Nuclear Protein Changes in Rat Hepatomas Correlating with Growth Rate

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SUMMARY

The nature of nuclear proteins that are soluble in 8 M urea-50 mM phosphate, pH 7.6, was compared in rat liver and Morris hepatomas. Isoelectric focusing, using carrier ampholytes for a pH gradient of 3.5 to 10, indicated that with increasing growth rate of the hepatomas there was a progressive tendency for a decrease in nonhistone nuclear proteins with isoelectric points in the range 7.5 to 8.9 and an increase in the range 5.1 to 6.7. Studies on the influence of time on the pH gradient revealed that a nonuniform drift provided a better resolution of the pH range 7.5 to 8.9 at 7 hr than at 24 hr, while the latter time for electrofocusing gave an improved resolution of the pH range 5.1 to 6.7. Polycrylamide gel electrophoresis in a urea-acetic acid system showed that 8 M urea-50 mM phosphate, pH 7.6, extracted a small part of the histones from nuclei of both liver and hepatomas. There was less extraction of histones from the hepatoma nuclei, especially in two rapidly growing hepatomas with the most notable difference being seen in the lysine-rich H1 histone. The results suggested that in addition to qualitative or quantitative changes in nonhistone nuclear proteins in liver cancer there are alterations in the binding of histones to chromatin.

INTRODUCTION

Past research on the biochemistry of cancer has revealed the abnormal production of normal cellular constituents rather than the synthesis of unique products. Such alterations might arise from changes in the regulation of genetic expression resulting from mutations in regulatory genes or altered association with regulatory molecules. The nuclear proteins have frequently been suggested as serving as regulators of genetic expression, and their nature has been compared in normal and neoplastic tissues (1-3, 7, 10, 13, 16, 17). Because the Morris spectrum of hepatomas provides the opportunity to correlate the biochemical properties of tumors with their degree of differentiation and growth rate, they have served as a model system in which to study histone (11) and non-histone chromosomal proteins (1, 3, 10, 17). Separation of the non-histone chromosomal proteins has usually been achieved by the technique of sodium dodecyl sulfate-polyacrylamide gel electrophoresis which distinguishes the proteins according to their molecular weights. We have chosen to examine the procedure of Gronow and Griffiths (6) which uses isoelectric focusing of nuclear proteins that are soluble in 8 M urea-50 mM phosphate, pH 7.6. In this way, proteins are separated according to their isoelectric points. The objective of this work was to examine any progressive changes that correlate with the growth rate of the tumors and to identify thereby any protein fractions that merit further resolution by more discriminating techniques. A preliminary communication of this work has been presented (10).

MATERIALS AND METHODS

Animals. Experiments were routinely performed with male Buffalo rats. Hepatomas were transplanted bilaterally s.c. The histology and growth properties of the tumors were described previously (12). Partial hepatectomies involved the removal of approximately 66% of the liver according to the technique of Higgins and Anderson (8).

Extraction of Nuclear Proteins. Tissues were homogenized with 9 volumes of 0.32 M sucrose-3 mM MgCl2-5 mM NaHSO4. After filtering through cheesecloth, the homogenate was centrifuged at 800 x g for 8 min. The crude nuclear pellet was suspended in 2.2 M sucrose-5 mM MgCl2-5 mM NaHSO4 and centrifuged in the No. 30 rotor of the Beckman Model L ultracentrifuge at 25,000 rpm for 60 min. The nuclear precipitate was washed 3 times with 0.25 M sucrose-3 mM MgCl2-5 mM NaHSO4 and centrifuged in the No. 30 rotor of the Beckman Model L ultracentrifuge. The acid extract was washed 3 times with 1 ml 8 M urea-50 mM sodium phosphate, pH 7.6, and the extracts were combined. Acid-soluble proteins were extracted from the nuclear residue with 0.24 N HCl.

Isoelectric Focusing and Electrophoresis. Isoelectric focusing was performed essentially according to the procedure of Gronow and Griffiths (6). In agreement with the observation of Hnilica (9), the reaction of the proteins with N-ethylmaleimide was not found to have a significant effect on the protein profile and that step was omitted. The gels contained 200 µg protein in a total volume of 2 ml. The pH gradient was measured by pH determinations of 2-ml aqueous extracts of 4-mm gel slices. Polycrylamide gel
electrophoresis in 6.25 M urea-0.9 M acetic acid was used for separation of histones (14). Densitometry was performed with a Beckman Acta spectrophotometer equipped with a linear transport.

RESULTS

In our preliminary studies we observed that isoelectric focusing for either 5 or 7 hr gave a close correspondence of the protein bands obtained with nuclear proteins that were soluble in 8 M urea-50 mM phosphate, pH 7.6. However, a different pattern was observed when the electrofocusing time was increased to 24 hr. The nature of the pH gradients, determined after 7 and 24 hr, is presented in Chart 1. The pH gradient is less linear after 24 hr. The effect of this change in pH gradient on protein distribution in polyacrylamide gels is shown in Fig. 1 for host liver and the rapidly growing and poorly differentiated hepatoma 7777. The 24-hr period for isoelectric focusing gave an improved resolution at pH 5.1 to 6.7, but this is achieved at the expense of a compression of the pH range 7.5 to 8.9. The 7-hr period for isoelectric focusing shows a decrease in the tumor of certain proteins with isoelectric points at pH 7.5 to 8.9. The 24-hr run provides improved resolution of proteins with isoelectric points in the range of pH 5.1 to 6.7, where there is an increase in the hepatoma, but there is a less satisfactory discrimination of the proteins with high isoelectric points. Densitometric tracings of the nuclear proteins from host livers and 3 slowly growing hepatomas after 24-hr isoelectric focusing are given in Chart 2. The differences among the 3 host livers shown in Chart 2 were not greater than those we have observed in different rats bearing the same tumor. We have not detected consistent differences between livers from normal rats and those bearing tumors, nor have we noted significant differences in male and female rats. Similar profiles were obtained for normal liver and regenerating liver 24 hr after partial hepatectomy. The densitometric recordings of gels shown in Chart 3 are for 24-hr runs with nuclear proteins from 3 rapidly growing tumors and their respective host livers. The greater propor-

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Chart 1. Influence of isoelectric focusing time on the pH gradients in polyacrylamide gels using pH 3.5 to 10 ampholytes.

Fig. 1. Isoelectric focusing in polyacrylamide gels of nuclear proteins soluble in 8 M urea-50 mM phosphate, pH 7.5. The protein bands after the 2 focusing periods are for hepatoma 7777 (T) and host liver (L). Arrows, pH at different points along the gels.

Chart 2. Densitometric tracings of nuclear proteins (soluble in 8 M urea-50 mM phosphate, pH 7.6) after 24-hr isoelectric focusing in polyacrylamide gels. The 3 hepatomas had transplant generation times greater than 2 months. The tracing for each tumor is to the left of the respective host liver. The proteins were stained with Coomassie blue.
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Polyacrylamide gels. The 3 hepatomas had transplant generation times less than 2 months. The tracing for each tumor is to the left of the respective host liver. The proteins were stained with Coomassie blue.

The prominent band at the upper (cathode) end of the gels after isoelectric focusing of nuclear proteins, soluble in 8 M urea-50 mM phosphate, pH 7.6, was generally more intense in preparations from liver than from tumors. Although histones would be expected to run off the end of the gel (6), we have obtained evidence that this band is at least in part due to histones, which presumably precipitate as they approach their isoelectric points. When isoelectric focusing was performed with 200 µg calf thymus histones (Sigma Chemical Co., St. Louis, Mo.), a band was obtained in the same position. Only a minor portion of the histones were extracted by the 8 M urea-50 mM phosphate, pH 7.6, was the H4, as noted by Gronow and Griffiths (6), under our conditions there was also significant extraction of other histones particularly with normal or host livers. The least extraction of histones by the urea solution was seen with the rapidly growing hepatomas 9618A2 and 7777 (Chart 4). The histones extracted from the nuclei of the slowly growing hepatoma 20 more closely resembled the normal liver with a significant extraction of histone H1, which was not seen in the rapidly growing hepatomas. Comparison of the protein extracted from nuclei from 2, 3 or 5 g hepatoma 7777 or host liver revealed that the amount of protein was proportional to the weight of tissue. In addition, the banding pattern on polyacrylamide gel electrophoresis was the same for a given tissue over this range. Gels A and B in Fig. 3 show the histones extracted by 8 M urea-50 mM phosphate in 2 control rats. Gels C and D, which represent histones extracted under the same conditions from regenerating liver of 2 rats at 24 hr after partial hepatectomy, indicate that, in common with the control livers, there was extraction of significant H1 histone from the nuclei.

DISCUSSION

The plateau phenomenon in isoelectric focusing was discussed by Righetti and Drysdale (15). As seen in our experiments after a 24-hr electrofocusing, it results in a flattening of the pH gradient at the center of the gel with an improved resolution of proteins that have an isoelectric point in this region. Thus proteins with isoelectric points between pH 5.1 and 6.7 were distributed over a 21-mm portion of the gel after 7 hr electrofocusing, but over a 33-mm region after 24 hr. It was in this region that an increased proportion of nonhistone nuclear proteins were seen in the more rapidly growing hepatomas. On the other hand, proteins with isoelectric points between 7.5 and 8.9 were distributed over a 20-mm region of the gel after 7 hr electrofocusing, but only over 12 mm after 24 hr. In this region, therefore, in which there was a progressive loss of some protein bands with increasing tumor growth rate, the shorter period for electrofocusing was necessary to give adequate resolution. An increase in the proportion of nonhistone nuclear proteins with low isoelectric points was also reported by Gronow and Thackrah (7) for liver tumors induced with diethylnitrosamine. The present work showed that this trend is difficult to detect in the well-differentiated and slowly growing Morris hepatomas 20 and 9618A. The acid shift appears to be one of a group of changes that may be classified as showing a correlation with hepatoma growth rate rather than being a consistent change in all liver tumors (19).

An increase in the ratio of nonhistone chromosomal protein to DNA has been well documented for hepatomas of the rat, and this contrasts with the apparent constancy of the ratio of histone to DNA. If the nonhistone chromosomal proteins alter the transcriptional activity of chromatin by

Chart 3. Densitometric tracings of nuclear proteins (soluble in 8 M urea-50 mM phosphate, pH 7.6) after 24-hr isoelectric focusing in polyacrylamide gels. The 3 hepatomas had transplant generation times less than 2 months. The tracing for each tumor is to the left of the respective host liver. The proteins were stained with Coomassie blue.

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altering the association between histone and DNA as considered, for example, by Olson et al. (13), then the dissociation must involve only a portion of the molecule. In view of the clustering of charged and hydrophobic side chains on the histone molecule, it is possible that a partial dissociation occurs. On the other hand, Arnold et al. (1) did not find altered in vitro template activity for chromatin of hepatoma 9618A despite the increased content of nonhistone protein. They were also unable to detect qualitative changes in the electrophoretic pattern of the nonhistone chromosomal proteins from this slowly growing liver tumor with sodium dodecyl sulfate-polyacrylamide gels. This work emphasizes the similarity in the profile of nonhistone protein isoelectric points in liver and slowly growing hepatomas, which is in line with the similar molecular weight profiles that were demonstrated previously (1, 3). If the acidic nature of most nonhistone chromosomal proteins is of significance in their influence on template activity, then the acid shift in the proportions of nuclear proteins in rapidly growing hepatomas may have particular importance for the altered transcriptional activity in those tumors.

Comparisons between nonhistone chromosomal proteins of liver and hepatomas have been criticized by Baserga (2) on the grounds that changes in cellular composition exist, and differences in gel electrophoretic profiles were reported by Gonzalez-Mujica and Mathias (5) for stromal and parenchymal nuclear proteins. If major differences are found among the nuclear proteins of liver cell types, it is surprising that this is not reflected in comparisons of normal liver and slowly growing tumors of parenchymal cell origin which have relatively few stromal cells. In collaborative studies with B. Wilson, G. Vidali, and V. G. Allfrey, we are at present investigating this discrepancy.

Another area that merits further study is the binding of histones to chromatin. Stein et al. (18) reported that histones are more tenaciously bound to chromatin of HeLa S2-cells in mitosis than during S phase. Hepatomas have a greater proportion of their cells in a dividing state than does the normal liver in which the cells are chiefly in a G1 or G0 state. Temporal studies with the regenerating liver, which has a partial synchronization of cell division, should provide some insight of the extent to which increased cellular proliferation affects histone binding in hepatic chromatin and the extent to which changes in hepatomas are related to increased growth or are peculiar to the neoplastic transformation. A small (7%) but significant decrease in the histone to DNA mass ratio has been reported within 8 hr of partial hepatectomy (4).
Nuclear Proteins of Hepatomas

Fig. 3. Polyacrylamide gel electrophoresis in 0.9 N acetic acid-6.25 M urea of 50 \( \mu \)g nuclear protein, soluble in 8 M urea-50 mM phosphate, pH 7.6. The proteins were extracted from liver nuclei of 2 control rats (Gels A and B) or 24 hr after partial hepatectomy (Gels C and D). Arrow, position of the H1 histone. The proteins were stained with Amido black.

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