THE SELECTIVE LOSS OF DNA SATELLITES ON DEPROTEINIZATION WITH PHENOL[1]

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Received July 19, 1967

DNA isolated from several tissues of the land crab, Gecarcinus lateralis, contains three components (Skinner, 1967): a main band (78% of the total DNA; density (ρ) = 1.701 gm/cm^3), a satellite rich in guanylate and cytidylate residues (d(G+C)-rich satellite; 4% of the total; ρ = 1.721 gm/cm^3) and a satellite composed of more than 90% alternating adenylate and thymidylate residues (d(A-T) satellite; 18% of the total; ρ = 1.677 gm/cm^3; Fig. 1a). Such DNA preparations (Type I) are prepared with isoamyl alcohol:chloroform (1:24, v/v; Sevag et al., 1938; Marmur, 1961) as the protein denaturant (see Method I, below). If phenol is substituted for isoamyl alcohol:chloroform (Method II, below), the resulting DNA preparations (Type II) are completely devoid of the d(A-T) satellite. We have done a series of experiments to investigate this phenomenon in greater detail.

DNA was prepared from either crab testes, epidermis, or midgut gland (hepatopancreas). The procedures used, together with alternate methods at various steps, are outlined in Table 1. The procedures are modified from the methods of Sevag et al. (1938), Marmur (1961), Smith (1963, 1964), Kirby (1957, 1959), and Massie and Zimm (1965). The solutions in which the tissues were homogenized contained more EDTA[2] than has been used by previous authors because of the relatively high concentrations of Ca^{++} and Mg^{++} in the crab (Skinner et al., 1965).

[1] Supported by USPHS Grant No. AM-06268 and by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; SSC, 0.15 M NaCl, 0.015 M Na citrate, pH 7.

Table 1

Procedure for Isolation of DNA from Crab Tissues

Step	Purpose	Procedure	Alternate Procedure
1	Potter-Elvehjem homogenization	Suspend in sucrose (0.5 M)-EDTA (0.2 M), pH 8	Suspend in NaCl (0.1 M)-EDTA (0.2 M), pH 8
2	Concentration of organelles	Spin 27,000 X g 10 min, 4°C, pellet resuspended in saline-EDTA	Centrifugation sometimes omitted with testes preparations
3	Disruption of organelles	Suspensions brought to 4% SDS	
4	Deproteinization	Isoamyl alcohol:chloroform (Method I, see text)	Phenol (Method II, see text)
5	Recovery of aqueous phase	Spin 27,000 X g 10 min	
6	Removal of denaturant and uric acid*	Dialyze against SSC/100	Sometimes omit after isoamyl alcohol:chloroform deproteinization
7	Concentration of nucleic acids	Ppt. with ethanol, redissolve in SSC/10	
8	Hydrolysis of RNA	Pancreatic RNase,† 50 µg/ml; 37°C, 30 min	
9	Hydrolysis of residual protein	Pronase,‡ 50 µg/ml; 37°C, 2 hr	
10	Removal of pronase	Add SDS to 0.5%, add 1/10 vol 10X SSC, add equal vol isoamyl alcohol:chloroform; shake 15 min, 22°C, spin 27,000 X g 10 min	
11	Precipitation of DNA	Ppt. aqueous phase with 2.5 vol ethanol, dissolve ppt. in 0.01 Tris, pH 8	

*Large concentrations of uric acid occur in certain crustacean tissues.

†Worthington Biochemical; heated 100°C, 10 min.

‡Calbiochem, B grade.

Proteins were denatured by one of two methods. In Method I, the SDS-containing mixture (Table 1) was shaken on a wrist-action shaker at room temperature for 30 min after which time solid sodium chloride was added to 5.5 M and shaking was continued for 1 hr.

An equal volume of the isoamyl alcohol:chloroform was added and the mixture was shaken for an additional 1 to 2 hr at room temperature and then centrifuged (Sevag, 1938; Marmur, 1961). In Method II, the SDS-containing mixtures were made 1 M in sodium chloride and were heated at 55°C for 10 min after which time an equal volume of hot phenol, equilibrated with 0.01 M Tris, pH 9, was added and the mixture was held at 55°C for an additional 10 min with occasional stirring (Massie and Zimm, 1965). With p-aminosalicylate present during deproteinization with phenol (Kirby, 1959), the method failed to yield any DNA with crab tissues. Because of the temperature sensitivity of the pH of Tris buffer (K. B. Jacobson, personal communication), in some experiments we equilibrated the phenol with 0.01 M potassium phosphate buffer, pH 7. Since the same results were obtained irrespective of the buffer used, we do not believe that the loss of d(A-T) on phenol extraction is due to extreme pH shifts with temperature.

At five steps in the procedure, including the selection of the tissue, alternate methods were used. The only step at which the choice of alternatives significantly affected the results was in the deproteinization.

Two to 3 μg (unless otherwise noted) of DNA in 0.01 M Tris were subjected to isopycnic CsCl (7.7 Molal) equilibrium centrifugation at pH 8 in the Model E analytical centrifuge (Meselson et al., 1957). The three components, typical of Type I preparations, are evident in Fig. 1a. DNA treated with hot phenol contains none of the d(A-T) satellite (Fig. 1b), even though the relative amounts of the main band DNA and of the d(G+C)-rich satellite were the same in Type I and Type II preparations. The constancy of the amount of d(G+C)-rich satellite, which is less than 1/4 the magnitude of the d(A-T) satellite, shows that the loss of the d(A-T) satellite is specific and is not due to the relatively poorer yield of DNA from the phenol-treated preparations.

Since the two standard methods of denaturing protein differ in the duration of exposure to SDS and salt as well as in the salt concentrations used, a Type II preparation was made under Type I SDS and salt conditions. Nevertheless, the DNA finally isolated contained only main band DNA and d(G+C)-rich satellite and no d(A-T) satellite.

Since phenol lowers the melting temperature of DNA (Massie and Zimm, 1965), a homogenate was treated with SDS and 1 M NaCl at room temperature and then shaken in the presence of phenol for 2 hr at room temperature rather than for 10 min at 55°C. The yield of DNA was only 20% that recovered after hot phenol treatment. Only main band DNA was recovered; even though 5 times the usual amount of DNA was analyzed in the CsCl centrifugation, neither satellite could be detected.

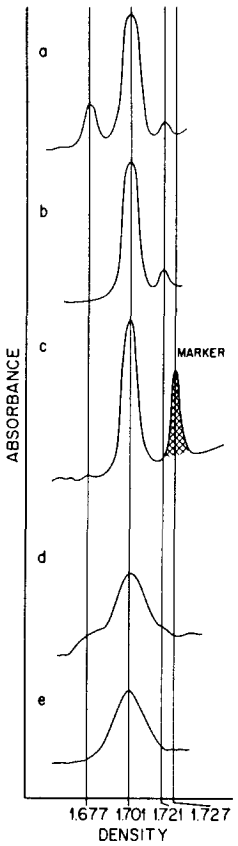

Fig. 1.

Microdensitometer tracings of photographs of crab DNA centrifuged to equilibrium (more than 20 hr at 44,770 RPM) in CsCl (7.7 M) at 25°C in the Spinco Model E analytical ultracentrifuge. The DNA's were isolated from testes. In 1a) isoamyl alcohol:chloroform (1:24) was used to denature protein (Type I). In 1b) hot (55°C) phenol which was 0.1% in hydroxyquinoline and had been saturated with 0.01 M Tris, pH 9, was used to denature protein (Type II). 1c) is a tracing of the DNA recovered from a sample of purified DNA (Type I) which had subsequently been treated with hot phenol; this sample also contains DNA from Micrococcus lysodeikticus (ρ = 1.727) as a marker. 1d) Type I purified DNA, 200 µgm/ml, after sonication, 1e) followed by treatment with hot phenol.

In order to see whether the d(A-T) satellite, once freed from other tissue components, would withstand the phenol extraction, we subjected a purified Type I preparation (200 µg in 1 ml saline-EDTA or sucrose-EDTA) to phenol as in Method II above. The reisolated DNA again was composed solely of main band material (Fig. 1c).

To test whether the size of DNA affects its behavior towards phenol, we sonicated a Type I preparation (200 µg in 1 ml; Biosonik at a power setting of 100 for 1 min) and then centrifuged it in CsCl before and after treatment with hot phenol (Fig. 1d, 1e). The shoulder on the light side of the main band (Fig. 1d) aligns with d(A-T) and that on the heavy side with the d(G+C)-rich satellite of a Type I preparation. All the peaks are greatly broadened, indicating a substantial reduction in molecular size. Phenol treatment of the sonicated DNA appears to remove both satellites, leaving a main band which is everywhere superimposable on the main band of the preparation prior to phenol treatment except in the satellite regions (compare Fig. 1d and 1e). We conclude that the degraded DNA molecules of the main band were not removed. Furthermore, the band-width of the

sonicated main band DNA is 2.9 X greater than that of the d(A-T) prior to sonication; hence the molecular size of the sonicated main band is approximately 1/8 that of the non-sonicated d(A-T); nevertheless, the nonsonicated (as well as sonicated) d(A-T) is lost on phenol extraction, whereas the smaller material in the sonicated main band is not. Therefore, the size of the DNA is not the factor which controls its behavior towards phenol.

Ben-Porat et al. (1962) have reported that newly synthesized DNA remains at the interphase during isolation in low salt with isoamyl alcohol:chloroform as the protein denaturant. This phenomenon has been ascribed to the binding of the DNA and a protein, possibly DNA polymerase (Rolfe, 1963). We find that our Type I DNA preparations contain less than 1 μg protein/25 μg DNA (method of Lowry et al., 1951). Moreover, at the time of phenol extraction, the salt concentration is usually 1 M and in some experiments, as high as 5.5 M, and SDS and EDTA are present. Under such conditions even the DNA-dependent RNA polymerase is freed from the DNA to which it specifically binds (Jones and Berg, 1966). Finally, d(A-T) which has been purified by Method I isolation followed by two centrifugations in 7.7 M CsCl (Flamm et al., 1966) is lost from the aqueous phase on subsequent phenol treatment. If proteins bound specifically to the satellites are responsible for their loss during phenol treatment, the protein-DNA binding must be very stable.

Unless they are specifically degraded by phenol or a phenol contaminant the satellite(s) which are lost must be localized either in the phenol layer and/or in the interphase. Therefore, we reextracted the interphase from a Type II preparation. The material was washed with ethanol, solublilized in SSC/10, dialyzed, and digested with pronase (200 μg/ml, 4 hr, 37°C). The reaction was stopped with SDS and SSC as before, and the mixture was reextracted with isoamyl alcohol:chloroform. Although the DNA so reextracted contained all three discrete bands on CsCl analysis, the d(A-T) was present only in trace amounts. Since the yield of d(A-T) satellite from the interphase was too small to account for the amount lost, we extracted the phenol layer. Again, three bands were found but again d(A-T) was present in relatively less amount (14% of the total DNA) than we routinely obtained in Type I preparations (18%).

Thus, the d(A-T) which is selectively lost from the aqueous layer of a phenol-treated preparation is not reextractable in significant quantity from either of the other layers into a new aqueous phase. Although the specific loss of the satellite is readily and reproducibly demonstrable, the reasons for the loss remain unclear.

In several bacteria, Rolfe (1963) has found satellite DNA's associated with replication after deproteinization with phenol but not with isoamyl alcohol:chloroform. Therefore, both

methods of deproteinization should be tried in any search for satellite DNA's before a conclusion is drawn as to their presence or absence.

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