Isolation of Heavy Satellite Deoxyribonucleic Acid from Sea-Urchin Spermatozoa

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Two different methods were used to isolate high-molecular-weight heavy satellite DNA from the spermatozoa of the sea-urchin *Lytechinus variegatus*. Purification of satellite rDNA (ribosomal DNA) by selective denaturation of the spermatozoal DNA followed by a separation of the native and denatured DNA in an aqueous mixture of poly(ethylene glycol)/dextran is affected by the molecular weight of the spermatozoal DNA. Enrichment of satellite rDNA by selective precipitation of main-band DNA by poly-L-lysine is cumbersome but more suitable.

There are several methods available for the isolation and purification of heavy satellite DNA (Flamm, 1972; Brown & Stern, 1974). One of them is the selective heat denaturation of main-band DNA followed by a separation of the denatured DNA from the native DNA in an aqueous mixture of poly(ethylene glycol)/dextran (Albertsson, 1960; Patterson & Stafford, 1970). This method has been successfully used by Patterson & Stafford (1970) to isolate satellite rDNA of sea-urchin (*Lytechinus variegatus*) spermatozoa. The quality of satellite rDNA isolated by this method has been questioned by several workers (Birnstiel *et al.*, 1971; Walker, 1971).

Another method of enrichment of satellite rDNA is the selective precipitation of the (A+T)-rich main-band DNA (Dawid *et al.*, 1970; Polito *et al.*, 1971). In the present work, both methods have been used for the isolation of high-molecular-weight satellite rDNA. The usefulness of these two methods is discussed.

Materials and Methods

Isolation of spermatozoal DNA

Freshly collected spermatozoa from *Lytechinus variegatus* (1 ml) were suspended in 20 ml of 0.05 M-EDTA/1% sodium dodecyl sulphate. The suspension was shaken gently for a few minutes. The lysed spermatozoa were shaken for 1 h in an equal volume of phenol/*m*-cresol/8-hydroxyquinoline (Kirby *et al.*, 1967). The protein precipitate was removed and two more such extractions were carried out. Phenol was removed by dialysing against 0.01 M-sodium phosphate, pH 6.8 (prepared by mixing equimolar

amounts of monobasic and dibasic sodium phosphate), and DNA was treated with nuclease-free Pronase (Calbiochem, Los Angeles, CA, U.S.A.). An equal volume of chloroform/3-methylbutan-1-ol (24:1, v/v) was added to the mixture and gently shaken for 20 min. A total of three extractions was carried out with chloroform/3-methylbutan-1-ol. It was then dialysed for 24 h against four changes of 0.01 M-sodium phosphate, pH 6.8. The DNA was stored at 4°C with a few drops of chloroform.

Sedimentation velocity

Sedimentation coefficients were determined in a Spinco model E analytical ultracentrifuge equipped with u.v. optics by using a 12 mm Kel-F band-forming centrepiece. Photographs were traced with a Joyce-Loebl microdensitometer. Equations and correction factors used for the calculation of molecular weight were taken from Studier (1965).

Shearing of spermatozoal DNA

The DNA was sheared by rapid and repeated (5 times) passage from a 5 ml syringe through a no. 27 hypodermic needle.

Isolation of satellite DNA by the poly(ethylene glycol)/dextran method

The phase components were prepared as described by Alberts (1967) and Patterson & Stafford (1970). Dextran T500 (lots 3202 and 4376) was purchased from Pharmacia, Uppsala, Sweden, and poly(ethylene glycol) (Carbowax 6000) was purchased from Union Carbide Chemicals, New York, NY, U.S.A. Dextran T500 (16.8%, w/w) and poly(ethylene glycol) 6000 (9.2%, w/w) were mixed in an aqueous solution and kept frozen.

Partition coefficients of the native and completely heat-denatured spermatozoal DNA were obtained

Abbreviations used: rDNA, deoxyribonucleic acid containing base sequences coding for rRNA; rRNA, ribosomal ribonucleic acid.

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for different concentrations of DNA by using different amounts of the poly(ethylene glycol)/dextran mixture.

For the isolation of satellite rDNA, spermatozoal DNA in 0.01M-sodium phosphate (pH 6.8) was heated to $79 \pm 0.2^\circ\text{C}$ in a 1-litre flat-bottomed flask for 10 min in a temperature-regulated water bath. This DNA was immediately poured into an ice-chilled flask and an appropriate amount of ice-cold poly(ethylene glycol)/dextran was added. After thorough mixing in the cold, the two phases were separated by centrifugation at 6000g for 30 min. The poly(ethylene glycol)-rich top phase was carefully pipetted off. The native DNA present in the top phase was separated from poly(ethylene glycol) by adding (23%, w/v) equimolar amounts of monobasic and dibasic potassium phosphate. This DNA was subsequently dialysed for 4 h against 0.01M-sodium phosphate, pH 6.8, and centrifuged at 55000 rev./min for 12 h in a SW 65 rotor (Spinco model L preparative ultracentrifuge). The pellets were dissolved in 0.01M-sodium phosphate, pH 6.8. A small amount of this DNA was centrifuged for 24 h at 44770 rev./min and 25°C in a Spinco model E analytical ultracentrifuge.

Enrichment of satellite rDNA by using poly-L-lysine

A stock solution of poly-L-lysine was made by dissolving 2 mg of poly-L-lysine hydrobromide (mol.wt. 14 200; Miles-Yeda, Elkhart, IN, U.S.A.) in 1 ml of 1M-NaCl/0.01M-Tris/HCl, pH 7.8. By calibration experiment it was found that 0.7 mg of poly-L-lysine added to 1 mg of DNA would precipitate 96% of the latter. The concentration of DNA was 160–200 $\mu\text{g/ml}$ in 3M-NaCl containing 0.01M-Tris/HCl, pH 7.8, and 0.7 mg of poly-L-lysine/mg of DNA was used for the first step of precipitation of DNA. The DNA–poly-L-lysine complex was dialysed against three changes of 1M-NaCl/0.01M-Tris/HCl, pH 7.8. The DNA–poly-L-lysine complex, which formed a precipitate, was removed by centrifugation at 20000g for 20 min at 25°C . The supernatant DNA was concentrated 4-fold in a rotary evaporator. The concentrated DNA was further dialysed against 1M-NaCl, and additional DNA–poly-L-lysine precipitate removed by centrifugation at 20000g for 20 min at 25°C . The supernatant was further concentrated 4-fold. A second calibration experiment was performed at this stage, showing that 0.5 mg of poly-L-lysine/mg of DNA would bring about maximum enrichment of satellite DNA. After a second addition of poly-L-lysine further treatment was carried out as described above. The supernatant containing DNA was made 3M in NaCl and passed through a column (2 cm $\times$ 3 cm) of Dowex 50 (Na^+ form) to remove any unbound poly-L-lysine.

Results and Discussion

DNA isolated from freshly collected spermatozoa of the sea urchin was of high molecular weight. The molecular weight of the batch of DNA used for the isolation of satellite DNA was determined in 1M-NaCl, by the method of Studier (1965). The rate of sedimentation was determined at 25980 rev./min. The relationship between $\ln r$ and time in min was linear. This batch of DNA had $s_{20,w}$ 27.7 S, corresponding to mol.wt. of 16.6×10^6 . Fig. 1 shows the partition coefficient of completely heat-denatured DNA, native DNA and partially heat-denatured (79°C) DNA in the poly(ethylene glycol)/dextran two-phase system; the higher the volume of poly(ethylene glycol)/dextran, the greater is the tendency of DNA to stay in the bottom phase. The concentration of DNA used in the present experiment ranged from 50 to 974 $\mu\text{g/ml}$. The native DNA that collected in the top phase, after selective denaturation of spermatozoal DNA at 79°C , was centrifuged to equilibrium in CsCl in a Spinco model E analytical ultracentrifuge. Fig. 2 shows the relative amount of main-band DNA (density 1.695 g/ml) and satellite rDNA (density 1.722 g/ml) present in the top phase. The amount of main-band DNA is still considerably greater than that of satellite DNA, and in this case, further separation of the two DNA species would require two cycles of preparative CsCl centrifugation. The main purpose of this experiment is to bring about

Fig. 1. Partition of different concentrations of native and heat-denatured DNA of *Lytechinus variegatus* spermatozoa as a function of different amounts of poly(ethylene glycol)/dextran

■, Native DNA; ○, completely denatured DNA.
1, 50 $\mu\text{g/ml}$; 2, 100 $\mu\text{g/ml}$; 3, 300 $\mu\text{g/ml}$; 4, 974 $\mu\text{g/ml}$.
●, 974 μg of DNA/ml denatured at 79°C .

Fig. 2. Microdensitometer tracings of photographic records obtained for analytical centrifugation of DNA samples DNA (300 μ g/ml) from the spermatozoa of *Lytechinus variegatus*, in 0.01 M-sodium phosphate, pH 6.8, was heated at 79°C for 10 min followed by rapid cooling and addition of: —, 0.7 ml of poly(ethylene glycol)/dextran/ml of DNA; ----, 0.7 ml of poly(ethylene glycol)/dextran/ml of DNA (sheared); - · - ·, 0.5 ml of poly(ethylene glycol)/dextran/ml of DNA (sheared). DNA present in 1 ml of poly(ethylene glycol)-rich top phase was centrifuged to equilibrium in CsCl (Spinco model E analytical ultracentrifuge). In addition to the heavy satellite DNA (density 1.722 g/ml) there is another satellite (density 1.710 g/ml) that appears in some preparations. The buoyant density of main-band DNA is 1.695 g/ml.

Fig. 3. *Enrichment of satellite rDNA by precipitation with poly-L-lysine*

Samples of the spermatozoal DNA that remained in solution after each of three successive precipitations with poly-L-lysine were centrifuged to equilibrium in CsCl (Spinco model E analytical ultracentrifuge). *Tetrahymena* DNA with a buoyant density of 1.685 g/ml was used as a marker in these samples. —, DNA remaining after first poly-L-lysine precipitation; ----, DNA remaining after second poly-L-lysine precipitation; - · - ·, DNA remaining after third poly-L-lysine precipitation.

sufficient enrichment of satellite DNA and simultaneous decrease in main-band DNA so that the former could be purified by one cycle of CsCl centrifugation, but when high-molecular-weight spermato-

zoal DNA is used for the isolation of satellite DNA the enrichment does not appear to be satisfactory.

If the DNA recovered in the top phase is centrifuged to equilibrium in CsCl in a 60Ti rotor (Spinco model

L preparative ultracentrifuge), the resolution of satellite rDNA is not satisfactory. This is because the resolution of satellite DNA by CsCl centrifugation depends on both the molecular weight and concentration of DNA (Brown & Weber, 1968).

For physical and chemical characterization of a gene, it must first be isolated and purified by methods that do not bring about any contamination. To study the multiple copies of a gene, it must be purified in a high-molecular-weight form. Isolation of heavy satellite DNA by the poly(ethylene glycol)/dextran two-phase system requires heating of DNA to high temperature. In the present experiment, spermatozoal DNA was heated to 79°C for 10 min in 0.01M-sodium phosphate, pH 6.8. This leads to considerable denaturation of low (G+C)-containing main-band DNA and some denaturation to low (G+C)-containing regions of satellite rDNA (N. K. Mishra, unpublished work). Rapid cooling of this DNA might not enable the denatured sites of satellite rDNA to reanneal properly in 0.01M-sodium phosphate. In this case, there is a risk of isolating satellite DNA in which there are small metastable segments. There is a possibility that heat will deplete satellite DNA (Tamm *et al.*, 1952) and thus create single-strand breaks (Walker, 1971; Ginoza & Guild, 1961; Ginoza & Zimm, 1961). Moreover, the poly(ethylene glycol)/dextran two-phase system depends on the separation of single- and double-stranded DNA in the two phases, i.e., on their distribution coefficient, K . As

$$K = \exp - \left(\frac{M\lambda}{kT} \right)$$

where M is molecular weight, k the Boltzmann constant, λ a constant, T temperature in K, it is obvious that the partition coefficient (K) also depends on the molecular weight of DNA. Before the isolation of satellite DNA by this method is undertaken, fresh calibration of the ratio of poly(ethylene glycol)/dextran/ml of DNA has to be done because the concentrations of DNA and its molecular weight are different for a particular batch of isolated DNA. Patterson & Stafford (1970) used 1 mg of DNA/ml for their experiment. It is very difficult to obtain such a high concentration of high-molecular-weight DNA because of the viscosity problem.

Dextran has a high affinity for single-stranded DNA (Graves, 1968) and it is possible that some denatured sites of satellite DNA might trap dextran. If dextran is bound to DNA, it will further complicate the experiment because its removal may pose another problem. With high-molecular-weight DNA, a considerable amount of DNA is trapped in the interphase of the poly(ethylene glycol)/dextran two-phase system. Thus a certain amount of satellite DNA present in the interphase will have to be

sacrificed. To sum up, it may be said that the enrichment of high-molecular-weight heavy satellite DNA by using poly(ethylene glycol)/dextran cannot always be deemed a method of choice. This method is extremely sensitive to a number of parameters involved in the process of enrichment of high-density satellite DNA. There is also a risk of contaminating satellite DNA by poly(ethylene glycol)/dextran (Birnstiel *et al.*, 1971; Walker, 1971).

Fig. 3 shows the enrichment of satellite rDNA by selective precipitation of the main-band DNA with poly-L-lysine. The enrichment of rDNA by this experiment is greater than that achieved by the poly(ethylene glycol)/dextran method. For the poly-L-lysine-preparative method, a calibration experiment for each batch of poly-L-lysine and DNA stock is needed. This method is certainly tedious, as it involves several steps, but it is not sensitive to certain parameters such as concentration and molecular weight of DNA, and it does not require heating of DNA to high temperature.

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