Metzger, H., Wofsy, L., and Singer, S. J. (1963), *Biochemistry 2*, 979.
Metzger, H., Wofsy, L., and Singer, S. J. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 612.
Miller, F., and Metzger, H. (1965), *J. Biol. Chem. 240*, 4740.
Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.
Nisonoff, A., Wissler, F. C., and Woernley, D. L. (1960), *Arch. Biochem. Biophys. 88*, 241.
Porath, J., Axen, R., and Ernback, S. (1967), *Nature 215*, 1491.
Porter, R. R. (1950), *Methods Med. Res. 3*, 256.
Porter, R. R. (1959), *Biochem. J. 73*, 119.
Raftery, M. A., and Cole, R. D. (1963), *Biochem. Biophys. Res. Commun. 10*, 467.
Scheidegger, J. J. (1955), *Intern. Arch. Allergy 7*, 103.
Singer, S. J., and Thorpe, N. O. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 1371.
Thorpe, N. O., and Singer, S. J. (1969), *Biochemistry 8*, 4523.
Traylor, P. S., and Singer, S. J. (1967), *Biochemistry 6*, 881.
Wofsy, L., Kimura, J., Bing, D. H., and Parker, D. C. (1967), *Biochemistry 6*, 1981.
Wofsy, L., Metzger, H., and Singer, S. J. (1962), *Biochemistry 1*, 1031.
Wofsy, L., and Parker, D. C. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 111.
Wofsy, L., Parker, D. C., Corneil, I., and Burr, B. (1969), *in* Developmental Aspects of Antibody Structure and Function, Sterzl, J., and Rhia, H., Ed., New York, N. Y., Academic (in press).

Sea Urchin Satellite Deoxyribonucleic Acid. Its Large-Scale Isolation and Hybridization with Homologous Ribosomal Ribonucleic Acid*

James B. Patterson and Darrel W. Stafford

ABSTRACT: A satellite deoxyribonucleic acid (DNA) has been isolated from sperm of the sea urchin *Lytechinus variegatus*. This satellite DNA has a density of 1.722 g/cc as compared with a density of 1.695 g/cc for main band DNA.

The satellite was isolated by selective denaturation of main band DNA followed by separation of the native and denatured DNA in a polyethylene glycol–dextran two-phase system. The satellite was purified 700-fold by this step alone. Preparative CsCl density gradient centrifugation further purified the satellite. The yield of satellite DNA was 45% of the total amount determined present in the purified whole sperm DNA. The sedimentation coefficient was 23.3 S, corresponding to a molecular weight of about 1×10^7 daltons. The satellite was shown to contain sequences which hybridize with homologous ribosomal ribonucleic acid.

The first evidence that the genes coding for rRNA could be physically separated from the rest of the genome in *Xenopus laevis* was presented by Birnstiel and coworkers (1966). There has subsequently been much work on the characterization of this (Birnstiel *et al.*, 1968; Brown and Dawid, 1968; Brown and Weber, 1968a,b; Brown *et al.*, 1967) and other similar satellite DNAs (Lima-de-Faria *et al.*, 1969; Matsudo and Siegel, 1967; Skinner, 1969; Stafford and Guild, 1969). Much of the work has been hampered by the difficulty and expense of obtaining sufficient satellite DNA.

We recently reported the presence of a G + C rich satellite DNA from sperm of the sea urchin *Lytechinus variegatus*. The satellite DNA was partially purified (Stafford and Guild, 1969) by thermal chromatography on hydroxylapatite (Miyazawa and Thomas, 1965) and it was shown that the G + C rich fraction contained sequences specific for rRNA. However, we could not show that the sequences specific for rRNA were definitely contained in the satellite DNA.

We report here a method for large-scale purification of the satellite DNA and show that it does contain sequences specific for rRNA.

Materials and Methods

Reagents. Nitrocellulose membrane filters (Type B-6, 25 mm diameter) were purchased from Carl Schleicher & Schuell Co. Dextran T500 was purchased from Pharmacia. Polyethylene glycol (Carbowax 6000) was purchased from Union Carbide Chemicals. Cesium chloride (optical grade) was purchased from Harshaw Chemical Co. Scintillation vials were purchased from Packard. Kieselguhr (Hyflo Supercel) was purchased from Johns Manville Products Corp. Isoamyl alcohol and toluene were purchased from Allied Chemical. Chloroform, phenol, and 95% sodium dodecyl sulfate were purchased from Matheson, Coleman & Bell. Pronase

* From the Department of Zoology, the University of North Carolina, Chapel Hill, North Carolina 27514. *Received October 15, 1969.* The work was supported by a predoctoral traineeship from the National Science Foundation (J. B. P.) and Grant GB-8334 from the National Science Foundation (D. W. S.).

grade B and dichlorodimethylsilane were purchased from Calbiochem. Diphenylamine, 8-hydroxyquinoline, and *m*-cresol, were purchased from Fisher Scientific Co. Sodium 4-aminosalicylate was purchased from The British Drug Houses, Ltd. Sarkosyl NL30 was a gift from Geigy Industrial Chemicals. Triton X-100 was purchased from Rohm & Haas. Penicillin "G" (potassium) and streptomycin sulfate were purchased from General Biochemicals. Ribonuclease A was purchased from Worthington Biochemical Corp. [^{32}P]-Monosodium (1000 mCi/mmole) and Omnifluor were purchased from New England Nuclear Corp. Dichlorodimethylsilane (1%) in benzene was prepared as described by Calbiochem (Calbiochem, 1968, Biochemical Data, p 17) for use in coating hybridization vials. Phenol and *m*-cresol were redistilled before use. Diphenylamine was recrystallized before use. All water used in these experiments was redistilled in glass. All phosphate buffers were prepared by mixing equal molar amounts of monobasic and dibasic sodium phosphate. The phase system components were prepared as described by Alberts (1967). Dextran T500 (16.8% w/w) (lots 3202 and 4376) and polyethylene glycol 6000 (9.2% w/w) were mixed in aqueous solution and frozen. The CsCl stock solution was prepared by dissolving 26 g of solid CsCl in 14 ml of 0.02 M Tris-HCl (pH 8.5) (Mandel *et al.*, 1968). Ribonuclease A was heat treated at 95° for 10 min at 2 mg/ml in 2X SSC (Landy and Spiegelman, 1968). Scintillation counting fluid was prepared by making toluene 0.4% with Omnifluor. Standard saline citrate (1X SSC)[1] was composed of 0.15 M sodium chloride and 0.015 M trisodium citrate.

DNA Isolation. Sea urchin DNA was isolated from sperm collected at Miami, Fla. The sperm were washed three times in 3X SSC–0.1 M EDTA (pH 8.8) and frozen until use. For DNA isolation, the sperm were thawed and pronase was added to a concentration of 7 mg/10 g of sperm suspension. The concentration of DNA ranged from 2.5 to 2.7 mg per ml. The mixture was placed into dialysis tubing which had been treated with $ZnCl_2$ to increase its pore size (McBain and Stuewer, 1936). It was then dialyzed against 0.01 M sodium phosphate buffer containing 0.1 M EDTA (pH 8.8) at about 25.° Dialysis was continued for 24 hr with four buffer changes. The pronase treatment was repeated until a homogeneous mixture free of lumps was obtained. The sperm lysate was then made 6% (w/v) sodium 4-aminosalicylate and 1% (w/v) sodium dodecyl sulfate. An equal volume of 24:1 chloroform–isoamyl alcohol was added to the mixture and gently rocked or swirled for at least 20 min (Marmur, 1961). A total of four extractions were made with the chloroform–isoamyl alcohol; these were followed by two extractions with equal volumes of phenol–cresol–8-hydroxyquinoline (Kirby *et al.*, 1967) saturated with 0.1 M EDTA (pH 8.8) and 0.01 M sodium phosphate. The DNA was finally dialyzed exhaustively against 0.01 M sodium phosphate and diluted to 1 mg/ml with 0.01 M sodium phosphate. The optical density spectra of such preparations showed OD_{260}/OD_{280} = 1.84–1.90 and OD_{260}/OD_{230} = 2.2–2.5.

Satellite DNA Isolation. Satellite DNA was isolated by selective denaturation of main band DNA followed by separation of the denatured and native DNA in the polyethylene glycol–dextran two-phase system (Albertsson, 1962; Pettijohn, 1967; Alberts, 1967). The DNA at a concentration of 1 mg/ml in 0.01 M sodium phosphate was heated at 79 ± 0.2° for 10 min. It was then chilled by pouring into a large surface area flask in an ice-water bath. Polyethylene glycol–dextran phase components were added to a concentration of 0.9/1.0 ml of DNA in 290-ml polycarbonate centrifuge bottles. After thorough mixing in the cold, the two phases were separated by centrifugation at 6000*g* for 30 min. The polyethylene glycol rich top phase, containing native DNA, was pipetted off, very carefully excluding any lower phase material. The top phase was then made 23% (w/v) equimolar potassium phosphate to separate the DNA from polyethylene glycol (modified from Alberts, 1967). The polyethylene glycol formed a thin liquid top layer which was easily separated by low-speed centrifugation. The DNA was then dialyzed against 0.1 M sodium phosphate for 4–6 hr to lower the salt concentration and centrifuged in a SW65 rotor (Spinco Model L preparative ultracentrifuge) at 55,000 rpm for 20 hr to pellet the DNA. The clear pellets were resuspended in 1 ml of 0.01 M sodium phosphate and the optical density spectra were observed. The OD_{260}/OD_{280} of the resuspended top phase DNA = 1.70–1.91 and OD_{260}/OD_{230} = 1.89–2.16.

T_m Approximation by Partition Coefficient. Samples (1 ml) of sea urchin sperm DNA at 1 mg/ml in 0.01 M sodium phosphate were heated at various selected temperatures and partitioned in the polyethylene glycol–dextran two-phase system. The phase components were added at 0.9/1.0 ml of DNA. Dilutions of 1:10 were made before OD_{260} determinations were carried out. The partition coefficient in two experiments was equal to 1.0 at 68°.

RNA Isolation. Radioactive sea urchin rRNA was isolated from ribosomes of larvae at the pluteus stage of development. The larvae were grown in Millipore-filtered sea water made 50 μg/ml with respect to streptomycin and 300 units/ml with respect to penicillin (Glišin and Glišin, 1964). The larvae were labeled at the prism stage with 20–30 μCi/ml of [^{32}P]monosodium and allowed to grow for 4–5 hr. Larvae from the eggs of one gravid female were resuspended in 40 ml of Hultin's homogenization buffer (Hultin, 1961) and homogenized in a Dounce homogenizer with tight-fitting pestle. The postmitochondrial supernatant fluid was made 1% with respect to Triton X-100. The ribosomes were pelleted through sucrose, resuspended in 3 ml of Hultin's homogenization buffer, and immediately frozen on Dry Ice. rRNA was prepared by extracting the ribosomes three times with equal volumes of phenol–cresol–8-hydroxyquinoline mixture (Kirby *et al.*, 1967) plus one-eighth total volume of 24:1 chloroform–isoamyl alcohol (Marmur, 1961). The clear aqueous phase was pipetted off and combined with two volumes of cold 95% ethanol and the precipitate was pelleted. The pellet was redissolved in 1 ml of water, made 4 M with respect to sodium chloride, and refrigerated overnight. The precipitate was pelleted and washed with 4 M sodium acetate. The pellet was then washed with 95% ethanol and resuspended in 0.1 M sodium chloride and 0.05 M sodium phosphate (pH 6.8). The RNA was free of labeled DNA as all radioactivity was rendered acid soluble by hydrolysis with sodium hydroxide.

The rRNA was further purified by methylated albumin kieselguhr column chromatography (Mandell and Hershey,

[1] Abbreviations used are: SSC, standard saline citrate; MAK, methylated albumin kieselguhr.

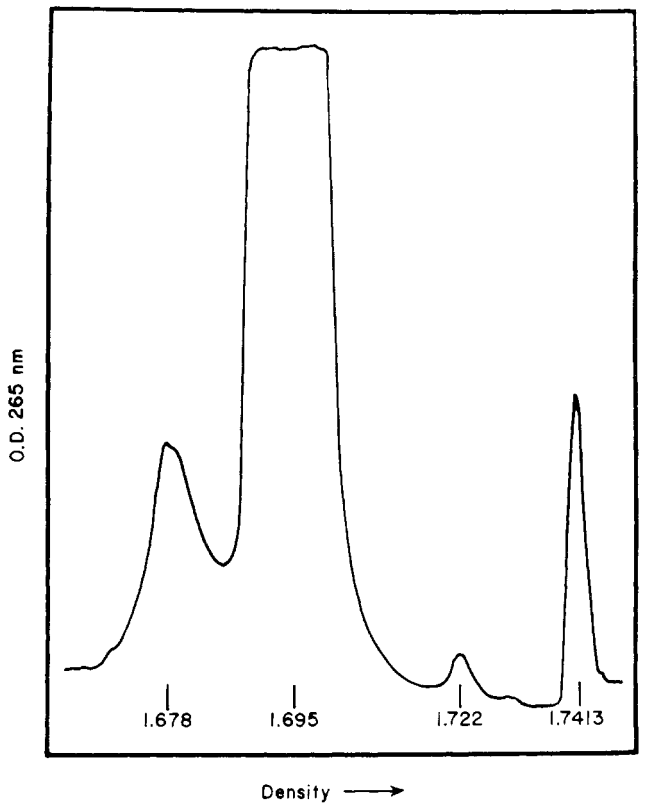

FIGURE 1: Satellite DNA in sea urchin sperm. The polyethylene glycol rich top phase (20 μl) of the two-phase system containing 100 μg of native unfractionated sea urchin DNA in 0.01 M sodium phosphate plus 170 μl of water were added to 700 μl of CsCl stock solution. Synthetic poly d(A-T) (density 1.678 g/cc) and SP82 DNA (density 1.7413 g/cc) on the scale where *E. coli* DNA is 1.710 g/cc (Schildkraut *et al.*, 1962) were added as reference standards. The main band (density 1.695 g/cc) was heavily overloaded. This same pattern is obtained with DNA which has not been put through the phase system. Density gradients were established by centrifugation at 44,700 rpm and 25° for 22 hr in a Spinco Model E analytical ultracentrifuge. Samples were photographed at 265 nm and films were traced by a Joyce-Loebl microdensitometer.

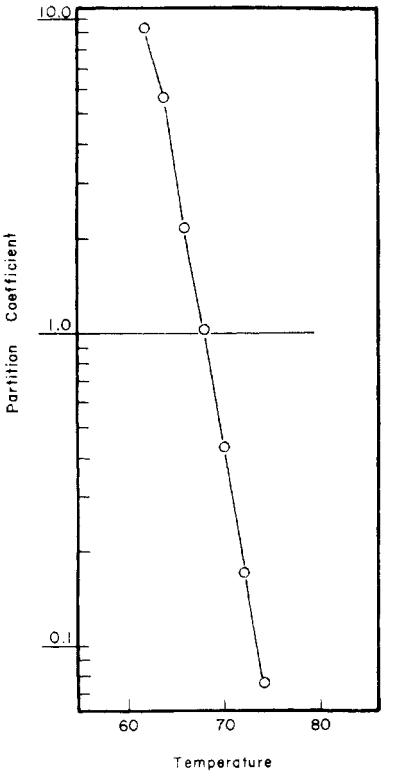

FIGURE 2: Denaturation of sea urchin DNA. Partition of DNA heated to various selected temperatures in the polyethylene glycol–dextran two-phase system.

1960). The MAK column was first washed with 0.05 M sodium phosphate and 0.5 M sodium chloride until the eluate showed no $OD_{260\,nm}$. Only that fraction eluting between 0.5 M sodium chloride–0.05 M sodium phosphate and 0.9 M sodium chloride–0.05 M sodium phosphate was used in the experiment. The OD_{260}:OD_{230} ratio was greater than 2.

Radioactive *Escherichia coli* rRNA was a gift from Charles Caldwell, University of North Carolina Biochemistry Department. It was precipitated with 4 M sodium chloride before use and had an OD_{260}:OD_{230} ratio greater than 2.

Hybridization. Sea urchin sperm DNA was prepared as described above and placed in a preparative CsCl density gradient. The DNA (0.1–0.15 mg in 0.01 M sodium phosphate) was added to 3.93 ml of CsCl stock solution to give a final volume of 5.00 ml. The solution was centrifuged at 60,000 rpm for 4 hr, then at 40,000 rpm for 38 hr in a Spinco SW65 angle rotor under a layer of paraffin oil (Flamm *et al.*, 1966). Drop fractions were collected, diluted to 0.3 ml with water, and the OD_{260} was determined for each fraction. Each DNA fraction was then diluted to 4 ml with water, denatured at 98° for 10 min, quickly chilled, and made 6X SSC by adding 1 ml of ice-cold 30X SSC. Each fraction was collected on a nitrocellulose membrane filter which had been presoaked in 6X SSC for 4 hr. The filters were washed with 100 ml of 6X SSC, dried at room temperature for 2 hr, and then dried *in vacuo* for 12 hr at 60° (Gillespie and Spiegelman, 1965).

Sea urchin ^{32}P-labeled rRNA with a specific activity of 348 cpm/μg was prepared as described above. *E. coli* ^{3}H-labeled rRNA had a specific activity of 2320 cpm/μg.

Filters upon which DNA had been adsorbed were placed flat, DNA up, on the bottom of coated scintillation vials. One filter was placed in each vial and 7.3 μg of sea urchin rRNA or 4.4 μg of *E. coli* rRNA in 0.83 ml of 2X SSC was pipetted onto it. A small square of Parafilm was placed over the vial and the top was screwed on tightly to prevent evaporation. Incubation at 66.5° for 12 hr was terminated by incubation of each filter with 8 ml of heat-treated ribonuclease A at a concentration of 30 μg/ml for 40 min. After ribonuclease treatment each filter was washed on each side with 50 ml of 2X SSC at about 65°, dried, and counted by liquid scintillation spectrometry for 30 min.

Sedimentation Velocity. The sedimentation coefficient was determined by band sedimentation in a 12-mm Kel-F band-forming centerpiece using 1.0 M NaCl as the bulk solution. Rotor speed was 25,980 rpm (Studier, 1965).

Results

The main band DNA of *Lytechinus variegatus* has a density of 1.695 g/cc as compared with the satellite DNA density

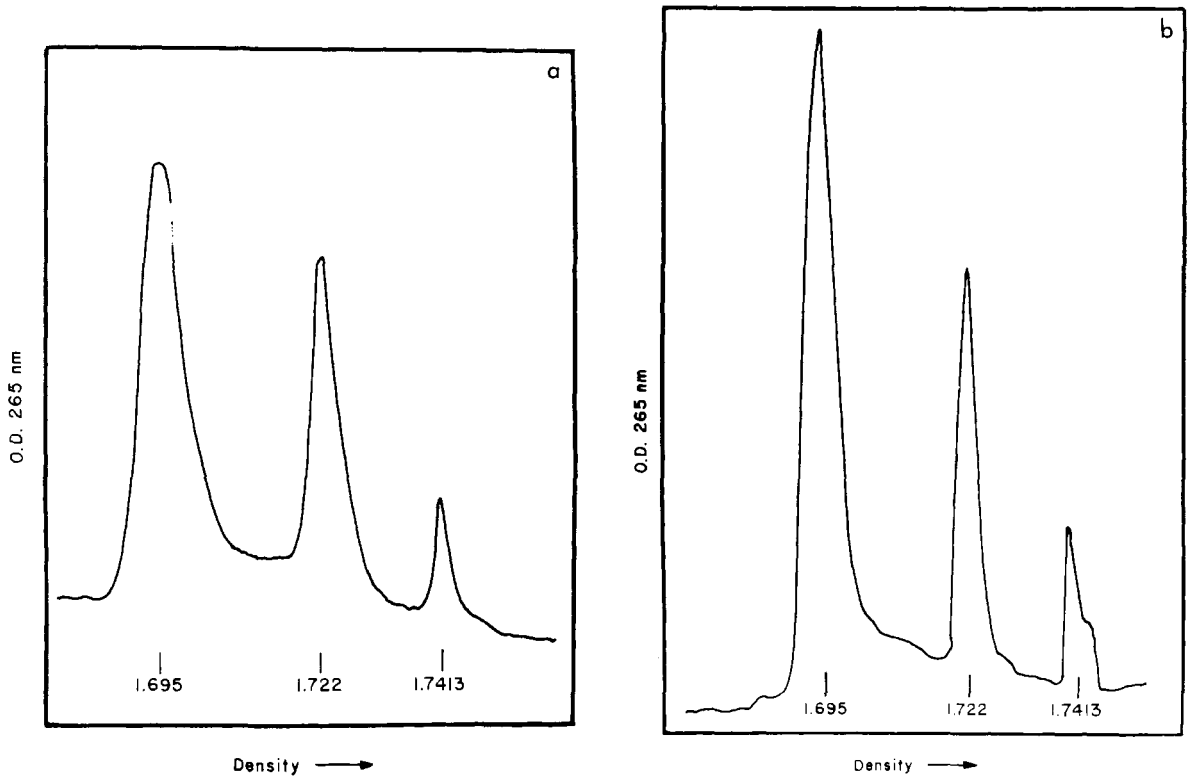

FIGURE 3: Analytical CsCl density gradient banding pattern of sea urchin sperm DNA. The DNA was selectively denatured at 79 and 80° and separated into the polyethylene glycol rich top phase as described in the Methods section of the text. The reference standard was SP82 DNA (density 1.7413 g/cc). Centrifugation was achieved as in Figure 1. (a) DNA (2.5 μg) isolated in the polyethylene glycol rich phase after selective denaturation at 79° was loaded onto the CsCl in 25 μl. Satellite DNA comprised about 21% of the total sea urchin DNA sample, an enrichment of about 700-fold; (b) 80°-treated DNA. DNA (2.5 μg) was loaded onto the CsCl in 50 μl. Satellite DNA comprised about 36% of the total sea urchin DNA sample, an enrichment of about 1200-fold. About twice as much satellite DNA is lost in the 80°-treated sample. Densitometer tracings of the film records are shown.

of 1.722 g/cc (Stafford and Guild, 1969; Figure 1); this corresponds to a G + C composition of 35 and 63%, respectively (Schildkraut *et al.*, 1962).

There are numerous methods available which should effectively separate components of such widely differing G + C composition. The excellent separation of double-stranded and single-stranded DNA obtained by Alberts (1967) using a polyethylene glycol–dextran two-phase system prompted us to attempt the isolation of the satellite DNA by this procedure. We selectively denatured the DNA at various temperatures and separated it into its single-stranded and double-stranded components in the polyethylene glycol–dextran two-phase system. Although the temperature of denaturation which gives a partition coefficient of one is not a true T_m, it should be a related function and approximate the T_m. Figure 2 shows that the partition coefficient is one at 68°. The expected T_m at this temperature is 66° (Schildkraut and Lifson, 1965).

Because of the observed difference in G + C composition between the main band and satellite DNA, we expected a T_m difference of 11.5° (Schildkraut and Lifson, 1965). We therefore selected four temperatures above the temperature of equal distribution of the main band DNA between the polyethylene glycol rich and dextran-rich phases and below the expected temperature of equal distribution of the satellite DNA. We performed selective denaturation of the DNA as described in the Materials and Methods section of the text. As expected, when the temperature was increased the proportion of satellite DNA in the polyethylene glycol rich phase was increased. Figure 3 shows the analytical CsCl density gradient results. Although the proportion of satellite DNA was higher in the 80°-treated sample (35% compared with 20% in the 79°-treated fraction), the total recovery of satellite DNA was greater in the 79°-treated sample (45% compared to 23% in the 80°-treated fraction). Since the enrichment at either temperature was sufficient to allow adequate separation on preparative CsCl density gradient centrifugation, we used 79° treatment for our isolation procedure.

The modal sedimentation coefficient of the satellite peak was determined by band sedimentation velocity to be 23.3 S, which corresponds to a molecular weight of about 1×10^7 daltons.

Figure 4a shows the results of hybridizing fractions of 79°-treated DNA from a preparative CsCl gradient with ^{32}P-labeled sea urchin rRNA. The radioactivity profile clearly follows the OD_{260} profile of the satellite DNA and there is little or no hybridization in the main band DNA area. Control experiments were performed by incubating nitrocellulose filters containing bull sperm DNA and nitrocellulose filters alone with ^{32}P-labeled sea urchin rRNA. The counts per minute for bull sperm DNA and no DNA were the same

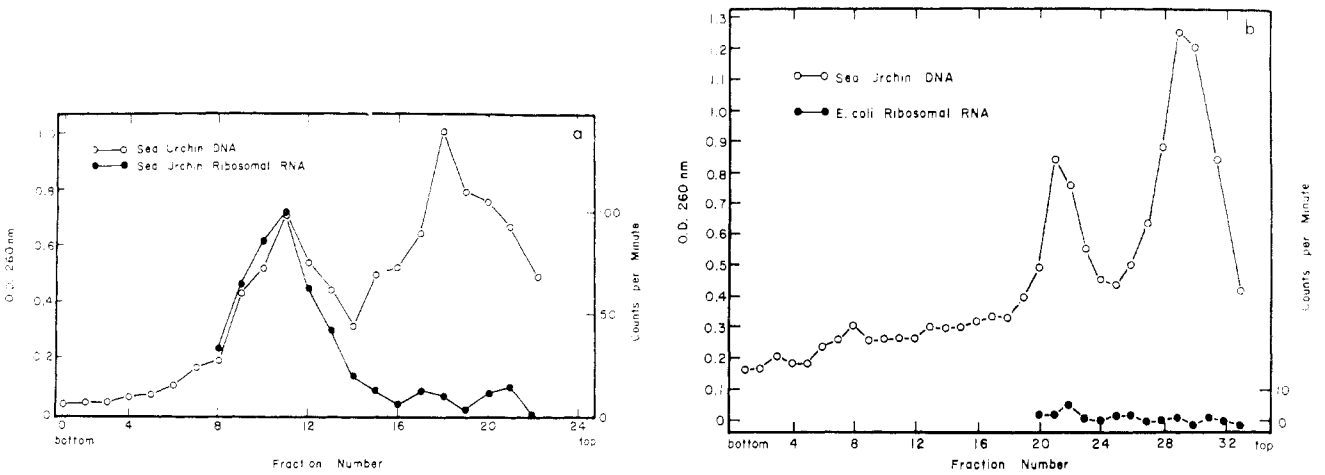

FIGURE 4: Hybridization of rRNA to sea urchin satellite DNA. The DNA and RNA were prepared as described in the Materials and Methods section of the text. DNA from the 79° isolation in the polyethylene glycol–dextran two-phase system was centrifuged to equilibrium in parallel preparative CsCl density gradients and fractions were collected for hybridization. The counts per minute shown represent the total counts per minute minus the control counts per minute. (a) Hybridization with 7.3 μg of sea urchin rRNA. The specific activity of the RNA was 348 cpm/μg. (b) Hybridization with 4.4 μg of *E. coli* rRNA. The specific activity of the RNA was 2320 cpm/μg.

and were 10 cpm above background. In Figure 4a the counts per minute shown represent the total counts per minute minus the control counts per minute.

Figure 4b shows the results of performing the same experiment except that heterologous ^{3}H-labeled *E. coli* rRNA was used for the hybridizations. No hybridization occurred. This control is even more meaningful when one considers that the specific activity of the *E. coli* rRNA was about seven times that of the sea urchin rRNA. Similar controls were performed in this experiment using filters containing bull sperm DNA and blank filters incubated with *E. coli* rRNA. The counts per minute for the bull sperm DNA and no DNA were the same and were 3 cpm above background. The values shown in Figure 4b represent the total counts per minute minus the control counts per minute.

Discussion

Our results clearly demonstrate that sequences complementary to rRNA are present in the satellite DNA. However, we cannot yet quantitate the proportion of satellite DNA which hybridizes with rRNA. Saturation and kinetic experiments will be required to answer this question. By extrapolating to the similar system from *Xenopus laevis* it is unlikely we are near saturation levels of rRNA (Birnstiel *et al.*, 1968).

The main purpose of this paper is to demonstrate the applicability of the polyethylene glycol–dextran two-phase system to large-scale isolation of the satellite. This method has several advantages over some of the other potentially useful methods. Preparative CsCl density gradient centrifugation has been used (Birnstiel *et al.*, 1966; Brown *et al.*, 1967; Skinner, 1969); however, this technique is expensive and the amount of material which can be processed is limited. Thermal chromatography on hydroxylapatite would seem to provide a means by which to effectively separate the two components. We found, however, that there was extensive degradation at the temperatures required to elute the DNA of lower G + C composition (Stafford and Guild, 1969). This difficulty could possibly be circumvented by use of formamide to reduce the temperature of elution.

The advantage of the polyethylene glycol–dextran system is that it allows the separation of about 1.3 mg of satellite from 1 g of starting DNA in one rather simple operation (based on one run in the GSA rotor of the Sorvall centrifuge). Moreover, the conditions used, 79° for 10 min, are relatively mild. It is unlikely that the fractionation procedure results in any degradation of the satellite. The density of the satellite is 1.722 g/cc both before and after the fractionation. The conditions used for bulk DNA isolation, chloroform and phenol extractions, result in DNA of about the size range obtained in these experiments.

The large-scale isolation of a satellite DNA containing the genes for rRNA should allow meaningful experiments on the chemistry and function of this satellite to be performed. Further experiments in these areas are now in progress in our laboratory.

Acknowledgment

We thank Dr. Walter Guild for the use of his microdensitometer and for help in analyzing the sedimentation velocity data.

References

Alberts, B. M. (1967), *Biochemistry 6*, 2527.

Albertsson, P. A. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 264.

Birnstiel, M. L., Speirs, J., Purdom, I., Jones, K., and Loening, U. E. (1968), *Nature 219*, 454.

Birnstiel, M. L., Wallace, H., Sirlin, J. L., and Fischberg, M. (1966), *Natl. Cancer Inst. Monograph 23*, 431.

Brown, D. D., and Dawid, I. B. (1968), *Science 160*, 272.

Brown, D. D., and Weber, C. S. (1968a), *J. Mol. Biol. 34*, 661.

Brown, D. D., and Weber, C. S. (1968b), *J. Mol. Biol. 34*, 681.

Brown, D. D., Weber, C. S., and Sinclair, J. H. (1967), *Carnegie Inst. Wash. Yearbook 66*, 580.
Flamm, W. G., Burr, H. E., and Bond, H. E. (1966), *Biochim. Biophys. Acta 129*, 310.
Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol. 12*, 829.
Glišin, V. R., and Glišin, M. V. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1548.
Hultin, T. (1961), *Exptl. Cell Res. 25*, 405.
Kirby, K. S., Fox-Carter, E., and Guest, M. (1967), *Biochem. J. 104*, 258.
Landy, A., and Spiegelman, S. (1968), *Biochemistry 7*, 585.
Lima-de-Faria, A., Birnstiel, M., and Jaworska, H. (1969), *Genetics Suppl. 61*, 145.
Mandel, M., Schildkraut, C., and Marmur, J. (1968), *Methods Enzymol. 12*, 184.
Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.
Marmur, J. (1961), *J. Mol. Biol. 3*, 208.
Matsuda, K., and Siegel, A. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 673.
McBain, J. W., and Stuewer, R. F. (1936), *J. Phys. Chem. 40*, 1157.
Miyazawa, Y., and Thomas, C. (1965), *J. Mol. Biol. 11*, 223.
Pettijohn, D. (1967), *European J. Biochem. 3*, 25.
Schildkraut, C., and Lifson, S. (1965), *Biopolymers 3*, 195.
Schildkraut, C. L., Marmur, J., and Doty, P. (1962), *J. Mol. Biol. 4*, 430.
Skinner, D. M. (1969), *Biochemistry 8*, 1467.
Stafford, D. W., and Guild, W. R. (1969), *Exptl. Cell Res. 55*, 347.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.