Denaturation map of the ribosomal DNA of *Lytechinus variegatus* sperm[1]

N.K. Mishra[2]

Department of Zoology, University of North Carolina, Chapel Hill (N.C., USA), 10 October 1978

Summary. Electron microscopy of the partially heat denatured ribosomal DNA (rDNA) from sea urchin *(Lytechinus variegatus)* sperm has demonstrated that it consists of repeating units of 3.6±0.2 μm, corresponding to a mol.wt of $7.2\pm0.4\times10^6$. Based on differential denaturability, each repeat unit is divided into 2 regions. The larger region of 2.47±0.11 μm (mol.wt $4.9\pm0.22\times10^6$) corresponds in length to the ribosomal precursor RNA of sea urchins and the smaller, GC-rich, subunit of 1.16±0.09 μm (mol.wt $2.3\pm0.18\times10^6$) is presumed to contain non-transcribed spacer sequences.

The ribosomal DNA (rDNA) of eukaryotes consists of repeating units, each of which contains a transcribed region and a non-transcribed spacer. While transcribed part of the rDNA exhibit considerable sequential homology of all eukaryotic species, both transcribed and non-transcribed spacer DNA contain sequential as well as length heterogeneity[3-9]. Extensive information is available on the structure of rDNA of *Xenopus laevis*[6-8,10-16]. Relatively little is known about the structure of sea urchin rDNA. Earlier investigations have shown that the rDNA from sea urchin *(Lytechinus variegatus)* sperm is composed of uniform repeating units of mol.wt 5.2×10^6 which includes sequences complementary to 18S and 28S ribosomal RNA, and there is no non-transcribed spacer[17-20]. The present investigation is designed to study the structure of rDNA from *L. variegatus* sperm using the technique of denaturation mapping[15,21].

Material and methods. Isolation of DNA from the spermatozoa of *L. variegatus* and purification of high mol.wt rDNA satellite by repeated cycles of CsCl centrifugation have been previously described[22,23].

Electron microscopy. The rDNA was prepared for electron microscopy using the basic protein film technique with some modification[24,25]. Purified rDNA was dialyzed against 0.02 M NaCl - 0.005 M EDTA (pH 7.5) for 24 h. 5 μl of rDNA was mixed with 5 μl 36% HCHO (previously neutralized, boiled and filtered) in a Pyrex glass tube. It was sealed and heated to denaturation temperature for 20 min in the cuvette chamber of Gilford 2000 spectrophotometer. The temperature of the cuvette chamber was equilibrated by a flow of glycerol-water from a temperature regulated water bath. The equilibrium time was 30 min. The control of temperature, minitored by a linear thermosensor, was recorded on the chart of the spectrophotometer. Partially heat-denatured rDNA was then immediately chilled in ice and 10 μl of cold 2 M Tris - 0.02 M EDTA (pH 8.5) buffer containing 20 μg/ml cytochrome-c (Calbiochem) was added. After adding 20 μl of redistilled formamide (Fisher Company, reagent grade), it was carefully mixed and the solution was immediately spread on a hypophase. The hypophase was 0.2 Mammonium acetate in a 90-mm square plastic petri dish. The glass slide was rinsed with 0.2 M ammonium acetate and allowed to drain dry just before its use as a ramp. The cytochrome-c-DNA film was picked up near the ramp solution boundary within 30-40 sec. The copper grid (200 mesh) was used to pick up the film. It was coated with Parlodion film from a 1% w/v solution in isoamylacetate. Parlodion coated grids were used within 24 h. The DNA-cytochrome-c film was stained by dipping the grid into 5×10^{-5} M uranyl acetate - 0.05×10^{-3} M HCl in 90% ethanol for 30 sec. The stain solution was prepared fresh and used within 15 min. The stock solution was stored in the dark. The stock solution was 5.0×10^{-2} M uranyl acetate - 0.05 M HCl.

Micrographs were taken with a Zeiss EM 9S2 electron microscope at a magnification ×4570 and the resulting negatives were then enlarged ×21 on a screen; molecules were traced on paper. These tracings were measured with a Keuffel and Esser 620 300 map measurer. The magnification of the microscope was determined from micrographs of a grating replica (54,864 lines/inch; E. Fullam). Negatives were printed on No. 6 Kodabromide paper.

Results. Purification of rDNA satellite. Figure 1 shows the profile of 3rd CsCl centrifugation of the pooled heavy fractions of the sperm DNA. Fraction No. 14 of this gradient was further centrifuged to equilibrium in a Spinco Model E analytical ultracentrifuge. The banding profile (figure 2) thus obtained shows that this fraction contains only rDNA (density 1.722 g/cm^3) with no detectable

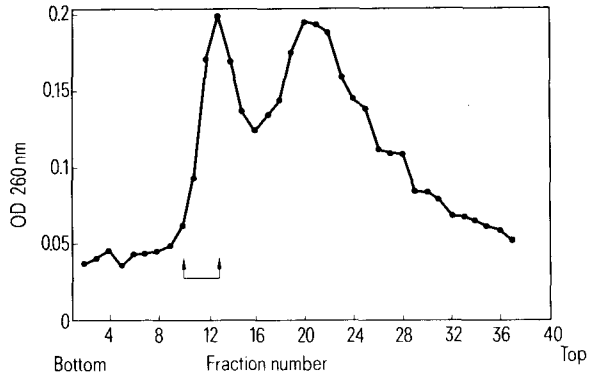

Fig. 1: Profile of 3rd CsCl centrifugation of the sperm DNA from *L. variegatus* to purify the rDNA satellite. After 1st CsCl centrifugation, heavy fractions were pooled and subsequently centrifuged twice to obtain this profile. 4 ml gradient was overlayered with paraffin oil and centrifuged in a 65 rotor for 72 h. in a Model L ultra centrifuge at 25 °C. 0.1 ml fractions were collected.

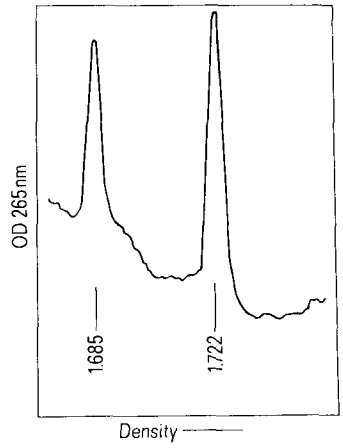

Fig. 2. Analytical centrifugation of the fraction No. 14 of figure 1 in a Model E analytical ultracentrifuge to determine the purity of rDNA (density 1.722 g/cm^3). The marker is *Tetrahymena* DNA (density 1.685 g/cm^3).

amount of any non-rDNA. Fractions 10–13 were pooled and the rDNA contained in them were used for electron microscopy.

Analysis of the structure of rDNA satellite by electron microscopy. From 68.7±0.25 °C to 71.0 °C, 6 different stages of denaturation were obtained. A regular pattern of denaturation seemed to recur in some of the molecules denatured at 70 °C. These molecules showed an average of 33% denaturation (figure 3). Each repeating pattern of denaturation showed 2 general regions, one contained several denatured sites and the other remained mostly native. The beginning and the end of these 2 regions in each repeating unit are marked by arrows in the accompanying trace (figure 3). This pattern of denaturation was found to recur in about 27% of the rDNA molecules. From this population, 12 molecules each exhibiting at least 2 repeating patterns were carefully aligned with respect to native and denatured regions, and their denaturation map was constructed by measuring the denatured sites at an interval of 0.05 μm[26] (figure 4). An average length of the repeat was calculated from 24 repeats and this was found to be 3.6±0.2 μm.

The relationship of the contour length of rDNA to its mol.wt was determined by using slightly nicked ∅ × 174 RF DNA molecules under similar conditions of heat denaturation. An average contour length of ∅ × 174 RF DNA was found to be 1.64±0.06 μm. Therefore, the relationship between the contour length and mol.wt is such that 1 μm of DNA length would contain 2.0×10^6 of DNA. Using this factor, the mean length of the rDNA repeat can be converted to a mol.wt of $7.2\pm0.4\times10^6$.

Denaturation maps within rDNA repeat. Pattern of denaturation within the rDNA repeat has also been obtained for the temperatures 68.7, 69.0, 70.3 and 71.0 °C and these have been represented in figures B–F. By looking at these histograms, it becomes obvious that 1 region of about 1 μm within the repeat remains native even in highly denatured samples. A gradient of denaturation pattern resulting from heating rDNA in the presence of formaldehyde at the temperatures mentioned above allows the construction of the map shown in figure 5B. Here the different parts of the repeat unit have been designated as *a, b, c* and *d* in order of increasing resistance to denaturation.

Discussion. Recurrence of a pattern of denaturation along the rDNA molecule of *L. variegatus* suggests the mol.wt of the repeat unit to be $7.2\pm0.4\times10^6$. Furthermore, the pattern of denaturation within the repeat unit allows its subdivision into 2 regions: 1 with a mol.wt of $4.9\pm0.22\times10^6$ and the other with a mol.wt of $2.3\pm0.18\times10^6$ (figure 5A). Studies on ribosomal RNA of sea urchins, *Paracentrotus lividus*[27] and *L. Pictus*[28], seem to suggest that the transcribed part of sea urchin rDNA should have a mol.wt close to 5.0×10^6 with an approximate GC content of 57%. On this assumption the larger part of the repeat unit may be designated as the transcribed part and the smaller part as the non-transcribed spacer. Since the mol.wt of the repeat unit has been calculated to be $7.2\pm0.4\times10^6$ and its GC content 63%[17], taking the GC content of the transcribed part to be 57%, the GC content of the non-transcribed spacer is calculated to be 76%. Biphasic melting curve of the rDNA at 260 nm and the results of the hyperchromic spectral analysis also point to the existence of a late melting component of 76–77% GC content[29]. Information on the generalized structure of the repeat unit where *g* refers to the transcribed part and *s* to non-transcribed spacer is given in figure 5A. The region *a* of the repeat unit is of highest AT content and the region *d* of highest GC content (figure 5B). The regions *c* and *d* belong to the so-called non-transcribed spacer *s*.

These results, however, are not in agreement with those of Patterson and Stafford[19,20] who found the mol.wt of the repeat unit to be 5.2×10^6. In their studies, determination of the mol.wt of rDNA repeating unit was based on saturation hybridization studies and renaturation kinetics. The relative inaccuracy of the percentage rRNA hybridized coupled with less stringent control of renaturation conditions may

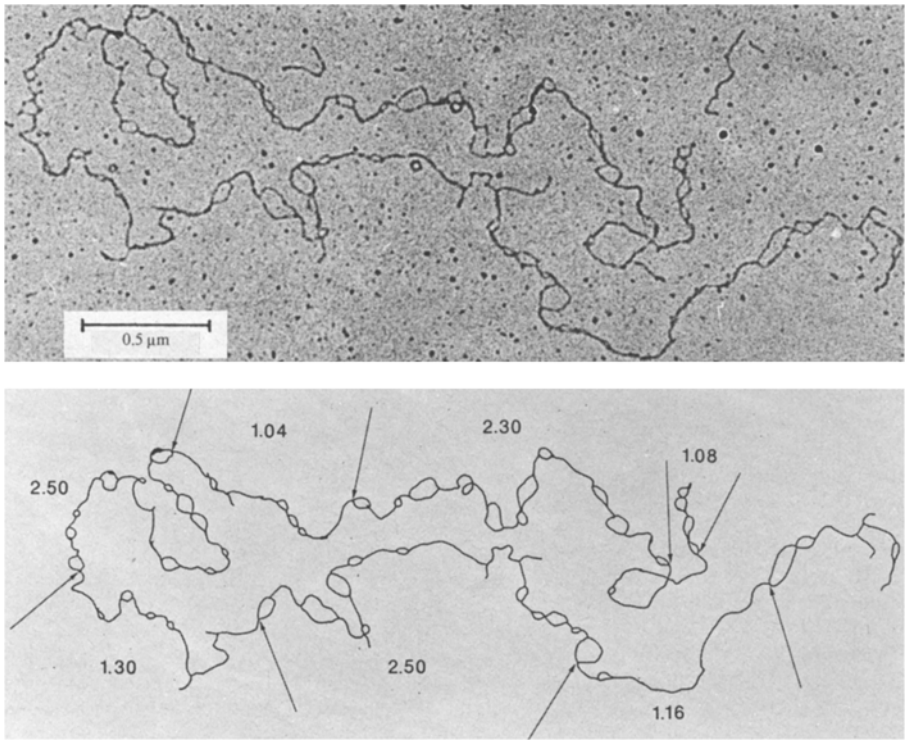

Fig. 3. Electron micrograph of the rDNA of *L. variegatus* denatured at 70 °C. The arrows in the accompanying trace point to 2 general regions in the rDNA that differ in their degree of denaturation. The numbers indicate the length in μm of the rDNA between arrows.

be the possible sources of error in the computation of the mol.wt in earlier studies[19,20]. Moreover, hyperchromic spectral analysis of Patterson and Stafford[19,20] cannot give any plausible explanation of the size of the rDNA subunits because of the wrong selection of parameters[29]. Purification of the rDNA by plyethylene glycol-dextran 2-phase system is also considered unsatisfactory[23].

In the present experiment, it is difficult to explain the occurrence of irregular denaturation pattern in a number of rDNA molecules. It is possible, but not proved, that there is some length heterogeneity among rDNA repeats of *L. variegatus* and this may account for irregular denaturation pattern. Results of Wellauer et al.[7] indicate that most, if not all, tandem repeats along a single molecule of amplified rDNA are equal in length. In contrast, between 50–68% of the adjacent repeats in a given molecule of chromosomal rDNA differ in length. It implies that intramolecular arrangement of length heterogeneity is different in amplified and chromosomal rDNA. In amplified rDNA, considerable length heterogeneity is maintained[7]. In the present work, rDNA has been purified from sperm where little or no amplification of rDNA is expected, although some amplification has been reported in the spermatogonia[30]. If sperm rDNA of *L. variegatus* is assumed to be mostly chromosomal, scrambled arrangement of the heterogeneous repeat lengths may be expected to occur in these molecules. This might mask the recurrence of clear pattern of denaturation of uniform length.

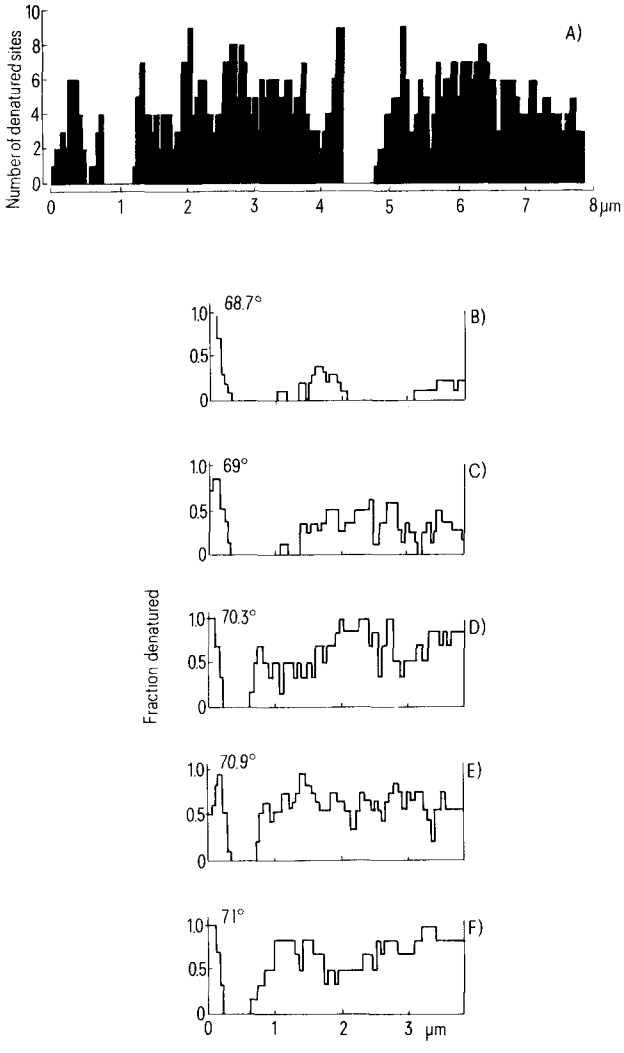

Fig. 4. Denaturation map of the rDNA molecules obtained at 70 °C. *A* Denaturated sites of 12 aligned molecules were counted at the interval of 0.05 µm; *B–F*, denaturation map of the rDNA repeat unit obtained for different temperatures mentioned against each set. Fraction denaturation was computed after dividing the repeat unit at the interval of 0.05 µm.

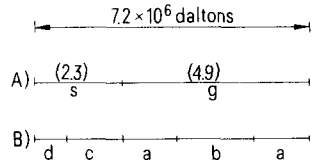

Fig. 5. Diagram of the generalized structure of the rDNA of *L. variegatus* based on denaturation mapping. In 5A, region *g* corresponds to the transcribed part of the gene and *s* to the non-transcribed spacer. The mol.wt ($\times 10^{-6}$) of these 2 parts are given in parentheses. In 5B, the repeat unit has been divided into regions of increasing resistance to denaturation: *a*, *b*, *c* and *d*. The sub-regions *c* and *d* belong to the non-transcribed spacer.

1 Acknowledgments. This paper is dedicated to the memory of Dr D.P. Costello whose inspiration made this work possible. I gratefully acknowledge the valuable advice of Drs D.W. Stafford and M.A. Bleyman.
2 Present address: Department of Zoology, Patna University, Patna 800005 (Bihar, India).
3 O.L. Miller, Jr, and B.R. Beatty, Genetics *61*, (suppl.) 133 (1969).
4 J.H. Sinclair and D.D. Brown, Biochemistry *10*, 2761 (1970).
5 D.D. Brown, P.C. Wensink and E.J. Jordan, J. molec. Biol. *63*, 57 (1972).
6 P.K. Wellauer, R.H. Reeder, D. Carroll, D.D. Brown, A. Deutch, T. Higashinakagawa and I.B. Dawid, Proc. natl Acad. Sci. USA *71*, 2823 (1974).
7 P.K. Wellauer, R.H. Reeder, I.B. Dawid and D.D. Brown, J. molec. Biol. *195*, 487 (1976).
8 R.R. Reeder, D.D. Brown, P.K. Wellauer and I.B. Dawid, J. molec. Biol. *105*, 507 (1976).
9 P.K. Wellauer and I.B. Dawid, Cell *10*, 193 (1977).
10 M.L. Birnstiel, H.J. Wallace and M. Fishberg, Natl Cancer Inst. Monogr. *23*, 431 (1966).
11 M.L. Birnstiel, J. Spiers, I. Purdom, K. Jones and U.E. Loening, Nature *219*, 454 (1968).
12 D.D. Brown and C.S. Weber, J. molec. Biol. *34*, 661 (1968).
13 D.D. Brown and C.S. Weber, J. molec. Biol. *34*, 681 (1968).
14 I.B. Dawid, D.D. Brown and R.H. Reeder, J. molec. Biol. *51*, 341 (1970).
15 P.C. Wensink and D.D. Brown, J. molec. Biol. *60*, 235 (1971).
16 A.B. Forsheit, N. Davidson and D.D. Brown, J. molec. Biol. *90*, 301 (1974).
17 D.W. Stafford and W.R. Guild, Exptl Cell. Res. *55*, 347 (1969).
18 J.P. Patterson and D.W. Stafford, Biochemistry *9*, 1278 (1970).
19 J.P. Patterson and D.W. Stafford, Biochemistry *10*, 2775 (1971).
20 J.B. Patterson,, Thesis, University of North Carolina, Chapel Hill, 1971.
21 R.B. Inman, J. molec. Biol. *18*, 464 (1966).
22 N.K. Mishra, Experientia *34*, 846 (1978).
23 N.K. Mishra, Biochem. J. *175*, 15 (1978).
24 A.K. Kleinschmidt, D. Lang, D. Jacherts and R.K. Zahn, Biochim. biophys. Acta *61*, 857 (1962).
25 R.B. Inman and G. Bertani, J. molec. Biol. *44*, 533 (1969).
26 R.B. Inman and M. Schnös, J. molec. Biol. *49*, 93 (1970).
27 G. Sconzo, E. Vitrano, A. Bono, L. di Giovanni, V. Mutolo and G. Guidice, Biochim. biophys. Acta *232*, 132 (1971).
28 V.R. Glisin and M.V. Glisin, Proc. natl Acad. Sci. USA *52*, 1548 (1964).
29 N.K. Mishra, Molec. biol. Reps. *4*, 241 (1978).
30 M.R. Kalt and J.G. Gall, J. molec. Biol. *62*, 460 (1974).