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Non-Histone Chromosomal Proteins of Chemically Transformed Neoplastic Cells in Tissue Culture[1]

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SUMMARY

Chromatin proteins from control and dimethylnitrosamine-transformed baby hamster kidney cells were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Our results indicate that non-histone chromosomal proteins from transformed cells contained protein components of low and intermediate electrophoretic mobility, which were deficient in normal cells. Comparison of the relative amount of incorporation of labeled amino acids into non-histone chromosomal proteins showed that protein components with a molecular weight of about 60,000 M.W. had a markedly increased labeling activity in the chemically transformed cells. These results suggest that changes in non-histone chromosomal proteins are associated with neoplastic transformation by chemical carcinogens.

INTRODUCTION

Chromosomal proteins in eukaryotic cells are classified into histones and non-histone proteins. The histones are made up of 5 major classes of proteins, some of which are believed to occur in repeating units in chromatin (13, 19). On the other hand, NHCP[5] are comprised of a large number of heterogeneous proteins (3, 19, 24), which are metabolically unstable (24), are synthesized differentially throughout the cell cycle (3, 24), and possess both species and tissue specificity (9, 26). These properties have led to the speculation that NHCP may play a role in the regulation of gene expression in higher organisms. Indeed, direct experimental evidence demonstrating alterations in the composition of NHCP during differentiation (16, 21) and cell proliferation (2, 19, 22, 23, 27) has been provided.

A number of studies have compared the NHCP of "normal" and malignant cell populations and in many instances interesting differences have been reported (1, 5–7, 10, 15, 17, 20, 25). Most studies with cells in tissue culture have utilized tumor cell lines transformed by oncogenic viruses, and hence, in our view, carry the attendant uncertainty that the changes observed may reflect activity of the viral genome. We have investigated NHCP in BHK cells ($BHK_{21}Cl_{13}$) and BHK cells transformed by the chemical carcinogen DMN. Our results indicate significant and reporducible differences in the composition of NHCP in these 2 cell lines based on electrophoretic analyses.

MATERIALS AND METHODS

Cell Culture

BHK cells ($BHK_{21}Cl_{13}$) and DMN-transformed BHK cells [DMN-BHK (8)] were generously donated by Dr. G. Di Mayorca of the University of Illinois, Medical Center, Chicago, Ill. As indicated by Di Mayorca *et al.* (8), the growth rate of clones of the chemically transformed cells is not significantly different from that of the BHK control. However, whereas at 32° the former cells have a normal clonal morphology and do not clone in soft agar, at 38.5° their clonal morphology and capacity to clone in soft agar are indicative of neoplastic transformation. All clones tested by Di Mayorca *et al.* (8) that plated in soft agar produced tumors in hamsters within 30 days after inoculation of 10^3 cells/animal. At this cell density BHK cells did not provoke tumor formation. The temperature-dependent capacity to clone in agar was confirmed with samples of cells received, and suitable quantities of both the control and chemically transformed lines were stored under liquid nitrogen. The basic culture medium used was Eagle's basal medium buffered to pH 7.4 with *N*-2-hydroxyethylpyperazine-*N'*-2 ethane-sulfonic acid/HCO_3 (29 mM) to which 2 mM glutamine had been added. This was supplemented with 10% tryptose phosphate broth and with either 10% heat-inactivated calf serum or 10% donor calf serum (Flow Laboratories, Inc., Rockville, Md.). Culture media contained penicillin (100 units/ml) and streptomycin (100 μg/ml), and in Method B (below) fungizone (0.25 μg/ml) was also added. Cells were grown in either Falcon or Lux T-75 flasks at 38 or 32°.

Isotopic Labeling of Cells

Cells were labeled by the addition to culture medium of [^{3}H]leucine (1 μCi/ml; specific activity, 46 Ci/mmole; Schwarz/Mann, Orangeburg, N. Y.) or [^{14}C]leucine (0.15 μCi/ml; specific activity, 300 mCi/mmole; New England Nuclear, Boston, Mass.) for 48 to 72 hr. Cells were harvested at confluency by trypsinization, washed with 0.25 M sucrose-

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[5] The abbreviations used are: NHCP, non-histone chromosomal proteins; BHK, baby hamster kidney; DMN, dimethylnitrosamine; SDS, sodium dodecyl sulfate.

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2 mM $MgCl_2$ (pH 8), counted in a hemocytometer, and stored at −20° until use. In double-label experiments, 2 sets of cells were labeled separately with [^{3}H]leucine or [^{14}C]leucine, and NHCP was extracted from the mixture of the 2 populations.

Isolation of Chromosomal Proteins

Chromosomal proteins were isolated from BHK and DMN-BHK cells at 0–4° using 1 of 2 different procedures.

Method A. Frozen cells were thawed in 0.25 M sucrose-2.0 mM $MgCl_2$-50 mM Tris-HCl buffer (pH 8.0) containing 5 mM $NaHSO_3$. The latter chemical was included in all phases of the isolation procedure in order to inhibit proteolysis. Thawed cells were disrupted in a Dounce homogenizer with a tight pestle (100 to 200 strokes), and nuclei were precipitated by centrifugation at 700 × *g* for 10 min. Isolated nuclei were washed once in 0.5% Triton X-100-0.25 M sucrose-2 mM $MgCl_2$ (pH 8.0), and then in 0.25 M sucrose-2 mM $MgCl_2$ (pH 8.0). The nuclei were lysed in 50 mM Tris-HCl (pH 8.0) and 20 mM EDTA and centrifuged at 2,500 × *g* for 15 min. The chromatin pellet was washed 2 times in 50 mM Tris-HCl (pH 8.0). The gelatinous chromatin was dissolved in 3 N NaCl-7 M urea-50 mM Tris-HCl (pH 8.0) and stirred overnight with a magnetic stirrer. The solution was centrifuged in a type 60 Ti fixed-angle rotor at 100,000 × g_{max} for 44 hr in a Beckman ultracentrifuge. The supernatant, containing total chromosomal proteins, was dialyzed extensively against 2 mM Tris-HCl (pH 8.0)-0.1% SDS-0.1% 2-mercaptoethanol and then lyophilized.

Method B. Cell pellets were homogenized by shearing 10 to 15 times with a TenBroek hand homogenizer in 0.25 M sucrose-1 mM $MgCl_2$. The sheared pellet was resuspended in 10 ml of 0.5% Triton X-100-0.25 M sucrose-1 mM $MgCl_2$ (pH 7.9) and centrifuged for 15 min at 900 × *g* in a Sorvall SS-34 rotor. The resulting pellet was washed 2 times in the above solution and resuspended in 5 ml of 75 mM NaCl-24 mM EDTA (pH 7.9). Following centrifugation at 3,500 × *g* for 10 min, the precipitate was repeatedly washed with decreasing molarities of Tris-HCl buffer (50 to 5 mM at pH 7.9). The clear gelatinous mass was then dispersed in 3 ml of 6 M urea-0.4 M guanidine-HCl-5 mM phosphate buffer (pH 7.0)-5 mM $NaHSO_3$-0.83% 2-mercaptoethanol. The chromatin solution was layered onto a 4 M CsCl shelf in polyallomer tubes and was centrifuged for at least 19 hr in the Spinco SW 56 rotor at 35,000 rpm (*g*, 120,000). The supernatant, containing most of the chromosomal proteins, was dialyzed exhaustively against 0.5 mM phosphate buffer and lyophilized.

Electrophoresis of Chromosomal Proteins

Chromosomal proteins were analyzed by SDS-polyacrylamide gel electrophoresis following the method of King and Laemmli (11) in a slab of gel (14 cm × 12 cm × 1.5 mm thick). The separating gel contained 10% acrylamide, 0.27% *N,N'*-methylene bisacrylamide, 0.1% SDS, and 375 mM Tris-HCl buffer (pH 8.8). The upper stacking gel was made of 3% acrylamide-0.08% *N,N'*-methylene bisacrylamide-0.1% SDS-125 mM Tris-HCl buffer (pH 6.8). Both gels were polymerized by the addition of *N,N,N',N'*-tetramethylethylenediamine (25 μl/100 ml of gel) and of 10% ammonium persulfate (0.4 ml/100 ml of gel). The electrode buffer contained 192 mM glycine-0.1% SDS-25 mM Tris-HCl buffer (pH 8.3).

The lyophilized chromosomal proteins were dissolved in 0.5 ml of 2% SDS-5% ME-60 mM Tris-HCl (pH 6.8) and heated in a water bath at 90° for 10 min. Aliquots (10 to 20 μl) containing 10% glycerol and tracking dye (0.001% bromophenol blue) were layered in wells of the stacking gel. Electrophoresis was carried out at a constant current of 35 ma for 5 hr at room temperature until the tracking dye reached the bottom of the gel. Gels were stained overnight in 0.1% Coomassie blue, 7% acetic acid, and 50% methanol and destained in 7% acetic acid with 10% methanol. In experiments using radioactively labeled NHCP, duplicate samples were subjected to electrophoresis. One of the samples was stained as described above. The other was cut into a 1.0-cm-wide strip and sliced into the 1.0 mm sections using a gel slicer. These were incubated overnight with 0.5 ml of 30% H_2O_2 at 37°. To each fraction of gel, 10.0 ml of a scintillation mixture consisting of 2 parts of toluene with Omnifluor (4.0 g/liter of toluene) and 1 part Triton X-100 were added. Radioactivity was measured in a Beckman Model LS-250 liquid scintillation spectrometer. Automatic quench correction was applied with settings determined by reference to appropriate standards.

Analytical Procedures

Protein was assayed by the method of Lowry *et al.* (18), and DNA was determined by the diphenylamine procedure of Burton (4). Electrophoretically separated and stained bands on the gels were traced on a Transidyne general RFT scanning densitometer at 550 nm.

RESULTS

Isolation of Nuclei and Chromosomal Proteins. Neoplastically transformed BHK cells were significantly more resistant to disruption by homogenization than were control cells. With the latter, about 80 strokes with the Dounce homogenizer yielded over 95% of the nuclei as monitored by phase microscopy. DMN-BHK cells required up to 200 strokes to obtain similar results. The separation of protein from DNA by Method A is indicated in Table 1. This procedure yields at least 95% of the total chromatin protein in the supernatant fraction and virtually all of the DNA in the pellet. With Method B a similar yield of protein was

Table 1

Dissociation of chromosomal protein from DNA

Chromatin prepared from isolated nuclei of BHK and DMN-BHK cells grown at 38° was dissolved in 3 N NaCl-7 M urea-50 mM Tris-HCl-5 mM $NaHSO_3$ (pH 8) and centrifuged at 100,000 × *g* for 44 hr as detailed in "Materials and Methods" (Method A).

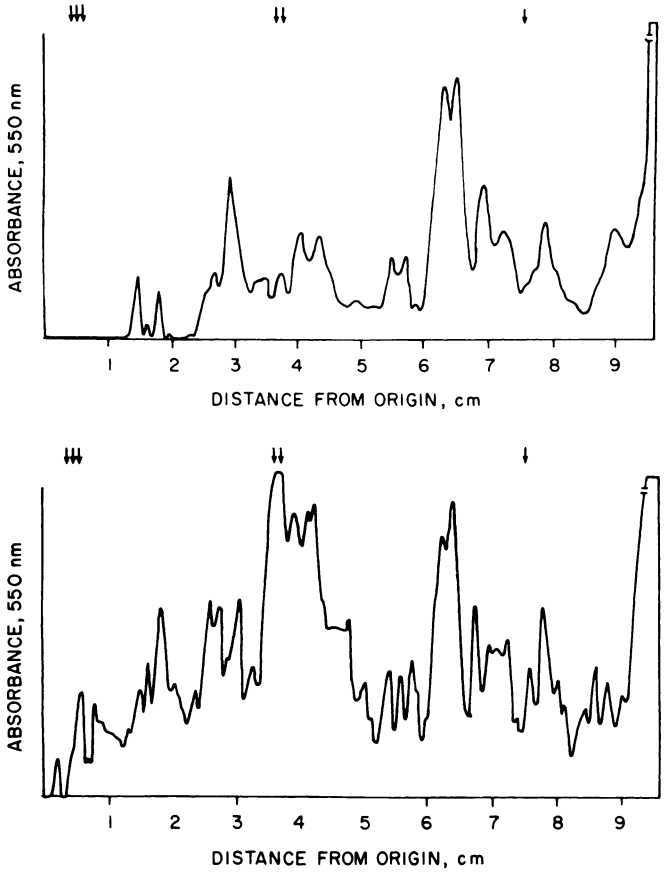
Cell lines	Fractions	Protein (mg)	DNA (mg)
BHK	Supernatant	6.0	0.07
	Pellet	0.4	2.8
DMN-BHK	Supernatant	5.3	0.05
	Pellet	0.2	2.4

achieved; however, contamination of the protein fraction with DNA was significantly greater since retrieval of the entire aqueous phase following centrifugation was invariably accompanied by inclusion of some of the CsCl shelf. The results obtained by electrophoresis of chromatin proteins (see below) were comparable with proteins prepared by either method of protein extraction. In our experiments, histones were not separated from NHCP in order to avoid any possible losses of the latter during histone extraction. The presence of histones did not seriously interfere with our analyses of NHCP, since the major histone fractions are of low molecular weight and migrate near to the bottom of the gel during electrophoresis.

Electrophoretic Characterization of NHCP in BHK and DMN-BHK Cells. Fig. 1 shows the results of a typical electrophoretic separation of chromosomal proteins. A complex array of stained bands numbering between 50 and 80 was discernible with preparations from both BHK and DMN-BHK cells grown at 38°. Representative scanning profiles at 550 nm are shown in Charts 1 and 2, which demonstrate several striking differences in the composition of NHCP from the 2 cell populations examined. The single most conspicuous difference is the presence in the chromatin from DMN-BHK cells of a cluster of NHCP components in the molecular weight range between 60,000 and 70,000 daltons, which are significantly reduced in the profiles obtained from BHK cells. In addition, in the region of low electrophoretic mobility (high molecular weight), several components of NHCP from DMN-BHK cells are clearly visible but noticeably reduced in the profiles of BHK proteins. The coelectrophoresing bands of molecular weight, about 20,000 daltons in both types of cells, are presumed to be Histone I (f1) (19). The rest of the major histone fractions were subjected to electrophoresis to the bottom quarter of the gel. No significant differences in the profiles of NHCP of DMN-BHK cells were observed when the cells were grown at 32 or 38° (not shown).

Radioactively Labeled NHCP. The difference in absorbance profiles of NHCP between BHK and DMN-BHK cells could have resulted from unknown artifacts such as differential proteolysis during chromatin preparation. To evaluate this possibility, BHK cells were labeled with [^{3}H]leucine, DMN-BHK cells were labeled with [^{14}C]leucine, and NHCP was prepared from the mixture of the 2 types of cells. Chart 3*A* shows the normalized electrophoretic radioactivity profiles. It is clear that slicing the gel and determining the radioactivity in each slice results in a loss of the resolution of many of the individual bands that can be observed in stained gels. Nonetheless, the major peaks observed by optical scanning of stained gels coincide closely with those observed by measuring radioactivity, indicating no significant differential proteolysis in the 2 cell populations. Furthermore, as shown in Chart 3*B*, a comparison of the ratio of radioactivity (^{14}C/^{3}H) in individual gel slices indicates significantly increased incorporation of label in NHCP of DMN-BHK cells in Slices 31 to 34, a region in the 60,000 molecular weight range, as determined by standard molecular-weight markers.

In order to be certain that the species in the molecular weight region of 60,000 daltons are indeed NHCP, DMN-



Charts 1 and 2. Absorbance profiles of total chromosomal proteins from BHK cells (Chart 1) and DMN-BHK cells (Chart 2). Total chromosomal proteins were extracted from 2 cell lines at confluency after growth at 38° by Method A and were subjected to electrophoresis on 10% SDS-polyacrylamide gel and stained with Coomassie blue. Direction of migration is from *left* to *right*. The gel was traced on a scanning densitometer at 550 nm. *One arrow*, M.W. 23,000; *2 arrows*, M.W. 69,000; *3 arrows*, M.W. 175,000; as determined by the use of molecular weight standards.

BHK cells were labeled with [^{3}H]tryptophan, which essentially excludes labeling of histones (15). The prominent peak of interest was observed once again following electrophoresis of extracted chromosomal proteins (not shown). The results of comparative studies of NHCP from DMN-BHK cells grown at 32 and 38° did not argue for any consistent differences in the relative incorporation of labeled amino acid into chromosomal proteins (not shown).

DISCUSSION

The results of our studies demonstrate that the electrophoretic profiles of NHCP of BHK cells are distinguishable from those of DMN-transformed cells. There have been several recent studies that compare the NHCP of normal and closely related malignant tumors. Arnold *et al.* (1) showed that the NHCP from hepatoma 5123C cells differ in electrophoretic pattern from those of normal rat liver cells. Weisenthal and Ruddon (25) have reported that human leukemia cells of various types could be distinguished from one another by the banding pattern of non-histone nuclear

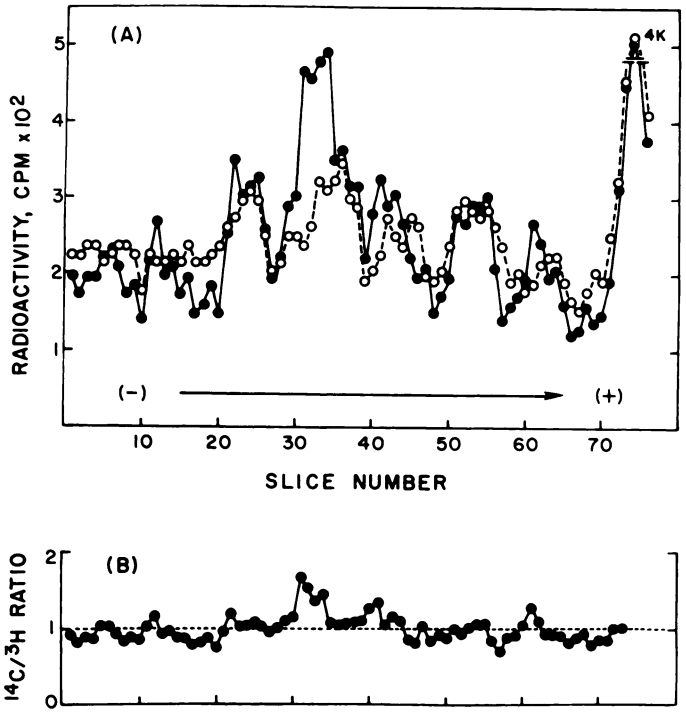


Chart 3. Electrophoretic radioactivity profiles of total chromosomal proteins prepared by Method A from BHK cells labeled with [³H]leucine (○) and from DMN-BHK cells labeled with [¹⁴C]leucine (●) for 48 to 72 hr (*A*). Both cell lines were grown at 38°. Chromosomal proteins were analyzed on SDS-polyacrylamide gel, and the gel fractions were counted in a scintillation counter, as described in "Materials and Methods." The relative ratios of $^{14}C/^{3}H$ for each fraction are shown in *B*.

proteins in polyacrylamide gels. Similarly, differences were reported in electrophoretic banding patterns of NHCP from normal and neoplastic mammary (10) and liver (5, 6, 20) cells of mice and rats. Other investigators have reported qualitative or quantitative differences in NHCP between normal and SV40-transformed human fibroblasts in culture (7, 15, 17). NHCP of normal and transformed cells have also been distinguished on the basis of immunospecificity (28). The significance of the present studies is that the DMN-BHK cells were neoplastically transformed *in vitro* by a chemical carcinogen and not by an oncogenic virus. This makes it unlikely that the differences in NHCP components observed are a function of viral activities.

In so far as was possible, we attempted to eliminate trivial explanations for these differences. Cell cultures were found to be free of bacterial and *Mycoplasma* contamination. Both cell populations were harvested at confluency, nuclei were washed with 0.5% Triton to eliminate cytoplasmic contamination, and inhibitors of proteolysis were used in the chromatin extracts. In addition, we have utilized 2 different procedures for the extraction of NHCP that specifically avoid harsh treatments, such as extremes of pH. We therefore suggest that our results reflect real alterations in the synthesis of various NHCP in BHK cells that have undergone neoplastic transformation by a chemical carcinogen. In line with recent evidence implicating the role of NHCP in gene regulation (12, 14, 21–24), it is possible that the differences in the electrophoretic profiles observed in our studies reflect altered states of gene expression in neoplasia. More detailed biochemical studies are required in order to establish whether the NHCP components characteristic for DMN-BHK cells are specific and whether the alterations observed precede the onset of neoplastic phenotype following treatment of cells with carcinogens.

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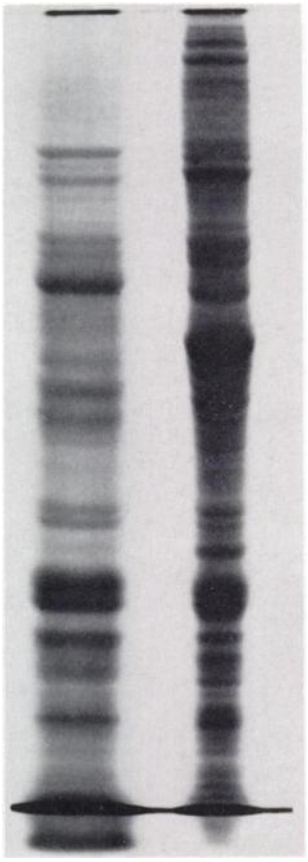

Fig. 1. Electrophoretic patterns of total chromosomal proteins from BHK cells (*left*) and DMN-BHK cells (*right*) in 10% SDS-polyacrylamide gel prepared by Method A. Both cell populations were grown at 38°. About 100 μg of protein were applied for each load, and the gel was stained with 0.1% Coomassie blue. Direction of migration is from *top* to *bottom*. Major histone fractions migrated to the bottom quarter of the gel.