Antigenic Similarity of Nonhistone Chromosomal Proteins in Cultured Rat Liver Epithelial Cells, Fetal Liver, and Transplantable Tumors

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ABSTRACT

The cell and tissue specificity of antisera prepared to chromatin fractions from a nontumorigenic adult rat liver-derived clonal epithelial cell line (ARL-15Ci) was characterized with immunotransfer analysis and immunoblotting experiments. These antisera reacted with a range of high-molecular-weight chromosomal proteins greater than \( M_r 100,000 \). Extensive immunoblotting-immunoblocking studies indicated antigenic homologies between the chromatin of the nontumorigenic ARL-15Ci cell line, a transformed, tumorigenic cell line (ARL-16T3), fetal liver, and several transplantable malignant tumors. Considerably less homology was observed for normal adult and regenerating liver chromatin. The antigenic similarity between the ARL-15Ci line and fetal rat liver was confirmed by immunoblotting experiments with antisera to fetal rat liver chromatin fractions. The apparent changes in antigenic specificity suggest that retrodifferentiation occurred in the cultured liver cells, and our findings identify several high-molecular-weight nonhistone proteins which may be useful markers of developmental expression.

INTRODUCTION

Cellular differentiation is often defined as the progressive and selective manifestation of specialized functions and phenotypes particular to each cell type of a given organism. Since differentiation ensues without dramatic quantitative or qualitative changes in cellular DNA, specific nuclear proteins may serve as gene regulators during these transitions (8, 17, 36). Implicit in this view is that different cellular phenotypes, proliferative states, or specialized cell functions may be a reflection of the presence or absence of cell-specific nuclear proteins.

Two classes of nuclear proteins, the histones and HMG3 proteins have been relatively well-characterized (14, 25). Substantial evidence indicates the octameric histone complex to serve as the basic repeating unit of chromatin structure (20), which correlates well with the evolutionary conservation of this class of nuclear proteins. Although the HMG proteins have been linked with active gene expression of cell-specific protein products (39, 42), the ubiquitous presence of HMGs in all somatic nuclei implies that additional elements may serve to modulate the genome during cellular maturation and differentiation. Hence, neither histones nor HMGs demonstrate sufficient cell specificity that might be expected of proteins controlling aspects of specific gene expression. On the other hand, nonhistone proteins which bind DNA and form protein:DNA complexes have been suspected to fulfill a critical role in these processes. Nevertheless, attempts to identify and characterize regulatory proteins in these nonhistone fractions have been hampered due to their extreme insolubility and heterogeneity.

To circumvent these problems, antibodies have been used to probe the organization and composition of nonhistone proteins (for a recent review, see Ref. 16). A variety of immunological studies have implicated cell-specific nonhistones or nonhistone:DNA complexes to be associated with normal cell differentiation, hormone stimulation, or the expression of neoplasia (5-7, 16, 29, 35, 40, 46).

For some time, our laboratory has been interested in the identification of cell-specific nonhistone proteins as markers for developmental gene expression and transformation. In the course of examining nonhistone antigenic profiles of cultured nontumorigenic and tumorigenic adult rat liver-derived cells, we discovered an interesting cell specificity. By polyacrylamide gel electrophoresis and nitrocellulose electroblot methods, the nontumorigenic cells were demonstrated to possess antigenic similarities to fetal liver and several neoplastic lines in their content of nonhistone chromatin proteins.

MATERIALS AND METHODS

Sources of Cell Lines and Tissues. Epithelial cell lines were derived from the livers of F344 male rats (44) and cultivated in Williams Medium E (45). A nontumorigenic clone (ARL-15Ci) and a line (ARL-16T3) derived from a tumor which was produced by inoculation of a transformed line ARL-16 (26, 27) were studied. Walker 256 solid tumor carcinosarcoma and 13762 MAT-B rat breast adenocarcinoma were obtained from Dr. A. Bogden, Mason Research Institute (Worcester, MA), and transplanted into healthy 150-g female Sprague-Dawley or Fisher 344 hosts, respectively. NAH was maintained by weekly transplantations in 150-g male Sprague-Dawley rats.

Normal tissues were obtained from healthy donors, immediately washed in ice-cold PBS, and processed as described below. Fetal rat liver and brain were obtained from Sprague-Dawley 18-day-old fetuses. For regenerating rat liver, 150-g male Sprague-Dawley rats were partially hepatectomized (15) under light ether anesthesia, and regenerating livers were then used 24 or 48 hr after surgery.

Isolation of Nuclei and Chromatin. All of the following procedures were performed on ice at 0–2°C. Tissue culture and ascites cells were washed 3 times in PBS before use. Nuclei from NAH were obtained by hypotonic shock in 10 mM Tris-HCl (pH 7.5):0.1 mM PMSF as described previously (43). Walker or MAT-B solid tumors were prepared using a modification of procedures described before (30). Tumors were disrupted by low-speed homogenization with a Polytron in 250 mM sucrose:50 mM Tris-HCl:25 mM KCl:5 mM MgCl2 (pH 7.5) (buffer:tissue, 1:4, v/v). Homogenates were then filtered and pressed through a 50-mesh stainless-steel sieve (A. H. Thomas Co., Philadelphia, PA), centrifuged at low speed, and used for subsequent analyses.

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3 The abbreviations used are: HMG, high-mobility-group; ARL-15Ci, nontumorigenic rat liver epithelial cells; ARL-16T3, a line established from carcinoma produced by transformed rat liver epithelial cells; NAH, Novikoff ascites hepatoma; PBS, phosphate-buffered saline (0.01 M sodium phosphate:0.14 M sodium chloride, pH 7.2); SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; PAP, peroxidase-antiperoxidase.

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speed (800 x g for 10 min) and resuspended in 10 mM Tris-HCl:250 mM sucrose: 1 mM MgCl₂ and homogenized using a tightly fitting Teflon-glass homogenizer. The crude nuclei were again centrifuged at low speed, resuspended in 2.2 mM sucrose: 10 mM Tris: 5 mM MgCl₂ (pH 7.5), and purified by ultracentrifugation (100,000 x g for 60 min). Nuclei from ARL-15Cl, or ARL-16T₂ cells and normal tissues were prepared by the method of Blobel andPotter (1). All steps of nuclei isolation were assessed routinely with phase-contrast microscopy. Purified nuclei were washed once (resuspension and centrifugation at 1000 x g for 10 min) with 10 mM Tris-HCl:0.1 mM PMSF:250 mM sucrose:0.2% (v/v) Triton X-100 (pH 7.5) before chromatin preparation.

Chromatin was prepared using the method of Bonner et al. (2) as modified in our laboratory (33). Isolated chromatin was washed once in 0.3 M NaCl before rehydration in 100-fold-diluted standard saline citrate (140 mM NaCl: 14 mM sodium citrate; pH 7.0). Where indicated, cytosol was prepared from the initial homogenates of tissue and cells as described previously (31).

Antisera and Immunoadsorbents. Chromatin prepared from ARL-15Cl, cells was dehistonized in 5 mM urea:2.0 mM NaCl:phosphate buffer (pH 6.0), as detailed by Spelsberg et al. (34). Dehistonized chromatin was recovered, rehydrated in 2 mM Tris-HCl:0.1 mM PMSF (pH 7.5), and 250-μg samples (as DNA) were mixed with Freund’s adjuvant and used to immunize New Zealand White rabbits (7). One or two 50-μg (i.v.) booster injections were given per month, and blood was collected 7 days later. Serum were decomplemented at 56° for 0.5 hr and stored at -20°. Antiser to dehistonized chromatin from fetal rat liver were prepared exactly as described by Kilianska et al. (19).

Immunoadsorption-immunoblocking studies were conducted using a modification of our previous procedure (31). Isolated chromatins were suspended in 2 mM Tris (pH 7.5), 1 mM MgCl₂, 1 mM PMSF, and were treated for 1 hr (on ice) with DNase I (50 μg/ml; Worthington, Freehold, NJ). Samples were then solubilized in 5 mM guanidine-HCl:50 mM Tris-HCl (pH 8.0) and dialyzed against 3 changes of 2 mM Tris buffer (pH 7.5), adjusted for protein concentration, and lyophilized. Samples of lyophilized protein were then mixed in the ratio of 2 mg chromosomal protein to 1.0 ml of 1:10 antiserum (diluted in PBS) and incubated for 1 to 2 hr on ice. After centrifugation (10,000 x g for 15 min), the supernatant was then mixed and incubated with another lyophilized sample of chromosomal protein as described above. Following centrifugation, the supernatant was then immunoadsorbed at least one additional time with undenatured chromatin exactly as described previously (31).

SDS-Polyacrylamide Gel Electrophoresis. Electrophoresis supplies were purchased from Bio-Rad Laboratories (Richmond, CA). Chromosomal proteins were prepared for SDS gel electrophoresis using DNase I digestion of DNA as described previously (30). Electrophoresis was conducted using the procedure of Laemmli (21) with a 3% stacking gel and a 7.5% running gel. Proteins were visualized by Coomassie brilliant blue staining (9).

Proteins separated with SDS-polyacrylamide gel electrophoresis were electroblotted to nitrocellulose as described by Towbin et al. (38). Immunoreactive proteins immobilized on nitrocellulose sheets were then visualized using the PAP method of Sternberger (37), as adapted by Glass et al. (13). All immunoreactive bands were confirmed by parallel assays with preimmune sera. Antigen molecular weights were calculated from semilogarithmic plots of the mobilities of high-molecular-weight standards after electrophoresis, transfer to nitrocellulose, and staining with Amido black (28).

RESULTS

Chromatins prepared from a variety of sources exhibited quantitative and qualitative differences in Coomassie-stained protein bands among the various chromatins. Chromatins enriched in antigenic DNA concentrations, it cannot be argued that the immunoreactivity was a reflection, per se, of different protein:DNA ratios among the various chromatins. Chromatins enriched in antigenic-staining such as ARL-15Cl₁, fetal liver, and MAT-B all contained slightly less amounts of nuclear protein (on a DNA basis) than regenerating and normal adult liver samples; yet the latter chromatins were noticeably diminished in immunoreactivity. Nevertheless, in order to minimize simple quantitative differences in the antigenic species, the immunoadsorption-immunoblocking experiments (described below) were conducted with antibodies and chromatins at a relatively constant protein ratio.

When proteins from the homologous ARL-15Cl₁ chromatin were used in the immunoadsorption-immunoblocking protocol (see "Materials and Methods") prior to immunotransfer staining, all detectable immunoreactivity was removed (Fig. 2B), indicating that all of the reactivities visualized were found in ARL-15Cl₁ chromatin. Treatment of these antisera with chromosomal proteins from ARL-16T₂ cells demonstrated that the cultured tumorigenic cells also contained nonhistone antigens similar to the ARL-15Cl₁ cells (Fig. 2C). In contrast, normal adult liver chromosomal proteins were poor immunoadsorption-immunoblocking agents for these antibodies (Fig. 2D).

Surprisingly, fetal liver chromosomal proteins were quite effective in the immunoadsorption-immunoblocking of these antibodies and were nearly equivalent to preparations from the ARL-15Cl₁ line in that only reactivity for a faint band at M, 200,000 (Fig. 2E). Furthermore, this immunoreactivity was not
apparently related to the more rapid proliferation rate of fetal hepatocytes as compared to adult liver, since proteins from regenerating liver chromatin were only slightly better than normal liver proteins as immunoblock-immunoabsorption-immunoblocking agents (Fig. 2F).

The ability of chromosomal proteins from the transplantable neoplastic lines, Walker 256 and 13762 MAT-B, to act as immunoblocking agents for these antisera was also tested (Fig. 2, G and H, respectively). Proteins from both lines showed a greater ability to block immunoreactivity than proteins from normal liver, although neither line was quite as effective as fetal liver. Treatment of these antisera with chromosomal proteins from NAH also gave similar results (data not shown).

It is of note that our former immunoblocking procedure (31), which used native, undenatured chromatin, was only partially effective with antisera to ARL-15CI dehistonized chromatin. These antisera could only be blocked with native chromatin from ARL-15CI, ARL-16T2, fetal liver or, to a lesser extent, the transplantable tumors, while the other chromatin assayed required prior denaturation to effect reduction in immunoreactivity, even for staining in the sample lane of the respective chromatin (data not shown). Apparently, some of the antigenic sites recognized by these antisera may exist in a conformation inaccessible to some antibodies during absorptions with native chromatin; these unabsorbed antibodies then persist to react with the fully denatured site on the immunotransfer. Other denaturants such as urea and SDS were also used in preliminary experiments with moderate success; however, none was as effective as guanidine-HCl.

The interesting immunoreactivity of the liver-derived cell line ARL-15CI was examined further using antisera prepared to fetal liver dehistonized chromatin. Immunoblot analysis revealed that these antibodies recognized a heterogeneous collection of antigens with molecular weights greater than M, 40,000 (Fig. 3A). There are many quantitative and apparent qualitative differences in these antigens among the various chromatinics which we have discussed in detail recently (19). Significantly, however, when ARL-15CI chromosomal proteins were used in immunoblocking-immunoabsorption experiments prior to immunotransfer staining, nearly all immunoreactivity was removed (Fig. 3E). Moreover, the immunoblocking pattern was virtually identical with that of the homologous tissue, fetal liver (Fig. 3C). The inability of normal liver or NAH chromatin to completely absorb these antibodies has been demonstrated previously (19).

DISCUSSION

Establishment of in vitro cultivation conditions for liver cells has received considerable experimental attention due to the attractiveness of using such cultures for studies in toxicology and carcinogenesis (3). A variety of techniques has been used to increase the fidelity of hepatocyte primary cultures, including enriched media formulations, culture on surfaces treated with attachment proteins, and natural or synthetic matrices (for reviews, see Refs. 3 and 23). From hepatocyte primary cultures, proliferating epithelial cell lines can be established for study of neoplastic transformation. These systems provide a means to study and characterize developmental gene products and identify early markers of cell transformation and differentiation.

In the present study, antisera to dehistonized chromatin from ARL-15CI cells and immunotransfer methodology were used to characterize the tissue and cell specificity of the antigens recognized by these antisera. A range of high-molecular-weight species was identified, and most antigenic proteins could be detected, with varying degrees of intensity, in every chromatin examined. Further experiments used chromosomal proteins as immunoblocking-immunoabsorption agents, and these studies disclosed distinct antigenic homologies between the ARL-15CI line, fetal liver, and several transplantable neoplastic lines. The interesting antigenic relationship between the ARL-15CI cells and fetal liver was confirmed with antisera to fetal liver dehistonized chromatin. Recently, we have reported that these latter antisera exhibit considerable specificity for fetal liver antigens, some comparable cross-reactivity with hepatoma antigens, and even less cross-reactivity with adult liver or kidney antigens (19).

Since the ARL-15CI line was originally derived from cultured adult hepatocytes (26, 27), our data suggest that the cells have undergone developmental changes in the overall composition of nonhistone proteins such that those of a more fetal phenotype are now present. While the reasons for this transition remain to be determined, in all likelihood, the changes are a result of adaptive responses in the differentiated state due to the culture conditions.

It is significant that the adult rat liver tumor-derived line ARL-16T2 also contained all of the antigens found in the nontumorigenic ARL-15CI line, indicating that malignant transformation did not detectably alter these nuclear proteins. While the relationship of these antigens to other physiological and phenotypic parameters in these cell lines requires further experimentation, recent characterization of both the tumorigenic and nontumorigenic lines showed clear differences in the potential of each line to form in vivo tumors in nude mice or neonatal rats and the ability to grow in soft agar cultures (26, 27). Moreover, cytochemical and biochemical assays of γ-glutamyl transpeptidase, a marker for fetal hepatocytes, carcinogenesis, and neoplastic cells in culture (10, 11, 18, 22), demonstrated the ARL-15CI cells to have low, nearly undetectable activity, while high γ-glutamyl transpeptidase activities were observed in the ARL-16T2 cells (27). Therefore, it is probable that the nonhistone antigens revealed by our experiments are not connected with expression of this enzyme, nor are they likely key oncodevelopmental alterations which may give rise to tumorigenicity.

Recent reports by several laboratories have indicated that hepatocytes maintained in vitro eventually revert to a more fetal phenotype (24, 32, 41). Sirica et al. (32) and Leffert et al. (24) have examined morphological and cytochemical changes in adult rat hepatocytes maintained in primary culture. The ultrastructure of such cells appears nearly adult or normal as do many secretion products; however, progressive increases occur in fetal isozyme activity and α1-fetoprotein production which appear to be temporally linked to proliferation and DNA synthesis. Although the cause of such retrodifferentiation remains unclear, recent studies by Freeman et al. (12) and Carlsson et al. (4) suggest that the attachment substrate may markedly affect in vitro differentiation. Whatever the cause of the retrodifferentiation of cultured hepatocytes, our studies provide additional evidence for its occurrence in cell lines and also reveal a range of nonhistone antigens which potentially may be useful markers of this progression.

Since chromosomal proteins from regenerating liver were relatively poor immunoblocking-immunoblocking agents for antibodies to ARL-15CI dehistonized chromatin, differences in cellular proliferation between the adult liver and cultured cells are
not likely responsible for the expression of these nonhistone antigens. Since some cytotoxic markers of the fetal phenotype are preferentially expressed during log-phase growth of hepatocytes (24), it should be mentioned that we have compared the antigenic profiles of semiconfluent, proliferating, and postconfluent, quiescent cells. No overt changes were observed in the nonhistone antigens of these cell populations. Although these experiments do not eliminate the possibility of involvement of these antigens in cellular proliferation, they do indicate that severe changes in the quantity and immunogenicity of these proteins are not required for the process.

In several past reports, we have used immunosorption procedures to confirm the cell and tissue specificity of antigens recognized by polyclonal antisera (29, 31). Since most of the antigens identified with the antisera to ARL-15CI, dehistonized chromatin could be detected in every chromatin assayed, although with varying degrees of intensity, the immunoabsorption-immunoblocking experiments were the most informative concerning the overall specificity of these antibodies. Small quantitative differences in the antibodies among the chromatins are not likely responsible for the results, since our immunoblocking conditions were designed to minimize this possibility.

The polyclonal antisera used here likely contain many antibodies for each individual antigen, and antigenic modifications perhaps predominate in a differentiated state. This may also contribute to the immunogenicity. Furthermore, we observed significant differences in the ability of deoxyribonucleic acid from the various sources to block immunoreactivity, suggesting that the exposure of antigenic sites in the native state may be variable. Although our experiments do not establish the reasons for the individual antigenic differences in these chromatins, they do demonstrate the existence of considerable trends and homologies among the various chromatins. Overall, homologies in the complement of nonhistone protein antigen may prove to be important in the appraisal of a cellular phenotype. At this time, we are uncertain as to the function of the antigens revealed by these studies and as to why they would exhibit immunological changes apparently in response to in vitro growth conditions. It is a major advantage for future studies that these changes can be correlated with an in vivo tissue type which ultimately may provide clues to the identity and function of these proteins.

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Fig. 1. SDS-polyacrylamide gel electrophoresis of chromosomal proteins from various sources. A, Coomassie-stained gel of various chromatins. Chromatin was prepared for SDS-polyacrylamide gel electrophoresis as described in "Materials and Methods," electrophoresed, and stained with Coomassie brilliant blue. Each lane contained 25 μg of each chromatin (as DNA). Lane 1, ARL-15Cl R; Lane 2, ARL-16T; Lane 3, normal adult liver; Lane 4, regenerating liver; Lane 5, fetal liver; Lane 6, fetal brain; Lane 7, Novikoff ascites hepatoma; Lane 8, Walker 256; Lane 9, MAT-B; Lane 10, M, standards (Bio-Rad) (myosin, M, 200,000; β-galactosidase, M, 116,500; phosphorylase b, M, 97,500; bovine serum albumin, M, 68,000; ovalbumin, M, 43,000). b, Amido black staining (28) of chromosomal proteins separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose (36). Lanes are exactly as indicated in a, with the omission of Lane 10, M, standards.
Fig. 2 a–f
Fig. 2. Immunoidentification of chromosomal protein antigens recognized by antisera to ARL-15Cit, dehistonized chromatin. Nitrocellulose sheets containing SDS-polyacrylamide gel electrophoresis separated proteins were incubated with antisera and then immunoreactive antigens visualized with the PAP reaction. Antisera were extensively immunoblocked-immunoabsorbed as detailed in "Materials and Methods." Lanes are exactly as indicated in Fig. 1 with the omission of Lane 10, M, standards. a, untreated control; b, immunoblocked-immunoabsorbed with chromosomal proteins from ARL-15Cit; c, ARL-16Ts; d, normal adult liver; e, fetal liver; f, regenerating liver; g, Walker 256; h, 13762-MAT-B.

Fig. 3. Immunoidentification of chromosomal protein antigens recognized by antisera to fetal liver dehistonized chromatin (19). Nitrocellulose sheets containing SDS-polyacrylamide gel electrophoresis-separated proteins were incubated with antisera and then immunoreactive antigens were visualized with the PAP reaction. Antisera were extensively immunoblocked-immunoabsorbed as detailed in "Materials and Methods." Lanes are as indicated in Fig. 1 with the omission of Lane 10, M, standards. a, Untreated control; b, immunoblocked-immunoabsorbed with chromosomal proteins from ARL-15Cit; c, fetal liver.
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