PURIFIED CYTOPLASMIC DNA FROM HELA CELLS: RESISTANCE TO INHIBITION BY HYDROXYUREA

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Received March 24, 1969

SUMMARY

Cytoplasmic DNA has been prepared from HeLa cells using detergent for cell fractionation. The DNA is mostly circular by the CsCl-Ethidium buoyant density shift criterion. The labeling of cytoplasmic DNA appears relatively resistant to inhibition by hydroxyurea.

INTRODUCTION

DNA synthesis is inhibited by hydroxyurea in bacteria (Rosencranz, et al., 1966), algae (Heilporn-Pohl and Limbosh-Rolin, 1969), and animal cells (Young and Hodas, 1964; Yarbro, et al., 1965). Synthesis resumes upon removal of the drug (Young and Hodas, 1964; Yarbro, et al., 1965). The drug has found a chemotherapeutic application in neoplastic diseases, especially leukemias (Karkoff, et al., 1964; Bloedow, 1964).

The effect of hydroxyurea on DNA synthesis in HeLa cells has been studied in these experiments. Thymidine incorporation into nuclear DNA is strongly inhibited, but incorporation into cytoplasmic DNA is relatively resistant.

The method of cell fractionation yields undegraded (i.e. circular) cytoplasmic DNA apparently free of nuclear contamination.

MATERIALS AND METHODS

Cells: HeLa cells type S3, were grown in suspension as previously described (Penman, 1966). Cell concentration was 4 X 10^5/ml during growth and 2 X 10^6/ml during the experiments. Initially, cells were fractionated by rupturing in hypotonic buffer with a Dounce homogenizer and mitochondria were prepared by differential centrifugation. A large amount of DNA of apparent nuclear origin contaminated the preparations. In these experiments cells were resuspended in 1 ml RSB and the suspension was made 0.5% NP40 (Nonidet, Shell Oil Co.). After stirring vigorously for 30 seconds, nuclei were separated from cytoplasm by centrifuging at 1000 X g for 3 minutes. This method is essentially that described by Borum, et al., 1967.

Assays of radioactivity: The nuclear pellets were resuspended by sonication in a buffer containing 0.5 M NaCl, 0.01 M TRIS, pH 7.4. Suitable aliquots were dissolved in SDS buffer (0.5% SDS, 0.1 M NaCl, 0.001 M EDTA, 0.01 M TRIS, pH 7.4) precipitated with 5% TCA and the radioactivity measured in a scintillation system. Samples of mitochondria or cytoplasmic suspensions were also dissolved in SDS buffer, precipitated and assayed as above.

Analysis of cytoplasmic DNA: This was carried out by sedimentation in 15-30% (W/W) sucrose gradients made in SDS buffer as previously described (Gilbert, 1963) and by CsCl-Ethidium bromide gradients (Radloff, et al., 1967). The fractions from these gradients were precipitated with TCA and assayed for radioactivity. Densities were obtained from the refractive index measured with a Bausch & Lomb refractometer. Ethidium bromide was obtained from Calbiochem, Los Angeles, Cal. Cesium Chloride was from Varlacoid Chem. Co., Elizabeth, N.J. Hydroxyurea was obtained from Aldrich Chem. Co., Milwakee, Wis. ^{3}H-thymidine and ^{14}C-thymidine were obtained from New England Nuclear Corp.

RESULTS AND DISCUSSION

Figure 1 shows the incorporation of ^{3}H-thymidine in HeLa cells in the presence of various concentrations of hydroxyurea. Cells were incubated for 4 hours with the radioactive nucleotide and the drug. Nuclei and cytoplasm were separated using detergent (Borun, et al., 1967) (see Methods) and assayed for radioactivity.

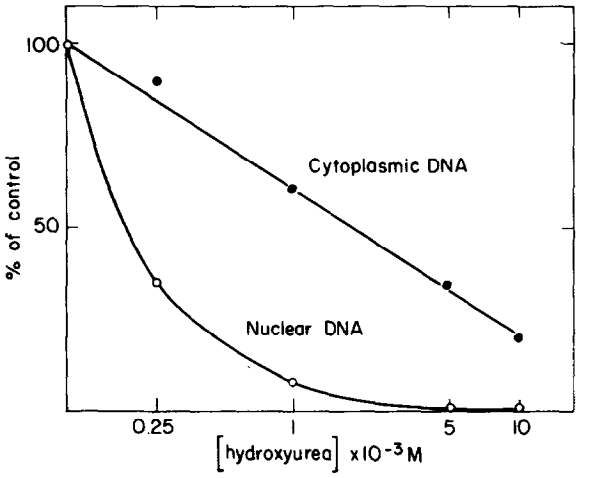

FIGURE 1: Dose-response curve of thymidine incorporation in HeLa cells. 4 X 10^{6} cells (each point) were incubated for 4 hours with 5 μC/ml of ^{3}H-thymidine (11C/m mole) in the presence of the indicated concentrations of hydroxyurea. Cells were then washed once with cold Earle's saline, resuspended in 1 ml of RSB and made 0.5% Nonidet P40. The suspension was stirred vigorously for 30 seconds. Nuclei were separated by centrifuging at 1000 X g for 3 minutes. An aliquot from the cytoplasmic supernatant and from the nuclear pellet (the latter homogenized by sonication) was diluted in 1 ml of SDS buffer, precipitated with 5% TCA and the radioactivity measured. The points show the percent radioactivity incorporated of the control, which was incubated in absence of hydroxyurea.

About 1% of cellular DNA is found in the cytoplasm. At 10^{-3} M hydroxyurea, thymidine incorporation into nuclear DNA is less than 10% of the control whereas incorporation into cytoplasmic DNA is still 60% of control. 5 X 10^{-3} M hydroxyurea decreases thymidine incorporation into nuclear and cytoplasmic DNA to 2% and 35% of control, respectively. These data indicate that hydroxyurea has a clearly different inhibitory effect on the two systems of DNA synthesis. The mechanism responsible for such a difference is presently unknown.

Two technical problems are encountered in the measurement of ^{3}H-thymidine incorporation into cytoplasmic DNA. Contamination of the cytoplasmic fraction with nuclear DNA occurs if the cells are fractionated with the Dounce homogenizer. The cytoplasmic DNA contains a rapidly sedimenting component which bands with nuclear DNA in CsCl-Ethidium bromide gradients and shows the same sensitivity to hydroxyurea as nuclear DNA. This component is absent from the cytoplasm when cells are fractionated with NP40.

The second problem is due to the incorporation of ^{3}H-thymidine into TCA precipitable material which is not DNA. The same or a very similar pseudoincorporation has been already described by others (Counts and Flamm, 1966). The error caused by simple precipitation with TCA is not significant when the radioactivity incorporated into the nuclear fraction is determined. The amount of thymidine in non-DNA is large when compared to cytoplasmic (or mitochondrial) DNA incorporation. The addition of SDS prior to TCA renders over 90% of the non-DNA radioactivity acid-soluble. The remaining material is sensitive to DNAse.

Cytoplasmic DNA labeled with radioactive thymidine in presence and absence of hydroxyurea (10^{-3}M) was characterized by sedimentation in sucrose-SDS gradients, shown in Fig. 2, and by equilibrium centrifugation in CsCl-Ethidium bromide, shown in Fig. 3 (Radloff, et al., 1967).

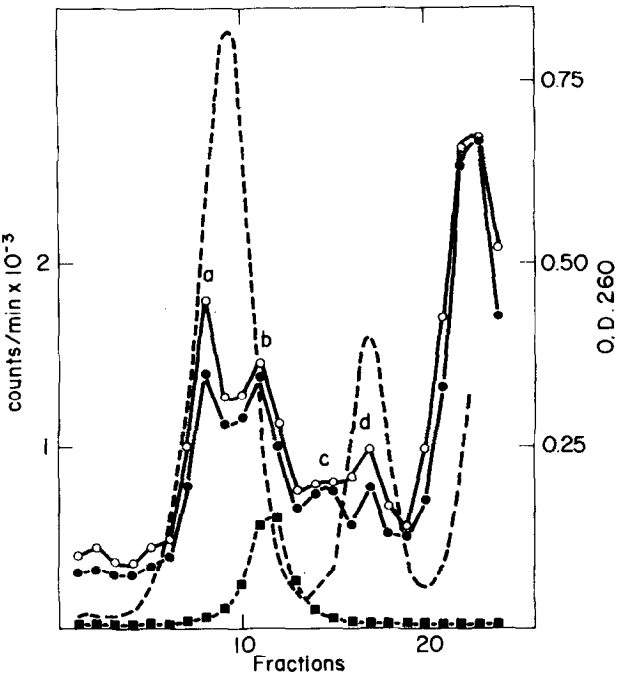

FIGURE 2: Sucrose gradient analysis of ^{3}H-thymidine labeled cytoplasmic DNA. 2 X 10^{7} cells were resuspended in 10 ml of medium. 50 µC of ^{3}H-thymidine were added and the culture was quickly divided into two parts, one of which was made 10^{-3} M hydroxyurea. At the end of the incubation cytoplasms were prepared as described in Figure 1 and made 1% in SDS and 10^{-2} M EDTA. An aliquot from each sample was mixed with T7 phages labeled with ^{14}C-thymidine (a kind gift of Dr. Gail Sonenshein) and lysed in 1% SDS at 60° for 10 minutes. These samples and the remaining portions of the cytoplasms were centrifuged in 15-30% sucrose-SDS gradients at 25,000 rpm, at 25°C for 16 hours in the SW 25.3 Spinco rotor. The fractions collected from the double-labeled samples were precipitated with 5% TCA and assayed for radioactivity. Fractions 1-19 of the samples containing only ^{3}H-labeled cytoplasmic DNA were

pooled and precipitated with alcohol in order to analyze the material in CsCl-Ethidium bromide gradients. The direction of sedimentation in the figure is from right to left. ---- $O.D._{260}$ (profile of ribosomal RNA); O——O, ^{3}H-thymidine incorporated in the control; ●——●, ^{3}H-thymidine incorporated into cytoplasmic DNA in the presence of 10^{-3} M hydroxyurea; ■■■■----■■■■ ^{14}C-thymidine incorporated into T7 phage DNA.

Cytoplasm from cells labeled 3 hours with ^{3}H-thymidine was prepared with the NP40 method and made 0.001 M EDTA and 1% SDS before layering onto sucrose-SDS gradients. Several peaks, not well separated, can be observed: the most prominent (labeled a) has a nominal value of 36S compared to T7 phage DNA (nominal value 32S). Under the ionic conditions used here, the "39S" DNA peak co-sediments with 28S ribosomal RNA. The other peaks (b,c,d) are always present in this type of preparation though the relative amounts vary. Three DNA components, a,c,d, may correspond to the different forms of mitochondrial circular DNA described by others (Borst, et al., 1967): closed circles, open circles and linear molecules, respectively. Component b, approximately co-sedimenting with T7 DNA cannot be identified without further investigations.

Isopycnic centrifugation in CsCl and ethidium bromide can be used to identify circular DNA. The distribution of labeled cytoplasmic DNA in such a gradient is shown in Figure 3. The peak of cytoplasmic DNA has a density of approximately 1.59, which is the value expected for closed circles of mitochondrial DNA in presence of ethidium bromide (Radloff, et al., 1967). ^{14}C-labeled nuclear DNA, added as a reference, is clearly separated under these conditions. The buoyant density of both cytoplasmic and nuclear

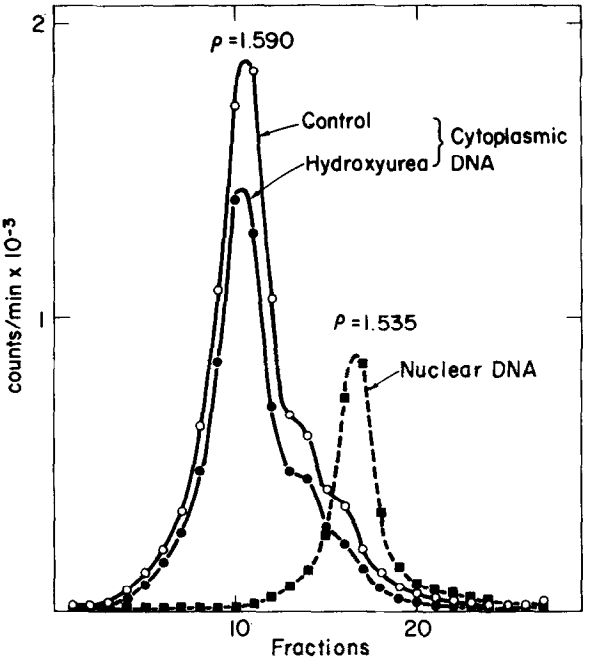

FIGURE 3: CsCl-Ethidium bromide analysis of cytoplasmic DNA labeled in presence and absence of hydroxyurea. Cytoplasmic DNA labeled with ^{3}H-thymidine in presence and absence of 10^{-3} M hydroxyurea was obtained by reprecipitating with alcohol the fractions 1-19 from the sucrose gradients, as described in Figure 2. The DNA of each sample was resuspended in 2 ml of 10^{-2} M TRIS, pH 7.4 and 10^{-3} M EDTA, with 500 µg of ethidium bromide and CsCl to a density of 1.550. A small amount of nuclear DNA from cells labeled for one generation with ^{14}C-thymidine was added as a reference. Centrifugation was for 36 hours at 43,000 rpm 20°C in the SW 65 Spinco rotor. Two drops fractions were collected, precipitated with TCA and assayed for radioactivity.

DNA from HeLa cells is approximately 1.70 in CsCl if ethidium bromide is omitted. Figure 3 also shows that cytoplasmic DNA labeled in the presence of 10^{-3} M hydroxyurea has essentially the same distribution as DNA from control cells.

Most of the cytoplasmic DNA bands at a higher density than the linear DNA marker in ethidium and thus appears to be in the form of closed circles. The NP40 fractionation method apparently minimizes both nuclear leakage and the introduction of nicks into cellular DNA compared to more lengthy procedures. Finally, by comparing the radioactivity distribution obtained from the same cytoplasmic DNA in sucrose gradients and in CsCl-Ethidium bromide gradients, it appears that the main band with a density of 1.59 in Figure 3 cannot be accounted for by only the 39S component (labeled a) in Figure 2. Presumably both species a and b behave in the same manner in CsCl-Ethidium bromide gradients.

ACKNOWLEDGEMENT

This work was supported by awards CA-08416-04 from the National Institutes of Health and GB-8515 of the National Science Foundation. Sheldon Penman is a Career Development Awardee of the U.S. Public Health Service GM-16127-04. It is a pleasure to acknowledge the excellent technical assistance of Maria Penman, Deana Fowler and Elizabeth Loutrel.

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