A Family of Three Related Satellite DNAs in Drosophila virilis

(hybridization/ultracentrifugation/embryos)

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ABSTRACT Isolated single DNA strands of satellites II or III of Drosophila virilis form hybrid duplexes with the complementary single strands of satellite I. The hybrids denature at a higher temperature than controls, renature rapidly, and form bands of hybrid density in neutral CsCl. Isolated single strands of satellite II do not form clear-cut duplexes with the complementary strands of satellite III. Mixtures of satellites II and III denature at the same temperature as controls, and do not form bands in neutral CsCl. Therefore, satellite I-II and 1-III complexes are extensively base paired, while satellite II-III complexes are minimally base paired. These experiments demonstrate partial homology among the three satellites, and suggest an evolutionary relationship among them. They also suggest that satellites and other repeated sequences from related species, which do not form hybrids in vitro, could have a common evolutionary origin, but have accumulated enough base substitutions to lose interspecific homology demonstrable by hybrid duplex formation.

Satellite DNAs and other repeated nucleotide sequences are present in the nuclear DNAs of virtually all eukaryotes, and are localized in centromeric heterochromatin (1–7). Their functions are unknown. Satellites and other repeated sequences from related organisms failed to form molecular hybrids in vitro, suggesting that they are unrelated in nucleotide sequence (8–11). This apparent species specificity, coupled with their short repeat length and high reiteration, has led to the hypothesis that repeated sequences arose independently many times during evolution (12) by a process termed salutary replication (13, 14).

This hypothesis related directly to attempts to understand the origin of repeated sequences. We therefore sought to determine whether satellite DNAs evolved from preexisting forms or whether they arose independently by studying hybrid formation in vitro among the three satellite DNAs in the genome of Drosophila virilis. The experiments suggest an evolutionary relationship among the three satellites, and also suggest that apparently dissimilar sequences in related organisms may be related.

MATERIALS AND METHODS

* Collection of D. virilis Embryos. Young adults (35–40 g) were emptied into a large Plexiglas cage and incubated at 24°. Embryos were collected daily as described (15), dechorionated, and frozen.

Abbreviations: H and L strands, heavy and light strands, respectively; standard saline citrate, 0.15 M NaCl-0.015 M Na citrate (pH 7.0); Tn, midpoint of thermal denaturation.

Preparation of DNA. Dechorionated embryos (2–5 g) were homogenized in 40 ml of 75 mM NaCl-30 mM EDTA, sodium salt (pH 7.5) and centrifuged (2000 × g, 10 min). The pellet was suspended in 13 ml of 0.15 M NaCl-0.10 M EDTA (pH 8.0). The suspension was made 2 M with respect to NaCl and 2% with respect to sodium dodecyl sulfate, deproteinized by shaking with an equal volume of chloroform–octanol 24:1, and centrifuged (10,000 × g, 10 min). DNA was precipitated from the aqueous phase by addition of two volumes of 95% ethanol, and dissolved in 0.1 × standard saline citrate [standard saline citrate is 0.15 M NaCl-0.015 M sodium citrate (pH 7.0)]. The solution was treated with ribonuclease (16) and deproteinized with chloroform–octanol; the DNA was precipitated and dissolved in 5 mM Na2B407 (pH 9.2).

Analytical Ultracentrifugation. DNA samples (1–2 µg) were centrifuged in neutral or alkaline CsCl in a Beckman model E centrifuge (17). Initial CsCl densities were checked with a refractometer (17). In neutral CsCl (pH = 7.0, ρ = 1.70–1.71 g/cm³), Escherichia coli DNA (ρ = 1.703) or poly[dA-dT]·poly[dA-dT] (ρ = 1.672) (17, 18) were used as internal density standards. Translation to a value for E. coli DNA of 1.710 (17) can be made by addition of 0.007 g/cm³ to the values reported here. In alkaline CsCl, (pH > 12.5, ρ = 1.72–1.73 g/cm³) poly[dA-dT] (ρ = 1.722) was used as an internal density standard. At equilibrium (44,000 rpm; 18–20 hr; 25°), negatives were made on Kodak Professional Film. These were scanned with a Joyce–Loebl microdensitometer, and band densities were calculated (17).

D. virilis Satellite DNAs Were Purified by ultracentrifugation in Cs2SO4-HgCl2 gradients (7, 15, 19). Each gradient contained 400 µg of DNA, 13–15 µmol of HgCl2, 5 mM Na2B407 (pH 9.2), and Cs2SO4 (ρ = 1.49 g/cm³). Samples (7 ml; covered with 3 ml of paraffin oil) were centrifuged to equilibrium (45,000 rpm; 20 hr; 20°) in cellulose nitrate tubes in a Spinco 65 rotor. Fractions (0.15 ml) were collected by dripping, diluted with 0.8 ml of borate buffer (pH 9.2), and measured spectrophotometrically at 260 nm. Fractions containing the A260 peaks were pooled separately, diluted with 1/20 volume of 20 × standard saline citrate, dialyzed exhaustively against 0.1 × standard saline citrate, and concentrated to 0.5–1.5 A260 units/ml by ultrafiltration through Millipore Pellicon PSED 10 filters. The identity of each peak was established by determining its density in neutral CsCl.

Purification of Complementary Strands. Satellites (60–100 µg) were separated into complementary heavy (H) and light...
RESULTS

Purification of satellite DNAs
Neutral CsCl pycnography resolved DNA of *D. virilis* embryos into four discrete bands (3, 22) (Fig. 1A). Their densities, relative to *E. coli* DNA (ρ = 1.703) are: main band, ρ = 1.693; satellite I, ρ = 1.685; satellite II, ρ = 1.680; satellite III, ρ = 1.664. The designation of bands used here corresponds to that suggested by Gall et al. (3). Blumenfeld and Forrest (22) designated the main band as fraction I and satellites I, II, and III as fractions II, III, and IV, respectively. They represent, respectively, 60, 23, 9, and 8% of the diploid DNA (3, 22).

CsSO₄-HgCl₂ centrifugation also resolved *D. virilis* DNA into four bands (Fig. 1B) and provided one-step purification of each of the three satellite DNAs. Since Hg²⁺ selectively increases the densities of certain AT-rich DNAs in CsSO₄ gradients (19), it was thought that the order of collection from the bottom of the gradient would be III, II, I, main band. However, the order was II, III, main band, I. This departure from expectation may reflect the presence of unusual bases or unusual base sequences in the satellites (17).

Separation of satellite DNAs into complementary strands
Alkaline CsCl centrifugation separates each satellite into complementary, heavy and light strands (Fig. 2; Table 1; also, ref. 3). The great differences in the densities of the complementary strands, which may reflect the short repeat length of the satellites and the nonrandom distribution of the bases along each strand (10, 20), made it possible to isolate each strand of each satellite by one round of preparative centrifugation.

The CsCl densities of the single strands are higher at pH 12.5 than at pH 7.0 (Table 1), as is true for most artificial polynucleotides of defined sequence (18). The light strands of satellites II and III have identical buoyant densities under alkaline conditions, and have identical but lower densities under neutral conditions, while the heavy strands of these satellites have different densities under both sets of conditions.

### Table 1. CsCl densities and thermal stabilities of *D. virilis* satellite DNAs

<table>
<thead>
<tr>
<th>Satellite</th>
<th>Buoyant density in CsCl (pH 7.0)</th>
<th>Tm in standard saline citrate</th>
<th>Strand identity</th>
<th>pH &gt; 12.5</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.685</td>
<td>81</td>
<td>I H</td>
<td>1.788</td>
<td>1.760</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>I L</td>
<td>1.679</td>
<td>1.660</td>
</tr>
<tr>
<td>II</td>
<td>1.680</td>
<td>74</td>
<td>II H</td>
<td>1.759</td>
<td>1.711</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>II L</td>
<td>1.689</td>
<td>1.665</td>
</tr>
<tr>
<td>III</td>
<td>1.664</td>
<td>76</td>
<td>III H</td>
<td>1.754</td>
<td>1.694</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>III L</td>
<td>1.689</td>
<td>1.665</td>
</tr>
</tbody>
</table>
FIG. 3. Thermal denaturation of satellite DNAs and satellite DNA hybrids in standard saline citrate. (A) Denaturation of hybrids I H + II L; and I L + II H) and satellites I, II, and III. (B) Denaturation of hybrids (I H + III L; and I L + III H) of reassociated satellites. (C) Denaturation of hybrids (II H + III L; and II L + III H). (D) Denaturation of controls (II H + III H; I H; and I L).

Thermal denaturation of satellite DNAs
The hyperchromicity of different satellite DNA preparations was 33-40%. The $T_{ms}$ of satellites I, II, and III in standard saline citrate were 81°, 74°, and 76°, respectively (Fig. 3A; Table I). The breadth of the thermal transition for each satellite was 3-4°. When the denatured satellites were cooled below their $T_{ms}$, they renatured rapidly. Their $C_{8H/9}$ (13) was less than $10^{-4}$. When they were remelted, their $T_{ms}$ were 79, 72, and 74°, respectively (Fig. 3B; Table 2).

Satellite II denatured at a lower temperature than satellite III, even though its density in neutral CsCl is higher. We confirmed this surprising result by denaturing mixtures of I and II and I and III and standardizing the $T_{ms}$ of satellites II and III against that of I (81°). Apparently, the relative GC contents of light satellite DNAs cannot be inferred from buoyant density or $T_{ms}$ measurements.

Characterization of satellite DNA hybrids
Hybrids were characterized according to three criteria: (1) their $T_{ms}$ as compared to those of single-strand controls; (2) their rate of renaturation after denaturation; and (3) formation of bands of hybrid density in neutral CsCl. By these criteria, I and II and I and III form hybrids, while II and III do not form hybrids.

$T_{ms}$ (Fig. 5; Table 2) and Hybrid Renaturation. The hyperchromicity of hybrids between satellites I and II or I and III was 20-25%. The breadth of the thermal transition was 5-8° (Fig. 3A and B). Hybrids containing I H or I L had indistinguishable $T_{ms}$ (I H + II L = I H + III L = 62°; I L + II H = I L + III H = 49°); the reciprocal hybrids had different $T_{ms}$ [I H + II L (or III L) = 62°; I L + II H (or III H) = 49°].

I-II and I-III complexes have higher $T_{ms}$ and sharper thermal transitions than do controls, and therefore contain some complementary base-paired regions. They have lower $T_{ms}$ and broader thermal transitions than satellites reconstituted from complementary single strands (Fig. 3A and B; Table 2) and therefore contain some mismatched regions. II-III complexes have $T_{ms}$ and thermal transitions indistinguishable from those of controls (Fig. 3C and D; Table 2) and therefore contain a minimal amount of complementary base pairing.

When dissolved I-II or I-III complexes were cooled below their $T_{ms}$, they rapidly reestablished complementary base pairing, as shown by an abrupt decrease in absorbance. Under the conditions used, renaturation was complete in less than 5 min. Their $C_{8H/9}$ was less than $10^{-4}$. Precise measurements of $C_{8H/9}$ were not attempted.

FIG. 4. Denitometric tracings after neutral CsCl equilibrium centrifugation of hybrids I H + II L; I H + III L; I L + II H; and I L + III H. E. coli DNA ($\rho = 1.703$) and poly(dA-dT)-poly(dA-dT) ($\rho = 1.672$) were used as density standards.
Buoyant Density in Neutral CsCl. Hybrid bands were detected between satellites I and II, and I and III but were not detected between satellites II and III (Fig. 4, Table 2). I + II H, I + III H, and I + II L had densities lower than the average of their component strands, while the density of I H + III L was 0.003 g/cm³ above the average of its component strands. When a control mixture of II H + III H was analyzed in neutral CsCl, both components banded at their normal densities and did not interact (Table 2). Therefore, hybrid bands result from complementary base pairing, and not from nonspecific strand–strand interactions.

**DISCUSSION**

The single strands of satellite DNAs II or III of *D. virilis* form hybrid duplexes with the complementary single strands of satellite DNA I. However, the single strands of satellite II do not form clear-cut duplexes with the complementary strands of satellite III. Therefore, I–II and I–III complexes are extensively base paired, while II–III complexes are minimally base paired. These results suggest that satellites II and III are related to satellite I, but are less closely related to each other. Gall has shown that the repeating unit of each *D. virilis* satellite is a heptanucleotide, that the repeating unit of I differs from the repeating units of II and III by one base substitution each, and that the repeating unit of II differs from the repeating unit of III by two base substitutions (23). From both lines of evidence, it appears that the three satellites constitute a family of related, tandemly repeated sequences, and that the members of the family share a common ancestor.

Members of this family of satellite DNAs can appear “unrelated” when their single strands are hybridized in vitro to one another. For instance, the single strands of satellite DNAs II and III, which according to Gall’s sequence data are 71% homologous, fail to form hybrids in vitro with each other. Clearly, when the single strands of different satellite DNAs fail to form hybrids, they are not automatically unrelated to one another.

The single strands of satellite DNAs from closely related rodents generally do not form hybrids in vitro with each other (8–11). Since by this criterion it would appear that satellites are species specific, as well as tandemly repeated, it has been suggested that they arose independently by the serial reiteration of unrelated, unrepeatd nucleotide sequences (12–14). However, as demonstrated here, some, if not all, of these observations can be interpreted as involving partial homologies not detectable by hybridization in vitro. It is therefore possible, and in our view probable, that apparently unrelated repeated sequences of related species are in fact related, even though the partial homology of their respective nucleotide sequences cannot be demonstrated by molecular hybridization.

The experiments described here suggest that satellite DNAs evolved from preexisting satellites, and consequently can be placed on a family tree. Some of the family’s members have accumulated comparatively few base substitutions and therefore retain sufficient homology to be demonstrable by hybridization [for instance, satellites I and II or I and III in *D. virilis*; or, the satellite DNAs of closely related species of mice (24)]. Others have accumulated extensive base substitutions and therefore have lost homology demonstrable by hybridization [for instance, satellites II and III in *D. virilis*; or the satellite DNAs of guinea pigs and mice (9, 25)]. Since many “species-specific” repeated sequences could be related in an evolutionary sense, their common localization in heterochromatin could reflect their common evolutionary origin.

Reciprocal hybrids constructed with the satellite DNAs of closely related species of mice have the same thermal stabilities (24). It was therefore surprising to discover that reciprocal hybrids constructed with the satellite DNAs of *D. virilis* had different *Tₘₚₜ* and buoyant densities. However, Gall (personal communication) has pointed out that hybrids containing I H (I H + II L; I H + III L) have a G-T mismatch at every seventh position along their length, while the reciprocal hybrids containing I L (I L + II H; I L + III H) have an A-C mismatch at the corresponding position. The different *Tₘₚₜ* of the reciprocals thus reflect the fact that a G-T mismatch has a higher thermal stability than an A-C mismatch. Different mismatches would also explain the dramatic differences in buoyant density observed in reciprocal hybrids.

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