Isolation and Some Biochemical Characteristics of Nuclei from AH-66 Hepatoma Cells

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SUMMARY

Cetylpyridinium chloride uniquely facilitated the isolation of nuclei from AH-66 hepatoma ascites cells in an isotonic medium without homogenization because of its strong solubilization of their plasma membranes, which were resistant to mechanical shearing with the commonly used non-ionic detergents such as Triton X-100, Nonidet P-40, and Tween 80. Virtually all the nuclei in a population of AH-66 cells (10^6/ml) can be isolated with 0.2% cetylpyridinium chloride. The isolated nuclei were free of adherent cytoplasm, maintained satisfactory morphology, and had high activity of nicotinamide adenine dinucleotide pyrophosphorylase. Two-dimensional polyacrylamide gel electrophoresis of the acid-soluble nuclear proteins of the AH-66 hepatoma nuclei isolated by the cetylpyridinium chloride procedure as well as by the citric acid procedure revealed that Spots Ac and C16-C18 were significantly intense in the gel pattern. Unexpectedly, Spot A10 was absent from the gel pattern of AH-66 hepatoma nuclei.

INTRODUCTION

Isolation of nuclei from transplantable tumor cells has been difficult because of the persistence of cytoplasmic tags in most instances. For removal of this adherent cytoplasm, tumor cells frequently have been homogenized in the presence of non-ionic detergents (2, 7, 8, 12, 13, 22, 23, 27) or citric acid (1, 6, 10, 17). Although these conditions are not mild enough to prevent loss of some nuclear components, enzymatically active nuclei were obtained from RPC 20 mouse plasma tumor cells (13), Krebs-2 tumor cells (8), and HeLa cells (2) with the use of non-ionic detergents. While most of the enzymatic activities of nuclei isolated with citric acid would be lost due to the low pH of the medium, the citric acid procedure has proved to be useful for the analyses of RNA (10) and nuclear proteins (30) of tumor cells.

Difficulties were encountered in the preparation of nuclei from AH-66 hepatoma ascites cells, a rat hepatoma originally induced by dimethylaminoazobenzene. The plasma membranes of AH-66 cells suspended in an isotonic medium resulted in swelling and breakage of nuclei. During the study of effects of cationic detergents containing hydrocarbon side chains longer than 16 or more carbon atoms on water-soluble proteins (19, 20, 29) and membranes, it was found that plasma membranes of AH-66 cells were easily solubilized by CPC but that nuclei of the cells remained intact. With the use of this phenomenon, a method for isolation of nuclei from AH-66 cells was devised. The nuclei isolated by this procedure were free from adherent cytoplasm, maintained satisfactory morphology, and retained high NAD pyrophosphorylase activity. The acid-soluble nuclear proteins of the AH-66 hepatoma nuclei isolated by this method as well as by the citric acid method were subjected to 2-dimensional polyacrylamide gel electrophoresis, and the gel pattern of the AH-66 hepatoma nuclei was compared with the gel pattern of other tumor nuclei reported thus far.

MATERIALS AND METHODS

Isolation of Nuclei from AH-66 Cells. AH-66 hepatoma ascites cells were grown in the cavity of Donryu rats and obtained by drainage of the ascites fluid 7 days after the implantation of the tumors. The cells were suspended in 0.25 M sucrose containing 10 mM Tris (pH 7.4) and 1 mM MgCl₂ and washed several times by centrifugation at 100 × g for 5 min until the cells were largely free of RBC. The washed cells (10^9/ml) were suspended in 0.25 M sucrose containing 10 mM MgCl₂ and centrifuged at 600 × g for 5 min. The crude nuclear sediment was resuspended in 0.25 M sucrose containing 1 mM MgCl₂ and centrifuged at 600 × g for 5 min at 4°C. The pellets were resuspended in 0.33 M sucrose and further purified by layering over 0.88 M sucrose and centrifugation at 1100 × g for 15 min. When nuclear proteins of AH-66 cells were examined, the nuclei were also prepared in 0.5% citric acid according to the procedure of Higashi et al. (10).

Isolation of Nuclei from Livers. Nuclei of rat liver were isolated by (a) a modification of 2.2 M sucrose method of Chauveau et al. (5) and by (b) the CPC procedure as described above. (a) The perfused rat liver tissue was chopped and homogenized in 9 volumes of 0.25 M sucrose containing 1 mM MgCl₂ in a loosely fitting Teflon:glass homogenizer. The suspension was centrifuged at 900 × g for 15 min. The pellets were resuspended in 2.2 M sucrose containing 1 mM MgCl₂ and centrifuged at 40,000 × g for 1 hr.

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The medium contained, in 1.0 ml: 1 @mole of Tmis-HCl, 4 @moles each of ATP, GTP, and UTP; 0.04 @mole of MgCl2; 20 @moles of KCl; 250 @moles of ammonium sulfate; 12 @moles of MgCl2; 40 @moles of glycine buffer, pH 7.4; and nuclear suspension. After incubation for 2 min at 38°, the reaction was stopped by addition of 0.2 ml of 5 N perchloric acid at 0°. After centrifugation, the precipitate was washed successively with 5% perchloric acid, absolute ethanol, ethanol:ether (3:1), and ether. The precipitate was hydrolyzed with 0.3 N KOH at 37° for 18 hr. The hydrolysate was cooled and acidified by adding an equal volume of cold 10% perchloric acid to precipitate DNA and protein. The supernatant was analyzed for RNA by the orcinol method (18). The precipitate was heated with 5% perchloric acid at 70° for 15 min, cooled, and centrifuged. The supernatant was used for the determination of DNA by the diphenylamine method (4).

**Enzyme Assays.** NAD pyrophosphorylase activity was measured according to Hogeboom and Schneider (11). The medium contained, in 0.8 ml, 5 @moles of ATP; 3 @moles of nicotinamide mononucleotide; 300 @moles of nicotinamide; 12 @moles of MgCl2; 40 @moles of glycine buffer, pH 7.4; and nuclear suspension. After incubation for 2 min at 38°, the reaction was stopped by addition of 0.2 ml of 5 N perchloric acid at 0°. After centrifugation, the precipitate was diluted to 20 ml and adjusted to pH 8 with NaOH. The concentration of NAD was calculated from the increase in absorbance at 340 nm on addition of 0.1 ml of ethanol, with the use of the value of 6.22 x 10³ M⁻¹ cm⁻¹ as the molecular extinction coefficient of the reduced form of NAD.

RNA polymerase activity was measured by counting the radioactivity of [³H]CTP incorporated into RNA fraction (14). The medium contained, in 1.0 ml: 1 @mole of Tris-HCl buffer, pH 8.0; 8 @moles of β-mercaptoethanol; 12 @moles of MgCl2; 20 @moles of KCl; 250 @moles of ammonium sulfate; 4 @moles each of ATP, GTP, and UTP; 0.04 @mole of [³H]CTP (23 @Ci/µmole); and nuclear suspension. After incubation for 15 min at 37°, the reaction was stopped by addition of 2.5 ml of chilled 10% trichloroacetic acid.

**Electron Microscopy.** Nuclei isolated from the AH-66 cells in 0.2% CPC were fixed at 0° for 4 hr with 4.2% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, and postfixed for 1 hr in 2% osmium-tetroxide. Dehydration of the pellets was performed in increasing concentrations of ethanol. The specimens were embedded in Epon 812 according to the method of Luft (16). Ultrathin sections were cut with a Porter Blum II ultramicrotome and stained with uranyl acetate followed by lead citrate. The specimens were observed with a Hitachi HU-12 electron microscope.

**Two-Dimensional Gel Electrophoresis.** Acid-soluble proteins in nuclei were extracted with sulfuric acid according to the method of Yeoman et al. (30). The nuclei were suspended in 10 volumes of 0.4 N sulfuric acid containing 2 mM phenylmethylsulfonyl fluoride and homogenized with a Teflon:glass homogenizer. The supernatant, after centrifugation at 5000 x g for 20 min, was dialyzed against 0.01 N HCl for 12 hr, dialyzed against deionized water for 16 hr, and lyophilized. Extracted proteins were analyzed by 2-dimensional gel electrophoresis. Samples were run in the 1st dimension on a slab gel of 10% acrylamide, 6 M urea, and 0.9 N acetic acid at 80 V for 8 hr. After electrophoresis, the slab gel was extruded and bisected longitudinally. The gel slice was adapted to 0.1% sodium dodecyl sulfate, 6 M urea, 5 mM dithiothreitol, and 0.1 M phosphate buffer (pH 7.1) and placed on a slab gel of 12% acrylamide, 0.1% sodium dodecyl sulfate, 6 M urea, and 0.1 M phosphate buffer. The gel was run for 14 hr at 50 ma/slab. Gels were stained with Coomassie brilliant blue.

**RESULTS**

**Isolation of Nuclei from AH-66 Cells.** When AH-66 hepatoma ascites cells were suspended in a CPC solution containing 0.25 m sucrose and 1 mM MgCl2, plasma membranes were solubilized and nuclei were released as judged by phase microscopy. Homogenization was unnecessary for disruption of the plasma membranes and removal of cytoplasm from the nuclei. The amount of nuclei released depends on the relative concentration of CPC to the number of the cells. Chart 1 shows the effect of various concentrations of CPC on 10⁶ cells/ml suspended in the isotonic solution containing 0.25 m sucrose and 1 mM MgCl2 (pH 7.4). The addition of CPC below 0.01% brought about a shrinkage of cells, but no cells were disrupted as judged by phase microscopy. With increase of the CPC concentration above 0.01%, plasma membrane forms disappeared, and the nuclei with no adherent cytoplasm increased markedly. In 0.2% CPC, 98% of the nuclei were free of cytoplasm as counted with the aid of phase microscopy. By contrast, the commonly used non-ionic detergents for isolation of nuclei such as Triton X-100, Nonidet P-40, and Tween 80 were not effective in solubilizing the plasma membranes of AH-66 cells under the same conditions. The AH-66 cells suspended in the isotonic solution were not disrupted by homogenization even in the presence of 1% of the non-ionic detergents described above. Among the cationic detergents, only those with hydrocarbon side chains longer than tetradecyl group were effective in the solubilization. In further experi-
ments, therefore, nuclei were prepared from 10⁶ AH-66 cells/ml in 0.2% CPC solution containing 0.25 M sucrose and 1 mM MgCl₂, as described in “Materials and Methods.” The critical micelle concentration of CPC in 0.25 M sucrose solution was 0.01% as measured by spectral change of methyl orange.

The nuclei prepared from AH-66 cells by the CPC procedure were further studied by electron microscopy. It can be seen from Fig. 1 that the nuclei are virtually free from any remnants of cytoplasm. While the outer layer of the nuclear membrane together with attached ribosomes are completely removed, the structure of chromatin and nucleoli appears to be reasonably stable to the treatment with CPC. The chromatin network is well preserved, although the condensed chromatin appears to have a slightly reduced electron opacity.

Nucleic Acid and Protein Content of Nuclei Isolated with CPC. Table 1 shows the chemical compositions of nuclei isolated from AH-66 cells with CPC as compared to those of nuclei of rat liver isolated both by the 2.2 M sucrose procedure and by the CPC procedure described in “Materials and Methods.” The recovery of nuclei isolated from AH-66 cells by the CPC procedure was as much as 76% on the basis of DNA content. The RNA:DNA ratio of the AH-66 cells was 0.44, which is similar to the values reported for tumor nuclei, i.e., 0.48 for Walker 256 carcinosarcoma (10), 0.24 for HeLa cell (2), 0.32 for Morris hepatoma 78000 (27), and 0.64 for AH-130 hepatoma (21). The RNA:DNA ratio of rat liver nuclei isolated by the CPC procedure, 0.17, is in good agreement with the ratios of 0.11 to 0.22 reported for those isolated by different methods (3, 13). It is generally believed that the nuclei of various cancer cells contain more RNA than do the nuclei of normal cells. The protein:DNA ratio of 3.40 obtained for the nuclei from AH-66 cells is also in the range reported previously (13, 22, 27).

Effect of CPC on NAD Pyrophosphorylase. The effect of CPC treatment on enzymatic activities of nuclei was examined for NAD pyrophosphorylase, which is known to be localized within the nuclei (11). The specific activity of NAD pyrophosphorylase of nuclei isolated from AH-66 cells by the CPC procedure was 0.111 μmole NAD per hr per mg, which is almost equal to 0.104 for that of the rat liver nuclei isolated by the sucrose procedure. The result indicates that CPC had no deleterious effect on NAD pyrophosphorylase.

Table 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>DNA (mg)</th>
<th>RNA (mg)</th>
<th>RNA:DNA</th>
<th>Protein:DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH-66 hepatoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact cells</td>
<td>1.71</td>
<td>3.13</td>
<td>1.82</td>
<td>24.34</td>
</tr>
<tr>
<td>Nuclei isolated with CPC</td>
<td>1.30</td>
<td>0.57</td>
<td>0.44</td>
<td>3.40</td>
</tr>
<tr>
<td>Rat liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude nuclear fraction</td>
<td>7.14</td>
<td>7.14</td>
<td>1.00</td>
<td>18.18</td>
</tr>
<tr>
<td>Nuclei isolated with sucrose</td>
<td>2.99</td>
<td>0.34</td>
<td>0.12</td>
<td>3.88</td>
</tr>
<tr>
<td>Nuclei isolated with CPC</td>
<td>7.78</td>
<td>0.80</td>
<td>0.17</td>
<td>2.99</td>
</tr>
</tbody>
</table>

- Averages of 3 experiments.
- Averages of 2 experiments.
- Crude nuclear fraction represents the sediment obtained after centrifugation of homogenate at 900 × g for 15 min.

DISCUSSION

While AH-66 hepatoma ascites cells suspended in an isotonic medium were resistant to mechanical shearing in the presence of the non-ionic detergents commonly used for isolation of nuclei, the plasma membranes of the cells were easily solubilized by a low concentration of CPC. Although the molecular mechanism of the lysis of the AH-66 cells is not yet clear, the solubilization seems to proceed in several stages. Stage 1 is binding of CPC to the membranes. Stage 2 is penetration of CPC into the membrane, where the...
detergent induces a change in molecular organization, resulting in the shrinkage of the cells. The final stage is removal of membrane proteins, lipids, and presumably also detergent as lipoprotein-detergent complexes (9, 25). Since the shrinkage occurs at concentrations well below the critical micelle concentration of CPC, it seems quite clear that it is primarily the monomer form of the detergent that is bound to the membrane. The fact that only cationic detergents with hydrocarbon side chains longer than 14 carbon atoms are effective for the solubilization of the plasma membranes suggests the importance of hydrophobic interactions between the side chains of the detergents and the lipid bilayer.

The nuclei isolated from AH-66 cells by CPC were essentially free of adherent cytoplasm. Moreover, the RNA:DNA ratios of the nuclei were in good agreement with the values reported for other tumor nuclei. The removal of the outer nuclear membranes from nuclei isolated by the CPC procedure may be inevitable when detergents are used for the isolation of nuclei. The use of nonionic detergents or citric acid usually provides nuclei devoid of the outer nuclear membranes (2, 12, 28). Another advantage of the CPC procedure is that the structures of chromatin and nucleoli are well preserved. In nuclei isolated with citric acid, the nucleoli are enlarged and less well organized and the chromatin is condensed into clumps (28).

Since the preservation of nuclear structure seems to be better with the CPC procedure, we examined the possibility that the CPC procedure might also preserve the integrity of nuclear RNA. Total nuclear RNA's were extracted with phenol-sodium dodecyl sulfate and examined on 5 to 47% sucrose gradients according to the method of Higashi et al. (10). The sucrose gradient profile of the RNA extracted from the nuclei prepared from AH-66 cells by the CPC procedure showed the presence of peaks of 18, 28, and 45 S RNA and a shoulder at 35 S RNA (result not shown). However, by comparison with the result obtained with the citric acid procedure (10, 28), the amounts of 35 and 45 S RNA were appreciably reduced and the amounts of 18 and 28 S RNA were proportionally increased. Since CPC has no inhibitory effect on RNase, it would appear that some degradation of nuclear RNA occurred in the course of the extraction of RNA. Accordingly, the citric acid procedure would be better than the CPC procedure for the isolation of high-molecular nuclear RNA.

The other advantage of the CPC procedure is that the nuclei isolated by this procedure retained high activity of NAD pyrophosphorylase. In addition, we examined the effect of RNA polymerase of the nuclei isolated from AH-66 cells by the CPC procedure and found that the level of RNA polymerase activity, 2.9 μmoles [3H]CTP incorporated per mg nuclear protein, was the same order of magnitude as that of liver nuclei prepared by the sucrose procedure. These mild effects of CPC on the nuclear enzymes were expected from our previous studies (19, 20, 29) between water-soluble proteins and cationic detergents with the cetyl group. We have previously found that the conformational change of myoglobin caused by CPC and inhibition of tryptophan activity by CPC are completely reversed by removing the detergents (19, 20). On the other hand, it has been reported that membrane proteins such as glucose-6-phosphatase (26), NADH oxidase (21), and p-nitrophenylphosphatase (21) were denatured by cetyltrimethylammonium chloride. Therefore, we could not still exclude the possibility that some nuclear enzymes other than NAD pyrophosphorylase and RNA polymerase may be denatured by treatment with CPC.

A disadvantage of the CPC procedure is that part of particular nuclear proteins such as Spots A17, A18, A25, Ar, As, Cc, and C16-C18 are selectively extracted by the detergent. A similar selective extraction was also reported for the preparation of normal liver nuclei isolated with citric acid. Taylor et al. (28) have demonstrated that Spots A1, A2, A17, A18, B7, B23-B25, and C23-C25 are extracted from normal liver nuclei with 5% citric acid. We observed that Spots A17, A18, and A19 were extracted selectively when nuclei were prepared from normal liver with 0.5% citric acid (result not shown). In addition, comparison of the gel patterns of AH-66 hepatoma nuclei isolated by 2 different procedures demonstrated that Spot Ac is partially extracted by 0.5% citric acid. Therefore, we think that it is necessary to isolate nuclei by at least 2 different methods for the investigation of protein components of tumor cell nuclei. In this study, the AH-66 hepatoma nuclei were prepared both by the CPC procedure and by the citric acid procedure and subjected to the 2-dimensional gel electrophoresis analysis.

It is interesting to compare the 2-dimensional gel patterns of acid-extractable nuclear proteins of AH-66 hepatoma nuclei with those reported for other tumor nuclei. Spot A10 was reported to be uniquely present in the patterns of Novikoff hepatoma nuclei (30), Morris 9618A hepatoma nuclei (31), and Walker 256 carcinosarcoma nuclei (31). In the AH-66 hepatoma pattern, however, Spot A10 was absent. Therefore, we cannot consider that this spot is characteristic of tumor nuclei. Yeoman et al. (31) have especially stressed that Spots C16-C18 are much more dense in hepatoma nuclei than in normal liver nuclei. We can observe this trend of high density of Spots C16-C18 only in the gel pattern of AH-66 hepatoma nuclei isolated with the use of citric acid. It appears that the citric acid procedure is suitable for the analysis of nuclear proteins in the vicinity of Spots C16-C18. Another quantitative difference between tumor cell and normal cell nuclei observed by Yeoman et al. (30, 31) is the greater intensity of Spot Ac in the hepatoma pattern as compared to that in the normal liver pattern. In agreement with them, Spot Ac was present in the AH-66 hepatoma pattern but was not found in normal liver patterns (result not shown). We think that accumulation of data with regard to Spots A10, Ac, and C16-C18 in various tumor nuclei other than examined thus far is necessary to determine whether these proteins are characteristic of tumor nuclei.

REFERENCES

Fig. 1. Electron micrographs of nuclei isolated from AH-66 hepatoma cells by CPC procedure. A. x 3600; B. x 9000.

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis pattern of sulfuric acid-extracted proteins from AH-66 hepatoma nuclei isolated by CPC procedure. Samples (250 µg) were run in the 1st dimension on gels of 10% acrylamide, 6 M urea, and 0.9 M acetic acid at 80 V for 6 hr. For the 2nd dimension, a 15% acrylamide slab gel gel was run for 14 hr at 50 mA/slab. Horizontal arrow, direction of electrophoresis of the 1st dimension; vertical arrow, direction of electrophoresis of the 2nd dimension. Gels were stained with Coomassie brilliant blue. The numbering of proteins is adopted from the system of Yeoman et al. (30). The position of Spot A10, which is absent from the gel, is indicated by the dotted circle. x 1.5.

Fig. 3. Two-dimensional gel electrophoresis pattern of sulfuric acid-extracted proteins from AH-66 hepatoma nuclei isolated with use of 0.5% citric acid according to the method of Taylor et al. (28). See Fig. 3 for conditions of electrophoresis. x 1.5.

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