

Antigenically Active Nonhistone Chromatin Proteins in Cancer Cells¹

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Summary

Two-dimensional polyacrylamide gel electrophoresis shows that in nuclei of Novikoff hepatoma ascites cells there are approximately 75 proteins in the chromatin fraction soluble in 3 M NaCl:7 M urea. Dialysis of this fraction to an ionic strength of 0.15 produces a soluble fraction and a precipitate. The proteins in the soluble fraction have been reported to be active in gene control. Antibodies to the soluble fraction distribute diffusely throughout the nucleus, and antibodies to the precipitate localized primarily in the nucleolus and the nuclear ribonucleoprotein network. The nucleolar proteins differ from the extranucleolar proteins in antigenicity and labeling patterns.

The development of methods for isolation, purification, and identification of nuclear proteins provided the opportunity for analysis of chromatin antigens in tumor cells. Utilizing two-dimensional preparative polyacrylamide gel techniques as well as conventional procedures, several nuclear proteins have been isolated in electrophoretically homogeneous states including protein A-24, a histone-like nonhistone protein; C-14, a protein that stimulates nucleolar RNA polymerase; and a chromatin antigen soluble in 3 M NaCl:7 M urea that remains soluble after dialysis to 0.15 M NaCl to precipitate the histones and the DNA. This antigen has been found in the chromatin of both the Novikoff hepatoma and the Walker 256 carcinosarcoma but not in the chromatin of either normal or regenerating liver. It is a nonhistone nuclear protein as indicated by its amino acid analysis in which the ratio of the number of acidic to basic amino acids is approximately 1.4. Further studies are in progress on the function and structure of this chromatin protein. As an approach to analysis of relative rates of synthesis of this antigen and other proteins, the products of translation of messenger RNA of Novikoff hepatoma and normal liver are being analyzed by autoradiography of two-dimensional electrophoretic gels.

Introduction

The evidence for an important role of NHP⁴ in gene con-

trol is the subject of reviews (26, 27, 40). Recent reports show a multiplicity of species of these molecules (5, 26, 39) as well as alterations of gene readouts in their presence (19, 28, 30, 31, 38). It was also shown (26-28, 40) that addition of protein fractions from specific tissues to chromatin fractions of other tissues produces alterations of gene readouts (20, 21, 30-32).

The evidence that there is an increased readout of mRNA_{ova} as a result of interactions between specific cytoplasmic proteins or specific "receptor proteins" (28) and chromatin (or DNA) has resulted in a search for the precise mechanisms involved in this and other gene activation reactions. Despite the suggestive evidence that definitive interactions between some protein fractions and chromatin cause the production of specific mRNA species (1, 15, 20, 21, 32, 35-37), no specific example of interaction has yet been demonstrated between a highly purified "gene activator" protein and chromatin that produces a single species of mRNA. In addition, it has been pointed out that the RNA polymerases generally used could not have had high fidelity in terms of reading the correct DNA strands (15) and, further, that the RNA produced was only of low C_t (highly reiterated) rather than of "unique sequence" or specific mRNA. Moreover, the large number of nuclear proteins found in studies in this and other laboratories (26, 27, 43) have not yet been categorized with respect to function, but it is certain that many will be shown to have enzymatic and structural roles.

Evidence has been presented that gene control activities are not generally distributed throughout the group of nuclear proteins but are extracted with salt solutions of high ionic strength (19, 20, 30, 31, 35-38). However, Fujitani and Holoubek (11, 12) and Kostraba *et al.* (18) have indicated that the proteins extracted from chromatin with 0.35 and 0.60 M NaCl are similar to those extracted with the higher salt concentrations as shown by 1-dimensional polyacrylamide gel electrophoretic analysis. The present studies were designed to analyze the selectivity of the extraction with high salt by 2-dimensional electrophoresis of the proteins and also to provide immunological comparisons of these proteins with those of other fractions (7, 9). For these comparisons, techniques were developed for purification of individual nuclear proteins in good yield.

In the experiments on proteins that might be important in regulation of growth, it was of interest to examine such normal events as nucleolar hypertrophy, an apparent necessary precursor to growth in both a dividing tissue such as regenerating liver and a nondividing tissue such as the thioacetamide-treated liver cell. A number of proteins, par-

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⁴ The abbreviations used are: NHP, nonhistone nuclear proteins; PMSF, phenylmethylsulfonyl fluoride, *i.d.*, intradermally.

ticularly proteins of the nuclear ribonucleoprotein particles (8, 25), were shown markedly to increase in amount during the nucleolar hypertrophy. In addition, there are several proteins that decrease in amount, and of these one of the most notable was protein A-24 (3, 4). To assess the effects of A-24 in *in vitro* systems, efforts were made to purify this protein utilizing salt extraction procedures followed by preparative gel electrophoresis in 2 dimensions. The purity of the protein and its amino terminal have already been reported (13). This protein is especially interesting because it appears to contain virtually the entire sequence of histone 2A and an additional polypeptide the structure of which is currently under study (M. O. J. Olson, I. L. Goldknopf, K. Guetzow, T. C. Hawkins, and H. Busch, manuscript in preparation).

Protein C-14 is another nonhistone protein that has recently been purified and subjected to preliminary analysis (G. T. James, S. Matsui, L. C. Yeoman, A. Goldberg, and H. Busch, manuscript in preparation). The methods used for its purification differed from those of protein A-24 only by the initial use of ammonium sulfate fractionation as a preliminary step. Its amino acid composition and amino terminal have also been defined. In preliminary studies, protein C-14 produced an apparent increase in the activity of RNA polymerase I. By comparison with control systems, the uptake of [³H]UTP isotope was increased by approximately 30 to 50% (G. T. James, S. Matsui, L. C. Yeoman, A. Goldberg, and H. Busch, manuscript in preparation) suggesting a possible accelerator role for this protein.

The initial portion of our immunological studies proceeded as part of a search for identification mechanisms for trace quantities of nuclear products, particularly using antibodies as a test for the presence of specific nucleolar and nuclear elements in tumor cells and other cells (7, 9).

Materials and Methods

Isolation of Nuclei and Preparation of Chromatin. Nuclei were isolated by the citric acid or NP-40 method (8, 41) from 0.13 M NaCl:0.005 M KCl:0.008 M MgCl₂-washed Novikoff hepatoma ascites cells transplanted in normal male albino rats (Holtzman Co., Madison, Wis.). The nuclei were purified in sucrose solutions containing 0.1 mM PMSF. Similar preparations of liver nuclei were obtained (8, 41). Chromatin was prepared from these nuclei by the method of Marushige and Bonner (24), and the extractions were made with 0.35 M NaCl, 0.60 M NaCl, or 3 M NaCl:7 M urea containing 0.01 M Tris, pH 8, and 0.1 mM PMSF, either directly or successively (2, 6, 21, 39).

Fractions Obtained from the Chromatin. The fractions obtained from these extractions were the soluble proteins and an insoluble residue (2). Some of the fractions, such as those extracted with either 2 M NaCl or 3 M NaCl:7 M urea were dialyzed against sufficient 0.01 M Tris buffer to reduce the ionic strength of 0.15 to precipitate the DNA-histone and associated residual proteins (2, 21, 39).

Preparation of 0.35 or 0.6 M NaCl-soluble Chromatin Proteins. Chromatin from rat liver citric acid nuclei was extracted 3 times with buffer containing 0.01 M Tris-HCl (pH 8.0), 0.35 or 0.6 M NaCl, and 0.1 M PMSF at a ratio of 10 ml

of extraction solution per g of nuclei (2). The pooled extract was centrifuged at 142,000 × g for 24 hr.

The soluble proteins were concentrated by an Amicon apparatus fitted with a UM-10 filter or by precipitation with ethanol (3:1, v/v); taken up in a solution containing 0.9 M acetic acid, 10 M urea, and 1% β-mercaptoethanol; and then dialyzed against 2 changes of the same buffer for 16 hr or against 0.05 M Tris-HCl, pH 7.8. Protein was determined by the method of Lowry *et al.*, (23) with bovine serum albumin as a standard (Miles Laboratories, Kankakee, Ill.).

Two-Dimensional Gel Electrophoresis of the Proteins. The samples were analyzed by the 2-dimensional electrophoretic procedure described previously (10, 29) with the modification that the gel used for the 1st dimension contained 6% polyacrylamide and that of the 2nd dimension contained 8% polyacrylamide (10).

Preparation of Antibodies. Protein samples (10 to 50 mg) were injected i.d. and i.m. in 3 weekly doses into New Zealand White rabbits in 1 ml Freund's adjuvant diluted 1:2 with 0.15 M NaCl. The blood was collected from the ear veins 7 to 10 days after the 3rd inoculation (7). The procedure used for detecting immunofluorescence was a modification of that of Higlens *et al.* (14). Double immunodiffusion disc assays were carried out on plates purchased from Miles Laboratories (Elkhart, Ind.).

Chromatographic Purification of Antigens. Chromatography was carried out on whole 0.6 M NaCl-soluble proteins at 4° on a 5- x 150-cm Sephadex G-150 column. The fractions were pooled and assayed for precipitin band formation. Polyacrylamide gel electrophoresis of Sephadex G-150 Fraction IV was carried out on a nondenaturing disc gel. Stained sidetracks of preparative slab gels were used to aid in cutting the antigen-containing slice from the slab. Antigen was subjected to electrophoresis from the slices into tubes fitted with dialysis membrane for further studies.

Results

Nucleolar Antigens. In studies on antinucleolar antibodies, antinucleolar antisera were produced in rabbits immunized with whole isolated nucleoli from normal rat liver and Novikoff hepatoma ascites cells. These antisera produced positive nucleolar fluorescence of varying degrees in nucleoli and nuclei (7) of Novikoff tumor, rat liver, kidney, and Walker tumor cells (Fig. 1). Novikoff hepatoma and liver antinucleolar antisera fixed complement when combined with 0.15 M NaCl-soluble proteins extracted from the hepatoma and liver nucleoli. Nucleolar specificity of the antibodies was demonstrated by inhibition of fluorescence or complement fixation after pretreatment of the immune sera with whole nucleoli or nucleolar 0.15 M NaCl-soluble protein fractions (7).

Chromatin Antibodies. To ascertain whether there were immunological differences in the chromatin, antibodies were prepared in rabbits, and their interactions with nuclei were studied by immunofluorescence (Fig. 2, A and B). Unlike antinucleolar antibodies (Fig. 1), which exhibited a higher order of specificity in localization to nucleoli, antibodies to the soluble fraction of chromatin proteins distributed generally throughout the nucleus (Fig. 2A) as a whole

without preferential staining of nucleoli. The multiple small regions of local density may reflect binding to some chromocenters. Antibodies to the fraction insoluble at an ionic strength of 0.14 were localized primarily to the nucleolus and the fibers of the nuclear ribonucleoprotein network. The localization of the antibodies resembled stained pictures of the network fibers that were described in earlier reports (8).

In whole cells the antichromatin protein antibodies did not distribute generally through the nucleus. Instead, they appeared to be localized in regions of linear density, mainly distributed in the center of the nucleus with varying degrees of immunofluorescence. These results (Fig. 2B) suggest that there are "wall effects" that limit the observable distribution of the chromatin proteins in the nucleus, *i.e.*, large portions of the chromatin either do not contain these proteins or are structurally prevented from interaction with the antibodies.

Two-Dimensional Gel Electrophoresis of Chromatin Proteins. Before the separation of the proteins of the chromatin, 2-dimensional electrophoretic analyses were made (Fig. 3). Of the extraction solutions used to solubilize chromatin proteins and DNA, 3 M NaCl:7 M urea is probably more efficient than the other NaCl solutions used. This extraction is usually preceded by initial nuclear extractions with 0.075 M NaCl:0.025 M sodium EDTA, pH 7.4 to 8.0, and intermediate swelling with 0.01 M Tris, pH 7.4 to 8.0. Addition of 0.1 mM PMSF effectively prevents proteolysis in these experiments; NaHSO₃ was shown to be ineffective (2, 6). An alternative procedure for obtaining NHP from "dehistonized" chromatin is the procedure of Wilson and Spelsberg (42). In this procedure, chromatin extracted first with 0.075 M NaCl, 0.025 M EDTA, and 0.01 M Tris is then extracted with 0.4 N H₂SO₄ to remove histones and small amounts of the acid-soluble nonhistone proteins. The remainder of the proteins

in the chromatin are extracted by treatment with DNase I and subsequent precipitation with 0.4 N HClO₄. There are marked differences in the chromatin protein patterns of normal liver, regenerating liver, and Novikoff hepatoma (Fig. 3) which have been dealt with in earlier publications on this subject (43).

Immunological Studies on a Chromatin Antigen of Novikoff Hepatoma Cells. For immunodiffusion assays rabbit antiserum to chromatin proteins of Novikoff hepatoma cells was placed in the center well, and proteins of a 0.6 M NaCl chromatin extract were placed in one of the side wells; precipitin bands formed as shown in Fig. 4. It was of interest that a similar band was also formed with the chromatin of the Walker tumor, which is completely different in origin and is a solid tumor. As a control, the antiserum was preadsorbed with 0.6 M NaCl extract from 18-hr regenerating rat liver chromatin; this preadsorbed antiserum still recognized the antigen in the Novikoff hepatoma chromatin extract by formation of a precipitin band.

To eliminate the possibility that contaminating serum proteins were responsible for the antigenicity, the serum of normal and tumor-bearing rats, as well as tumor cell membranes, were collected and analyzed by double diffusion analysis. With these samples, no precipitin bands were detected (L. C. Yeoman, J. J. Jordan, R. K. Busch, C. W. Taylor, H. Savage, and H. Busch, manuscript in preparation).

Antigen isolated as described in "Materials and Methods" was lyophilized and dissolved in 0.9 N acetic acid, 10 M urea, and 1% β -mercaptoethanol and subjected to 2-dimensional gel electrophoresis (Fig. 5). A single dense spot was found along with the molecular weight standards. On the migration plots, a molecular weight of 26,000 daltons was calculated for the antigen.

Characterization of Isolated Antigen. The amino acid

Table 1
Amino acid compositions of purified nuclear proteins

Amino acid	Protein analyzed			
	NHP C-14	NHP A-24	Histone 2A	Chromatin antigen
Alanine	8.2	9.6	13.4	7.1
Arginine	4.7	7.4	9.3	5.8
Aspartic acid	11.7	7.3	6.2	9.6
Glutamic acid	13.7	12.3	9.3	13.2
Glycine	8.9	9.2	10.9	9.3
Histidine	1.3	2.4	3.1	2.6
Isoleucine	4.8	5.8	4.7	3.8
Leucine	7.6	10.9	12.4	6.1
Lysine	8.0	11.3	10.9	7.5
Methionine	1.7	0.3	0.0	1.6
Phenylalanine	3.4	0.9	0.8	3.9
Proline	4.2	5.6	3.9	6.5
Serine	7.4	4.5	3.1	8.5
Threonine	6.9	6.5	3.9	6.2
Tryptophan	0.0	0.0	0.0	0.0
Tyrosine	2.2	1.3	2.3	2.1
Valine	5.2	4.9	6.2	6.5
Acidic:basic	1.8	0.93	0.67	1.4
Amino terminus	Lysine	Methionine and acetylserine	Acetylserine	Lysine

analysis (Table 1) shows the antigen is an acidic protein, *i.e.*, the ratio of acidic to basic amino acids is 1:4 (6). Hydrolysis with mercaptoethanesulfonic acid did not result in a tryptophan peak. The amino-terminal amino acid was identified as lysine. The single terminal in a protein migrating as a single spot on 2-dimensional gel electrophoresis indicated that the antigen is highly purified. Table 1 also shows analyses for other proteins.

Analysis of Protein Products Translated from Polyadenylate + mRNA of Novikoff Hepatoma and Normal Liver by 2-Dimensional Gel Electrophoresis. In an effort to develop methods for isolation of the mRNA for synthesis of the chromatin antigen, purification of mRNA was carried out by procedures using binding of the 3'-terminal polyadenylate portion on polyuridylyate-Sepharose (16) which have provided an important inroad into the problem of purification of mRNA species. With the addition of the recent technique of Kabat *et al.* (17, 22), the task of purification of mRNA has been markedly simplified.

To ascertain whether specific antigenic proteins of tumors could be defined in translational systems, the wheat germ system of Roberts and Paterson (33) was utilized initially with ovalbumin mRNA and then with mRNA of normal liver and Novikoff hepatoma. After sedimentation of the wheat germ factors and the other insoluble elements of the system, the supernatant protein-containing fraction was subjected to 2-dimensional gel electrophoresis as shown in Fig. 6. The tracers used for these autoradiographic studies were [³⁵S]methionine with a specific activity of approximately 300 Ci/mmmole.

The patterns revealed intense labeling in 3 major proteins (M-1, M-2, and B-X) in the normal liver and 6 major proteins including M-1, M-2, and several "r" proteins in the Novikoff hepatoma. The corresponding pattern for the wheat germ alone shows a much lower level of isotope incorporation and distribution in very different protein spots. Of the proteins labeled in the normal liver, one (B-X) appears to be normal serum albumin; the others, M-1 and M-2, appear to be identical in mobility with M-1 and M-2 in the Novikoff hepatoma. It is important that very large proteins (M. W. 70,000 to 80,000) are labeled in both of these patterns, indicating that the mRNA species are probably totally translated in this system.

The most remarkable effect is the difference in these patterns for the large concentration of small proteins shown as "r" translated from Novikoff hepatoma mRNA. These proteins migrate in the same region that ribosomal proteins migrate, and studies are in progress to establish the similarities and differences of these proteins. In addition, a protein shown as B-3 in this pattern was found in the tumor pattern but not in the liver pattern. This protein may be one of the antigenically active proteins produced by the Novikoff hepatoma. It is too early in these studies to establish whether there are qualitative differences in the proteins of the tumor and liver patterns, but it is clear that there are quantitative differences in labeling of these proteins.

Discussion

An antigen that forms a precipitin band was detected in

the 0.6 M NaCl extracts of Novikoff hepatoma chromatin and Walker 256 carcinosarcoma with antibodies to 3 M NaCl:7 M urea-soluble chromatin proteins. This antigen was purified to electrophoretic homogeneity by conditions that maintain its nativeness as shown by formation of a precipitin band after purification. Its molecular weight on denaturing 2-dimensional gel electrophoresis is approximately 26,000 which is consistent with a single polypeptide chain protein. Its single-lysine amino terminus is also consistent with a single-chain protein unless contaminants also have lysine termini or blocked termini. Double immunodiffusion assays on sera of normal and tumor-bearing rats have eliminated the possibility that this antigen is from serum proteins. However, some cytoplasmic fractions contain an immunological antigen identical to the nuclear antigen.

The immunological evidence that indicates that this protein is common to the Novikoff hepatoma and the Walker 256 carcinosarcoma is important since these 2 tumors are of widely dissimilar origins. This antigen may be present in other tumors, but it is absent from normal and regenerating liver. The absence of this antigen from regenerating rat liver suggests that it is not a growth-specific antigen. The regenerating rat liver was used at 18 hr after hepatectomy because it has enhanced amounts of C-region nonhistone chromosomal proteins CP, C-21, C-18, CQ, C-15, and CT, recently described for "Chromatin Fraction II" (43).

One of the most intriguing aspects of this is whether it will now be possible to isolate the polysomes that provide the template for synthesis of this antibody. Studies currently in progress have indicated that with the matrix method (34) it will be possible to purify the antibodies for this antigen. With these antibodies, it should then be possible to isolate and purify the mRNA for its synthesis from the Walker and Novikoff cells. If a satisfactory complementary DNA can be made, hybridization studies will be carried out to determine whether the gene segment that produces these RNA species exists in the tumors and the nontumor tissues or whether it is possible to limit it to the tumors. The possibility exists that its product represents the presence of an exogenous gene segment that has been integrated into the genome. On the other hand, it may represent an embryonic DNA that is only activated in neoplasia. A great deal of meticulous research is required to elucidate these important points.

One point that is clear is that the antibody does not produce surface phenomena such as agglutination of cells. When antibodies to tumor ribosomes were prepared, they caused agglutination of Novikoff hepatoma cells (9). Corresponding antibodies to liver ribosomes did not exhibit surface phenomena and neither did the chromatin antibodies. Accordingly, there seems little doubt that these antibodies are directed against intracellular proteins rather than against any of the surface proteins of such cells.

A goal of future studies on the antigens is to ascertain whether the DNA involved in their production is endogenous or exogenous. For this purpose, the translational studies that have been initiated have shown quantitative differences in products of mRNA of the Novikoff hepatoma and the normal liver. The matrix method (34) is being tested for selective isolation of polysomes synthesizing these antigens. If it is feasible selectively to isolate the polysomes that

produce these antigens, their mRNA will be extracted and utilized for synthesis of the corresponding complementary DNA. It will then be possible to determine by hybridization whether corresponding DNA exists in both tumor and liver cells and whether any unique DNA exists in the tumor cells that codes for these antigens.

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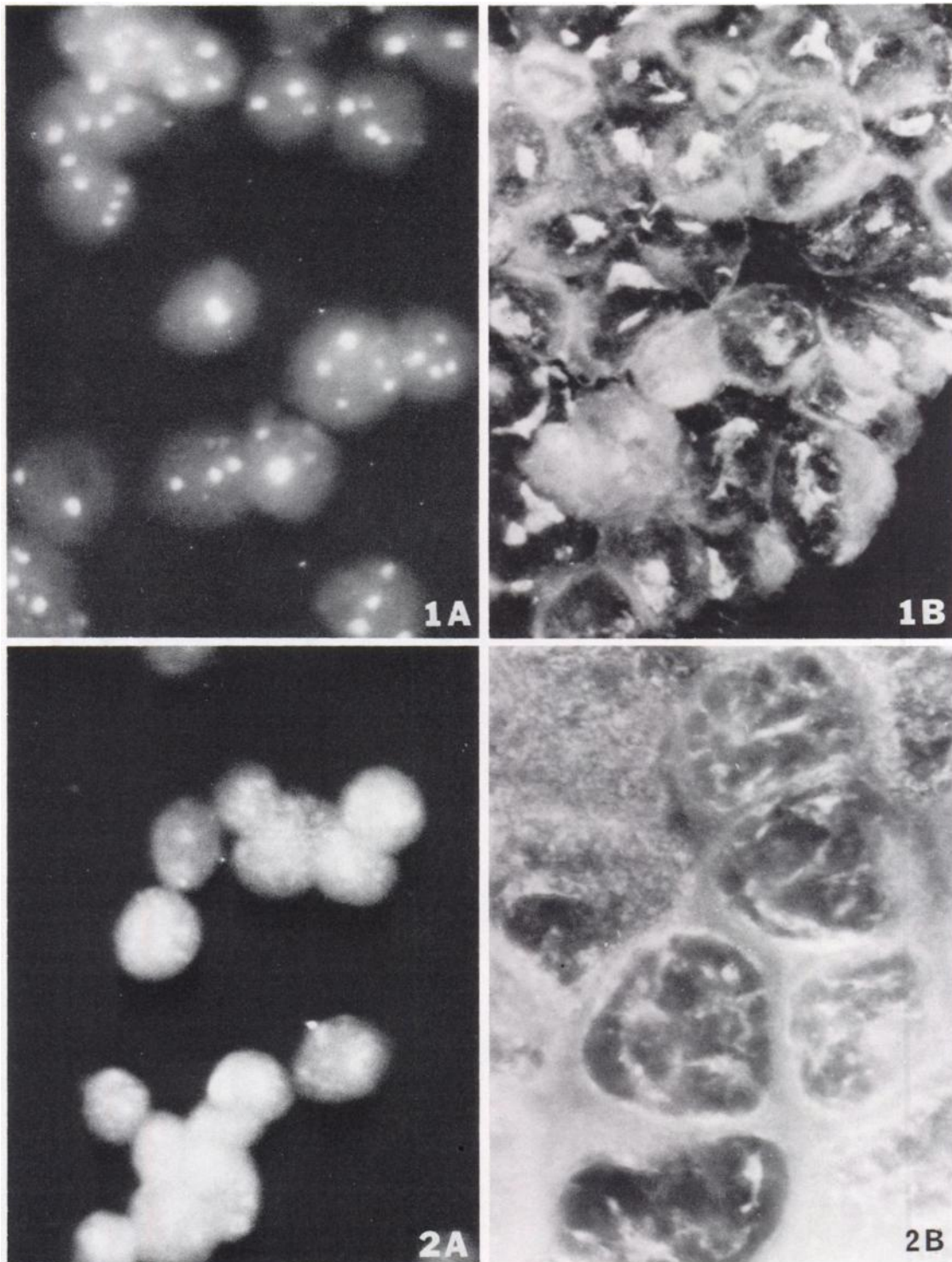


Fig. 1. *A*, immunofluorescence produced in nuclei isolated by the citric acid method on exposure to rabbit antinucleolar antibodies and fluorescein-labeled goat antirabbit antibodies. *B*, immunofluorescence produced in cells after exposure to rabbit antinucleolar antibodies and fluorescein-labeled goat antirabbit antibodies.

Fig. 2. *A*, immunofluorescence produced in nuclei isolated by the citric acid method with antibodies to the chromatin fraction soluble in 3 M NaCl:7 M urea after reduction of the ionic strength to 0.15. Antibody localization was detected by indirect immunofluorescence, *i.e.*, by staining with fluorescein-labeled goat antirabbit antibodies. *B*, immunofluorescence produced in cells after exposure to antichromatin antibodies.

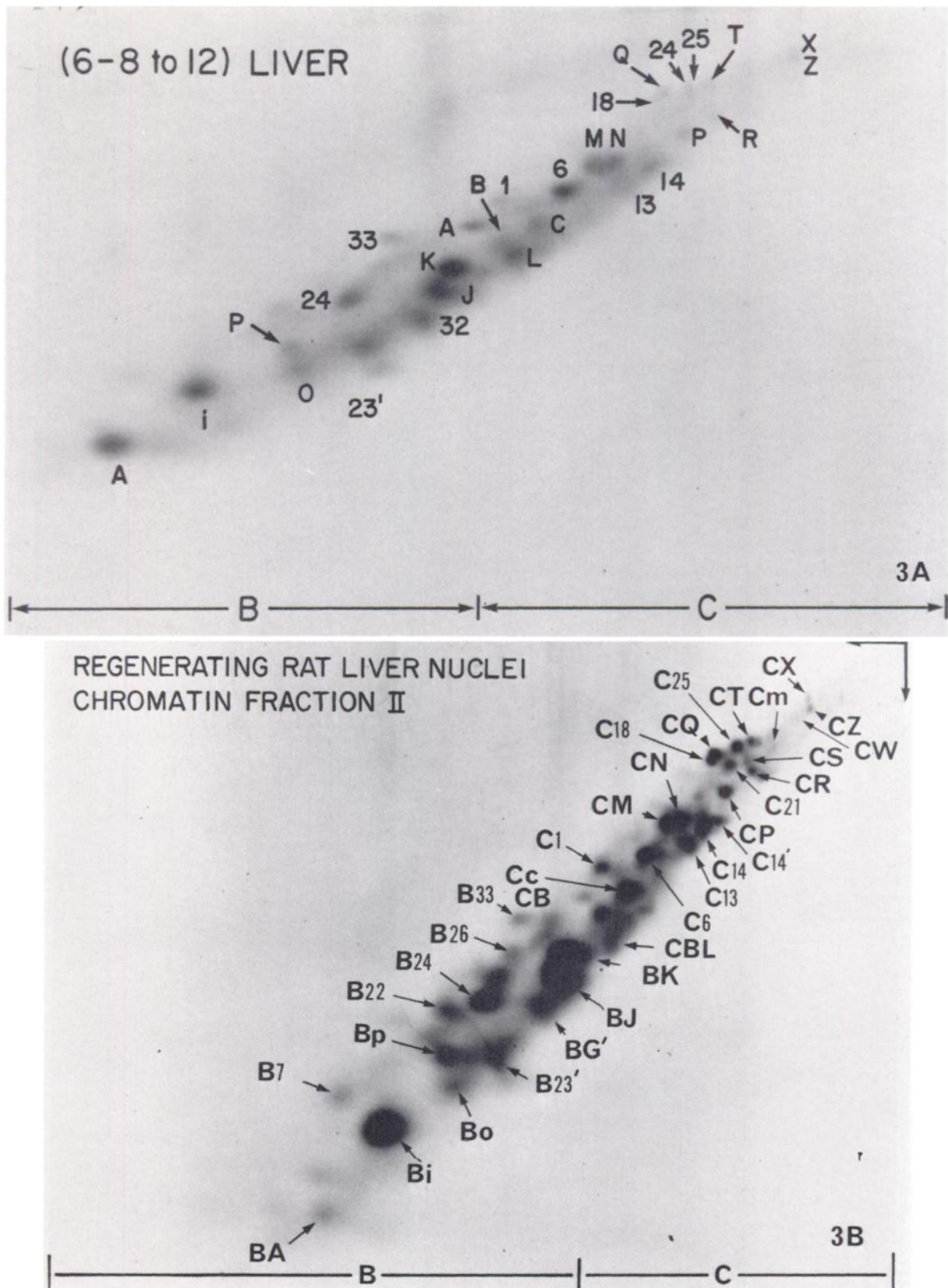


Fig. 3. Two-dimensional electrophoretic patterns of nonhistone proteins extracted from chromatin of normal liver (A), regenerating liver (B), and Novikoff hepatoma (C) by the method of Wilson and Spelsberg (42).

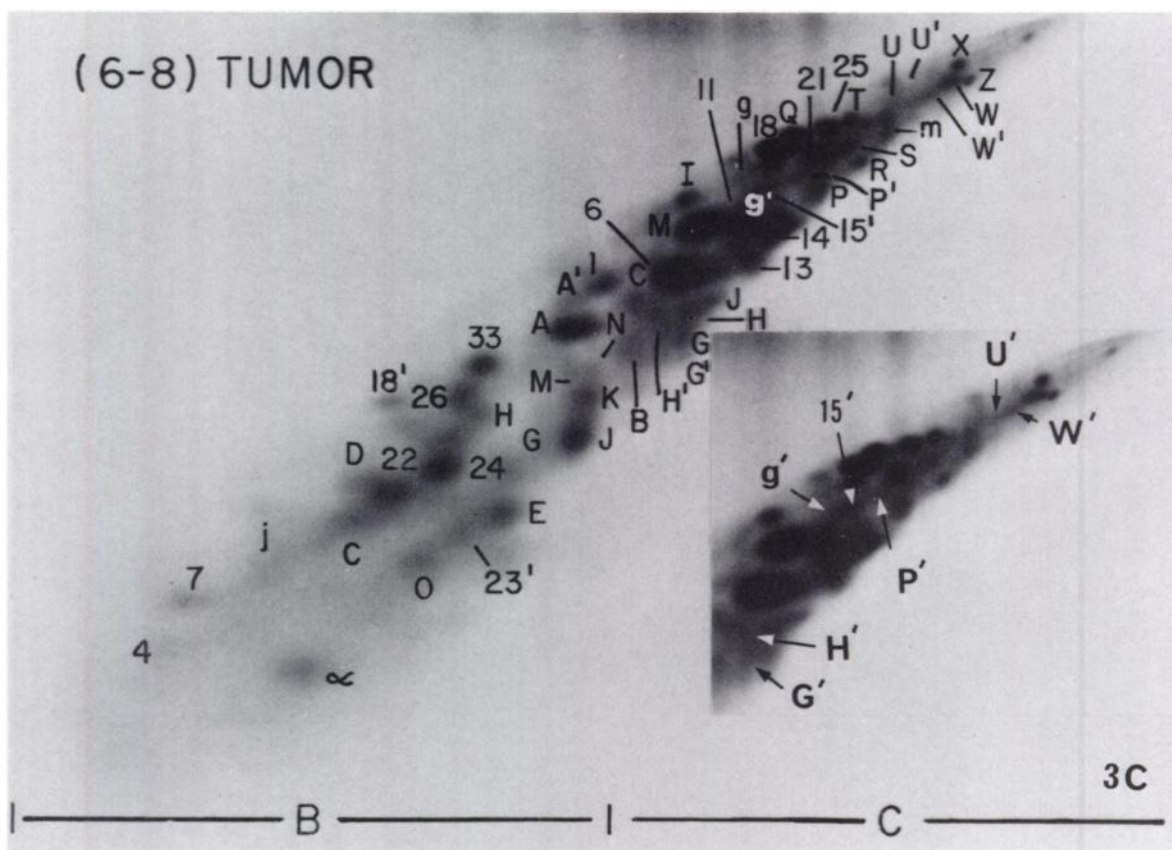


Fig. 3c

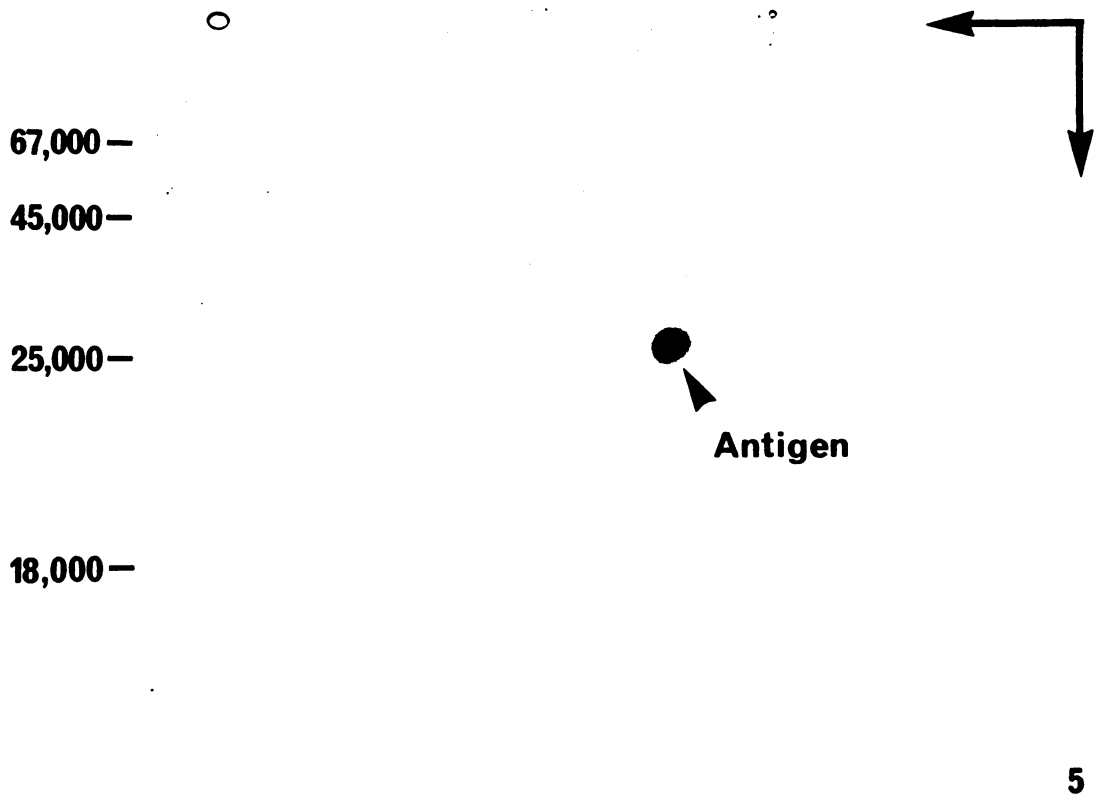
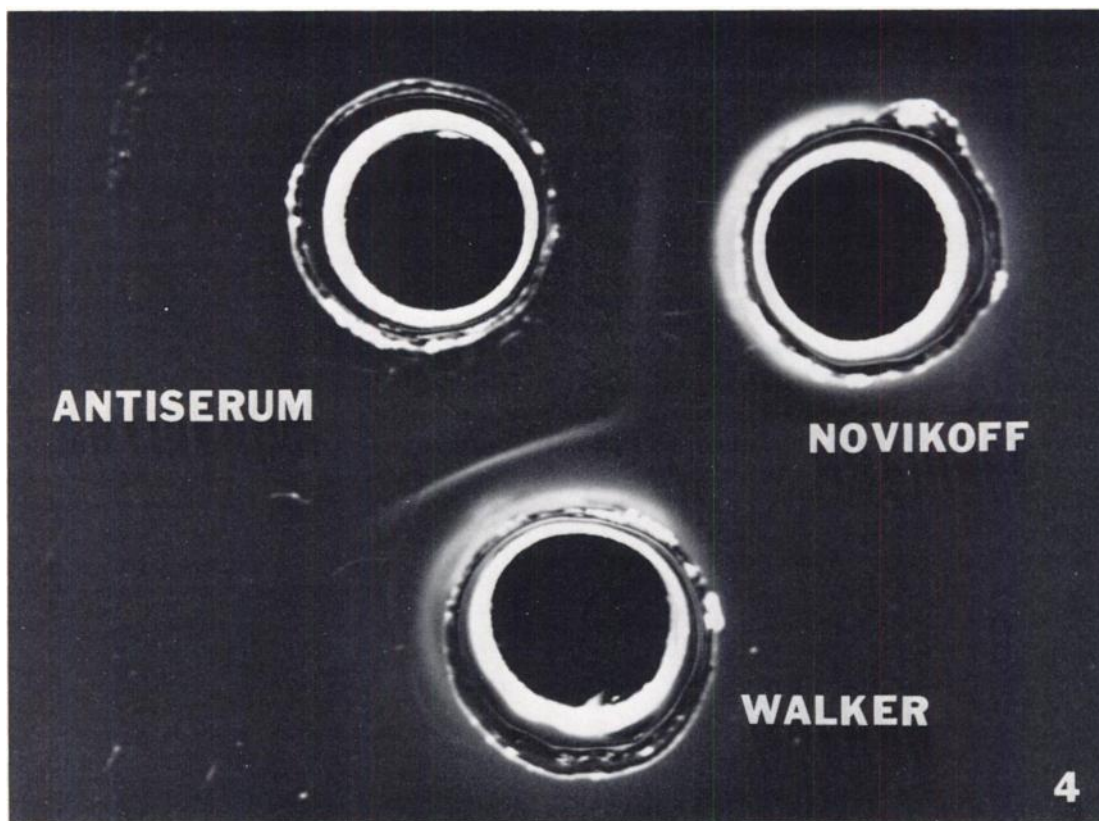


Fig. 4. Double diffusion analysis of 0.6 M NaCl extracts from Novikoff hepatoma and Walker 256 carcinosarcoma chromatin. A 40- μ l aliquot of the Novikoff hepatoma 0.6 M NaCl extract was placed in the well labeled "Novikoff;" a 40- μ l aliquot of the Walker 256 carcinosarcoma 0.6 M NaCl extract was placed in the well labeled "Walker." Antiserum (40 μ l) was placed in the well labeled "Antiserum." Protein concentrations were 15 mg/ml. Precipitin bands were detected after 18 hr of diffusion in a moist chamber.

Fig. 5. Two-dimensional polyacrylamide gel electrophoresis of highly purified antigen. Purified antigen (7 μ g) was subjected to electrophoresis with 10 μ g myoglobin, α -chymotrypsinogen, ovalbumin, and albumin on a 2-dimensional polyacrylamide gel. The 1st-dimension tube gel of 10% acrylamide:4.5 M urea:0.9 N acetic acid was run at 120 V for 6 hr. The 2nd-dimension slab gel of 12% acrylamide:0.1% sodium dodecyl sulfate:0.1 M phosphate was run at 50 ma/slab for 16 hr. Gels were stained with Coomassie brilliant blue R in acetic acid:methanol:water (1:5:5) for 6 hr. Positions of molecular weight markers are shown at the left.

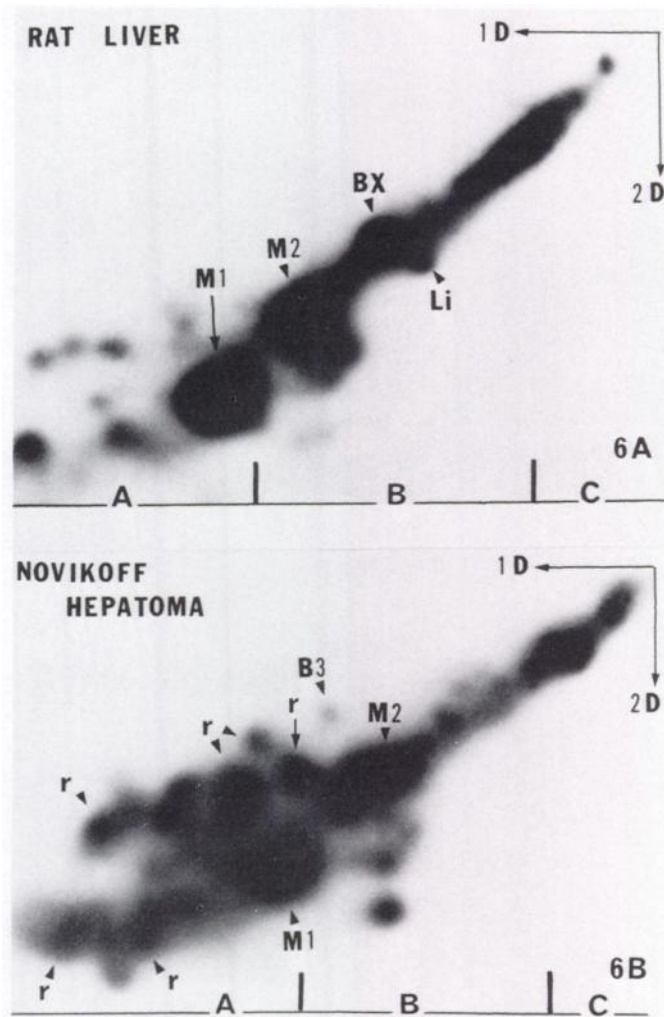


Fig. 6. Autoradiography of [35 S]methionine-labeled proteins synthesized *in vitro* by the wheat germ system to which polyadenylate (+) mRNA of liver (A) and Novikoff hepatoma (B) were added as a template. Several proteins labeled to a large extent in the liver are insignificantly labeled in the tumor; further, several proteins of low molecular weight are highly labeled in the tumor and insignificantly labeled in the liver. It remains to be seen whether any of these proteins is a specific product of the tumor or the liver.