Reiteration Frequency of the Protamine Genes in Rainbow Trout (*Salmo gairdnerii*)

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Protamine mRNA was isolated in a very pure form from trout testis and used as a template for the synthesis of a complementary DNA of high specific activity. The cDNA represented a full copy of the mRNA template and was used in hybridization reactions with purified trout DNA to determine the number of genes for protamines in trout testis. Our results indicate that there are less than four genes for each protamine polypeptide per the content of DNA in the male gamete and that the control of protamine synthesis in trout testis cells does not involve specific amplification of the protamine genes.

The degree of reiteration of genes coding for specific proteins has been studied in various systems, including that of ovalbumin in chicken oviduct (1, 2), globin in avian and mouse red cells (3, 4), silk fibroin in the posterior silk gland of the silkworm (5, 6), and immunoglobulin light chain in antibody-producing cells (7). With the exception of the genes coding for histones which are known to be reiterated to different degrees in the DNA of several eukaryotes (8-11), the genes for all other proteins so far studied seem to be present in only one or at most a very few copies per genome.

The process of spermatogenesis in trout testis offers a good system for the study of cell differentiation and gene function. One of the characteristic features of the later stages of spermatogenesis is the synthesis of protamines in the spermatid cells of the testis (12, 13). These proteins comprise four polypeptides of very similar amino acid sequence. The corresponding mRNA or group of mRNAs have recently been purified and extensively characterized in our laboratory (14-18). The presence of a polyadenylic acid segment at the 3' end of the protamine mRNAs has allowed, by the use of viral reverse transcriptase, the synthesis of a highly radioactive cDNA probe for protamine gene sequences. In the present report, this cDNA probe has been employed in hybridization reactions to determine the number of protamine genes present in the DNA from trout testis.

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MATERIALS AND METHODS

Trout testis were collected at a late stage of maturation (19, 20) from freshly killed trout (Danterout, Brände, Denmark), immediately frozen on dry ice and stored frozen at -70°C.

Preparation of Protamine mRNA and cDNA: Protamine mRNA was purified from trout testis as described by Gedamu and Dixon (15) and Iatrou and Dixon (16). cDNA complementary to the gel purified mRNA was synthesized as described by Iatrou and Dixon (16). The cDNA, after purification, behaved on denaturing acrylamide gels as a full length transcript of the mRNA.

Preparation of Cytoplasmic Polyadenylated RNA: Total cytoplasmic polyadenylated RNA from trout testis was prepared as described previously (19-21).

Purification of Trout Testis DNA: Trout testis nuclei were prepared as previously described (20). The clean nuclear pellet was resuspended by homogenization in a volume of Buffer A (0.1 M NaCl, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA) and 1% SDS. An equal volume of phenol/chloroform (1:1) was added. After shaking the mixture for 20 min at room temperature and separating phases by slow speed centrifugation, the organic phase was removed and the aqueous phase reextracted three times more with chloroform/isoamyl alcohol (24:1). The aqueous phase was then transferred to a beaker, the salt concentration was adjusted to 0.1 M NaCl, and 2 volumes of cold ethanol were added. The DNA was scooped out with a glass rod and dissolved in 10 mM Tris/HCl, 1 mM EDTA (pH 8.5) (Buffer B). The viscous DNA solution was then fragmented by shearing at 16,000 p.s.i. in a French pressure cell, and precipitated with ethanol as before. The DNA pellet was recovered by centrifugation at 12,000 × g at 4°C, resuspended in Buffer B, and incubated for 1 h with 0.3 M NaOH at 37°C to hydrolyze any contaminating RNA. After hydrolysis, the DNA was neutralized by the addition of HCl, reprecipitated with ethanol, and resuspended in Buffer B at a concentration of 8 mg/ml. The length of the DNA fragments obtained in this manner was found to be between 300 and 400 nucleotides both by electrophoresis on 4% polyacrylamide gels containing 7 M urea and centrifugation on alkaline sucrose gradients in comparison with markers of known size (19) (data not shown).

cDNA/DNA Hybridization Reactions: cDNA/DNA reactions were performed as previously described (19-21). Samples, in triplicate, containing some 2000 cpm of [3H]cDNA (specific activity, 10^6 cpm/μg) and protamine mRNA at a concentration of 0.1 μg/ml or total polyadenylated cytoplasmic RNA at a concentration of 50 μg/ml were annealed at 70°C in a buffer containing 0.5 M NaCl, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, and 0.01% SDS. After incubation for the desired periods of time, the content of each capillary was ejected into 1 ml of S, nuclease buffer. S, nuclease (500 units) (Miles) was added and incubation was for 1 h at 45°C. The samples were then precipitated by the addition of cold 20% trichloroacetic acid, filtered, and counted.

cDNA/DNA Reassociation Reactions: In DNA excess reactions, the amount of DNA used in each capillary was 1 mg. Reactions were performed as previously described (19-21). Samples, in triplicate, containing some 2000 cpm of [3H]cDNA (specific activity, 10^6 cpm/μg) and protamine mRNA or total polyadenylated cytoplasmic RNA at a concentration of 0.1 μg/ml Total polyadenylated cytoplasmic RNA at a concentration of 50 μg/ml were annealed at 70°C in a buffer containing 0.5 M NaCl, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, and 0.01% SDS. After incubation for the desired periods of time, the content of each capillary was ejected into 1 ml of S, nuclease buffer. S, nuclease (500 units) (Miles) was added and incubation was for 1 h at 45°C. The samples were then precipitated by the addition of cold 20% trichloroacetic acid, filtered, and counted.
were performed in triplicate in 200-μl capillaries in a buffer containing 0.5 M NaCl, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA. In the DNA excess reactions, some 50 ng of cDNA (2500 cpm) were used per point. The extent of self-annealing of the cDNA was determined as previously described (19-21) by including in each experiment a zero time control. In DNA excess experiments, the samples, after incubation, were flushed into 4 ml of S1 buffer and incubated with 15,000 units of S1 nuclease for 90 min at 45°.

RESULTS

Characterization of the cDNA Probe: Hybridization with its Template mRNA—In Fig. 1, the hybridization of protamine cDNA to its template RNA is shown. Under our experimental conditions of salt and temperature (0.5 M NaCl, 70°), the cDNA hybridizes to the mRNA with a $R_s^{1/2}$ value of 1.2 x 10^-5 M s. This value is very close to that previously reported (16). In this experiment, the percentage of hybridization at zero time was 15%. This rather high value is due to self-annealing of the protamine cDNA to form double-stranded structures, a phenomenon that occurs readily in diluted solutions as has been discussed in detail (16). In order to determine the hybridizing sequence length of the cDNA probe (and therefore, its purity), we compared the $R_s^{1/2}$ value of the protamine cDNA/mRNA reaction ($1.2 \times 10^{-5}$) with that of globin cDNA (prepared from an approximately equimolar mixture of α- and β-globin mRNA) to its template (2.3 x 10^-5) under the same experimental conditions of salt and temperature (data not shown). This comparison yields a calculated hybridizing sequence length for protamine cDNA of 750 nucleotides or 250,000 daltons. This is in agreement with previous results from our laboratory (16). Since the mean size of protamine mRNA has been determined as 290 nucleotides (17), this value suggests that the cDNA includes sequences complementary to all four protamine mRNA subcomponents whose nucleotide sequences are slightly different (16).

To estimate the concentration of protamine mRNA sequences in the population of cytoplasmic polyadenylated RNA from trout testis, we performed hybridization experiments between protamine cDNA and an excess of cytoplasmic poly(A+) RNA. The results are shown in Fig. 1. The reaction driven by the total poly(A+) RNA is displaced by a factor of ~15-fold to high $R_s$ values as compared to the protamine cDNA/pure protamine mRNA reaction ($R_s^{1/2}$ of 7.8 x 10^-2 versus 1.2 x 10^-5), indicating that protamine mRNA sequences represent some 7 to 8% of the total cytoplasmic poly(A+) RNA from trout testis, as detected by our assay.

Similar experiments were performed with cytoplasmic poly(A+) RNA from two other batches of testis representative of an earlier and later stage of development, respectively (21). Protamine mRNA represented some 5% of the polyadenylated RNA of the earlier batch and some 10% of the RNA from the later stage. Therefore, protamine mRNA can be regarded as an abundant mRNA in trout testis. These data are consistent with previous data from our laboratory where it was shown that protamine mRNA is a major translatable mRNA species in trout testis (14).

Reassociation of Trout DNA Fragments—It has been shown that the parameter $C_M^{1/2}$ is proportional to the haploid genome size of the DNA of an organism, in the absence of repeated sequences (22). We have previously studied the kinetics of renaturation of trout DNA fragments of 350 to 450 nucleotides in length. Under our experimental conditions, unique sequences of trout DNA renature with a $C_M^{1/2}$ value of 2000 M s. By comparison, the kinetics of renaturation of Drosophila DNA fragments of approximately the same length (400 to 500 nucleotides) yield a value of 100 for the $C_M^{1/2}$ of renaturation of unique sequences (23, 24). Knowing the size of the haploid Drosophila genome to be 10^6 daltons, we estimate the trout genome size to be 2 x 10^9 daltons. This estimate is in good agreement with our previous direct measurement of the DNA in a single rainbow trout spermatid cell (12) as 2.45 x 10^12 g or 2.45 x 10^12 M x 1.47 x 10^12 daltons.

Reassociation of Protamine cDNA with Trout DNA—To determine the reiteration frequency of protamine genes in the trout genome, we followed the kinetics of reassociation of highly labeled single-stranded protamine cDNA, with an excess of unlabeled trout DNA fragments of an average length of 400 nucleotides. The results are shown in Fig. 2. We obtained a $C_M^{1/2}$ value of 250 for the cDNA reaction, which would give us four copies of a sequence of about 800 nucleotides. In other words, the $C_M^{1/2}$ value of 250 indicates that there would be four copies of each of the genes for the protamine mRNA components. However, the data must be interpreted with caution. The accuracy in the determination of the gene number depends on the achievement of a vast DNA excess in the reassociation reaction. This consideration is particularly important where the gene(s) to be measured is (are) not highly reiterated, since in that case, very large excess of cellular DNA is required to supply sufficient unlabeled sequences to hybridize with the probe. If the reaction contained insufficient cellular DNA, the labeled cDNA probe would not be completely annealed at completion of the reaction. If we look at the data in Fig. 2, we observe that the

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reaction achieves completion at 58% saturation of the cDNA probe. This experiment has been repeated many times, using different batches of 3H- or 32P-labeled cDNA and also trout DNA, and on no occasion have we obtained a higher saturation value. However, this cDNA probe reacts up to 79% with its template mRNA (Fig. 1), suggesting, therefore, that the low saturation plateau value obtained in the reassociation reaction, might be due to our inability to achieve an excess of trout DNA sequences complementary to the probe. This observation, however, provides an alternative method, independent of C$ analysis, for the determination of gene number.

Calculation of Reiteration Frequency of Protamine Genes using Percentage of Hybridization at Completion of Reannealing Following Method of Ross et al. (25) — We define reiteration frequency as the number of copies of a given DNA sequence per haploid genome.

We define the following parameters: experimental ratio $(Er) =$ unlabeled cellular DNA in reaction mixture (mg/ml)/[32P]cDNA protamine in reaction mixture (mg/ml):

$$ Er = \frac{1 \text{ mg/200 ml}}{40 \times 10^{-9} \text{ mg/200 ml}} = 2.5 \times 10^7 $$

We consider that only 80% of the input cDNA is hybridizable.) $f$ is the fraction of the haploid trout genome corresponding to one copy of the cDNA sequence. $f =$ number of bases in protamine cDNA/number of bases in haploid trout genome:

$$ f = \frac{750 \text{ bases}}{3 \times 10^6 \text{ bases}} = 2.5 \times 10^{-7} $$

This result indicates that the sequences represented in the protamine cDNA, are reiterated only 0.4 times in the trout genome.

We have obtained two independent estimates of the reiteration frequency of the genes coding for protamine in the trout. The determination based on comparative C$ analysis gives a reiteration frequency of 4; the value based on percentage of hybridization at completion of the reaction is 0.4.

**DISCUSSION**

Protamine cDNA of high specific activity prepared with a template of purified protamine mRNA has been hybridized to trout DNA, sheared to an average length of ~350 to 450 nucleotides in an attempt to estimate the reiteration frequency of protamine genes in the rainbow trout genome. The DNA excess approach allowed us to make two independent estimates of the gene number, a direct value based on comparative C$ analysis obtained from the data in Fig. 2 of some four genes for each protamine polypeptide represented in the cDNA, and an indirect estimate, based on the percentage of hybridization at completion of a DNA driven reaction (25) which gave a value of 0.4 for the reiteration frequency of protamine genes.

It is clear that hybridization is not sufficiently precise to take the values obtained as absolute figures for the numbers of protamine genes in trout DNA. Nevertheless, it is evident that the number is small, certainly less than four, and perhaps as little as one copy.

We have been careful to relate our estimates of the gene number to the amount of DNA in the trout sperm rather than to the haploid trout genome since there is a variety of evidence (26, 27) indicating that rainbow trout, like other salmonid fishes, is a tetraploid species. Thus the trout sperm would contain a diploid amount of DNA. Our estimates of the number of genes for each protamine polypeptide per the
amount of DNA present in the sperm range from 0.4 to 4 and would be entirely consistent with an estimate of 2 genes per the diploid content of DNA in the sperm or 1 gene per haploid genome.

Protamines, therefore, would constitute another example of a protein product which is produced in large amounts in a specialized tissue but whose preferential synthesis in testis cells cannot be accounted for by gene amplification. The provision of the large amounts of the protamines required at the spermatid stage of testis development for the complete replacement of the nuclear histones appears to be achieved by the prior synthesis over a long time period of stable protamine mRNAs which are stored as 16 to 18 S mRNPs in the postribosomal supernatant fraction of prespermatid cells until, at the early spermatid stage, the protamine mRNP bind to the spermatid polysomes (16, 28) and translation is activated. Thus, the selective expression of the protamine genes appears to be controlled in large degree at the translational level.

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