Cellular Distribution of the Hamster Liver Specific Nucleolar Antigens

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Abstract. The immunochemical localization of hamster liver nucleolar antigens in subcellular fractions (nuclei, 10,000 × *g* pellet, 100,000 × *g* pellet and supernatant), nuclear substructures (chromatin, nuclear matrix, nuclear envelope, nucleoli, RNP particles and nucleosomes), and three classes of nonhistone chromosomal proteins with different affinities to DNA (NHCP1, NHCP2 and NHCP3) from nuclease-sensitive and nuclease-resistant chromatin fractions of hamster liver were studied. Six main nucleolar antigens with mol. wts 27,000; 29,000; 30,000; 36,000; 45,000; and 46,000 were found in subcellular fractions, nuclear substructures and classes of non-histone proteins of hamster liver. The antigens with mol. wts of approx. 27,000; 29,000; and 36,000 which were absent in hamster pancreas, spleen and Kirkman—Robbins hepatoma nuclei, seem specific for liver tissue.

Key words: Nucleolar antigens — Immunochemical localization — Cellular distribution — Hamster liver

Introduction

Studies on the nuclear antigens of tumour and normal tissues indicated that there might exist important differences in these proteins (Yeoman et al. 1978; Yeoman 1978; Hnilica and Briggs 1980; Wojtkowiak et al. 1982). Nucleolus, being recognized as hyperactive and highly pleomorphic in cells of malignant tissues unlike in most normal tissues (Busch and Smetana 1970), is widely used in the search for antigens which distinguish cancer cells. Earlier attempts involved the isolation of tumour nucleoli as the source of antigens and subsequent immunization to produce polyclonal antibodies able to recognize tumour but not normal cells. Chan et al. (1980, 1981, 1982) purified from tumour cells nucleolar proteins with M_r 54,000; 61,000; and 68,000 not detectable in normal

human tissues. Recently monoclonal antibodies were used to identify a number of nucleolar antigens (p40,p120,p145) in proliferating, but not normal resting cells (Chatterjee et al. 1987; Freeman et al. 1988; Ochs et al. 1988; Freeman et al. 1986, 1987). Antigens specific for different normal tissues were also detected (Bray et al. 1980) including the antigen specific for rat liver absent in Novikoff hepatoma (Abelev et al. 1979; Busch et al. 1979).

The role of normal liver specific antigens in the structure and function of the nuclei is poorly understood. Therefore we tried to localize these proteins and characterize them partly.

The aim of this study was the immunochemical localization of hamster liver nucleolar antigens in hamster liver subcellular fractions (nuclei, $10{,}000 \times g$ pellet, $100{,}000 \times g$ pellet and supernatant), nuclear substructures (chromatin, nuclear matrix, nuclear envelope, nucleoli, RNP particles and nucleosomes), and three classes of non-histone chromosomal proteins (NHCP1, NHCP2 and NHCP3) with different sensitivities to nuclease treatment and different affinities to DNA. Hamster pancreas, spleen and Kirkman—Robbins hepatoma nuclei were used for comparative purposes.

Polyacrylamide gel electrophoresis (Laemmli 1970), electrophoretic transfer of separated proteins to nitrocellulose (Towbin et al. 1979) and the PAP method (Sternberger 1979) were used for the immunochemical detection of antigens. Antigenic properties of non-histone chromatin proteins (Wakabayashi and Hnilica 1973) and sensitivity, as well as selectivity of the immunochemical methods used (Glass et al. 1981; Wojtkowiak et al. 1982, 1983; Wojtkowiak and Kłyszejko—Stefanowicz 1985) appeared to be particularly useful in the investigation of nuclear structures.

Material and Methods

Liver of 8-week Golden Syrian hamster and Kirkman—Robbins hepatoma were used. All preparative work was done at 4°C, and all solutions used contained 1 mmol/l phenylmethylsulphonyl fluoride (PMSF). The inhibitor was added as 100 mmol/l solution in isopropyl alcohol immediately before use. The nuclei of hamster liver (L) and Kirkman—Robbins hepatoma (H) as well as those from hamster pancreas (P) and spleen (S) were isolated by the method of Chauveau et al. (1956)) with additional treatment of nuclei with 0,4% Triton X-100.

The cytoplasmic fractions from hamster liver were centrifuged twice at $1000 \times g$ for 10 min to remove traces of nuclei. The supernatant was centrifuged at $10{,}000 \times g$ for 30 min ($10{,}000 \times g$ pellet — 10P). The second supernatant was centrifuged at $100{,}000 \times g$ for 1 h, and the pellet and the supernatant were isolated ($100{,}00 \times g$ pellet — 100P and $100{,}000 \times g$ supernatant — 100S).

Nuclear matrix (M) was isolated according to the method of Berezney and Coffey (1977) by extraction of nuclei with 0.2 mmol/l $MgCl_2$, 10 mmol/l Tris.HCl, pH 7.4 buffer, 2 mol/l NaCl and 1% Triton X-100. The nuclei were digested with DNaseI before final washing with 10 mmol/l Tris. HCl, pH 7.4, containing 0.25 mol/l sucrose and 5 mmol/l $MgCl_2$.

Chromatin (Ch) was isolated according to Spelsberg and Hnilica (1971).

Inner layer of nuclear envelope (E) was obtained by the method according to Berezney and Coffey (1977) with addition of heparin as described by Widmer and Parish (1980).

Nucleoli (No) were isolated by the procedure according to Busch (1967). Hamster liver nuclei were sonicated in 0.34 mol/l sucrose, 1 mmol/l $CaCl_2$ buffered with 5 mmol/l Tris. HCl, pH 7.4, until almost all nuclei were broken. Nucleoli were purified by centrifugation through 0.88 mol/l sucrose.

The RNP particles (RNP) were released from nuclei also by sonication (Pederson 1974). The sonicate was centrifuged at $16{,}000 \times g$ for 15 min to collect the RNP particles (Walker et al. 1980).

Monomeric nucleosomes (Nu) were obtained by digesting isolated nuclei with micrococcal nuclease followed by extraction of the digested nuclei with 0.2 mmol/l EDTA and differential centrifugation according to Whittaker et al. (1979).

The nuclease-sensitive and nuclease-resistant chromatin fractions were obtained from hamster liver nuclei. The nuclear pellet was suspended in 10 mmol/l Tris.HCl, pH 7.5, 0.35 mol/l sucrose, 0.25 mmol/l $CaCl_2$ and 100 mmol/l NaCl at a DNA concentration of 2 mg/ml and digested for 150 s at 25°C with micrococcal nuclease (Sigma), 1 unit of enzyme per 1 mg DNA. The reaction was terminated by adding EDTA to a final concentration of 10 mmol/l. The digested nuclei were centrifuged at $12{,}000 \times g$ for 10 min yielding supernatant, i.e., nuclease-sensitive (NS), and pellet, i.e., nuclease-resistant (NR) chromatin fractions. Non-histone chromosomal proteins from both fractions were fractionated according to Kiliańska et al. (1980). The method is based on the separation of nuclear proteins from DNA with a polyethylene glycol-dextran mixture and fractionation of the proteins by hydroxyapatite chromatography. The non-histone chromosomal protein fractions NHCP1, NHCP2 and NHCP3 were eluted from hydroxyapatite with 50 mmol/l (Na^+ form), 100 and 200 mmol/l (K^+ form) phosphate buffer, pH 6.8 in 2 mol/l NaCl, 8% glycerol, respectively.

Two New Zealand rabbits were immunized with nucleoli suspended in 2 mmol/l Tris.HCl, pH 7.5 following the schedule of Wakabayashi and Hnilica (1973). Briefly, rabbits were injected intradermally at weekly intervals for 5 weeks with 0.4 mg protein emulsified with equal volume of complete (twice) and/or incomplete (thrice) Freund's adjuvant. Blood was obtained by marginal ear venopuncture 7 days after the last injection. The serum was heat inactivated at 56°C for 30 min and stored at −20°C.

Electrophoresis in polyacrylamide gels was carried out according to Laemmli (1970). Samples containing 1 mg DNA were incubated with 20 μg of DNaseI in 10 mmol/l Tris.HCl, 1 mmol/l $MgCl_2$, pH 7.4 at 37°C for 1 h. The digested samples were mixed with 0.9 vol. of solution containing 4.44% sodium dodecyl sulphate (SDS), 22.2% glycerol, pyronin Y 25 μg/ml, 0.139 mol/l Tris.HCl, pH 6.8, and 0.1 vol. of β-mercaptoethanol, and heated for 2 min in a boiling-water bath, and left to stand overnight at room temperature. The samples not containing DNA were mixed and heated without DNase digestion. The solubilized samples were applied to vertical 3% stacking and 11% running gels. Molecular weights were determined by comparing relative mobilities of proteins bands to those of standard proteins (Low Molecular Weight Calibration Kit, Pharmacia).

The electrophoretically separated proteins in parallel gels were either stained with Coomassie Brilliant Blue R-250 or transferred to nitrocellulose sheets (Towbin et al. 1979; Glass et al. 1981). The transfers were placed in 10 mmol/l sodium phosphate-buffered 0.14 mol/l NaCl, pH 7.2, containing 3% bovine serum albumin. After gentle shaking at 40°C for 1 h, the sheets were incubated at room temperature for 3 h with diluted (1:100) rabbit antiserum to hamster liver nucleoli. Next, the nitrocellulose sheets were washed with 4 changes of phosphate-buffered saline (PBS; 50 ml for 10 min each) and incubated for 1 h with antiserum to rabbit immunoglobulins (Gibco) diluted 1:200. Finally, the sheets were placed into peroxidase-antiperoxidase (Miles) diluted 1:200 for 30 min and washed as above. The antigens were detected by staining with 0.03% diaminobenzidine and 0.005% H_2O_2 in 50 mmol/l Tris.HCl buffer, pH 7.5 (Sternberger 1979).

Protein and DNA contents were determined according to Lowry et al. (1951) and Burton (1956), respectively.

Results

Electrophoretically separated proteins from hamster liver subcellular fractions, nuclear substructures and three classes of non-histone chromosomal proteins were transferred to nitrocellulose and immunochemically stained with antiserum raised against hamster liver nucleoli. Immunochemical localization of the nucleolar antigens showed that though at trace amounts they are strongly immunologically reactive. They are visible in whole nuclei and enriched in some nuclear substructures, but poorly represented in nucleoli preparations. This can be due to different availability of antigenic determinants and/or the quantity of antigens in these substructures. The major hamster liver nucleolar antigens are characterized by mol. wts 27,000; 29,000; 30,000; 36,000; 45,000; and 46,000. The bands of mol. wts 27,000; 29,000; 36,000; and 46,000 visible in liver nuclei preparations were absent in hepatoma nuclei (Fig. 1). The antigens of mol. wts

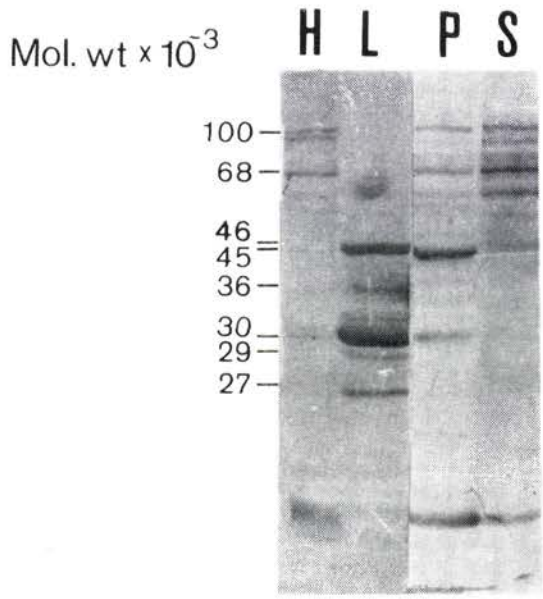

Fig. 1. Electrophoretical separation in SDS polyacrylamide gel of hamster proteins from Kirkman—Robbins hepatoma nuclei (H), liver nuclei (L), pancreas nuclei (P) and spleen nuclei (S). The separated proteins were transferred to nitrocellulose sheets and stained by PAP method with antiserum to hamster liver nucleoli.

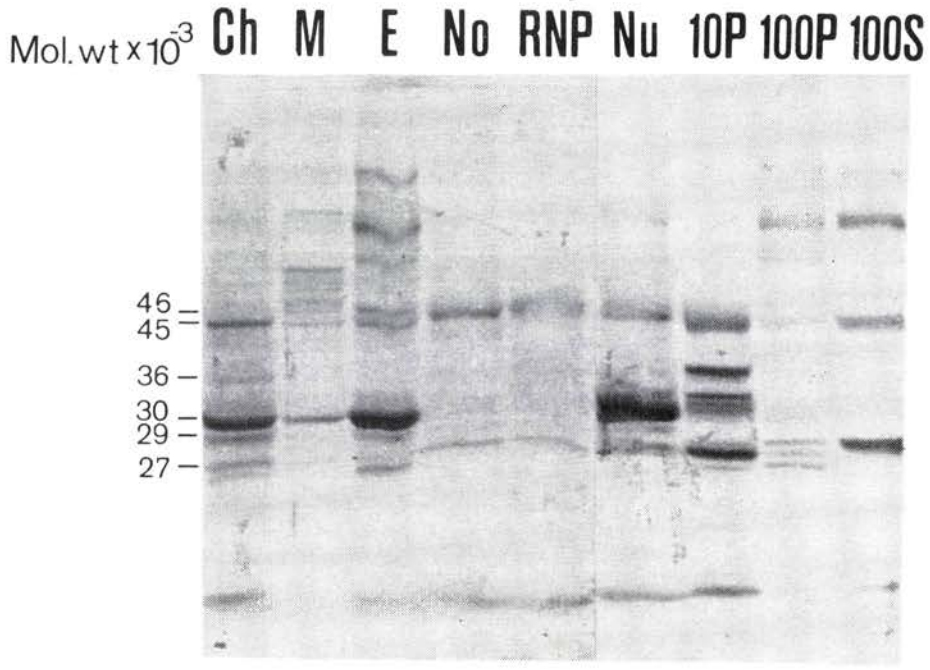

Fig. 2. Electrophoretical separation in SDS polyacrylamide gel of hamster liver substructure proteins from chromatin (Ch), nuclear matrix (M), nuclear envelope (E), nucleoli (No), RNP particles (RNP), nucleosomes (Nu), 10,000 × *g* cytoplasmic pellet (10P), 100,000 × *g* cytoplasmic pellet (100P) and 100,000 × *g* cytoplasmic supernatant (100S). The separated proteins were transferred to nitrocellulose sheets and stained by PAP method with antiserum to hamster liver nucleoli.

27,000; 29,000; and 36,000 not present in pancreas and spleen nuclear proteins are likely specific for liver tissue. The protein of mol. wt 46,000 present also in spleen may be characteristic for normal hamster tissues.

The antigen of mol. wt 27,000 was present in chromatin, nucleosomes, inner layer of nuclear envelope, and cytoplasmic fractions (only weakly visible in nucleoli and RNP particles). This antigen is associated with transcriptionally active chromatin as confirmed by its presence in the micrococcal nuclease-sensitive chromatin fraction. Being mostly accumulated in the NHCP1 fraction it is likely loosely bound to DNA (Figs. 2 and 3).

The antigens of mol. wts 29,000 and 30,000 occurred in chromatin, nuclear envelope, weakly in nuclear matrix and mainly in nucleosomes. The lack of differences in the distribution of these antigens between the three classes of NHCP of both nuclease-sensitive and nuclease-resistant chromatin fractions confirmed their presence in many nuclear substructures (Figs. 2 and 3).

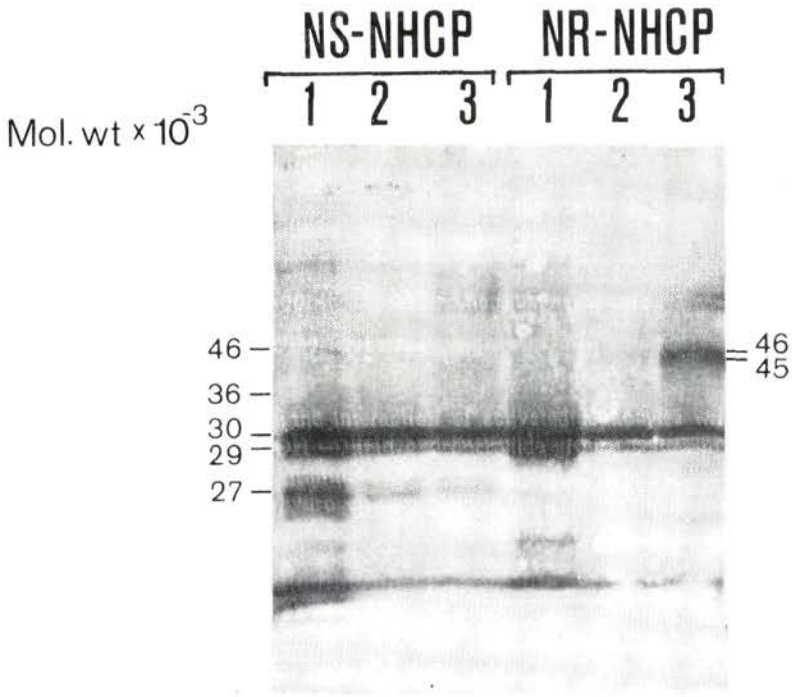

Fig. 3. Electrophoretical separation in SDS polyacrylamide gel of hamster liver non-histone proteins NHCP1, NHCP2 and NHCP3 from nuclease-sensitive (NS) and nuclease-resistant chromatin (NR). The separated proteins were transferred to nitrocellulose sheets and stained by PAP method with antiserum to hamster liver nucleoli.

Another nucleolar antigen occurring in the band for mol. wt of approx. 36,000 could only be detected in chromatin and $10{,}000 \times g$ cytoplasmic pellet. This antigen most probably is a cytoplasmic contamination of chromatin as it was absent in the three classes of NHCP of both nuclease-sensitive and nuclease--resistant fractions of chromatin (with the exception of traces found in NS-NHCP) (Figs. 2 and 3).

The antigen of mol. wt 45,000 was visible in the preparations of chromatin, nuclear matrix, nuclear envelope and in the $10{,}000 \times g$ cytoplasmic pellet and the $100{,}000 \times g$ cytoplasmic supernatant. The antigen of mol. wt 46,000 could also be detected in nuclear matrix, nuclear envelope, nucleosomes and additionally in RNP particles and nucleoli. The participation of both antigens in the skeleton structures of nuclei seems to be confirmed by their accumulation in the NHCP3 fraction from the hamster liver nuclease-resistant part of chromatin. Thus it is

clear that they are tightly associated with the nuclear heterochromatin (Figs. 2 and 3).

Discussion

The main finding of the present investigation has been the immunochemical localization of six hamster liver nucleolar antigens in subcellular fractions (nuclei, 10,000 × *g* pellet, 100,000 × *g* pellet and supernatant), nuclear substructures (chromatin, nuclear matrix, nuclear envelope, nucleoli, RNP particles and nucleosomes) as well as in the three classes of non-histone chromatin proteins with different affinities to DNA (NHCP1, NHCP2 and NHCP3) from nuclease-sensitive and nuclease-resistant chromatin fractions of hamster liver.

These antigens had mol. wts 27,000; 29,000; 30,000; 36,000; 45,000; and 46,000 (Figs. 1 and 2). The antigen with mol. wt of 27,000 was found in chromatin, nucleosomes, inner layer of nuclear envelope and cytoplasmic fractions. The position of the three bands for mol. wt of about 27,000 in NHCP1 of nuclease-sensitive chromatin fraction (Fig. 3) was identical with that for non-histone protein BA identified by Bennet et al. (1986) as glutatione S-Transferase. Non-histone protein BA was present in chromatin isolated from normal liver and lymphocytes but almost undetectable in chromatin isolated from various neoplastic tissues (Yeoman et al. 1975; 1978). Antibody to HMG-1 protein (with mol. wt similar to that of the 27,000 antigen described in this paper) did not significantly stain the nuclei, but reacted predominantly with the cytoplasm and nucleoli of several mammalian cell lines (Bustin and Neihart 1979).

We observed the presence of nucleolar antigens of mol. wt 29,000 and 30,000 in chromatin, nuclear envelope, nucleosomes and nuclear matrix. It should be noted that similar proteins were revealed by Comings and Peters (1981), in rat liver nucleoli (mol. wts 28,500; 29,500; 30,000; 30,500; and 33,000). Also Williams et al. (1982) found soluble phosphoprotein of nuclear sap (nucleoplasmin — mol. wt 30,000) from *Xenopus laevis* oocytes.

Another hamster liver nucleolar antigen occurring in the band at mol. wt of approx. 36,000 was detected in chromatin and in 10,000 × *g* cytoplasmic pellet. Proteins with identical or similar molecular weights were reported by Banville and Simard (1982) as CHO cells nucleolar phosphoprotein (mol. wt 38,000), by Comings and Peters (1981) in rat liver nuclear RNP particles and by Pieck et al. (1985) in bovine liver chromosomal residual structure polypeptides (mol. wt 37,000) after extraction with 2 mol/l NaCl.

The nucleolar antigen of mol. wt 45,000 was found in our preparations of

chromatin, nuclear matrix, nuclear envelope and in the $10,000 \times g$ cytoplasmic pellet and the $100,000 \times g$ cytoplasmic supernatant. The antigen of mol. wt 46,000 was observed in nuclear matrix, nuclear envelope, nucleosomes, and additionally in RNP particles and nucleoli. Closely spaced doublet bands were found in rat liver chromatin and nuclear matrix proteins (mol. wt 47,000 and 48,000) by Comings and Peters (1981). Additionally protein of mol. wt 47,000 was detected in chromosomal residual structure proteins from bovine liver cells (Pieck et al. 1985). We found traces of mol. wt 46,000 component in NHCP1 fraction from hamster liver nuclease-sensitive portion of chromatin (Fig. 3). Bands of similar molecular weights (44,500 and 45,200) were revealed in rat liver polypeptides released by nuclease or DNaseI treatment (Inoue et al. 1983).

The localization of liver-specific nucleolar antigens of approximate mol. wts 27,000; 29,000; and 36,000 was described; however, further characterization is required to explain the role of these antigens in the structure and function of nuclei.

Abbreviations used: PMSF — phenylmethylsulphonyl fluoride
SDS — sodium dodecyl suphate
PAP — peroxidase-antiperoxidase
HMG-1 — high-mobility-group protein 1
CHO — Chinese Hamster ovary cells
EDTA — ethylenediaminetetraacetic acid disodium salt

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