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Renaturation kinetics of cDNA complementary to cytoplasmic polyadenylated RNA from rainbow trout testis. Accessibility of transcribed genes to pancreatic DNase

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ABSTRACT

We have determined the fraction of polyadenylated cytoplasmic RNA from trout testis complementary to unique and repetitive DNA. Some 21% of the cDNA probe representative of this RNA population renatures with rapid kinetics, characteristic of repetitive sequences. The major proportion of the cDNA renatures with unique sequence DNA. Experiments with fractionated cDNA probes allow us to conclude that, in trout testis, the most abundant polyadenylated mRNAs are not preferentially transcribed from repetitive DNA, as it has shown to be the case in two eukaryotic cell lines.

Treatment of trout testis nuclei with DNase I, under conditions in which 10% of the total DNA is digested, preferentially depletes the DNA of sequences being transcribed into polyadenylated mRNA. These data confirm the results of H. Weintraub and M. Groudine [(1976) Science 193, 848-856] and those of A. Garel and R. Axel [(1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3966-3970] and suggest that the conformation of DNA in the active genes of chromatin is such that it is more susceptible to digestion by DNaseI.

INTRODUCTION

The DNA in higher cells is organized in such a way that repetitive and unique sequences alternate at regular intervals throughout the genome[1-2-3]. These patterns of organization are likely to be of functional significance[2] and, therefore, it is of interest to try to ascertain experimentally, the roles of the different sequences. Previous studies indicate that most eukaryotic mRNA sequences are derived from unique DNA[2, 4-10]. A notable exception is the set of histone genes[11-12]. In several instances, a fraction of the mRNA population constituting some 15-30% of the total mRNA in mammalian cells[13-17] has been observed to be complementary to repetitive DNA. In the present report, we present the results of experiments dealing with the representation of repetitive and unique sequences of DNA in a heterogeneous population of polyadenylated cytoplasmic RNA from trout testis. We show that both abundant and rare mRNAs are reiterated to the same degree in the trout genome.

Several lines of evidence suggest that the structures of regions of the genome active and inactive in transcription of RNA are different. The

observation that *E. coli* RNA polymerase is capable of selective transcription from chromatin templates[18-24], even though there is no evidence that the enzyme can properly recognize initiation and termination signals in eukaryotic DNA, together with the enhanced susceptibility of transcribed genes to DNase I[25-26] and DNase II[27], make it likely that heterologous polymerases can recognize some alteration in chromatin structure, correlated with transcriptional activity. Recently, Weintraub and Groudine[25] have demonstrated in elegant experiments that specific globin sequences from erythroid nuclei, and also DNA sequences complementary to nuclear RNA are preferentially digested by DNase I. In addition, the same authors have shown that the ovalbumin gene is resistant to digestion in cells in which the gene is inactive. Garel and Axel[26] have extended these studies by showing that, similar treatment of hen oviduct nuclei with the DNase I, selectively digests the ovalbumin genes, actively being expressed in that tissue.

These observations have led to the conclusion that the conformation of active genes in a given tissue is such that it renders the DNA particularly accessible to DNase I.

In an attempt to broaden these studies we have asked whether the sensitivity to DNase I is a common feature of all DNA sequences which are transcribed in a given tissue. To this end, we have used, as a general probe, a cDNA representative of the population of cytoplasmic polyadenylated RNA from trout testis. Our results agree with those of Weintraub and Groudine[25] and Garel and Axel[26] and show that the DNA sequences involved in the production of polyadenylated mRNA are very susceptible to digestion by DNase I.

METHODS

Trout Testis

Testis were collected at a late stage of maturation (October 1974) from freshly killed trout (Dantrout, Brande, Denmark), immediately frozen on dry ice and stored frozen at -70°C.

Preparation of Cytoplasmic Polyadenylated RNA

Cytoplasmic RNA was prepared as previously described[28-29]. Polyadenylated RNA was prepared by two cycles of chromatography on oligo-dT cellulose, essentially as described by Aviv and Leder[30]. Polyadenylated RNA eluting with H_2O after the second oligo-dT column was adjusted to 0.2 M ammonium acetate and precipitated overnight with 2 volumes of 95% ethanol at -40°C. The RNA was recovered by centrifugation at 10,000 rpm for 1 hour at -10°C in a Sorvall HB-4 rotor.

Synthesis of Complementary DNA

cDNA complementary to polyadenylated cytoplasmic RNA was synthesized as described before[28-29] and purified as described by Iatrou and Dixon (1977)[31].

Preparation of Trout Testis Nuclei

Trout testis nuclei were prepared as described previously[28-29]. The nuclear pellet was washed twice with buffer RSB (0.01 M Tris-HCl pH 7.4, 0.01 M NaCl, 3 mM $MgCl_2$) and used immediately for DNA extraction or DNase digestion experiments. Microscopic examination of the nuclear suspension revealed nuclei free of cytoplasmic tabs.

DNase Digestion of Trout Testis Nuclei

Nuclei were resuspended in RSB buffer, at a DNA concentration of 1 mg/ml (20 $O.D._{260}$/ml) and incubated for the desired periods of time at 37°C with pancreatic DNase I (Worthington DPFF, 2070 units/mg) at a concentration of 20 µg/ml.

DNA Extraction From Testis Nuclei

The clean nuclear pellet was resuspended by homogenization in 5 volumes of NTE buffer (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 25 minutes at room temperature and separating phases by slow speed centrifugation, the organic phase was removed and the aqueous phase re-extracted three more times with chloroform-isoamyl alcohol (24:1). The aqueous phase was finally transferred to a beaker, the salt concentration adjusted to 0.1 M NaCl and 2 volumes of cold ethanol were added. The DNA was spooled out with a glass rod and dissolved in 10 mM Tris-HCl, 1 mM EDTA (pH 8.5), (TE). The viscous DNA solution was then fragmented by shearing at 16,000 psi through a French pressure cell and precipitated with ethanol as before. The DNA pellet was recovered by centrifugation at 12,000 x g at 4°C, resuspended in buffer TE and incubated for 16 hours with 0.3 N 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 TE at a concentration of 9 mg/ml to be used in further experiments. The size of the DNA fragments obtained in this manner was found to be between 300-400 nucleotides, both by electrophoresis on 4% polyacrylamide gels, containing 7 M urea and centrifugation on alkaline sucrose gradients and comparison with markers of known size[28-29] (data not shown).

Isolation of DNA Fragments Resistant to DNase I

After incubation with DNase I, the nuclear suspension was pelleted by centrifugation for 5 min. at 3000 rpm on a Sorvall SS-34 rotor. The pellet

was used as a source of resistant DNA fragments. The DNA was extracted from the pellet as described above.

In vitro Labeling of Trout DNA

Trout DNA fragments of about 350 nucleotides in length were labeled *in vitro* as described by Summers, 1975[34].

cDNA/RNA Hybridization Reactions

cDNA/RNA hybridization reactions were carried out in 0.5 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA buffer, containing 0.01% SDS. Samples, in triplicate, containing 1000 cpm of cDNA (S.A. = 10^7 cpm/µg) and a 2000 fold excess of unlabeled RNA, were sealed in 5 µl capillaries, heated for 10 min. at 95°C and incubated at 70°C for various time periods. At the end of each incubation period, samples (in triplicate) were diluted with 1 ml of S_1 nuclease buffer (0.3 M NaCl, 0.03 M sodium acetate, 3 mM $ZnCl_2$, pH 4.5) containing also 20 µg/ml of denatured calf thymus DNA and digested with 1000 units of S_1 nuclease (Miles) for 45 min. at 45°C. After incubation, the samples were precipitated by the addition of 20% TCA, filtered onto glass fibre filters and counted. A zero time control sample (in triplicate) was included in every experiment. The counts remaining after S_1 nuclease digestion of this control and representing self-annealing of the cDNA were routinely subtracted from all the experimental points. The extent of self-annealing varied between 6-12%.

cDNA/DNA Renaturation Reactions

Renaturation reactions were carried out essentially as described above, except that the buffer contained NaCl at a concentration of 0.8 M. The reactions were carried out in 50 µl capillaries, and the DNA concentration was 5 mg/ml.

Fractionation of cDNA

Fractionated cDNA probes, representing the most abundant or rare mRNA sequences were obtained by partial annealing of the cDNA to mRNA to a Rot value of 0.24 M sec., followed by chromatography on hydroxylapatite to separate single and double-stranded species as previously described[28-29].

RESULTS

Characterization of the cDNA Probe. Hybridization with its Template RNA

Previous studies in our laboratory have demonstrated that, polyadenylated cytoplasmic RNA from testis at this particular stage of spermatogenesis and obtained by chromatography on poly U Sepharose, has a complexity of about 3.6 x 10^9 daltons[29], corresponding to some 6000 different sequences of average length, 600,000 daltons[28]. Individual mRNAs occur at widely different

frequencies.

We have recently found that the yields of mRNA obtained by using oligo-dT cellulose instead of poly U Sepharose, are more satisfactory and very reproducible and therefore we have chosen to use this method as a routine step in our mRNA preparations. In order to be certain that oligo-dT cellulose yields an identical population of mRNA sequences as the previously characterized one, we analyzed the complexity of this oligo-dT purified polyadenylated RNA, by hybridization of a cDNA representative of this population, with a vast excess (~ 2000 fold) of template RNA. The results are shown in Fig. 1 and demonstrate that this population of mRNA is identical to that previously studied in terms of diversity of sequences. The cDNA probe, representative of this population of polyadenylated cytoplasmic RNA was used in the following experiments described below.

Renaturation of cDNA with Trout DNA

To determine the fraction of polyadenylated cytoplasmic RNA derived from repetitive and non-repetitive DNA sequences, the cDNA was reassociated with a vast excess (~ 600 fold sequence excess) of trout DNA, sheared to a mean

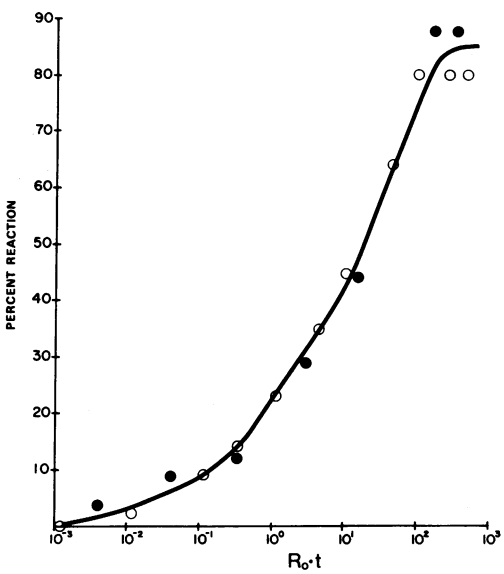

FIGURE 1. Complexity of the trout testis polyadenylated cytoplasmic RNA. Hybridization between cDNA and a vast excess of polyadenylated RNA prepared either by chromatography on poly U Sepharose (O) or oligo-dT cellulose (●), was performed as described in Methods. The points represent the mean value of 6 determinations. The mean size of the cDNA used in this hybridization was determined by alkaline sucrose gradient centrifugation to be approximately 7S (ca. 400 nucleotides).

average length of about 350 nucleotides.

As an internal control and to define the renaturation kinetic behavior of trout DNA fragments of this length we performed a reassociation experiment of the trout DNA by itself. To monitor the reaction, we chose to label *in vitro* the DNA fragments by the method of Summers[34] i.e., repair synthesis by DNA polymerase I, using calf thymus primers. Using ^{32}P-triphosphates, the DNA can be labeled to high specific activity (~ 10^7 cpm/μg). The labeled DNA fragments were incubated with an excess of unlabeled DNA. As illustrated in Fig. 2, under fairly stringent conditions of salt and temperature (i.e., 0.8 M NaCl, pH 8.0 and 70°C), approximately 45% of the trout DNA of this length renatures prior to a Cot value of 300 M sec. and can be designated as repeated sequences and the remaining 45% of the DNA is designated as unique sequences, although it may well contain two or more copies of a given gene sequence. On the other hand, some 21% of the cDNA representative of the polyadenylated cytoplasmic RNA population (the fraction that reacts up to a Cot of 300 M. sec.) exhibits kinetics characteristic of repeated sequences and the remaining 59% of the cDNA is complementary to unique DNA sequences. This finding is similar to that of Ryffel

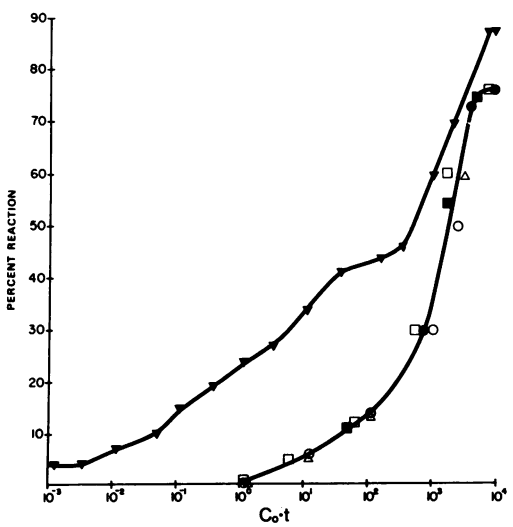

FIGURE 2. Renaturation kinetics of trout testis DNA and cDNA. Aliquots of *in vitro* labeled trout DNA or cDNA were reassociated with an excess of unlabeled trout DNA. (▼) Reassociation of *in vitro* labeled trout DNA, (●, ○, □, ■, △) reassociation of the cDNA.

and McCarthy, in L cells[17], Bishop *et al.*, in HeLa cells[15] and Levy W. and McCarthy, in Drosophila cells[16].

Renaturation Studies on cDNA Representing the Most Abundant or Rare Polyadenylated mRNA Sequences

McCarthy and co-workers in both mouse L cells[17] and Drosophila cells[16], demonstrated that the most abundant mRNAs of the cell are preferentially derived from repetitive DNA. Based on these results, we wished to establish the extent to which abundant polyadenylated mRNAs from trout testis would react with repetitive DNA. To this end, testis cDNA was fractionated into abundant and rare cDNA probes, by partial hybridization to testis mRNA and purification of single-stranded and double-stranded species on hydroxylapatite as described in Methods. Successful fractionation of the cDNA was evidenced by a much faster reaction kinetics displayed by testis abundant cDNA, upon hybridization with mRNA (Fig. 3). Both abundant and rare cDNA probes were then renatured with an excess of trout DNA (Fig. 4). To our surprise, both cDNAs reacted with essentially identical kinetics, demonstrating that both abundant and rare mRNAs of trout testis are reiterated to the same extent in the trout genome and that there is no preferential reaction of abundant mRNAs with repetitive DNA. A significant fraction (~ 25%) of both cDNA probes renatures with repetitive DNA.

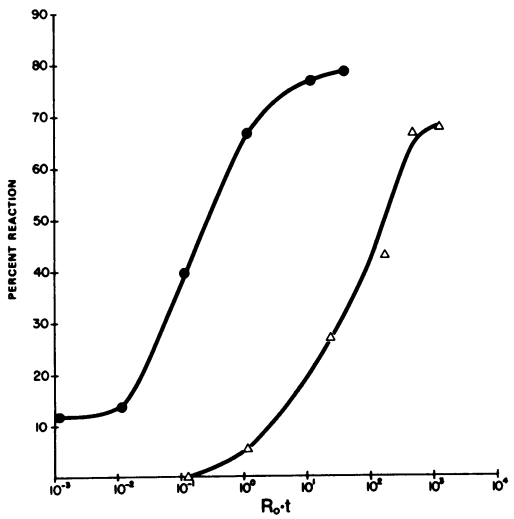

FIGURE 3. Hybridization of abundant and rare cDNA to an excess of polyadenylated RNA. (●) Reaction of the abundant cDNA, (△) reaction of the rare cDNA.

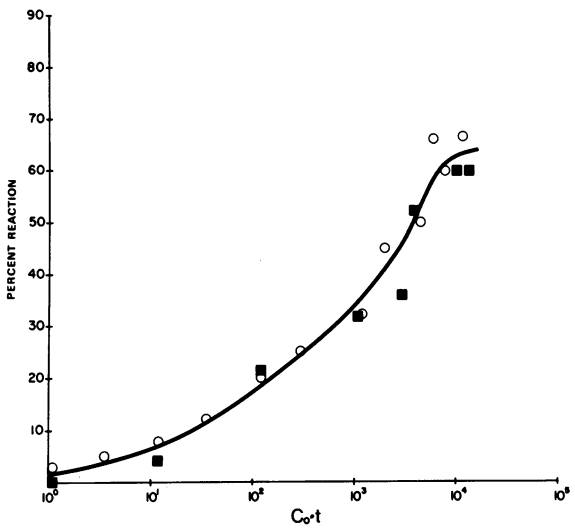

FIGURE 4. Reassociation of abundant and rare cDNAs with an excess of unlabeled trout DNA. (○) Reaction of the abundant cDNA, (■) reaction of the rare cDNA.

Digestion of Trout Testis Nuclei by DNase I

The kinetics of digestion of trout testis nuclei by pancreatic DNase are shown in Fig. 5. We observe a rapid initial digestion of about 18% of the DNA, followed by a slower digestion that levels off at about 28%. This behavior is similar to that observed in chicken erythrocyte nuclei[25], except that the plateau value achieved is different. This difference in the fraction of DNA susceptible to the enzyme is likely to be due to the fact that, in trout testis, at a relatively late stage of spermatogenesis, the DNA becomes tightly complexed with protamines, in such a way that it renders it inaccessible to nucleases[35].

We then examined the sensitivity to DNase I of those genes which give rise to cytoplasmic polyadenylated RNA. Trout testis nuclei were incubated with DNase I for 10 min. and the DNase resistant DNA purified as described in Methods. The DNA obtained in this manner, was used as a driver in renaturation experiments with the cDNA representative of the polyadenylated RNA population. As a control, we examined the kinetics of the reaction of DNA extracted from nuclei which were incubated under identical conditions, but in the absence of DNase I. Fig. 6 shows the results of these experiments. The

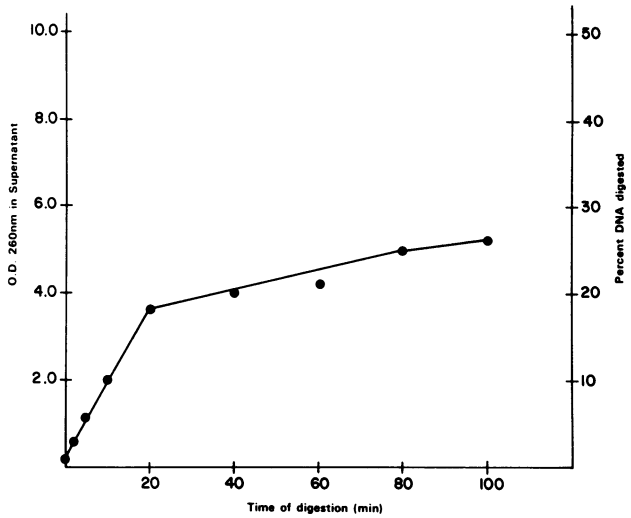

FIGURE 5. Kinetic of the digestion of trout testis nuclei by pancreatic DNase. The reaction was performed as described in Methods. The testis used in this experiments consisted of 30-40% late spermatid and sperm cells in which the histones had been replaced by protamine[29].

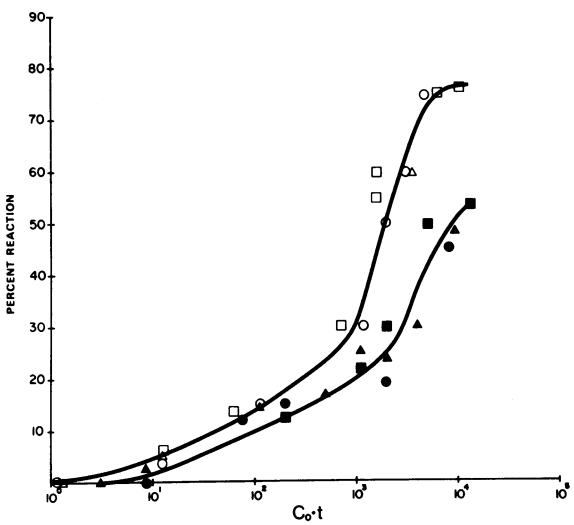

FIGURE 6. Reassociation kinetics of cDNA with DNase resistant DNA. The reactions were performed as described in Methods. Each symbol represents a different experiment with a different batch of DNA. (○, △, □) Reassociation of the cDNA with DNA from untreated nuclei. (●, ▲, ■) Reaction of the cDNA with DNase resistant DNA. Both fractions of DNA used to drive the reactions were found to be 300-400 nucleotides in length by polyacrylamide gel analysis.

kinetics of the reaction driven by the DNase resistant DNA is slower than that of the control reaction, as evidenced by a displacement to higher Cot values. At a Cot value of 10^4 M. sec., at which the control reaction has achieved completion at 77% saturation of the cDNA, the reaction of the resistant DNA has reached only 50%. Even though we were unable to achieve higher saturation values than 55%, after carrying the reaction to Cot values higher than 20,000, we are not certain whether this represents a real plateau since we observed a decrease in the counts of our controls without S_1 nuclease when the reaction was carried out to Cot values higher than 2×10^4 M. sec., indicating that the long period of incubation at 70°C (needed to reach such high Cot values due to the large analytical complexity of the trout genome) had caused some degradation of our cDNA probe.

In any event, even if this reaction were likely to achieve the same level of saturation as the control reaction, when carried out at much higher Cot values, our data still show that, upon DNase I treatment in which 10% of the total DNA is digested, there is a depletion of sequences coding for cytoplasmic polyadenylated RNA in the DNase resistant DNA. Unfortunately, the lack of a suitable probe representative of an inactive genome in our system has not allowed us to perform experiments to study the DNase sensitivity of unexpressed genes. Therefore, we cannot extend our conclusions to say that "only" those genes in an active conformation in trout testis nuclei are preferentially attacked by DNase I. Instead, we can say that, in trout testis, a great proportion of those genes which are actively transcribed, giving rise to cytoplasmic polyadenylated RNA, are very susceptible to DNase I attack.

These studies extend and confirm the results of Weintraub and Groudine[25], for the globin genes, and those of Garel and Axel[26], for the ovalbumin genes and reveal that the template active segments are organized in chromatin in such a way that they are very sensitive to DNase I attack.

The availability of cDNA probes complementary to either the most abundant or the most rare mRNA sequences from trout testis allowed us to extend these studies by asking whether we could observe a differential behavior of DNase I towards DNA sequences engaged in the production of abundant or rare polyadenylated RNAs. To elucidate this question, we compared the renaturation kinetic behavior of abundant and rare cDNA probes when driven by DNA obtained from DNase digested trout testis nuclei. Fig. 7 shows the renaturation of the abundant cDNA and Fig. 8, the reaction of the rare cDNA. We observe that the kinetic curve of the reaction of the abundant cDNA is almost identical to that observed in the control reaction with total DNA,

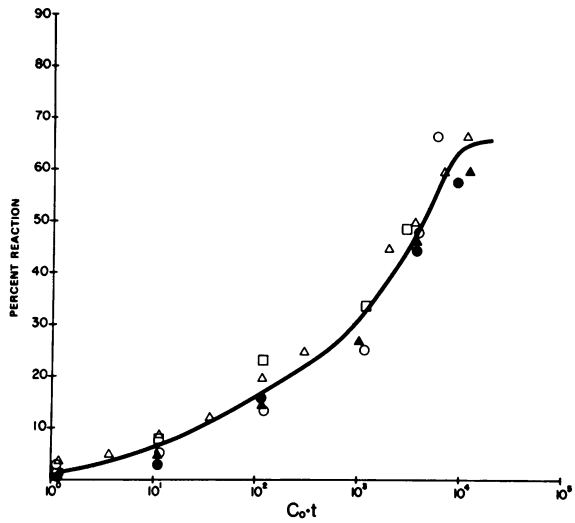

FIGURE 7. Reassociation of abundant cDNA with control and DNased DNA. (○, □, △) Reaction with total DNA, (●, ▲) reaction with DNased DNA.

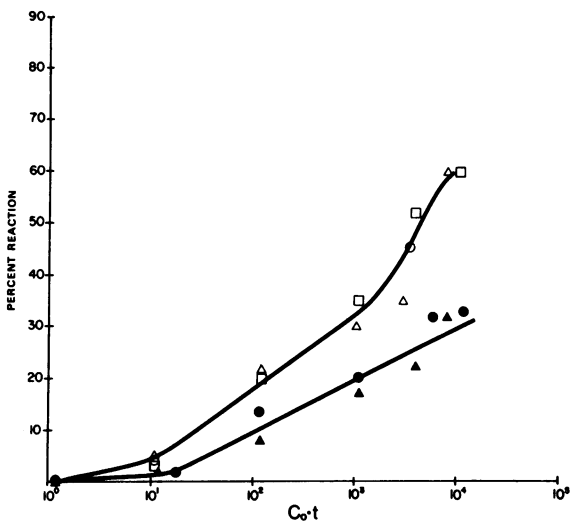

FIGURE 8. Reassociation of rare cDNA with control and DNased DNA. (○, △, □) Reaction with total DNA, (●, ▲) reaction with DNased DNA.

indicating that the DNase resistant DNA has not been depleted of sequences giving rise to abundant polyadenylated RNA. In contrast, the slower renaturation reaction of the rare cDNA probe with DNase I treated DNA as

compared to the control reaction demonstrates that a significant fraction of the sequences coding for rare mRNAs has been removed from the DNA upon digestion with the DNase. Therefore we can conclude that DNA sequences in the trout genome coding for polyadenylated mRNAs whose concentration in the cell is low, are accessible to digestion by DNase I.

DISCUSSION

We have examined the renaturation kinetics of trout cDNA, complementary to cytoplasmic polyadenylated RNA from trout testis at a late stage of spermatogenesis. The validity of our conclusions in all experiments regarding the mRNA population is, therefore, limited to those sequences represented in the cDNA and may not accurately reflect the total polyadenylated RNA population, since it is not clear to what extent all polyadenylated molecules are copied by the reverse transcriptase[15,16]. However, the good agreement encountered between the results of experiments using unique sequence DNA/RNA and cDNA/RNA hybridizations[32,33,36] give support to the cDNA approach.

Non-polyadenylated cytoplasmic mRNAs, known to exist in various cell types[37,38,39,40], are also not dealt with in our study.

Our results show that, even though a major fraction of the cDNA (some 59%) is complementary to unique DNA, a significant fraction (some 21%) of our probe renatures with intermediate repetitive DNA. It should be mentioned, that the designations of repetitive and unique sequence DNA are operationally defined by the conditions of ionic strength and temperature[41] and DNA fragment size[42]. Our findings are in close agreement with similar findings in other cell types[13-17].

The studies with fractionated cDNA probes demonstrate that, in trout testis, the relative abundance of a polyadenylated RNA in the cell, bears no relationship to its degree of reiteration in the genome. These findings differ from previous studies in cultured cells[16-17], in which it was found that the most abundant mRNAs were preferentially derived from repetitive DNA.

The significance of the repetitive sequence content of the cDNA cannot be clearly assessed at present. Several possibilities can be suggested. The most attractive one would imply that rapidly renaturing sequences would reflect multiple copies of gene sequences, such as those coding for histones[11-12]. The recent discovery of polyadenylated histone mRNAs in *Xenopus*[43] makes the histone gene sequences a likely component of our polyadenylated RNA fraction that exhibits rapid reassociation rates. A second possible source of repetitive transcripts is the mitochondrial genome, as it has been discussed by Ryffel and McCarthy[17]. The renaturation kinetics could also be explained by

internal repetition of sequences within genes[44] or sequences common to several genes. However, none of the possibilities suggested above are mutually exclusive.

The organization of proteins within actively transcribed genes is likely to be different from that about non-transcribed regions of the genome. The convincing experiments of Weintraub and Groudine[25], which demonstrate the selective digestion of globin genes by DNase I in erythroid tissues together with those of Garel and Axel[26], which showed the selective digestion of ovalbumin genes in hen oviduct by DNase I, provide strong support to this idea.

Our data obtained by digestion of trout testis nuclei with DNase I, further support the belief that active genes exist in a special conformation that is selectively sensitive to DNase I. Upon digestion of trout DNA, to the extent of 10%, the genome is depleted of sequences which normally give rise to cytoplasmic polyadenylated RNA, as measured by hybridization of the resistant DNA to our cDNA probe. The reduced but still significant rate of annealing between the cDNA and the DNase I depleted DNA may be the result of the heterogeneity of cell types present in our tissue. In some cell types, those genes represented in the cDNA might be inactive and, therefore, less susceptible to DNase I[25-26]. The batch of testes employed in our present studies is not homogeneous since individual testes were collected from a random population of naturally maturing fish, at a determined period of the year. However, because trout testis undergoing spermatogenesis is a relatively synchronous tissue in which one cell type predominates at each stage of development, we should bear in mind that it is also possible that the DNase might be cleaving active genes from all cell types to small fragments that still participate in reassociation reactions, but at a reduced rate.

Our results with fractionated cDNA probes indicate that DNA sequences coding for mRNAs whose concentration in the cell is low are more sensitive to the DNase than those sequences which give rise to abundant mRNAs. This finding is very difficult to interpret, especially since, from our results on renaturation of fractionated cDNAs (Fig. 4) we cannot argue that the sequences coding for abundant mRNAs are reiterated to a greater extent in the genome, and therefore, more resistant to DNase on the mere basis of a concentration effect. Moreover, we do not know at present, whether the entire cytoplasmic polyadenylated RNA population corresponds to messenger RNA. If the abundant poly A-containing RNA represented another non-messenger class of RNA[45] it is perhaps possible that the chromatin from which it was transcribed, might exist in a different, i.e., DNase I resistant conformation.

One should also bear in mind that, in these studies with complex mRNA populations, where heterogeneity of lifetimes exists, abundant mRNA species may arise not only as a result of increased transcription of particular sets of genes but also because of more efficient processing of their nuclear precursors, longer lifetimes of these mRNAs, due to more extensive secondary structure or due to association with proteins in mRNPs, etc.

Very little information is currently available as to the nature of the factors responsible for the conformation of active regions of the genome. Therefore, the enhanced susceptibility to DNase I of certain regions of chromatin cannot easily be explained in simple terms. The observation that adult globin genes remain susceptible to DNase I in mature erythrocytes[25], in which globin RNA synthesis has ceased, make it unlikely that the presence of the transcriptional complex upon a given region of chromatin is the only factor responsible for the conformational differences.

The observation that active genes exist in a conformation in which they are very sensitive to DNase I attack seems to be of wide occurrence. In the pioneer work by Weintraub and Groudine[25] the structure of globin genes in erythroid cells was examined. These studies were supported by those of Garel and Axel[26], on the DNase I sensitivity of ovalbumin genes in oviduct nuclei. Our studies extend these observations to the whole population of genes engaged in the production of cytoplasmic polyadenylated RNA from trout testis. The general validity of this phenomenon indicates that a major fraction of the genes transcribed into poly A-containing RNA in a particular tissue share the perturbation of structure which enables DNase I to digest their constituent DNA.

ABBREVIATIONS USED: mRNA, polyadenylated cytoplasmic RNA; SDS, dodecyl-sulfate; Cot, product of the DNA concentration (M) versus time (sec); Rot, product of the RNA concentration (M) versus time (sec); DNase, deoxyribo-nuclease; mRNP's, messenger-ribonucleoprotein complexes.

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