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Diversity of Sequences of Polyadenylated Cytoplasmic RNA from Rainbow Trout (*Salmo gairdnerii*) Testis and Liver†

Beatriz Levy W. and Gordon H. Dixon*

ABSTRACT: We have compared the sequence complexity and diversity of polyadenylated cytoplasmic RNA derived from two differentiated trout tissues: liver and testis. The kinetics of hybridization of polyadenylated RNA from each of these tissues with complementary DNA synthesized by reverse transcriptase revealed three abundance classes for liver RNA, the first comprising 4 sequences, the second 120, and the third, 20 000; in contrast, testis RNA showed only two abundance classes containing 6 and 6100 different RNA sequences, respectively, and of average length 6×10^5 daltons. The extent of overlapping among those two RNA populations was further studied by performing heterologous annealing reactions between cDNA and a vast excess of mRNA. Liver mRNA was complementary to 80% of the testis cDNA. Conversely, testis mRNA reacted with only 25% of the liver cDNA. Experiments with fractionated cDNA probes indicated that the unshared sequences belonged mainly to the less frequent, most complex, class of mRNAs.

The mechanisms by which gene expression is regulated in eukaryotes remain unclear. The active genes in a given tissue may be divided into two sets: those genes required for functions common to all cells of the organism, e.g., the common enzymes of metabolism, membrane components, etc., which may be termed "housekeeping genes", and those which determine the specialized functions of the cell. The relative sizes of these two sets of genes could vary widely so that, at one extreme, the set of "housekeeping genes" would be large in relation to those for specialized functions, while, at the other, this set could be small in relation to the set of specialized genes (Galau et al., 1976).

One approach to this problem involves a comparative study of the messenger RNA populations of different specialized tissues. Two experimental procedures are currently available for the study of sequence complexity of RNA populations. In the first, hybridization reactions are allowed to take place to saturation between radioactive unique sequence DNA and RNA (Hahn and Laird, 1971; Levy W. et al., 1976a,b). The amount of DNA in hybrid form at saturation gives information about the extent of transcription from this particular class of DNA. The second procedure involves synthesis of highly radioactive DNA copies of polyadenylated RNA fractions by reverse transcriptase in the presence of oligo(dT) primers and hybridization of the cDNA to a vast excess of RNA template (Bishop et al., 1974; Birnie et al., 1974; Ryffel and McCarthy, 1975; Levy W. and McCarthy, 1975; Axel et al., 1976). The kinetics of hybridization allows not only a measurement of the number of different sequences present as mRNA, but also gives information regarding the relative abundance of these sequences within the RNA population. This method has the additional advantage of dealing with transcripts from both repetitive and unique DNA.

In this report, we have used the second procedure to analyze the diversity of sequences of polyadenylated RNA from two specialized tissues of the trout: liver and testis.

Materials and Methods

Preparation of Liver and Testis Cytoplasmic RNA. Livers were removed from rainbow trout (*Salmo gairdnerii*), maintained in the laboratory aquarium at 12–13 °C, and stored frozen at −70 °C for short periods of time before utilization.

Testis were collected at a late stage of maturation (in October 1973) from freshly killed trout (Dantrout, Brande, Denmark), immediately frozen on dry ice, and stored frozen at −70 °C.

For a typical RNA preparation, 20 g of frozen tissue (liver or testis) was broken into small pieces, and then allowed to thaw at room temperature for about 10 min. Two volumes of a solution of TMKS buffer (0.25 M sucrose, 40 mM Tris[1]-HCl, pH 7.4, 25 mM KCl, and 5 mM $MgCl_2$) were added and the tissue was first homogenized (at 4 °C) in a mortar and then in a glass homogenizer with a Teflon pestle at a slow speed (three to four strokes). The homogenate was filtered through four layers of gauze and subsequently centrifuged at 800*g* for 7 min at 4 °C. The nuclear pellet was kept for the isolation of DNA.

The cytoplasmic supernatant was adjusted to 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5% sodium dodecyl sulfate, and the RNA was extracted with phenol-chloroform and chloroform–isoamyl alcohol as described

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[1] Abbreviations used are: mRNA, polyadenylated cytoplasmic RNA; cDNA, complementary DNA; poly(A), polyadenylic acid; rRNA, ribosomal RNA; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

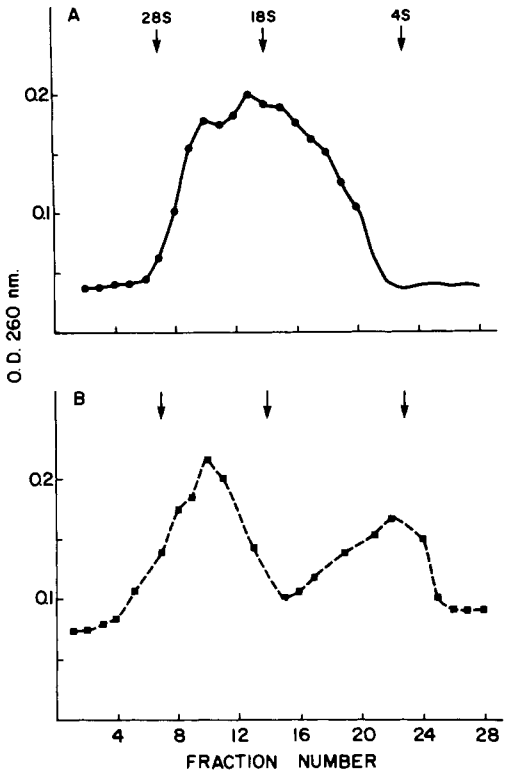

FIGURE 1: Sucrose gradient analysis of liver and testis polyadenylated cytoplasmic RNA. Liver and testis polyadenylated RNA were prepared as described under Methods. One sample of each preparation was analyzed on a 15–30% sucrose gradient in 0.1 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA containing 0.5% sodium dodecyl sulfate. Centrifugation was for 19 h at 25 000 rpm in a Beckman SW 41 rotor at 22 °C. The S values were taken from 4, 18, and 28 S trout testis rRNA markers run on a parallel gradient. (A, ●) Liver mRNA; (B, ■) late testis mRNA.

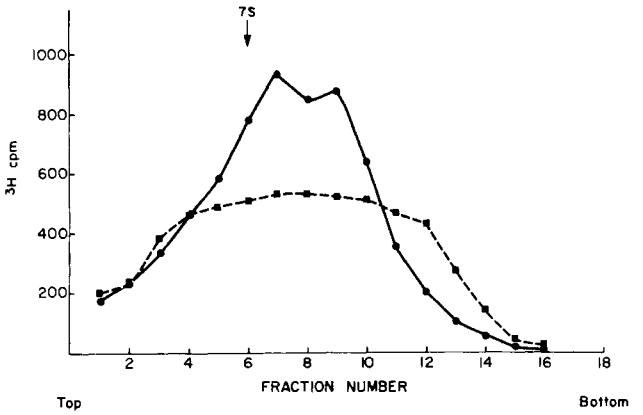

FIGURE 2: Sedimentation profile of liver and testis cDNAs on alkaline sucrose gradients. Liver and testis cDNAs were synthesized by reverse transcriptase as described under Methods. A sample of each was analyzed on a 10–30% sucrose gradient in 0.9 M NaCl, 0.1 M NaOH, 5 mM EDTA. Centrifugation was for 20 h at 30 000 rpm in a SW 41 Beckman rotor at 10 °C (Levy W., 1975). A sample of mouse single-stranded [^{3}H]DNA sedimenting at 7 S was run as a standard on a parallel gradient. (■) Liver cDNA; (●) testis cDNA.

previously (Levy W. and McCarthy, 1975). After the last extraction, the RNA in the aqueous phase was precipitated by the addition of 2 volumes of 95% ethanol.

Polyadenylated RNA was obtained by chromatography on poly(U)-Sepharose (two cycles) as described previously (Levy W. and McCarthy, 1975).

Synthesis of cDNA. The conditions described by Iatrou and Dixon (1977) for synthesis of the cDNA for purified protamine mRNA were used. The complete reaction mixture, 100 μL, contained the following final concentrations: 50 mM Tris-HCl, pH 8.3; 10 mM dithiothreitol (DTT); 6.5 mM $MgCl_2$; 45 mM KCl; 100 μg/mL actinomycin D (Boehringer-Mannheim); 0.6 mM dATP, dTTP, and dGTP; 200 μCi/mL [^{3}H]dCTP (Schwarz/Mann, specific activity 25.46 Ci/mmol); 9 μg/mL oligo(dT)$_{12-18}$; 10 μg/mL polyadenylated RNA; and 160 units/mL AMV reverse transcriptase (kindly supplied by Dr. J. W. Beard).

Incubation was for 50 min at 40 °C. After incubation, the cDNA was treated with NaOH as described previously (Levy W. and McCarthy, 1975) and further purified as reported by Iatrou and Dixon (1977).

Hybridization Reactions. Hybridization reactions were carried out in 0.5 M NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA buffer containing 0.01% sodium dodecyl sulfate. Samples (in triplicate) containing about 1000 cpm of cDNA (specific activity ~10^7 cpm/μg) and 100 μg/mL RNA were sealed in 5-μL capillaries, boiled for 10 min, and incubated at 70 °C for various time periods. At the end of each incubation period, duplicate samples were diluted with 0.25 mL of S_1 nuclease buffer (0.3 M NaCl, 0.03 M NaOAc, 3 mM $ZnCl_2$, pH 4.5) and digested with S_1 nuclease for 45 min at 45 °C. After incubation, the samples were precipitated by the addition of 20% Cl_3CCOOH, filtered onto glass fiber filters, and counted.

A zero time control sample (in duplicate) 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 was consistently about 7–10% with liver cDNA and between 5 and 10% with testis cDNA.

Fractionation of cDNA Probes. Fractionated cDNA probes were obtained by partial annealing of the cDNA with RNA to a desired R_0t value followed by chromatography on hydroxylapatite to separate single- and double-stranded species as previously described (Levy W. and McCarthy, 1975).

Results

Preparation of Liver and Testis Polyadenylated mRNA and Complementary DNA. Total and polyadenylated cytoplasmic RNA were prepared from trout liver and late testis as described under Methods. A sucrose gradient profile of both mRNAs is shown in Figure 1. The mean sedimentation value for the trout liver polyadenylated RNA was about 18 S (Figure 1A). A similar result was obtained by Ryffel (1976) in *Xenopus* liver. The corresponding testis RNA showed a biphasic sedimentation behavior with one peak between 18 and 28 S and the other with a mean value of about 6 S (Figure 1B). This sedimentation behavior of testis mRNA was very reproducible, as checked by using different batches of mRNA and also with mRNA from three different stages of development of the testis (Levy W. and Dixon, 1977).

Both liver and testis mRNA populations were used as templates for the synthesis of complementary DNA probes by reverse transcriptase. In both cases, reactions were totally dependent upon the addition of oligo(dT), implying that transcription of sequences adjacent to poly(A) had taken place. Figure 2 shows alkaline sucrose gradient profiles of liver and testis cDNA: both cDNAs sediment as a series of components having a broad range of sizes.

Complexity of Liver and Testis mRNA. The complexity of both RNA populations was elucidated by an analysis of the kinetics of hybridization of both liver and testis cDNA to large

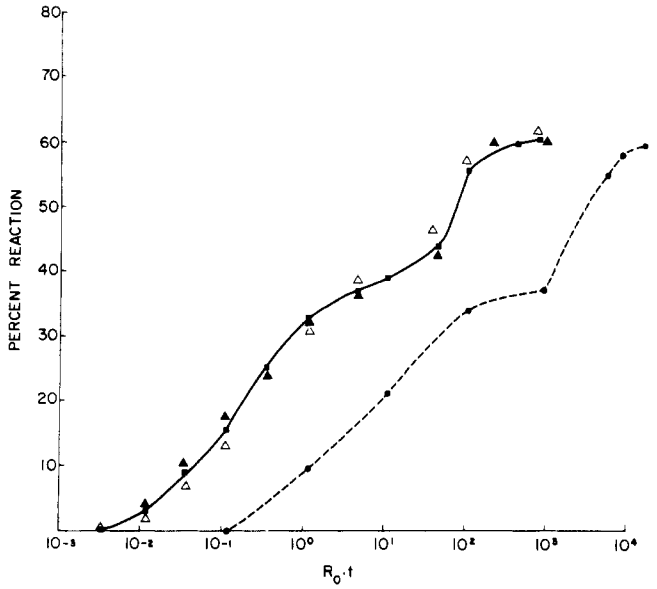

FIGURE 3: Hybridization between liver cDNA and polyadenylated and total cytoplasmic liver RNA. cDNA was transcribed on cytoplasmic polyadenylated liver RNA as template was hybridized with polyadenylated liver RNA (△, ■, ▲) and total cytoplasmic RNA (●) as described under Methods. Reproducibility was checked by performing the experiment three times with different batches of cDNAs and RNAs. Each point represents the mean value of three determinations.

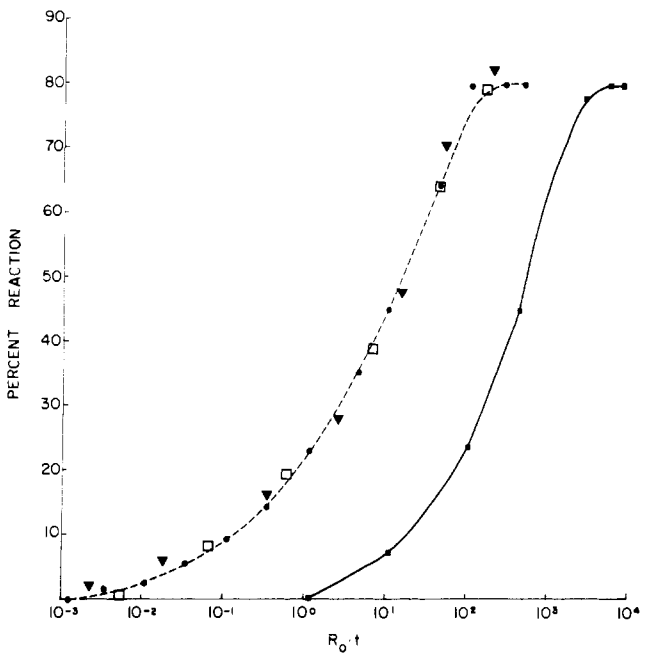

FIGURE 4: Hybridization between testis cDNA and polyadenylated and total cytoplasmic RNA from testis. cDNA synthesized on a template of testis polyadenylated cytoplasmic RNA was annealed with its template (▼, □, ●) and total cytoplasmic testis RNA (■). Reproducibility was checked as described in the legend to Figure 3.

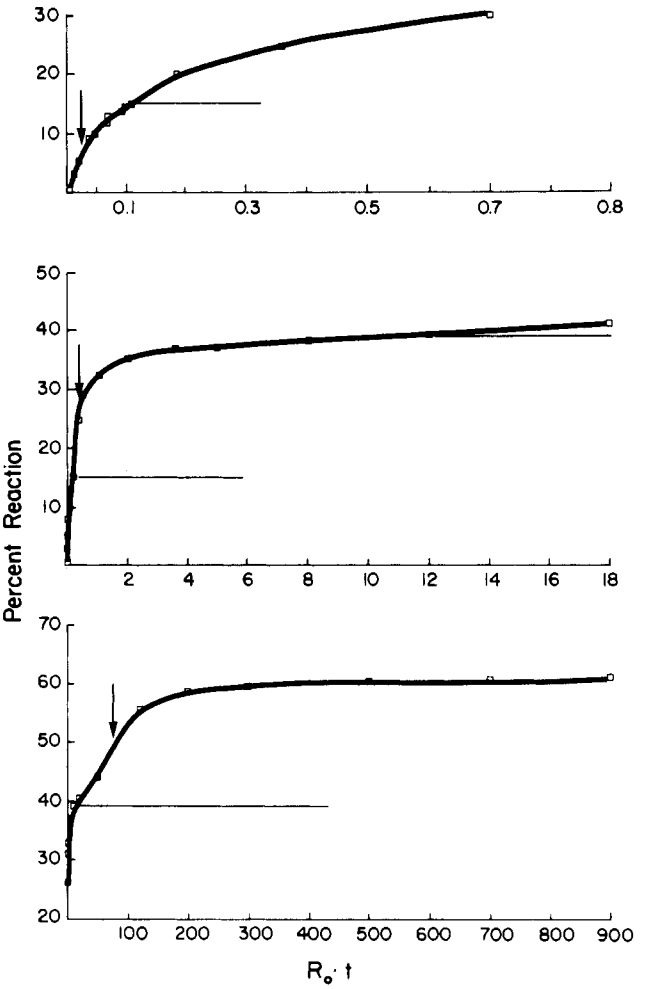

FIGURE 5: Linear plots of hybridization between liver cDNA and its template RNA. The data are taken from Figure 3.

excesses (2500-fold) of liver and testis mRNAs, respectively. Figure 3 shows the reaction of liver cDNA with its template, and Figure 4 shows the reaction of testis cDNA with testis mRNA. Clearly, in both cases, the hybridization kinetics are complex, extending over several log units of R_0t. This behavior can be explained by the presence of RNA molecules occurring at widely different frequencies in the cell (Bishop et al., 1974; Ryffel and McCarthy, 1975; Levy W. and McCarthy, 1975).

Abundant polyadenylated RNA hybridizes more rapidly to its cDNA than is the case for rare species. The frequency distribution within the messenger population can, in principle, be calculated by calibrating the hybridization kinetics with that of a pure species of mRNA and its cDNA. We have obtained a $R_0t_{1/2}$ value of 1×10^{-3} for the reaction between purified ovalbumin mRNA of 600 000 daltons and its cDNA (data not shown) (Levy W., 1975).

When semilog R_0t plots are complex, the resolution of individual kinetic components may be achieved through use of a plot linear in R_0t (Bishop et al., 1974) or through the use of a computer program designed to obtain best fit of the data (Levy W. and McCarthy, 1975; Axel et al., 1976). We have chosen to resolve the data into the minimum number of components necessary to describe adequately the complete reaction, by using the former alternative (whose only pitfall is the somewhat subjective extrapolation of an asymptotic plot), since our main interest in the present communication is a comparison of the RNA populations and not an exact determination of the number of sequences in each case.

Figure 5 shows a linear plot of the data of Figure 3. Three saturation plateaus are seen with midpoints ($R_0t_{1/2}$, M s) of 0.025, 0.5, and 75. Figure 6 shows a linear plot for the data of Figure 4. In this case, two saturation plateaus are obtained with $R_0t_{1/2}$ values of 0.04 and 10. To calculate the number of different RNAs in each class, the $R_0t_{1/2}$ values obtained were corrected to those for pure components and divided by $1 \times$

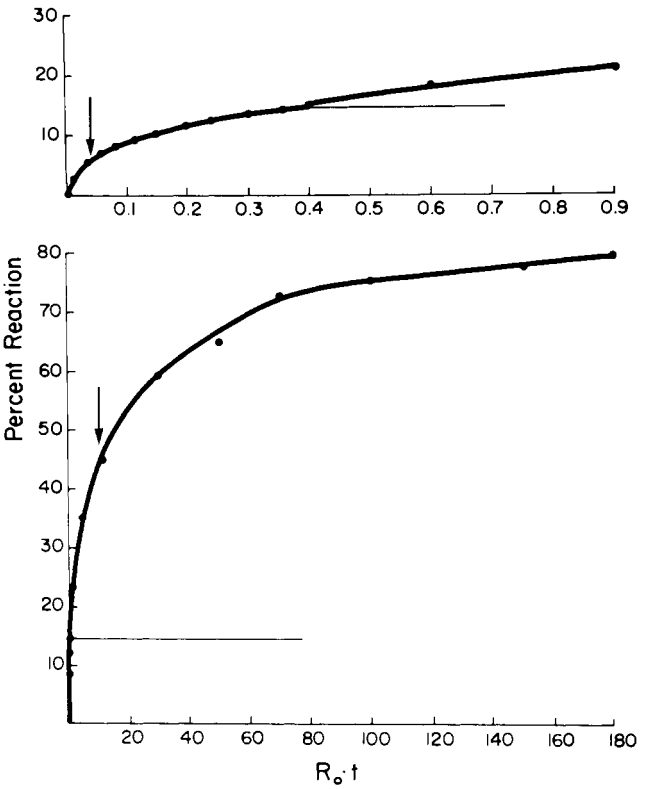

FIGURE 6: Linear plots of hybridization between testis cDNA and its template RNA. The data are taken from Figure 4.

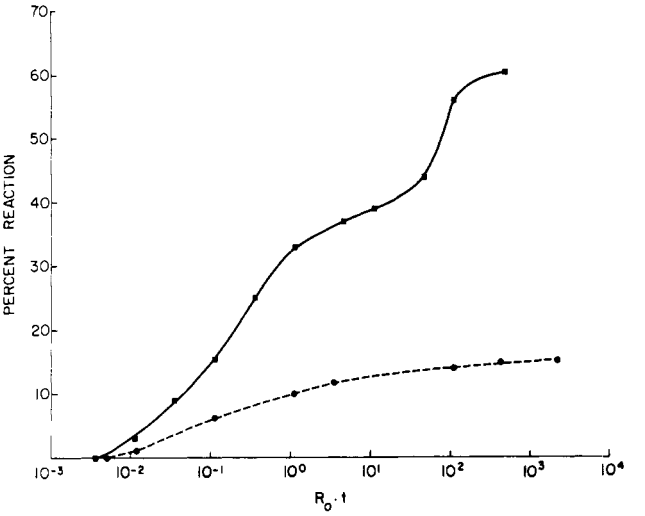

FIGURE 7: Hybridization between liver cDNA and testis cytoplasmic polyadenylated RNA. Liver cDNA was annealed to an excess of testis mRNA as described under Methods. (●) Reaction of liver cDNA with testis mRNA; (■) reaction of liver cDNA with liver mRNA.

TABLE I: Frequency Classes of Trout Liver mRNA.[a]

Transition	% cDNA Hybridized	Obsd $R_0t_{1/2}$	Corr $R_0t_{1/2}$	No. of Different Sequences
1	14	0.025	0.0035	4
2	24	0.5	0.12	120
3	27	75	20.2	20 000

[a] The data are obtained from Figure 5.

TABLE II: Frequency Classes of Trout Testis mRNA.

Transition	% cDNA Hybridized	Obsd $R_0t_{1/2}$	Corr $R_0t_{1/2}$	No. of Different Sequences
1	14	0.04	0.0056	6
2	61	10	6.1	6100

[a] The data are obtained from Figure 6.

10^{-3}, the $R_0t_{1/2}$ obtained for our standard, ovalbumin RNA, with molecular weight 6×10^5 daltons.

Table I shows that, in liver, the first class, representing 14% of the total cDNA hybridized, includes approximately 4 different sequences, the second (24%) about 120, and the last (27%) about 20 000 different RNA sequences of this average size. These results are in reasonable agreement with those of Axel et al. (1976) and Ryffel (1976) for chicken liver cells and *Xenopus* liver cells, respectively.

The data in Table II indicate that, in late testis, the first component, comprising 14% of the total cDNA, includes about 6 different sequences and the remaining component (61%) about 6100 sequences. Even though the mean average length of a polyadenylated RNA sequence of late testis cytoplasm is smaller than that of liver mRNA, we have calculated the numbers of sequences, assuming the same average length for both RNA populations, in order to facilitate comparison.

It should be stressed that the data could be resolved into a much larger number of components but this would not significantly improve the degree of fit. Furthermore, the division into abundance classes is only a crude approximation, since there is no reason to assume that all molecules in each class are represented in exactly equal numbers. The greatest contribution to the total base sequence complexity of an RNA population is from the slowest kinetic component. Because we cannot discard the possibility that a very low abundance component of high complexity may have been missed, we regard the data in Tables I and II as minimum estimates.

Both liver and testis cDNA were then annealed with total cytoplasmic RNA, rather than the poly(A)-containing fraction alone from these tissues. In the case of liver cDNA (Figure 3), the reaction was shifted by a factor of 50 to higher R_0t values from that driven by polyadenylated RNA, implying that only about 2% of the liver cytoplasmic RNA is polyadenylated. This estimate agrees well with that reported for chicken liver (Axel et al., 1976) and *Xenopus* liver (Ryffel, 1976). A similar result was found when late testis cytoplasmic RNA was employed to drive testis cDNA (Figure 4). This reaction was displaced by almost 100-fold to higher R_0t values, indicating that approximately 1% of the cytoplasmic RNA is polyadenylated in late testis.

Extent of Sequence Homology Among These Two RNA Populations. Having demonstrated the presence of about 20 000 different polyadenylated mRNA sequences in liver and about 6000 different RNA sequences in testis, of average length 600 000 daltons, we proceeded to determine the extent to which those sequences were shared by both tissues. To this end, we examined the hybridization kinetics of cDNA complementary to the mRNA of one tissue when driven by the RNA from the heterologous tissue. The extent of overlap between the sequences from the two RNA populations can be estimated from the saturation value attained, while the kinetics

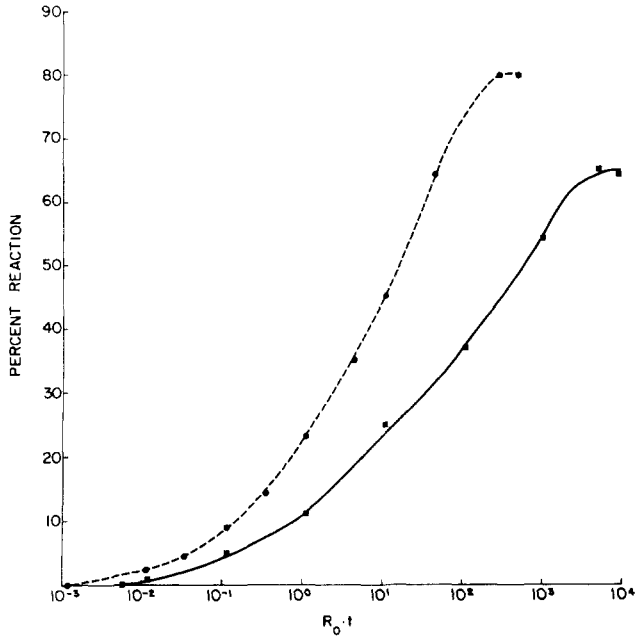

FIGURE 8: Hybridization between testis cDNA and liver cytoplasmic polyadenylated RNA. Testis cDNA was annealed to liver mRNA as described under Methods. (■) Reaction of testis cDNA and liver mRNA; (●) reaction of testis cDNA and its template.

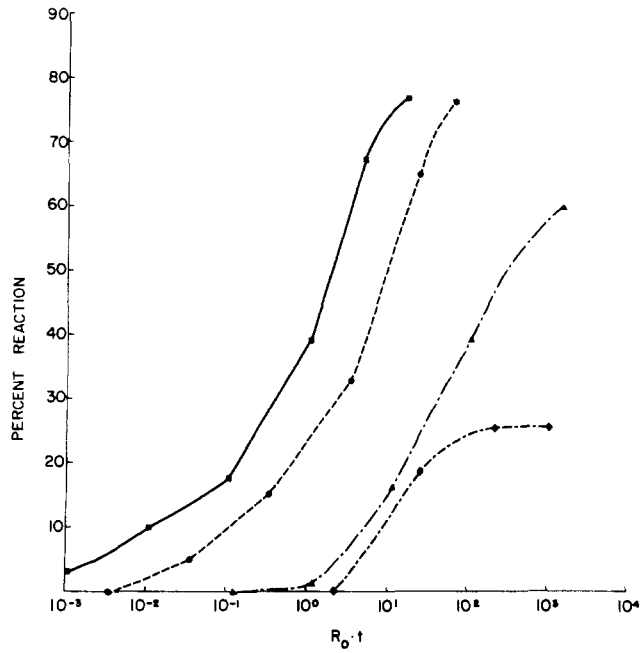

FIGURE 9: Reaction of frequent and infrequent testis cDNA with testis and liver polyadenylated RNA. cDNA probes, representing frequent and infrequent testis mRNAs, were obtained as indicated under Methods. Samples in triplicate, containing about 1000 cpm of cDNA were annealed with RNA. (■) Reaction of frequent testis cDNA with testis mRNA; (●) reaction of frequent testis cDNA with liver mRNA; (▲) reaction of infrequent testis cDNA with testis mRNA; (♦) reaction of infrequent testis cDNA with liver mRNA.

of the reaction yields information concerning the relative abundance of the overlapping sequences in the two tissues.

The reaction between a vast excess of testis mRNA and liver cDNA is shown in Figure 7. To facilitate comparison, we have included in the figure the reaction of the cDNA with the homologous RNA. The testis mRNA driven reaction approaches saturation at about 15% hybridization. In other words, testis mRNA is complementary to 25% (15/60) of the liver cDNA.

The results obtained by hybridization of liver mRNA to testis cDNA are shown in Figure 8. Again, we have included in the figure the homologous annealing reaction for comparison.

This reaction approaches a value of 64% hybridization at saturation, compared to 80% observed in homologous reaction. Therefore, we conclude that 80% (64/80) of the testis cDNA is complementary to liver polyadenylated RNA. The kinetics of hybridization show that, for instance, at a R_0t of 30 about 30% of the cDNA reacts with the heterologous RNA, while at the same R_0t value the homologous reaction reaches 60%. In addition, the bulk of the liver mRNA sequences also present in testis mRNA react with a $R_0t_{1/2}$ value of 20 indicating that their relative abundance is quite low.

The availability of purified cDNA probes representing frequent and infrequent sequences in late testis mRNA (Levy W. and Dixon, 1977), allowed us to investigate the question of the relative abundance of the RNA sequences shared by testis and liver. Testis frequent and infrequent cDNA probes were hybridized to vast excesses of testis and liver polyadenylated RNA. The results of these experiments (Figure 9) show that liver mRNA reacts to the same extent as testis mRNA with frequent testis cDNA, proving, therefore, that all of the most frequent sequences in testis cDNA are also represented in the liver mRNA population. However, the relative concentration of these common sequences is lower in the liver RNA population, as indicated by a fivefold shift to higher R_0t values. In contrast, liver mRNA reacted only with ~25% of the infrequent testis cDNA, thus demonstrating that the great majority of the testis-specific sequences absent in liver belong to the infrequent, most complex, class of mRNAs. Converse experiments employing fractionated liver cDNA probes were not performed, because from inspection of the kinetics of reaction of the total liver cDNA with testis mRNA it seemed very likely that most of the sequences missing in testis would also belong to the rare class of liver mRNA sequences.

Discussion

We have studied the distribution of the relative numbers of individual polyadenylated RNAs within the populations of cells from two trout tissues: liver and testis. Furthermore, we have determined the extent to which the population of these mRNAs is shared between these tissues.

The experimental procedure involved hybridization of cDNA to an excess of RNA. The limitations of this method have been extensively discussed (Levy W. and McCarthy, 1975; Ryffel and McCarthy, 1975; Bishop et al., 1974; Birnie et al., 1974; Axel et al., 1976; Levy W. and Dixon, 1977). The main assumption is that there is random copying of mRNAs by reverse transcriptase, regardless of their relative abundance (Bishop et al., 1974).

Estimation of the number of active genes depends on obtaining an accurate value of the $R_0t_{1/2}$ for the most slowly annealing component. This is very difficult to obtain because cDNA reactions (in which only a fraction of the population of cDNAs represent complete copies of the templates) do not go to completion (Weiss et al., 1976, submitted for publication) and, when the final transition represents a small proportion of the total reaction, the accuracy in the determination of small increments of hybridization is limited. For these reasons, very precise estimates of gene number are not possible using this method.

Our experimental results indicate that there are wide variations in the number of copies of different polyadenylated RNAs in both trout liver and testis.

For liver, this distribution can be approximated by three frequency classes, one representing a few genes, the second a few hundred genes, and the third several thousand genes. These results are in close agreement with those of Ryffel (1976) in *Xenopus* liver and Axel et al. (1976) in chicken liver and imply the existence of some 14 000–20 000 different polyadenylated RNA sequences of average length in the liver of eukaryotic organisms.

In contrast, the number of mRNA species present in testis is smaller. Only about 6000 different polyadenylated RNAs of 600 000 daltons in length exist in mature testis. In this case, their distribution can be best approximated by two frequency classes, representing some 6 and 6100 active genes, respectively. This result is not surprising, since trout testis is known to be a tissue in which RNA synthesis declines rapidly at late stages of maturation (Ando and Hashimoto, 1958).

Those experiments which were designed to determine the extent of overlap between polyadenylated RNAs from the two tissues show that 80% of the liver poly(A) RNA is complementary to testis cDNA and that only 25% of testis poly(A) RNA reacts with liver cDNA. In other words, only 20% of the testis cDNA is unable to anneal with liver mRNA, while a much greater proportion (about 75%) of the liver cDNA cannot anneal with testis mRNA.

The results of experiments performed with cDNA probes representative of the most frequent and most infrequent sequences of testis mRNA indicate that most of the sequences common to liver and testis belong to a high- and middle-frequency class, while the unshared sequences belong to the less abundant class.

We should bear in mind that these experiments are subject to the uncertainty regarding the extent to which all polyadenylated RNAs are copied by the reverse transcriptase. Therefore, the assessment of shared sequences is limited to those represented in the cDNA and may not accurately reflect the total mRNA population. Also, the possibility that a significant fraction of mRNAs devoid of poly(A) may exist in these tissues cannot be eliminated (Milcarek et al., 1974).

The numbers obtained encourage one to suggest that in specialized tissues a correlation may exist between the diversity of mRNA species and their metabolic functions. Liver, with its multiple functions, would require for proper operation the activation of a much larger set of genes than a very specialized tissue, like mature testis. The high proportion of genes known to be expressed in a tissue of complex functions, such as brain, would tend to support this view (Hahn and Laird, 1971; Grouse et al., 1972; Ryffel and McCarthy, 1975; Bantle and Hahn, 1976).

It is also possible to obtain some information regarding the number of housekeeping genes from our data. For example, about 80% of the liver mRNA reacts with testis cDNA, implying that those shared mRNA sequences may represent common housekeeping functions for both types of cell populations.

Even though we cannot prove with absolute certainty from our data that 80% of the sequences in testis are also present in liver and that 25% of the liver sequences are common to testis mRNA, it is reasonable to assume that the majority of the unshared sequences belong to the highest complexity class. With this assumption in mind, some simple calculations can yield a very approximate estimation of the number of common functions shared by these two tissues. For instance, 75% of the liver sequences are not present in testis, implying that liver would require the expression of some (75/100)20 000 = 15 000 different sequences for its characteristic functions. On the other hand, about 20% of the testis sequences are not present in liver, implying the existence of some 1200 testis-specific functions. At this point, it should be stessed that, up to date, there is no reason to believe that all polyadenylated RNA sequences in the cytoplasm represent mRNAs, in the sense of being translatable and giving rise to proteins. Indeed, experiments by one of us in *Drosophila* cultured cells suggest that a significant fraction of polyadenylated cytoplasmic RNA (~35%) is not associated with polysomes (Levy W., 1975; Levy W. and Rizzino, 1977).

From the above calculations, we are left with some 4800 sequences shared by both tissues. Again, we should stress that because the majority of the shared sequences belong to the most abundant class these numbers are only speculative. Furthermore, since we have used testis at a rather late stage of maturation, we have examined a limited number of mRNAs (Levy W. and Dixon, 1977). In an earlier stage of development of the testis, the situation might be different and the percentage of shared sequences may vary slightly. Nevertheless, it is not unreasonable that some few thousand sequences would be required to perform the basic functions common to every cell type in the fish.

Acknowledgments

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Visualization of Ribosome–Single-Stranded DNA Complexes in the Electron Microscope†

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ABSTRACT: A procedure is described which allows ribosomes bound to single-stranded DNA to be visualized in the electron microscope. The number of bound ribosomes may be determined and the position of the bound ribosomes may be readily measured along the DNA. The distribution of ribosomes bound to separated *l* and *r* strands of λ DNA was shown to conform to the pattern predicted for binding at specific sites. The procedure should allow mapping of ribosome binding sites for the determination of genetic maps and may also be useful for studying translational control and relative binding affinities for ribosomes.

Mapping the location of genes, protein binding sites, and other interesting nucleotide sequences along DNA is now commonly done by electron microscopy. Procedures have become very versatile and include DNA–DNA heteroduplex analysis to map deletions and substitutions, RNA–DNA hybridization to map genes directly, visualization of transcription complexes to map promotor and termination sites, and direct observation of proteins bound to the DNA. (For a review, see Younghusband and Inman, 1974.) There is no direct procedure at present for determining the location of ribosome binding sites, although the positions can be inferred, for example, by sequencing the RNA or by assuming that the site lies immediately adjacent to the DNA sequences coding for the protein. We have developed a procedure for visualizing ribosomes bound to single-stranded DNA which we expect to be useful in mapping the location of ribosome binding sites and in investigating the factor requirements and binding affinities of different initiation sites.

Single-stranded DNA is of course not the natural polynucleotide for ribosome binding, but DNA can act as a messenger for polypeptide synthesis in the presence of the antibiotic neomycin (McCarthy and Holland, 1965; Thorpe and Ihler, 1974). The role of neomycin is not understood, but Bretscher (1969) has shown that neomycin is not required for binding of the ribosome or for formation of the first few peptide bonds. Ihler and Nakada (1970) found that ribosomes bound preferentially to the strand of T7 DNA that contained the same sequences as in vivo T7 mRNA. Robertson (1975) studied the specificity of DNA–ribosome interactions and showed that only a small number of specific fragments could be protected by ribosomes from pancreatic DNase digestion within both f1 and φX174 DNAs. Barrell et al. (1975) sequenced the major ribosome protected fragment of φX174 DNA and showed that it corresponded to the initiation region for the spike protein (gene G). This protein had previously been known to comprise more than one-half of the total product of protein synthesis stimulated in vitro by φX174 duplex DNA in a coupled transcription–translation system (Gelfand and Hayashi, 1969).

The use of single-stranded DNA in place of mRNA potentially offers a number of technical advantages in certain kinds of experiments because the DNA is easy to purify and is less susceptible to nucleolytic attack than RNA. For the purposes of mapping, the use of DNA is essential since the mRNA in general would contain the binding sites for only a few of the proteins. The procedure discussed here allows ribosome–DNA complexes to be readily visualized and the locations of the complexes along the DNA to be accurately measured.

Experimental Procedures

Preparation of DNA and Ribosomes. φX174 DNA and [^{3}H]-φX174 DNA (specific activity, 2×10^4 cpm/μg) were obtained by phenol extraction of phage using amber 3 mutants purified by the method of Pagano and Hutchinson (1971). Single-stranded T7 DNA was obtained directly from CsCl banded wild type phage by simultaneous lysis and denaturation with 0.2 M NaOH. Phage proteins were not removed before the denatured DNA was used in binding reactions. Separated strands of $\lambda_{bb\text{-}kan\ 2}$ DNA were prepared from purified phage by the poly(U,G) method of Szybalski et al. (1971); poly(U,G) was removed as described by Ihler and Nakada (1970).

E. coli strain 238 (DNase I$^-$, B1$^-$) were used for the preparation of ribosomes, initiation factors, and charging enzymes, essentially as described by Anderson et al. (1967). Crude and salt washed (1 M NH_4Cl) ribosomes were prepared by the standard alumina grinding procedure except that the addition of pancreatic DNase immediately after cell breakage was omitted. Ribosomes were resuspended in ribosome buffer (100 mM NH_4Cl, 10 mM magnesium acetate, 20 mM Tris[1]-HCl,

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.