ISOLATION OF CHROMATIN BEARING NASCENT RNA FROM NUCLEI OF SEA URCHIN EMBRYOS[1]

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Received June 23, 1971

SUMMARY

Sea urchin embryos were labeled with C^{14} thymidine and H^3 Uridine, the nuclei isolated, and a chromatin preparation partially deproteinized in salt and detergent. After banding this preparation in a Cs_2SO_4 gradient, the nascent RNA is associated with a small fraction of the chromatin at a density lighter than the bulk chromatin.

INTRODUCTION

Much of the RNA transcribed in metazoon cells is degraded without export to the cytoplasm (1). We have been concerned with the details of the sites of synthesis, degradation and processing of this labile nuclear RNA (2, 3) in the belief this approach would allow one to construct testable models of its function. A distinctive feature of these experiments is the utilization of the blastula of the sea urchin embryo, the cells of which synthesize little ribosomal RNA but large amounts of high molecular weight nuclear RNA. During the course of the experiments procedures were devised for the fractionation of nuclear desoxyribonucleoprotein into a class which bears newly synthesized RNA and one that does not.

METHODS

Embryos of the sea urchin, Lytechinus pictus, were cultured in the presence of 2- C^{14} thymidine from the 2 cell to 128 cell stage, which allowed detection of small amounts of DNA during the fractionation. At the hatching blastula stage (400 cells) the embryos were pulsed with 5- H^3 uridine, quickly

[1]Research supported by Grant GM 13882 from the US Public Health Service.

collected and washed in ice cold 1.5 M dextrose. Nuclei were isolated from homogenates of embryos (4) by centrifuging them through 2.2 M sucrose (containing 0.002 Mg); the nuclei were diluted with 3 volumes of 1 M dextrose, and desoxycholate was added to a final concentration of 0.43% and Tween 40 was added to a final concentration of 0.86% (5). The detergent treatment removes all the outer nuclear membrane and its adnexa as well as most of the inner nuclear membrane. The resultant chromatin still retains > 98% of its DNA and > 90% of the pulse labeled RNA. After collecting the chromatin by centrifugation at 17,000 x g for 10 minutes, the pellet was resuspended in 1 M dextrose containing 1% deoxycholate, 0.5% Lubrol W; to this was added an equal volume of 1 M NaCl, 0.025 M Mg acetate, 0.02 M Tris, pH 7.6, and the mixture was given 1 stroke in a loose fitting Dounce homogenizer. The combination of detergents and 0.5 M salt "releases" more than 90% of the DNA and labeled RNA, i.e., they do not sediment at 12,000 x g in 10 minutes. The preparation was then subjected to isopycnic centrifugation by layering over a preformed gradient of Cs_2SO_4 (1.65 gm/cc to 1.25 gm/cc) dissolved in 0.01 M NaCl, 0.0015 M Mg acetate, 0.01 M Tris, pH 7.6 containing 0.01% Triton-x-100 (6). Centrifugation in a Spinco SW 39 rotor was carried out for 16 hours, and fractions collected by tube puncture. Refractive indices of every fourth fraction were determined, and samples precipitated with acid, collected on membrane filters, and counted in a toluene based fluor containing the commercial solubilizer, NCS (2%). The walls and bottom of the centrifuge tube were scraped for radioactivity, and this is figured as fraction 0. Since uridine is converted to precursors for DNA synthesis, portions of each sample were hydrolyzed in 0.5 N NaOH at 37°C. for 2 hours prior to acid precipitation to determine the alkaline resistant H^3 counts in DNA. This value was subtracted from the figured H^3 radioactivity.

RESULTS AND DISCUSSION

The results of centrifugation of such a preparation in Cs_2SO_4 is shown in fig. 1. In this instance the RNA label was added to the embryos for 1.5

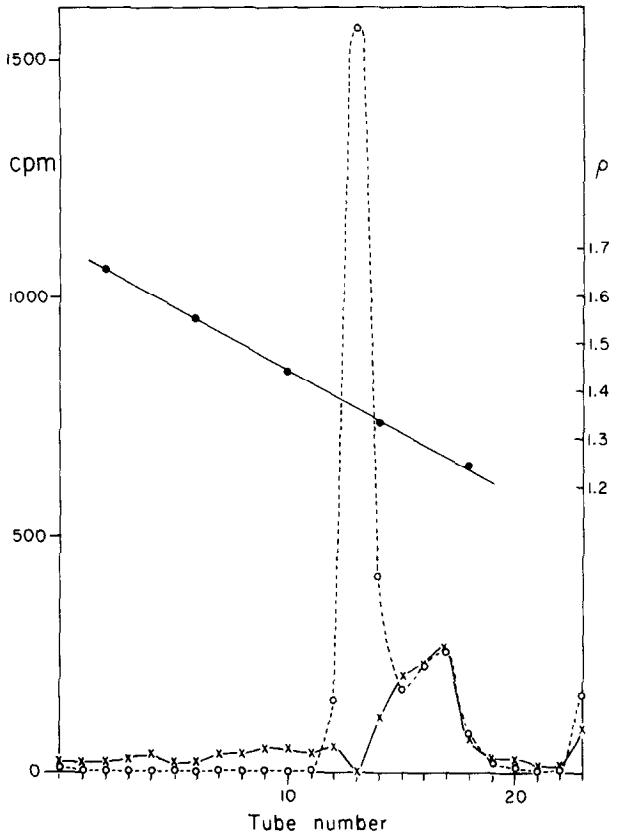

Fig. 1. One ml. of Lytechinus pictus eggs were suspended in 100 ml of millipore filtered sea water containing 50 μg/ml of streptomycin, fertilized, and 4 μc of 2-C^{14} thymidine (47.9 mC/mM, Schwarz) was added. At the hatching blastula stage 200 μc of 5-H^3 uridine (24.8 C/mM, Schwarz) was added, and 1.5 minutes thereafter the embryos were quickly chilled, washed, and nuclei prepared. One ml. of a "dispersed chromatin" preparation was layered over a preformed gradient of Cs_2SO_4. ------ C^{14} thymidine. ——— H^3 uridine in RNA.

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minutes prior to embryo collection. The DNA bands as a sharp peak at a buoyant density of about 1.37 gm/cc. Pure DNA bands at 1.42 gm/cc and protein at about 1.15 gm/cc. If one ignores the contribution of any RNA to the density, one may conclude the major DNA peak is deproteinized chromatin composed of 80% DNA, 20% protein. Little or none of the pulse labeled RNA is associated with this chromatin fraction. About 10% of the chromatin DNA bands broadly at a higher density centering about 1.28 gm/cc, and is presumably composed of 50% DNA and 50% protein. Almost all the labeled RNA is associated with this less dense chromatin. Some labeled RNA may also be seen spread throughout denser

protions of the gradient at 1.60-1.40 gm/cc. Since pure RNA bands at about 1.62 gm/cc, it is presumed these rather dense structures are ribonucleoprotein with widely varying protein contents. While not all the chromatin in the less dense fraction may be engaged in transcription, apparently most of the DNA in the heavy chromatin is inactive; the above procedure may represent a considerable purification of the portion of the genome active in transcription.

There are, however, a number of possible artefacts. Could the association of RNA with light chromatin be due to adventitious binding? The use of high salt concentration and absence of aldehyde fixatives should eliminate this possibility. It is more likely the structures observed are the results of loss of associated proteins rather than adventitious binding, and the observed densities are consistent with this interpretation. The addition of purified DNA, RNA and protein (labeled with appropriate precursors) to unlabeled nuclei at the time of dispersal of chromatin in 0.5 M NaCl and detergents did not lead to formation of any of the types of structures shown in figure 1; rather, the labeled nucleic acids and proteins banded at their characteristic densities. Furthermore, if extracts are subjected to sedimention through sucrose density gradients (15-30% sucrose in 0.5 M NaCl, 0.013 Mg acetate, 0.02 Tris buffer, pH 7.6), the less dense chromatin and nascent RNA seem to sediment together (heterodisperse from about 30 to 200 s) and the more dense chromatin sediments sharply at about 20-25 s without any associated radioactive RNA.

Could the absence of RNA from the major heavy chromatin peak be due to detachment of nascent RNA? This seems unlikely; if the dispersed chromatin is fixed in 6% glutaraldehyde immediately prior to centrifugation (6), the pattern of chromatin fractionation is unchanged (fig. 2). After fixation the ribonucleoprotein not associated with chromatin remains near the top of the gradient, indicating binding of additional protein to the RNA by the fixative. The small amount of DNA at the top of the gradient is probably due to trapping in the thick proteinaceous pad at the top, and DNA in fraction 0 may come from the same material recovered from the walls of the tube. The absence of very much

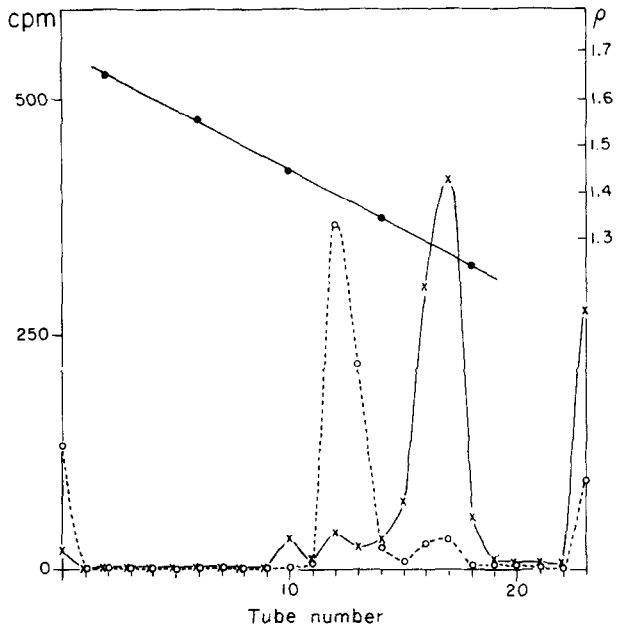

Fig. 2. The experimental conditions are identical to figure 1 except the pulse labeling with uridine was carried out for 10 minutes. Prior to centrifugation glutaraldehyde (final concentration, 6%) was added to the chromatin. ------ C^{14} thymidine. ——— H^3 uridine in RNA.

RNA free of chromatin at early pulse labeling times also militates against selective loss of RNA from dense chromatin. Some release of nascent RNA from the more dense chromatin could occur during dispersion of nuclei in salt and detergent. To examine this possibility, chromatin spheres were suspended in 0.1 M KCl, 0.01 M Mg acetate, 0.02 M Tris, pH 7.6, 1% deoxycholate, 0.5% Lubrol W and sonicated for 5 seconds to disperse the preparation at lower ionic strengths (2, 3). When this extract was subjected to centrifugation in Cs_2SO_4, with or without glutaraldehyde fixation, levels of non-chromatin bound ribonucleoprotein were found similar to that observed after dispersion of nuclei in 0.5 M salt and detergent. Hence, the selective detachment of ribonucleoprotein in 0.5 M NaCl from more dense chromatin seems unlikely.

The approximate size of the DNA in the chromatin fractions has been determined (7). The DNA in the major dense chromatin peak sediments broadly with a peak about 23 s in neutral sucrose gradients, indicating a molecular

weight on the order of 10^7. The DNA in the minor less dense peak sediments more sharply at about 45 s and has a molecular weight approximating 6 x 10^7. The RNA associated with the less dense chromatin is removed from it by treatment with sodium dodecyl sulfate (0.2% at 15°C for 10 minutes) or pronase (100 μg/ml at 15°C for 10 minutes); consequently, the association of RNA with chromatin seems to be due to protein and not to extensive hydrogen bonding.

Other methods of fractionating chromatin have often been based on separation of euchromatin and heterochromatin by centrifugation (8). The present method offers another alternative which is rapid, simple and reproducible. It is likely the observed structures are formed by partial deproteinization of chromatin, which because of their different functional states results in separable fractions. The less dense fraction seems to bear the DNA active in transcription. The procedure should allow detailed characterization of some of the proteins responsible for binding nascent RNA to DNA, allow separation and characterization of DNA which is active as a template at any given time from the remainder of the genome, as well as provide a tool for dissecting precursor-product relationships of chromatin bound RNA, "nucleoplasmic" ribonucleoprotein, and cytoplasmic messenger RNA.

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