Comparison of Nuclear Nonhistone Phosphoproteins of Rat Liver and Novikoff Hepatoma

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SUMMARY

As part of a continuing comparison of nuclear proteins of tumors and other tissues, $^{32}$P-labeled nuclear proteins were extracted successively with 0.15 and 0.35 M NaCl from the nuclei of normal, regenerating, and thioacetamide-treated rat liver as well as Novikoff hepatoma 3 hr after injection of $^{32}$P, into rats. Separation of proteins of these fractions with aqueous phenol was carried out before two-dimensional electrophoresis on polyacrylamide gels. By autoradiography many common spots were found, but four $^{32}$P-labeled protein spots, CU', C13p, C21p, and CMp, were found in the Novikoff hepatoma and not in the various liver samples studied. Two spots, B6 and B10, were found in the liver patterns and not in the tumor. Spot B33 was very dense in regenerating liver but was only a faint spot in thioacetamide-treated liver. The greater density of Spots CU', C13p, C21p, and CMp in the tumor patterns is consistent with the increased density reported earlier for spots of the C-region of a variety of tumors.

INTRODUCTION

Of the possible control mechanisms that regulate normal gene transcription, increasing studies have been made on chromosomal proteins, particularly phosphoproteins, as likely regulatory molecules (7, 8, 21, 27, 29, 30, 31, 37, 48, 50, 51, 59, 60, 62, 64). Histones are now considered to be primarily present in "Nu" particles or "nucleosomes" (8, 19, 23, 44). The nonhistone proteins which exhibit considerable heterogeneity and tissue specificity (10, 35, 37, 53, 62, 65) have roles in regulation of gene transcription (8, 48, 60). Recent attention has been particularly focused on a possible regulatory function of the nonhistone proteins soluble in 0.35 M NaCl (14, 18, 37-39, 54).

It has been suggested that control of gene expression might occur through selective phosphorylation and dephosphorylation of the nuclear acidic proteins (3, 20, 32, 35, 36, 60). Phosphorylation of this heterogeneous group of proteins varies during growth and differentiation (5, 20, 24, 33, 42, 52, 57), hormonal stimulation (2, 3, 13, 25, 59, 63), and malignant transformation (12, 14, 26, 40).

The goal of the present study was to compare the nuclear phosphoproteins of normal and thioacetamide-treated rat liver, which are nondividing tissues, with regenerating rat liver and Novikoff hepatoma, which are growing and dividing tissues. Thioacetamide treatment was utilized to induce nucleolar hypertrophy (1, 9).

With the increased resolution of the 2-dimensional electrophoresis method, (6, 49), the phosphoproteins soluble in 0.15 and 0.35 M NaCl were separated on polyacrylamide slab gels and subjected to autoradiography. In the Novikoff hepatoma patterns, there were 4 spots in the C-region, CU', C13p, C21p, and CMp, which were not found in the other tissues studied.

MATERIALS AND METHODS

Tumor Cells and Labeling. The Novikoff hepatoma ascites cells were transplanted 6 days before the experiments in male 200-g albino rats obtained from the Holtzman Co., Madison, Wis. The nuclear proteins were labeled by i.p. injection of 20 mCi of carrier-free $^{32}$P (Union Carbide Corp., Tuxedo, N. Y.) into each rat 3 hr before sacrifice. The ascites cells were drained from the abdominal cavity, filtered through cheesecloth, and washed 2 times with ice-cold 0.13 M NaCl:0.005 M KCl:0.008 M MgCl$_2$ (60). For studies with thioacetamide, each rat was treated daily (i.p.) for 9 days with a dose of 50 mg thioacetamide per kg rat body weight (1). Regenerating rat livers were obtained after partial hepatectomy (22, 43). About 70% of the liver from each rat was removed, and the remaining 30% was allowed to regenerate for 18 hr. The livers were cut into small pieces and washed twice with 0.13 M NaCl:0.005 M KCl:0.008 M MgCl$_2$ buffer by mild homogenization.

Isolation of Nuclei and Extraction of 0.15 and 0.35 M NaCl-soluble Proteins. Nuclei were isolated from the livers and Novikoff hepatoma ascites cells by using 0.5% citric acid (61, 69) and purified by centrifugation through 2.2 M sucrose containing 3.3 mM calcium acetate (9, 41). Proteins were extracted from nuclei as described below.

Nuclei were extracted twice with 10 volumes of 0.01 M Tris-HCl buffer, pH 7.0, containing 0.15 M NaCl, 0.001 M MgCl$_2$, and 1 mM phenylmethylsulfonyl fluoride (4) to remove the nuclear sap proteins. Following this, nuclei were extracted 3 times with the same buffer at pH 8.0 (58), followed by 3 extractions with 10 volumes of 0.35 M NaCl in 0.01 M Tris-HCl buffer, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride. After each extraction, samples were centrifuged at 8,000 x g for 10 min. Supernatants were
Preparation of Phenol-soluble Nuclear Proteins. Phenol-soluble proteins were isolated from the 0.15 and 0.35 M NaCl fractions following the procedure of Teng et al. (62). Aliquots of the NaCl fractions were dialyzed against 0.1 M Tris-HCl, pH 8.4, containing 0.01 M EDTA and 0.14 M β-mercaptoethanol for 24 hr (37). The proteins were extracted by adding an equal volume of buffer-saturated phenol, and the mixture was allowed to stand at 4°C for 12 hr. The resulting suspension was centrifuged at 12,000 × g for 10 min, and the aqueous phase was extracted again with phenol. The 2 phenol extracts were pooled and dialyzed against 0.1 M acetic acid containing 0.14 M β-mercaptoethanol until the volume of the phenol extract was reduced to one-fifth. The phenol fraction was subsequently dialyzed against 0.05 M acetic acid: 9.0 M urea: 0.14 M β-mercaptoethanol for 24 hr, followed by 0.01 M Tris-HCl (pH 8.4): 8.6 M urea: 0.01 M EDTA: 0.14 M β-mercaptoethanol for 2 hr (62).

Two-Dimensional Gel Electrophoresis and Autoradiography. Both the NaCl and phenol fractions were concentrated with polyethylene glycol to a protein concentration of about 5 mg/ml. The concentrated samples were dialyzed overnight against sample buffer containing 0.9 M acetic acid, 9 M urea, and 1% β-mercaptoethanol. Two-dimensional polyacrylamide gels were prepared and run essentially by methods developed in this laboratory (6, 49); 6% acrylamide was used for the 1st dimension, and 8% was used for the 2nd dimension in order to obtain better resolution of the high-molecular-weight nonhistone proteins (6) in the B- and C-regions of the 2-dimensional gels. In preliminary studies it was found that very few 32P-labeled proteins were present in the A-region (49). Approximately 400 to 500 μg of protein (100 to 200 × 10^3 cpm) were in each sample. After electrophoresis, the gels were dried under vacuum and exposed to X-ray film for 8 to 10 days (28, 46).

RESULTS

0.15 M NaCl-soluble Nuclear Phosphoproteins. The 2-dimensional labeling patterns and composite drawing of liver and hepatoma 0.15 M NaCl extracts (Fig. 1) show that 32P-containing protein spots were resolved in the B- and C-regions of the gel (6). Spots B6 and B10, which were dense in the liver pattern, were not detected in the tumor pattern. In the tumor, 8 spots were found that were absent from the liver, i.e., B2, B7, B13, Cmp, C13p, C21p, CU, and CU'.

Phenol-soluble, 0.15 M NaCl-soluble Nuclear Phosphoproteins. The autographic pattern for the phenol extract of the 0.15 M NaCl fraction for the Novikoff hepatoma (Fig. 2) was very similar to that of the whole 0.15 M NaCl extract (Fig. 1). Quantitative differences were noted in spot density between the patterns for the phenol-soluble fraction and the whole 0.15 M NaCl fraction of the liver (Figs. 1A and 2A). Spots B24p and C3p were less dense in the phenol extract and Spots B5p, B13, CT, and C18 were more dense.

0.35 M NaCl-soluble Nuclear Phosphoproteins. As reported earlier (56), the phosphoproteins in the 0.35 M NaCl extract of rat liver nuclei (Fig. 3A) differed from those of the 0.15 M NaCl fraction (Fig. 1A). In the 0.35 M NaCl-soluble fraction the most densely labeled spots were C18 and C3p; B6, B10, and B24p were less dense than in the 0.15 M NaCl fraction.

The pattern for the 0.35 M NaCl-soluble phosphoproteins of the Novikoff hepatoma was very similar to the corresponding 0.15 M NaCl-solution fraction (Figs. 1A and 3B). These results show that there are marked differences in the protein spots of 2-dimensional gels for the liver and hepatoma 0.35 M NaCl-soluble fractions. The tumor contains Spots B2, B7, B23, Cmp, C13p, C21p, CU, and CU', which were faint or absent in the normal liver pattern.

Phenol-soluble, 0.35 M NaCl-soluble Nuclear Phosphoproteins. Fig. 4, A and B, shows autoradiograms of the 2-dimensional gel separation of the phenol-extracted phosphoproteins for the 0.35 M NaCl extract of liver and Novikoff hepatoma. All the spots of the whole 0.35 M NaCl extract of the tumor were present in the phenol extract (Figs. 3B and 4B). The pattern of the phenol-soluble, 0.35 M NaCl-extracted proteins of liver was different from that of the whole 0.35 M NaCl extract in that Spots B6, B7, B10, B33, C2p, CC, C5, CM, and CT were relatively concentrated in this fraction (Table 1).

Fig. 4, A and B, shows the differences in phenol-soluble 0.35 M NaCl-soluble proteins of liver and tumor. Table 1 shows that the 0.35 M NaCl-soluble nuclear protein fraction of Novikoff hepatoma contains Proteins B23, Cmp, C13p, C21p, CU, and CU', which were not in the normal liver patterns.

0.35 M NaCl-soluble Phosphoproteins of Regenerating and Thioacetamide-treated Rat Liver. For comparison with the fraction that showed the largest differences between the normal liver and the Novikoff hepatoma, the phenol-soluble 0.35 M NaCl fraction was analyzed for regenerating rat liver (Fig. 5) and thioacetamide-treated liver (Fig. 6). The pattern for regenerating liver contained all the spots of the normal liver. In addition, Spots B23 and CU are present in this pattern.

Fig. 6 shows the pattern for the phenol-soluble phosphoproteins of thioacetamide-treated rat liver. In addition to the spots of the regenerating rat liver, Spot B2 was present. Spot B33 was much less labeled than in regenerating liver. Although the regenerating liver and thioacetamide-treated liver contained several phosphoproteins that were absent from normal liver, neither contained Spots C13p, C21p, Cmp, or CU', which were found in the Novikoff hepatoma patterns (Table 1).

DISCUSSION

The 2-dimensional gel method (6, 49) provided an opportunity for more refined comparisons of nuclear, nucleolar, and chromatin proteins of the Novikoff hepatoma and other tumors and the corresponding patterns of nontumorous tissues. Initial studies on chromatin proteins (6, 72) in tumors showed that the C-region contained many more dense spots than were found in normal liver. These results agreed well with the reports of increased concentrations of high-molecular-weight chromatin proteins by Weisenthal and...
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### Table 1

<table>
<thead>
<tr>
<th>Spot</th>
<th>Normal liver NaCl-soluble, 0.35 M</th>
<th>Novikoff hepatoma NaCl-soluble, 0.35 M</th>
<th>Regenerating rat liver NaCl-soluble, 0.35 M</th>
<th>Thioacetamide-treated rat liver NaCl-soluble, 0.35 M</th>
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Ruddon (66) and with the more recent studies on carcinogenesis in vitro of Forger et al. (16). Some of these proteins, such as C18, C21, and CQ, were found in the nucleolus (47). However, 5 proteins were found in the tumors that were not found in rat nontumorous tissues, including CP', C15', CG', CH', and CG' (61, 68, 72). In human leukemic cells, CH' and CG' were found; Proteins CP', C15', Cg', and C15' were found as found in rat nontumorous tissues, including CP', C15', Cg', and fetal tissues but not in regenerating or normal liver or cytoplasts, indicating that they may be involved in normal growth processes (68).

In efforts to probe further for proteins present in smaller amounts, antigenically active proteins were investigated. Recently, the chromatin antigen NAg-1 was found in tumors and fetal tissues but not in regenerating or normal liver or other nontumorous tissues (11, 67).

The use of the 32P label provided an additional approach to detection of nuclear and chromatin proteins present in small amounts. Several previous studies in this laboratory have established some differences in localization of nuclear and nucleolar phosphoproteins (45, 47, 48) as well as tissue differences in the phosphoproteins of the Chromatin II fraction (15). The Novikoff hepatoma chromatin contained the 32P-labeled proteins CN and CQ that were not detected in normal and regenerating liver or rat heart (15).

The present study was carried out on the 0.15 and 0.35 M NaCl-soluble phosphoproteins to determine whether the same or different proteins were present in the 2-dimensional gel patterns of normal liver and Novikoff hepatoma. The separation of liver nuclear phosphoproteins achieved by extractions with these 2 salt concentrations was shown earlier by Prestayko et al. (54). The additional use of the phenol procedure (9, 62) produced significant concentration of several proteins in the phenol phase of both the 0.15 and 0.35 M NaCl extracts of liver nuclei. Proteins B6, B10, and CT were concentrated in the phenol fractions. However, in the Novikoff hepatoma patterns, relatively little fractionation was achieved by the phenol procedure (14, 17, 18, 37). Only Protein CU' appeared to be selectively concentrated in the phenol phase in comparison with the whole extracts.

Inasmuch as the 0.35 M NaCl-soluble fractions contained the largest number of proteins, comparisons were also made for this fraction for regenerating liver and thioacetamide-treated liver. Although these samples contained a few more spots than did normal liver, only the Novikoff hepatoma contained Spots CU', C21p, C13p, and Cmp. These proteins may be related to increased synthesis of nuclear and cytoplasmic proteins found earlier in the tumors (8, 24, 36, 60, 68, 72).

Some 32P-labeled proteins were found in the liver and not in the Novikoff hepatoma, including Proteins B6 and B10. In earlier studies of nucleolar proteins (49), Spot B5L was found in the liver patterns and not in the Novikoff hepatoma. Also, in studies on Chromatin Fraction II, the liver pattern contained Proteins CB, Bp, BA, and Bi (71). In addition, regenerating liver contained several high-molecular-weight proteins in increased concentrations in both the acid-soluble and the Chromatin II fractions (70, 73).

The role of the nuclear phosphoproteins is not yet well defined. Earlier studies in this laboratory (55, 56) showed that some of the phosphorylated proteins are found in the nucleolar ribonucleoprotein particles including Proteins B13, B23, and C3p (47). Proteins B2, B3, and B6 were found earlier in ribosomes (47, 55), and Spot B24 is the major protein of informer particles (54, 58). Further characterization will be required to determine whether proteins containing 32P are identical to those seen earlier in the stained patterns (55).

In studies in which differences were demonstrated between proteins of tumors and other tissues, several possible explanations for their presence arise: (a) these proteins may be fetal or "oncodevelopmental" proteins, (b) the proteins may be isozymes or subunits that represent products other than those in the parent tissue, (c) the proteins may be "phase-specific" products of cell division, (d) the proteins may represent "tumor-related" products that are normally absent or present only in small amounts in nontumorous tissues, and (e) new 32P-labeled spots may reflect altered phosphorylation of preexisting proteins.

Among the questions emerging from the evidence that tumors contain a variety of proteins not usually found in nontumorous tissues is, "What temporal relationships exist between these proteins, i.e., do some appear at earlier times than others in cell development, and in turn do some result in expression of genes that produce others (67)?" Another question is "Do 1 or more of these proteins specifically produce the continuing growth of tumors?" During the course of mitosis a critical concentration of some key protein "triggers" may throw the dividing cell into G1. Extended studies on these proteins in both primary and human tumors are clearly essential for further insights into these questions.

### REFERENCES

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Fig. 1. A, 2-dimensional autoradiogram of normal rat liver nuclear 0.15 M NaCl-soluble proteins. Samples were run in the 1st dimension on 9.5-cm tube gels of 6% acrylamide:4.5 M urea:0.9 N acetic acid at 120 V for 5.5 hr. The 2nd dimension was an 8% acrylamide:0.1% sodium dodecyl sulfate:0.1 M phosphate (pH 7.1) slab gel. Gels were stained with Coomassie Brilliant Blue R after 15 hr of electrophoresis at 50 ma/slab. After destaining, the gels were subsequently dried under vacuum and exposed to X-ray film for 5 to 15 days. After development, the stained spots were matched with spots on the film. Numbers followed by p indicate radioactive spots that do not co-migrate with stained spots. B, two-dimensional autoradiogram of Novikoff hepatoma nuclear proteins soluble in 0.15 M NaCl:0.01 M Tris:0.001 M MgCl₂:0.001 M phenylmethylsulfonyl fluoride.

Fig. 2. Two-dimensional autoradiogram of phenol-soluble 0.15 M nuclear proteins of rat liver (A) and Novikoff hepatoma (B). See Figure 1A for conditions.

Fig. 3. Two-dimensional autoradiogram of 0.35 M NaCl-soluble nuclear proteins of rat liver (A), and Novikoff hepatoma (B). See Figure 1A for conditions.

Fig. 4. Two-dimensional autoradiogram of phenol-soluble 0.35 M nuclear proteins of rat liver (A) and Novikoff hepatoma (B). See Figure 1A for conditions.

Fig. 5. Two-dimensional autoradiogram of phenol-soluble 0.35 M nuclear proteins of thioacetamide-treated rat liver. See Figure 1A for conditions.

Fig. 6. Two-dimensional autoradiogram of phenol-soluble 0.35 M nuclear proteins of thioacetamide-treated rat liver. See Figure 1A for conditions.
Nuclear Phosphoproteins

0.15M NaCl-Soluble Nuclear Phosphoproteins
Rat Liver

0.15M NaCl-Soluble Nuclear Phosphoproteins
Novikoff Hepatoma
Nuclear Phosphoproteins

0.35M NaCl-Soluble Nuclear Phosphoproteins
Rat Liver

3A

0.35M NaCl-Soluble Nuclear Phosphoproteins
Novikoff Hepatoma

3B
Phenol-Soluble 0.35M NaCl
Soluble Phosphoproteins
Rat Liver

Phenol-Soluble 0.35M
Nuclear Phosphoproteins
Novikoff Hepatoma
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Phenol-Soluble 0.35M NaCl Soluble Phosphoproteins
Thioacetamide-Treated Rat Liver

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