Journal of Cellular Biochemistry 45:303–310 (1991)

Hepatoma-Associated Nuclear Matrix Nonhistone Antigens

Zofia Kilianska, Wanda M. Krajewska, Ronglin Xie, Leokadia Klyszejko-Stefanowicz, and Jen-Fu Chiu

Department of Cytobiochemistry, Institute of Biochemistry, University of Lodz, 90-237 Lodz, Poland (Z.K., W.M.K., L.K.-S.); Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405 (R.X., J.-F.C.)

Abstract Polyclonal antibodies generated against a group of high molecular weight nonhistone proteins from Morris hepatoma 7777 were used in immunological studies of hepatoma-associated nonhistone proteins in rat and hamster. We revealed the presence of cross-reactive antigens in rat Morris hepatomas 7777 and 8994, and in hamster Kirkman-Robbins hepatoma, but not in normal rat or hamster livers. These specific nonhistone proteins were found to be preferentially localized in the nuclear matrix of rat Morris hepatoma 7777 as well as hamster Kirkman-Robbins hepatoma.

Key words: hepatoma-associated antigens, nonhistone proteins, nuclear matrix, polyclonal antibodies, Morris hepatoma 7777, Kirkman-Robbins hepatomas

Nonhistone proteins (NHPs) that are specifically expressed in neoplastic cells have been identified in several laboratories [1–6]. This class of nuclear proteins is thought to be involved in the regulation of gene activity and to have a structural function in chromatin [7,8]. There is evidence that the organization of genetic material into the dynamic structure of chromatin is important for nuclear function [9,10]. In nuclei of a variety of eukaryotic cell types, chromatin is anchored to structural proteins called the nuclear matrix. The nuclear matrix is a substructure composed predominantly of nonhistone proteins which resist extraction by detergents, nucleases, and low and high ionic strength buffers [11,12]. The nuclear matrix has been increasingly implicated in dynamic processes, such as chromatin organization, DNA replication, gene transcription, and RNA processing [13–16]. Specific DNA sequences within defined cellular genes have also been identified which exhibit highly specific interactions with protein components of the nuclear matrix, suggesting that such proteins may delineate both structural and functional DNA domains [14,17–19]. Such a diversity in the functional activities associated with the nuclear matrix makes it necessary to identify differences in the expression of its protein components. The nuclear matrix proteins, localized in the interior of the nucleus, vary in a cell-type-specific manner and have been shown to be altered during transformation [20–24].

Attempts in the search for possible hepatoma specific nonhistone proteins revealed the changes in nuclear protein composition accompanying liver carcinogenesis [25–34]. A group of nonhistone proteins with mol. wt. ranging from 160,000 to 200,000 daltons has been found to be specifically associated with rat hepatomas [35,36].

In this study we have examined the specificity of the above proteins in relation to a hamster hepatoma, i.e., Kirkman-Robbins hepatoma. In order to investigate the role of these proteins in nuclear functions, their localization in the nuclear matrix was analyzed.

MATERIALS AND METHODS

Animals and Tumors

Transplantable Morris hepatoma 7777 and 8994 cells were grown in the thighs of Buffalo rats. Hepatomas were harvested when tumors reached 1–2 cm in diameter.

Kirkman-Robbins hamster hepatoma was obtained from the Department of Oncology, School of Medicine in Lodz. A 0.2 ml sample of a me-

Received June 25, 1990; accepted November 9, 1990.

Address reprint requests to Zofia Kilianska at the address given above.

chanically dispersed tumor cell suspension taken from 9 day-old cancer was inoculated subcutaneously above the axilla of Syrian hamsters.

Chemically induced hepatomas were produced by feeding male Fisher rats (Charles River Breeding Laboratories, Wilmington, MA) of 120–150 g initial weight with Wayne Laboratory pellets (Allied Mills, Inc., Chicago, Ill.) containing 10% corn oil (Mazola) and 0.06% 3′-methyl-4-dimethylaminoazobenzene (3′-MDAB, Eastman Kodak Co., Rochester, NY) according to Chiu et al. [37]. The rats in control groups were fed pellets with 10% corn oil.

Livers of normal rats and hamsters were also used as reference tissues.

Preparation of High Molecular Weight NHPs

Nuclei were isolated from hepatomas and livers using the modified procedure of Burkhardt et al. [35]. Chromatin was obtained according to Chiu et al. [38]. Dehistonized chromatin was prepared as described [37]. Briefly, freshly prepared chromatin was resuspended in 2.5 M NaCl, 5 M urea, 50 mM sodium acetate (pH 5.0), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and stirred for 2 h at 4°C. The chromatin was then centrifuged at 100,000*g* for 36 h. The pellet was then resuspended in 10 mM Tris-HCl (pH 7.6), 0.1 mM $MgCl_2$, 0.5 mM PMSF, and stirred for 14 h at 4°C. Undissolved material was removed by centrifugation at 1,000*g* for 5 min. The supernatant, containing NHPs-DNA complexes, was adjusted to 1 mM $MgCl_2$ and digested with pancreatic DNase I (50 μg/mg DNA) for 30 min at 37°C. After adjustment to final concentration of 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, and 0.0625 M Tris-HCl (pH 6.8), the digestions were applied to a column (diameter 2.5 cm) containing 120 cm of Sephadex G-100 and 30 cm of Sephacryl S-100 and equilibrated with the above buffer. NHPs were eluted with the same buffer and fractions of 2 ml were collected at a flow rate of 6 ml/h [36]. The presence of NHPs with high molecular weight was estimated by one-dimensional SDS-polyacrylamide gel electrophoresis [39].

Immunization of Rabbits

New Zealand white rabbits were immunized with Morris hepatoma 7777 high molecular weight NHPs according to Chytil [40]. Antisera were subjected to immunoabsorption with normal rat liver chromatin as described by Chiu et al. [38]. Immunoglobulins were precipitated with 40% saturated ammonium sulfate and were further purified by DEAE-cellulose [41].

Fractionation of Nuclei

Nuclei of Morris hepatoma 7777 and Kirkman-Robbins hepatoma (2 mg DNA/ml) were suspended in 0.25 M sucrose, 5 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.4), 1 mM PMSF, and endogeneously digested for 45 min at 37°C. The suspension was spun down at 780*g* for 20 min. The pellet was extracted twice with low salt extraction buffer (10 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$, 1 mM PMSF) and four times with high salt buffer (2 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$, 1 mM PMSF) according to the procedure of Berezney [12,42]. The residual pellet was then washed with low salt buffer and the remaining fraction was nuclear matrix.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel (8% acrylamide/0.8% bis-acrylamide running gel) was performed according to Laemmli [39]. The gels were stained with silver nitrate as described by Wray et al. [43].

Immunoblotting Assay

Chromatins (1 mg/ml as DNA) were digested with 50 μg/ml pancreatic DNase I in 10 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 0.5 mM PMSF for 1 h at 2°C [35]. The digestion was stopped by addition of EDTA to 2 mM. Protein concentration was determined by the method of Lowry et al. [44]. Samples were made 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.0625 M Tris-HCl (pH 6.8) and 25 μg/ml pyronin Y, and boiled for 5 min. Proteins in the gel were electrophoretically transferred to nitrocellulose paper according to method of Towbin et al. [45]. For immunodetection, the nitrocellulose was incubated in 10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 50 mM NaCl, 1 × Denhardt's solution (0.02% each of bovine serum albumin, Ficoll, and polyvinyl pyrrolidone) for 40 min at 37°C. The paper was then washed three times in 100 ml phosphate-buffered saline (PBS, pH 7.2), 0.05% Tween, for 10 min intervals at 37°C. The paper was then incubated for 30 min at 37°C in 3% BSA in PBS to saturate nonspecific protein-binding sites, washed as described, and incubated with antisera at 1/100 dilution in 1% BSA in PBS overnight at 0–4°C. The nitrocellulose was washed as described and incubated with goat anti-rabbit

immunoglobulin conjugated to alkaline phosphatase (Jackson Lab.) at a 1/5,000 dilution in 1% BSA in PBS for 4 h at room temperature. Following the wash procedure, the paper was stained in 0.1 M $NaHCO_3$/0.001 M $MgCl_2$ (pH 9.8) containing 0.03% nitro blue tetrazolium (NBT) and 0.015% 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Histological and Immunohistochemical Examinations

The fixation of liver tissue, tissue processing, sectioning, and mounting were performed as described by Nakane and Pierce [46]. Serial tissue sections were either stained with hematoxylin/eosin or subjected to immunohistochemical staining by the ABC method following the procedure provided by the supplier of the assay kit (Vector Laboratories, Inc.).

RESULTS

Using antisera against NHPs from Morris hepatoma 7777 and an immunoblotting technique, we have identified a group of high molecular weight NHPs which are present in hepatomas but not in normal rat liver [35]. These hepatoma-associated NHPs appeared and increased in rats treated with the carcinogen 3′-MDAB. 3′-MDAB is a powerful hepatocarcinogen which can elicit hepatomas in rats [47]. In this study, we treated rats with 3′-MDAB for 80 days. At this time point, multiple hyperplastic nodules were observed in the liver of experimental rats (Fig. 1B) while no neoplastic changes could be observed in the liver of control rats (Fig. 1A). The localization of hepatoma-associated NHPs in liver sections was determined by indirect immunohistochemical examination. As shown in Figure 1D and 1E the antigenic hepatoma-associated NHPs are localized only in the nuclei of neoplastic growing foci. No antigens are detected in liver sections of control rats (Fig. 1C).

The specificity of rat hepatoma-associated NHPs was compared with hamster chromatin to determine species specificity as shown in Figure 2. The electrophoretic patterns of total chromatin proteins obtained from rat Morris hepatoma 7777 and Kirkman-Robbins hepatoma as well as control livers are shown in Figure 2A. The immunoreactivities of these proteins were also studied by immunoblotting techniques. As shown in Figure 2B, chromatin preparations from both Morris hepatomas, i.e., 7777 and 8994, and Kirkman-Robbins hepatoma demonstrated reactivity to the antibodies generated to Morris hepatoma 7777 high molecular weight NHPs (their molecular weights are larger than 80,000 daltons, for detail see ref. [36]). The antigenic hepatoma-associated NHPs were found in the NHPs of all hepatoma chromatins ranging from mol. wt. 80,000 to 100,000, 120,000 to 140,000, and 160,000 to 230,000 (lanes 2′, 2 and 4). No hepatoma-associated NHPs were detected in normal rat or hamster livers (lanes 1 and 3).

Recently Wen et al. [48] demonstrated that hepatoma nonhistone protein antigens were associated with active genes. The preferential association of active gene sequences with the nuclear matrix has implicated this structure in the organization of chromatin and in gene expression [49,50]. To determine if antigenic hepatoma-associated NHPs were present in nuclear matrix, nuclei were fractionated into nuclear matrices and various salt-soluble NHPs from rat and hamster hepatomas as shown in Figures 3 and 4. Nuclear constituents of Morris hepatoma 7777 and Kirkman-Robbins hepatoma were separated into four distinct fractions, i.e., nuclear digest, low and high salt extracts, and nuclear matrix according to a method developed in Berezney's laboratory [12,42]. Using an immunoblotting assay we detected hepatoma-associated nonhistone protein antigens preferentially concentrated in the nuclear matrix fraction originating from both rat and hamster hepatomas. However, some high molecular weight and low molecular weight immunoreactive bands were also detected in the endogeneous digestion and salt extraction fractions. These proteins are probably associated with active genes or loosely bound to genomic DNA as described in our earlier paper [48]. It is interesting to find that the species of high molecular weight proteins are different from those present in the nuclear matrix.

DISCUSSION

Abundant evidence exists in the literature [2,4–7,51] which demonstrates changes in nuclear NHPs of cells while they are transformed from normal to neoplastic states in vivo or in vitro. However most of the changes in NHPs were quantatively detected by biochemical methods such as column chromatography and SDS-polyacrylamide gel eletrophoresis. Unique proteins characteristic for neoplastic process have

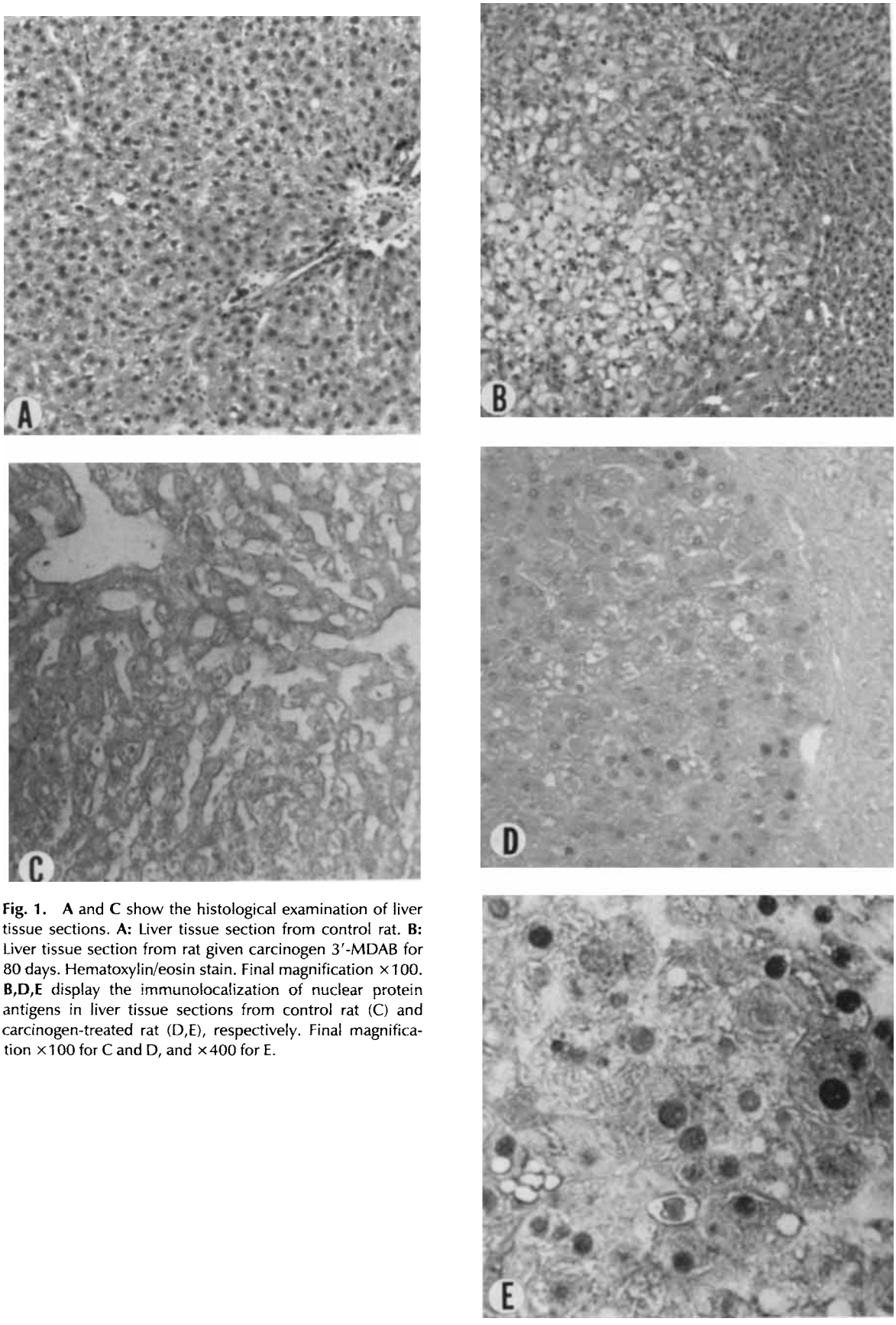

Fig. 1. **A** and **C** show the histological examination of liver tissue sections. **A:** Liver tissue section from control rat. **B:** Liver tissue section from rat given carcinogen 3′-MDAB for 80 days. Hematoxylin/eosin stain. Final magnification ×100. **B,D,E** display the immunolocalization of nuclear protein antigens in liver tissue sections from control rat (C) and carcinogen-treated rat (D,E), respectively. Final magnification ×100 for C and D, and ×400 for E.

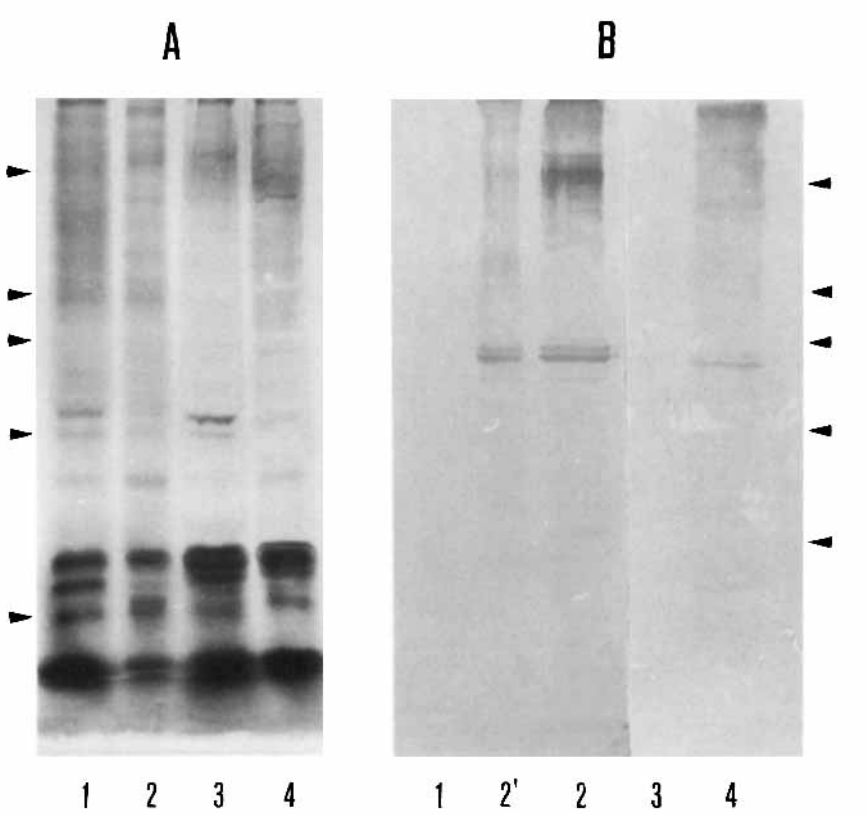

Fig. 2. SDS-polyacrylamide gel electrophoresis profiles (**A**) and immuno-identification (**B**) of chromatin proteins from rat liver (**lane 1**); Morris hepatoma 7777 (**lane 2**); Morris hepatoma 8994 (**lane 2'**); hamster liver (**lane 3**); and Kirkman-Robbins hepatoma (**lane 4**). The antibodies were recognized by antisera to Morris hepatoma 7777 high molecular weight NHPs. Arrowheads indicate the positions of molecular weight markers: myosin (Mr 205,000), β-galactosidase (Mr 116,000), β-phosphorylase (Mr 97,000), bovine serum albumin (Mr 66,000), and ovalbumin (Mr 45,000).

recently been observed using immunochemical detection [25,29,31,33–35,52].

The results of this study revealed that hepatoma-associated nonhistone protein antigens in rat hepatoma cells described recently by this laboratory [36] are also present in other rodent hepatoma, i.e., hamster Kirkman-Robbins hepatoma. Both hepatomas are transplantable and comprise poorly differentiated and very malignant tumor cells. Morris hepatoma 7777 was originally cultured from a Buffalo rat hepatoma induced by N-2-fluorenylphthalamic acid. Kirkman-Robbins hepatoma cells originated from a hamster hepatoma induced by testosterone [53].

These antigenic proteins are tightly bound to DNA. They cannot be dissociated from DNA by dehistonization procedure, DNase I treatment, or salt extraction. It requires 2% SDS, 5% 2-mercaptoethanol to solubilize these proteins from chromatin. As was shown in Figure 2A, the chromatin proteins from Morris hepatoma 7777 and Kirkman-Robbins hepatoma represent a highly heterogeneous complex of polypeptides from about 35,000 to 250,000 with a predominant protein species in two regions, i.e., 35,000–55,000 and 100,000–230,000. Molecular characterization of Morris hepatoma 8994 chromatin proteins were very similar to those from Morris hepatoma 7777 as was shown previously [36]. Polyclonal antisera raised to high molecular weight NHPs of Morris hepatoma 7777 reacted strongly with both rat and hamster hepatoma NHPs ranging from 80,000 to 100,000, 120,000 to 140,000, and 160,000 to 230,000, but not with those NHPs from normal liver of rat and hamster (Fig. 2B).

Treatment of nuclei from eukaryotic cells with a combination of low and high salt concentrations following endogeneous nuclease digestion (37°C, 45 min) as recommended by Berezney and associates [12,42] resulted in an insoluble, proteinaceous pellet called nuclear matrix. Despite the lack of a precise definition, numer-

ous studies have revealed evidence implicating this structure in many aspects of the nuclear physiology of normal and pathological cells [12–14,23,24].

As shown by SDS-polyacrylamide gel electrophoresis, nuclear matrix proteins of rat and hamster hepatomas ranged in molecular size from about 35,000 to 230,000. It is interesting to note that hepatoma-associated antigens are mainly concentrated in the nuclear matrix of rat and hamster hepatomas (Figs. 3, 4).

As was demonstrated previously by one of our laboratories [35,54] hepatoma-associated NHPs are expressed not only in hepatoma cells but also in hepatocyte cells committed to carcinogenesis. These antigenic nuclear proteins appeared at an early stage of hepatocarcinogenesis and steadily increased during the occurrence of the marked histological alterations that take place during chemically induced hepatocarcinogenesis. The antigenic nuclear proteins were found only in the nuclei of neoplastic growing foci (Fig. 1). The appearance of hepatoma-associated NHPs at early stages of tumor promotion during hepatocarcinogenesis suggests that these nuclear proteins are related either to a general cell proliferation or to a process specific to hepatocarcinogenesis. However, our previous results [35] demonstrated that proliferative fetal rat liver and regenerating liver did not contain these antigenic NHPs. The results presented in this paper also demonstrate that these specific NHPs are present in both rat and hamster hepatoma. The possibility that these antigenic nuclear proteins are associated with the process specific to hepatocarcinogenesis is very high. The possible functional role of such NHPs in neoplastic transformation of liver cells requires further investigation.

ACKNOWLEDGMENTS

This work was partially supported by Polish Programme CPBR 11.5 grant 88 and by USPHS grant CA 42969 awarded by the National Cancer Institute. The authors wish to express their

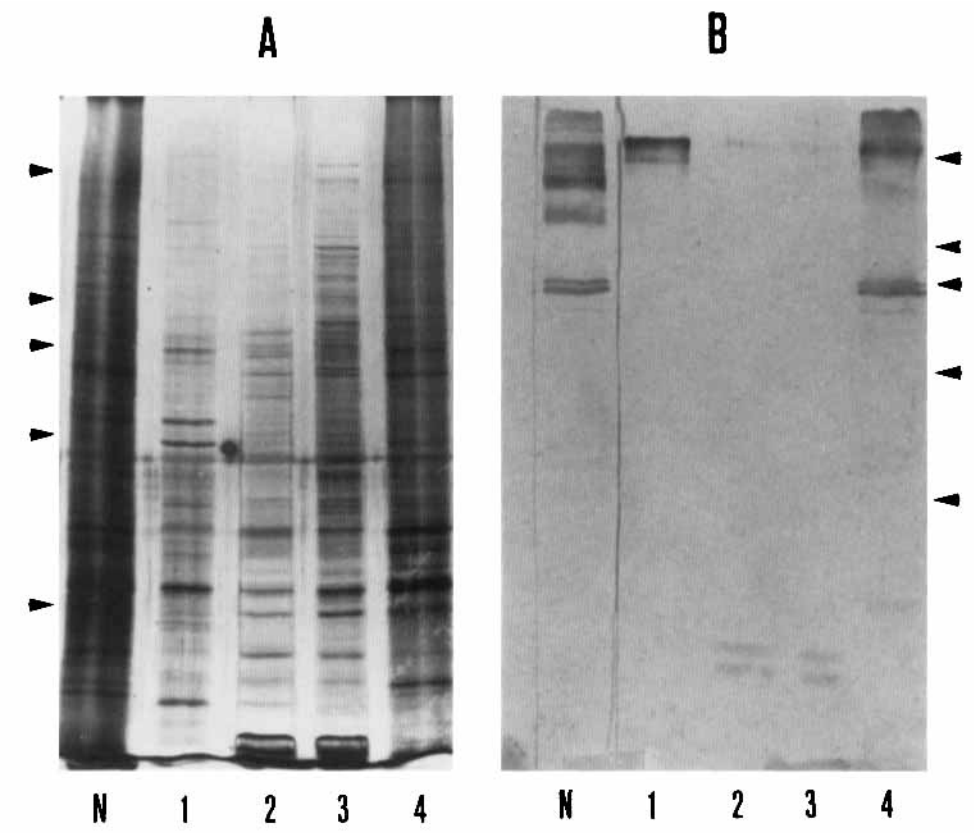

Fig. 3. SDS-polyacrylamide gel electrophoresis profile (**A**) and immuno-identification (**B**) of Morris hepatoma 7777 nuclear protein fractions: total nuclear protein fractions (**lane N**); nuclear endogeneous DNase digestion (**lane 1**); low salt extract (**lane 2**); high salt extract (**lane 3**); and nuclear matrix (**lane 4**). The antigens were recognized by antisera to Morris hepatoma 7777 high molecular weight NHPs. Molecular markers are the same as in Figure 2.

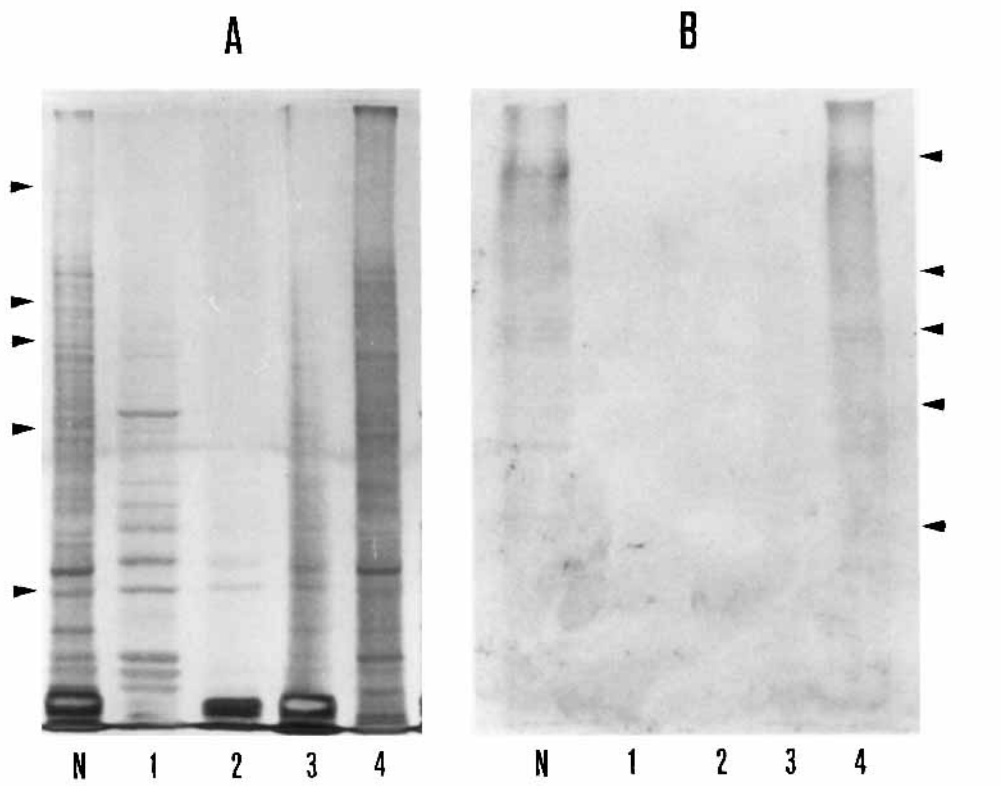

Fig. 4. SDS-polyacrylamide gel electrophoresis (**A**) and immuno-identification (**B**) of Kirkman-Robbins hepatoma nuclear protein fractions: total nuclear proteins (**lane N**); nuclear endogeneous DNase digestion (**lane 1**); low salt extract (**lane 2**); high salt extract (**lane 3**) and nuclear matrix (**lane 4**). The antigens were recognized by antisera to Morris hepatoma 7777 high molecular weight NHPs. Molecular weight standards are the same as in Figure 2.

thanks to P. DiTullio for assistance in immunization of the rabbits, to M. Radwan for preparing the photographs, and to J. Gierak for assistance in preparing this manuscript. We also thank Jack Egan for critical review of this manuscript.

REFERENCES

1. Hnilica LS, Briggs RC: In Sell S (ed): "Cancer Markers." Clifton: The Humana Press, 1980, p 463.
2. Durban E, Roll D, Beckner G, Busch H: Cancer Res 41:537, 1981.
3. Busch H, Busch RK, Chan P-K, Isenberg W, Weigand R, Russo J, Furmanski P: Clin Immunol Immunopathol 18:155, 1981.
4. Smith GJ: Cell Biol Int Rep 5:635, 1981.
5. Lea MA: Int J Biochem 15:767, 1983.
6. Menzel A, Unteregger G: Electrophoresis 10:554, 1989.
7. Stein GS, Plumb M, Stein JL, Phillips IR, Shephard EA: In Hnilica LS (ed): "Chromosomal Nonhistone Proteins," Vol. I. Boca Raton, FL: CRC Press Inc, 1983, p 127.
8. Halikowski MJ, Liew CC: Biochem Cell Biol 66:349, 1988.
9. Newport JW, Forbes DJ: Annu Rev Biochem 56:535, 1987.
10. Wildom J: Ann Biophys Chem 18:365, 1989.
11. Berezney R, Coffey DS: Biochem Biophys Res Commun 60:1410, 1974.
12. Berezney R: In Hnilica LS (ed): "Chromosomal Nonhistone Proteins," Vol. IV. Boca Raton, FL: CRC Press, 1984, p 119.
13. Kaufmann H, Fields AP, Shaper JH: Methods Achiev Exp Pathol 12:141, 1986.
14. Van der Velden HMW, Wanka F: Mol Biol Rep 12:69, 1987.
15. Bodnar JW: J Theor Biol 132:479, 1988.
16. Cook DR: J Cell Sci 90:1, 1988.
17. Small D, Nelkin B, Vogelstein B: Nucleic Acids Res 13:2413, 1985.
18. Mirkovitch J, Gasser SM, Laemmli UK: J Mol Biol 200:101, 1988.
19. Phi-Van L, Strätling WH: EMBO J 7:655, 1988.
20. Zbarsky IB: Mol Biol Rep 7:139, 1981.
21. Tas S, Rodriguez LJ, Drewinko B, Trujillo JM: Int J Cancer 34:329, 1984.
22. Bladon T, Brasch K, Brown DL, Setlerfield G: Biochem Cell Biol 66:40, 1988.
23. Fey EG, Penman S: Proc Natl Acad Sci USA 85:121, 1988.
24. Pienta KJ, Partin AW, Coffey DS: Cancer Res 49:2525, 1989.
25. Schmidt WN, Hnilica LS: Cancer Res 42:1441, 1982.
26. Schmidt WN, Gronert BJ, Page DL, Briggs RC, Hnilica LS: Cancer Res 42:3164, 1982.
27. Kilianska Z, Schmidt WN, Hnilica LS: Cell Differ 13: 225, 1983.

28. Krajewska WM, Kilianska Z, Lipinska A, Klyszejko-Stefanowicz L: Cell Biochem Funct 3:53, 1985.
29. Krajewska WM, Schmidt WN, Hnilica LS: Mol Cell Biochem 70:77, 1986.
30. Black A, Freeman JW, Zhou G, Busch H: Cancer Res 47:3266, 1987.
31. Babin JK, Anderson JN: Cancer Res 48:5495, 1988.
32. Freeman JW, Busch RK, Gyorkey F, Gyorkey P, Ross BE, Busch H: Cancer Res 48:1244, 1988.
33. Kilianska Z, Krajewska WM, Gaczynski M, Marszalek M, Klyszejko-Stefanowicz L: Int J Biochem 21:873, 1989.
34. Krajewska WM, Lipinska A, Marszalek M, Kilianska Z, Wojtkowiak Z, Klyszejko-Stefanowicz L: Cell Biochem Funct 8:79, 1990.
35. Burkhardt AL, Huang D-P, Chiu J-F: Biochim Biophys Acta 781:165, 1984.
36. Kilianska Z, Ditullio P, Chiu J-F: Int J Biochem 22:969, 1990.
37. Chiu J-F, Hunt M, Hnilica LS: Cancer Res 35:913, 1975.
38. Chiu J-F, Fujitani H, Hnilica LS: Methods Cell Biol 14:283, 1977.
39. Laemmli UK: Nature 227:680, 1970.
40. Chytil F: Methods Cell Biol 18:123, 1978.
41. Good A, Wofsy L, Kimura J, Henry C: In Mishell B, Shiigi S (eds): "Selected Methods in Cellular Immunology." San Francisco, CA: Freeman and Co. 1980., p 278.
42. Tubo RA, Berezney R: J Biol Chem 262:1148, 1987.
43. Wray W, Boulikas T, Wray VP, Hancock R: Anal Biochem 118:197, 1981.
44. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
45. Towbin H, Staehelin T, Gordon J: Proc Natl Acad Sci USA 76:4350, 1979.
46. Nakane PK, Pierce GB: J Cell Biol 33:307, 1967.
47. Price JM, Harman JW, Miller EC, Miller JA: Cancer Res 12:192, 1952.
48. Wen B-G, Burkhardt AL, Cote GJ, Huang D-P, Chiu J-F: Mol Cell Biochem 76:75, 1987.
49. Robinson SI, Nelkin BD, Vogelstein B: Cell 28:99, 1982.
50. Ciejek EM, Tsai MJ, O'Malley BW: Nature 306:607, 1983.
51. Sudhakar S, Johnston DA, Becker FF, Rodriguez LV: Cell Mol Biol 30:275, 1984.
52. Dihn B-L, Temblay M, Paradis D, Roy P-E, Tetu B: Int J Cancer 41:201, 1988.
53. Kirkman H, Robbins M: Proc Am Assoc Cancer Res 2:38, 1955.
54. Dong J-M, Kilianska Z, Fukuda K, Chiu J-F: Cancer Lett 51:259, 1990