNA by nick translation and used it as an internal standard for the single-copy DNA in the total DNA vast-excess experiments. Furthermore, to attain a true vast excess of driving total DNA, which is required for an accurate determination of $C_0t_{1/2}$ values for the unique DNA, we prepared cDNA with a very high specific activity ($>1 \times 10^8$ cpm/μg), with which an excess of 3.5×10^7 cDNA has been obtained for the driver concentration.

Total DNA vast-excess hybridization was carried out at two different temperatures, 60 and 70 °C, and the hybrids were assayed with two different systems, nuclease S1 and hydroxylapatite column chromatography. The former procedure detected the net amount of sequences in the hybrid structure and the latter all the molecules in the hybrid with flanking sequences.

Figure 5 shows the results of hybridization of protamine cDNA with vast-excess total DNA at 70 °C. At this temperature, the nuclease S1 assay (Figure 5a) and the hydroxylapatite column assay (Figure 5b) showed essentially the same pattern. When nuclease S1 was used to detect the hybrids, $C_0t_{1/2}$ values of unique [^{3}H]DNA and [^{32}P]cDNA were about 2×10^3 and 1.8×10^3, respectively. With the hydroxylapatite column method, unique [^{3}H]DNA had a $C_0t_{1/2}$ of 2.4×10^3 and [^{32}P]cDNA a $C_0t_{1/2}$ of 2×10^3. In both cases, unique [^{3}H]DNA and [^{32}P]cDNA hybridized with similar reassociation kinetics. This indicated that the protamine gene frequency was probably unique in the haploid genome.

When a lower temperature was used for hybridization, however, a different situation was revealed (Figure 6). At 60 °C, unique [^{3}H]DNA hybridized with $C_0t_{1/2}$ values of 2×10^3 and 3.5×10^3, with the nuclease S1 and hydroxylapatite column assay, respectively. These results showed that the unique DNA reassociated with similar kinetics at 60 °C as at 70 °C. In contrast to unique DNA, the hybridization pattern of protamine cDNA at this temperature was different from that at 70 °C. When nuclease S1 was used in 60 °C hybridization, protamine-[^{32}P]cDNA hybridized apparently with two transition curves, one with an approximate $C_0t_{1/2}$ of about six times smaller than that of the unique DNA and the other somewhat larger than that of the unique DNA (Figure 6). This biphasic pattern was highly reproducible, and the fast-reassociated portion was generally five to six times faster than the unique DNA. These C_0t curves suggest that there are two families of protamine gene sequences, one being unique and the other five to six times reiterated compared to unique DNA. The reiterated sequences must have a lower degree of homology, since they cannot reassociate under more stringent conditions of 70 °C (Figure 5). This was confirmed by the fol-

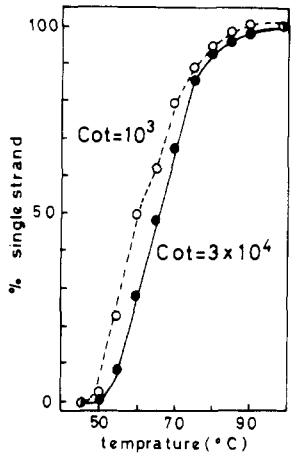

FIGURE 7: Melting profiles of total DNA-cDNA duplexes. Protamine cDNA was hybridized with total DNA up to indicated C_0t values, and melting profiles of the duplexes were drawn as described in the legend of Figure 4: (○) duplexes hybridized up to a C_0t of 1×10^3, (●) duplexes hybridized up to a C_0t of 3×10^4.

lowing experiment in which fast and slowly reassociated fractions were tested for their melting behavior (Figure 7). The melting temperature of slowly reassociated duplexes was about 5 °C higher than that of rapidly reassociated duplexes, indicating that some 7% or more mismatches of base pairing appear to exist in the former (Laird et al., 1969).

Two interpretations are possible for the biphasic nature of the C_0t curve at 60 °C, in contrast to the monophasic pattern at 70 °C. First, protamine genes for individual species have completely independent sequences, but each gene is repeated several times with such differences that the DNAs can partially cross-hybridize with each other at 60 °C but not at 70 °C. Second, protamine genes for individual species are unique but have a partially homologous region, which cross-hybridize at 60 °C but not at 70 °C. However, the results of mRNA-cDNA hybridization (Figure 3), which suggest that there are more than two complexity classes of mRNA sequences, support the latter possibility.

When a hydroxylapatite column was used in the assay of hybridization, we have obtained a single transition pattern with a $C_0t_{1/2}$ of about 6×10^2, similar to that for the rapidly hybridized portion of the nuclease S1 assay (Figure 6b). The ratio of the $C_0t_{1/2}$ values for [^{32}P]cDNA and unique [^{3}H]DNA marker was reproducibly about 6. Since partially double-stranded DNA behaved as totally double-stranded DNA on the hydroxylapatite column under these conditions, these findings indicated that the unique sequence in the cDNA was covalently linked to the reiterated sequence. In addition, all the hybridizable cDNA were hybridized with a $C_0t_{1/2}$ of 6×10^2, indicating that almost all the protamine cDNAs have a common sequence. Therefore, we conclude that the protamine mRNAs contain about six unique species but they have a similar sequence which can be hybridized under less stringent conditions in some parts of the molecule. The possibility that the biphasic pattern was due to the contamination of some other mRNAs could also be ruled out by the difference observed between nuclease S1 and hydroxylapatite column procedures.

Discussion

In the present study, protamine mRNAs were purified from rainbow trout testis and their gene reiterations estimated with cDNA made on these mRNA templates. Polyacrylamide gel electrophoresis and the wheat germ cell-free protein-synthesizing system suggested that this mRNA preparation had a high purity and contained several mRNA sequences coding for different protamine peptides (Figure 1; also see Gedamu and Dixon, 1976b).

cDNA-mRNA hybridization kinetics suggested that the protamine mRNA preparation contained some unique and several times more abundant sequences. These data suggest the presence of several species of protamine mRNA whose sequences are partially homologous.

This inference was substantiated by the presence in the cDNA-total DNA hybridization at 60 °C of a considerable amount of components hybridizing about six times faster than the other which behaved apparently as a unique gene (Figure 6a). At 70 °C, all the cDNA hybridized with a $C_0t_{1/2}$ of the unique gene, suggesting that the fast-hybridizing fraction at 60 °C could not form stable hybrids at this higher temperature (Figure 5a). That these two components were linked to each other, probably covalently, to form a mRNA was demonstrated by the difference in hybridization kinetics assayed with a hydroxylapatite column and nuclease S1 (Figure 6b). Moreover, the rapidly reassociating fraction was found to have more mismatched sequences than the unique sequences, as measured by the thermal stability of the hybrids (Figure 7). This is to be expected from the difference in the C_0t curve observed at 60 and 70 °C as described above. All these data strongly suggest that the genes for protamine mRNA are probably unique for each protamine species, but the mRNA must have a homologous region common to most of the different species. Incidentally, we have calculated the gene frequency from the equation[2] proposed by Ross et al. (1974), using the percentage of hybridization at completion. This has been attempted by Levy and Dixon (1977a) with the result that the gene frequency was 0.4, a conclusion obtained by the lower completion level of hybridization. Our calculated value was very close to 1, supporting the conclusion that the protamine genes are indeed unique. If we assume that all of the protamine mRNAs have a homologous region whose cDNAs are cross-hybridizable with each other, the number of protamine mRNA species may be estimated to be about six from the ratio of $C_0t_{1/2}$ values of rapidly and slowly hybridizing components. Gedamu et al. (1977) separated the protamine mRNA lacking poly(A) into four components by polyacrylamide gel electrophoresis and hybridized it with total protamine cDNA. They found that mRNA from each of the four bands on the gel was hybridized with 45-55% of the total protamine cDNA, indicating either that each mRNA species contained a common sequence or that each band comprised more than one species of mRNA, or both. These results are compatible with ours. Ando and Watanabe (1969) have identified at least four protamines in rainbow trout testis, i.e., iridine Ia, Ib, II, and III. Although the first three peptides were already sequenced, the last peak (III) in their carboxymethylcellulose column was not well characterized. This peak may well contain more than one, possibly two or three, different peptides, considering its rather broad shape.

[2] Reiteration frequency = $R/Er \cdot f$ (Ross et al., 1974), where Er = unlabeled cellular DNA per labeled cDNA, f = the fraction of the haploid trout cell geneome corresponding to one copy of the cDNA sequence which equals the number of bases in cDNA per number of bases in the haploid genome, and R = number of unlabeled cellular protamine genes in the reaction mixture per number of labeled cDNA sequences in the reaction mixture. For the reaction in Figure 5a, percent hybridization at completion was 65%. Under the present conditions, hybridizable cDNA was only 80% (Figure 3). Therefore, the value of percent hybridization at completion was corrected by this factor: $65/80 \times 100 = 81$. This value corresponds to $R = 4.1$ according to Ross' figure. Since $Er = 0.725/0.02 \times 10^{-6} = 3.6 \times 10^7$, $f = 300/3 \times 10^9$ (Levy and Dixon 1977) $= 1 \times 10^{-7}$. Reiteration frequency, $R/Er \cdot f = 4.1/3.6 \times 10^7 \times 10^{-7} = 1.1$.

The presence of five to six different protamine peptides is thus not unreasonable. Whether the cross-hybridizable sequences belong to the coding region of these mRNAs or to the non-coding region is particularly interesting with respect to the function and evolution of these regions. Although the close similarity of amino acid sequences among the rainbow trout protamines may suggest the possible homology in the amino acid coding region, this is by no means conclusive in view of the degeneracy of the amino acid code.

Localization of coding and space regions in mRNA, their nucleotide sequences, and the arrangement of different protamine genes in the genome will be the subjects of future studies.

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