# Murine primitive neuroectodermal tumor: nucleolar isolation and RNA polymerase inhibition

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*Summary.* A nucleolar isolation procedure was developed for the murine primitive neuroectodermal tumor originally induced with methylcholanthrene. This procedure utilizes sonication for breakage of the nuclei at a magnesium concentration which disperses the extranucleolar chromatin. The nucleoli retain RNA synthetic activity which is inhibited to about 90% by actinomycin D and the chemotherapeutic drug adriamycin.

In studying nuclear RNA synthesis of a series of chemically induced rodent brain tumors, it was found that this activity in the methylcholanthrene induced murine primitive neuroectodermal tumor was particularly sensitive to the drug adriamycin[2]. The study of the effect of adriamycin on RNA synthesis carried out by RNA polymerase I would be of considerable interest in elucidating its mechanism of action as a chemotherapeutic agent. The development of a method for isolation of contamination free, synthetically active nucleoli from the neuroectodermal tumor model would be useful for this study.

The various techniques which have been used to disrupt cell nuclei include sonication, pressure disruption, and chemical and enzymatic treatment[3–5]. Dispersal of heterochromatin with maintenance of nucleolar integrity has largely been accomplished by selective control of the divalent cation concentration. Divalent cations can act reversibly on nuclear morphology[6]. Nucleoli and extranucleolar chromatin exhibit a differential behavior with respect to the concentration of divalent cations[7]. Calcium or magnesium was most commonly used for this purpose[5,8]. Differential centrifugation is then carried out to isolate nucleoli from the dispersed chromatin residue.

The majority of the current techniques described in the literature were developed using liver or hepatoma tissue. Because of variation in the susceptibility to breakage of the nuclear membrane and in the ionic conditions necessary for heterochromatin dispersion, it is frequently found that good results are obtained only with the biological material for which the procedure was specifically developed. We report here a procedure using conditions adapted for the methylcholanthrene induced neuroectodermal tumor which gives purified cell nucleoli that retain their RNA synthetic capability. The isolation of an intact, functioning cell nucleolar preparation which still maintains the macromolecular organization could be used for the study of synthesis, control, and inhibition of ribosomal RNA by RNA polymerase I.

*Materials and methods.* The primitive neuroectodermal mouse tumor used in the following experiments was described as a mutant substrain (ependymoblastoma A)[9] originating from the methylcholanthrene induced mouse ependymoblastoma[10]. This tumor, originally obtained from Hazelton Laboratories, was maintained by s.c. passage in the flank of C57BL mice at 14-day intervals. Flank tumors used in the experiment were aseptically removed from

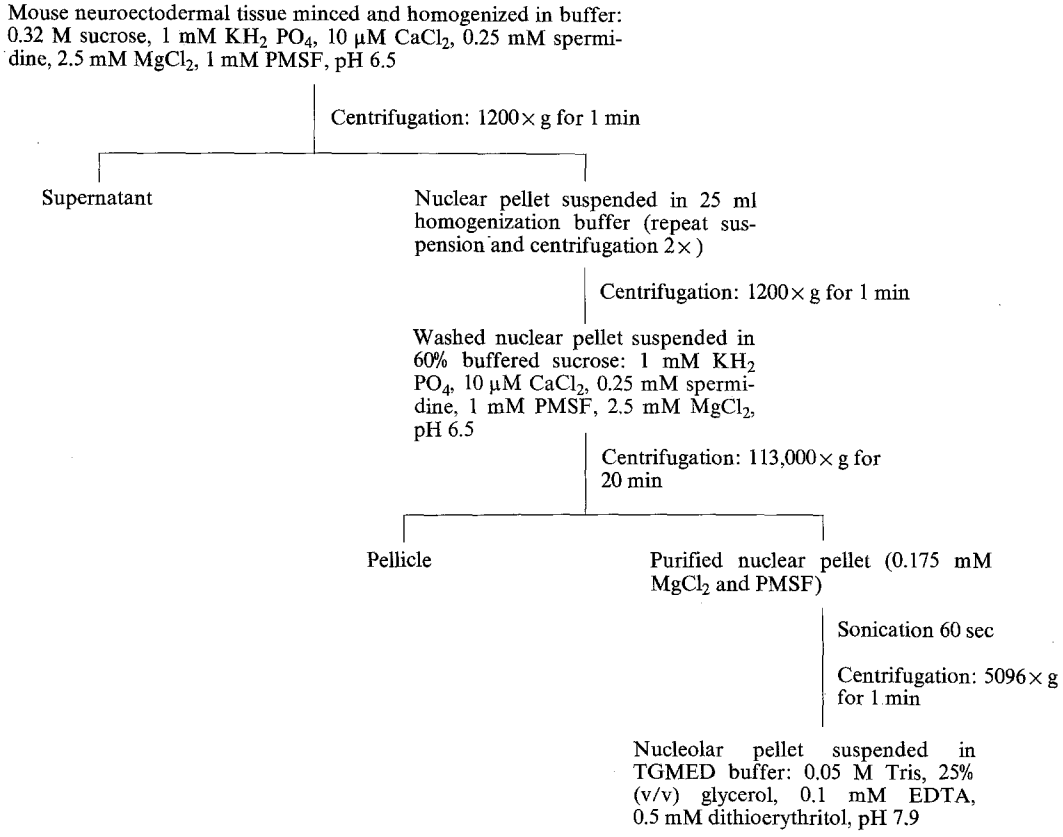


Fig. 1. Scheme summarizing the nucleolar isolation procedure for murine primitive neuroectodermal tumor tissue.

diethyl ether anesthetized mice. Grossly necrotic and connective tissue were removed from tumor tissue used in the experiment.

Nuclear isolation was carried out using a procedure described previously[2,11] with the exception that the magnesium chloride concentration was changed to 2.5 mM from 1 mM. The purified nuclear pellet was suspended in 0.525 ml per g of original tumor wet weight in buffer containing 0.32 M sucrose, 0.001 M $KH_2PO_4$, 0.001 M $MgCl_2$, and 0.001 M phenylmethylsulfonylfluoride adjusted to pH 6.5. Nuclei were allowed to stand for 5 min for the purpose of cation equilibration. The magnesium concentration was then lowered to approximately 0.175 mM by the addition of 2.475 ml per g of original tumor wet weight of a solution containing 0.32 M sucrose and 0.001 M $KH_2PO_4$. After 10 min the nuclear chromatin dispersion was monitored by viewing in the phase microscope. The nuclear suspension contained about 1 g wet weight of nuclei in 18.75 ml of solution was sonicated with samples having a 3-ml volume in a 20-ml glass vial using a 1-cm diameter probe. A sustained burst of 45 sec was followed by 3-, 5-sec bursts alternating with 5-sec pauses. The postsonicate was centrifuged for 1 min at 5000× g in a Sorvall SS-34 rotor. The supernatant was decanted from a pellet containing the purified nucleoli. The nucleolar pellet was suspended in a buffer having the following composition: 0.05 M tris-HCl (pH 7.9), 25% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM EDTA and 0.5 mM dithiothreitol (TGMED). The protein concentration of the final suspension was about 100 µg/ml.

In the RNA polymerase assay a 20-µl volume of nucleolar suspension was added to 30 µl of assay solution making a total volume of 50 µl having the following composition in µmoles/50 µl: Tris-HCl 3.5, glycerol 10% (v/v), $MgCl_2$ 0.1, EDTA 0.02, dithiothreitol 0.01, $MnCl_2$ 0.075, nucleotidetriphosphates: GTP, CTP and ATP 0.03, UTP unlabeled 0.0025, $(NH_4)_2SO_4$ 5, calf thymus DNA 5 µg and ($^3$H) UTP 2 µCi/assay (21 Ci/µmole, New England Nuclear Corp., Boston, Mass.). The assay solution in the inhibition experiment contained either 21.0 µg actinomycin D, 0.024 µg α-amanitin, or 4.12 µg adriamycin in addition to the components listed above. Samples were incubated for 20 min at 25 °C with continuous shaking. The reaction was terminated by pipetting the 50-µl assay solution onto DEAE (DE81, Whatman) cellulose disks. The disks were washed for 4 min per time in the following sequence: 6× in 5% $Na_2HPO_4$, 2× glass distilled water, 2× 95% ethanol, 1× diethyl ether and air dried. The disks were then placed in vials and counted in 10 ml of scintillation fluor (Permafluor, Packard Instruments Co., Downers Grove, Ill.) containing 5 g 2,5-diphenyloxazole (PPO) and 0.1 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per 1 of toluene. Samples were counted in a Packard TriCarb (3375) liquid scintillation spectrophotometer.

*Results.* The nucleolar isolation procedure developed for the murine neuroectodermal tumor (figure 1) was carried out in a phosphate buffered sucrose solution containing magnesium chloride and a small amount of calcium chloride which acts to stabilize the nuclear membrane during the isolation of the nuclear pellet. Phenylmethylsulfonylfluoride which has been reported to inhibit the release of RNA polymerase I from isolated nuclei and nucleoli[12] was also used throughout the isolation. Prior to sonication the concentration of magnesium in the nuclear pellet was reduced to a concentration threshold for magnesium ions[8]. At this magnesium concentration which differs for nuclei of different cells, a transition takes place in which the extranucleolar chromatin changes from an aggregated to a more dispersed state. This can be seen and monitored in the phase microscope. A phase photomicrograph (figure 2, A) of a nuclear pellet suspended in the homogenization solution containing 1.0 mM $MgCl_2$ shows the nuclei are intact with prominent nucleoli and heterochromatin which is visible in the phase microscope because of its light diffraction property. A similar photomicrograph of the nuclear pellet at a magnesium chloride concentration reduced to 0.3–0.2 mM (figure 2, B) shows the heterochromatin at this concentration no longer contains enough aggregates to diffract the light and give a phase contrast image. The nucleolus at this concentration is still intact. Sonication under these conditions degrades the nuclear membrane and nucleoplasm, leaving the nucleoli intact. The nucleolar pellet (figure 3) has a nuclear to nucleolar ratio of greater than 1 to 170. Their structural integrity and RNA synthetic activity are maintained.

The RNA synthetic activity was measured per µg of protein in the cell homogenate, nuclear suspension and in the

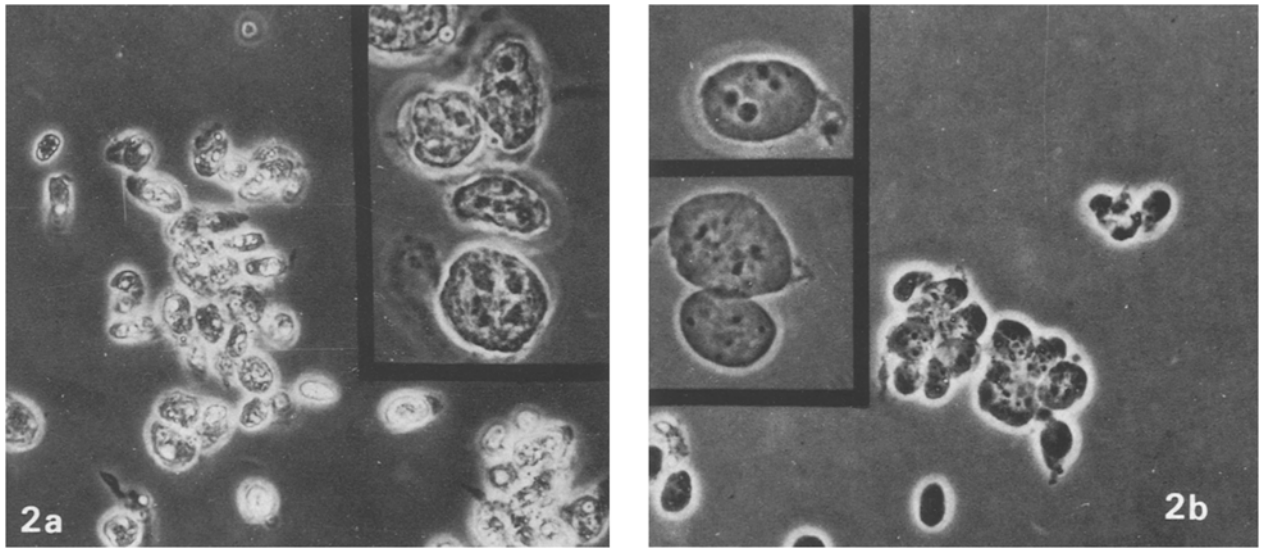


Fig. 2. Phase photomicrograph of nuclei at a magnesium chloride concentration reduced to 1.0 mM (A) and 0.175 mM (B). At the lower magnesium concentrations the extranucleolar chromatin changes from an aggregated form, refractile in the phase microscope to a more dispersed, nonrefractile form ready for sonication. × 500. Insets × 1120.

Nucleolar RNA polymerase activity and inhibition in the mouse primitive neuroectodermal tumor

| Sample | μg protein/assay | cpm/μg protein[a] × $10^2$ | Inhibition (%) |
|---|---|---|---|
| Cell homogenate | 160 | 1080 | - |
| Nuclear suspension | 46 | 1680 | - |
| Nucleolar suspension[b] | 200 | 840 | - |
| Nucleolar suspension + amanitin[c] | 200 | 556 | 34 |
| Nucleolar suspension + actinomycin d[d] | 200 | 105 | 87 |
| Nucleolar suspension + adriamycin[e] | 200 | 63 | 93 |

[a] Assay contained 1.5 mM $Mg^{2+}$ and 0.1 M ammonium sulfate. [b] Assay contained 5 μg calf thymus DNA. [c] Amanitin concentration is 0.024 μg/assay. [d] Actinomycin concentration is 21.1 μg/assay. [e] Adriamycin concentration is 4.12 μg/assay.

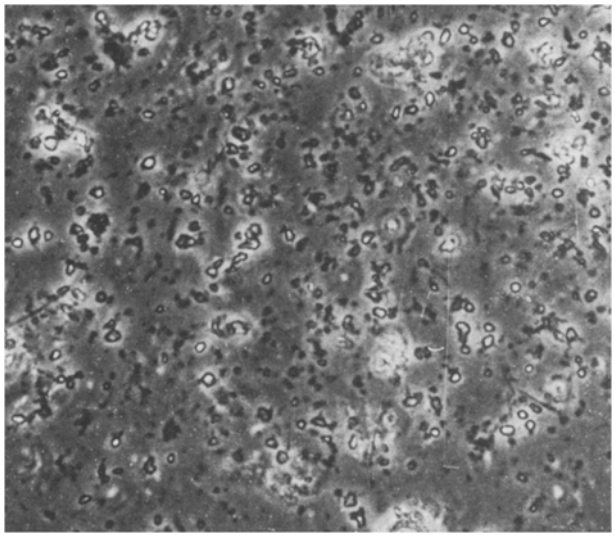

Fig. 3. Phase photomicrograph of the isolated nucleoli after nuclear sonication and centrifugation. The nuclear to nucleoli ratio in this area is 1 to 171. × 500.

isolated nucleolar pellet (table). The activity in the nuclear pellet was one-third greater than the cell homogenate and about twice as great as the nucleolar pellet. This is an expected result since the nuclear pellet contains both RNA polymerase I and II. The nucleolar pellet synthetic activity is inhibited about 90% by both adriamycin and actinomycin D. α-amanitin inhibits this activity by about 30%.

*Discussion.* A current review of methods used for nucleolar isolation discussed several experimental techniques which have been used to disrupt isolated mammalian nuclei[13]. Among these are deoxyribonuclease digestion, sonication, French pressure press, and the use of high concentration of magnesium ions with homogenization. The optimum nucleolar isolation procedure would be one in which the rRNA transcription unit remained intact. A procedure which did this would require the preservation of the RNA polymerase enzyme activity, the complete rDNA template, rRNA products, and associated proteins important in nucleolar structural integrity and in the regulation of rRNA synthesis. We have chosen to use a sonication procedure combined with a decreased divalent cation concentration[8]. In the study of the influence of magnesium ion concentration on the structure of nuclear chromatin and the nucleolus in isolated nuclei the threshold magnesium concentration ($T_{Mg}$) is defined as the concentration below which the nucleoli swell and cease to appear dense and refrastile in the phase microscope. The nuclear chromatin and the nucleolus are differentially affected by the decrease in divalent cation concentration. As the magnesium ion concentration is lowered to the threshold concentration the heterochromatin is the first component to be dispersed. In the current molecular view of chromatin structure this could represent a change in the aggregation of the nucleosome units brought about by decreased screening by magnesium ions of the electron charge on the DNA polyanion. Chromatin which is in the dispersed state is more quickly broken up by sonication and allows a more rapid release of the nucleoli. For nucleoli isolation a magnesium concentration below that which is necessary for chromatin dispersion but above the threshold concentration is optimum. One of the advantages of using decreased magnesium concentration is that it minimizes the sonication time necessary for nuclear breakage and perhaps limits its deleterious effects on nucleolar macromolecules.

Actinomycin D and adriamycin both inhibit to about 90% the endogenous RNA polymerase activity of the mouse primitive neuroectodermal tumor nucleoli. Actinomycin D is a well-characterized inhibitor of rRNA synthesis at low concentration and of total RNA synthesis at high concentrations. Adriamycin inhibits this nucleolar RNA synthesis to the same extent. Adriamycin, an anthracycline amino sugar, reacts with nucleic acids by intercalation between the nucleotide base of the nucleic acid structure[14]. In view of this the most plausible molecular mechanism of the inhibitory action on RNA polymerase I is its interaction with rDNA template to prevent RNA polymerase I from carrying out its normal synthetic function.

The nucleolar fraction was inhibited about 30% by α-amanitin. An earlier study showed that RNA polymerase I activity was not affected by even high concentrations of α-amanitin[15]. This inhibition is most probably due to the presence in the nucleolar fraction of RNA polymerase II released from extranucleolar chromatin.

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