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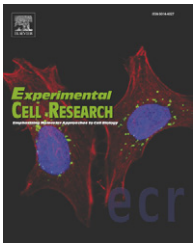

Research Article

# Proteomic analysis of the nuclear matrix in the early stages of rat liver carcinogenesis: Identification of differentially expressed and MAR-binding proteins

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ABSTRACT

Tumor progression is characterized by definite changes in the protein composition of the nuclear matrix (NM). The interactions of chromatin with the NM occur via specific DNA sequences called MARs (matrix attachment regions). In the present study, we applied a proteomic approach along with a Southwestern assay to detect both differentially expressed and MAR-binding NM proteins, in persistent hepatocyte nodules (PHN) in respect with normal hepatocytes (NH). In PHN, the NM undergoes changes both in morphology and in protein composition. We detected over 500 protein spots in each two dimensional map and 44 spots were identified. Twenty-three proteins were differentially expressed; among these, 15 spots were under-expressed and 8 spots were over-expressed in PHN compared to NH. These changes were synchronous with several modifications in both NM morphology and the ability of NM proteins to bind nuclear RNA and/or DNA containing MARs sequences. In PHN, we observed a general decrease in the expression of the basic proteins that bound nuclear RNA and the over-expression of two species of Mw 135 kDa and 81 kDa and pI 6.7–7.0 and 6.2–7.4, respectively, which exclusively bind to MARs. These results suggest that the deregulated expression of these species might be related to large-scale chromatin reorganization observed in the process of carcinogenesis by modulating the interaction between MARs and the scaffold structure.


## Introduction

It has long been recognized that the process of carcinogenesis involves several alterations in the nuclear organization and in the amount and distribution of heterochromatin [1,2]. The levels of condensation of the chromatin domains are determined by the complex balance of: the conformational effects arising from the binding of trans-acting factors; the post-translational modification of the histone complements; and, finally, the interaction of chromatin with the nuclear matrix (NM) [3]. The NM is composed of 2% DNA, 4% RNA and 94% proteins; the

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*Abbreviations:* 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; BUR, base-unpairing regions; CK, cytokeratin; hnRNP, heterogeneous nuclear ribonucleoprotein; HSP, heat shock protein; MARs, matrix attachment regions; MS, mass spectrometry; NH, normal hepatocytes; NM, nuclear matrix; NuMA, nuclear mitotic apparatus protein; PHN, persistent hepatocyte nodules; SWB, Southwestern blotting; WB, Western blotting

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transcription machinery, specific transcription factors and some enzymes involved in the modulation of the structure and transcriptional competence of chromatin (histone acetyltransferases, deacetylases and topoisomerases) have been co-isolated with the NM. In 1979 Berezney et al., [4] first characterized the NM from hepatoma and normal liver found several quantitative difference in protein composition that were specific for the hepatoma and independent of proliferating state of the cells. Changes in NM protein composition were subsequently found in many human tumors including prostate [5], bladder [6], renal [7], colon [8] and these changes were frequently associated with pathological stage [9,10]. Using differential scanning calorimetry in combination with ultrastructural observations on chromatin (both isolated and in situ), and along with the molecular characterization both of genomic DNA and the histone complement, we have observed that in interphase nuclei, the chromatin may assume different structures (with different levels of condensation) depending on the functional state of the cell. In particular the chromatin of transformed rat hepatocytes is characterized by the progressive unfolding of the higher-order structure [11] synchronous with specific alterations in the protein composition of the NM-intermediate filaments complex [12]. This finding strongly suggests the existence of a causal relationship between these phenomena.

The interactions of chromatin with the NM occur via specific DNA sequences called MARs (matrix attachment regions). It has been shown that the MARs are placed close to the transcriptionally active regions of chromatin so that transcription is initiated in the regions of chromatin anchored to the NM. The binding of certain MARs to the NM depends of cell-type or cell cycle, therefore the attachment of MARs to the NM is a dynamic event [13].

Several studies on unraveling the protein composition of the internal NM have confirmed that lamins and nuclear mitotic apparatus protein (NuMA) are the major structural components of the internal NM [14–16]. Work carried out in our laboratory using ultrastructural, immunoelectron and confocal microscopy [17,18], provided evidence for the existence of a RNA-stabilized lamin/NuMA frame, consisting of a web of thin lamin filaments to which NuMA is anchored mainly in the form of discrete islands.

In order to clarify the basic molecular mechanism of chromatin remodeling in cancer cells, we initiated a comparative study of the NM proteins expressed in normal (NH) and persistent hepatocyte nodules (PHN), before the onset of hepatocellular carcinoma, using a proteomic approach together with two-dimensional Southwestern blotting (SWB) analysis to identify the differentially expressed NM proteins and to find the role of the NM protein-MARs bond and/or the nuclear RNA bond in cellular transformation.

The results presented here show that, in PHN, the NM undergoes changes in morphology. These changes were synchronous with several modifications in the protein composition and in the ability to bind nuclear RNA and/or DNA containing MARs sequences, suggesting that modifications of the NM might be related to the large-scale chromatin reorganization observed in the process of carcinogenesis by modulating the interaction between MARs and the scaffold structure.

## Materials and methods

### *Induction of PHN*

Nodules were induced in male Fisher F-344 rats (Charles River, Como, Italy) following the Semple-Roberts et al., [19] procedure, as already reported [11]. Isolation and characterization of a persistent population of transformed hepatocytes was carried out at 32 weeks from initiation according to standard criteria, relying on the determination of the number and size of glutathione transferase placental positive nodules. At this post-initiation time the lesions have acquired the capacity to persist and grow actively, even in the absence of a mitogenic stimulus, and do not undergo spontaneous remodeling. These persistent nodules maintained a synchrony development and progress in a one to two month period to hepatocellular carcinoma. Therefore, they represent a good tool for investigating on early stages of tumor development [20].

Control hepatocytes were obtained from non-treated rats of the same strain and age of those utilized to induce nodules.

### *Isolation of the NM*

Hepatocytes both normal and transformed were prepared as reported in a previous paper [11] and nuclei were isolated on a sucrose cushion. The NM was isolated according to Barboro et al., [17], with minor modifications. The nuclear pellet was resuspended in digestion buffer consisting of 10 mM NaCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.8) (all from Sigma, St. Louis, MO), and 2 mM vanadyl ribonucleoside complex (VRC) (BioLabs, New England) was added to prevent the activation of endogenous RNase. The digestion by the $Ca^{2+}/Mg^{2+}$-dependent endogenous nuclease was allowed to proceed for 18 h. Chromatin fragments were extracted by adding $(NH_4)_2SO_4$ to a final concentration of 0.25 M. The NM was recovered by centrifugation at 6500 ×*g* for 15 min and extracted again with a large excess of digestion buffer containing 0.25 M $(NH_4)_2SO_4$. The sample was again pelleted and solubilized for one- or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The NM retained about 2% of nuclear DNA and 4% RNA. In some preparations the nuclei were digested in the absence of VRC.

### *Preparation of DNA and RNA probes*

The NM, isolated in the presence of VRC from HN, was resuspended in 0.5% SDS, 10 mM Tris–HCl (pH 7.8), 5 mM $Na_2EDTA$ and incubated overnight with proteinase K (50 μg/ml) at 37 °C. The nucleic acids were purified by standard phenol/chloroform/isoamylalcohol extraction and ethanol precipitation. The precipitate was rinsed with 70% ethanol, dissolved in 10 mM Tris–HCl (pH 8.0), 1 mM $Na_2EDTA$ and split into two aliquots. The first aliquot, containing both DNA and RNA co-isolated with the NM (NM–DNA, RNA fragments), was immediately stored at −80 °C; the second was incubated with RNase A (0.4 mg/ml) at 37 °C for 1 h. Then, the extraction with phenol/chloroform/isoamylalcohol was repeated. This procedure yielded high purity DNA and from now on we will demoninate it NM–DNA fragments.

A highly repetitive DNA sequence of 370-bp (XmnI) was also used as a probe for DNA binding experiments. The XmnI sequence was obtained from the S/MAR transaction DataBase [http://smartdb.bioinf.med.uni-goettingen.de], release 2.3 (accession number SM0000134). Plasmid pUC18 containing the XmnI sequence was constructed by GenScript Corporation (Piscatway, NJ, USA). *Escherichia coli* DH5α was transformed with pUC18 and transformants were selected on agar plates supplemented with ampicillin. Plasmids were isolated using a Qiagen Plasmid Maxi kit,