THE RIBOSOMES OF DROSOPHILA. III. RNA AND PROTEIN HOMOLOGY BETWEEN D. MELANOGASTER AND D. VIRILIS

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ABSTRACT

The extent of interspecific homology between D. melanogaster and D. virilis for ribosomal RNA and ribosomal protein was examined using the techniques of two-dimensional gel electrophoresis, and RNA-DNA filter hybridization. Only 2 of the 71 ribosomal proteins resolved were found to be species specific, while comparisons of soluble larval hemolymph protein patterns showed little similarity. Depending on the technique employed, the sequence homology for 18S + 28S ribosomal RNA was found to be between 83–94%, and sequence homology for 5S rRNA was judged to be complete.

THE rate of molecular evolution is not uniform throughout the genome. For example, amino acid substitutions in the histone IV sequence occur at only 2% of the rate of cytochrome-c, and 0.2% of the rate of fibrinopeptide-A (Birnstiel, Weinberg and Pardue 1974). Similarly, nucleic acid hybridization studies suggest that unique sequence DNA of related species tends to diverge at a more rapid rate than certain classes of repetitive sequences (Kohne 1968, Entingh 1970; Laird and McCarthy 1968). The ribosomal genes in particular seem to contain sequences that have been conserved throughout the evolution of eukaryotic organisms (Sinclair and Brown 1971; Gerbi, in preparation). Since ribosomal RNA interacts, either directly or indirectly, with more than 70 proteins to form a functional ribosome it is of interest to consider the evolution of both the RNA and the protein components of the ribosome.

Recently, evidence has accumulated suggesting that ribosomal proteins represent a fairly conservative class of macromolecules. In Drosophila, (Steffensen 1973; Berger and Weber 1974) and in Neurospora (Alberghina and Suskind 1967) electrophoretic comparisons of ribosomal proteins have failed to uncover intraspecific variations while similar comparisons of soluble proteins (O'Brien and Macintyre 1969; Berger 1971) typically define intraspecific mobility variations at 40–50% of the gene-protein systems identified.

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In the present study we simultaneously examine the extent of homology for ribosomal proteins, soluble proteins, and ribosomal RNAs between *D. melanogaster* and a distantly related Drosophila, *D. virilis*. Using one- and two-dimensional electrophoresis, we show that the electrophoretic properties of the ribosomal subunit proteins are virtually identical in the two species. On the other hand, we find that extensive base substitution has occurred within the sequence of 18S + 28S RNA.

**MATERIALS AND METHODS**

a) *Drosophila*: The *Drosophila melanogaster* strains used in this study were Swedish-B, obtained by H. Krider, University of Connecticut. *Drosophila virilis* (Texmalucan) was provided by L. Throckmorton, University of Chicago. These stocks were maintained on banana-agar medium or cornmeal-agar.

b) Electrophoretic analysis of ribosomal and soluble proteins: The isolation of proteins from dissociated ribosomal subunits (Berger 1974) and the one- and two-dimensional gel electrophoresis systems used to resolve the proteins (Howard and Traut 1973; Berger and Weber 1974) have been described previously. Soluble proteins isolated from a post-ribosomal supernatant fraction were studied according to the electrophoretic procedure of Hubby and Lewontin (1966).

c) Isolation of RNA: Tritium-labelled RNA was isolated from larvae labelled according to Steffensen and Wimber (1971). The larvae were washed with water, frozen on dry ice, and pulverized. The frozen powder was poured into an emulsion consisting of equal volumes of phenol and a buffer containing 0.5% SDS, 0.1 M NaCl, 0.01 M sodium acetate, pH 5.0 and 10 µg/ml polyvinyl-sulfate and the mixture was vigorously stirred with a magnetic stir bar for 20 minutes at 4°. The aqueous phase, recovered after centrifugation at 12,000 × g for 10 minutes, was re-extracted with phenol twice more and the RNA recovered by ethanol precipitation. The RNA precipitate was rinsed with 70% ethanol, dissolved in 0.2 M NaCl, 0.05 M Tris, pH 7.1, and bound to a MAK column (Mandell and Hershey 1960). Low molecular weight RNA was eluted from the column with buffer containing 0.6 M NaCl, followed by 1.0 M NaCl, which releases high molecular weight RNA. Both fractions were precipitated with ethanol and redissolved in buffer containing 0.2% SDS, 0.02 M NaAc, 0.002 M EDTA, 0.04 M Tris-acetate, pH 7.4 (Loening 1967). The RNA which eluted in the 1.0 M NaCl fraction was repurified on 10–30% sucrose gradients centrifuged in an IEC SB-110 rotor at 25,000 RPM for 18 hours at 23°. The fractions containing 18S and 28S RNA were combined and precipitated with ethanol. A procedure similar to that described by Tartof and Perry (1970) was used to purify 5S RNA on 7.5% acrylamide gels. The 5S RNA was recovered from the appropriate region of the gel by electroelution from a dialysis membrane followed by ethanol precipitation. Kinetic hybridization experiments showed that the 5S RNA preparation was free of contaminating hybridizable sequences except for traces of 18S + 28S RNA degradation products which could be eliminated from the reaction by the addition of unlabelled RNA.

The specific activity of the *D. virilis* 18S + 28S RNA used in this study was 2,200 cpm/µg. *D. melanogaster* 18S + 28S and 5S RNA were 25,000 cpm/µg and 103,000 cpm/µg, respectively.

d) Hybridization: DNA prepared from *D. melanogaster* and *D. virilis* adults, according to a procedure to be described elsewhere (Weber et al. in preparation), was loaded onto nitrocellulose filters (Schleicher and Schuell, type B-6) according to the method of Vincent et al. (1969). The amount of DNA which bound to the filter was estimated optically at 260 nm, by measuring the optical density of the DNA solution before and after filtration. The filters were dried at 80° in a vacuum oven and stored desiccated at —10°.

Filter hybridization (Gillespie and Spiegelman 1965) was carried out for 12 hours in 3 ml of 4 × SSC, 0.1% SDS, at 67°. The reactions were terminated by chilling the vials on ice. The filters (usually 2 DNA filters and 1 blank per incubation) were removed, rinsed in 2 × SSC and treated with RNase according to a modification of the batch procedure of Birnstiel et al. (1968).
RNase treatment was for 20 minutes at 25° using an RNase A concentration of 10 μg/ml in 2 × SSC except where specified otherwise. Radioactivity in the hybrid was measured following the addition of toluene-liquifluor. Background controls for nonspecific binding of RNA using either blank filters or filters containing B. subtilis DNA showed a "noise" level of less than 0.01% of the RNA input.

The formation of 5S RNA-DNA hybrids was carried out for 4 hours at 47° in solutions containing 6 × SSC and 50% formamide (Eastman). Unlabelled 18S + 28S RNA was present at 100-fold excess over 3H-labelled 5S RNA in order to eliminate the hybridization of contaminating high molecular weight RNA degradation products.

The amount of DNA bound to each filter was determined chemically after scintillation counting. The filters were rinsed free fromfluors with 3 washes in chloroform. DNA was then hydrolyzed and eluted with 1 N PCA at 70° for 30 minutes. The amount of DNA released by each filter was measured by the method of GILES and MYERS (1965) using calf thymus DNA (Sigma) as a standard.

e) Thermal dissociation of RNA-DNA hybrids: Hybrids were formed using saturating concentrations of RNA in all cases. The filters were washed as described above except that when RNase-treated hybrids were studied the RNase treatment was followed by an incubation in 2 × SSC containing 0.1% diethylpyrocarbonate for 30 minutes at room temperature to remove nuclease activity (BIRNSTIEL, CHIPCHASE and SPIERS 1972). The filters were then rinsed exhaustively in 2 × SSC. To determine the thermal stability of the hybrids, the filters were placed in 0.1 × SSC in plastic scintillation vials and heated in a Haake circulating water bath in temperature increments of about 5°. The bath was allowed to rise to a given temperature and the incubation was then timed for 5 minutes. The solution was then rapidly withdrawn from the vial and replaced with 2 ml of fresh 0.1 × SSC. The radioactivity of the released RNA was measured by scintillation counting after the addition of 3 volumes of Aquasol (New England Nuclear).

RESULTS

a) Homology of ribosomal and soluble proteins—Comparisons of ribosomal subunit protein homology in the two species were made using one- and two-dimensional gel electrophoresis. In electropherograms of small subunit proteins (Figures 1a and 1b) the mobilities of all but one of the 33 proteins resolved in

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**Figure 1a.**—Acid-urea disc gel electrophoresis of 40S ribosomal subunit proteins from (1) D. melanogaster; (2) D. virilis; (3) a mixture of proteins from the two species. Arrow indicates species difference.
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Figure 1b.—Two-dimensional gel electrophoresis of 40S ribosomal subunit proteins from (1) D. melanogaster; and (2) D. virilis. Coelectrophoresis of proteins from the 2 species (not shown) shows no additional species variations. Electrophoresis was carried out in the first dimension in disc gels containing 4% acrylamide, 0.133% bisacrylamide, 6M Urea in a Tris-Borate-EDTA buffer, pH 8.7. Running buffer contained Na2EDTA, 2.4 g/l; boric acid, 4.8 g/l; Tris, 7.25 g/l; final pH 8.2. Second-dimension electrophoresis was carried out in slabs containing 18% acrylamide, 0.25% bisacrylamide, 6M Urea in an acetic acid-KOH buffer, final pH 4.5. The running buffer, at pH 4.0, contained glycine, 14g/l; glacial acetic acid, 1.5 ml/l. Additional details are found in Howard and Traut (1973) and Berger and Weber (1974).

each species were indistinguishable. A single mobility difference was clearly detected in one-dimensional acid-urea gels (Figures 1a) but was poorly resolved in two-dimensional gels. Electrophoresis of the large subunit proteins in 1 dimension produced a complex pattern which in two-dimensional gels resolved into 38 protein spots in each species. Only a single protein with a species-specific mobility was detected (Figure 2). The electrophoretic similarity of the ribosomal proteins was contrasted by the striking differences seen when soluble proteins extracted from third instar larvae were studied by one-dimensional electrophoresis (Figure 3). Here, as in other studies of distantly related Drosophila (Hubby and Throckmorton (1968) most of the proteins showed species specific mobility.

b) Homology of ribosomal RNA—Three independent procedures involving nucleic acid hybridization were used to estimate the degree of base sequence homology for 18S + 28S rRNA between D. melanogaster and D. virilis. The first method was to quantitate the amount of RNase-resistant hybrid which would form when each type of DNA was hybridized with saturating amounts of either homologous or heterologous rRNA. The accuracy of this type of comparison depends upon the ability of RNase to remove unpaired regions of the duplex without digesting bona fide hybrid. Since heterologous RNA-DNA hybrids would be expected to contain more mispaired RNA than homologous hybrids, the kinetics of RNase A digestion on homologous and heterologous hybrids were examined (Figure 4). In either case, following digestion with 10 μg/ml or 20
**Figure 2.**—Two-dimensional gel electrophoresis of 55S ribosomal subunit protein of (1) *D. virilis*; (2) *D. melanogaster*; (3) a mixture of *D. virilis* and *D. melanogaster*. Approximately 200 μg of protein were applied for each gel. Arrow indicates species difference.

μg/ml of enzyme for 10 minutes, the level of resistant RNA in the hybrid remained essentially the same for up to 1 hour. Figure 5 summarizes the results of saturation experiments where the hybrids were treated with RNase as described in MATERIALS AND METHODS. *D. melanogaster* forms hybrids with about 0.42% of the homologous DNA (Figure 5), a value similar to that reported by Spear and Gall (1973) and Tartof (1971). However, a saturation plateau of only 0.37% was obtained when *D. virilis* 18S + 28S RNA was hybridized with *D. melanogaster* DNA. A straightforward interpretation of these results is that *D. melanogaster* rDNA contains sequence homologous to only 88% of *D. virilis* 18S + 28S RNA.

The reciprocal experiments (Figure 5) in which *D. virilis* DNA was used showed a saturation plateau of 0.36% of the DNA for homologous RNA and 0.295% for *D. melanogaster* 18S + 28S RNA. These results again indicate that some portion of the RNA sequence has diverged between the two species. In this
Figure 3.—Gel electrophoresis of larval hemolymph proteins from *D. virilis* (a, b), *D. melanogaster* (c, d) *D. pseudoobscura* (e, f), and *D. funebris* (g, h) stained with Coomassie Blue. Method of Hubby and Lewontin (1966) was used.

Figure 4.—The kinetics of RNAse treatment of homologous (continuous line) and heterologous (dashed line) rRNA-DNA hybrids. $^3$H labelled 18S + 28S RNA from either *D. virilis* (2200 CPM/µg) or *D. melanogaster* (25,000 CPM/µg) were incubated with *D. virilis* DNA filters (45 µg/filter) for 12 hours. The filters were rinsed and treated with RNAse A at 10 µg/ml (circles) or 20 µg/ml (triangle) in 2 × SSC. At indicated times the filters were rinsed in 2 × SSC and the RNA remaining in the hybrid was determined by scintillation counting.
FIGURE 5.—The saturation of D. melanogaster DNA (top panel) and D. virilis DNA (lower panel) with \(^{3}H\)-labelled 18S + 28S from D. melanogaster (filled circles) or D. virilis (open circles). The filters were treated with RNAse and the resistant radioactivity was determined. DNA content per filter was measured chemically after scintillation counting. Points are averages of duplicate filters and specific activities of RNAs are described in legend of Figure 4.

case about 83% homology is suggested. The reciprocity of these experiments excludes the possibility that the difference seen between homologous and heterologous saturation levels result from errors in specific activity determination.

Competition experiments were then carried out to test the ability of unlabelled heterologous RNA to compete for DNA binding sites with radioactive homologous
RNA. The results (Figure 6) essentially agree with the saturation experiments and again demonstrate that the base sequence of *D. melanogaster* and *D. virilis* 18S + 28S RNA still hybridizes to the extent of 11% of the uncompeted saturation value (Figure 6). The same amount of unlabelled homologous competitor reduced the level of hybridization to 1.5%. Figure 6 also shows the effect of homologous competitor RNA which was isolated from purified 80S ribosomes rather than from whole organisms. This preparation also competed to the level of 1% of the uncompeted control value. This verifies that these experiments are showing differences in the 18S + 28S RNA sequence and are not the results of small amounts of contaminating heterogeneous RNA sequences which might be highly species specific. Figure 6 suggests that *D. melanogaster* 18S + 28S RNA does not compete for about 9% of the sequence found in *D. virilis* 18S + 28S RNA.

The third method used to estimate base sequence homology was to compare the extent of base pairing in homologous and heterologous duplexes by measuring the thermal stabilities of the hybrids. Figure 7 shows that when RNase treatment is omitted, the homologous *D. melanogaster* 18S + 28S RNA-DNA hybrid had a mean thermal stability ($T_m$) of 72.5° in 0.1 × SSC while the heterologous hybrid, formed using *D. virilis* DNA, had a $T_m$ of 66.5°. According to the relationship

![Figure 6. Competition between (left panel) $^3$H-labelled *D. melanogaster* 18S + 28S and unlabelled *D. melanogaster* (open triangle) or *D. virilis* (open circle) RNA for *D. melanogaster* DNA. Filters containing approximately 45 μg/ml DNA. Filters were hybridized with 2 μg/ml of homologous $^3$H-18S + 28S RNA in the presence of unlabelled competitor at varying concentrations. Plot with closed circles represents competition with unlabelled 18S + 28S RNA isolated from sucrose gradient purified ribosomes. Right panel depicts reciprocal experiment using *D. virilis* DNA as the filter bound material.](https://academic.oup.com/genetics/article/84/3/573/5991838)
Figure 7.—The thermal stability of *D. melanogaster* 3H-labelled 18S+28S RNA-DNA hybrids formed with either *D. melanogaster* (open circles) or *D. virilis* (closed circles) DNA without (top panel) or with (bottom panel) RNAse treatment. Hybrids were formed by incubating 3 µg/ml of labelled RNA (10⁶ CPM/µg) with DNA filters. Experimental details are cited in METHODS section.

Figure 8.—The thermal stabilities of *D. melanogaster* 5S RNA-DNA hybrids formed with either *D. melanogaster* (filled circles) or *D. virilis* (open circles) DNA. Hybrids were formed using 0.5 µg/ml 3H-5S RNA and 100 µg of DNA per filter. RNAse treatment was omitted.
between thermal stability and base pairing suggested by Britten, Graham and Neufeld (1974), a decrease in \( T_m \) of one degree indicates 1% mismatching of bases. Therefore, these results suggest that the heterologous hybrid contains about 6% mispaired bases.

The effect of RNase treatment on the thermal stability of hybrids is also shown in Figure 7. The \( T_m \) of the homologous hybrid was reduced by 4.5° relative to the untreated hybrid while the \( T_m \) of the heterologous hybrid was essentially unchanged. The differences in \( T_m \) between the homologous and heterologous RNase-treated hybrid was only 2°. It should be noted, however, that the heterologous hybrid seemed to melt over a broader temperature range than before RNase treatment. A portion of the hybrid appears to be somewhat more stable than the homologous hybrid.

The base-sequence homology of the 5S ribosomal RNA components was compared between the two species by examining the thermal stability of the hybrids formed using \( D. \) melanogaster 5S RNA and either \( D. \) melanogaster or \( D. \) virilis DNA (Figure 8). A \( T_m \) of 72.5° was determined in each case which suggests that the base sequence of this RNA has been conserved in both species.

DISCUSSION

An electrophoretic analysis of the ribosomal subunit proteins from several distantly related species of Drosophila using our two-dimensional procedure has demonstrated great similarity, perhaps identity, among these proteins (Berger and Vaslet, unpublished results). We decided to begin a more detailed comparison of ribosomal RNA and protein components with \( D. \) melanogaster and \( D. \) virilis because the genomes of both these species have been studied and they are known to have diverged extensively at both the morphological and molecular level. The base sequences of the centromeric satellite DNA is known to be different in these two species (Gall, Cohen and Atherton 1973) and only 40% of the unique DNA sequences in their genomes will cross-react under nonstringent conditions for DNA reassociation (Entingh 1970). Throckmorton (1975) estimates that they are separated by 50 million years of evolution.

In marked contrast to the soluble hemolymph proteins, only two differences were observed among ribosomal proteins with the others showing great similarity. We suggest that most, possibly all, of the other proteins have identical mobilities. These results for Drosophila species are distinct from what is seen in bacteria. Geisser et al. (1973a, 1973b), using an electrophoretic technique similar to ours, have observed large differences in the electrophoretic mobilities of most of the ribosomal proteins within Enterobacteriaceae species and among several species of Bacillus. Despite the electrophoretic differences, however, physical, serological and functional studies have shown that a high degree of structural similarity has been conserved during the evolution of bacterial ribosomal proteins (Sun, Bickle and Traut 1972; Isono, Isono and Stoffler 1973; Geisser et al. 1973b; Nomura, Traub and Beckmann 1968).
Base-sequence homology between the 18S + 28S rRNAs was found to be between 83–94%, depending on the technique employed. Thus between 450 and 1200 nucleotide substitutions seem to have taken place among the 7000 nucleotides comprising both chains. Although the techniques employed cannot provide a detailed topological picture of conserved or nonconserved regions within the RNA molecule, several observations may bear on this question. For instance, the thermal stability studies revealed that following RNase treatment the Tm values of both homologous and heterologous hybrids decreased, but that the absolute values became quite similar (only 2° apart). This suggests that some regions of homology may be, on the average, fairly long, for the production of only very short regions of duplex (as would be anticipated if substitutions were randomly distributed), would be expected to substantially lower Tm (CROTHERS, KALLENBACH and ZIMM 1965; THOMAS and DANCIS 1973). The broad melting profile of RNase-resistant heterologous hybrid, furthermore, indicates that there are regions of duplex both more and less stable than the homologous hybrid. The less stable regions may be rather short regions of homology, or longer regions relatively rich in less stable A + U. The more stable component cannot be reasonably ascribed to regions of duplex longer than in the homologous hybrid, but must be the result of localized regions which are richer, on the average, in G + C. We suspect, and others have shown (MARMUR and DOTY 1962; FELSENFELD and SANDEEN 1962), that these G + C rich regions once removed from cooperative covalent and stacking interactions with adjacent low G + C regions would be more thermostable.

The 5S RNA component of both D. melanogaster and D. virilis ribosomes are probably identical. This molecule is only about 120 nucleotides long, so the mispairing of more than 1 or 2 bases should have been detected in the melting profile. This finding is not surprising considering the astonishing evolutionary conservation of the 5S RNA sequence in higher organisms. The nucleotide sequence of the 5S RNA from several mammals has been found to be identical and the sequence of Xenopus laevis somatic cell 5S RNA differs from the mammalian sequence by only eight bases (MONIER 1974).

LITERATURE CITED


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