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Characterization of Mitochondrial and Nuclear Satellite Deoxyribonucleic Acids of Five Species of Crustacea*

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ABSTRACT: DNAs localized in nuclei purified from ovaries or midgut glands of five different crustacean species have been characterized. In addition to the main band, three or four species of crab had the poly[d(A-T)] satellite while the fourth had a (dA + dT)-rich satellite with the same buoyant density in CsCl but with other physical characteristics differing from poly[d(A-T)]. The lobster and two of the crabs had distinct (dG + dC)-rich satellites. DNA isolated from the mitochondria of each of the five species has a density of 1.688 g/cm^3, and mitochondrial DNA from at least one species separates into two distinct bands in alkaline CsCl gradients. The reassociation characteristics of mitochondrial DNA from all species are similar to those of vertebrate mitochondrial DNAs.

Satellite DNAs have been described in many species of plants and animals. The poly[d(A-T)][1] DNA found in various crustacea is one of the most remarkable of these in that more than 90% of it is alternating adenylate and thymidylate residues (Swartz *et al.*, 1962; Skinner, 1967). Among the true crabs, this satellite is more widespread than was originally suspected; various publications describe its isolation from a total of at least seven species of *Cancer* (Sueoka, 1961; Smith, 1963, 1964; Pochon *et al.*, 1966) and two other crab genera (Skinner, 1967; Skinner *et al.*, 1970).

To our knowledge poly[d(A-T)] has not been described in any group of animals other than the crustaceans, although the (dA + dT)-rich satellite DNA of *Drosophila melanogaster* (Fansler *et al.*, 1970) and the mtDNA of a strain of petite yeast (Bernardi *et al.*, 1968, 1970) have a number of physical properties similar to those of poly[d(A-T)]. In contrast to the mitochondrial localization of the yeast d(A-T)-like satellite, crab poly[d(A-T)] has been reported to be localized in the nuclei of testicular cells of *Cancer productus* (Astell *et al.*, 1969). Since the mitochondria of crab spermatocytes either degenerate or are incorporated into the nuclear membrane during maturation (Langreth, 1969), it was possible that DNA from nuclei of such cells might contain mtDNA as well. It seemed desirable to determine the subcellular localization of the satellite in tissues containing large numbers of intact mitochondria so that nuclei and mitochondria could be separated and their DNAs compared.

To that end, we have developed methods for the purification of crustacean nuclei and mitochondria and have characterized the DNAs from these organelles isolated from midgut glands (hepatopancreas) and ovaries of five crustacean species. Three of them have the poly[d(A-T)] satellite, and in each case it is localized in the nucleus, confirming the conclusion of Astell *et al.* (1969). In addition we find the (dA + dT)-rich satellite of *Callinectes* as well as three (dG + dC)-rich satellites from several other animal species are also localized in the nucleus. By various manipulations, we can enrich the amount of satellite in relation to main-band DNA in a manner which suggests that the ratio of satellite to main band is not the same for all nuclei. The mitochondria of all five crustacean species contain a DNA of ρ = 1.688 g/cm^3. In at least one species mtDNA separates into two distinct bands in alkali. Its reassociation characteristics are similar to those of the mtDNAs of rat liver (Leffler *et al.*, 1969, 1970) and several amphibia as well as chicken and yeast (Dawid and Wolstenholme, 1967, 1968).

Materials and Methods

Animals. Specimens of the Bermuda land crab, *Gecarcinus lateralis*, were obtained and maintained in the laboratory as previously described (Skinner, 1962). The marine crabs—

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[1] Abbreviations used are: poly[d(A-T)], naturally occurring satellite DNA composed of more than 90% alternating adenylate and thymidylate residues and some guanylate and cytidylate residues. Some thymidylate and adenylate residues are also present in nonalternating sequences (Skinner, 1967). mtDNA, mitochondrial DNA; SSC/10, 0.01 M NaCl–0.0015 M sodium citrate (pH 7).

Cancer borealis (Jonah crab), *Callinectes sapidus* (edible blue crab), and *Libinia dubia* (spider crab)—were obtained from the Marine Biological Laboratory, Woods Hole, Mass., and kept in tanks of artificial sea water at 12° (Kerr, 1969). Lobsters (*Homarus americanus*) were obtained from either Woods Hole or a local restaurant. For some experiments, specimens of the land crab were injected with 0.75 mCi of [^{3}H]thymidine (1.9 or 6.7 Ci per mmole), 24 μCi of [^{14}C]deoxycytosine (30 Ci/mole), and/or 12 μCi of [^{14}C]thymidine (59 Ci/mole). Four days later, the animals were bled and their midgut glands, ovaries, or regenerating limb buds were removed. To prevent clotting, hemolymph was collected into 0.166 M EDTA–0.333 M NaCl (pH 7.5). The final concentration of EDTA at the end of the collection was greater than 0.0166 (M. S. Kerr, in preparation, 1971). Hemocytes were pelleted from the hemolymph by centrifugation at 300*g* in the cold.

The tissues selected for study depended on the demands of the experiment: *e.g.*, testis is rich in DNA, ovary and midgut glands are rich in mitochondria, and regenerating limb buds or hemocytes incorporate radioactive DNA precursors most rapidly. The specific tissues are identified either in the text or in the figure legends.

Preparative Isopycnic CsCl Gradients. In preparation for alkaline CsCl gradients, ovaries, hemocytes, or regenerating limb buds were lysed in 0.5 N NaOH at room temperature in nitrocellulose tubes. In preparation for neutral CsCl gradients tissues were homogenized gently (five strokes in a loose-fitting Dounce homogenizer) in a solution of 0.05% sodium dodecyl sulfate, 0.025% sodium deoxycholate, 0.15 M NaCl, 0.01 M EDTA, and 0.1 M Tris (pH 7.4) (Holt and Gurney, 1969). The cells lysed in 0.5 N NaOH were centrifuged in alkaline CsCl buffered with 0.04 M phosphate (pH 12.45). To achieve the desired density (1.734 g/cm^3), samples plus NaOH were brought to 0.2 ml, and 4.8 ml of a standard CsCl solution of ρ = 1.765 g/cm^3 was added. For neutral gradients, 1.25 or 1.13 ml of sample plus buffer was added to either 3.23 or 3.37 ml of CsCl (ρ = 1.9 g/cm^3), which yielded final densities of 1.65 or 1.675 g per cm^3. Both alkaline and neutral samples were overlaid with light mineral oil and centrifuged at 33,000 rpm for 66 hr at 25° in a Ti-50 rotor of a Spinco Model L2-65B centrifuge (Flamm *et al.*, 1966; Skinner, 1967). Fifteen-drop (about 100 μl) samples were collected through a pinhole punched in the bottom of the tube. Aliquots (50 μl) were then pipetted onto Whatman No. 3MM filter paper disks and precipitated in cold trichloroacetic acid (Bollum, 1959). The disks were counted in a scintillation counter, then washed in toluene, treated with hot trichloroacetic acid to hydrolyze nucleic acids, and recounted.

Determination of the Molecular Weight of DNAs. The molecular weights were determined by sedimentation velocity in 5–20% sucrose gradients (Burgi and Hershey, 1963). Varying amounts of either main band, poly[d(A-T)] or total DNA, were run with less than 1 μg of radioactive marker DNAs. Either ^{14}C-labeled T_4 or ^{3}H-labeled λ phage was used.

Analytical Ultracentrifugations. Selected fractions from the CsCl preparative gradients were pooled. Neutral gradients were then buffered to pH 8, and alkaline gradients were made 0.04 M in phosphate (pH 12.45) (Vinograd *et al.*, 1963). The CsCl concentration was adjusted, and the gradients were centrifuged at 25° in a Model E analytical ultracentrifuge at 44,770 rpm for 20 hr. Tracings of the ultraviolet photographs were made with a Joyce-Loebl microdensitometer. Usually *Micrococcus luteus* (*lysodeikticus*) DNA was used as a marker; its density was taken as 1.727 g/cm^3 in neutral CsCl and 1.788 g/cm^3 in alkaline CsCl (Vinograd *et al.*, 1963). In a few cases, poly[d(A-T)] (ρ = 1.677 g/cm^3; Skinner, 1967) was used as a marker. Standard DNAs are shown as shaded peaks in the figures. The per cent of each DNA in a preparation was determined from areas of the Joyce-Loebl scans.

Cell Fractionation. Ovaries or midgut glands were homogenized in two volumes of one of two media: *high Mg^{2+} medium*—0.3 M sucrose–0.1 M $MgCl_2$–0.05 M $CaCl_2$ buffered at pH 7.4 with 0.02 M Tris containing 10 mg/ml of bovine serum albumin; *KCl–EDTA medium*—same as high Mg^{2+} medium, except that $MgCl_2$ was replaced with 0.1 M KCl and 0.01 M EDTA added. After the homogenates were filtered through several layers of cheesecloth, nuclei and whole cells were pelleted by centrifugation at 300*g* in a refrigerated Sorvall. The pellets from this first low-speed spin were suspended in homogenizing medium and centrifuged again at 300*g* and resuspended. The suspension of nuclei and whole cells was layered on 27-ml discontinuous sucrose gradients containing 5 ml of 66% sucrose, 8 ml of 60% sucrose, 5 ml of 45% sucrose, 4.5 ml of 40% sucrose, and 4.5 ml of 35% sucrose buffered to pH 7.4 with 0.02 M Tris. Nuclei were found concentrated at the 60–66% sucrose interface (see below). After the supernatant fractions from the first two 300*g* centrifugations were combined and further purified by two more low-speed (300*g*) centrifugations, the final supernatant fraction was centrifuged at 9700*g*. The pellet was then suspended in homogenizing medium and layered on other sucrose gradients containing: 4 ml of 60% sucrose, 9 ml of 45% sucrose, 8 ml of 40% sucrose, and 6 ml of 35% sucrose buffered with Tris as above. In some of our experiments, the 35 and 45% sucrose layers were omitted and the 30 and 40% sucrose layers were increased to 10 ml each. The gradients were centrifuged at 25,000 rpm for 30–60 min at 4° in an SW25.1 rotor of a Spinco Model L2-65B centrifuge or the SB110 rotor of an International B60 centrifuge. Fractions which collected at each interface were removed by pipetting from the top. Each fraction was diluted to approximately 1 M sucrose by the addition of 0.1 M phosphate buffer (pH 7) and pelleted by centrifugation at 27,000*g* at 4°. In some experiments, the pellet from the 60–66% interface (nuclei) was lysed directly (see below); in others it was resuspended in 0.3 M sucrose, 10 mg/ml of bovine serum albumin, and 0.02 M Tris (pH 7.4) and centrifuged at 300*g*. The resulting pellet was washed again in the same manner. The particles in the other interfaces (45–60 and 40–45) of those gradients, and those of the gradients on which the low-speed supernatant fraction had been centrifuged, were suspended in 1 M sucrose, 0.005 M $MgCl_2$, and 0.02 M Tris (pH 7.4), and treated with DNase (20 μg/ml) for 1 hr at 0°. In most experiments, a second aliquot of DNase was added after the first 30 min. The samples were washed once in 1 M sucrose and twice in 0.15 M citrate–0.3 M sucrose (pH 7.8), and collected each time by centrifugation in the cold (Kalf and Grece, 1967). For the determination of cytochrome oxidase activity (Smith, 1955), fractions were disrupted by freezing twice at −20° and thawing at 0°. They were then diluted with a known volume of 0.1 M phosphate buffer (pH 7), frozen, and thawed again.

Whole tissues or fresh samples of each fraction collected from the discontinuous sucrose gradients were stained with acetocarmine and examined with phase optics. Several mitochondrial fractions, either treated with DNase or untreated, were pelleted, fixed in 1 M sucrose containing 0.5% glutaraldehyde, postfixed in 1 M sucrose containing osmium tetroxide, washed numerous times by suspension in cold 1 M sucrose, and centrifuged at 27,000*g*. Then the pellets were dehydrated and embedded in Epon. The blocks were sectioned

TABLE I: Densities in Neutral CsCl and Fractional Concentrations of Crustacean DNAs.

Animal	Density[a] (g/cm³) and % of Total DNA[b]			
	Light Satellite	Main Band	Heavy Satellite	mtDNA
Gecarcinus lateralis (Bermuda land crab)	1.677 (18)	1.701 (79)	1.721 (3)	1.688 (<1)
Libinia dubia[c] (spider crab)	1.675 (6)	1.698 (92)	1.714 (2)	1.688 (<1)
Callinectes sapidus (edible blue crab)	1.676 (13)	1.698 (87)	Trace	1.688 (<1)
Cancer borealis (Jonah crab)	1.677 (23)	1.698 (77)	None detectable	1.688 (<1)
Homarus americanus (lobster)	None detectable	1.700 (90)	1.712 (10)	1.688 (<1)

[a] Densities in neutral CsCl were calculated from microdensitometer tracings of analytical ultracentrifugations in a Model E centrifuge using poly[d(A-T)] or *M. luteus* DNA as markers. [b] Percentages were determined by outlining Joyce-Loebl microdensitometer scans with a planimeter or by tracing and weighing them. [c] Except for *Libinia* each calculation is the average of two or more Model E analyses of two or more DNA preparations.

on a Porter-Blum microtome and examined with a Phillips electron microscope.

Isolation of DNA from Subcellular Fractions. Three methods were used to isolate DNA from subcellular fractions. The results obtained were independent of the method used for the extraction of DNA.

SALINE–EDTA–ISOAMYL ALCOHOL–CHLOROFORM. Our modifications of the method of Marmur (1961) have been described (Skinner, 1967). In those experiments in which the final preparations were colored, the DNA was purified further by centrifugation through saturated NaCl for 18 hr at 38,000 rpm at 20° (Wells and Birnstiel, 1969).

DIETHYL PYROCARBONATE. See Solymosy *et al.* (1968). Fractions were suspended in 3 volumes of either 0.05 M Tris (pH 7.6)–0.1% sodium dodecyl sulfate–0.005 M $MgCl_2$ or in 0.1 M NaCl–0.05 M EDTA (pH 8). Diethyl pyrocarbonate (0.03 ml/ml of solution) was added. The mixture was heated at 37° for 5 min and centrifuged at 7710*g* for 15 min at room temperature. The pellet was discarded, and 0.1 g of NaCl/ml was dissolved in the supernatant fraction. After the mixture was incubated at 37° for 5 min, it was centrifuged at 10,000*g* for 20 min at 4°. Nucleic acids were precipitated from the supernatant by the addition of 2.5 volumes cold 95% ethanol.

CsCl–ETHIDUM BROMIDE. The methods of Radloff *et al.* (1967) and Nass (1969) were used. Subcellular fractions were lysed in 0.1 M EDTA, 0.01 M Tris (pH 8), 0.8% sodium dodecyl sulfate, and 0.15 M NaCl, dialyzed against 0.02 M Tris (pH 8) and 0.01 M EDTA, and centrifuged for 48 hr at 20° in 5 ml of CsCl (ρ = 1.65 g/cm³) gradients in the presence of ethidium bromide (200 μg/ml) to facilitate the identification of DNA bands. Fractions were collected through a hole in the bottom of the tube.

Results

Characterization of Total DNAs. Table I summarizes data on the buoyant densities of the DNAs of the five species examined (see also Skinner *et al.*, 1970). A (dA + dT)-rich satellite is found in all four of the true crabs (Brachyura) but not in the lobster. Although the (dA + dT)-rich satellite of *Callinectes* has the same density in CsCl as poly[d(A-T)], further characterization has shown it to be distinctly different (Skinner *et al.*, 1970). The (dA + dT)-rich satellites in the other three species have characteristics typical of poly [d(A-T)].

Buoyant Densities of DNAs from Whole Cell Lysates. Our first experiments were directed to the question of whether the poly[d(A-T)] satellite represented long, specific sections of alternating adenylate and thymidylate that were broken off from main band DNA pieces during preparation. We therefore isolated DNA by gentle procedures that minimized shear. Standard techniques that employ phenol (Kirby, 1957) without stirring (Massie and Zimm, 1965) are not suitable in this case since the poly[d(A-T)] satellite is soluble in phenol (Skinner and Triplett, 1967; Skinner *et al.*, 1970) as is synthetic alternating poly [d(A-T)] (Morgan and Wells, 1968).

In the experiments with radioactive precursors we used only rapidly replicating tissues from the land crab. When either hemocytes, ovaries, or minced regenerating limb buds from crabs injected with [^{14}C]thymidine and/or [^{14}C]deoxycytidine were lysed directly in alkali and the lysates centrifuged to equilibrium in alkaline isopycnic CsCl gradients, the DNA formed two distinct bands. Moreover, two peaks having densities characteristic of main-band DNA and poly[d(A-T)] were also obtained in neutral CsCl preparative gradients when the tissues were disrupted by gentle homogenization in a loose-fitting Dounce homogenizer and transferred to gradient tubes by pipetting through a wide-bore (1.5 mm) pipet (Figure 1).

That the two bands were DNA was established by the following criteria. (1) Fractions 7–13 (Figure 1, fraction I) and 21–31 (fraction II) were pooled, dialyzed, and analyzed spectrophotometrically. Their scans were typical of nucleic acids with peaks at 257 nm. (2) The radioactivity in the two peaks is insoluble in cold trichloroacetic acid but soluble in hot trichloroacetic acid. (3) When analyzed in the Model E ultracentrifuge, fraction I had the same density as a sample of purified main-band DNA (Figure 2a). Fraction II had a density of 1.677 g/cm (Figure 2b) typical of the poly[d(A-T)] satellite.

It should be noted that the (dG + dC)-rich satellite is not readily resolved from the main band in such experiments be-

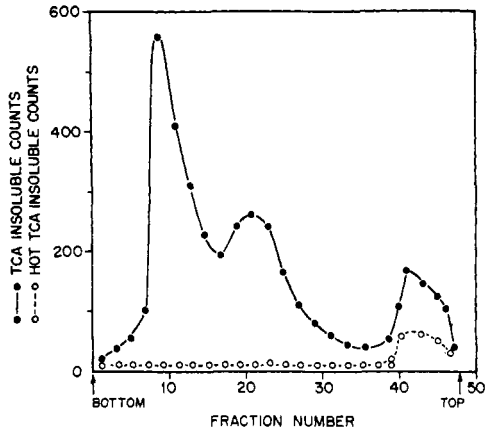

FIGURE 1: Preparative neutral CsCl gradient of ovary from crab injected with [^{3}H]thymidine (750 μCi). Pieces of ovary (162 mg) were homogenized in the lysis medium of Holt and Gurney (1969). CsCl was added (final calculated ρ = 1.65 g/cm^3). Centrifugation was at 33,000 rpm for 66 hr at 25° in a Ti 50 rotor of a Spinco.

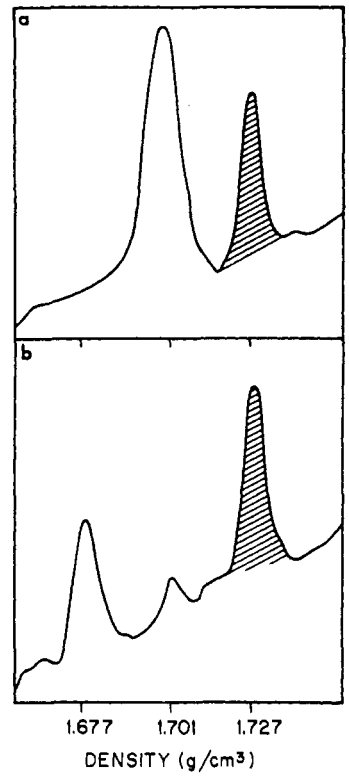

FIGURE 2: Analytical ultracentrifugation patterns of fractions I and II from preparative CsCl gradient. Fractions 7–13 and 21–31 were pooled, dialyzed against SSC/100, and centrifuged in CsCl (ρ = 1.705 g/cm^3) in the Model E analytical ultracentrifuge at 44,770 rpm at 25° for 20 hr. The shaded peaks are *M. luteus* DNA ρ = 1.727 g/cm^3 added as a marker. (a) Fraction I and (b) fraction II.

cause it is present in such small quantities and would presumably be found lower than tube 7 in Figure 1.

These results show that with minimal manipulation the DNA of three different tissues of the land crab forms two distinct bands with densities characteristic of main-band DNA and satellite poly[d(A-T)]. It is possible that the poly[d(A-T)] exists *in vivo* in long stretches of DNA and that, despite the gentle techniques used in handling the cells, the DNA was broken into pieces substantially smaller than the runs of poly[d(A-T)]. In this way, many molecules of poly[d(A-T)] could be generated without the linkage to the main-band molecules necessarily being a particularly labile one.

We have determined the molecular weights in neutral sucrose gradients of highly purified (including centrifugations through neutral preparative CsCl gradients) samples of main band and poly[d(A-T)]. Both had molecular weights (Studier, 1965) of 5–6 $\times$ 10^6 or about 10,000 nucleotide pairs. Therefore if the satellite arises from random breaks within long stretches of poly[d(A-T)], such stretches must be at least several tens of thousands of nucleotides in length.

Our data are also consistent with several other possibilities: the bond joining the segments rich in dA-dT to the remainder of the DNA is labile; the two classes of DNA are physically separated *in vivo* but are located in the same organelle; or the two classes of DNA are located in different subcellular organelles. To determine if the last possibility is correct, we have fractionated tissues into subcellular fractions and characterized the DNA from each.

Characterization of Subcellular Fractions. The densities of mitochondria and nuclei of most organisms are such that they collect at approximately 45 and 65% sucrose, respectively (Dawid, 1966; L. H. Elrod, personal communication, 1970). In discontinuous sucrose gradients on which the low-speed supernatant fraction had been centrifuged, we found that the interface between the 40 and 60% sucrose layers (interface M) had 60–80 times as much cytochrome oxidase activity as the fraction which collected at the interface between the 60 and 66% layers on the other series of gradients (interface N). In addition to having a high cytochrome oxidase activity, such fractions, on microscopic examination, were found to contain few if any nuclei as judged by particle morphology and acetocarmine staining. We conclude, therefore, that mitochondria are chiefly localized in interface M. Electron micrographs showed that fraction M contained granules with the morphological appearance of mitochondria (Plate I). Although fraction M also contained membranes and small particles which may be microsomes, the contamination with nuclei appeared to be insignificant.

In our best preparations, the material that collected between the 60 and 66% sucrose layers of the series of gradients on which the low-speed pellet had been centrifuged had less than 2% of the cytochrome oxidase activity as compared to fraction M. Microscopic examination revealed acetocarmine-staining structures with the appearance of typical nuclei. Acetocarmine-negative acellular sheets, similar to those that cover the projections of the midgut gland, were the only other structures present in significant quantity in the midgut gland preparations. We therefore concluded that this fraction was composed chiefly of nuclei.

Characterization of DNA Isolated from Purified Mitochondria. We used discontinuous gradients composed of 30, 35, 40, 45, and 60% sucrose for further purification of the low-speed supernatant fraction. When we treated those interfaces presumably containing mitochondria with DNase to remove adsorbed DNA, we found a substantial loss in particle-bound cytochrome oxidase activity, indicating that any purification at this step was achieved at the expense of intact mitochondria. We have extracted DNA from DNase-treated mitochondria isolated from midgut glands or ovaries of all five species of crustacea (Figure 3); all these mtDNAs have a density of 1.688 g/cm^3 in neutral CsCl. The DNA isolated from the interfaces between the three most dense layers formed a single

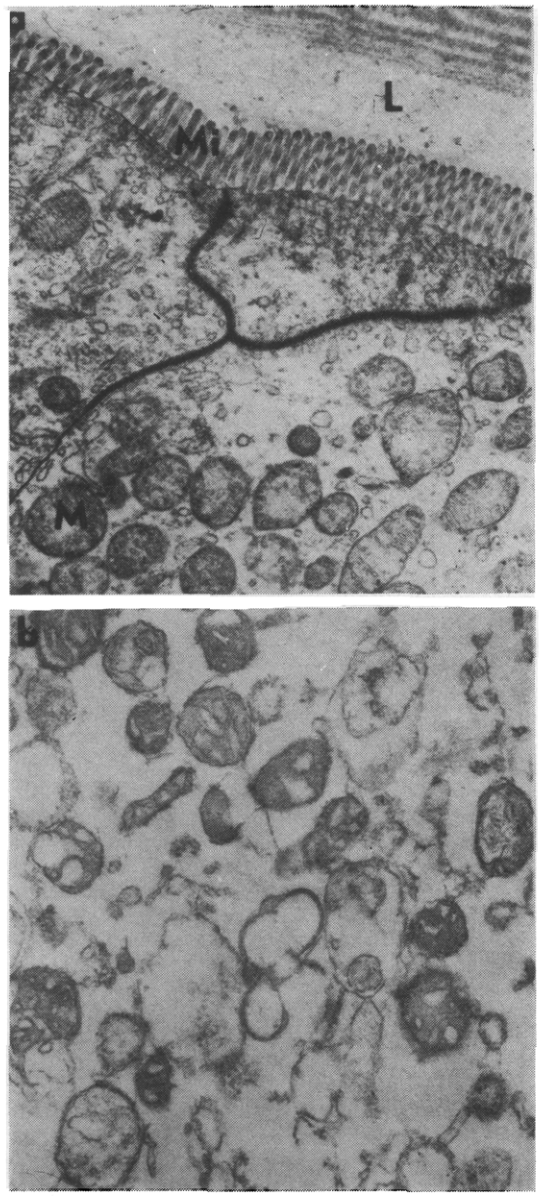

PLATE I: Electron micrographs of mitochondria of midgut gland of *Cancer borealis*. (a) Section of whole tissue: parts of 3 cells. Mi = microvilli; M = mitochondrion; L = lumen of midgut gland. (b) DNased mitochondria from the interface between 40 and 45% sucrose layers of a discontinuous gradient.

band of $\rho = 1.688$ g/cm^3 in neutral CsCl. In order to increase our yields of this DNA, we subsequently reduced the number of layers to include only 30, 40, and 60% sucrose and collected the material at the 40–60% interface as described above. The yields of mtDNA differed considerably from species to species and tissue to tissue. The purest preparations consistently came from the midgut gland. Frequently, at the end of the procedures used to obtain highly purified preparations, there was as little as 0.3–1 μg of DNA from as much as 25 g of tissue.

Figure 4a shows that mtDNA of *Callinectes* separates into distinct bands of $\rho = 1.741$ and 1.754 g per cm^3 in alkaline CsCl; the mean of these two is 0.060 g/cm^3 more dense than the native mtDNA, as expected (Vinograd *et al.*, 1963). Similar resolution into two peaks in alkaline CsCl has also been observed with mtDNA from other species (see, *e.g.*, Leffler *et al.*, 1969). When *Callinectes* mtDNA is denatured in 0.1 M NaOH, neutralized with 0.15 M NaH_2PO_4 15 min later, and centrifuged immediately in neutral CsCl, a single band at $\rho = 1.705$ g/cm^3 is found (Figure 4b). The absence of a second band at $\rho = 1.688$ g/cm^3 indicates that there has been no

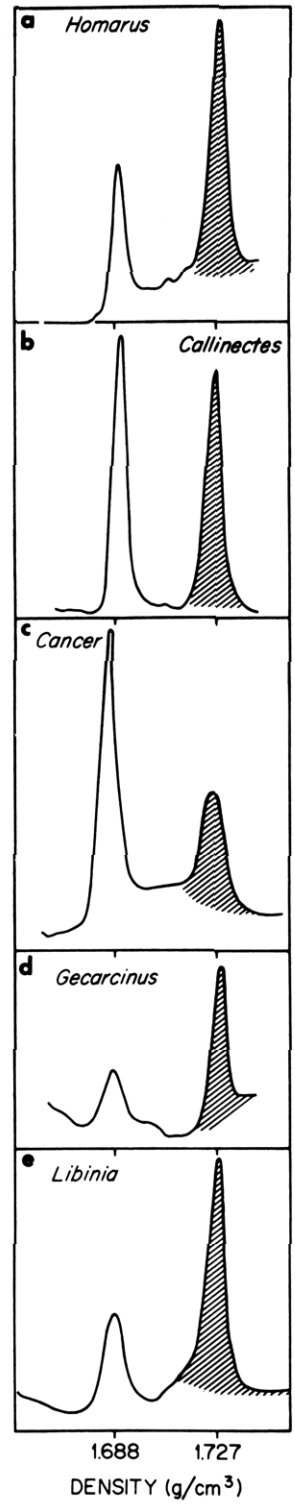

FIGURE 3: Analytical ultracentrifugation patterns of mtDNA of five crustacean species. DNase-treated mitochondria isolated from (a) *Homarus* (40–60), (b) *Callinectes* (45–60), (c) *Cancer borealis* (40–45), (d) *Gecarcinus* (40–45), and (e) *Libinia dubia* (45–60) in either high Mg^{2+} or KCl and EDTA media. DNA from each species was purified by lysing material in the interface indicated in parentheses, centrifuging through CsCl in the presence of ethidium bromide as described, and removing the ethidium bromide by dialysis. The shaded peaks are *M. luteus* DNA added as marker in the Model E centrifugations.

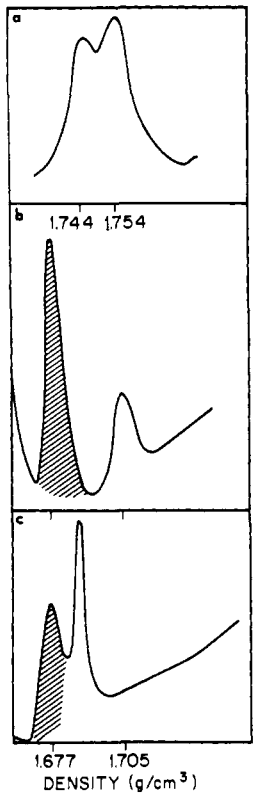

FIGURE 4: Analytical ultracentrifugation patterns of mtDNA. (a) *Callinectes* mtDNA in alkaline CsCl; (b) after alkaline denaturation and neutralization with 0.15 M NaH_2PO_4; (c) *Cancer* mtDNA after alkaline denaturation, neutralization, and subsequent heating for 2 hr at 65°. Parts b and c were neutral CsCl gradients. The shaded peaks are ρ = 1.677 g/cm³, added as a marker.

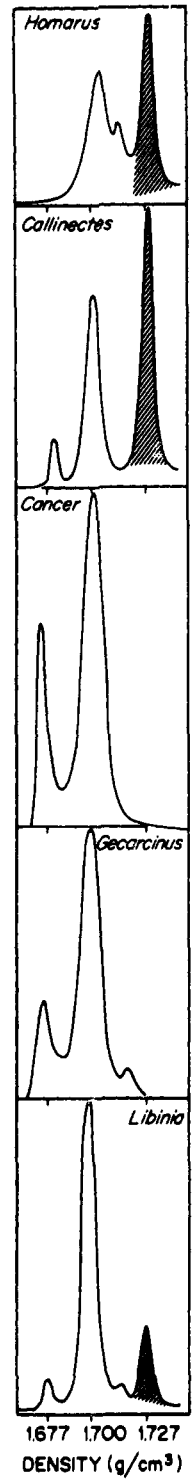

FIGURE 5: Analytical ultracentrifugation patterns of DNA isolated from nuclei of crustacean species. Nuclei were isolated in the KCl–EDTA medium, lysed, dialyzed as described, and centrifuged at 40,000 rpm through CsCl in the presence of ethidium bromide (as in Figure 3). Ovaries of *Cancer* and *Libinia* and the midgut glands of the other three species were used. The shaded peaks are *M. luteus* DNA added as a marker.

"snap back" to the original configuration and density as is seen with circular DNA molecules (Dawid and Wolstenholme, 1967). However, if the alkaline-denatured material is, after neutralization, held for 2 hr at 65° (20° less than the calculated T_m in 0.15 M phosphate buffer), a very sharp peak at the original density is found in neutral CsCl (Figure 4c). This reassociation into aggregates of high molecular weight is characteristic of mtDNA from other species (Dawid and Wolstenholme, 1967, 1968; Leffler *et al.*, 1970; Nass, 1969).

Occasionally a putative mitochondrial band from the sucrose gradients yielded two ultraviolet-absorbing bands, one at the usual density of 1.688 g/cm³ and a second between ρ = 1.670 and 1.675 g per cm³. The latter band was found to be DNase resistant and amylase sensitive. It may be glycogen, which bands in this region of a CsCl isopycnic gradient (Klett *et al.*, 1969; Counts and Flamm, 1966), or possibly another polysaccharide with a similar density (Piko, 1970).

To establish that the DNA isolated from the mitochondrial preparations was not due to contaminating microorganisms, we plated aliquots of each fraction from the sucrose gradients on nutrient agar. If we assume that any contaminating bacteria were viable and were able to grow on this medium and that the DNA content of a single log phase bacterium is 1.4–2 × 10^{-8} μg (Gillies and Alper, 1960), then the number of bacteria isolated from the mitochondrial fraction was too low by three orders of magnitude to account for the amount of DNA isolated from that fraction. As a further control, we grew the contaminating bacteria in liquid culture and isolated the DNA. It banded in CsCl as a single peak of ρ = 1.715 g/cm³, distinctly different from crustacean mtDNA. The dissociation and reassociation characteristics coupled with these data confirm our contention that the 1.688 g/cm³ DNA is mitochondrial.

Characterization of DNA from Purified Nuclei. When midgut glands were removed from crabs injected with both [^{14}C]-

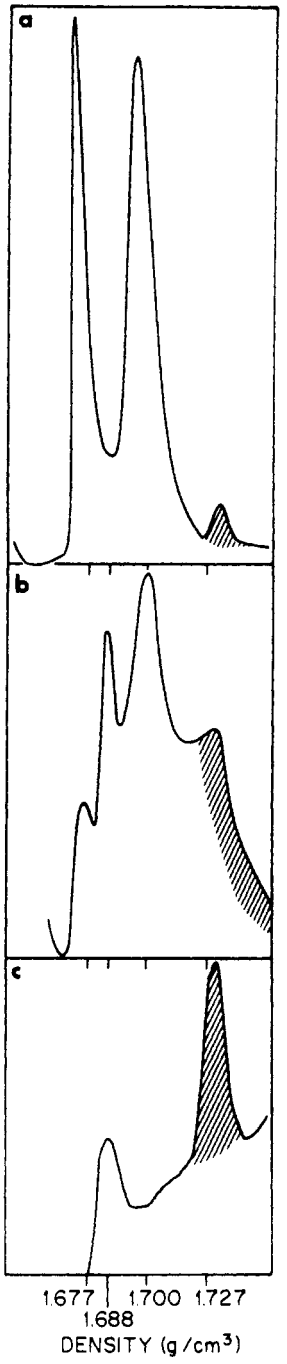

FIGURE 6: Analytical ultracentrifugation pattern of DNA isolated from fractions of *Cancer borealis* ovary. (a) Nuclear DNA, enriched for poly[d(A-T)] as described in the text. (b) DNA from a fraction containing predominantly mitochondria plus a few nuclei. (c) mtDNA isolated from part b after mitochondria had been purified on a discontinuous sucrose gradient. All figures are from scans of neutral CsCl gradients. The shaded peaks are *M. luteus* DNA added as a marker.

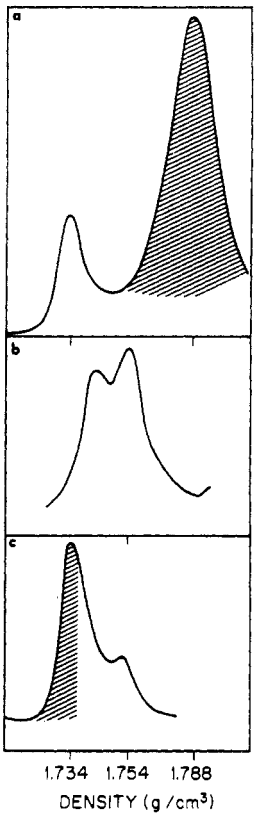

FIGURE 7: Analytical ultracentrifugation patterns of crab poly[d-(A-T)] and mtDNA in alkaline CsCl. (a) poly[d(A-T)] cocentrifuged with *M. luteus* shaded peak (ρ = 1.788 g/cm³); (b) *Callinectes* mtDNA; (c) poly[d(A-T)] (shaded peak) cocentrifuged with *Callinectes* mtDNA.

thymidine and [^{14}C]cytidine, and the nuclei were isolated in Mg^{2+}-rich medium (see Methods), lysed in alkali, and centrifuged to equilibrium in alkaline CsCl gradients, their DNA formed a single band with a density slightly less than that of denatured *Escherichia coli* DNA (Kerr and Skinner, 1969). The pattern differed from Figure 1 in that the denatured main-band DNA was substantially broader and the poly-[d(A-T)] satellite, if present at all, formed no more than a small shoulder on the light side of the main band. In none of these experiments did we recover detectable amounts of the poly[d(A-T)] satellite as judged by subsequent analyses in the analytical ultracentrifuge. DNA isolated by several procedures (Marmur, 1961; Skinner, 1967; Solymosy *et al.*, 1969) from ovarian or midgut gland nuclei prepared in medium containing 0.1 M Mg^{2+} also formed a single ultraviolet-absorbing band with a density characteristic of native main-band DNA.

Because of the broadness of the band, it seemed possible that the DNA might be degraded, even though the nuclei appeared morphologically normal under phase optics and we expected that crab DNase would be inhibited by Mg^{2+} at these high concentrations (Georgatsos, 1965). We therefore substituted a medium containing 0.1 M KCl instead of $MgCl_2$ and added 0.01 M EDTA to inhibit nucleases. DNAs isolated from nuclei prepared in this medium were composed of a narrow main band and one or two satellite bands of varying densities, depending on the animal from which the tissue was taken (Figure 5). These preparations yielded all the satellites found previously in total DNA preparations (Table I; Skinner *et al.*, 1970). Despite the absence of detectable satellites in nuclei isolated in high Mg^{2+} medium, we conclude that the poly[d(A-T)], the (dA + dT)-rich satellite of *Callinectes*, and the (dG + dC)-rich satellites are in fact localized in the nuclear fraction. This conclusion is consistent with the previous suggestion (Johnson and Laskowski, 1970) that the poly-[d(A-T)] satellites are more sensitive to nucleases. The absence of satellites that we observed in our early experiments is presumably an artifact of preparation of nuclei in the high Mg^{2+} medium.

We have been able to enrich for the poly[d(A-T)] satellite without evidence of selective degradation of any DNA component (*i.e.*, broadening of the bands). Homogenates of *Cancer borealis* ovaries were centrifuged twice at 300*g* (spin *a*). The resulting supernatant fractions containing mitochondria and some nuclei were centrifuged at 9700*g* (spin *b*). The pellets were resuspended in homogenizing medium and centrifuged again at 300*g* (spin *c*); the low-speed centrifugations (spins *c*) and resuspensions were repeated four times. Microscopic examination of the final low-speed pellets revealed nuclei and sheets of acetocarmine-negative membranes. The poly[d(A-T)] was enriched in these fractions from 23% characteristic of total DNA of *Cancer borealis* to as much as 39% (Figure 6a). It appears that the initial low-speed centrifugation (spin *a*) had left in suspension those nuceli richer in the poly[d(A-T)] satellite. These were subsequently harvested in spin *b* and separated from mitochondria by spins *c*.

This conclusion is supported by a companion observation. The four supernatant fractions from the low-speed centrifugations (spins *c*) were pooled and centrifuged at 9700*g*. That pellet was treated with DNase, washed, and recollected. Microscopic examination revealed no acellular sheets and a trivial number of nuclei compared to the numbers of particles which appeared to be mitochondria. The DNA from this fraction (Figure 6b) is composed of a sharp band at $\rho = 1.688$ g/cm^3 characteristic of mtDNA and relatively small components of both main band and the poly[d(A-T)] satellite. Again the narrowness of the peaks argues against the action of endogenous DNases; nevertheless the poly[d(A-T)] satellite is considerably diminished relative to the main band (Figure 6b).

In this predominantly mitochondrial fraction containing a small number of nuclei, the distinction between the poly[d(A-T)] satellite and mtDNA is clearly discernible. When these mitochondria are further purified on sucrose gradients they are found to contain only the mtDNA (Figure 6c).

A final distinction can be made between poly[d(A-T)], crab mtDNA, and the (dA + dT)-like mtDNA described by Bernardi *et al.* (1970) in petite mutant yeast. Figure 7a shows the banding of poly[d(A-T)] at $\rho = 1.734$ g/cm^3 in alkaline CsCl. This value[2] is 0.012 g/cm^3 higher than that observed by Bernardi *et al.* for the yeast satellite in alkaline CsCl, and the crab satellite shows none of the bimodality observed with the mutant yeast mtDNA under these conditions. Figure 7b again shows the bimodality of crab (*Callinectes*) mtDNA in alkaline CsCl. When poly[d(A-T)] and *Callinectes* mtDNA were centrifuged together in alkaline CsCl (Figure 7c), the nuclear poly[d(A-T)] satellite overlaps the less dense of the mtDNA bands but is again found to be about 0.010 g/cm^3 less dense than the mean density of mtDNA.

Discussion

It is clear that in the five crustacean species studied, the satellites which are distinctly discernible in a total-tissue DNA preparation are, together with the main-band DNA, localized within the nucleus. In addition, each species has a mitochondrial DNA of $\rho = 1.688$ g/cm^3 present in such small quantities that it is not clearly evident in most total DNA preparations. Now that we are aware that such DNA exists, we are able to see suggestive indications of it in our earlier Model E analytical tracings (Skinner, 1967), but the peaks are not conspicuous enough for us to identify them with certainty. On the other hand, they are small enough compared to, say, the (dG + dC)-rich satellite of *Gecarcinus* that we may be certain that mtDNA comprises less than 1% of the total cellular DNA in all of the tissues examined.

Although we have used various tissues in these studies, in any given animal species we have been able to isolate as distinct entities all of the characteristic satellites, main-band DNA, and mtDNA from a single tissue (usually midgut gland).

Under certain circumstances a high-Mg^{2+} medium has proved useful for cellular fractionation studies (K. S. Chiang, personal communication), but its use in our initial experiments led us to conclude that nDNA of all five crustacean species was composed of only a single component having a density characteristic of the main-band DNA (Kerr and Skinner, 1969). The virtual disappearance of all satellite DNAs in such an isolation medium could be due to their greater sensitivity to DNases (Astell *et al.*, 1969) and/or the smaller amount of them in a total DNA preparation. The insensitivity of endogenous crustacean DNases to 0.1 M $MgCl_2$ is surprising in view of the usual suppression of DNase activity, including that of at least one crustacean DNase, by high concentrations of Mg^{2+} (Georgatsos, 1965).

The experiments in which the poly[d(A-T)] satellite has been greatly enriched with respect to main-band DNA may be due to the fractionation of nuclei at different stages of the cell cycle. Since the percentage of satellites in DNA isolated from total tissues of any one of the crustaceans is fairly constant, from tissue to tissue, it does not appear that the enrichment of satellite reflects a distinction between different cell types of a given tissue. Possibly, the enriched fractions may contain nuclei from a stage of the cell cycle in which relatively more of the poly[d(A-T)] has been synthesized.

Although the structure of the poly[d(A-T)] satellite has been fairly well characterized (Swartz *et al.*, 1962; Skinner, 1967; Hyman and Davidson, 1971), nothing is known of its biological function. It seems unlikely that it serves no other function than the storage of nucleotide residues since the fractional content of DNA isolated as satellite is relatively constant for each species of animal in which it is found (Cheng and Sueoka, 1964; Smith, 1963, 1964; Pochon *et al.*, 1966; Skinner, 1967; Skinner *et al.*, 1970). Certainly the coding properties of poly[d(A-T)] according to the present dogma are highly restricted, much more so than other (dA + dT)-rich satellites with more complex base sequences.

If a protein is coded for by the poly[d(A-T)] satellite, it would be predominantly alternating isoleucine and tyrosine. Such a protein would be highly insoluble and could possibly play a structural function in the arthropod exoskeleton. Alternatively, the mouse satellite has been localized in heterochromatin (Jones, 1970) and it is postulated that this satellite is not transcribed (Flamm *et al.*, 1969) and does not code for any protein. The localization of the mouse satellite near the centromeric region of most (all?) chromosomes is interesting in view of the fact that many crustaceans have very large numbers of chromosomes [$2n$ = 100–200 (Makino, 1951)]. It is conceivable that the appearance of a distinct satellite in many crustaceans may be related to the large numbers of centromeres per nucleus and may in fact play a role in chromosome pairing (see Walker *et al.*, 1969).

[2] Since Bernardi *et al.* (1970) took 1.731 g/cm^3 as the density of *Micrococcus luteus* DNA, which they used as their primary reference, whereas we took $\rho = 1.727$ g/cm^3 (Vinograd *et al.*, 1963), this value of 0.012 g/cm^3 is a minimum difference between our results with denatured crab poly[d(A-T)] and the (dA + dT)-rich mtDNA of the mutant yeast.

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